A P.M.R. STUDY OF THE CONFORMATION OF SOME DERIVATIVES OF DI-*O*-ISOPROPYLIDENE-ARABINOPYRANOSE

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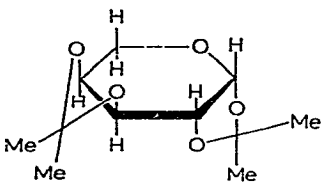
INTRODUCTION

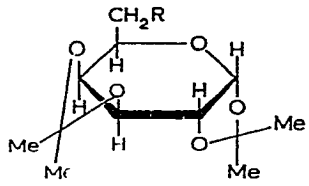
Considerable interest has been shown in the conformational effect of *cis*-fused isopropylidene rings on parent five- and six-membered rings. Thus *cis*-fusion of one isopropylidene ring to a cyclohexane[1] or pyranose[2] ring causes little distortion from the initial chair conformation except in the case of some 1,2-*O*-alkylidene-α-D-glucopyranose derivatives[3] when the pyranose ring appears to adopt a skew (or twist boat) conformation. Angyal and Hoskinson[1] showed from hydrogen-bonding studies that *cis*-fusion of two isopropylidene rings forces the cyclohexane ring to adopt a non-chair conformation, generally of the skew type. McCasland *et al*[4] have subsequently demonstrated from the p.m.r. spectrum that a dideoxy-di-*O*-isopropylidene-dithioinositol also adopts a skew conformation. On the basis of a limited amount of p.m.r. spectral information, Coxon and Hall[3] suggested that 6-*O*-acetyl-1,2:3,4-di-*O*-isopropylidene-α-D-galactopyranose (VI) is in a chair conformation in solution. Examination of some related compounds, in which the assignment of most or all of the vicinal coupling constants is unambiguous, has now shown that the vicinal coupling constants for the $H_{(2)}$–$H_{(3)}$ and $H_{(3)}$–$H_{(4)}$ interactions in the above galactose derivative must be reversed. The observed coupling constants for these di-*O*-isopropylidene derivatives are consistent with a non-chair conformation similar to the skew conformation proposed by Angyal and Hoskinson[1] for di-*O*-isopropylidene-inositols.

EXPERIMENTAL

The spectra were measured with a Varian A-60 spectrometer at its normal operating temperature. The carbohydrate derivatives were examined as 10% w/v solutions in deuterochloroform or acetone as indicated. Tetramethylsilane was used as the internal reference; 2–3 drops were added to each solution (0.4 ml or 0.5 ml). After the spectrum had been measured at 500 c.p.s. sweep width, the part of the spectrum containing the signals due to the ring hydrogens was reswept at 250 c.p.s. sweep width, with optimum values of the RF field and frequency band width. The 250 c.p.s. sweep-width spectrum was calibrated from 2 or 3 of the sharpest lines in the same part of the 500 c.p.s. sweep-width spectrum and the vicinal coupling constants and chemical shifts of the ring hydrogens measured on the former spectrum. Melting points were measured on a Kofler microstage apparatus.



(I)



(II) R = I (III) R = H
(IV) R = OH (V) R = OTs
(VI) R = OAc

1,2:3,4-Di-O-isopropylidene-β-L-arabinopyranose (I)

This ketal was prepared as described by Bell[5] but the neutralised solution was evaporated to a syrup, which was sublimed at 80–100° (bath)/*ca.* 1.5 mm. The syrupy crystals were pressed dry on a filter and recrystallised twice from light petroleum (b.p. 100–120°). They had m.p. 37–40° (Bell[5] reports 41–42°).

6-Deoxy-1,2:3,4-di-O-isopropylidene-α-D-galactopyranose (III)

The method of Freudenberg and Raschig[6] was used but the product was not further purified after distillation at 12–15 mm. It was colourless and crystalline with m.p. 30–35° (Freudenberg and Raschig[6] report m.p. 37°, after an elaborate purification process).

1,2:3,4-Di-O-isopropylidene-α-D-galactopyranose (IV)

The commercially available syrup was used.

The remaining derivatives were pure crystalline compounds.

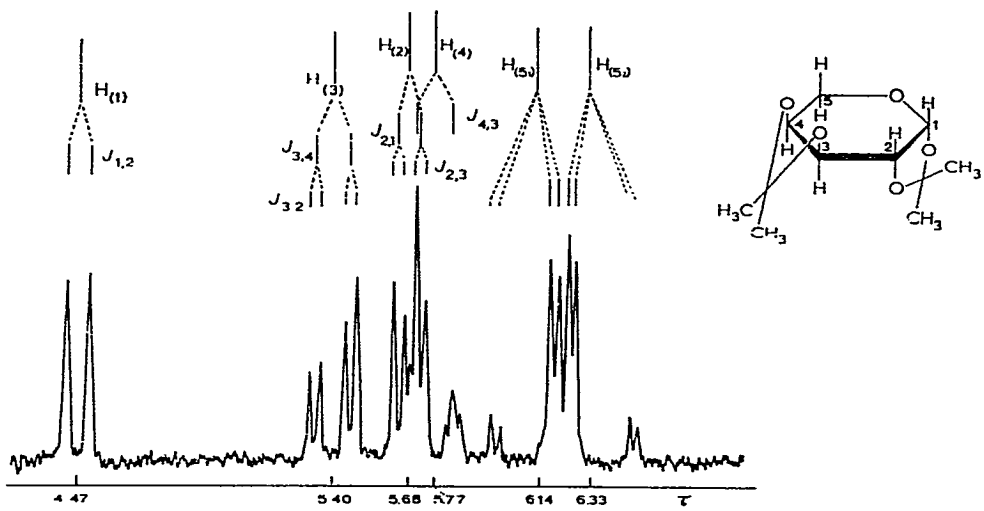


Fig. 1. P.m.r. spectrum of 1,2:3,4-di-*O*-isopropylidene-β-L-arabinopyranose. The methyl peaks are omitted.

SPECTRAL ASSIGNMENTS

1,2:3,4-Di-O-isopropylidene-β-L-arabinopyranose (I)

The doublet at low field was assigned to $H_{(1)}$ in the usual manner. The assignment of the $H_{(2)}$ and $H_{(3)}$ quartets was then obvious. Three of the six $H_{(4)}$ lines were overlapped with the $H_{(2)}$ lines. The separation between the two $H_{(4)}$triplets was $J_{3,4}$. The AB lines could be unambiguously assigned; the coupling constants between $H_{(4)}$, $H_{(5_1)}$, and $H_{(5_2)}$ are discussed later. The spectrum and assignments are shown in Fig. 1. The methyl resonances are not reproduced; two of these were superimposed.

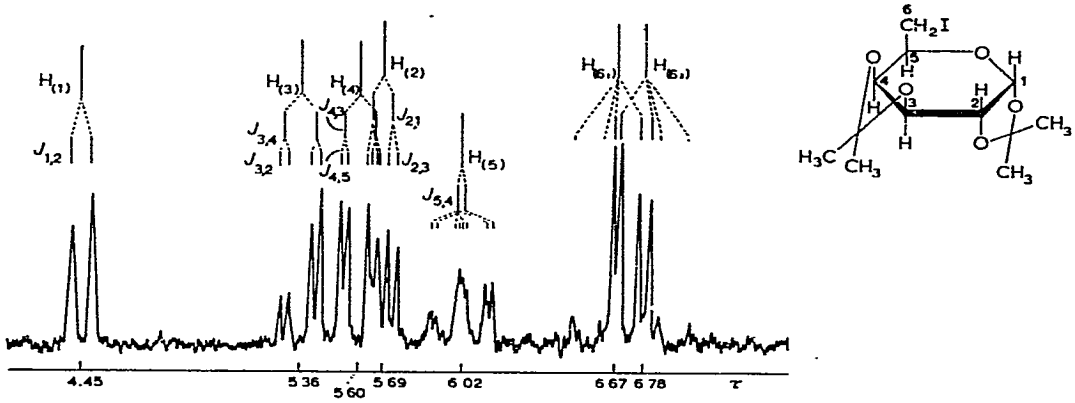


Fig. 2. P.m.r. spectrum of 6-deoxy-6-iodo-di-*O*-isopropylidene-α-D-galactopyranose. The methyl peaks are omitted.

6-Deoxy-6-iodo-1,2:3,4-di-O-isopropylidene-α-D-galactopyranose (II)

The spectrum was similar to that of (I); the $H_{(2)}$ and $H_{(4)}$ quartets were partly overlapped while the $H_{(5)}$ signal was at higher field. The intensities of the $H_{(3)}$ quartet were not equal; the chemical shifts given in Table I for $H_{(3)}$ are the multiplet centres and are only approximations of the true chemical shifts. The remainder of the spectrum shown in Fig. 2 was analysed as an ABX spectrum[7] where each X-line is symmetrically split by coupling with $H_{(4)}$. The calculated line spectrum is given in the Figure. The values obtained in both acetone and deuterochloroform solution are given in the Tables to show that there is almost no change of coupling constant with change of solvent.

6-Deoxy-1,2:3,4-di-O-isopropylidene-α-D-galactopyranose (III)

The signal for $H_{(5)}$ in this compound was a quartet (approx. 1:3:3:1) of doublets partly overlapped with the signal for $H_{(4)}$. As the latter occurred at highest field of any of the compounds studied, the $H_{(3)}$ signal was more symmetrical for this compound. The coupling between $H_{(5)}$ and the methyl group ($J_{5,6}$ = 6.8 c.p.s.) is a little larger than previously observed couplings of this type. Thus, in 3,4,6-trideoxy-3-dimethylamino-D-*xylo*-hexopyranose[8] $J_{5,6}$ = 6.3 c.p.s., and in 2,6-dideoxy-3-*C*-methyl-β-L-*ribo*-hexopyranose and its 3-*O*-methyl ether[9] $J_{5,6}$ = 6.1 c.p.s.

TABLE I

CHEMICAL SHIFTS (τ-VALUES)

Compound	*Solvent*	H_1	H_2	H_3^a	H_4	H_5	H_6	*C–Me*
I	$CDCl_3$	4.48	5.68	5.40	5.77	6.14,6.33		8.47,8.50,8.56(2×)
II	$CDCl_3$	4.45	5.69	5.36	5.60	6.02	6.67,6.78	8.45,8.55,8.65(2×)
II	Me_2CO	4.49	5.64	5.32	5.57	6.01		8.52,8.60,8.67(2×)
III	Me_2CO	4.56	5.72	5.40	5.92	6.08	8.93	8.55,8.63,8.69(2×)
IV[b]	$CDCl_3$	4.40	5.64[c]	5.34	5.71[c]	[d]	[d]	8.46,8.54,8.65(2×)
V[e]	$CDCl_3$	4.53	5.58[c]	5.23				8.50,8.66,8.68,8.72
VI[f]	Me_2CO	4.49	5.19[c]	5.32	*ca.* 5.70[c]			8.53,8.60,8.67(2×)

[a] Multiplet centre, see experimental.
[b] OH, τ 7.22.
[c] Tentative, see experimental.
[d] Complex multiplet 6.0–6.4.
[e] Arom., τ 2.03–2.77; arom. Me, τ 7.55.
[f] OAc, τ 7.94 (in $CDCl_3$).

1,2:3,4-Di-O-isopropylidene-α-D-galactopyranose (IV)

The signal for $H_{(4)}$ in this compound had no lines separated to the base line. The 6-hydroxyl peak (a broad singlet) was removed by shaking the sample with deuterium oxide (*ca.* 0.3 ml) and allowing the layers to separate out. The spectra of the 6-*O*-toluene-*p*-sulphonyl derivative (V) and the 6-*O*-acetyl derivative (VI) gave clear multiplets for $H_{(1)}$ and $H_{(3)}$ only. The lines from $H_{(2)}$ (V and VI) and $H_{(4)}$ (VI) were tentatively assigned by analogy with the other compounds.

TABLE II COUPLING CONSTANTS (C.P.S.)

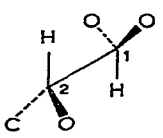
Compound	*Solvent*	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	
I	$CDCl_3$	5.0	2.4	7.8		$J_{4,5_1} = 2.0$; $J_{4,5_2} = 1.4$; $J_{5_1,5_2} = 13.0$
II	$CDCl_3$	5.0	2.4	7.8	1.6	$J_{5,6_1} = 5.9$; $J_{5,6_2} = 7.7$; $J_{6_1,6_2} = 9.8$
II	Me_2CO	5.2	2.4	7.8	1.8	$J_{5,6_1} + J_{5,6_2} = 13.6$
III	Me_2CO	5.2	2.4	7.8	2.0	$J_{5,6} = 6.8$
IV	$CDCl_3$	5.0	2.4	8.0	1.4[a]	
V	$CDCl_3$	5.0	2.4	7.8		
VI	Me_2CO	5.0	2.4	8.0	*ca.* 1.5[a]	

[a] Tentative, see experimental.

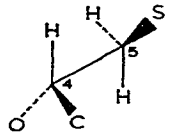
DISCUSSION

Diaxial coupling constants for vicinal hydrogen substituents on six-membered rings in a chair conformation have been shown to be large. Typical values for the fragment (A) of a pyranose ring (*i.e.* $J_{1a,2a}$) are 5 to 8 c.p.s. for a series of pyranose acetates[10] and 6 to 7 c.p.s. for some of the corresponding aldopyranoses in dimethyl

sulphoxide solution[11]. For fragments in which one of the oxygen atoms has been replaced by a less electronegative atom the values may be higher. Thus for β-D-xylopyranose tetraacetate, Lemieux *et al.*[10] found $J_{4a,5a}$ to be 8 c.p.s. and for some 2-deoxypyranosides, Lemieux and Levine[12] found $J_{1a,2a}$ to be 7.8 to 9.9 c.p.s. and $J_{2a,3a}$ to be 11.0 to 11.4 c.p.s., while for methyl (methyl 4-deoxy-β-L-*arabino*-hexopyranosid)uronate Schmidt and Neukom[13] found $J_{2a,3a}$ to be 7.2 c.p.s., and



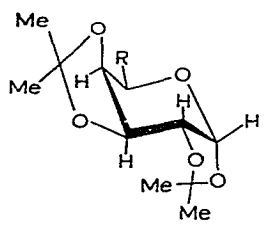
(A)



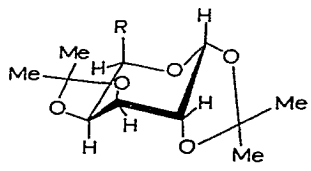
(B)

$J_{3a,4a}$ to be 8.0 c.p.s. A similar trend can be seen in the 5-thioxylopyranose derivatives whose p.m.r. spectra were obtained by Rao *et al.*[14]. Thus, β-D-xylopyranose and methyl β-D-xylopyranoside have $J_{1a,2a}$ values of 7.2 c.p.s. whereas 5-thio-β-D-xylopyranose has a $J_{1a,2a}$ value of 8.2 c.p.s. and methyl 5-thio-β-D-xylopyranoside one of 8.4 c.p.s. The latter compound has $J_{2a,3a} = J_{3a,4a} = 8.6$ c.p.s., and $J_{4a,5a} = 11.2$ c.p.s. for the fragment (B).

The conclusion drawn from such results, and from similar results for other six-membered rings[15] is that a coupling constant in the range 0–4 c.p.s. does not correspond to a diaxial relationship for hydrogen substituents on a six-membered ring. If the arabinopyranose derivatives studied in this work adopted the *C1* chair conformation (C) then the coupling constant ($J_{2,3}$) would be at least twice that observed; we can therefore rule out this conformation. The alternative *1C* chair conformation (D) is *a priori* less likely due to the Δ-2 effect,[16] and the 3,5-diaxial interaction in the galactose derivatives; furthermore this conformation does not fit the p.m.r. spectral data. The arabinose derivative and the galactose derivatives are clearly conformationally very similar and the value of $J_{4,5_1} + J_{4,5_2}$ found for the arabinose derivative rules out a diaxial relationship between $H_{(4)}$ and $H_{(5_1)}$ or $H_{(5_2)}$.



(C)



(D)

The second conclusion that has been drawn from values for diaxial coupling constants is that the variation observed is not entirely due to conformational differences and must be partly due to the electronegativities of substituent atoms or groups on the fragment studied. Williamson[17] has shown from his studies of a rigid bicyclic

system that increased substituent electronegativities lead to smaller coupling constants. Laszlo and Schleyer[18] have confirmed this result for a similar system. This has been applied by Coxon and Fletcher[19] to explain their observation that the value for $J_{1a,2a}$ in 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl cyanide (10 c.p.s.) is considerably larger than the corresponding values found for ordinary glycosides. Rao *et al.*[14] contrast their values for the anomeric diaxial coupling in the 5-thioxylopyranose ring with the corresponding coupling in the xylopyranose ring in terms of conformational change alone. However, sulphur is considerably less electronegative than oxygen and at least part of the difference observed must be due to the different substitution pattern. The parameters of Lenz and Heeschen[20] used by Rao *et al.*[14] do not allow for a vicinal coupling constant as large as that observed for the $H_{(4a)}$-$H_{(5a)}$ interaction (11.2 c.p.s.). Recently, Williams and Bhacca[21] have suggested that the orientation of an electronegative substituent may have an effect on the coupling constants observed in a cyclic system. In view of this possibility, and the clearly demonstrated effect of the electronegativity of substituents on coupling constants, the application of the Karplus equation[22] with a single set of parameters to a cyclic compound such as a pyranose ring is clearly an approximation. However, carefully used, it can give information which will continue to be useful until a survey of more spectral data than is available at present leads to a more precise approach.

Since a chair conformation is clearly unlikely for the di-*O*-isopropylidene-arabinopyranose ring, the most likely conformation is one which approximates to a skew conformation. Angyal and Hoskinson[1] have shown that on theoretical grounds the most stable skew conformation is one in which the bridgehead angles for both isopropylidene rings are 33°. In view of previous p.m.r. studies of dioxolanes[2,3,23,24] a bridgehead angle of 30 to 40° is to be expected for both isopropylidene rings.

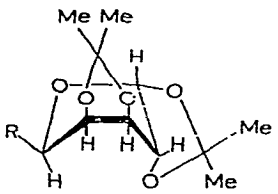
TABLE III

DIHEDRAL ANGLES OF HYDROGEN SUBSTITUENTS ON THE α-GALACTOPYRANOSE RING FOR THE CONFORMATIONS IN THE FLEXIBLE CYCLE

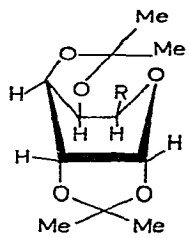
Conformation[a]	$\phi_{1,2}$°	$\phi_{2,3}$°	$\phi_{3,4}$°	$\phi_{4,5}$°
B_1E	60	120	60	60
$S_{1,5}E$	71	87	33	71
B_2E(E)	60	60	0	60
$S_{2,0}$(G)	33	49	33	33
B_3A(F)	0	60	60	0
$S_{1,3}A$	33	87	71	33
B_1A	60	120	60	60
$S_{2,4}A$	71	153	33	71
B_2A	60	180	0	60
$S_{3,5}$	33	169	33	33
B_3E	0	180	60	0
$S_{4,0}E$	33	153	71	33

[a] The conformations are described by the symbols of Isbell and Tipson[28] where B denotes a boat and S a skew conformation. The values were calculated arithmetically from the angles quoted by Angyal and Hoskinson[1].

Examination of Table III in which the angles for all the boat and intermediate skew conformations of the α-galactopyranose ring (strictly the angles for the corresponding cyclohexane derivatives) are given, shows that if we eliminate all conformations with either two bridgehead angles of 60° or one bridgehead angle of 71°, together with all conformations with a dihedral angle between $H_{(2)}$ and $H_{(3)}$ of 169° or 180°, then only three ideal conformations of the boat cycle are left. These are the boat conformations (E) and (F), and the skew conformation (G). For these three ideal conformations, the coupling constants derived from the Karplus relationship with the parameters of Abraham *et al.*[25] are given in Table IV. Comparison with the results shown in Table II shows that to a first approximation the skew conformation (G) is adopted by these molecules. To the extent to which it is valid to apply these parameters, the comparison also indicates that the actual conformation is intermediate between the skew (G) and the boat (E). It is significant that this conformation is precisely that which best fits the 1,2-*O*-alkylidene-α-D-glucopyranose derivatives studied by Coxon and Hall[3]. The large value for $J_{3,4}$ in these compounds indicates that the bridgehead hydrogens of the second isopropylidene ring must have a considerably smaller dihedral angle than for any other such ring previously studied by p.m.r. spectroscopy.



(E)



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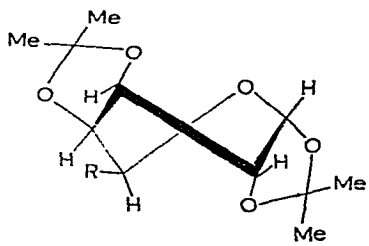

(G)

The only discrepancy is the small value for $J_{4,5}$. In 2,3:4,6-tetra-*O*-acetyl-β-D-galactopyranosyl cyanide Coxon and Fletcher[19] found a small value of 0.7 c.p.s. for $J_{4,5}$ whilst Hall *et al.*[26] found $J_{4,5}$ to be 0.5 c.p.s. in two diethylsulphonylgalactopyranosylmethane derivatives. Although these latter compounds are probably in a chair form, the small coupling constants are also difficult to explain. The considerably

TABLE IV

CALCULATED COUPLING CONSTANTS (C.P.S.)

Conformation	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$
(E)	2.0	2.0	9.0	2.0
(G)	6.3	3.7	6.3	6.3
(F)	9.0	2.0	2.0	9.0

greater variation in the value of $J_{4,5}$ than that observed for the other ring coupling constants is probably, at least in part, due to the different substituents on C–5.

Examination of experimentally-determined bond angles for simple iodoalkanes[27] suggests that the bond angles for the $C_{(5)}$–$C_{(6)}$ fragment of the 6-deoxy-6-iodogalactose derivative are fairly close to the tetrahedral bond angle and that the projected valence angle $H_{(6_1)}$–$C_{(6)}$–$H_{(6_2)}$ ($\phi_{6_1,6_2}$) should be approximately 120°. Application of the parameters of Abraham *et al.*[25] gives the values *ca.* 35° or 140° for $\phi_{5,6_1}$ and *ca.* 22° or 151° for $\phi_{5,6_2}$. The difference between these, either *ca.* 116° or 118°, is thus the experimentally determined value for $\phi_{6_1,6_2}$ if these parameters are applicable. In view of the earlier discussion, this measure of agreement is encouraging. Similar application of the parameters of Abraham *et al.*[25] to the $C_{(4)}$–$C_{(5)}$ fragment of di-*O*-isopropylidene-arabinose gives values of 60° for $\phi_{4,5_1}$ and 65° for $\phi_{4,5_2}$. This gives an experimental value of 125° for $\phi_{5_1,5_2}$ which is also reasonably close to the expected value of 120° considering the approximations involved. A tentative assignment of $H_{(5_1)}$ as the hydrogen directed below the ring and $H_{(5_2)}$ as the hydrogen directed above the ring is also possible.

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SUMMARY

The p.m.r. spectra of a series of 1,2:3,4-di-*O*-isopropylidene-arabinopyranose derivatives have been measured at 60 Mc.p.s. The derived vicinal coupling constants show that the pyranose ring in these derivatives adopts a non-chair conformation.

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STUDIES ON CYCLIC POLYOLS

PART IV. NUCLEAR MAGNETIC RESONANCE SPECTRA AND CONFIGURATIONAL ASSIGNMENTS OF POLYHYDROXYCYCLOPENTANES*

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INTRODUCTION

Previous publications[1-3] described the preparation of a series of isomeric cyclopentanetetrols, a number of related cyclitols, halo-cyclitols, and epoxycyclitols, as well as derivatives of many of these compounds. The configurational assignments were based on the structures of the starting materials and the presumed stereospecificity of subsequent reactions, by analogy with similar reactions in the cyclohexane series. In certain cases the course of reaction was different from the supposedly analogous reaction in the cyclohexane series, and in addition there was some controversy about the configurational assignment of two diols used as starting materials[1]. A completely independent means of determining the configuration was therefore essential. The successful application of n.m.r.†† spectroscopy to carbohydrates[4-6] and to cyclitols [7] as well as the intrinsic value of an n.m.r. study of the cyclopentane series indicated that useful results could be obtained. The assignment of all the ring proton resonances has been accomplished in most of the cases studied in this communication, and even when individual O–C–H resonances could not be assigned, the spectrum of the methylene protons gave the desired information. All assignments of configuration previously based on chemical grounds have been confirmed by the n.m.r. study. Definite parallels have been found between the cyclopentane series and the hexachlorobicyclo[2.2.1]heptenes[8] and related norbornene compounds[9] with respect to the internal chemical shifts between the methylene protons and adjacent O–C–H or Br–C–H protons.

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††The following abbreviations are used: n.m.r., nuclear magnetic resonance; p.p.m., parts per million; c.p.s., cycles per second; TMS, tetramethylsilane; W_h width at half-height.

MATERIALS AND METHODS

N.m.r. spectra were recorded with a Varian Associates DP-60 n.m.r. Spectrometer with TMS as internal standard. Spectral data are reported as follows: chemical shifts are expressed on the δ-scale in p.p.m. downfield from TMS (the value of δ is positive in this direction); coupling constants (J) are expressed in c.p.s. Unless otherwise stated solutions of the samples were 10% or less (w/v) in CCl_4, $CDCl_3$, or CD_3OD. Most of the values of δ and J were obtained by direct measurement of the spectral lines. Line positions were reproducible to $\pm$ 0.01 p.p.m. or better, and coupling constants were reproducible to $\pm$ 0.2 c.p.s. or better. In four cases (VIIa, b and IXa,b) the spectra were measured at three different concentrations and the parameters extrapolated to zero concentration. The exact values of δ and J were obtained with an IBM 7090 computer using the program of Swalen and Reilly[10]. Bulk susceptibility effect due to the compound being studied was negligible in all cases. The values of δ and J obtained by direct measurement and by computation were in close agreement.

The configurations of variously substituted cyclopentane derivatives are shown in Fig. 1. Two compounds of known structure were included in the study. These are

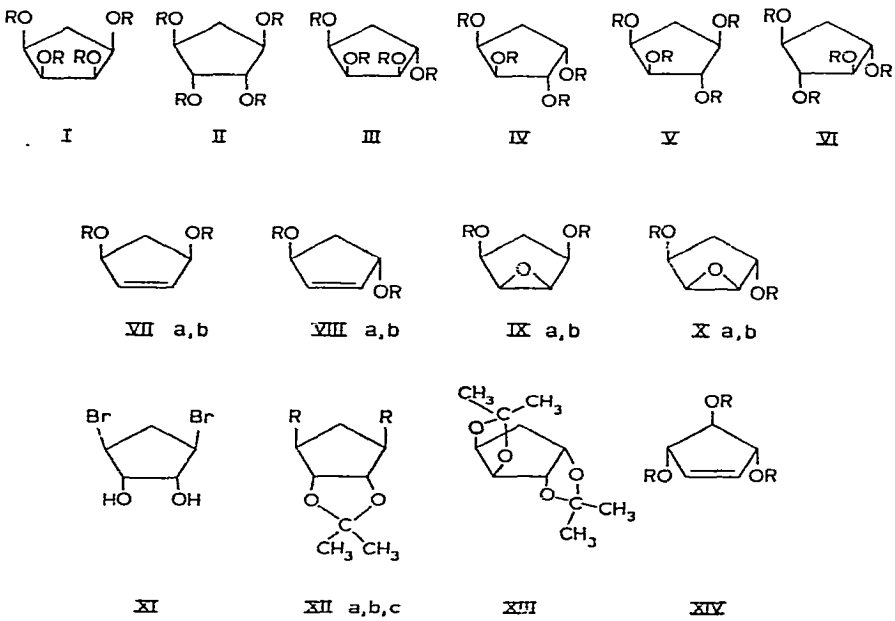

Fig. 1. Compounds studied. Compound I is unknown but the configuration is included for the sake of completeness. II–VI, R = Bz. VII–X, R = H for the "a" series and R = Bz for the "b" series. XIIa, b, c, R = Br, OH, OBz respectively. XIV, R = Bz.

the two bromo compounds XI and XIIa which were described by Thiele[11] and by Young *et al*[12]. All the other substances shown (except I, which is still unknown) are described in the other papers of this series[1–3,13].

The numbering of ring protons is shown in Fig. 2. All the compounds studied except XIV have two methylene protons and four other ring protons. The methylene protons are numbered so that H_1 and H_C always have a *cis*-relationship.

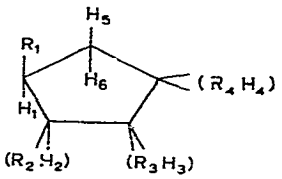

Fig. 2. Numbering of ring protons.

RESULTS

Bromo compounds

The spectra of the two compounds of known configuration (XI, XIIa) were measured first. The spectral parameters are summarized in Table I, and the spectrum

TABLE I

N.M.R. SPECTRA OF THE SYMMETRICAL 1,4-DIBROMO COMPOUNDS

Compound	*Protons*	*Appearance*	δ	J_{12}	J_{15}	J_{16}	J_{56}
XI	$H_{1,4}$	Multiplet	4.14	total width 18.2 c.p.s.			
	$H_{2,3}$	Doublet	4.37	4.5			
	H_5	Doublet of triplets	2.34		6.1		14.9
	H_6	5-Line multiplet	3.22			7.6	15.0
	OH	Singlet	4.56				
XIIa	$H_{1,4}$	Doublet of doublets	4.27	0	3.2	6.2	
	$H_{2,3}$	Singlet	5.01	0			
	H_5	Doublet of triplets	2.46		3.2		16.1
	H_6	Doublet of triplets	3.12			6.2	16.0
	CH_3-*endo*[a]	Singlet	1.45				
	CH_3-*exo*[a]	Singlet	1.31				

[a] The assignment of the two methyl signals to the *endo*- and *exo*-methyl groups is based on an unpublished study by the authors[16].

of XIIa is shown in Fig. 3. In each of these cases the higher field signal in the methylene region has been assigned to H_5 and the other signal to H_6. Each of these signals consists of 6 lines. The small tripling is due to coupling of the methylene proton with the two equivalent protons H_1 and H_4, and the larger doubling represents the geminal coupling J_{56}. The 5-line multiplet observed for H_6 of XI arises from superimposition of the inner lines of the two triplets. The two methylene protons and the Br–C–H protons form an ABX_2 system[14]. The assignment of H_5 as "A" and H_6 as "B" seems justified on two grounds: (*a*) the angular dependence of the internal chemical shifts between the methylene protons and the Br–C–H protons; (*b*) the magnitude of the

coupling constants J_{AX} and J_{BX} (see below). In a study of 2-substituted bicyclo-[2.2.1]heptenes, Williamson[8] observed that the lower field signal in the 3-methylene region belonged to the proton which was *trans* to the substituent on the adjacent carbon atom. He also determined that the internal chemical shifts δ_X-δ_A and δ_X-δ_B were linearly related to the electronegativity of the substituent. Laszlo and Schleyer[9] reported similar results. The findings of these authors show that in a system H–C–C–R

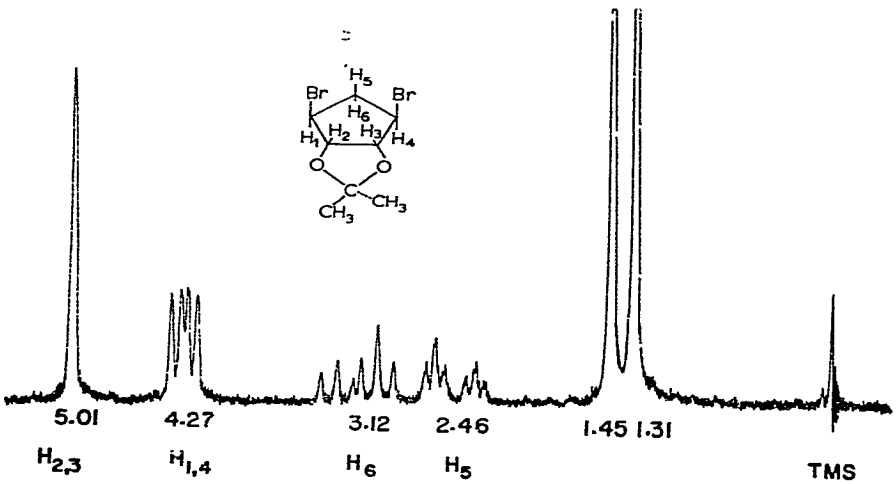

Fig. 3. 60Mc.p.s. n.m.r. spectrum of (1, 4/2, 3)-*O*-isopropylidene-1,4-dibromo-2,3-cyclopentanediol, XIIa, in $CDCl_3$. Numbers are chemical shifts in p.p.m. on the δ-scale.

the deshielding effect of R is stronger when R and H are *trans* to one another than when they are *cis*. The internal chemical shifts observed in our series agree well with those reported for the norbornenes, when the assignments are made as indicated above. In addition, in all compounds in our series except the epoxides J_{BX} is larger than J_{AX}. The conformations of substituted cyclopentanes are not precisely certain, but based on approximate dihedral angles H_1–C–C–H_5 and H_1–C–C–H_6 of 120° and 0° respectively, the expected values of J_{15} and J_{16} are approximately 2–3 c.p.s. and 8–10 c.p.s. according to the treatment of Karplus[15] (See Discussion concerning the conformational factors).

The 3,5 Diols

The distinction between the *cis* and *trans* isomers VII and VIII was made very easy, since an ABX_2 spectrum would identify structure VII. The spectral data for the diols and dibenzoates are summarized in Table II. In our earlier work the *cis* configuration was assigned to the crystalline diol, m.p. 59–60° and the *trans* configuration to the liquid diol. These assignments agreed with results obtained by Schenck and Dunlap[17], Henbest *et al.*[18], and Brown and Zweifel[19]. The spectra of the solid diol and its dibenzoate show the expected ABX_2 pattern, consistent with the proposed structure VII. The spectra of the liquid diol and its dibenzoate show a pattern characteristic of a degenerate or deceptively simple A_2X_2 spectrum[20,21] consistent only

with the proposed structure VIII. These spectral studies therefore confirm the earlier assignments made in several laboratories[1,12,17–19].

TABLE II

N.M.R. SPECTRA OF THE 3,5-CYCLOPENTENEDIOLS AND DIBENZOATES[a]

Compound	*Protons*	*Appearance*	δ	J_{12}	J_{15}	J_{16}	J_{56}
VIIa	$H_{1,4}$	Doublet of doublets	4.59	0	5.3	6.9	13.70
	$H_{2,3}$	Tall singlet	5.87				
	H_5	Doublet of triplets	1.41				
	H_6	Doublet of triplets	2.73				
	OH	Broad-based singlet	4.82				
VIIb	$H_{1,4}$	Doublet of doublets	5.84	0	4.2	7.5	14.5
	$H_{2,3}$	Tall singlet	6.26				
	H_5	Doublet of triplets	2.03				
	H_6	5-line multiplet	3.09				
VIIIa	$H_{1,4}$	Triplet–wide lines	4.93	?	"J_{AX}" = 5.3		
	$H_{2,3}$	Tall narrow doublet with widening at base	5.94				
	$H_{5,6}$	Triplet–narrow lines	1.96				
	OH	Broad singlet	4.83				
VIIIb	$H_{1,4}$	Triplet	6.04	?	"J_{AX}" = 4.8		
	$H_{2,3}$	Singlet	6.24				
	$H_{5,6}$	Triplet	2.46				

[a] The value of J_{AX} is an average coupling constant and not the true J_{15} or J_{16}, compare refs. 20, 21, 22. The other values given are the averages measured from the different signals in the spectra.

Epoxides

Treatment of VIIa and VIIIa with peroxybenzoic acid produces the corresponding epoxides IXa and Xa. The structure proof of IXa and a preliminary study of its n.m.r. spectrum have been reported[3]. The methylene protons and adjacent O–C–H protons (H_1 and H_4) form the expected ABX_2 system. In this case J_{AX} and J_{BX} are nearly equal and the expected doublet of doublets which should represent H_1 (H_4) therefore appears as a triplet with a broadened inner line. $J_{H_1H_2}$ is too small to cause splitting of the spectral lines in both IXa and IXb. Such small or zero splitting has also been observed in a number of other epoxycyclopentanes. The spectrum of Xb shows complicated patterns in the methylene, ester O–C–H, and oxirane O–C–H regions. Spin–spin decoupling by nuclear double resonance techniques will be required to simplify the spectrum before it can be analyzed. One such experiment suggests that $J_{H_2H_3}$ is smaller than predicted for the dihedral angle of 0°. More refined experiments of this type are needed, however, before the spectrum can be analyzed properly. A detailed examination of the n.m.r. spectra of these and other epoxycyclopentanes will be presented in a later publication.

Tetrols

The most serious configurational uncertainty concerned the tetrols. Configurations III and V seemed firmly established on chemical grounds; the former because it was produced by *cis*-hydroxylation of VIII, and the latter because it was made by acid hydrolysis of IX. The other three configurations could only be considered tentative because each of them was produced in a manner that could lead to two different structures (see Fig. 4). This uncertainty has been resolved by study of the n.m.r.

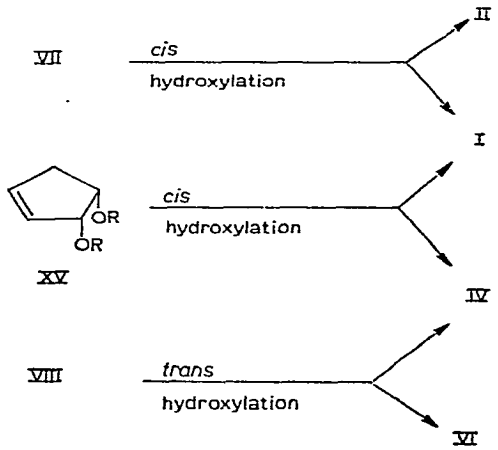

Fig. 4. Configurational uncertainties due to alternate possible hydroxylation reactions. R = H in each case.

spectra of the tetrabenzoates of the tetrols. Structures I and II have the same kind of methylene-group symmetry as VII, IX, XI and XII, and the methylene protons should therefore give signals characteristic of the AB portion of an ABX_2 system. On the other hand structures IV and VI have the same type of methylene-group symmetry as VIII, and the methylene protons should form the A_2 portion of an A_2X_2 system. The spectral measurements are summarized in Table III.

The substance presumed to have structure II shows the expected doublet of triplets for each of the methylene protons. (The 5-line appearance of the H_6 signal is due to the fortuitous superimposition of the inner lines of the two triplets.) The choice between configurations I and II is based on J_{12}. On the basis of dihedral angles H_1–C–C–H_2 of 0° (I) and 120° (II) J_{12} should be 8–10 c.p.s. (*cis*) and 2–3 c.p.s. (*trans*). The observed value of 2.8 c.p.s. proves that II is the correct configuration. Direct measurement of this value is possible because $H_1(H_4)$ and $H_2(H_3)$ form a separate AB system* and the splitting of the H_2 signal is caused only by J_{12}. The multiplet representing $H_1(H_4)$ shows fewer than the expected 8 lines, but the total width of the pattern closely approximates $J_{12} + J_{15} + J_{16}$.

*Strictly speaking the O–C–H protons are an A_2B_2 system in which $J_{12}(J_{AB}) = J_{43}(J_{A'B'})$. The couplings $J_{AA'}$, $J_{AB'}$, $J_{A'B}$, and $J_{BB'}$, do not cause observable splitting of the spectral lines.

TABLE III

N.M.R. SPECTRA OF CYCLOPENTANETETROL TETRABENZOATES[a]

Configuration	*Protons*	*Appearance*	δ	J_{15}	J_{12}	J_{16}	J_{56}
II[b]	$H_{1,4}$	5-Line multiplet, width 14.8 c.p.s.	5.72				
	$H_{2,3}$	Doublet	6.03	2.8	4.7	7.6	15.6
	H_5	Doublet of triplets	2.12				
	H_6	5-Line multiplet	3.30				
IV	H_5,P	Triplet	2.70	"J_{AX}" = 4.5 c.p.s.			
	All O–C–H	Unresolved envelope, width 11 c.p.s.	~5.95				
VI	H_5,P	Triplet	2.76	"J_{AX}" = 6.1 c.p.s.			
	All O–C–H	Unresolved envelope width 22 c.p.s.	~5.78				

[a] See footnote to Table II regarding "J_{AX}".
[b] The coupling constants for compound II are averages based on measurement of the different signals in the spectrum, since these couplings may appear in more than one signal.

The substance formed from XV, which could have been I or IV, shows the A_2 portion of an A_2X_2 system in the methylene region and is thus proved to be IV. The substance formed from VIII by *trans*-hydroxylation, which could have been IV or VI, also shows the same kind of methylene signal. Since structure IV belongs to the product obtained from XV, the *trans*-hydroxylation of VIII has produced VI.

The two remaining tetrols III and V have also been examined. In each case the methylene protons give broad, complicated signals of the ABXY type which have not yet been analyzed, but which are consistent with the lack of symmetry of the molecules.

Isopropylidene Tetrols

Confirmation of the configurational assignments was obtained from the spectra of a series of isopropylidene derivatives of the tetrols. Acetonation of the 1,4/2,3-tetrol (II, R = H) produced a monoisopropylidene derivative XIIb, which was benzoylated to give XIIc[13]. The spectra of both compounds showed the expected ABX_2 patterns, and J_{12} in each case was either zero or very small, thus confirming the *trans* relation of H_1 and H_2. The spectral parameters are not reported in detail here, since a number of features of these spectra are still under investigation[16]. Acetonation of the 1,2/3,4-tetrol (IV, R = H) produced only a diisopropylidene derivative XIII. The spectrum of this substance, shown in Fig. 5 is another example of a deceptively simple spectrum. Much of the spectral simplification is probably due to the high degree of symmetry of the molecule. The equivalences $H_1 = H_4$, $H_2 = H_3$ and $H_5 = H_6$ verify the configurational assignment. The following explanation seems reasonable*. H_5 and H_6

*This explanation was suggested by Dr. Roy W. King.

appear as a triplet at $\delta = 2.13$ ($J = 5.0$) because they form the A_2 portion of an A_2X_2 system. J_{23} may be assumed safely to be negligible on the basis of the small or zero J_{12} found in XIIabc. H_2 is a doublet ($\delta = 4.49$) due to splitting by H_1. H_1 is a

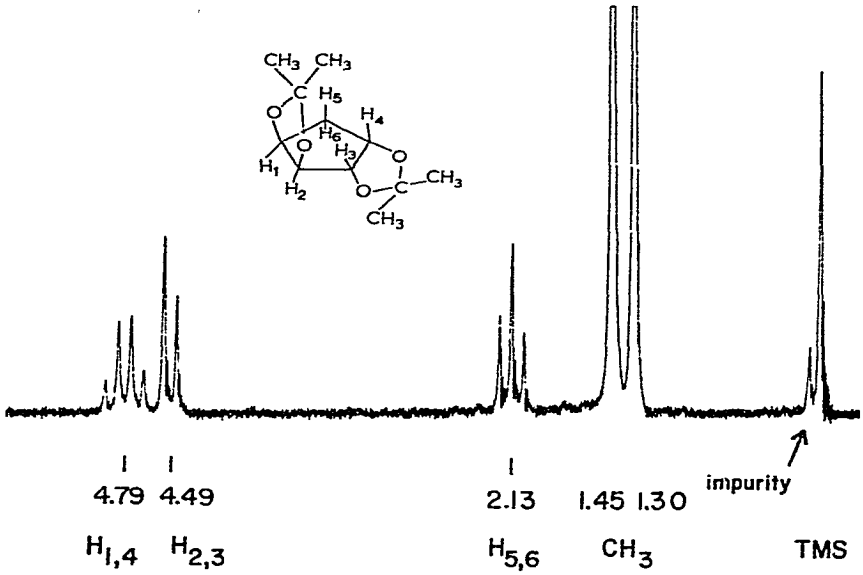

Fig. 5. 60Mc.p.s. n.m.r. spectrum of (1,2/3,4)-di-*O*-isopropylidenecyclopentanetetrol XIII, in $CDCl_3$. Numbers are chemical shifts in p.p.m. on the δ-scale.

quartet ($\delta = 4.79$) due to the splitting by H_2 and "virtual coupling"[23] equally to H_5 and H_6 with a splitting fortuitously equal to J_{12}. (See the Discussion section for the relation of this effect to the virtual coupling described by Musher and Corey).

1,3/2-Cyclopentenetriol (XIV)

The configuration of this substance was established unequivocally by Gaoni[24] who first synthesized it. The C=C–H proton signal is a single line at $\delta = 6.23$ p.p.m. and the O–C–H protons appear at $\delta = 5.92$ p.p.m. as a single line with some unsymmetrical broadening at the base ($W_h = 2.2$ c.p.s., width of base = 9.6 c.p.s.). The O–C–H protons form an AB_2 system with nearly identical chemical shifts. In such systems the intensity of the satellite lines diminishes as the ratio* J/δ increases. Since in the present case J/δ is extremely large, the satellite lines are not observable. Consequently no statement can be made about the coupling constants in this system[25].

DISCUSSION

N.m.r. spectra of cyclopentane derivatives cannot be interpreted as easily as can the spectra of the corresponding cyclohexanes. In the latter case the molecule may be

*In this ratio the denominator is the difference in chemical shifts of the two nuclei.

considered to exist in one or other of the various chair or boat conformations. The conformation of cyclopentane derivatives is more complicated. Cyclopentane itself is in constant wriggling motion, rapidly changing shape among the various possible conformations in which one or two carbon atoms are out of the plane of the others. The same may be true of substituted cyclopentanes, in which case the coupling constants determined are really time-average values. On the other hand Brutcher and Bauer[26] suggest that polysubstituted cyclopentanes exist in a favored conformation. Possibly this question may be resolved by a study of vicinal coupling constants when a much larger body of data becomes available. As noted above, in many cases the observed line splittings are not true coupling constants, but are averages of two related couplings affecting the proton in question. This is probably true in all the cases in which the A_2X_2 patterns were observed. It is interesting that in spite of these considerations, the methylene signals of related cyclohexanetetrols also appear as the A_2 portions of A_2X_2 systems[7], presumably because of rapid interchange between two equivalent conformations.

Virtual coupling, which is probably concerned in many of the spectra reported here, has also been a feature of the spectra of many of the other cyclopentane derivatives under investigation[16]. Virtual coupling, as described by Musher and Corey[23] involves the case of H_p–C–C(–H_q)–C–H_r in which H_p and H_r are coupled either very weakly or not at all, whereas strong coupling H_pH_q and H_qH_r exists. In this situation the signal for H_r may appear as a triplet, and the (unjustified) first-order analysis indicates $J_{pr} = J_{qr}$. H_p and H_r are separated by four bonds. The present case is different, since the separations H_1H_6 and H_1H_5 are each three bonds.* Nevertheless the effect is probably the same as that described above. H_1 and H_6 are strongly coupled, H_1 and H_5 are weakly coupled and H_5 and H_6 are strongly coupled. This system also has some of the characteristics of an AA'X system which gives the same spectrum as an A_2X with an apparent coupling constant $= 1/2\,(J_{AX} + J_{A'X})$. Several examples of such spectra in variously substituted bornanes are reported by Flautt and Erman[27]. It should be noted that in XIIa,b,c, and XIII, $J_{H_1H_2}$ seems to be zero, whereas in II and XI $J_{H_1H_2}$ is measurable, and is of the order of 2–4 c.p.s. This discrepancy may be related to differences in conformation of the compounds or electronegativity of the substituents, but there is inadequate experimental evidence for deciding between these alternatives at present.

The appearance of H_6 at lower field than H_5 is a consistent finding in the various derivatives whose spectra we have been able to evaluate. Both H_5 and H_6 are deshielded by the substituent on the neighboring carbon. The deshielding effect on H_6 is always greater than that on H_5. This finding is in agreement with similar observations in a variety of substituted norbornenes[8,9] in which an angular dependence of the deshielding effect, and a linear relationship between the electronegativity of the substituent and the internal chemical shifts δ_X-δ_A and δ_X-δ_B, were found. In the present case (see the notation in Fig. 2) H_X, H_A, H_B and R are $H_{1(4)}$, H_5, H_6, and R_1

*Musher and Corey actually mention such a system, but emphasize the system described above.

respectively. Table IV shows the internal shifts observed in our series of compounds. The individual chemical shifts are quite variable because of the differing shielding effects of the other substituents in the molecules and the spatial relationships of these substituents to the protons under consideration. The variability of chemical shifts in our series is illustrated further by the difference of 0.4 p.p.m. in the location of the vinyl proton signals between VIIa and VIIb. The reason for this shift is probably related to the anisotropy of the phenyl ring. An effect of the same magnitude is seen in the signals of the oxirane O–C–H protons of IXa and IXb. Detailed analysis of this effect is now under investigation. In spite of this variability, the agreement between our series and the norbornenes is remarkably good and justifies the assign-

TABLE IV

VARIATION OF INTERNAL CHEMICAL SHIFTS WITH SUBSTITUENT ELECTRONEGATIVITY[a]

R	*Compound*	δ_X	δ_A	δ_B	$\delta_X-\delta_A$	$\delta_X-\delta_B$	*Literature values for norbornenes*	
	VIIa	4.59	1.41	2.73	3.18	1.86		
	XIIb	4.06	1.68	2.08	2.38	1.98	2.73[b]	1.82[b]
OH	IXa	4.01	1.27	2.08	2.74	1.93		
	Average				2.77	1.92		
	II	5.72	2.12	3.30	3.60	2.42		
	VIIb	5.84	2.03	3.09	3.81	2.75		
OBz	XIIc	5.45	2.20	2.68	3.25	2.77	3.58[b]	2.53[b]
	IXb	5.39	1.88	2.68	3.51	2.71	3.06[c]	2.05[c]
	Average				3.54	2.66		
Br	XI	4.14	2.34	3.22	1.80	0.92		
	XIIa	4.27	2.46	3.12	1.81	1.15	1.98[c]	1.13[c]
	Average				1.81	1.04		

[a] The values for R = OBz are compared with published values for R = OAc in the norbornene series.
[b] See ref. 8.
[c] See ref. 9.

ments of the H_5 and H_6 signals in the spectra. On the other hand we are unable to observe a regular effect of electronegativity of the substituent on J_{AX} and J_{BX} such as was observed in the norbornenes. The simplest explanation for this discrepancy is that in the norbornenes the dihedral angles H_A–C–C–H_X and H_B–C–C–H_X vary only slightly, whereas in our series of compounds the dihedral angles would be expected to vary considerably under the influence of such factors as intramolecular hydrogen bonding, electrostatic repulsion, and crowding of bulky substituents.

When this study was undertaken only three of the tetrols were available in crystalline form, and it was necessary to study crystalline derivatives. A great deal of

information was obtained from the signals of the methylene protons. This was possible largely because *O*-benzoyl rather than *O*-acetyl derivatives were available. Almost certainly some of the methylene signals would have been obscured by the acetate methyl signals had *O*-acetyl derivatives been studied. On the other hand much information can also be obtained from study of the acetyl methyl signals of carbohydrate acetates[28], and obviously no single type of derivative can be used to obtain all the information in all cases. Our study of cyclopentane systems is now being extended to include acetates, aminocyclitols and unsubstituted cyclitols. N.m.r. studies of these compounds, as well as certain long-range shielding effects will be the subject of future communications.

Note added in proof. In the spectrum of XIII (Fig. 5) the signal for $H_2(H_3)$ appears at higher field than the signal for $H_1(H_4)$. This may be due to long-range deshielding of H_1 or long-range shielding of H_2 by the oxygen atoms on the same side of the plane of the ring. Preliminary study of models suggests that the principal effect is probably long-range shielding of H_2 by O_3.

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The assistance of Herbert Grossman who measured the n.m.r. spectra is acknowledged. The senior author wishes to acknowledge a number of stimulating and informative discussions, in person and by correspondence, with Dr. Roy W. King of Iowa State University and with Dr. G.E. McCasland of the University of San Francisco.

SUMMARY

The configurations of five cyclopentanetetrols and both 3,5-cyclopentenediols have been established by examination of their n.m.r. spectra. The configurational assignments are based to a large extent on the nature of the methylene signals (A_2X_2, ABX_2 or ABXY), and in one case the magnitude of the vicinal coupling constant is used as the basis of assigning the 1,4/2,3 structure in preference to the all-*cis* configuration. The present conclusions confirm earlier assignments based on purely chemical grounds. Spectral data for the ring protons of the tetrol tetrabenzoates, diols, epoxydiols and corresponding dibenzoates, a dibromoglycol and several *O*-isopropylidene derivatives of cyclopentanepolyols are presented. The internal chemical shifts between the A, B and X protons of the ABX_2 system observed in many of these compounds have been studied. The internal shifts $\delta_X-\delta_A$ and $\delta_X-\delta_B$ agree well with the internal shifts reported for the ABX system in two series of substituted bicyclo[2.2.1]heptenes. The linear variation of internal chemical shift with electronegativity of the substituent and the angular dependence of deshielding found in the bicycloheptenes is thus also found in the cyclopentanes. Unlike the case of the bicycloheptenes, which are rigid, the coupling constants J_{AX}, J_{BX} and J_{AB} do not vary linearly with the electronegativity of the substituents, presumably because of

the additional factor of variability of the dihedral angles in the various cyclopentanes, due to the greater flexibility of these molecules.

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DIASTEREOISOMERIC FORMS OF METHYL 4,6-*O*-BENZYLIDENE-2,3-DI-*O*-METHYL-α-D-GLUCO- AND α- AND β-D-GALACTOPYRANOSIDE*

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INTRODUCTION

The acid-catalysed equilibration reaction of benzaldehyde with tetritols and higher polyhydric alcohols affords derivatives of 2-phenyl-1,3-dioxan in preference to those of 2-phenyl-1,3-dioxolan[2]; glycerol is exceptional in that this preference is reversed[3]. Whilst it is usual for the diastereoisomers of 2-phenyl-1,3-dioxolan derivatives to be formed to similar extents, there is no substantiated example of the occurrence of diastereoisomeric 2-phenyl-1,3-dioxan derivatives; supposed diastereoisomers are, in fact, dimorphs[4]. It has been predicted[5] and verified experimentally[6] that the preferred 2-phenyl-1,3-dioxan derivatives have the phenyl group equatorial in a chair form of the 1,3-dioxan ring. On the assumption that the kinetic control operative on benzylidenation under basic conditions might result in the formation of both diastereoisomers, the reaction of benzylidene halides with suitable sugar derivatives in the presence of potassium *tert*-butoxide was investigated.

There have been few reports of the reaction of *gem*-dihalides with diols. The reaction of a dihalogenomethane with a steroidal diol[7] and with vicinal dihydric phenols[8], each in the presence of a base, has been described, and we have reported on the methylenation of cyclohexane-*cis*- and *trans*-1,2-diol and certain methyl 4,6-*O*-benzylidenehexopyranosides using dibromomethane and sodium hydride[9]. Apparently, there has been no report of comparable reactions with benzylidene halides.

RESULTS AND DISCUSSION

When a mixture of methyl 2,3-di-*O*-methyl-α-D-glucopyranoside, benzylidene bromide, toluene, potassium *tert*-butoxide, and *tert*-butanol was boiled and the product subsequently fractionated on silica gel, elution with benzene–ether (9:1) gave approximately equal amounts (*ca.* 30% combined yield) of the diasteroisomeric forms (*A* and *B*) of methyl 4,6-*O*-benzylidene-2,3-di-*O*-methyl-α-D-glucopyranoside. Diastereoisomer *B* (m.p. 122-123°, $[\alpha]_D$ + 97°, acetone), eluted second, was identical with the product obtained by acid-catalysed benzylidenation of methyl α-D-gluco-

*For a preliminary report of some of these results, see ref. 1.

pyranoside followed by methylation[10]. Elemental analysis and molecular weight data indicated compound *A* (m.p. 125.5–127°, $[\alpha]_D$ + 184°, acetone) to be a methyl *O*-benzylidene-di-*O*-methylhexoside, and although it was isolated as a stable, crystalline solid, addition of hydrogen chloride to its solution in carbon tetrachloride at 25–30° caused smooth and complete isomerization into diastereoisomer *B*. The isomerization was readily followed by observation of the change in the pattern of the n.m.r. signals for the protons in the methoxyl groups (see below). Thus, compound *A* must be the second, diastereoisomeric form of methyl 4,6-*O*-benzylidene-2,3-di-*O*-methyl-α-D-glucopyranoside.

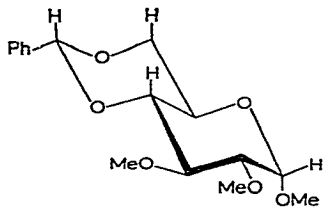
The complete conversion *A* → *B* indicates that diastereoisomer *A* is thermodynamically unstable in the presence of acid and explains the formation of only single diastereoisomers in the acid-catalysed conversion of glucopyranose derivatives into 4,6-*O*-benzylidene compounds.

Similar benzylidenation of methyl 2,3-di-*O*-methyl-α-D-galactopyranoside under basic conditions gave, in 34% combined yield, the known[11] 4,6-*O*-benzylidene derivative (*D*, m.p. 117-119°, $[\alpha]_D$ + 167°, chloroform) and a new diastereoisomer *C* (m.p.158–160°, $[\alpha]_D$ + 109°, chloroform). Addition of hydrogen chloride to a solution of diastereoisomer *C* in chloroform caused smooth and complete conversion into diastereoisomer *D*. A parallel sequence of reactions was carried out with methyl 2,3-di-*O*-methyl-β-D-galactopyranoside which gave the known 4,6-*O*-benzylidene derivative[12] (*F*, m.p. 142–144°, $[\alpha]_D$ + 18°, chloroform) and a new diastereoisomer *E* (m.p. 156.5–157.5°, $[\alpha]_D$ −27°, chloroform). The conversion *E* → *F* was effected with a chloroform–hydrogen chloride mixture. As with the D-glucopyranoside derivatives described above, the new isomer (*C* and *E*) in each pair of 4,6-*O*-benzylidene-D-galactopyranose derivatives was eluted first during chromatography on silica gel.

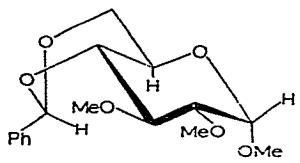
The facile conversions *C* → *D* and *E* → *F* indicate that diastereoisomers of the type *C* and *E* are thermodynamically unstable in the presence of acid, and are therefore unlikely to be present to a significant extent when the acid-catalysed conversion of galactopyranose derivatives into 4,6-*O*-benzylidene compounds has reached equilibrium.

The n.m.r. spectra of the methyl 4,6-*O*-benzylidene-2,3-di-*O*-methyl-α-D-glucopyranosides *A* and *B* in chloroform showed significant differences (see Table I), in particular a marked shift to low field of the benzyl proton signal in diastereoisomer *A*. This shift could be accounted for[13] by the existence in isomer *A* of the 1,3-dioxan ring in a chair form with an equatorial benzyl proton, but such a conformation would be markedly destabilised by the substantial, adverse, non-bonded interactions associated with the axial phenyl group[14]. The facile, acid-catalysed conversion *A* → *B* is clearly indicative of steric strain in isomer *A* such as would be present in conformation (II) which contains a boat form of the 1,3-dioxan ring. Several features of conformation (II) are noteworthy: (1) the close approach of the benzyl proton to the C–5 hydrogen atom results in substantial deshielding, (2) the phenyl group is in a sterically relatively favourable position, (3) because of the low steric requirement[15] of the lone pairs of electrons on oxygen, the non-bonded interactions between the lone pairs on the ring

oxygen atoms of the 1,3-dioxan ring and the substituents at C–4 and C–6 may be relatively low, and in consequence the boat form may not be energetically very unfavourable. Whilst the available evidence does not permit precise determination of the conformation of diastereoisomer *A*, it seems likely that a shape near to that of structure (II) is adopted.



I



II

The chemical shift of the benzyl proton signal for diastereoisomer *B* is typical[16] of an axial benzyl proton in the chair form of a 2-phenyl-1,3-dioxan ring with axial protons on the same side of the ring, and is consistent with the conformation (I).

TABLE I

NUCLEAR MAGNETIC RESONANCE SPECTROSCOPIC DATA[a] FOR SOME 4,6-*O*-BENZYLIDENEHEXOPYRANOSIDE DERIVATIVES[b] (τ SCALE)

Methyl 4,6-*O*-benzylidene-2,3-di-*O*-methyl-α-D-glucopyranoside (*ca.* 8% solutions in CCl_4)

	Ph.CH	*Glycosidic* H	*O-CH*$_3$
A	4.06	5.50 (doublet, *J. ca.* 3.5 c.p.s.)	6.37, 6.65, 6.67
B	4.62	5.36 (doublet, *J ca.* 3.5 c.p.s.)	6.47, 6.57, 6.64

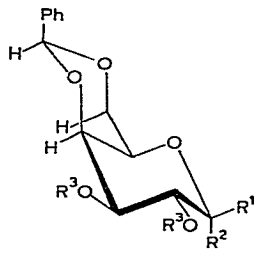
Methyl 4,6-*O*-benzylidene-2,3-di-*O*-methyl-α- and β-D-galactopyranoside (*ca.* 8% solutions in $CHCl_3$)

	Ph.CH	*Glycosidic* H	*O-CH*$_3$
C(α)	3.72	4.95 (doublet, *J ca.* 3.25 c.p.s.)	6.36, 6.39, 6.53
D(α)	4.43	4.98 (doublet, *J ca.* 2.25 c.p.s.)	6.42, 6.44, 6.51
E(β)	3.70		6.30, 6.37, 6.39
F(β)	4.39		6.32, 6.37, 6.44

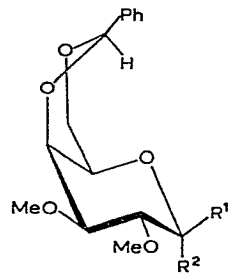
[a] Determined using a Varian A60 spectrometer with Me_4Si as external reference.
[b] The configuration[22] at the benzylidene carbon atoms is (*R*) in cyclic acetals *B*, *D*, and *F*, and (*S*) in the respective isomers *A*, *C*, and *E*.

Marked differences in the n.m.r. spectra (see Table I) were also observed for the methyl 4,6-*O*-benzylidene-2,3-di-*O*-methyl-α-D-galactopyranosides *C* and *D*. The most noteworthy of these was the marked shift to lower field of the benzyl proton signal in the sterically strained isomer *C*. Two double-chair structures of the *cis*-deca-

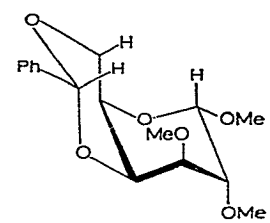
lin type ring system are possible in the 4,6-*O*-benzylidene-D-galactopyranose derivatives involving[5] "O"-inside (III) and "H"-inside structures (VII) with equatorial phenyl groups. The n.m.r. spectroscopic evidence which has been adduced[17] in favour of the predicted[5] "O"-inside structures with equatorial phenyl groups when related ring systems are formed under normal conditions of acid catalysis, applies to the 4,6-*O*-benzylidene-D-galactopyranoside case and the structure (III) may be confidently assigned to diastereoisomer *D*. However, the conformational situation is not so clear for the sterically-strained diastereoisomer *C*. The substantial shift to low field of the benzyl proton signal in the latter compound would be expected for the boat conformation (IV) [or a conformation near to this shape which is analogous to that (II) suggested for the D-glucopyranoside derivative *A*] or the "H"-inside conformation (VII); (IV) and (VII) are different conformations of the same structure.



III R^1 = H, R^2 = OMe, R^3 = Me
V R^1 = OMe, R^2 = H, R^3 = Me
VIII R^1 = H, R^2 = OMe, R^3 = H



IV R^1 = H, R^2 = OMe
VI R^1 = OMe, R^2 = H



VII

In conformation (IV), the benzyl proton is in close proximity to the ring oxygen atom of the tetrahydropyran ring, and in (VII), the benzyl proton is in a 1,3-diaxial relation with C–3; subsequent deshielding would be expected in each case. Also, each conformation would be associated with adverse, non-bonded interactions but conformation (VII) would be particularly destabilised by the *endo*-methoxyl group at C–3. The fact that diastereoisomers *C* and *D* each had a doublet for the glycosidic proton of similar chemical shift (see Table I) with only slightly different coupling constants, and methoxyl proton signals of similar chemical shifts suggests that the conformation of the tetrahydropyran ring is substantially the same in each case. By analogy with the results of Capon and Thacker[18], the inversion of the chair form of the tetrahydropyran ring in the conformational change (IV) → (VII) should give rise to a substantial upfield shift of the signal for the glycosidic proton which changes from an equatorial to an axial position. However, it should also be noted that in conformation (VII) the glycosidic proton and C–6 are in 1,3-diaxial relationship and a compensating downfield shift could result[19].

In the β-D-galactopyranoside series, the sterically strained diastereoisomer *E* also has the benzyl proton signal displaced to low field but, unfortunately, the signal for the glycosidic proton in each diastereoisomer was displaced upfield into the region of the ring proton signals and could not be identified. By analogy with the results in

the α-series, isomer *E* is tentatively assigned structure (VI) and isomer *F*, more certainly, structure (V).

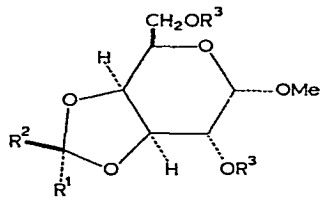
It is of interest to note that the members of each pair of diastereoisomers in Table I showed significantly different optical rotations, and the origin of these differences is being further investigated.

Although much use has been made in the past of benzylidenation of polyhydric alcohols in preparative procedures, little is known of the precise course of these reactions. Benzylidenation of diols under homogeneous conditions can be readily followed by observing the development of the n.m.r. signals for the benzyl protons in the formed acetals. Preliminary results[20] obtained on benzylidenation of 1,4-anhydroerythritol and cyclohexane-*cis*-1,2-diol in nitromethane or dimethylformamide with catalysis by toluene-*p*-sulphonic acid clearly showed the operation of kinetic control in the early stage of the reaction. In each case, under the conditions employed, there was rapid formation of the isomer with the benzyl proton *exo* in the respective fused ring system, followed by relatively slow isomerization to an approximately equimolar *exo*, *endo*-mixture. The marked difference in chemical shift of the benzyl proton signals for the diastereoisomers *C* and *D* of methyl 4,6-*O*-benzylidene-2,3-di-*O*-methyl-α-D-galactopyranoside prompted an examination of the homogeneous, acid-catalysed benzylidenation. By varying the acid concentration in the system methyl 2,3-di-*O*-methyl-α-D-galactopyranoside–benzaldehyde–nitromethane–toluene-*p*-sulphonic acid at 25–30°, it was possible to select conditions where benzylidenation occurred moderately slowly ($t\frac{1}{2}$ reaction *ca.* 2 h). The reaction proceeded to *ca.* 90% completion with the development of a signal at τ 5.10 due to diastereoisomer *D*; there was no detectable signal at 4.56 due to diastereoisomer *C*; the chemical shift of the latter signal was determined for this solvent system by the addition of diastereoisomer *C*. Thus, if formed at all, diastereoisomer *C* must undergo rapid isomerization to diastereoisomer *D* and hence does not accumulate.

With methyl α- or β-D-galactopyranoside, benzylidenation can occur in the 3,4- and/or 4,6-positions. There is no report of the direct formation of a 3,4-*O*-benzylidene derivative and the preceding results make it unlikely that the reported[21] variations in physical constants for methyl 4,6-*O*-benzylidene-α-D-galactopyranoside are indicative of diastereoisomers. None of the fractions of a reaction mixture obtained from a zinc chloride-catalysed benzylidenation of methyl α-D-galactopyranoside at room temperature showed other than the single benzyl proton signal associated with the 4,6-*O*-benzylidene derivative (m.p. 168–170°, $[\alpha]_D$ + 144°, chloroform, *cf.* m.p. 171°, $[\alpha]_D$ + 144°, chloroform, recorded by Fletcher *et al.*[21]) which may be assigned[17] the structure (VIII).

In the initial stage of benzylidenation of methyl α-D-galactopyranoside under homogeneous conditions using the system benzaldehyde–dimethylformamide–toluene-*p*-sulphonic acid, benzyl proton signals could be detected at 4.77, 4.51, and 4.26, the last signal being very weak. The signal at 4.77, which increased in strength with time and ultimately corresponded to *ca.* 90% reaction, may be assigned to the 4,6-*O*-benzylidene derivative (VIII), and the signals at 4.51 and 4.26, which reached

a steady although low intensity before diminishing and disappearing, may be assigned to the 3,4-*O*-benzylidene derivatives, (IX) and (X), respectively having *exo*- and *endo*-benzyl protons[16,19]. The operation of kinetic control in the early stages of the reaction is therefore demonstrated. Using the system benzaldehyde–nitromethane–toluene-*p*-sulphonic acid, methyl 2,6-di-*O*-methyl-α-D-galactopyranoside, for which benzylidene acetal formation can occur only at the 3,4-position, gave first a benzyl



IX R^1=H, R^2=Ph, R^3=H
X R^1=Ph, R^2=R^3=H
XI R^1=H, R^2=Ph, R^3=Me
XII R^1=Ph, R^2=H, R^3=Me

proton signal at 4.78 followed by a signal at 4.53 due, respectively, to the isomers (XI) and (XII) with *exo*- and *endo*-benzyl protons (assignment by analogy with other results[16,19]). When the nitromethane was replaced by *tert*-butanol only a single signal for benzyl protons appeared at 4.65 corresponding to the isomer (XI).

EXPERIMENTAL

Benzylidenations under basic conditions

(*a*) *Methyl 2,3-di-O-methyl-α-D-glucopyranoside*

A solution of the title compound[10] (2.7 g) in a mixture of a N-solution of potassium *tert*-butoxide in *tert*-butanol (40 ml) and toluene (40 ml) was added dropwise to a boiling solution of benzylidene bromide (3.5 g) in benzene (500 ml) during 3.5 h and the mixture was then boiled overnight. The cooled mixture was washed with water, dried ($MgSO_4$), and evaporated to give a residue (2.7 g) which was fractionated on silica gel (100 g, Davison). Elution with benzene–ether (9:1) gave a product (0.74 g), m.p. 95–102°, $[\alpha]_D$ + 143° (*c* 2.0, acetone), which showed components with R_F values 0.5 and 0.6 on examination by thin-layer chromatography on Kieselgel using benzene–methanol (9:1) and detection with vanillin–sulphuric acid[23] or iodine vapour. Subsequent elution with ethyl acetate afforded material (0.96 g) with R_F 0.3, presumably title compound.

The above product mixture (from two similar experiments) was refractionated on silica gel (150 g) using the same solvent mixture. The fractions (50 ml) were examined by thin-layer chromatography and n.m.r. spectroscopy (for benzyl proton signals). Fractions 17–26 contained a single component (0.5 g) with a benzyl proton signal at 4.06 (CCl_4), and recrystallisation from ether–light petroleum (b.p. 60–80°) gave

the 4,6-*O*-benzylidene derivative *A* (0.12 g), m.p. 125.5–127°, $[\alpha]_D^{27}$ + 184° (*c* 0.5, acetone) [Found: C, 62.1; H, 7.1; Mol.wt. (Rast in camphor) 285. $C_{16}H_{22}O_6$ calc.: C, 61.9; H, 7.2%; Mol. wt. 310].

Fractions 27–36 contained diastereoisomer *A* together with the known benzylidene derivative *B*. From the combined fractions pure diastereoisomer *B*, m.p. 122–123°, $[\alpha]_D$ + 97° in acetone, was isolated.

When diastereoisomer *A* (50 mg) was dissolved in carbon tetrachloride (0.6 ml) which was 0.07N with respect to hydrogen chloride, rearrangement was complete within 5 min [ascertained on the basis of the benzyl proton and methoxyl proton signals in the n.m.r. spectrum (see Table I)]. The solution was diluted with carbon tetrachloride, washed with aqueous sodium hydrogen carbonate, dried ($MgSO_4$), and concentrated. Recrystallisation of the residue from ether–light petroleum (b.p. 60–80°) gave diastereoisomer *B*, m.p. 121° (mixed m.p. 122°).

(*b*) *Methyl 2,3-di-*O*-methyl-α and β-*D*-galactopyranoside*

A solution of the title compound (α-anomer[11] 2 g) in a mixture of an N-solution of potassium *tert*-butoxide in *tert*-butanol (40 ml) and benzene (40 ml) was added dropwise to a boiling solution of benzylidene bromide (3.5 g) in benzene (500 ml) during 2.5 h. Boiling under reflux was continued for 20 h. The cooled mixture was washed with water (2 × 150 ml), then dried ($MgSO_4$), and concentrated. The residue (2.9 g) was fractionated on a column of silica gel (Davison, 100 g) by elution with ether–benzene (1:9). Eluted first was the 4,6-*O*-benzylidene derivative *C* (0.6 g), m.p. 158–160° [from ether–light petroleum (b.p. 60–80°)], $[\alpha]_D$ + 109° (*c* 1.0, chloroform) [Found: C, 62.0; H, 7.0; Mol. wt. (Rast in camphor) 272. $C_{16}H_{22}O_6$ calc.: C, 61.9; H, 7.2%; Mol. wt. 310]. Eluted second was the known[11] 4,6-*O*-benzylidene derivative (*D*, 0.35 g), m.p. 117–119°, $[\alpha]_D$ + 167° (*c*. 1.0, chloroform).

Using essentially the above conditions, methyl 2,3-di-*O*-methyl-β-D-galactopyranoside[12] (0.95 g) was benzylidenated. The crude, oily product (1.7 g) was fractionated on silica gel (50 g). Elution with benzene–ether (4:1) gave first the 4,6-*O*-benzylidene derivative *E* (0.26 g), m.p. 156.5–157.5°, $[\alpha]_D$ −27° (*c* 1.0, chloroform) (Found: C, 61.7; H, 7.3%), followed by the known[12] isomer (*F*, 140 mg), m.p. 142–144°, $[\alpha]_D$ + 18° (*c* 1.0, chloroform).

A solution of isomer *C* (m.p. 158–160°, 0.1 g) in chloroform (0.5 ml) was treated with a small amount of a saturated solution of hydrogen chloride in the same solvent. When isomerization was complete (20 min, followed by n.m.r. spectroscopy), the solution was diluted with chloroform, washed with aqueous sodium hydrogen carbonate, then dried ($MgSO_4$), and concentrated to give an almost quantitative yield of diastereoisomer *D*, m.p. 117–118° (mixed m.p.). Essentially similar results were obtained for the conversion *E* → *F*.

Homogeneous benzylidenations

(*a*) *Methyl 2,3-di-*O*-methyl-α-D-galactopyranoside*

A solution of the title compound (0.1 g) in nitromethane (0.5 ml) was treated with a solution of toluene-*p*-sulphonic acid (0.25 mg) in benzaldehyde (0.5 ml),

and the development of benzyl proton signals was followed at *ca.* 30°. The extent of reaction (90%) was determined by the addition of benzoin (221 mg) and comparison of the integrated areas of the peaks for the benzyl proton in the benzylidene acetal (τ 5.10) and in the benzoin (4.23).

(*b*) *Methyl* D-*galactopyranoside*

A solution of the α-glycoside (0.1 g) in dimethylformamide (0.5 ml) was treated with a solution of toluene-*p*-sulphonic acid (20 mg) in benzaldehyde (0.5 ml). The development of benzyl proton signals was followed at *ca.* 30°. The extent of reaction (*ca.* 90%) was determined as in (*a*). A similar reaction extent was observed for the β-glycoside.

(*c*) *Methyl 2,6-di-*O*-methyl-*α-D-*galactopyranoside*

A solution of the title compound[24] (0.1 g) in nitromethane (0.5 ml) was treated with a solution of toluene-*p*-sulphonic acid (0.5 mg) in benzaldehyde (0.5 ml) at *ca.* 30°. The benzyl proton signal at 4.78 appeared first followed by a signal at 4.53. When equilibrium had been reached in a reaction performed on a preparative scale, the solution was washed with aqueous sodium hydrogen carbonate and water, then dried ($MgSO_4$), and concentrated to give a residue which was freed from benzaldehyde by elution from silica gel (20 g) with benzene–ether (9:1). The product, $[\alpha]_D^{31.5}$ + 139.5° (*c* 1.0, chloroform), which failed to crystallise and could not be distilled was, presumably, a mixture of compounds (XI) and (XII).

In a parallel experiment, the nitromethane was replaced by an equal volume of *tert*-butanol. Under these conditions only one benzyl proton signal (4.65) appeared. Isolation of the product as described above, after dilution of the mixture with chloroform, gave isomer (XI), $[\alpha]_D^{31.5}$ + 144.5° (*c* 1.0, chloroform), which also failed to crystallise and could not be distilled.

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SUMMARY

Treatment of methyl 2,3-di-*O*-methyl-α-D-glucopyranoside with benzylidene bromide, toluene, potassium *tert*-butoxide, and *tert*-butanol gave two diastereoisomeric 4,6-*O*-benzylidene derivatives (*A* and *B*). The complete conversion $A \rightarrow B$ was effected with carbon tetrachloride–hydrogen chloride. Only diastereoisomer *B* was formed on acid-catalysed benzylidenation. Closely parallel results were obtained on benzylidenation of methyl 2,3-di-*O*-methyl-α- and β-D-galactopyranoside under basic conditions. The possible conformations of the benzylidene derivatives are considered on the basis of n.m.r. spectroscopic data. At equilibrium in the acid-catalysed benzylidenation of methyl α-D-galactopyranoside the only product present is the 4,6-*O*-benzylidene derivative, m.p. 168–170°, $[\alpha]_D$ + 144° in chloroform. In the

early, kinetically-controlled, stages of the reaction, small amounts of the diastereoisomeric 3,4-*O*-benzylidene derivatives were detected.

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ACYLATED OSAZONES AND ANHYDRO-DERIVATIVES

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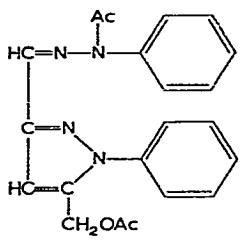
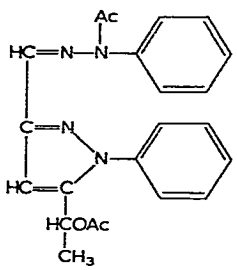
It has been shown[1,2,3] that monosaccharide phenylosazones when refluxed with acetic anhydride yield colorless dianhydrophenylosazone acetates. We have now extended this reaction to the phenylosazone acetates. For this purpose we prepared tri-*O*-acetyl-D-*erythro*- and D-*threo*-pentose phenylosazones, treated them with boiling acetic anhydride, and obtained 5-acetoxymethyl-3-formyl-1-phenylpyrazole *N*-acetylphenylhydrazone (Ia). Similarly, tri-*O*-acetyl-6-deoxy-D-*lyxo*-hexose phenylosazone was prepared and converted into 5-(D-*glycero*-1-acetoxyethyl)-3-formyl-1-phenylpyrazole *N*-acetylphenylhydrazone (Ib). Likewise, the tetra-*O*-acetyl-D-*lyxo*-hexose phenylosazone of Wolfrom *et al.*[4] gave 5-(D-*glycero*-1,2-diacetoxyethyl)-3-formyl-1-phenylpyrazole *N*-acetylphenylhydrazone (Ic). The identity of these compounds was established by comparison with authentic specimens we prepared earlier[1] by the action of boiling acetic anhydride on the free phenylosazones. It seems that the phenylosazone acetates are converted into the dianhydrophenylosazone acetates by the removal of two molecules of acetic acid. The yields, however, were considerably lower than when the free osazones were used, suggesting that on boiling the free osazone with acetic anhydride only a minor part of it is acetylated and subsequently converted into the dianhydrophenylosazone acetate, while the major portion undergoes dehydration first and then acetylation. This would account for the higher yield obtained on using the phenylosazones rather than their acetates.

We have also extended the dianhydrophenylosazone formation to substituted phenylosazones. Thus, on boiling D-*arabino*-hexose *p*-chlorophenylosazone with acetic anhydride we obtained 1-*p*-chlorophenyl-5-(D-*glycero*-1,2-diacetoxyethyl)-3-formylpyrazole *N*-acetyl-*p*-chlorophenylhydrazone (II, R = CH_2OAc), which was saponified to give 1-*p*-chlorophenyl-5-(D-*glycero*-1,2-dihydroxyethyl)-3-formylpyrazole *N*-acetyl-*p*-chlorophenylhydrazone. Similarly, L-*xylo*-hexose *p*-chlorophenylosazone yielded the enantiomorphous 1-*p*-chlorophenyl-5-(L-*glycero*-1,2-diacetoxyethyl)-3-formylpyrazole *N*-acetyl-*p*-chlorophenylhydrazone. Likewise, D-*erythro*-pentose *p*-chlorophenylosazone yielded 5-acetoxymethyl-1-*p*-chlorophenyl-3-formylpyrazole *N*-acetyl-*p*-chlorophenylhydrazone (II, R = H). These three fully acetylated compounds showed infrared spectral absorptions characteristic of the *O*- and *N*-acetyl groups, with the ester band at 1750 cm^{-1}, the amide band at 1675 cm^{-1}, and the C=N band at 1580–1600 cm^{-1}. The structure of the D-*glycero* isomer (II, R = CH_2 OAc) was further established by its nuclear magnetic resonance (n.m.r.) spectrum,

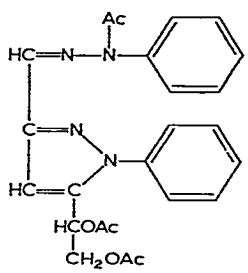
which was quite similar to that of the phenyl derivative reported earlier[3]. The spectrum showed resonance in the τ 2.4 region which was assigned to the aryl group protons and to the proton of the pyrazole ring, and a singlet of unit proton intensity at τ 2.7 was assigned to the strongly deshielded methine hydrogen of the hydrazone function. The methylene group from C-6 of the hexose precursor appeared as a two-proton doublet, τ 5.55, split by the adjacent proton, J = 6.0 c.p.s. The latter proton (at C-5 of the hexose) gave the expected triplet of unit proton intensity at τ 4.0, with J = 6.0 c.p.s. A singlet of three-proton intensity appeared at τ 7.35, and was assigned to the methyl protons of the *N*-acetyl group. Two singlets, each of three proton intensity, appeared at τ 7.94 and 7.97, and were assigned to the methyl protons of the two *O*-acetyl groups.



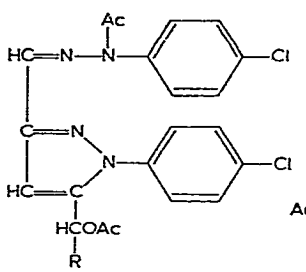
(Ia)



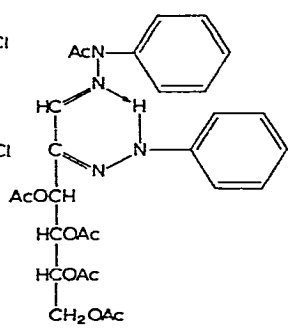
(Ib)



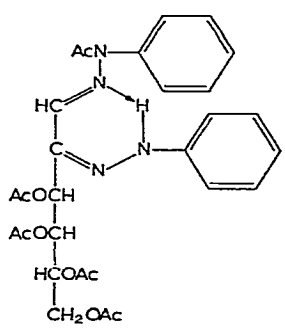
(Ic)



(II)



(III)



(IV)

Hexose phenylosazones were recently[5] found to react with benzoyl chloride yielding *N*-benzoyl-tetra-*O*-benzoyl derivatives. In a similar reaction, we treated the phenylosazones from D-glucose and D-galactose in pyridine with acetyl chloride, and obtained the expected pentaacetates. These showed in their infrared spectra an ester band at 1750, an amide band at 1675–1690 and a C=N band at 1610 cm^{-1}, denoting the presence of *O*- and *N*-acetyl groups. Accordingly, they were formulated

as *N*-acetyl-tetra-*O*-acetyl-D-*arabino*-hexose phenylosazone (III) and *N*-acetyl-tetra-*O*-acetyl-D-*lyxo*-hexose phenylosazone (IV).

We have also studied the benzoylation of phenylosazones with insufficient amounts of benzoyl chloride, and have obtained from D-*arabino*-hexose phenylosazone a crystalline tetrabenzoate which showed only one carbonyl band at 1725 cm^{-1}, denoting absence of the *N*-benzoyl group. We therefore suggest that it is the tetra-*O*-benzoyl derivative reported by Fischer and Freudenberg[6], and later by Engel[7]. These authors were unable to isolate it in a crystalline form. We have also obtained, by incomplete benzoylation of the phenylosazone from L-sorbose, a monobenzoate which is presumably the 6-benzoate.

EXPERIMENTAL

Infrared spectra were measured with a Perkin Elmer "Infracord" infrared spectrophotometer; nuclear magnetic resonance spectra were obtained with a Varian Associates Model A60 n.m.r. spectrometer, with deuteriochloroform as the solvent and tetramethylsilane ($\tau = 10.00$) as the internal reference standard. Microanalytical determinations were made by A. Bernhardt, Mülheim, W. Germany.

Arylosazone acetates (*Table I*)

The osazone (2 g) was dissolved in pyridine (15 ml), treated with acetic anhydride (10 ml), and the mixture kept overnight at room temperature. It was then poured onto crushed ice (200 g) and washed repeatedly with water. The D-xylose and D-fucose derivatives were crystallized from aqueous ethanol, and the others were purified by repeated precipitation from hot aqueous ethanol.

5-(Acetoxymethyl)-3-formyl-1-phenylpyrazole N-*acetylphenylhydrazone* (*Ia*)

(*a*) *From tri*-O-*acetyl*-D-erythro-*pentose phenylosazone.* Tri-*O*-acetyl-D-*erythro*-pentose phenylosazone (6 g) was refluxed for 30 min with acetic anhydride (65 ml). The pale brown solution was poured onto crushed ice (200 g) and left overnight. The aqueous layer was decanted and the residual oil was washed repeatedly with water until it solidified (one day). The solid was crystallized from aqueous ethanol as colorless needles, yield 0.6 g, m.p. 196°, undepressed on admixture with an authentic specimen obtained by refluxing D-*erythro*-pentose phenylosazone with acetic anhydride.

Anal. Calc. for $C_{21}H_{20}N_4O_3$: C, 67.02; H, 5.30; N, 14.90. Found: C, 66.64; H, 5.43; N, 14.61.

(*b*) *From tri*-O-*acetyl*-D-threo-*pentose phenylosazone.* Tri-*O*-acetyl-D-*threo*-pentose phenylosazone (4 g), treated with acetic anhydride (20 ml) as in the preceding experiment, yielded the same product, m.p. and mixed m.p. 196°.

Anal. Found: C, 67.10; H, 5.51.

TABLE I

ARYLOSAZONE ACETATES

Sugar	Aryl residue	m.p.	$[\alpha]_D$ (c 0.5, $CHCl_3$)	Formula	Calc.			Found		
					C	H	N	C	H	N
D-Glucose	m-C_6H_4Cl		+ 77°	$C_{26}H_{28}Cl_2N_4O_8$	52.43	4.71	9.41	52.61	4.75	9.35
D-Xylose	Ph	106–110°	+ 63°	$C_{23}H_{26}N_4O_6$	60.79	5.72	12.33	61.40	6.06	12.20
D-Arabinose	Ph		− 21°	$C_{23}H_{26}N_4O_6$			12.33			12.47
D-Fucose	Ph	178°	+ 73°	$C_{24}H_{28}N_4O_6$	61.55	5.98	11.97	61.36	6.29	11.73

*5-(*D-glycero-*1-Acetoxyethyl)-3-formyl-1-phenylpyrazole* N-*acetylphenylhydrazone* (*Ib*)

Tri-*O*-acetyl-6-deoxy-D-*lyxo*-hexose phenylosazone (0.8 g) was treated with boiling acetic anhydride (10 ml) as above, then poured onto crushed ice (100 g), and the crystalline precipitate was crystallized from aqueous ethanol, m.p. and mixed m.p. with an authentic specimen 173–174°.

*5-(*D-glycero-*1,2-Diacetoxyethyl)-3-formyl-1-phenylpyrazole* N-*acetylphenylhydrazone* (*Ic*)

Tetra-*O*-acetyl-D-*lyxo*-hexose phenylosazone (2 g) was refluxed with acetic anhydride (20 ml) for one hour, and processed as before. After three days the residual oil solidified, and was crystallized from aqueous ethanol as colorless needles, m.p. and mixed m.p. with an authentic specimen 130–131°.

*1-(*p-*Chlorophenyl)-5-(*D-glycero-*1,2-diacetoxyethyl)-3-formylpyrazole* N-*acetyl*-p-*chlorophenylhydrazone* (*II*, $R = CH_2OAc$)

D-*arabino*-Hexose *p*-chlorophenylosazone (5 g) was treated as above with acetic anhydride (70 ml) for one hour, poured onto crushed ice, and the residual oil washed repeatedly with water. After two days the oil solidified and was crystallized from aqueous ethanol as colorless needles, m.p. 149–151°, $[\alpha]_D^{20}$ + 48° (*c* 1, chloroform); soluble in ethanol and chloroform, insoluble in water, ν_{max}^{KBr} 1750 (OAc), 1675 (NAc), 1600 (C=N) cm^{-1}.

Anal. Calc. for $C_{24}H_{22}Cl_2N_4O_5$: C, 55.71; H, 4.26; N, 10.83. Found: C, 56.09; H, 4.62; N, 10.26.

*1-(*p-*Chlorophenyl)-5-(*L-glycero-*1,2-diacetoxyethyl)-3-formylpyrazole* N-*acetyl*-p-*chlorophenylhydrazone*

L-*xylo*-Hexose *p*-chlorophenylosazone (7 g) was similarly treated with acetic anhydride (100 ml) for 4 h, and then poured onto crushed ice (500 g). The aqueous solution was decanted, and the residual oil was washed repeatedly with water whereupon it solidified and was crystallized from aqueous ethanol as colorless needles, m.p. and mixed m.p. 151°, $[\alpha]_D^{20}$ —47° (*c* 0.13, chloroform).

Anal. Found: N, 10.48.

*1-(*p-*Chlorophenyl)-5-(*D-glycero-*1,2-dihydroxyethyl)-3-formylpyrazole* N-*acetyl*-p-*chlorophenylhydrazone*

1-(*p*-Chlorophenyl)-5-(D-*glycero*-1,2-diacetoxyethyl)-3-formylpyrazole *N*-acetyl-*p*-chlorophenylhydrazone (0.5 g) was saponified with 20% ethanolic ammonia (20 ml) overnight at room temperature. The product was evaporated nearly to dryness on the water bath, whereupon the *N*-acetyl derivative separated. It was purified by repeated precipitation from hot aqueous ethanol, ν_{max}^{KBr} 3325 (OH), 1675 (NAc), 1600 (C=N) cm^{-1}.

Anal. Calc. for $C_{20}H_{18}Cl_2N_4O_3$: C, 55.43; H, 4.16; N, 12.93. Found: C, 55.22; H, 4.26; N, 13.26.

5-(Acetoxymethyl)-1-(p-chlorophenyl)-3-formylpyrazole N-*acetyl*-p-*chlorophenylhydrazone* (*II*, $R = H$)

D-*erythro*-Pentose *p*-chlorophenylosazone (10 g) was refluxed for 4 h with acetic anhydride (100 ml), and the reaction mixture was poured onto crushed ice (1 kg). After two days the residual oil solidified, and was crystallized from aqueous ethanol as colorless needles, m.p. 158–160°, ν_{max}^{KBr} 1750 (OAc), 1675 (NAc), 1580 (C=N) cm^{-1}.

Anal. Calc. for $C_{21}H_{18}Cl_2N_4O_3$: C, 56.63; H, 4.05. Found: C, 56.37; H, 4.31.

N-*Acetyl-tetra*-O-*acetyl*-D-arabino-*hexose phenylosazone* (*III*)

D-*arabino*-Hexose phenylosazone (5 g) in cold, dry pyridine (50 ml) was treated while stirring with acetyl chloride (20 ml) during 2 h. The cold reaction mixture was poured onto crushed ice (500 g) and the aqueous solution was decanted. The residual yellowish brown acetate was then washed repeatedly with 2% aqueous sodium bicarbonate and water. The acetate was purified by repeated precipitation from hot aqueous ethanol, ν_{max}^{KBr} 1750 (OAc), 1675 (NAc), 1610 (C=N) cm^{-1}.

Anal. Calc. for $C_{28}H_{32}N_4O_9$: C, 59.15; H, 5.63; N, 9.85. Found: C, 59.60; H, 5.80; N, 9.80.

N-*Acetyl-tetra*-O-*acetyl*-D-lyxo-*hexose phenylosazone* (*IV*)

D-*lyxo*-Hexose phenylosazone was treated with acetyl chloride as above, and the product was crystallized from aqueous ethanol as yellow needles, m.p. 139–141°, $[\alpha]_D^{20} + 48°$ (*c* 0.8, chloroform), soluble in ethanol, methanol, and chloroform, and insoluble in water, ν_{max}^{KBr} 1750 (OAc), 1690 (NAc), 1610 (C=N) cm^{-1}.

Anal. Found: C, 58.78; H, 5.57; N, 9.32.

Tetra-O-*benzoyl*-D-arabino-*hexose phenylosazone*[8]

D-*arabino*-Hexose phenylosazone (1 g) in dry pyridine (10 ml) was treated with benzoyl chloride (2 ml). The temperature rose rapidly and an abundant precipitate of pyridine hydrochloride separated. After standing overnight at room temperature the reaction mixture was poured onto crushed ice (100 g) and stirred with frequent decantation of water. After 2 days the product solidified and was crystallized from methanol–benzene as yellow globules, m.p. 145–148°, $[\alpha]_D^{20} -24°$ (*c* 0.9, chloroform), ν_{max}^{KBr} 1725 (OBz), 1600 (C=N) cm^{-1}.

Anal. Calc. for $C_{46}H_{38}N_4O_8$: C, 71.52; H, 4.92; N, 7.24. Found: C, 72.05; H, 5.12; N, 7.10.

6-O-Benzoyl-L-xylo-*hexose phenylosazone*[8]

L-*xylo*-Hexose phenylosazone (1 g) in pyridine (10 ml) was benzoylated as above with benzoyl chloride (2 ml) overnight at room temperature. The mono-*O*-benzoyl derivative crystallized from methanol as yellow aggregates, m.p. 180–182° (dec.).

Anal. Calc. for $C_{25}H_{26}N_4O_5$: C, 64.93; H, 5.63; N, 12.12. Found: C, 65.35; H, 5.82; N, 12.29.

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SUMMARY

The phenylosazone acetates from pentoses, 6-deoxyhexoses and hexoses were boiled with acetic anhydride yielding dianhydrophenylosazone acetates of the type (Ia–c). Similarly, *p*-chlorophenylosazones from D-glucose, L-sorbose and D-arabinose yielded dianhydro-*p*-chlorophenylosazone acetates. Acetyl chloride reacted with the phenylosazones from D-glucose and D-galactose yielding the *N*-acetyl-tetra-*O*-acetyl derivatives (III) and (IV). The phenylosazones from D-glucose and L-sorbose gave a tetra- and a mono-*O*-benzoyl derivative, respectively, in crystalline form when an insufficient amount of benzoyl chloride was used during benzoylation.

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BORIC ACID DERIVATIVES AS REAGENTS IN CARBOHYDRATE CHEMISTRY

PART IV*. THE INTERACTION OF PHENYLBORONIC ACID WITH HEXOPYRANOID COMPOUNDS

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Previous workers investigated the interaction between equimolar amounts of methyl β-D-glucopyranoside and phenylboronic acid, and concluded that in boiling acetone part of the glucoside was fully esterified, part was unaltered, and products containing the reactants in the ratio 1:1 were not formed in significant amounts[2]. Since a discrete cyclic ester has now been obtained in nearly quantitative yield by carrying out the condensation in benzene solution, it seems probable that, in the experiment with acetone, the initial product was largely destroyed during the subsequent treatments.

The cyclic ester formed from methyl α-D-glucopyranoside was found to have the 4,6-cyclic structure, and with excess of the acid it formed a 2,3-(diphenylpyroboronate)[3]. The methyl β-D-glucopyranoside ester was required for comparison with the α-derivative, and since sites other than 4,6 could react with the acid (the 2,4-diol of the methyl xylopyranosides takes part in condensations[4]) it was subjected to structural analysis. On acetylation and benzoylation it afforded crystalline products which were identical with those obtained by treatment of methyl 2,3-di-*O*-acetyl- and methyl 2,3-di-*O*-benzoyl-β-D-glucopyranoside with phenylboronic acid, and therefore this cyclic ester also has the 4,6-structure. Further condensation with the acid caused the formation of methyl β-D-glucopyranoside 2,3-(diphenylpyroboronate) 4,6-phenylboronate which, like the corresponding polyester in the α-series, was exceedingly susceptible to hydrolysis. Methyl β-D-glucopyranoside 4,6-phenylboronate was isolated readily from the products of partial hydrolysis.

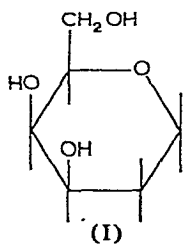
Methyl α- and β-D-glucopyranoside both react, therefore, at positions 4 and 6 with phenylboronic acid, in the same way as they do with borate anions[5]. In the latter case a significant difference exists between the stabilities of the complexes formed, since the M_G values obtained on electrophoresis of the α- and β-glycosides in borate electrolyte at pH 10 are 0.11 and 0.19, respectively. The non-bonded interactions between the axial methoxyl group and H-3 and H-5, which are held to be responsible for destabilising the α-complex[6], also occur in methyl α-D-glucopyranoside 4,6-phenylboronate, and so it was expected that this ester would show greater susceptibility to hydrolysis than the β-anomer. No difference could be detect-

*For Part III, see ref. 1.

ed by polarimetric methods; both esters were hydrolysed completely on the addition of 4% water to dioxan solutions (2%). One phenylboronate has been reported to require 30% water for hydrolysis[1].

3-*O*-Acetyl-D-glucal which was required for other studies has been prepared (in slightly impure form) by way of its phenylboronate. Acetylation of D-glucal 4,6-phenylboronate gave a crystalline product which, after hydrolysis of the boronate ring, did not reduce the periodate ion (see Table I) and was therefore 3-*O*-acetyl-D-glucal 4,6-phenylboronate. Propan-1,3-diol was added to a solution of this ester in acetone and, after removal of the solvent and extraction of the residue with light petroleum, syrupy 3-*O*-acetyl-D-glucal (contaminated with 5% of the initial phenylboronate) remained.

An analysis has been undertaken of the structures of the phenylboronates formed from a series of hexopyranoid compounds with the D-*lyxo*-configuration at C-3, C-4, and C-5 (*e.g.* I), which could condense at either the 4,6- or 3,4-sites. A surprising feature of this work is that high yields of pure products were obtained in every case, mixed products were not encountered. From the ester derived from methyl β-D-galactopyranoside, a crystalline dibenzoate was formed which was identical with methyl 2,3-di-*O*-benzoyl-β-D-galactoside 4,6-phenylboronate prepared from methyl 2,3-di-*O*-benzoyl-β-D-galactoside, and therefore the unsubstituted glycoside had reacted at the 4,6-positions. Subsequent analyses on the other esters of this series were carried out using a more convenient method. The unsubstituted hydroxyl groups in each ester were protected by acetylation and the products were subjected to periodate oxidation under conditions in which the boronate ester groups were completely hydrolysed. 3,4-Boronates liberated a vicinal diol and therefore reduced one molar equivalent of periodate; the 4,6-esters gave unreactive dihydroxy-compounds.



(I)

Like the methyl glucosides, methyl α- and β-D-galactopyranoside both formed a 4,6-cyclic ester, as did 1,5-anhydro-D-galactitol and its 2-deoxy derivative. Alternatively, D-galactal and methyl 2-deoxy-α-D-*lyxo*-hexopyranoside (methyl 2-deoxy-α-D-galactoside) condensed with the reagent to give esters which, after acetylation and hydrolysis, reduced 1 mol of periodate and were therefore 3,4-boronates. In galactal, the influence of the double bond is to decrease the angle subtended by the $C_{(3)}$–O and $C_{(4)}$–O bonds from the 60° of a perfect chair ($O_{(3)}$–$O_{(4)}$ distance 2.76 Å) to near zero ($O_{(3)}$–$O_{(4)}$ distance 2.48 Å), and since the O–O distance in phenylboronic acid will be near 2.38 Å (the corresponding distance in crystalline orthoboric acid[7] is 2.36 Å and in trimethyl borate[8] 2.38 Å) such a distortion would increase the availa-

bility of the 3,4-diol to the reagent. Molecular models show that any distortion from a perfect chair conformation in the case of the α-glycosides, brought about by repulsion between the axial methoxyl group and H-3 and H-5, would also cause ring flattening and decrease the $O_{(3)}$–$O_{(4)}$ distance. It is proposed that with methyl 2-deoxy-α-D-*lyxo*-hexopyranoside such a conformational effect accounts for the formation of the 3,4-ester, while with methyl α-D-galactoside this distortion would be inhibited by the presence of the 2-hydroxyl grouping which would be required to approach the aglycone. Support for this explanation comes from the finding that methyl 2-deoxy-β-D-*lyxo*-hexopyranoside, which is not susceptible to the "flattening" effect, gave the 4,6-cyclic ester. This same effect, brought about by 1,3-diaxial interactions, has been invoked to account for the variability in the n.m.r. chemical shifts of the anomeric protons of α-glucosides[9].

Periodic acid oxidations were carried out on the anomeric methyl 2-deoxy-D-*lyxo*-hexopyranosides in an attempt to confirm that the $O_{(3)}$–$O_{(4)}$ distance was less in the α- than in the β-compound; efforts to do this by n.m.r. and infrared spectroscopy were unsuccessful. The β-isomer was found to oxidise more rapidly than the α-compound (0.7 mol periodate reduced in 90 and 170 sec, respectively), and, since the 3,4-diol in the β-glycoside (assuming that the molecule adopts a nearly perfect chair conformation) would be almost ideally suited for cyclic dianion formation with the oxidising agent[10], this result is in accord with that to be expected if the $O_{(3)}$–$O_{(4)}$ distance is appreciably diminished in the case of the α-compound.

A similarity exists between the mode of action of acetone and of phenylboronic acid with methyl α-D-galactoside and D-galactal. In the presence of zinc chloride, which is believed to catalyse the formation of kinetically-controlled products[11], galactal gives mainly the 3,4-isopropylidene derivative[12], while the 4,6-ketal is formed in large amounts from the glycoside[11].

1,5-Anhydro-D-galactitol 2,3-(diphenylpyroboronate) 4,6-phenylboronate was prepared and, like the glucitol analogue, was found to exhibit none of the unusual instability of the methyl glucoside polyesters.

EXPERIMENTAL

Optical rotations of the phenylboronates were measured in dry dioxan within the concentration range 0.7–3%. Methyl 2-deoxy-α-D-*lyxo*-hexopyranoside was prepared from D-galactal[13], and the β-isomer was kindly supplied by Dr. J.C.P. Schwarz (University of Edinburgh). The preparations of the phenylboronates were carried out in boiling benzene[3]; on numerous occasions it was advantageous to add the carbohydrate component in methanolic solution.

Acetylations were carried out by dissolving the boronates in dry pyridine at 0° (20 ml/g of ester) and adding acetyl chloride (1 ml/g of ester), slowly with stirring. After 2 h benzene was added and the pyridinium chloride and solvents were removed. Distillation of the residues gave colourless viscous syrups which crystallised on treatment with light petroleum (b.p. 40–60°) or with light petroleum–benzene.

TABLE I

PHENYLBORONATES AND THEIR ACETYLATED DERIVATIVES

No.	*Phenylboronate derived from*	*Yield* %	*No.*	*Acetyl derivative of compound*	*Yield* %	*Periodate consumption (mol)*
1	Methyl β-D-glucopyranoside	98	10	1	82[a],34[b]	
2	Methyl α-D-galactopyranoside	93	11	2	77[a]	0.04
3	Methyl β-D-galactopyranoside	98	12	3	89[a],66[b]	0.02
4	Methyl 2-deoxy-α-D-*lyxo*-hexopyranoside	93	13	4	26[b]	1.00
5	Methyl 2-deoxy-β-D-*lyxo*-hexopyranoside	99	14	5	96[a],36[b]	0.05
6	1,5-Anhydro-2-deoxy-D-*lyxo*-hexitol	88	15	6	29[b]	0.05
7	1,5-Anhydro-D-galactitol	83	16	7	43[b]	0.02
8	D-Galactal	83	17	8	69[b]	1.07
9	D-Glucal	82	18	9	72[a],40[b]	0.09

[a] Crude products.
[b] Pure crystalline products.

No.	*M.p. (degrees)*	$[\alpha]_D$ *(degrees)*	*Found (%)* C	H	B	OMe	*Formula*	*Calc. (%)* C	H	B	OMe
1	185–186	− 99	55.8	6.3	3.9						
2	119–120	+ 147	55.9	6.4	4.0	11.1	$C_{13}H_{17}BO_6$	55.7	6.1	3.9	11.1
3	176–177	− 28	55.8	6.3	3.8	10.9					
4	159	+ 114	58.9	6.3	4.1	11.9	$C_{13}H_{17}BO_5$	59.2	6.5	4.1	11.8
5	188	− 71	59.7	6.6	4.4	11.5					
6	114–115	+ 60	61.3	6.3	4.8		$C_{12}H_{15}BO_4$	61.6	6.5	4.6	
7	141–142	+ 69	57.8	6.0	4.3		$C_{12}H_{15}BO_5$	57.6	6.0	4.3	
8	104	− 87	62.0	5.7	4.6		$C_{12}H_{13}BO_4$	62.1	5.6	4.7	
9	128	− 53	62.6	5.7	4.6						
10	123–124	− 99	56.1	5.8	3.0	8.7					
11	166–167	+ 232	56.2	5.8	3.0	8.7	$C_{17}H_{21}BO_8$	56.1	5.8	3.0	8.5
12	145	+ 75	56.4	6.0	3.0	8.2					
13	132–133	+ 39	58.7	6.6	3.7	10.5	$C_{15}H_{19}BO_6$	58.9	6.3	3.5	10.1
14	131	− 87	58.2	6.5	3.7	10.3					
15	89	+ 172	60.2	6.2	4.2		$C_{14}H_{17}BO_5$	60.9	6.2	3.9	
16	143–144	+ 177	57.7	5.7	3.4		$C_{16}H_{19}BO_7$	57.5	5.7	3.2	
17	98–99	− 74	61.1	5.9	4.0		$C_{14}H_{15}BO_5$	61.3	5.5	3.9	
18	88–89	− 90	61.6	5.4	4.1						

Methyl 2,3-di-O-benzoyl-β-D-glucopyranoside 4,6-phenylboronate

The phenylboronate (2.0 g) was dissolved in dry pyridine (25 ml) at 0° and benzoyl chloride (2.0 ml) was added slowly with stirring. After 16 h at room temperature, dry benzene (20 ml) was added and the pyridinium chloride and solvents were removed. Extraction of the residue with dry benzene and crystallisation from benzene–light petroleum (b.p. 40–60°) gave the benzoate boronate (2.0 g, 57%), m.p. 123–124°,

$[\alpha]_D$ −2.6° (Found: C, 66.7; H, 5.1; B, 2.1; OMe, 6.4. $C_{27}H_{25}BO_8$ calcd.: C, 66.4; H, 5.2; B, 2.2; OMe, 6.4%). It was identical (m.p., mixed m.p., infrared spectrum, and optical rotation) with the ester formed by condensing methyl 2,3-di-*O*-benzoyl-β-D-glucopyranoside with phenylboronic acid. In addition, methyl 2,3-di-*O*-acetyl-β-D-glucopyranoside afforded the same compound as was obtained by acetylation of methyl β-D-glucoside boronate (m.p., mixed m.p., optical rotation, and infrared spectrum).

Methyl 2,3-di-O-benzoyl-β-D-galactopyranoside 4,6-phenylboronate

Similar benzoylation of methyl β-D-galactopyranoside 4,6-phenylboronate (1.0 g) afforded the dibenzoate (0.5 g, 29%), m.p. 161–162°, $[\alpha]_D$ + 125° (Found: C, 65.5; H, 4.8; B, 2.3; OMe, 6.5%), identical (m.p., mixed m.p., optical rotation, and infrared spectrum) with the ester formed from methyl 2,3-di-*O*-benzoyl-β-D-galactopyranoside.

3-O-Acetyl-D-glucal

The acetylated boronate (1.18 g, see Table I) was dissolved in acetone (25 ml) and propan-1,3-diol (0.326 g, 1.0 mol) was added. The optical rotation of the solution changed immediately (−4.0 → −2.5°) and after 1 h the solvent was removed. The residue (0.617 g, 77%), after extraction with cold light petroleum (b.p. 40–60°; 3 × 25 ml), had $[\alpha]_D$ −65° (*c* 3, MeOH) (Found: B, 0.20%).

Methyl β-D-glucopyranoside 2,3-(diphenylpyroboronate) 4,6-phenylboronate

Treatment of methyl β-D-glucopyranoside with triphenylboroxole (1.0 mol) or of the 4,6-phenylboronate with the anhydride (0.66 mol) afforded the polyester (83% from the glycoside), m.p. 185–186°, $[\alpha]_D$ −127° (Found: C, 62.7; H, 5.5; B, 6.9. $C_{25}H_{25}B_3O_7$ calc.: C, 63.8; H, 5.4; B, 6.9%). Particular care had to be taken to avoid hydrolysis during recrystallisation from carbon tetrachloride–light petroleum (b.p. 40–60°).

1,5-Anhydro-D-galactitol 2,3-(diphenylpyroboronate) 4,6-phenylboronate

Condensation of the anhydrohexitol or its 4,6-phenylboronate with appropriate amounts of phenylboronic acid gave the polyester (78% from the anhydride), m.p. 201–203°, $[\alpha]_D$ + 177° (Found: C, 65.8; H, 5.1; B, 7.2. $C_{24}H_{23}B_3O_6$ calc.: C, 65.5; H, 5.3; B, 7.4%). This compound could be recrystallised from benzene saturated with water.

Periodate oxidations

(a) Phenylboronate acetates

The reduction of the periodate ion was followed spectrophotometrically[14], using appropriate control solutions in the "blank" cells. Oxidations were performed by adding an aqueous solution of the reagent (0.03M) to an equal volume of ester solution (0.025M in aqueous dioxan 30:70). In each case it was demonstrated by

polarimetric examination that, under the conditions of the oxidations, the boronate ester linkages were completely hydrolysed. The values of periodate consumption given in Table I are those found after 12 h, in which time methyl α-D-glucopyranoside reduced 2.1 mol of the reagent under the same conditions. In those cases where no oxidation occurred, the periodate solution was shown to be active by subsequent addition of methyl α-D-glucopyranoside to the solutions of boronates.

(*b*) *Methyl 2-deoxy-α-* and *-β-*D-lyxo-*hexopyranosides*

Oxidations were carried out in aqueous solution using 0.0075M reagent.

ACKNOWLEDGEMENTS

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SUMMARY

Methyl β-D-glucopyranoside, like the α-anomer, reacts smoothly with phenylboronic acid (1 mol) to give a 4,6-cyclic boronate which, with excess of reagent, forms a 2,3-pyroboronate. The nature of the products obtained from hexopyranoid derivatives which possess the D-*lyxo*-configuration at C-3, C-4 and C-5 was found to be dependent upon the substituents at C-1 and C-2. Whereas methyl α- and β-D-galactopyranoside, methyl 2-deoxy-β-D-*lyxo*-hexopyranoside, 1,5-anhydro-D-galactitol, and 1,5-anhydro-2-deoxy-D-*lyxo*-hexitol afforded 4,6-cyclic esters in good yield, the only products obtained from D-galactal and methyl 2-deoxy-α-D-*lyxo*-hexopyranoside possessed the 3,4-cyclic structures.

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SIALIC ACID AND RELATED SUBSTANCES

PART II. A COMPARATIVE ASSAY OF *N*-ACETYLNEURAMINIC ACID

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INTRODUCTION

Members of the sialic acid family have the same basic skeleton of neuraminic acid, which may be regarded as a condensed product of pyruvic acid with 2-amino-2-deoxy-D-mannose. Our previous work[1] described the condensation of oxalacetic acid with *N*-acetyl-D-galactosamine (2-acetamido-2-deoxy-D-galactopyranose).

The assay of sialic acid has been carried out mainly with colorimetric procedures, such as the thiobarbituric acid[2–4], resorcinol[5,6], direct Ehrlich[7], alkali-Ehrlich[3], and diphenylamine[8] methods. Among these methods, more commonly employed procedures are the thiobarbituric acid, resorcinol, and direct Ehrlich methods. It has often been observed that analytical results on the same biological material depend on the assay method employed. Therefore, it is of interest and importance to make comparative assays with these methods, especially with reference to a statistical procedure, in order to find the method most suitable for the analysis of sialic acid in biological materials. The statistical method has recently been used in many fields and satisfactory results have been reported[9].

Thus, the present paper describes a comparison of the factors influencing color formation, with use of the three methods in the presence and absence of some other substances.

MATERIALS

N-Acetylneuraminic acid was isolated from cow colostrum according to a procedure reported by Clark *et al.*[10] with a slight modification as follows: 1,400 ml of fresh cow colostrum was defatted by centrifugation and 2.0 volumes of acetone were added to give about 100 g of precipitate, which was washed with acetone and dried. The acetone powder was hydrolyzed and passed through a Dowex-1X10 (acetate form) column (5 × 55 cm, 50–200 mesh). The column was eluted with 0.3 N-phosphoric acid. The eluates, which were positive for the direct Ehrlich reaction[7], were carefully neutralized with N-sodium hydroxide and concentrated under reduced pressure to give a dried material. This was fractionated into six fractions with water, methanol, and ether. Of these, the two fractions which were relatively strongly positive for the direct Ehrlich reaction were dissolved in a minimum

volume of water, and five volumes of acetic acid were added. The mixture was stored in a refrigerator overnight. The resultant crystals were recrystallized twice from water–acetic acid (1:5, v/v) to give about 200 mg of *N*-acetylneuraminic acid, m.p. 185–186° (decomp.), $[\alpha]_D^{17}$ −31° (*c* 0.1, water). (Found: C, 42.27; H, 6.20; N, 4.13. $C_{11}H_{19}NO_9$ calc.: C, 42.72; H, 6.19; N, 4.53%). The reported values[11] are m.p. 185–186° (dec.), $[\alpha]_D^{22}$ −32° in water. Additional crude crystals (*ca.* 100 mg) were obtained from the remaining fractions. The infrared spectrum of the product was identical with that of authentic *N*-acetylneuraminic acid, and paper-chromatographic examination showed no contamination with *N*-glycolylneuraminic acid. The product was allowed to react with *p*-dimethylaminobenzaldehyde, with thiobarbituric acid following oxidation with sodium metaperiodate, and with resorcinol, to afford the characteristic absorption spectra of sialic acid.

Heparin, sodium salt, was a gift from the Upjohn Company, Kalamazoo, Michigan, U.S.A.

Keratosulfuric acid, calcium salt, was a gift from Prof. K. Meyer, Columbia University, New York, N.Y., U.S.A.

Hyaluronic acid, calcium salt, isolated from bovine brain[12], had $[\alpha]_D^{17}$ −62° (*c* 1.0, water). [Found: uronic acid (by carbazole reaction), 40.6; hexosamine, 36.5; N, 3.84; SO_4^{2-}, negative. $(C_{11}H_{20}NO_{11}.\frac{1}{2}Ca)_n$ calc.: uronic acid, 44.2; hexosamine, 40.2; N, 3.54%].

METHODS

In the assay of *N*-acetylneuraminic acid, some fundamental factors were chosen for study, from various steps of each of the three procedures. It was intended to determine the effect of these factors on color formation as measured by optical density values. The factors for the thiobarbituric acid method are: IA, the concentration of buffer solution (1 M and 2 M); IB, the amount of sodium metaperiodate (IB_1, 20 μmoles; IB_2, 40 μmoles; and IB_3, 60 μmoles); IC, the time and temperature of periodate oxidation (0, 5, 10, 15, 20, and 40 min and 15, 25, and 30°), IE, the concentration of sodium arsenite (IE_0, 5%; IE_1, 10%; and IE_2, 20%); IJ, the concentration of thiobarbituric acid (IJ_0, 0.3%; IJ_1, 0.6%; and IJ_2, 1.2%); and IG, the cooling time and conditions after the completion of the reaction with thiobarbituric acid (5 min in an ice bath, 30 min at 25°, and 60 min at 25°). Those for the resorcinol method are: IIA, the amount of resorcinol (0.1, 0.2, and 0.4% solutions); IIB, the time of the reaction with resorcinol (10, 30, 40, 60, 90, and 120 min); IIC, the cooling time after the completion of the reaction (15, 30, and 60 min); and IID, the chilling time before centrifugation. Those for the direct Ehrlich method are: IIIA, the amount of *p*-dimethylaminobenzaldehyde (25, 50, and 100 mg); IIIB, the time of the reaction with *p*-dimethylaminobenzaldehyde (10, 30, 60, 90, and 120 min); and IIIC, the cooling time after the completion of the reaction (10, 20, 30, and 90 min). Analyses of the factors were carried out by dividing the experiments into five groups for the thiobarbituric acid method, and into three groups for the resorcinol and direct Ehrlich

methods. The factors consisted of two to six levels, and their main effect was analyzed. Analysis of the factors was assigned to $L8(2^6)$ and $L4(2^3)$ for the thiobarbituric acid method, $L9(3^4)$ for the resorcinol and direct Ehrlich methods according to the orthogonal arrangements. Optical density was measured as a characteristic value in all the experiments. Each assay was performed in duplicate, and, therefore, 16 assays in $L8(2^6)$, 8 assays in $L4(2^3)$, and 18 assays in $L9(3^4)$ were carried out in all.

For setting up the corresponding calibration curve using the new conditions suggested by the study, there were carried out a total of 38 assays for the thiobarbituric acid method, a total of 33 assays for the resorcinol method, and a total of 8 assays for the direct Ehrlich method. Each assay was repeated three to six times on each aliquot against each reagent blank, and each calibration curve was calculated by the method of least squares.

For tests of interference with color formation, several acidic substances which are adsorbed on cation-exchange resin were examined, and the experiment was performed by assaying 1,000 μg of each substance in the presence of 9.646 μg of *N*-acetylneuraminic acid for the thiobarbituric acid method, 53.47 μg of *N*-acetylneuraminic acid for the resorcinol method, and 127.5 μg of *N*-acetylneuraminic acid for the direct Ehrlich method.

Acid hydrolysis of biological materials was carried out with 0.1 N-sulfuric acid for 90 min*, and purification was performed according to the procedure using a Dowex-2x8 column[13].

A Hitachi photoelectric spectrometer (EPU-2A) was used in the experiments.

RESULTS AND DISCUSSION

The graphic representation of the effect of some main factors on color formation is shown in Fig. 1. This is based on the analytical results of the variations for the thiobarbituric acid, resorcinol, and direct Ehrlich methods.

The thiobarbituric acid method

Tests of importance in color formation indicate that factors IB_1B_2, IJ_1J_2, IE_1E_2, and IC are significant in this order at the critical rate of 0.5%, factors IB_2B_3 and IG at the critical rate of 5.0%, and all other factors do not strongly affect color formation. With respect to factor IB, Warren[2] proposed 0.1 ml (20 μmoles) of 0.2 M-sodium metaperiodate in 9 M-phosphoric acid. Aminoff[3] reported a condition for maximum color formation at pH 1.2 in 0.125 N-sulfuric acid. Our experiment showed that the use of 0.2 ml (40 μmoles) of the reagent resulted in the maximum optical density. In the case of factor C, higher optical density was observed by allowing to stand for 10–15 min at 25°, and treatment at higher temperatures resulted

*A test of acid hydrolysis of the acetone powder prepared from cow colostrum indicated that hydrolysis for 90 min gave maximum optical density as assayed with the thiobarbituric acid and resorcinol methods, and the optical density did not change up to 240 min. Therefore, hydrolysis for 90 min was employed in the experiment.

in a decrease of optical density. Factor IG resulted in higher optical density on storage for 30 min at 25° than that on storage for 60 min at 25° or for 5 min in an ice bath. The latter condition was proposed by both Warren[2] and Aminoff[3]. Factors

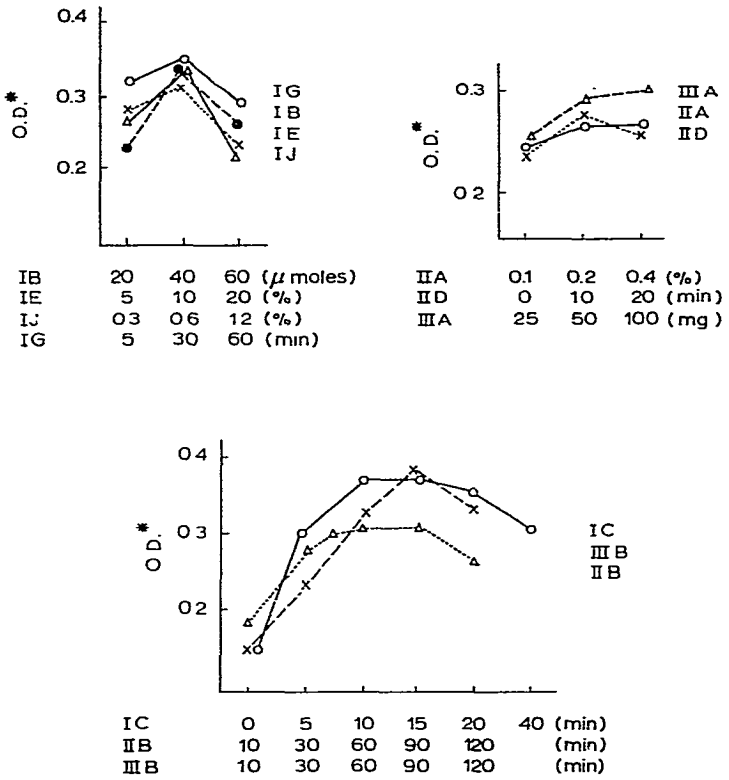

Fig. 1. Graphical representation of the effect of some factors on color formation. I, Thiobarbituric acid method (IB, the amount of sodium metaperiodate; IE, the concentration of sodium arsenite; IJ, the concentration of thiobarbituric acid; IG, the cooling time, and conditions after the completion of the reaction with thiobarbituric acid; IC, the time and temperature of periodate oxidation). II, Resorcinol method (IIA, the amount of resorcinol; IID, the chilling time before centrifuging). III, Direct Ehrlich method (IIIA; the amount of *p*-dimethylaminobenzaldehyde; IIIB, the time of the reaction with *p*-dimethylaminobenzaldehyde). O.D.* does not represent the direct reading of optical density, and it is a value obtained by the direct sum in order to examine the comparative effect of each factor.

IE and IJ gave the maximum color formation under the same conditions as those in the original one. A little higher optical density was observed with use of cyclohexanone than that with use of butanol which was proposed by Aminoff[3]. Factor IA did not have much effect on color formation.

The resorcinol method

Tests of significance in color formation indicate that factors IIB, IID, and IIA are important in this order at the critical rate of 0.5%, and that factor IIC does not

exert much influence on color formation. With respect to factor IIB, the maximum optical absorbancy was observed on heating for 60 min. Factor IIA resulted in the maximum optical absorbancy with use of 2 ml of 0.2% resorcinol solution, and factor IID gave the maximum optical density on chilling the tube for 10 min in an ice bath after addition of isopentyl alcohol. The conditions suggested by the study for factors IIA and IID are the same as those used originally by Svennerholm[5].

The direct Ehrlich method

Tests indicate that factors IIIB and IIIA influence color formation in this order at the critical rate of 0.5%. With respect to factor IIIB, the condition of the original procedure was that of heating for exactly 30 min in a boiling-water bath. In our experiments, heating for 90 min gave the maximum absorbancy. For factor IIIA, the use of excess reagent gave slightly higher optical density values, but factor IIIC did not exert much effect on color formation.

TABLE I

CALIBRATION CURVES FOR THE ASSAY OF *N*-ACETYLNEURAMINIC ACID BY THREE METHODS

Methods	*Calibration curves*[a]	*Analytical ranges* (μg)	*Standard error of Y* (%)
Thiobarbituric acid	$Y=25.90X-1.184$	3.5–16	4.5
Resorcinol	$Y=153.3X+3.242$	30–110	1.7
Direct Ehrlich	$Y=862.0X-69.07$	100–650	6.0

[a] Y is μg of *N*-acetylneuraminic acid and X is optical density.

A comparison was made in regard to the calibration curves, as shown in Table I. The resorcinol method gave a relatively lower standard error (1.7%) in comparison with those obtained with the thiobarbituric acid method (4.5%) and with the direct Ehrlich method (6.0%). Color formation is linearly proportional in the ranges 3.5–16.0 μg of *N*-acetylneuraminic acid for the thiobarbituric acid method, 30–110 μg of *N*-acetylneuraminic acid for the resorcinol method, and 100–650 μg of *N*-acetylneuraminic acid for the direct Ehrlich method.

In applying the methods to the assay of sialic acid in biological materials, it is of interest to determine if other substances interfere with color formation on use of these new conditions. There are several reports on interfering substances, especially neutral compounds, for the thiobarbituric acid method[2,3], for the resorcinol method[5], and for the direct Ehrlich method[14,15]. Alais and Jollès[16] and Eylar *et al.*[17] reported that the thiobarbituric acid method gave slightly lower values than those obtained by the resorcinol method, whereas Gibbons[18] noted excellent agreement between the two methods.

The results of our tests on the influence of acidic compounds are shown in Table II. The resorcinol method gave slightly higher results in the presence of other substances, and the thiobarbituric acid method showed interference effects similar

to that in the direct Ehrlich method. In regard to the thiobarbituric acid method, it is questionable whether an optimum amount of periodate will still be available to split *N*-acetylneuraminic acid in order to give a maximum color yield, because each of the compounds tested will take up varying amounts of periodate. Therefore, it is very important to remove other substances as completely as possible, by means of ion-exchange columns, in the assay of sialic acid.

TABLE II

EFFECTS OF SOME COMPOUNDS ON COLOR FORMATION[a]

Compounds[b]	*Resorcinol method, %*	*Thiobarbituric acid method, %*	*Direct Ehrlich method, %*
Hyaluronic acid (Na salt)	+ 13	+ 8	+ 13
Glycine	0	+ 5	+ 10
Heparin (Na salt)	+ 11	+ 12	+ 7
L-Glutamic acid	− 2	+ 7	+ 9
D-Glucuronolactone	+ 73	− 17	+ 10
Keratosulfuric acid (Ca salt)	+ 4	− 2	+ 7
Deoxyribonucleic acid	+ 37	− 25	+ 1
Ribonucleic acid	+ 55	− 1	− 10
Uridine 5-phosphate	+ 3	− 21	− 11
Adenosine 3-phosphate	+ 73	− 21	− 16
Cytidine 5-phosphate	+ 3	− 33	− 20
Guanosine 5-phosphate	+ 34	− 18	− 41
Adenosine 5-triphosphate	+ 62	− 19	− 48
None	0	0	0

[a] The experiment was performed with corresponding methods using the new conditions suggested in the study; + indicates increase in optical density and − indicates decrease in optical density in comparison with no addition of other compounds.

[b] One mg of each compound was examined in the presence of 9.65 μg of *N*-acetylneuraminic acid for the thiobarbituric acid method, of 53.5 μg of *N*-acetylneuraminic acid for the resorcinol method, and of 128 μg of *N*-acetylneuraminic acid for the direct Ehrlich method.

A comparison was made of the sialic acid contents of various biological tissues and fluids as determined by the three different methods using the new conditions, as shown in Table III. Differences between the values obtained with the resorcinol and thiobarbituric acid methods might arise, since the proportions of the different kinds of sialic acid in these biological materials are not known. However, the resorcinol method gave relatively little higher values in comparison with the thiobarbituric acid method, as observed in the test for interference in color formation. Body fluids of the silk worm, *Bombyx mori*, showed a positive color formation with the thiobarbituric acid method, but did not give any color formation with the resorcinol and direct Ehrlich methods; this effect is under investigation.

These experiments indicate that it is necessary to use at least two methods, such as the thiobarbituric acid and resorcinol methods, for estimating the sialic acid contents of biological materials.

TABLE III

SIALIC ACID CONTENTS OF SOME BIOLOGICAL MATERIALS ANALYZED BY THREE DIFFERENT METHODS[a]

Biological materials	*No. of samples*	*Thiobarbituric acid method*	*Resorcinol method*	*Direct Ehrlich method*
Human colostrum (second day after parturition)	1	294	339	
Human colostrum (fourth day after parturition)	5	138	167	
Human colostrum (sixth day after parturition)	4	67.7	147	
Acetone powder of human colostrum	2	1,110	1,705	
Cow colostrum (first day after parturition)	2	145	168	200
Cow colostrum (second day after parturition)	2	99.5	122	88.6
Cow milk	4	23.5	26.4	19.8
Acetone powder of cow colostrum	6	450	540	240
Body fluids of silkworm, *Bombyx mori*	3	21.3	0	0
Body fluids of silkworm, *Bombyx mori*, infected with polyhedrosis	3	84.3	0	0
Crude, bovine submaxillary gland	2	5,090	5,960	5,310
Egg white (hen)	2	15.6	18.4	22.0
Egg yolk (hen)	2	101	85.9	108

[a] Sialic acid (mg)/100 g of biological material. The sialic acid contents were estimated as *N*-acetylneuraminic acid.

ACKNOWLEDGEMENT

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SUMMARY

The assays of *N*-acetylneuraminic acid by the thiobarbituric acid, resorcinol, and direct Ehrlich methods were investigated comparatively by a statistical examination, in order to study the factors influencing color formation. Interference with color formation in the assay with the thiobarbituric acid method was similar to that with the direct Ehrlich method, and different from that with the resorcinol method.

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MUCOPOLYSACCHARIDES IN RAT SKIN

PART I. ISOLATION AND IDENTIFICATION

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INTRODUCTION

In recent years, the role of sulphated mucopolysaccharides in skin has excited much interest and there is evidence that, although present in very small amounts, they may be of considerable importance. For example, avitaminosis C causes reduction in the amounts of sulphated mucopolysaccharides and collagen formation[1]. Dramatic effects such as that induced in the epidermis *in vitro* by vitamin A, which inhibits keratin formation and causes accumulation of mucopolysaccharides in the cells, also indicate some vital function for these compounds[2,3].

However, the precise nature of the polysaccharides involved in these processes is not known. A mucopolysaccharide fraction was first isolated[4] from freshly-dissected pig skin by extraction with sodium hydroxide at 37°, and later from human skin by a similar method[5]. Digestion of pig skin with papain and trypsin at 37° to reduce contamination by protein[6], permitted the mucopolysaccharide fraction to be separated, by alcohol precipitation of the calcium salts, into hyaluronic acid and chondroitin sulphates B and C. Digestion of rat skin with papain at 60°, followed by trypsin at 37°, yielded mucopolysaccharide material which could be fractionated into chondroitin sulphate, hyaluronic acid, and heparin. This was achieved by utilising the differential solubility of the cetylpyridinium chloride (CPC) complexes followed by chromatography on an anion-exchange resin[7,8]. The individual chondroitin sulphates were not separated by this procedure.

These methods involved the use of very large amounts of tissue and, in many cases, did not give a clear-cut fractionation. In this study, all the skin polysaccharides were separated on Deacidite FF (Cl^- form). A column, once calibrated, can be used in conjunction with sulphate-labelling, to separate micro-amounts of mucopolysaccharide from biopsy specimens and hence to detect biologically-induced changes.

MATERIALS AND METHODS

The dorsal skin was removed from new-born rats which had been killed by cervical fracture. Each litter of 10–12 animals provided 4–5 g wet-weight of skin and

so 4–5 litters were required to provide the 20 g used for extraction purposes. After initial labelling, the skin was deep-frozen and bulked until the required weight had been collected.

For the individual labelling of the skin from each litter of rats, 1.0 mc of $Na_2{}^{35}SO_4$ was added to 20 ml of a tissue-culture medium consisting of 60% modified Hanks B.S.S., containing lactalbumen hydrolysate, antibiotics, and 40% horse serum. The final volume was divided between two petri dishes. The skin was cut into pieces (approximately 1 cm square) and placed with the dermis in contact with a sheet of lens tissue which lay upon a disc of stainless-steel gauze inside the petri dish. Support upon stainless steel in this manner, a modification of the method of Trowell[9], ensures that the skin remains at the surface of the tissue-culture medium. Incubation was carried out for 24 h at 37° in an atmosphere of 95% oxygen and 5% carbon dioxide.

The skin was minced with scissors, defatted with acetone followed by chloroform–methanol (2:1, v/v), air-dried, frozen in liquid nitrogen, and ground by mechanical means. It was then incubated for 20 h at 37°, with one fiftieth of its weight of the proteolytic enzyme ficin in the presence of its activator L-cysteine (0.007M), in 0.02M-acetate buffer (pH 5.8) on a slow shaker. Any residual protein was removed by shaking three times with chloroform–pentyl alcohol (10:1, v/v), followed by the addition of cold trichloroacetic acid to 10%. After standing at 4° for 30 min, the precipitate was removed by centrifugation and the solution dialysed against running water for 24 h before lyophilisation.

Deacidite FF (100–200 mesh; 7–9% cross-linking) was heated with 6N-hydrochloric acid followed by 6N-sodium hydroxide, and washed three times with 6N-hydrochloric acid. It was then suspended in water, packed as a slurry into a column (44 × 1 cm), and washed overnight with distilled water. The extract (5 mg) was placed onto the column and eluted (80 ml/h; 10 ml fractions) at ambient temperature using 20 ml water, 80 ml 0.5 M-NaCl, 80 ml 1.25 M-NaCl, 80 ml 1.5 M-NaCl, 80 ml 2.0 M-NaCl and 100 ml 4.0 M-NaCl (Fig. 1).

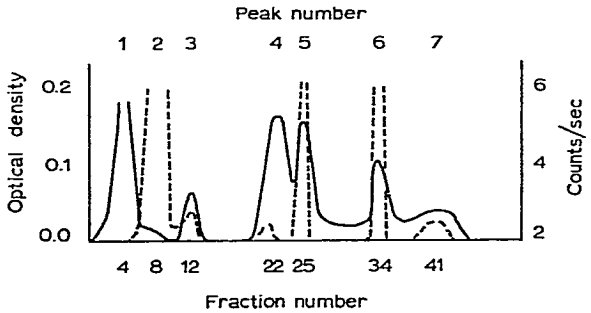

Fig. 1. Fractionation of mucopolysaccharide extract of rat skin on Deacidite FF. (——), orcinol reaction; (- - -), radioactivity.

Each fraction was dialysed against running water for 24 h to remove the sodium chloride, and the series scanned using the orcinol[10] and carbazole reagents[11]. In addition, each fraction was examined for radioactivity using fine filaments of NE102A

plastic scintillator (Nuclear Enterprises, Sighthill, Edinburgh). Positive fractions were bulked into individual peaks, freeze-dried, and dissolved in distilled water (10 ml). Extracts I, II and III (Tables I, III, and IV) refer to products from separate experiments which were fractionated as described above.

Identification methods

The following identification methods were employed:

(*a*) *Carbazole:orcinol ratio*

The uronic-acid contents of the fractions were assayed by the carbazole method of Dische[11] and the orcinol method of Svennerholm[10], using D-glucurono-6,3-lactone as a standard. Chondroitin sulphate B shows a depressed carbazole value due to the presence of L-iduronic and not D-glucuronic acid[12]. Chondroitin sulphates A and C, both of which contain D-glucuronic acid, have carbazole:orcinol ratios near to unity. Heparin[13], however, although containing D-glucuronic acid, gives a ratio greater than unity (Table I).

TABLE I

ANALYTICAL DATA ON RAT-SKIN MUCOPOLYSACCHARIDE COMPONENTS

Peak number	*Carbazole: orcinol ratio*	*N-sulphate: orcinol ratio*	*N-acetyl-oligo-saccharide[a]: orcinol ratio*	*Conclusion*
Extract I				
3	1.0	0.2:1	0.2:1	CS[b]–A
4	0.8	0.1:1	0.8:1	CS–C
5	0.3	0.3:1	0.3:1	CS–B
6	2.3 Carbazole at 430 mμ	0.9:1	0.3:1	Heparin
7	0.8	0.1:1	0.1:1	Keratosulphate
Extract II				
3	1.0		0.2:1	CS–A
4	1.0		0.8:1	CS–C
5	0.7		0.0:1	CS–B
6			0.0:1	
Standards				
Chondroitin sulphate (Evans Medical Ltd.)	1.0	0.1:1		
Heparin	2.1	0.9:1		
Keratosulphate	Carbazole at 430 mμ 0.8			

[a]Liberated by hyaluronidase and reactive to assay of Reissig *et al*[15].
[b]CS = Chondroitin sulphate.

The orcinol colorimetric method was used as a measure of the amount of mucopolysaccharide present. The intensity of colour produced by this reagent for uronic acid has been standardised for chondroitin sulphate and also for heparin (Table II).

TABLE II

THE QUANTITATIVE ORCINOL REACTION OF CHONDROITIN SULPHATE AND HEPARIN

Polysaccharide	*μg Equivalent glucurone*	*Calculated weight (μg) present*	*Actual weight (μg) present*
Chrondroitin sulphate	6.6	18.5	17.5
	6.5	18.2	17.5
Heparin	15.1	37.8	41.0
	17.6	44.0	41.0

This standard could not, however, be applied to keratosulphate which contains no uronic acid, but reacts with carbazole due to the galactose content. Maximum absorption appears at 430 mμ and not at 530 mμ.

(*b*) *Susceptibility to testicular hyaluronidase*

Chondroitin sulphates A and C are degraded when incubated with testicular hyaluronidase[14] and the oligosaccharides released may be estimated[15] using Ehrlich's reagent. As substitution in position 4 of the *N*-acetylgalactosamine moiety suppresses the colour formation in this reaction[16,17] the oligosaccharides of chondroitin sulphate A should not be detected, and any reaction can therefore be attributed to the presence of chondroitin sulphate C. Chondroitin sulphate B, heparin, and keratosulphate are not attacked by hyaluronidase.

(*c*) N-*Sulphate estimation*

Heparin carries *N*-sulphated hexosamine as part of the molecular chain. This may be estimated assuming that the mild conditions required to hydrolyse the *N*-sulphate group will not hydrolyse the *N*-acetyl groups present in the chondroitin sulphates[18].

(*d*) *Characterisation of amino-sugars after hydrolysis*

The hydrolysate (3N-hydrochloric acid for 6 h at 100°) obtained from whole rat tissue was fractionated on Dowex 50 (H^+ form)[19]. Elution with 0.3N-hydrochloric acid up to three times the volume required to elute glucosamine revealed after hexosamine assay that only glucosamine and galactosamine were present. The positive fractions were bulked, reduced to dryness, and the hexosamines further characterised by paper chromatography in butanol–pyridine–water (6:4:3)[20].

Heparin and keratosulphate contain 2-amino-2-deoxy-D-glucose (D-glucosamine) residues, while the three chondroitin sulphates contain 2-amino-2-deoxy-D-galactose (D-galactosamine). The two hexosamines may be distinguished by an adaptation[21] of the Elson–Morgan reaction[22]. The method depends upon the differing abilities of the amino-sugars to complex with borate, and thus suppress colour for-

mation with Ehrlich's reagent. With borate present, the colour intensity produced by glucosamine is reduced to between 24 and 30% of its original value, and that produced by galactosamine to between 50 and 65%. Since only these two hexosamines are present, they may be distinguished in this manner. For the quantitative determination of hexosamine, the Rondle and Morgan adaptation[23] of the Elson–Morgan reaction[22] was employed.

(*e*) *Uronic acid identification after hydrolysis*[24]

Glucuronic acid and iduronic acid were identified by their effluent positions upon a column of Dowex 1 (acetate form); elution was with 0.1N-sodium acetate. This resin is approximately equivalent to that used by Dziewiatkowski[24]. The iduronic acid used to calibrate the column was very kindly supplied as a gift from the Radiochemical Centre, Amersham, Bucks.

(*f*) *Sulphate estimation after hydrolysis*

Sulphate ion was estimated by the method of Dodgson[25].

(*g*) *Infrared spectra*

Infrared spectra of the separated fractions, and of reference substances where possible, were obtained using a Perkin–Elmer Model 21 spectrometer with rock-salt optics.

(*h*) *Column chromatography*

Samples of heparin, chondroitin sulphate, shark keratosulphate (a gift from Dr. Karl Meyer), and hyaluronic acid were passed down the column of Deacidite FF and eluted as described previously. Their effluent positions (Fig. 2) were found to correspond with those of certain of the skin-polysaccharide fractions. Adenosine 3-phosphate 5-phosphosulphate (PAPS), prepared from a guinea-pig liver by the method of Hilz and Lipmann[26], was eluted from the same column in fractions 6–10 and corresponds with peak 2 in the rat-skin separation (Fig. 1).

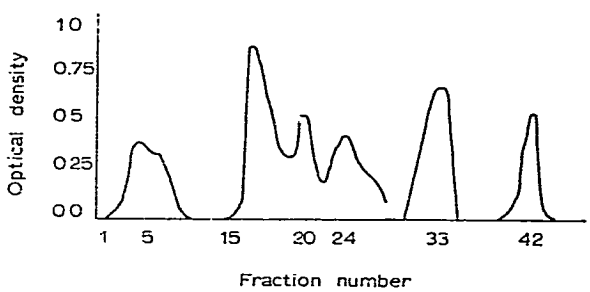

Fig. 2. Effluent position (fractions) on Deacidite FF of hyaluronic acid (5), chondroitin sulphate (15–24), heparin (33), and keratosulphate (42).

RESULTS

Fig. 1 indicates the effluent position of various peaks obtained from the mucopolysaccharide material of skin as shown by the orcinol reaction and the presence of radioactivity.

Peak 1 carried no sulphate and gave very high values with both the carbazole and the orcinol reagents, but also contained residual protein. It appeared in the position at which umbilical-cord hyaluronic acid was eluted, and was therefore regarded as a mixture of hyaluronic acid and protein.

Peak 2 did not occur when authentic samples of hyaluronic acid, chondroitin sulphate, heparin, or keratosulphate were passed down the column. It was also only slightly positive to the colour reagents, but carried the greater part of the radioactivity. When [^{32}P] phosphate and [^{35}S] sulphate were both incorporated into the tissue-culture medium prior to the incubation and extraction procedures, radioactive scanning showed that this fraction contained phosphate as well as sulphate. Since this fraction appeared to resemble "active sulphate" more closely than mucopolysaccharide it was investigated separately[27].

Peak 3 gave a carbazole:orcinol ratio of unity, consistent with the presence of glucuronic acid (Table I). The degree of stability shown by the *N*-substituent towards nitrous acid indicated *N*-acetylated hexosamine. This was confirmed by the presence of an N–H deformation mode in the infrared spectrum, which also indicated the presence of *O*-sulphate. In the fingerprint region (700–1000 cm^{-1}), the infrared spectrum was consistent[28] with the material being either chondroitin sulphate A or B.

The low value obtained in the detection of *N*-acetylhexosamine-containing oligosaccharides after incubation with testicular hyaluronidase indicated that either the molecule was unattacked by the enzyme or that it was substituted in position 4

TABLE III

GLUCOSAMINE AND GALACTOSAMINE CONTENT OF RAT-SKIN MUCOPOLYSACCHARIDE COMPONENTS

Concentration of galactosamine μg	*% Colour intensity[a] with borate present*	*Concentration of glucosamine μg*	*% Colour intensity[a] with borate present*
12.0	61	17.0	39
24.0	71	29.0	38
60.0	65	57.5	43
120.0	61	86.0	39
		115.0	39
Peak hydrolysate		*Peak hydrolysate*	
Extract I			
3	71	6	32
4	67	7	47
5	72		
Extract II			
4	73	6	42
		7	43

[a]Elson–Morgan test[21–23]

(Table I). The use of borate in conjunction with the Elson–Morgan reaction indicated that galactosamine was present (Table III). It was concluded that peak 3 was chondroitin sulphate A.

Peak 4 gave a carbazole:orcinol ratio of 0.8 (Table I) indicating the presence of glucuronic acid. The very low value obtained in the *N*-sulphated hexosamine determination precludes the possibility that the fraction contains heparin or heparitin sulphate. The material was susceptible to attack by testicular hyaluronidase and the liberated *N*-acetylhexosamine oligosaccharides were detectable by the assay of Reissig *et al.*[15] It was concluded that peak 4 must be mainly chondroitin sulphate C. The detection of galactosamine (Table III) and glucuronic acid in the hydrolysed material supported this conclusion. Finally, the infrared spectrum corresponded fairly closely with that reported by Mathews[28] for chondroitin sulphate C, with bands at 775 and 1000 cm^{-1}.

Peak 5 (carbazole:orcinol ratio well below unity) showed the depression of colour formation in the carbazole reaction associated with the iduronic acid present in chondroitin sulphate B.

In the estimation of *N*-sulphated hexosamine, the degree of lability shown by the *N*-substituent was somewhat surprising, but the molecule was fairly resistant to attack by testicular hyaluronidase. Furthermore, the detection of galactosamine by the method of Tracey[21] (Table III) and of iduronic acid by separation upon a column of Dowex 1 (acetate form) indicated the presence of chondroitin sulphate B. The infrared spectrum of this material showed bands at 855 and 920 cm^{-1} in the fingerprint region, resembling those reported by Mathews[28] for chondroitin sulphate B.

Peak 6 was eluted in a position similar to that of heparin (Figs. 1 and 2) and the carbazole:orcinol ratio exceeded unity as did that of an authentic sample of the polysaccharide. The heparin-like character of the material was confirmed by the high value obtained in the estimation of *N*-sulphated hexosamine. Glucosamine was detected in the borate–Elson–Morgan determination (Table III) and the column separation of uronic acids indicated mainly glucuronic acid with traces of other orcinol-positive materials, which were eluted subsequently to the main fraction. Peak 6 and an authentic sample of heparin had infrared bands in the fingerprint region at 940, 890 and 800 cm^{-1}.

Peak 7 contained hexose but no uronic acid (carbazole reaction, Table I). Because of this, none of the tests using the reaction of D-glucurono-6,3-lactone with orcinol as a quantitative standard was applicable. This material represented only a very small percentage of the extract and in effluent position it corresponded with the main band obtained from shark-cartilage keratosulphate. Glucosamine was detected after hydrolysis (Table III) and one sample, kindly tested for us with galactose oxidase by Dr. G.I. Pardoe, contained galactose. It was therefore concluded that peak 7 must be keratosulphate.

Further amounts of the major fractions from another extract were assayed for the ratios of their component moieties. Hydrolysis was in 3N-hydrochloric acid for 6 h at 100°. The results obtained are shown in Table IV.

TABLE IV

ANALYTICAL DATA FOR RAT-SKIN MUCOPOLYSACCHARIDE COMPONENTS

Peak number	*Carbazole:orcinol ratio*	*Molar ratio hexosamine:uronic acid (orcinol)*	*Molar ratio sulphate:uronic acid (orcinol)*
Extract III			
3	1.1	0.4:1	0.7:1
4	1.0	1.0:1	1.2:1
5	0.7	1.0:1	1.0:1
6	3.7	1.0:1	1.6:1
7	0.5	0.3:1	1.1:1

DISCUSSION

A technique for the simultaneous separation of all the polysaccharides of whole skin has been found to give consistent results with several extracts and, in part at least, this may be attributed to the thorough removal of protein by ficin. When trypsin, papain, pepsin, or sodium hydroxide-extraction were used in preliminary experiments, polysaccharide was obtained, but a high proportion of accompanying protein made separation and identification difficult. In the present method, residual protein was eluted together with hyaluronic acid, and the remaining polysaccharides contained only small amounts of bound peptides.

The presence of chondroitin sulphates B and C and heparin in rat skin has been confirmed, and in addition smaller amounts of chondroitin sulphate A and keratosulphate were detected. The extracts were prepared from samples (*ca.* 20 g) which were very much smaller than those used by previous workers[6,7].

The total amount of polysaccharide from the 20 g batches of tissue fractions was only about 20 mg, consisting of approximately 50% hyaluronic acid and 50% sulphated mucopolysaccharide, most of which was chondroitin sulphates B and C. Most of the techniques described above enable the individual polysaccharides to be characterised on this scale. Further experience with the fractionation technique and its application to other tissues, *e.g.* cartilage, has given us confirmation of the reproducibility of the method. When [^{14}C] iduronic acid was tissue-cultured with human skin and the resulting product submitted to the above fractionation, it was found that the only polysaccharide labelled after 4 h appeared on the column in the position of chondroitin sulphate B.

The method appears particularly promising when applied to biopsy specimens since the incorporation of $^{35}SO_4^{2-}$ by a tissue-culture method permits the study of even smaller amounts of tissue. For example, it is possible to obtain a separation pattern on the basis of scanning for radioactivity alone when there is insufficient material to scan with the colour reagents. This provides a method of further studying polysaccharide metabolism and tissue abnormalities. It has already been established that

substances such as vitamin A and hydrocortisone alter the amount of [^{35}S] sulphate taken up by skin and that more than 60% of that sulphate is incorporated into the mucopolysaccharide fraction[29]. By the use of [^{14}C] glucose instead of [^{35}S] sulphate it has also been established that the actual amount of mucopolysaccharide was increased, and that the vitamin A effect was not just due to over-sulphation.

It is therefore permissible to use $^{35}SO_4^{2-}$ incorporation as a reliable indicator of mucopolysaccharide changes. Changes in the amount of activity, however, are related not only to the amount of mucopolysaccharide but also to the rate of turnover. In some experiments the keratosulphate was only weakly active, thus reflecting a slow turnover rate.

It has been reported[30] that in cartilage the proportion of keratosulphate increased with age. It is therefore interesting that in the skin of new-born rats it should be detectable in small amounts. No data are yet available concerning the proportions of this polysaccharide in older skin.

SUMMARY

A technique has been devised for the separation of the polysaccharides of rat skin on a single column of Deacidite FF resin. A combination of micromethods in conjunction with $^{35}SO_4$ labelling has been employed to characterise the individual polysaccharides on a microgram scale.

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MUCOPOLYSACCHARIDES IN RAT SKIN

PART II[1]. CHARACTERISATION OF A NOVEL SULPHATED NUCLEOTIDE

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INTRODUCTION

From mucopolysaccharide extracts of [^{35}S] sulphate-labelled rat skin, fractionated on Deacidite FF (Cl^- form), material can be obtained[1] which contains phosphate and sulphate, but little carbohydrate as shown by reaction with the orcinol[2] and carbazole[3] reagents. The very high level of radioactivity in the material, and the lability of the sulphate, suggested that it might be a mucopolysaccharide intermediate, or a substance resembling adenosine 3-phosphate 5-phosphosulphate (PAPS).

Robbins and Lipmann[4] established that ATP-linked sulphate activation involved the production of PAPS, and several investigators have demonstrated the transfer of sulphate from the latter to various polysaccharide acceptors. Suzuki and Strominger[5] extracted from hen isthmus an enzyme which would catalyse the transfer of [^{35}S] sulphate from PAPS to chondroitin, and chondroitin sulphates A, B and C. Similarly, Spolter and Marx[6], working with mast-cell tumours, demonstrated that [^{35}S] PAPS served as a sulphate donor to heparin.

This study is concerned with the elucidation of the nature of a phosphate-containing fraction from rat skin, and the preparation of a similar material from guinea-pig skin. A comparison is made between these fractions and PAPS prepared from guinea-pig liver.

MATERIALS AND METHODS

Active phosphate-containing fractions were prepared from the skin of new-born rats and from adult guinea-pig skin, as described previously[1].

Preparation and purification of PAPS from guinea-pig liver

The ATP-sulphurylase and APS-kinase necessary to effect the biosynthesis of PAPS from adenosine 5-triphosphate (ATP) were obtained from guinea-pig liver by the method of Hilz and Lipmann[7]. The enzyme system also contains a phenol sulphokinase which enables the activity of the isolated protein mixture to be assessed

via the sulphation of *p*-nitrophenol, which has an absorption maximum in alkaline solution at 420 mμ. Phenol sulphokinase transfers sulphate from PAPS to various phenolic acceptors. *p*-Nitrophenol sulphate gives no colour in alkaline solution so the reaction provides a test for the presence and activity of the enzyme system. Once it had been established that the activation system was present, then a system was set up for the biosynthesis of PAPS.

The protein-free incubation mixture was placed onto a column (1.6 × 15 cm) of Dowex 1 (formate form, 200–400 mesh), prepared by the method of Siekevitz and Potter[8], and maintained at 4°. The column was washed with water (50 ml) followed by 4N-formic acid–0.3N-ammonium formate, until the optical density had fallen below 0.1 at 260 mμ. The PAPS was then eluted with 5N-formic acid–N-ammonium formate, 10 ml fractions being collected[4] (see Fig. 1).

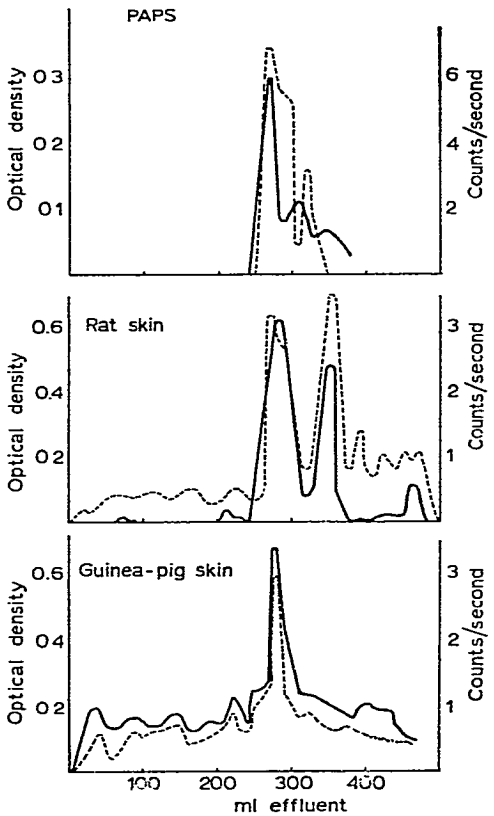

Fig. 1. Fractionation of nucleotide sulphates on Dowex 1 (formate form). (- - -), radioactivity; (——), optical density at 260 mμ.

Comparison of prepared PAPS and the phosphate-containing fraction

(a) High-voltage electrophoresis

Portions of each fraction were spotted onto Whatman No. 3MM paper along with $Na_2{}^{35}SO_4$ and a mixture of adenosine mono-, di-, and tri-phosphates as markers,

and subjected to electrophoresis at 28 volts per cm for 90 min. The buffer solution consisted[9] of 50% 0.05M-citrate–phosphate (pH 6.5) and 50% 0.1M-Tris-acetate (pH 6.5). After drying, the electrophoretogram was affixed to Kodirex X-ray film and exposed for 3 days. After development of the film, the electrophoretogram was exposed to ultraviolet light, and the positions of absorbing spots were marked.

(b) Low-voltage electrophoresis

Electrophoresis of prepared PAPS and the phosphate-containing fraction was carried out with the same markers at 8 volts per cm for 5 h. The individual materials were placed as bands across 2″ strips of Whatman No. 1 paper and separated in 0.1M-citrate buffer[10] (pH 5.5). On the dried strips, the radioactive material was visualised by exposure of an X-ray film, and u.v.-absorbing spots were marked as before.

(c) Two-way chromatography[11]

Whatman No. 1 paper was developed overnight in (1) 0.05M-ammonium formate (pH 4.3)–isopropanol (3:2), dried, turned through 90°, and irrigated for 17 h in (2) isobutyric acid–M-ammonium hydroxide–0.1M-sodium versenate (100:60:1.6). Radioactive and u.v.-active spots were detected as before.

(d) Column chromatography

A portion of the prepared PAPS was placed onto a column of Deacidite FF (Cl^-form) and eluted with increasing concentrations of sodium chloride, as described previously[1]. The eluate was scanned both for radioactivity and absorption at 260 mμ.

Further purification and study of the phosphate-containing fractions

The fraction was dissolved in a minimum of distilled water, placed onto a column of Dowex 1 (formate form) as described previously, and eluted with water (20 ml), 4N-formic acid–0.3N-ammonium formate (200 ml), and finally 5N-formic acid–1N-ammonium formate. The eluate was scanned for radioactivity, and u.v. absorption at 260 mμ (Fig. 1). Positive fractions were bulked, dialysed free of formate, lyophilised, dissolved in 10 ml of deionised water, and subjected to the following procedures.

*(1) Pentose and 2-deoxy-*D-erythro-*pentose determinations*

Portions of the separated peaks were hydrolysed with 10% trichloroacetic acid (0.5 ml) at 100° for 15 min, and separately analysed for pentose[12] and 2-deoxy-D-*erythro*-pentose[13].

(2) Phosphate determination

Orthophosphate was determined by the method of Berenblum and Chain[14] after hydrolysis with either N-hydrochloric acid at 100° for 30 min (30 min phosphate), or a mixture of 60% (w/w) perchloric acid (analytical grade) and conc. sulphuric acid (microanalytical grade) (2:3, v/v) for 5 min at 100° (total phosphate).

(*3*) *Sulphate determination*

Sulphate was determined, after hydrolysis with 2N-hydrochloric acid at 100° for 16 h, by the method of Jones and Letham[15]. In this method the phosphate is removed prior to the determination of sulphate, and so does not interfere.

(*4*) *Identification of nucleotide bases*

Purines can be released from nucleotide fragments during mild hydrolysis procedures, but are destroyed by vigorous treatment, whereas release of the pyrimidine bases requires a far more drastic hydrolysis. Two hydrolyses were performed. For the first, portions of each peak component were treated with N-hydrochloric acid at 100° for 1 h, then the acid was removed *in vacuo* over potassium hydroxide pellets, and the hydrolysate redissolved in a minimum of distilled water. Such treatment releases the purines, but the pyrimidines remain bound in cytidylic or uridylic acids.

A further portion of each peak was evaporated to dryness *in vacuo*, and hydrolysed in 72% perchloric acid (1 ml) for 1 h at 100°. The hydrolysate was neutralised with potassium hydroxide and concentrated to 0.5 ml, and the precipitated potassium perchlorate was centrifuged off; more potassium perchlorate was removed after further concentration and cooling to 0°. The hydrolysates were finally evaporated to dryness and redissolved in a minimum of distilled water.

Each hydrolysate was examined by paper chromatography using Whatman No. 1 paper, and development for 15 h at 27° with (1) a mixture of isopropanol (68 ml), conc. hydrochloric acid (16.4 ml), and water (15.6 ml)[16], or (2) water saturated with butanol[17]. The nucleotides bases were detected by exposing the paper to u.v. light. The u.v.-absorbing spots were eluted from the chromatograms with 0.1N-hydrochloric acid, and the u.v. spectrum of each was determined using a Unicam SP 500 spectrophotometer. The blanks were obtained by eluting from a similar strip of the developed chromatogram.

(*5*) *Identification of amino-acids*

The final portions of each peak component were hydrolysed in 6N-hydrochloric acid at 100° for 16 h, and the acid was removed *in vacuo* over potassium hydroxide. The hydrolysates were then subjected to two-way paper chromatography in butanol–ethyl methyl ketone–water–ammonia (5:3:1:1) followed by butanol–acetic acid–water (4:1:5)[18]. The chromatograms were developed with ninhydrin. In later work hydrolysates were subjected to automatic analysis on the Technicon Amino-acid Auto-analyser.

Sulphate donor ability

A portion of the phosphate-containing fraction from a Deacidite FF (Cl^--form) column was incorporated into a tissue-culture medium as the sole source of radioactivity. Several pieces of fine human-skin were then incubated upon this medium for 4 h. After removal from the medium, some portions of skin were treated with 0.15%

trypsin prior to separation into dermis and epidermis, while the others were retained as whole skin. All skin fragments were maintained at —70° for several minutes, dialysed against 0.1M-sodium sulphate, dried to constant weight, and counted[19]. The specific activity of each piece could then be calculated. To test that any apparent "donation" was not due solely to surface adsorption, the procedure was repeated with a piece of dead human-skin as recipient. Skin was regarded as dead after maintenance at —70° for several minutes, and such skin did not adsorb or incorporate activity from the medium.

RESULTS

Electrophoresis and Chromatography

The electrophoretic data obtained for both prepared PAPS and the phosphate-containing fraction obtained from rat skin are shown below. The fractions do not separate under the conditions employed.

	MOBILITY (SO_4^{2-} M = 1)		
Buffer	*ATP*	*Phosphate-containing fraction*	*PAPS*
0.1M-citrate (pH 5.5)	0.50–0.56	0.83	0.77–0.83
Tris-acetate/citrate–phosphate (pH 6.5)	0.50–0.53	0.83	0.77

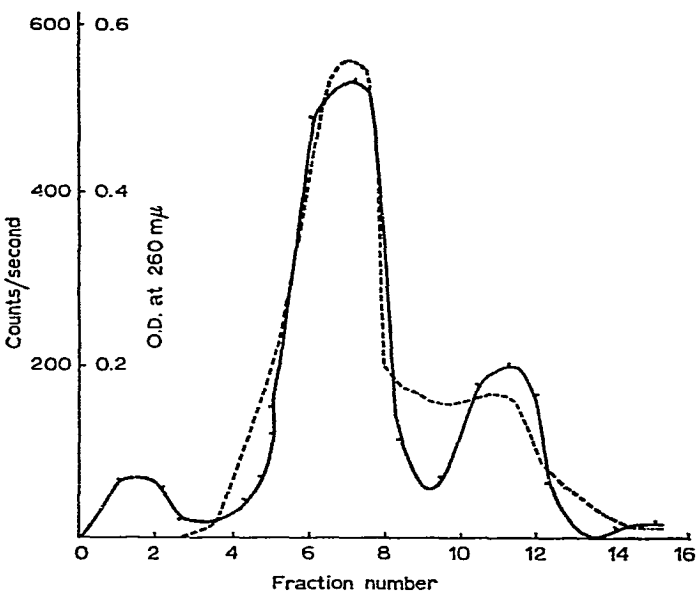

Fig. 2. Fractionation of guinea-pig liver PAPS on Deacidite FF (Cl^- form). (——), radioactivity; (.....), optical density at 260 mμ.

In the two-way chromatographic examination, PAPS had R_{ATP} 1.1 and 0.55, and the active phosphate-containing material R_{ATP} 1.0 and 0.45, in solvents 1 and 2, respectively.

Column chromatography of the prepared PAPS upon Deacidite FF (Cl^- form) exactly as carried out in the preparation of the phosphate-containing fraction, resulted in elution of a radioactive and u.v.-absorbing peak in fractions 6–8, the elution position of the peak containing active phosphate material (Fig. 2).

The active material from guinea-pig skin appeared in the same position as PAPS when eluted from a Dowex 1 (formate form) column. The corresponding material from rat skin appeared as two peaks, with the major one corresponding with PAPS, and the second peak five fractions later.

Table I summarises the analytical data for these peaks. The figures for peak 1 obtained from rat skin and for the peak from guinea-pig skin are in agreement with those quoted by Robbins and Lipmann[4] for their "active sulphate fractions". Peak 2 in rat skin contained cytosine, pentose, sulphate, and phosphate, but no adenine.

TABLE I

ANALYTICAL DATA FOR THE PHOSPHATE-CONTAINING PEAKS OBTAINED ON FRACTIONATION OF EXTRACTS FROM RAT AND GUINEA-PIG SKIN[a]

	Peak	*Phosphate:*	*Sulphate:*	*Ribose:*	*Adenine or: cytosine*	30 *minute phosphate*
Rat skin	1	1.8	1.0	1.1	0.75	1
	2	0.8	1.0	0.9	0.5	trace
Guinea-pig skin	1	1.6	1	0.8		

[a]Assays for deoxyribose were negative. Adenine and cytosine were determined after recovery from a paper chromatogram.

TABLE II

CHROMATOGRAPHY OF THE BASES RECOVERED FROM THE PHOSPHATE-CONTAINING PEAKS

Base	R_F *in solvent 1*	R_F *in solvent 2*	λ_{max}
Adenine	0.36	0.43	261
Guanine	0.24		
Uracil	0.70		259
Thymine	0.78		264
Cytosine	0.48	0.34	265
Adenosine	0.38	0.33	
Cytidylic acid	0.58		
Peak 1 (HCl hydrolysate)	0.32	0.38	260
Peak 1 ($HClO_4$ hydrolysate)	0.34	0.43	262
Peak 2 (HCl hydrolysate)	0.53		
Peak 2 ($HClO_4$ hydrolysate)	0.44	0.36	266

The R_F values obtained on paper chromatography of the nucleotide fragments present after both mild and rigorous hydrolysis are compared in Table II. The R_F values in solvent 2 were found to be very dependent on temperature. Further confirmation of the identity of the bases was obtained by plotting the ultraviolet spectra of the components eluted from the chromatogram. This latter procedure also permitted a quantitative estimation of base concentration, using $\varepsilon_{263} = 13.1 \times 10^3$ for adenine and $\varepsilon_{274} = 10.2 \times 10^3$ for cytosine. The values obtained are, however, dependent upon the achievement of efficient hydrolysis of the nucleotide, without destruction of the base.

The adenine-containing fractions from the skin of both new-born rat and guinea-pig ear contained the following amino-acids: aspartic acid, glutamic acid, glycine, serine, valine, alanine, and leucine. The cytosine-containing component from rat skin contained the same seven amino-acids, with the addition of threonine.

TABLE III

THE INCORPORATION OF $^{35}SO_4^{2-}$ INTO SKIN FROM $Na_2^{35}SO_4$ AND "ACTIVE FRACTION"

	Medium counts/ml/sec	*Skin counts/g/sec*
$Na_2^{35}SO_4$	70.0	119
	67.0	116
	65.4	109
"Active fraction"	27.0	430
	27.0	420

The phosphate-containing component was about ten times more efficient than sulphate ion as a sulphate donor to skin. The comparative experiments were performed with approximately the same amount of radioactivity added to the medium for either donor (Table III). Since the phosphate-containing fraction is the more efficient donor, it cannot donate sulphate ion via a dissociation process.

DISCUSSION

Material which is very similar to PAPS has been isolated from the skin of both rat and guinea-pig. When examined electrophoretically in two buffers, by paper chromatography, or by passage through two anion-exchange columns, it was indistinguishable from PAPS synthesised by the method of Hilz and Lipmann[7]. The material contained phosphate, adenosine, ribose, and sulphate, and acted as a very efficient sulphate-donor to skin.

The main difference between our material and PAPS was that the former was associated with several amino-acids. Both our material and the material prepared after Hilz and Lipmann[7] were largely retained in the sac during dialysis. Enough

activity still remained to mark an X-ray film after electrophoresis of the dialysed material. We have found no reports, other than that of Torii and Bandurski[20], describing dialysis of PAPS. These authors were working with an enzyme system which reduced the [^{35}S] sulphate of PAP^{35}S to [^{35}S] sulphite, and then coupled it to a long-chain moiety giving X-$^{35}SO_3^-$. They did not stop this reaction before commencing dialysis, and the PAP^{35}S activity may have been lost as dialysable sulphite. In fact, they continued the reaction long enough for all of the X-$^{35}SO_3^-$ activity to exchange with unlabelled SO_3^{2-} ion, and dialyse away.

Baddiley *et al.*[21] have achieved a chemical synthesis of both PAPS and adenosine-5-phosphosulphate (APS). We assume that our material contains PAPS which is weakly bound to a peptide chain. Since isolation of the material involves rigorous removal of protein [denaturing with chloroform–pentyl alcohol (10:1 v/v), followed by precipitation with trichloroacetic acid], the peptide chain must be very short. Since the material had already been incubated with the proteolytic enzyme ficin at an earlier stage, it may not occur thus *in vivo*.

The extreme lability of the sulphate group makes purification of the material very difficult. Passage through Dowex 1 (formate form) with elution by 5N-formic acid–M-ammonium formate caused 90% of the radioactivity to be lost, even when the operation was carried out at 4°. This difficulty has prevented the testing of individual subfractions for their ability to donate [^{35}S] sulphate to skin.

Hitherto, cytosine has not been reported as being involved with a precursor stage of mucopolysaccharide biosynthesis, but the base is a component of cytidine 5-(*N*-acetylneuraminic acid monophosphate) and the cytidine pyrophosphate derivatives of ribitol, glycerol, and choline, as well as occupying the terminal position in transfer ribonucleic acid. Derivatives of uracil which form the nucleotide–polysaccharide moiety involved in polysaccharide back-bone synthesis were, as one might expect, not detected. They would have been lost in the first dialysis step. The cytosine-containing fraction was detected only in the skin of new-born rats, not in the skin of the adult guinea-pig. The adenosine-containing fraction from the two types of skin appeared to be identical; even the same amino-acids were present in identifiable amounts.

SUMMARY

From rat skin and from guinea-pig skin a material has been isolated which is indistinguishable from adenosine 3-phosphate 5-phosphosulphate (PAPS). In addition, rat skin appears to contain a sulphated cytidine monophosphate.

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STUDIES ON THE BIOSYNTHESIS OF STARCH GRANULES

PART III*. THE PROPERTIES OF THE COMPONENTS OF STARCHES FROM THE GROWING POTATO TUBER

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INTRODUCTION

It is now well-established that during growth there are changes in the amount of starch, and the characteristics of the starch granules, stored in the plant. Such studies have been carried out on maize[1,2], sweet corn[3], barley[4], smooth-seeded and wrinkled-seeded peas[5], and tobacco leaves[6]. The general pattern resulting from this work is that as the plant matures, its starch-content increases, whilst there is concurrently an increase in both the average size of the granule and the amount of the amylose component that it contains. In the case of potato starch, the work of Halsall *et al.*[7] indicated that the percentage of amylose remained constant for two varieties of the growing tuber. There is evidence, however, that the amylose-content may vary with the botanical variety of tuber[8], and our earlier results on the starch from the tuber and shoots of the sprouting tuber[9] indicated that immature potato starch may contain less amylose than the mature granules.

In this work, therefore, we have studied the physical and chemical properties of starch isolated from the growing potato tuber. Furthermore, the properties of the amylose and amylopectin components have been studied, for apart from our work on pea starches[5], there is little evidence in the literature as to whether changes occur in the fine-structure of the components during growth. The occurrence and properties of starch material differing in character from conventional amylose and amylopectin[10] have also been investigated. Such results have an important bearing on theories of starch-biosynthesis.

EXPERIMENTAL

Growth and isolation of the starches

The potatoes (var. Pentland Crown) were grown at the Scottish Plant Breeding Station, Pentlandfield, Roslin, Midlothian, Scotland, in the 1962-season. Tubers were harvested at intervals of ten days after the appearance of leaves on the plants, and were separated into sizes varying from < 1 to 15 cm in longitudinal diameter. Starch was isolated from the different-sized tubers, and was purified by the method given earlier[9].

*For Part II, see ref. 5.

In a similar manner, starch was also isolated from the shoots (*i.e.* the underground stems devoid of tubers) and the original seed-tubers (6–7 cm diameter) from mature plants, the dry growing-season leaving the latter intact.

Estimation of the percentage of starch in the plant material

The fresh potato sample (200 mg) was ground with an equal weight of silver-sand, and then heated for 30 min at 98°. After cooling in ice, perchloric acid (72%; 6 ml) was added. The mixture was then extracted for 20 min before water (40 ml) was added and the whole was centrifuged. The residue was then re-extracted two or three times with perchloric acid until the washings gave no iodine-stain. The combined supernatant liquor and the washings were then made up to a standard volume (500 ml), and aliquots were taken for estimation of starch-content by the phenol–sulphuric acid method of Dubois *et al.*[11]. Duplicate estimations gave results agreeing to $\pm$ 3%.

Characterization of the starches

The granular size distribution, the number-average particle diameter, the percentage of phosphorus, the average gelatinization temperature, and the iodine affinity of the starches were determined as described by Banks and Greenwood[9].

Fractionation of the starches

The starch samples were pretreated with liquid ammonia[12], prior to their dispersion into water by boiling for 1 h under nitrogen, and the addition of thymol to precipitate the amylose. The amylose was purified as the butan-1-ol complex. The amylopectin was obtained by freeze-drying the supernatant liquors after removal of the amylose-complex. These methods have been given in detail elsewhere[12].

In each fractionation, the anomalous amylopectin fraction[10] was also isolated from the supernatant liquor from the recrystallization of the initial thymol-amylose complex.

Subfractionation of the anomalous amylopectin

Subfractions were obtained by the stepwise addition of ethanol to a 0.25% solution of the intermediate material from starch 6 in dimethyl sulphoxide at 35°. Precipitates were washed with ethanol, redispersed in water, and freeze-dried. Seven fractions were obtained.

Characterization of the fractionation products

Methods described in the earlier parts of this series[5,9] were used to measure:

(*i*) *Iodine affinity*, to obtain the purity.

(*ii*) *Percentage of phosphorus*, except that the optical density of the reduced-phosphomolybdate-complex was measured at its maximum of 820 mμ. The optical density was then almost twice that at the wavelength of 675 mμ used by Fogg and Wilkinson[13], and with this modification, the method using ascorbic acid as reducing agent was as sensitive as that suggested by Bartlett[14].

(*iii*) *Limiting viscosity number* [η] in M-potassium hydroxide at 25°.

(*iv*) *Percentage conversion into maltose* under (a) the action of purified β-amylase, and (b) the concurrent action of β-amylase and Z-enzyme.

(*v*) *Average length of unit-chain* of amylopectin-materials by periodate oxidations. (Our procedure has also been detailed elsewhere[15]).

(*vi*) *Sedimentation measurements* of aqueous solutions of amylose and amylopectin.

(*vii*) *Molecular weight by light-scattering* for amylopectins, except that Millipore filters were used for clarification [type VM (50 mμ) for solvent, and type RA (1.2 μ) for solutions], and the angular scattering was measured in cylindrical cells in the range 30–120°.

Separation of starch granules into fractions of differing size

Starch 6 (30 g) was separated into 5 fractions of granules of differing size by the technique of Decker and Hoeller[16], the granules being allowed to sediment through distilled water. The resultant distribution curves are shown in Fig. 1.

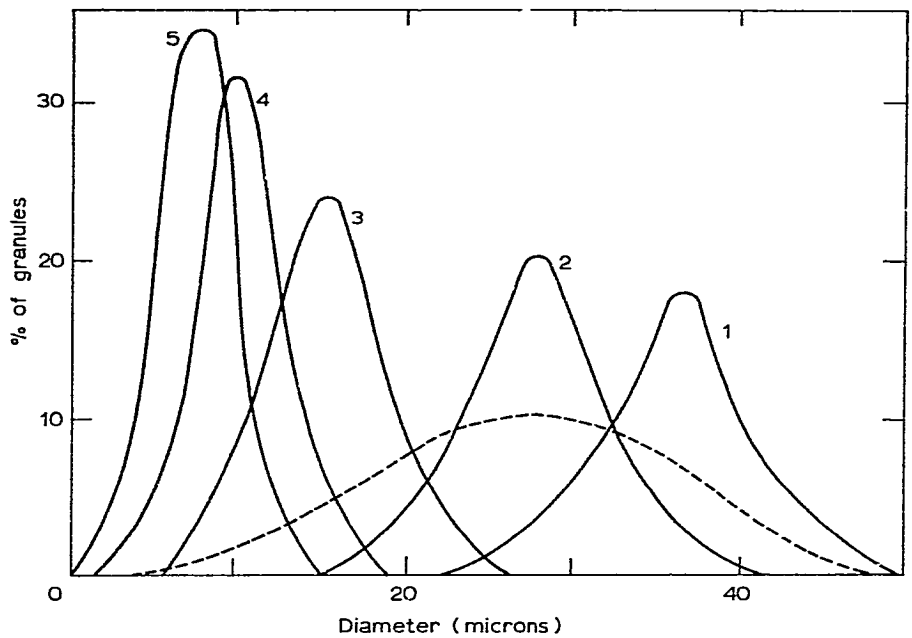

Fig. 1. Number-average-size distribution curves of the granules of starch 6(- - -) and its subfractions (curves 1–5).

RESULTS AND DISCUSSION

Properties of the granular starches

An exact criterion for assessing the maturity of potato starch is more difficult to establish than is the case for the cereal starches. In the first instance, we used the

size of the potato tuber. Although this may be influenced by unequal growth rates rather than differences in age, potato tubers harvested at different times were graded on a size-basis. Starch was also isolated from the underground roots and the residual seed-tuber. Table I shows that the starch content increased with increase in size of the tuber, from a lower value of 5% up to an apparent limit of about 18%. (The starch-content of the 0–1 cm tuber was equivalent to that for the shoots, whilst that for the seed-tuber was reduced to 3%.) Furthermore, the properties of the granular starches isolated from the tubers of increasing size show (Table I) the same general trends as have been attributed in previous work to changes in maturity (see Introduction).

The variation in the percentage of nitrogen present in the starches is purely random; the corresponding low protein-contents illustrate the efficiency of the purification procedure. (The higher values for starch R were due to the difficulty of separating the small amount of starch present from the fibrous, residual tuber-tissue). All the fifteen starch samples contained comparable amounts of phosphorus, and there was no apparent trend with increase in maturity of the tuber (*cf.*, however, ref. 17). Although phosphorus is thought to be present as the ester-phosphate, there is the possibility that some may arise from contaminating phosphorus-compounds, *e.g.* nucleic acid and phospholipids.

Fig. 2(a) shows that the increase in average granular size was directly proportional to the size of tuber; indeed, other evidence [see Fig. 2(b)] suggests that a more fundamental criterion for maturity is the size of the granule.

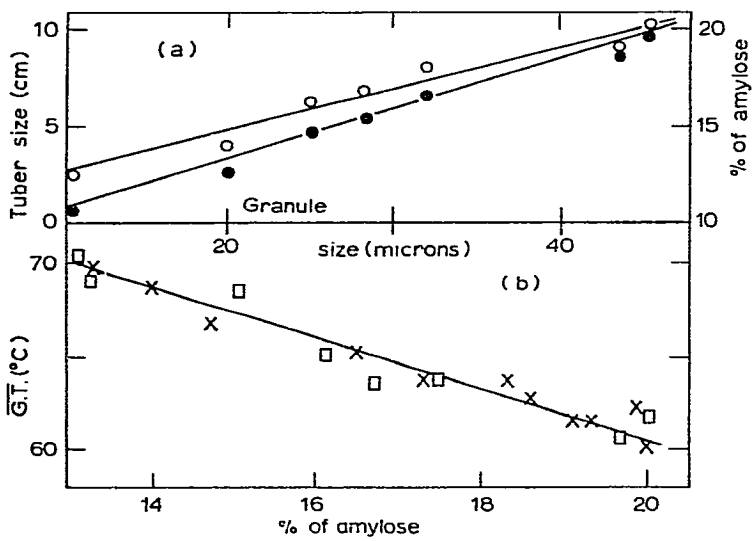

Fig. 2. (a) Variation of tuber size (-●-) and percentage of amylose (-○-) with average granule size. (b) Variation of average gelatinization temperature ($\overline{G.T.}$) with percentage of amylose in the starch (-×-) and the average granule size (-□-).

In agreement with our earlier observations on pea starches[5], the increase in maturity of the potato starch was associated with a large overall change in the iodine

TABLE I

PROPERTIES OF THE STARCH GRANULES

Average gelatinization temperature is the temperature at which 50% of the granules are gelatinized. Iodine affinities are expressed as: mg of iodine bound per 100 mg of starch.

Size of tuber (cm)	*Starch content (% fresh weight)*	*Starch sample*	*No.-av. granular diam. (μ)*	*Phosphorus (%)*[a]	*Nitrogen (%)*	*Av. gelatinization temp.*	*Iodine affinity*	*Amylose (%)*[b]
0–1	5.0	1	10.5	0.05	0.003	70.0	2.43	12.5
1–2	5.6	2	n.d.	0.05	0.010	69.5	2.56	13.2
2–3	6.4	3	20	0.03	0.012	68.5	2.70	13.9
3–4	8.3	4	n.d.	0.03	0.007	66.5	2.85	14.6
4–5	9.2	5	25	0.04	0.008	65.0	3.20	16.4
5–6	11.0	6	28	0.05	0.007	63.5	3.35	17.2
6–7	13.4	7	32	0.04	0.010	63.5	3.55	18.2
7–8	17.0	8	n.d.	0.05	0.011	62.5	3.60	18.5
8–9	17.5	9	43	0.04	0.008	61.5	3.70	19.0
9–10	17.5	10	n.d.	0.04	0.006	61.5	3.74	19.2
10–11	18.0	11	45	0.06	0.010	62.0	3.85	19.8
14–15	n.d.	12	n.d.	0.04	0.007	60.5	3.90	20.0
15–16	n.d.	13	n.d.	0.04	0.006	59.5	3.90	20.0
Residual seed-tuber	3.0	R	30	n.d.	0.050	61.5	3.80	19.5
Shoot	5.0	S	16	n.d.	n.d.	70	2.40	12.4

n.d. = not determined.

[a] This value was unchanged after exhaustive extraction with boiling 80% aqueous methanol.

[b] Calculated from [(iodine affinity ÷ 19.5) × 100].

affinity of the granules. Although iodine affinity cannot give an *exact* estimate of the percentage of amylose, relative changes can be accurately determined. In these experiments, the apparent amylose-content increased from 12.5% for starch 1 to an apparent limit of about 20% for starch 13. There is, in fact, a direct relation between the percentage of amylose and the granular size as shown in Fig. 2(a), and so the biosynthesis of starch cannot be a simple equilibrium process. This raises the problem of whether a granule is formed in the amyloplast, to an invariant size, or whether further growth can occur. Radioactive studies[18,19] indicating that the granule grows by apposition suggest that the latter may occur. If this is so, when small granules of low amylose-content form large granules—with an overall higher percentage of amylose—there must be more amylose in the outer layers of a granule than internally. However, some intussusception may also occur[20].

The average gelatinization temperature decreases directly in relation to the granular size, as shown in Fig. 2(b). This figure also shows that the gelatinization temperature is influenced directly by the percentage of amylose present in the granule.

The properties of the starch from the shoots (S) were similar to those for starch 1 (0–1 cm tuber), whilst those for residual seed-tuber starch (R) and a comparable-sized mature tuber were similar, in agreement with our earlier results[9].

Fractionation of the granular starches

Amylose and amylopectin components were obtained by conventional dispersion and fractionation techniques, after a liquid ammonia pretreatment of the granules to ensure complete dispersion. It was found that the initial thymol–amylose precipitates from starches 1 and S were difficult to isolate; the possible effect of this on the properties of the components will be discussed later.

Properties of the amylose components

Potentiometric iodine titrations indicated that all the samples were pure, this conclusion being substantiated by the complete conversion of the samples into maltose under the concurrent action of β-amylase and Z-enzyme[15].

The incomplete conversion with pure β-amylase (Table II) was thus not due to contaminating amylopectin, and the β-amylase limits indicate that, as the starch granules mature, the amylose component becomes less susceptible to degradation by the enzyme, *i.e.* some "barrier" is being introduced. This trend in β-amylolysis limits affords a strong indication that the barrier is a natural one and not an artificial product of the fractionation procedure. Since the ester-phosphate in amylose is a possible cause of the barrier[9,21], we carried out careful phosphorus-determinations on amylose-samples 5, 7, 10, and 12. The results showed that the apparent phosphorus-content was 0.002–0.003%, with no trend. In view of (*i*) the large effect of traces of amylopectin-impurity on the observed phosphorus-content, and (*ii*) the observed quantities being at the extreme limit of the experimental method, it is thought unlikely that an ester-phosphate group is the barrier. Although Radomski and Smith[22] reported a positive correlation between amylose β-amylolysis limits and the phos-

TABLE II

PROPERTIES OF THE AMYLOSE AND THE AMYLOPECTIN COMPONENTS ISOLATED FROM DISPERSIONS OF THE STARCHES

Iodine titrations showed that all the amylose samples were > 98% pure, and all the amylopectin samples were > 99.7% pure.

Amyloses				*Amylopectins*				
Starch	*β-Limit*[a]	[η] (*C in g/ml*)	*Degree of polymerization*	*Phosphorus* (%)	*β-Limit*[a]	*Chain-length*	*Internal chain-length*[b]	*Molecular weight* (× 10^{-6})
1	92	305[c]	2200	0.062	56	26	9	9
2	90	145	1100	0.052	56	26	9	n.d.
3	90	185	1400	0.055	55	26	9	n.d.
4	88	210	1600	0.050	54	24	9	23
5	88	276	2100	0.050	54	23	8	n.d.
6	87	345	2800	0.061	53	23	8	n.d.
7	85	415	3100	0.048	52	23	9	35
8	84	435	3200	0.053	52	24	9	n.d.
9	84	450	3300	0.059	51	23	8	n.d.
10	83	490	3600	0.050	51	22	8	37
11	83	505	3700	0.062	52	22	8	n.d.
12	81	540	4000	0.050	51	22	8	60
13	81	530	3900	0.048	52	22	8	130
R	86	310	3300	0.050	53	24	9	n.d.
S	89	390	2900	0.040	57	33	12	n.d.

[a] Percentage conversion into maltose on treatment with β-amylase.
[b] Calculated from chain-length − [(chain-length × β-limit) + 2.5], to nearest whole number.
[c] Average value from two independent fractionations when [η] = 300 and 310, respectively.
n.d. = not determined.

phorus-content, their phosphorus-contents were up to ten times greater, and their β-amylolysis limits were some 15–20% lower than any values we have found. Furthermore, these authors presented no evidence for the purity of their amylose samples, and it seems very likely that these were contaminated with amylopectin.

With the exception of the 0–1 cm tuber, an increase in maturity was accompanied by an increase in limiting viscosity number for the amylose. This indicates an increase in molecular size. Some indication of the magnitude of this change is shown by the values of number-average degree of polymerization calculated from the viscosity results, using the relation developed by Cowie and Greenwood[23].

The sedimentation coefficient and limiting viscosity number of amylose samples 3, 7, 11, and 13 were measured in 0.15M-potassium hydroxide. Weight-average molecular weights were then calculated from these values using the Scheraga–Mandelkern equation[24]. The results (2×10^5, 8×10^5, 1.2×10^6, and 1.3×10^6, respectively) confirmed that the molecular weight of the amylose is increasing with maturity, as we found in our work on pea amyloses[5].

The high $[\eta]$-value for amylose 1 was found in two independent fractionations. Comparable results were obtained for the shoot amylose, S. It is likely that in these samples loss of very low molecular-weight material occurred during fractionation of the starch.

It is of interest to note that the increase in $[\eta]$-value and the decrease in β-amylolysis limit for samples 2–13 were both directly proportional to the percentage of amylose in the parent starch (see Fig. 3); conversely, the $[\eta]$-value and the β-limit were directly related.

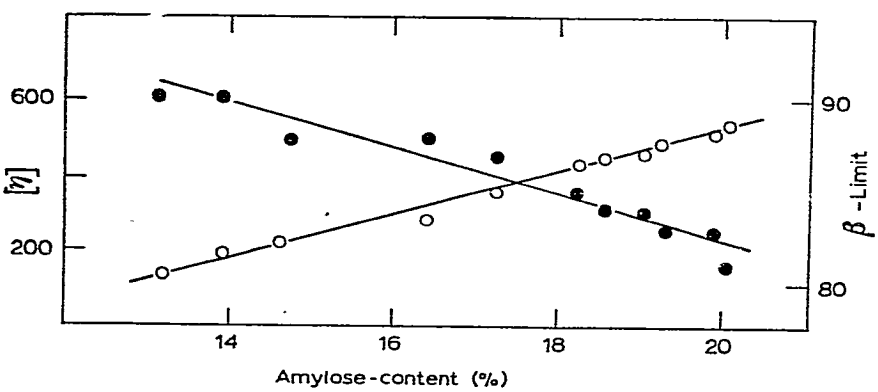

Fig. 3. Variation of $[\eta]$-value (- O-) and β-amylolysis limit (-●-) with amylose-content of the starch.

Properties of the amylopectin components

The phosphorus-contents of these samples varied randomly (see Table II), as did those for the parent starches. Iodine-affinity measurements showed that a high degree of purity (>99.7%) was obtained on fractionation. The average length of unit-chain and the β-amylolysis limit for the amylopectins from immature starches were significantly higher than those for the mature ones. This indicates a slight increase

in the degree of molecular branching, with the apparent internal chain-length remaining effectively constant. The values of chain-length and β-amylolysis limit for the mature starches were lower than those for other varieties of potato[9,15]. The shoot amylopectin, S, appears to have an unusually large average length of unit-chain.

The molecular weights of samples 1, 4, 7, 10, 12, and 13 were determined by light-scattering measurements in 0.1M-sodium chloride. In agreement with earlier work in these laboratories[5], the second virial coefficient in this solvent was negligible. Data were evaluated by the method of Zimm[25], and a typical result is shown in Fig. 4. Table II shows that there is a profound increase (from 9×10^6 to 130×10^6) in molecular weight of the amylopectin with increase in maturity. The root-mean-square end-to-end distance showed a corresponding increase from 1040 to 2120 Å.

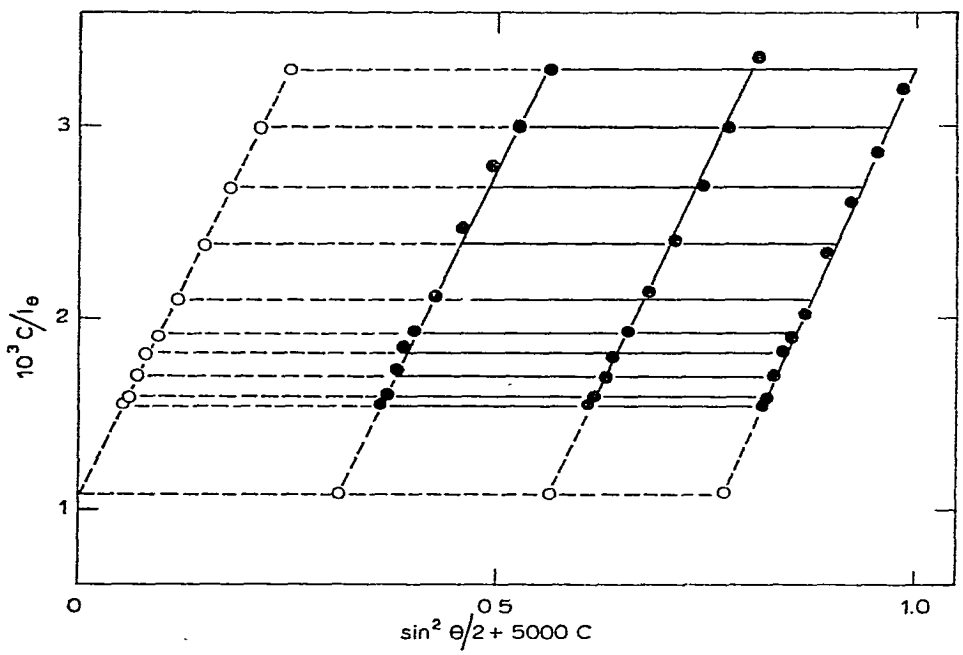

Fig. 4. Zimm-plot[25] for amylopectin 12 in 0.1M-aqueous sodium chloride.

Properties of the intermediate material

The yields of the intermediate material described by Banks and Greenwood[10] varied from 1–5% (see Table III). Iodine-affinity measurements indicated the essential absence of linear material. β-Amylolysis limits were generally less than those for the corresponding amylopectin-sample. The average lengths of unit-chain show a decrease, with increase in maturity, down to values of 12 and 13, in agreement with earlier results[10]. Samples 2, 6, 9, and 13 were examined in the ultracentrifuge. The sedimentation coefficients increased with maturity, but samples 2 and 6 were apparently homogeneous, whilst 9 and 13 were heterogeneous. However, the ultracentrifugation of a mixture of two components is complex. Johnston and Ogston[26] have

TABLE III

PROPERTIES OF INTERMEDIATE MATERIAL[10] ISOLATED FROM DISPERSIONS OF THE STARCHES

Starch sample	*Yield* (%)	*Amylose content* (%)[a]	*β-Limit*[b]	*Chain-length*	$10^{13}S$[c]
1	2.4	2.3	50	26	n.d.
2	0.8	1.3	n.d.	n.d.	3
3	2.6	1.9	50	n.d.	n.d.
5	4.6	2.1	52	19	n.d.
6	2.2	2.2	n.d.	n.d.	11
7	2.4	1.3	52	13	n.d.
9	2.0	1.3	52	n.d.	11 and 130
11	2.8	2.3	54	12	n.d.
13	2.5	3.4	53	n.d.	19 and 215
S	1.4	1.5	50	n.d.	n.d.

[a] Calculated from iodine-affinity measurements.
[b] Percentage conversion into maltose on treatment with β-amylase.
[c] Sedimentation coefficient at 0.3% concentration in 0.1M-NaCl.
n.d. = not determined.

shown that in a mixture of fast- and slow-moving components, the apparent concentration of the former is drastically reduced. We have shown recently[27] that this effect is very pronounced with starch-type materials. Sample 6 was subfractionated by the addition of ethanol to a dimethyl sulphoxide solution, but the fractions showed no trend in either β-amylolysis limit, or in λ_{max} (555 mμ) of the iodine-complex.

These intermediate materials appeared to have the properties of degraded amylopectin, in agreement with our earlier suggestion[10].

Separation of a starch sample into granules of different sizes, and the properties of these fractions

The above results indicated that the properties of both the starch and its separated components depend essentially on granular size. Starch 6—in the middle of the maturity series—was separated, therefore, into fractions varying in number-average granular diameter from 7 to 37μ (*cf.* Fig. 1). The gelatinization temperature and iodine affinity for these samples (Table IV) showed identical trends with increase in granular size, as did the starch samples graded on the basis of maturity (Table I). An analogous result was obtained when two of these starches were fractionated. The properties of the amylose and amylopectin in Table V show the same trend—with increase in granular size—as is found in Table II.

The properties of a starch and its components must depend essentially, therefore, on the size of the granule.

Biosynthesis of the starch granule

The above results show that with increase in maturity of potato starch there is—in addition to changes in the size and properties of the granule—a profound

TABLE IV

PROPERTIES OF THE STARCHES SEPARATED BY SEDIMENTATION

The starches were characterized as in Table I.

Starch sample	*Yield (%wt.)*	*No.-av. granular diam. (μ)*	*Av. gelatinization temp.*	*Iodine affinity*	*Amylose(%)*
6	100	28	63.5	3.35	17.2
6.1	28	37	62.5	3.80	19.5
6.2	38	28	63.5	3.50	18.0
6.3	21	16	64.5	3.25	16.7
6.4	6	10	65.5	3.10	16.0
6.5	4	7	67.5	2.80	14.4

TABLE V

PROPERTIES OF THE COMPONENTS OF STARCHES 6.1 AND 6.5[a]

Amyloses				*Amylopectins*			
Starch sample	*β-Limit[b]* (*i*)	(*ii*)	[η]	*Purity* (%)	*β-Limit* (*i*)[b]	*Chain-length*	*Internal chain-length*
6	87	101	345	99.7	53	23	8
6.1	85	99	400	99.6	52	22	8
6.5	91	101	310	99.8	59	25	8

[a] As in Table IV.

[b] Percentage conversion into maltose under the action of (*i*) pure β-amylase, and (*ii*) β-amylase and Z-enzyme.

alteration in the fine-structure of the components. The biosynthesis of amylose and amylopectin is, therefore, not a simple process[20]. Current theories of starch-biosynthesis are those of (*i*) Erlander[28], involving a glycogen-type precursor, and (*ii*) Whelan[29], involving the independent synthesis of amylose and amylopectin. Our results indicate that biosynthesis is more complicated than either of these theories would suggest. It is hoped to present a detailed discussion of this problem elsewhere.

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SUMMARY

Starch granules have been isolated from potato tubers of various stages of maturity, and their properties investigated. Increase in maturity was accompanied

by an increase in granule size and amylose-content, whilst the gelatinization-temperature decreased. The amylose-content and gelatinization-temperature were both directly related to granule size. The starches have been fractionated into their amylose and amylopectin components, and the fine-structure of these investigated. For both components, it was found that with increase in maturity there was (*i*) an increase in molecular size, and (*ii*) a decrease in the extent of conversion into maltose by β-amylase. The properties of an intermediate fraction have also been characterized. One starch sample was separated into granules of different sizes, and the properties of these and their component amylose and amylopectin were studied. The results indicated that the fundamental properties of a sample of potato starch are determined essentially by the size of the granules.

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Araucaria bidwilli GUM

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The characteristic resinous exudates of coniferous woods are terpenoid in character. In the course of an investigation of such a resin from the Australian bunya pine (*Araucaria bidwilli*) the presence of an accompanying polysaccharide was noted, and through the kindness of Professor A.J. Birch, F.R.S. and Dr. D.C.C. Smith, this material was placed at our disposal. As far as we are aware this is the first report of a polysaccharide exudate from a coniferous tree, and it was of particular interest to compare the structure of this polysaccharide with those of other exudate gums from deciduous trees and with those of polysaccharides from the woods of typical conifers.

RESULTS AND DISCUSSION

A dispersion of the exudate from *Araucaria bidwilli* had been poured into acidified ethanol to give an acidic polysaccharide with an equivalent weight of approximately 1,600. Galactose, arabinose, and rhamnose had been recognised as constituent sugars by paper chromatography of the hydrolysate.

Hydrolysis of a sample of the polysaccharide gave galactose, arabinose, and rhamnose in the molar proportions of 13:3.5:1, together with acidic sugars. A larger sample of the polysaccharide was hydrolysed and the hydrolysate was passed through diethylaminoethyl-Sephadex to adsorb acidic sugars. The neutral sugars were separated by filter-sheet chromatography and crystalline D-galactose, L-arabinose, and L-rhamnose hydrate were isolated. Desorption of the acidic sugars gave a mixture which contained glucuronic acid and two aldobiouronic acids, and the components were separated chromatographically. Aldobiouronic acid I was chromatographically indistinguishable from 6-*O*-(*β*-D-glucopyranosyluronic acid)-D-galactose. Hydrolysis of the disaccharide gave glucuronic acid and galactose, and reduction of the derived methyl ester methyl glycosides with potassium borohydride followed by hydrolysis gave glucose and galactose. The aldobiouronic acid was methylated and examination by gas chromatography of the methanolysis products from the methylated derivative showed the methyl glycosides of 2,3,4-tri-*O*-methylglucuronic acid, and 2,3,4- and 2,3,5-tri-*O*-methylgalactose. In a similar series of experiments aldobiouronic acid II was shown to be the methyl ether of I, namely, 6-*O*-(4-*O*-methyl-*β*-D-glucopyrano-

syluronic acid)-D-galactose. The optical rotations, $[\alpha]_D$ 0° and +3°, of the two aldobiouronic acids were consistent with the presence of β-glycosidic linkages.

β-D-GpA 1→6 D-Gal (I) 4-Me β-D-GpA 1→6 D-Gal (II)

Under mild conditions of hydrolysis three neutral oligosaccharides were detected with the chromatographic mobilities of 6-*O*-β-D-galactopyranosyl-D-galactose (major component), 3-*O*-β-D-galactopyranosyl-D-galactose (minor component), and 3-*O*-β-L-arabinopyranosyl-L-arabinose (trace component). The highly branched nature of the polysaccharide was further indicated by the gas chromatographic examination of the methanolysis products from the methylated polysaccharide which indicated the presence of methyl glycosides of the following sugars (approximate relative proportions in parenthesis); 2,3,4-tri-*O*-methylrhamnose (+), 2,3,4- (trace) and 2,3,5-tri- (++), and 2,5-di-*O*-methylarabinose (trace), 2,3,4,6-tetra- (+++), 2,3,4- (++) and 2,4,6-tri- (++), and 2,4-di-*O*-methylgalactose (+++), and 2,3,4-tri-*O*-methylglucuronic acid (++). In a separate experiment the acidic sugars formed on hydrolysis of the methylated polysaccharide were separated by adsorption on diethylaminoethyl-Sephadex. Treatment of the acidic sugars with methanolic hydrogen chloride furnished methyl glycosides of 2,3,4-tri-*O*-methylglucuronic acid and 2,3,4-tri-*O*-methylgalactose. Reduction of the methyl ester methyl glycosides with lithium aluminium hydride followed by hydrolysis afforded 2,3,4- tri-*O*-methylglucose and 2,3,4-tri-*O*-methylgalactose. These experiments indicate that the methylated acidic sugar fraction was composed largely of the fully etherified aldobiouronic acid, 6-*O*-(2,3,4-tri-*O*-methylglucopyranosyluronic acid)-2,3,4-tri-*O*-methylgalactose.

The results of these experiments show that the polysaccharide contains a branched framework of D-galactopyranose residues, mutually joined by 1→3 and 1→6 linkages, in which the outer chains are terminated by units of D-galactopyranose, 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactopyranose (approximately one third of the glucuronic acid residues are present as the 4-methyl ether), L-rhamnopyranose, and L-arabinofuranose residues. In addition, a small proportion of L-arabinose residues are probably present in units of 3-*O*-β-L-arabinopyranosyl-L-arabinofuranose. Evidence concerning the distribution of 1→3 and 1→6 linkages in the galactan framework was obtained by examination of the degraded polysaccharide formed by Smith's procedure[1]. Reduction of the periodate-oxidised polysaccharide with sodium borohydride followed by mild acid hydrolysis afforded degraded polysaccharide A. Further degradation in a similar manner furnished degraded polysaccharide B. Degraded polysaccharide A contained galactose and arabinose residues in the approximate proportion of 20:1, and degraded polysaccharide B was composed essentially of galactose residues only.

The degraded polysaccharides A and B were examined by (a) partial hydrolysis, (b) periodate oxidation, and (c) methylation. Partial hydrolysis of the degraded arabinogalactan A showed that 3-*O*-β-galactopyranosylgalactose was the disaccharide formed in greatest amount, with only small amounts of the 1,6-linked isomer; even smaller amounts of the latter disaccharide were formed on partial hydrolysis

of degraded galactan B. The degraded polysaccharides A and B consumed 0.27 and 0.15 mol of periodate per sugar residue, respectively, and these results show clearly that the two successive degradations had led to the formation of polysaccharides which approximated increasingly to linear 1→3-linked galactans. Complete methylation of the degraded polysaccharides could not be achieved. Jones and Reid[2] have also observed that a similar degraded polysaccharide obtained from mountain larch arabinogalactan was resistant to complete methylation. Despite incomplete methylation, however, examination of the cleavage products from the methylated polysaccharides showed 2,4,6-tri-*O*-methylgalactose to be the dominant component sugar. The additional recognition of 2,6-di-*O*-methylgalactose as an important cleavage product suggested that the resistance to methylation was associated primarily with the axial 4-hydroxyl group of 3-*O*-substituted galactose residues since no evidence for the presence of any substantial proportion of 4-*O*-substituted galactose residues in the original polysaccharide had been obtained from other experiments. Assuming that 2,6-di-*O*-methylgalactose and possibly also 2-*O*-methylgalactose were cleavage products arising from incomplete methylation of the degraded polysaccharides, and hence of little or no structural significance, Table I compares semiquantitatively the structural units present in the original gum and in the two degraded polysaccharides derived therefrom. It may be noted that the arabinose residues in degraded arabinogalactan A were detected only as furanose end-groups, which probably arose from the degradation of 3-*O*-β-arabinopyranosylarabinofuranose side-chains.

TABLE I

APPROXIMATE RELATIVE PROPORTIONS OF STRUCTURAL UNITS IN *Araucaria bidwilli* GUM AND DEGRADED POLYSACCHARIDES DERIVED THEREFROM

Structural unit	*Original gum*	*Degraded galactan A*	*Degraded galactan B*
Araf 1→	++	+	—
Araf 1→3 Araf 1→	+	—	—
Rhap 1→	+	—	—
Galp 1→	+++	+	+
→3 Galp 1→	++	+++	+++
→6 Galp 1→	++	+	trace
→3 Galp 1→ 6	+++	++	+
(4-Me) GpA 1→6 Galp 1→	++	—	—

On the basis of the present evidence the following partial structure may be proposed for the polysaccharide from *Araucaria bidwilli* gum. The principal chain is composed mainly, if not exclusively, of 1→3-linked D-galactopyranose residues to which are attached as side-chains other D-galactopyranose residues joined by 1→6 linkages. The side-chains are terminated by D-galactopyranose, D-glucuronic acid

(or its 4-methyl ether), L-rhamnopyranose, L-arabinofuranose, and, infrequently, L-arabinopyranose residues. The precise points of attachment of the various rhamnopyranose and arabinofuranose end groups are not yet known, nor is it possible to assess the length of the side-chains which are terminated by D-galactopyranose or D-glucuronic acid residues. Furthermore, some degree of multiple branching is indicated since the periodate degradations do not result in complete removal of all the side-chains from a comb-like structure. The polysaccharide clearly belongs to the same structural class as the arabinogalactans from the woods of other conifers, *e.g.* larches[2–5] and pines[6,7] in possessing similar arrangements of galactose residues. Likewise, *Araucaria bidwilli* gum resembles the exudate gums from *Acacia senegal* (gum arabic)[8] and *A. pycnantha*[9], but contains a lower proportion and less complex arrangement of arabinose and rhamnose residues on the periphery of the molecular structure. In contrast to gum arabic, where D-glucuronic acid residues carry end groups of L-rhamnopyranose[10], *Araucaria bidwilli* gum contains glucuronic acid residues as end groups only and the rhamnopyranose end groups must be attached elsewhere, probably to galactopyranose residues (see Fig. 1).

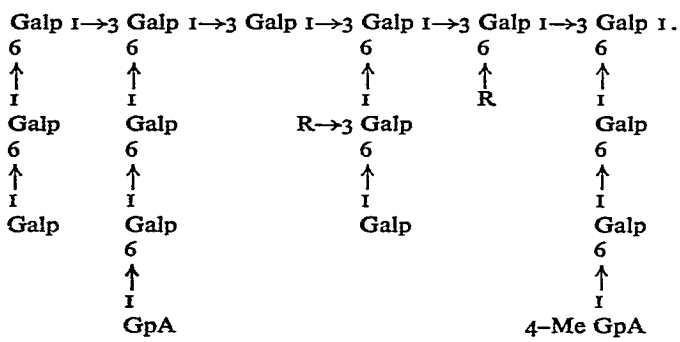

Fig. 1. Proposed structure for *Araucaria bidwilli* gum (R = Araf 1., Arap 1→3 Araf 1. (infrequently), or Rhap 1.).

EXPERIMENTAL

The polysaccharide was received as a white powder, $[\alpha]_D$ +11° (*c* 0.5, water) [Found: uronic anhydride (by decarboxylation), 10.1%]. Paper chromatography was carried out on Whatman No. 1 and 3MM papers using the following solvent systems (v/v): (A) ethyl acetate–pyridine–water (10 : 4 : 3); (B) ethyl acetate–pyridine–water (8 : 2 : 1); (C) ethyl acetate–acetic acid–formic acid–water (18 : 3 : 1 : 4); (D) butanol–ethanol–water (4 : 1 : 5, upper layer); (E) butanol–acetic acid–water (4 : 1 : 5, upper layer); (F) butan-2-one, half saturated with water. R_G values of methylated sugars refer to rates of movement relative to 2,3,4,6-tetra-*O*-methyl-D-glucose in solvent D. Gas-liquid chromatography was carried out on columns of (*a*) 10% by weight of butan-1,4-diol succinate polyester on Celite at 175°, and (*b*) 10% by weight of polyphenyl ether [*m*-bis(*m*-phenoxyphenoxy)benzene] on Celite at 200°. Retention

times (T) are quoted relative to methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucopyranoside as an internal standard[11]. Optical rotations were observed at *ca.* 18°.

Partial hydrolysis of the polysaccharide

The polysaccharide (10 mg) was heated with 0.5N-sulphuric acid (3 ml) for 1 h on the boiling-water bath, and the cooled solution was neutralised with Amberlite resin IR-4B(HO⁻form), filtered, and concentrated. Paper chromatography of the hydrolysate in solvent A showed arabinose, galactose, rhamnose, three neutral oligosaccharides with the mobilities of 6-*O*-β-D-galactopyranosyl-D-galactose ($R_{galactose}$ 0.39, major component), 3-*O*-β-D-galactopyranosyl-D-galactose ($R_{galactose}$ 0.53, minor component), and 3-*O*-β-L-arabinopyranosyl-L-arabinose ($R_{galactose}$ 0.82, trace), and unidentified higher oligosaccharides.

Characterisation of neutral and acidic sugar components

The polysaccharide (2 g) was hydrolysed with N-sulphuric acid (50 ml) for 4 h on the boiling-water bath. The cooled solution was partially neutralised with barium hydroxide, centrifuged, and fully neutralised by shaking with Amberlite resin LA–2 in chloroform (5% v/v, 5 × 20 ml), shaken with Amberlite resin IR-120 (H^+ form) and concentrated to a syrup (1.9 g). The syrup was adsorbed on a column of diethylaminoethyl-Sephadex A-25 (formate form, 6 × 3 cm). Elution with water gave neutral sugars (1.4 g) and elution with aqueous 3% formic acid gave acidic sugars (0.42 g). The neutral sugars were separated by filter-sheet chromatography in solvent B to give (i) D-galactose (1.1 g), m.p. and mixed m.p. 163°, $[\alpha]_D$ +79° (equil.) (*c* 0.8, water); (ii) L-arabinose (161 mg), m.p. and mixed m.p. 159°, $[\alpha]_D$ +104° (equil.) (*c* 0.9, water); and (iii) L-rhamnose monohydrate (50 mg), m.p. and mixed m.p. 93°, $[\alpha]_D$ +6.7° (equil.) (*c* 0.7, water). In a similar experiment, the neutral sugars were separated chromatographically in solvent B, and colorimetric estimation[12] showed the presence of galactose, arabinose, and rhamnose, in the molar proportions of 13 : 3.5 : 1.

Paper chromatography of the mixture of acidic sugars in solvent C showed glucuronic acid and two aldobiouronic acids ($R_{galactose}$ 0.20 and 0.61). Filter-sheet chromatography in solvent C furnished aldobiouronic acid I (216 mg) and aldobiouronic acid II (70 mg).

Aldobiouronic acid I ($R_{galactose}$ 0.20 in solvent C) was chromatographically indistinguishable from 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose and had $[\alpha]_D$ 0° (*c* 1.5, water). Hydrolysis with 2N-sulphuric acid gave glucuronic acid and galactose. Reduction of the derived methyl ester methyl glycosides with potassium borohydride followed by hydrolysis gave glucose and galactose, in approximately equal amounts. Methyl sulphate (1 ml) and aqueous 30% sodium hydroxide (1 ml) were added dropwise during 2 h to aldobiouronic acid I (100 mg) in water (5 ml), and the solution was stirred vigorously under nitrogen at 0° for 12 h. Methyl sulphate (3 ml) and aqueous sodium hydroxide (6 ml) were added with stirring at room temperature during 5 h, and similar additions of reagents were made on 4 successive

days. The reaction mixture was heated on a boiling-water bath for 30 min to destroy excess of methyl sulphate, cooled, acidified with dilute sulphuric acid, and poured into ethanol (4 vol.). The precipitated sodium sulphate was removed at the centrifuge, and the supernatant liquid and washings were concentrated to small volume (50 ml) and extracted with chloroform. The resulting methylated aldobiouronic acid was converted into the silver salt (120 mg) and further methylated twice with methyl iodide (10 ml) and silver oxide (1.5 g) to give methylated aldobiouronic acid I (70 mg). Hydrolysis of the methylated aldobiouronic acid gave 2,3,4-tri-*O*-methylglucuronic acid (R_G 0.87 in solvent E) and 2,3,4-tri-*O*-methylgalactose (R_G 0.69). The methylated aldobiouronic acid was heated in a sealed tube with methanolic 4% hydrogen chloride for 18 h at 100°. Examination of the resulting methyl glycosides by gas chromatography on columns *a* and *b* showed the presence of components having the retention times of methyl glycosides of 2,3,4-tri-*O*-methylglucuronic acid (*a*, *T* 2.50 and 3.21; *b*, *T* 1.78 and 2.20), 2,3,4-tri-*O*-methylgalactose (*a*, *T* 7.3; *b*, *T* 2.61 and 2.88), and, in small amount, 2,3,5-tri-*O*-methylgalactose (*a*, *T* 4.43).

Aldobiouronic acid II ($R_{galactose}$ 0.61 in solvent C) was chromatographically indistinguishable from 6-*O*-(4-*O*-methyl-β-D-glucopyranosyluronic acid)-D-galactose and had $[\alpha]_D$ +3° (*c* 0.9, water). Hydrolysis with 2N-sulphuric acid gave 4-*O*-methylglucuronic acid and galactose. The aldobiouronic acid (10 mg) was converted into the methyl ester methyl glycosides with methanolic hydrogen chloride, reduced with potassium borohydride, and hydrolysed to give approximately equal amounts of galactose and 4-*O*-methylglucose. A portion (3 mg) of the hydrolysate was oxidised with periodate[13], and paper chromatography of the oxidation products showed 2-*O*-methylerythrose (R_F 0.55 in solvent D), a characteristic product from 4-*O*-methylglucose. Aldobiouronic acid II (50 mg) was methylated as described for aldobiouronic acid I. Paper chromatography of the hydrolysis products and gas chromatography of the methanolysis products from the methylated derivative indicated the presence of the same cleavage products as those formed from methylated aldobiouronic acid I.

Methylation of the polysaccharide[14]

A suspension of the polysaccharide (400 mg) in ethereal 1% diazomethane (100 ml) was stirred vigorously for 18 h. The resulting polysaccharide methyl ester was dissolved in dimethyl sulphoxide (10 ml) and *NN*-dimethylformamide (10 ml), and barium hydroxide octahydrate (10 g) was added to the solution at 0°. With continuous stirring, portions (2 ml) of methyl sulphate were added after 30, 90, 120, and 180 min (the temperature being allowed to rise after 30 min), and stirring was continued for 2 days. The reaction mixture was dialysed against running tap-water for 5 days, treated with Amberlite resin IR-120 (H^+ form) to remove cations, and freeze-dried to give partially methylated polysaccharide (355 mg) (Found: OMe, 31.2%). Further methylation with methyl iodide (2 ml) and silver oxide (2 g) in *NN*-dimethylformamide (6 ml) furnished methylated polysaccharide (210 mg), $[\alpha]_D$ −49° (*c* 0.6, chloroform) (Found: OMe, 42.8%, not raised on further methylation).

A sample of the methylated polysaccharide was heated in a sealed tube with methanolic 4% hydrogen chloride for 18 h at 100°. Examination of the resulting methyl glycosides by gas chromatography on columns *a* and *b* showed the presence of components having the retention times of the methyl glycosides of the following sugars (figures in parenthesis indicate incomplete resolution of certain components on one column only): 2,3,4-tri-*O*-methylrhamnose (*a*, *T* 0.47; *b*, *T* (0.47)), 2,3,4- (*a*, *T* 1.06; *b*, *T* 0.83) and 2,3,5-tri-*O*-methylarabinose (*a*, *T* 0.56, 0.73; *b*, *T* (0.47), 0.60), 2,5-di-*O*-methylarabinose (*a*, not detected; *b*, 0.70), 2,3,4,6-tetra-*O*-methylgalactose (*a*, *T* 1.80; *b*, *T* 1.52, 1.60), 2,3,4-(*a*, *T* 7.5; *b*, *T* 2.60, 2.88) and 2,4,6-tri-*O*-methylgalactose (*a*, *T* 4.16, 4.74; *b*, *T* 2.07, 2.37), 2,4-di-*O*-methylgalactose (*b*, *T* 3.57, 4.24), and 2,3,4-tri-*O*-methylglucuronic acid (*a*, *T* 2.50, 3.24; *b*, *T* 1.77, 2.21).

The methylated polysaccharide (50 mg) was refluxed with methanolic 2% hydrogen chloride (5 ml) for 2 h, and the solution was neutralised with silver carbonate, filtered, and concentrated. The resulting syrup was hydrolysed with N-sulphuric acid for 4 h at 100°, and the solution was neutralised with barium hydroxide and barium carbonate, treated with Amberlite resin IR-120 (H^+ form) to remove barium ions, concentrated to small volume, and adsorbed on a column (4 × 1 cm) of diethylaminoethyl-Sephadex A-25 (formate form). Elution with water gave neutral sugars, examination of which by paper chromatography showed the presence of a trace of 2-*O*-methylgalactose, in addition to those sugars which had been previously characterised by gas chromatography of their methyl glycosides. Elution of the column with aqueous 3% formic acid furnished methylated acidic sugars which were heated in a sealed tube with methanolic 4% hydrogen chloride. Examination of a portion of the resulting methyl glycosides by gas chromatography on columns *a* and *b* showed the presence of components having the retention times of methyl glycosides of 2,3,4-tri-*O*-methylglucuronic acid (*a*, *T* 2.50, 3.22; *b*, *T* 1.77, 2.22) and 2,3,4-tri-*O*-methylgalactose (*a*, *T* 7.5; *b*, *T* 2.66, 2.94). The remainder of the methyl glycosides was reduced with potassium borohydride and hydrolysed. Paper chromatography of the resulting sugars in solvent D showed 2,3,4-tri-*O*-methylglucose (R_G 0.88) and 2,3,4-tri-*O*-methylgalactose (R_G 0.72). No dimethyl ethers of glucose or galactose were detected.

Periodate oxidation of the polysaccharide and isolation of degraded polysaccharides A and B

The polysaccharide (100 mg) was oxidised with sodium metaperiodate solution (10 ml, 0.15M), and the consumption of reagent was determined spectrophotometrically[15] and corresponded to 1.43 mol per sugar residue (constant after 90 h). The polysaccharide was oxidised with potassium metaperiodate according to the procedure of Halsall *et al.*[16], and the formic acid liberated corresponded to 0.64 mol per sugar residue.

The polysaccharide (8 g) was oxidised with 0.15M-sodium metaperiodate (800 ml) for 90 h. Ethylene glycol (7 g) was added to the solution to destroy excess of periodate, sodium ions were removed by passage through Amberlite resin IR-120

(H^+ form), and the resulting solution was neutralised with barium hydroxide and barium carbonate. Barium salts were removed by centrifugation and the combined centrifugate and washings were treated with potassium borohydride (11 g) for 2 days. The resulting solution was stirred with Amberlite resin IR-120 (H^+ form) to destroy excess of hydride and to remove potassium ions, filtered, and concentrated with repeated additions of methanol to remove boric acid as methyl borate. The residue was treated with N-sulphuric acid (200 ml) at room temperature for 3 h, neutralised, concentrated to small volume (50 ml), and poured into ethanol (200 ml). The precipitated, degraded polysaccharide A (2.07 g) was separated and concentration of the supernatant liquid afforded a syrup (2.8 g). Paper chromatography of the syrup in solvent C showed glycerol ($R_{galactose}$ 2.38) and small amounts of two non-reducing substances ($R_{galactose}$ 2.08 and 1.91), but no reducing sugars. Hydrolysis of a portion of the syrup gave in addition traces of galactose and arabinose. The degraded polysaccharide A (1.3 g) was degraded in a similar sequence of reactions involving periodate oxidation, reduction, and hydrolysis with cold dilute acid, and furnished degraded polysaccharide B (771 mg) and a syrup which contained the above-mentioned non-reducing substances ($R_{galactose}$ 2.38, 2.08, and 1.91) and gave traces of galactose on hydrolysis.

Examination of degraded polysaccharide A

Degraded polysaccharide A had $[\alpha]_D$ +26° (*c* 0.7, water) and colorimetric estimation of the sugars formed on hydrolysis with N-sulphuric acid for 4 h at 100° showed the presence of galactose and arabinose in the molar proportions of 20 : 1. A sample of the polysaccharide was heated with 0.5N-sulphuric acid for 1 h on the boiling-water bath, and paper chromatography of the hydrolysate in solvent A showed arabinose, galactose, 3-*O*-β-galactopyranosylgalactose ($R_{galactose}$ 0.53), and the polymer-homologous galactotriose ($R_{galactose}$ 0.23), with only relatively small amounts of 6-*O*-β-galactopyranosylgalactose ($R_{galactose}$ 0.35). Periodate oxidation of degraded polysaccharide A resulted in the consumption of 0.27 mol of reagent with the liberation of 0.09 mol of formic acid per sugar residue.

The degraded polysaccharide A (150 mg) was methylated (6 days) as described above for aldobiouronic acid I, acetone being added as required to maintain the partially methylated polysaccharide in solution. Further methylation of the product (120 mg) (Found: OMe, 32.1%) with methyl iodide and silver oxide, and with methyl iodide and silver oxide in *NN*-dimethylformamide afforded incompletely-methylated degraded-polysaccharide A (51 mg), $[\alpha]_D$ −16° (*c* 0.7, chloroform) (Found: OMe, 37.4%, not raised on further methylation). A portion of the methylated polysaccharide was heated with methanolic hydrogen chloride and examination of the resulting methyl glycosides by gas chromatography on columns *a* and *b* showed the presence of components having the retention times of methyl glycosides of 2,3,5-tri-*O*-methylarabinose (*a*, *T* 0.53, 0.70; *b*, *T* 0.47, 0.60), 2,3,4,6-tetra-*O*-methylgalactose (*a*, *T* 1.72; *b*, *T* 1.54), 2,3,4- (*a*, *T* 7.0) and 2,4,6-tri-*O*-methylgalactose (*a*, *T* 3.92, 4.46; *b*, *T* 1.98, 2.26), and 2,4- (*b*, *T* (3.46), 4.02) and 2,6-di-*O*-methylgalactose (*a*, *T* 9.5).

Hydrolysis of the methyl glycosides afforded sugars which were shown by paper chromatography in solvents D and F to include the above-mentioned sugars together with 2-*O*-methylgalactose. Paper chromatography in solvent F readily separated 2,4- and 2,6-di-*O*-methylgalactose.

Examination of degraded polysaccharide B

Degraded polysaccharide B had $[\alpha]_D$ +30° (*c* 0.6, water) and gave on hydrolysis galactose with only a trace of arabinose. Partial hydrolysis of the polysaccharide with 0.5N-sulphuric acid for 1 h on the boiling-water bath furnished 3-*O*-*β*-galactopyranosylgalactose and the polymer-homologous trisaccharide, and only traces of 6-*O*-*β*-galactopyranosylgalactose. Periodate oxidation of degraded polysaccharide B resulted in the consumption of 0.15 mol of reagent and in the liberation of 0.04 mol of formic acid per sugar residue.

Methylation of degraded polysaccharide B (150 mg), as described for degraded polysaccharide A, yielded incompletely-methylated degraded-polysaccharide B (56 mg), $[\alpha]_D$ −12° (*c* 0.8, chloroform) (Found: OMe, 36.6%). Examination by gas chromatography of the methyl glycosides formed on methanolysis of the methylated polysaccharide, and by paper chromatography of the sugars formed on subsequent hydrolysis showed the presence of tetra-, 2,3,4- and 2,4,6-tri-, 2,4- and 2,6-di-, and 2-*O*-methylgalactose as cleavage products.

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SUMMARY

The polysaccharide exudate from the gymnosperm, *Araucaria bidwilli*, contains residues of D-galactose, L-arabinose, L-rhamnose, and D-glucuronic acid (in part as the 4-methyl ether). Graded hydrolysis affords 6-*O*-(*β*-D-glucopyranosyluronic acid)-D-galactose and 6-*O*-(4-*O*-methyl-*β*-D-glucopyranosyluronic acid)-D-galactose. The methylated polysaccharide contains residues of 2,3,4,6-tetra-, 2,3,4- and 2,4,6-tri-, and 2,4-di-*O*-methylgalactose, 2,3,4-, and 2,3,5-tri-, and 2,5-di-*O*-methylarabinose, 2,3,4-tri-*O*-methylrhamnose, and 2,3,4-tri-*O*-methylglucuronic acid. Successive degradations by periodate oxidation, borohydride reduction, and mild acid hydrolysis, furnish arabinogalactan A and galactan B in which 1→3 linkages predominate. It is concluded that the polysaccharide possesses a highly branched structure with a main chain of 1→3-linked D-galactopyranose residues to which are attached side-chains carrying more D-galactopyranose residues in 1→6 linkages and terminated by D-glucuronic acid, D-galactopyranose, L-arabinofuranose, L-arabinopyranose, and L-rhamnopyranose residues.

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Preliminary communications

2-Deoxy sugars

PART X. 5,6-*O*-CARBONYL-2-DEOXY-3-*O*-*p*-NITROBENZOYL-D-*arabino*-HEXOSYL BROMIDE. A STABLE, CRYSTALLINE *O*-ACYLGLYCOFURANOSYL HALIDE OF A 2-DEOXYHEXOSE*

The "α,β-methyl-2-deoxy-D-glucofuranoside" of Hughes *et al.*[1] has now been shown, by paper chromatographic analysis, to consist of an anomerically pure methyl 2-deoxy-D-*arabino*-hexofuranoside (II), plus substantial amounts of an approximately equal mixture of methyl α- and β-2-deoxy-D-*arabino*-hexopyranosides (III and IV), and unconsumed "2-deoxy-D-glucose" (2-deoxy-D-*arabino*-hexose, I). *p*-Nitrobenzoylation of the syrupy mixture arising from the methyl glycosidation of "2-deoxy-D-glucose" (I) led to a mixture of *p*-nitrobenzoic esters from which, by fractional recrystallization, there was obtained in pure form a methyl 2-deoxy-3,5,6-tri-*O*-*p*-nitrobenzoyl-D-*arabino*-hexoside (V), m.p. 142–144° and also 168–169°; $[\alpha]_D^{24}$ −122.3° (*c* 1.00, chloroform). Calc. for $C_{28}H_{23}N_3O_{14}$: C, 53.72; H, 3.71; N, 6.72. Found: C, 53.70; H, 3.66; N, 6.49. Attempts to replace directly the severely hindered C-1 methoxyl group of V by hydrogen halide failed.

Methoxide-catalyzed saponification of V gave crystalline methyl 2-deoxy-D-*arabino*-hexofuranoside (II), m.p. 80–81°; $[\alpha]_D^{24}$ + 117.1° (*c* 0.99, ethanol). Calc. for $C_7H_{14}O_5$: C, 47.19; H, 7.92. Found: C, 47.20; H. 7.82. On conformational grounds and because of its strongly positive specific rotation, the α-D-anomeric configuration is provisionally assigned to II, as well as to V. Treatment of II with carbonyl chloride gave the 5,6-*O*-carbonyl derivative VI, m.p. 90–91°, $[\alpha]_D^{24}$ + 132.4° (*c* 1.00, ethanol). Calc. for $C_8H_{12}O_6$: C, 47.06; H, 5.92. Found: C, 47.21; H, 6.15. Nitrobenzoylation of VI gave methyl 5,6-*O*-carbonyl-2-deoxy-3-*O*-*p*-nitrobenzoyl-α-D-*arabino*-hexoside (VII), m.p. 213–214°; $[\alpha]_D^{24}$ + 22.4° (*c* 1.00, dichloromethane). Calc. for $C_{15}H_{15}NO_9$: C, 51.00; H, 4.28; N, 3.96. Found: C, 51.08; H, 4.10; N, 3.96. Replacement of the C-1 methoxyl group of VII by hydrogen bromide in dichloromethane was facile, yielding crystalline 5,6-*O*-carbonyl-2-deoxy-3-*O*-*p*-nitrobenzoyl-D-*arabino*-hexosyl bromide (VIII), m.p. 125–132° (dec.); $[\alpha]_D^{24}$ −35.1° (*c* 0.436, acetone). The anomeric configuration of the bromide VIII has not yet been determined, but is presumed to be α-D, in which the bromine atom occupies a position *trans* to the substituted side-chain at C-4 of the furanose ring.

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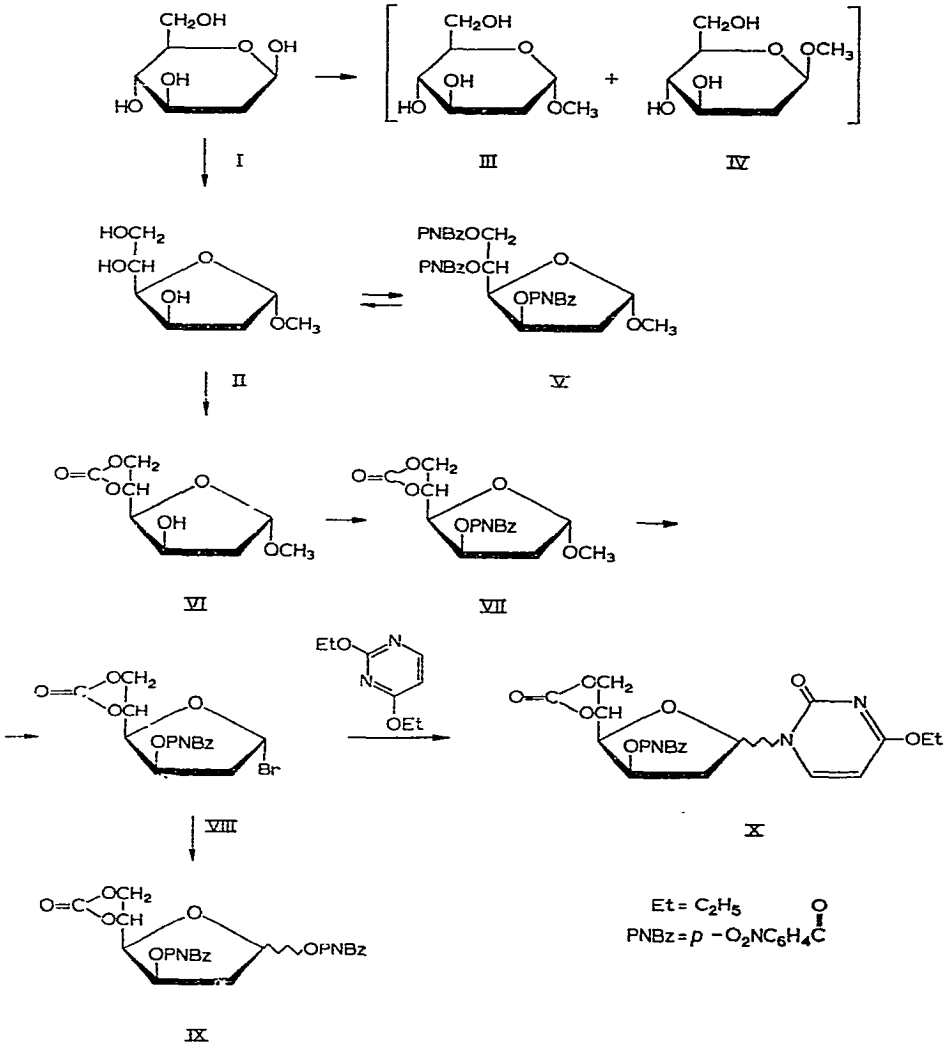
The bromide VIII reacted readily with silver *p*-nitrobenzoate to give 5,6-*O*-carbonyl-2-deoxy-1,3-di-*O*-*p*-nitrobenzoyl-D-*arabino*-hexose (IX), m.p. 204–205°; $[\alpha]_D^{24}$ −3.2° (*c* 0.286, dichloromethane). Calc. for $C_{21}H_{16}N_2O_{12}$: C, 51.64; H, 3.30; N, 5.74. Found: C, 51.82; H, 3.33; N, 5.65. The anomeric configuration of IX has



not yet been determined. The latter conversion indicated that the bromide VIII would have utility in the preparation of 2-deoxy-D-glucofuranosyl glycosides and 2-deoxy-D-glucofuranosyl nucleosides. As a test, we were successful in coupling VIII with 2,4-diethoxypyrimidine, by the Hilbert–Johnson procedure[2], to give 1-

(5,6-*O* carbonyl-2-deoxy-3-*O*-*p*-nitrobenzoyl-D-*arabino*-hexosyl)-4-ethoxy-2(1*H*)-pyrimidinone (X), m.p. 223–224.5°; $[\alpha]_D^{24}$ −7.4° (*c* 1.54, dichloromethane). Calc. for $C_{20}H_{19}N_3O_{10}$:C, 52.07; H, 4.15; N, 9.11. Found: C, 52.21; H, 4.22; N, 9.09.

To the best of our knowledge, crystalline methyl glycofuranosides and crystalline *O*-acylglycofuranosyl halides of 2-deoxyhexoses were unknown prior to this work, full details of which will be published at a later date.

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1 I.W. HUGHES, W.G. OVEREND, AND M. STACEY, *J. Chem. Soc.*, (1949) 2846.
2 G.E. HILBERT AND T.B. JOHNSON, *J. Am. Chem. Soc.*, 52 (1930) 2001, 4489.

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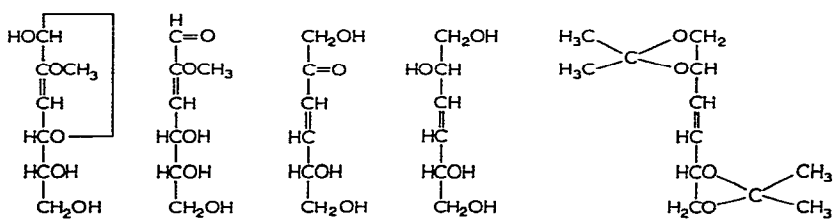
**To whom all enquiries regarding this paper should be addressed.

An unsaturated ketohexose

Although many unsaturated sugars have been prepared, the following is believed to be the first description of a simple unsaturated ketose.



I Ia II III IV

An aqueous solution of 3-deoxy-2-*O*-methyl-β-D-*erythro*-hexofuranos-2-ene (I)[1] was treated with sodium borohydride at 30–40° to reduce the aldehydo form (Ia) present in solution. Sodium ions were removed from the mixosture by an ion-exchange resin, and the boric acid by distillation with methanol. The mild, acid treatment invol-

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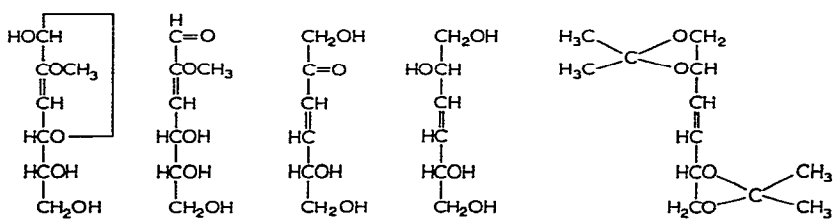

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ved in this procedure caused an allylic rearrangement[2], so that the product obtained was the unsaturated ketose (II) (3,4-dideoxy-*keto*-D-*glycero*-hexulos-*trans*-3-ene). The ketose was a syrup* that gave a single spot on chromatograms; the gas chromatography of its trimethylsilyl derivative showed only one component. The ultraviolet and infrared spectra were consistent with the structure (II) (λ_{max} 225 mμ, ε, 7000; a strong band at 1705 cm^{-1} with medium bands at 1645 and 975 cm^{-1}).

The p.m.r. spectrum of the ketose (II) was similar to that reported[3] for 3,4-dideoxy-D-*glycero*-hexosulos-*trans*-3-ene hydrate (an acyclic, unsaturated hexosone), except that the signal for the proton(s) at C-1 was now of intensity 2 and at τ 5.33. Furthermore, the spectrum of the ketose (II) showed no signs of any impurity or of other forms. The ketose consisted, therefore, of the *trans* isomer ($J_{3,4}$ = 16.4 c.p.s.) free of the *cis* isomer.

Sodium borohydride reduction, as above, of the ketose (II) yielded a syrup, from which a small proportion of *trans*-3-hexene-D-*threo*-1,2,5,6-tetrol (III) crystallized out. Several recrystallizations gave m.p. 114°, $[\alpha]_D$ −2° (water), λ_{max} 189 mμ, $J_{3,4}$ = 15.5 c.p.s. The syrup readily formed di-*O*-isopropylidene acetals, the isomer (IV) being readily obtained crystalline, m.p. 80–82°, $[\alpha]_D$ +55° (*c* 2, chloroform), $J_{3,4}$ = 15.4 c.p.s.: properties identical with those of the compound recently prepared by Haines[4] by another method. Mild, acid hydrolysis regenerated the tetrol (III) in good yield. The tetrol is of interest because a compound[5] with the same R_F value, believed to be a 3-hexene-1,2,5,6-tetrol[6], has been isolated from the alkaline degradation of blood-group substances.

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6 E.A. KABAT, private communication.

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*All the compounds mentioned in this paper gave satisfactory elemental analyses.

CARBOHYDRATE SPECIFICITY OF INDUCIBLE *Escherichia coli* AMYLOMALTASE

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INTRODUCTION

Cells of wild strains of *Escherichia coli* which have been grown in media not containing maltose are capable of attacking maltose only slightly, if at all. This weak action of some strains has been attributed to a constitutive maltase[1]. The presence of maltose in the growth medium elicits the formation of an enzyme, amylomaltase, which catalyses the reaction

$$n \text{ maltose} \rightleftharpoons (\text{glucose})_n + n \text{ glucose}$$

and there is evidence that the ability of induced cells to attack maltose is attributable almost exclusively to this enzyme[1,2]. The polymer products of the action of purified enzyme preparations ordinarily consist of short-chain, α-(1→4)-linked dextrins[3,4], but if glucose is removed continuously by glucose oxidase, amylose-like products can be obtained[5]. A detailed study of the properties of highly purified amylomaltase from *E. coli* ML 308 has been undertaken by Wiesmeyer and Cohn[6,7]. The induction of amylase-like enzymes in *E. coli* has not been studied, but Monod and Torriani[5] mentioned that partially purified preparations of amylomaltase had definite amylase activity. On the other hand, Welker and Campbell[8] have investigated the induction of amylase in *Bacillus stearothermophilus*, and have shown that maltodextrins are more effective inducers than maltose.

Research in the Department of Chemistry at Birmingham University has, over the years, yielded a number of compounds structurally related to maltose and maltodextrins. The availability of these compounds offered an opportunity to undertake a comprehensive survey of their potentialities as growth substrates for *Escherichia coli*, their ability to induce in this organism enzymes capable of attacking maltose, and their susceptibility to attack by cells induced with maltose.

METHODS

Organism and growth conditions

The amylomaltase-inducible ML 30 strain of *Escherichia coli* (kindly supplied by Professor J. Monod) was used throughout the work. The basal mineral composition of media was that recommended by Cohen and Rickenberg[9]. For growth tests,

carbon sources were included at a concentration of 0.1% (w/v) and media were inoculated with an aqueous suspension of cells grown on nutrient agar. For induction tests, the cells were pregrown in mineral medium containing 0.1 or 0.2% (w/v) succinic acid. Exponential phase cells were inoculated into fresh medium of the same composition, and when the cell density had reached 50 μg dry wt./ml the compound under test was added. After two further generations, the cells were harvested by centrifugation, washed, and assayed. Growth was measured in a Unicam SP 500 spectrophotometer at 650 mμ. Cell density was determined from a standard curve, relating dry weight concentration to optical density.

All cultures were incubated shaken at 37°. The volumes of cultures depended on the quantity of substrate available, but amounted to one-tenth or one-fifth of the flask volume.

Enzyme assay

Cell suspensions were first treated with toluene and sodium deoxycholate[10]. The assay mixture comprised treated cells, 0.5–2.0 mg dry wt./ml; substrate, 0.025–0.1 M (weight concentration for polysaccharides); 0.05 M-phosphate buffer (pH 7.0) The mixture was incubated shaken at 37°, usually for one hour, then a sample was withdrawn and placed in a boiling-water bath for 5 min to destroy enzyme activity, and finally cell debris was removed by centrifugation. For quantitative analyses, the glucose content of a suitably diluted sample of the supernatant was determined employing glucose oxidase under the conditions recommended by Dahlqvist[11]. For qualitative analyses paper chromatography was employed. Samples were placed on Whatman No. 1 filter paper and irrigated with pentyl alcohol–pyridine–water (1:1:1) for 20–24 h, and the spots detected with alkaline silver nitrate[12].

Enzyme preparation

Amylomaltase was prepared and partially purified as described by Monod and Torriani[5].

Maltose

Maltose was determined by the method of Dische[13].

Chemical preparations

Maltotriose, maltotetraose, maltopentaose, and maltohexaose were isolated as described by Barker *et al.*[14]. The isolation of the two isomeric trisaccharides, *O*-α-D-glucopyranosyl-(1→3)-*O*-α-D-glucopyranosyl-(1→4)-D-glucose (I) and *O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→3)-D-glucose (II) (for some experiments a mixture of the trisaccharides was employed), together with the homologous mixture of tetrasaccharides from nigeran, was as described by Barker *et al.*[15]. Maltitol and maltotetraitol were obtained from the corresponding sugars by reduction with sodium borohydride. Methyl β-maltoside was obtained by converting maltose into hepta-*O*-acetyl-α-maltosyl bromide[16], and then carrying out an exchange reaction

with methanol–Ag_2CO_3, followed by deacetylation by the method of Zemplén and Pacsu[17]. 1-Thiomaltose (Found: C, 39.7; H, 6.5; S, 8.9. $C_{12}H_{22}O_{10}S$ calc.: C, 40.2; H, 6.1; S, 8.9%) was obtained by reaction of the above glycosyl bromide with potassium polysulphide using methods similar to those adopted by Wrede and Hettche[18] for the preparation of 1-thiocellobiose. The reaction of 1-thiomaltose with alkaline hypoiodite[19] indicated a molecular weight of 353 (calc.: 358). Synthetic amylose and amylopectin were isolated as described by Barker *et al.*[20].

RESULTS

Amylomaltase activity of cells growing logarithmically on maltose

Whole cells attacked maltose, but glucose did not accumulate and was clearly utilised by the cells. Toluenisation destroyed the ability to utilise glucose and the production of glucose from maltose was approximately linear with time. Table I gives the results of a typical assay.

TABLE I

LIBERATION OF GLUCOSE FROM MALTOSE BY TOLUENISED PREPARATIONS OF MALTOSE-GROWN CELLS OF *E. coli*

Cells were grown on maltose, toluenised, and then included in an assay mixture of composition: maltose, 0.1M; cells, 2 mg dry wt./ml; 0.05M-phosphate buffer (pH 7.0). The mixture was incubated shaken at 37° and samples were removed at intervals, heated, and centrifuged, and the glucose concentration determined by means of glucose oxidase.

Time (*min*)	*Glucose liberated* (*μ mole/mg dry wt.cells*)
10	4.0
20	9.0
40	15.0
60	21.5

Inclusion of 0.001M-sodium iodoacetate had no effect, but 0.05M-sodium fluoride caused *ca.* 5% inhibition. If phosphate buffer was replaced by either tris or citrate buffers in the same concentration, the activity was reduced *ca.* 30%. Inclusion of 0.0025M-potassium dihydrogen phosphate in assay mixtures buffered with tris or citrate did not increase the activity.

Ability of compounds to support growth of E. coli

A wide variety of compounds was examined and Table II reveals that, of the compounds structurally related to maltose, only maltodextrins can serve as sole carbon source for growth. Compounds were tested serially and with each series a glucose control was included. A number of other substances, not related structurally to maltose, were also examined. The organism exhibited normal behaviour with common sugars and was able to grow on trehalose.

TABLE II

COMPOUNDS STRUCTURALLY RELATED TO MALTOSE, AND THE GROWTH OF *E. coli*

Compounds were included as sole carbon source at a concentration of 0.1% (w/v) in a basal mineral medium (10 ml) which was inoculated with 0.1 ml of an aqueous suspension of cells grown on nutrient agar. Cultures were incubated shaken at 37° and examined after 24 and 48 h.

Compounds utilised for growth	*Compounds not utilised for growth*
Maltose	Maltitol
Maltotriose	Methyl β-maltoside
Maltotetraose	Methyl hepta-*O*-methyl-β-maltoside
Maltopentaose	Nigeran trisaccharides
Maltohexaose	Nigeran tetrasaccharides

Ability of compounds to induce the formation of amylomaltase

The compounds listed in Table III were added to cultures growing on succinic acid. As some of the substances were available in only small amounts, inducing power at 0.0001M initial concentration was compared with that of maltose in the same initial concentration. The effect of a few compounds at 0.001M initial concentration was also examined.

TABLE III

COMPOUNDS TESTED AS INDUCERS FOR AMYLOMALTASE IN *E. coli*

Compounds were added at 0.0001M initial concentration, except compounds marked [a] which were added at 0.001M concentration, to cultures growing logarithmically at 37° on succinic acid. After two further generations, the cells were harvested and assayed for amylomaltase activity. The activity of the culture which had received maltose was taken as 100%.

Compound	*Amylomaltase activity*	*Compound*	*Amylomaltase activity*
Maltose	100	Synthetic amylopectin[b]	3
Maltotriose	109	Potato amylopectin[b]	6
Maltotetraose	100	Maltobionic acid	6
Maltopentaose	111	Methyl hepta-*O*-methyl-β-maltoside	6
Maltohexaose	20	Methyl β-maltoside	4
Trehalose	10	Melezitose	7
Maltotetraitol	6	Nigeran trisaccharides[a]	3
Maltitol	3	Nigeran tetrasaccharides[a]	3
Nigeran trisaccharide (I)	3	Methyl α-D-glucoside[a]	8
(II)	3	1-Thiomaltose[a]	3

[b]Added at an initial concentration of 36 mg/100 ml.

The effect of adding maltose at 0.002M initial concentration on the growth and enzyme activity of cells is shown in Table IV.

After the addition of maltose, the growth rate increases and formation of induced enzyme occurs apparently without lag in a manner similar to that described by Wiesmeyer and Cohn[6].

TABLE IV

EFFECT OF ADDITION OF MALTOSE ON GROWTH RATE AND AMYLOMALTASE ACTIVITY OF CELLS OF *E. coli* GROWING ON SUCCINIC ACID

A culture growing shaken at 37° on succinic acid was divided into two parts when the cell density had reached *ca.* 50 μg dry wt./ml. One part received maltose, (0.002M initial concentration) the other, no addition. Incubation was continued, and at intervals the growth of both cultures, and the maltose concentration and amylomaltose activity of the maltose-supplemented culture were determined.

Time (*h*)	*Culture growing on succinic acid. Concentration of cells* (*μg/ml*)	*Culture growing on succinic acid and maltose Concentration of cells* (*μg/ml*)	*Maltose concentration* (*mg/ml*)	*Amylomaltase activity. (μ moles glucose liberated/h/mg dry wt. cells)*
0	45	45	0.68	0.6[b]
1	75	90	0.54	18.8
2	139	183	0.20	24.2
3	231	375	0	29.6
4	285	475	[a]	[a]
5	285	490	[a]	26.5

[a]No determination made.
[b]Amylomaltase activity just prior to addition of maltose.

Maltodextrins added at approximately 0.0015M concentration also altered the growth rate and yield, whereas methyl β-maltoside, maltitol, or synthetic amylopectin altered neither of these parameters (Table V).

Effect of induced-cell preparations on various compounds

The action of toluenised preparations of maltose-grown cells on various compounds was examined qualitatively by paper chromatographic analysis. Glucose, together with its higher homologues was liberated from maltose, the maltodextrins (4–6 units long), the nigeran trisaccharide (I), synthetic amylose, and amylopectin. Quantitative estimations of activity at 0.025M substrate concentration revealed that the rates of attack on the maltodextrins were similar to that on maltose. Other compounds were attacked only slowly, if at all, but the synthetic polysaccharides were attacked at an appreciable rate (Table VI). For these experiments, cells which had been stored at 4° for 48 h were employed. The amylomaltase activity was lower than normal, but subsequent experiments with more active cultures consistently showed that maltose-induced cells were able to attack the synthetic polysaccharides. On the other hand, purified amylomaltase preparations had a low activity with these compounds.

TABLE V

EFFECT ON MEAN GENERATION TIME AND YIELD IN STATIONARY PHASE OF ADDITION OF COMPOUNDS TO CULTURES OF *E. coli* GROWING ON SUCCINIC ACID

Cultures of *E. coli* were grown shaken at 37° on succinic acid. When the cell concentration had reached *ca.* 50 μg/ml, the compounds listed below were added at an initial concentration of *ca.* 0.0015M.

Compound added	*Initial concentration (mg/100 ml)*	*Mean generation time in log phase after addition (min)*	*Yield of cells at stationary phase (μg/ml)*
Succinic acid[a]		75	174
Maltose	50	65	345
Maltotetraose	100	55	450
Maltopentaose	125	55	510
Methyl β-maltoside	50	75	164
Maltitol	50	75	182
Synthetic amylopectin	50	75	182

[a]Corresponds to a culture which received no addition.

TABLE VI

ACTION OF TOLUENISED PREPARATIONS OF MALTOSE-GROWN CELLS OF *E. coli* ON VARIOUS SUBSTANCES

Induced cells were toluenised and then suspended in 0.1M-phosphate buffer (pH 7.0) at a concentration of 4 mg dry wt./ml. To 0.5 ml of the cell preparation was added 0.5 ml of a 0.5M-solution of the substance to be tested and the mixture incubated at 37°. Finally, the mixture was heat-treated and centrifuged, and the glucose content determined.

Substance	*Glucose liberated (μmole/h/mg dry wt. cells)*	*Substance*	*Glucose liberated (μmole/h/mg dry wt. cells)*
Maltose	6.2	Panose	0
Maltotriose	5.0	Potato amylopectin[a]	1.5
Maltotetraose	6.5	Glycogen[a]	0.7
Maltopentaose	4.2	Dextran[a]	0.8
Maltohexaose	5.6	Methyl β-maltoside	0
Trehalose	2.2	Calcium maltobionate	0
Maltotetraitol	0.8	Synthetic amylose[a]	8.6
Nigeran trisaccharides	0.5	Synthetic amylopectin[a]	7.2
Nigeran tetrasaccharides	0		

[a]Concentration in reaction mixture, 85 mg/ml.

The ability to attack the synthetic polysaccharides appears to be directly related to maltose induction, since cells grown on succinic acid as sole carbon source attacked the polysaccharides at only a very low rate. The behaviour with trehalose was in agreement with the results obtained with purified preparations[5]. Cells induced with maltose yielded glucose from trehalose at *ca.* 30% of the rate at which maltose was attacked, but cells grown on trehalose as sole carbon source had less than 10% of the amylomaltase activity of cells grown on maltose.

Inhibition of enzyme action and enzyme formation

The presence of D-xylose, D-glucose, D-galactose, panose, trehalose, maltitol, methyl α-D-glucoside, or methyl β-maltoside at 0.05M concentration in assay mixtures with maltose at this same concentration had no effect on enzyme activity. 1-Thiomaltose at 0.05M concentration caused 80% inhibition, but was without effect at 0.01M and 0.005M concentration. 1-Thiomaltose at 0.001M concentration in a growth medium with maltose at this same concentration had no effect on maltose uptake, and also at 0.001M concentration caused no diminution in the degree of induction brought about by 0.0001M-maltose.

Utilisation of maltodextrins in the presence of maltose by growing cultures

When a mixture of maltose and maltotetraose (0.001M each) was added to a culture growing logarithmically on succinate, the chromatographic evidence strongly suggested that maltotetraose was utilised before maltose. A mixture of maltodextrins and maltose was added to a growing culture, but the fate of the higher oligosaccharides could not be adequately assessed chromatographically because of salt interference. However, maltotetraose was utilized before there was a qualitative diminution in the maltose concentration.

Action of purified enzyme preparations on radioactive substrates

The homologous series of [^{14}C] labelled maltodextrins from maltose to maltodecaose could be readily isolated after paper chromatographic separation of a digest obtained by incubation of [^{14}C] maltose (6.2 mg: 100 μc) with amylomaltase (0.5 ml) for 30 min at 37°. Each saccharide was dissolved in 0.1M-tris buffer pH 6.8 (0.5 ml), incubated with amylomaltase (0.5 ml) at 37°, and then analysed at intervals by paper chromatography and autoradiography. Maltotriose in three hours was completely converted into maltose and glucose, while maltotetraose gave maltotriose, maltose, and glucose. The tetrasaccharide disappeared after two hours and the trisaccharide after six hours. Maltopentaose rapidly gave the homologous series of lower saccharides as indeed did maltohexaose. It was significant, however, that even when the maltopentaose and maltose had disappeared a maltohexaose spot still persisted. After conversion of maltoheptaose into the lower homologous series of amylosaccharides, the maltopentaose and maltotetraose disappeared but the maltohexaose persisted. This persistence of maltohexaose in the digestion products was also noticed when malto-octaose, maltononaose, and even maltodecaose were incubated with amylomaltase. The initial breakdown patterns in these cases were $8 \rightarrow 6 + 2$; $9 \rightarrow 6 + 3$; $10 \rightarrow 6 + 4$. This preferential persistence of maltohexaose may be associated with helix formation. The [^{14}C] saccharides were at very low concentration (< 1 mg/ml) under the above conditions. When [^{14}C] maltotetraose was mixed with maltotetraose (5 mg) and incubated with the same amount of enzyme-buffer as above, the sole initial products (3–6 min) were maltopentaose and maltotriose, followed later (15–20 min) by maltohexaose and maltose.

DISCUSSION

The observation that cells grown in the presence of maltose, unlike cells grown on succinate as sole carbon source, are capable of attacking synthetic polysaccharides with the production of glucose suggests that an amylase or phosphorylase type of enzyme is induced concurrently with amylomaltase. Monod and Torriani[5] mentioned that partially purified preparations of amylomaltase can attack amylose, and they attributed this action to amylase or phosphorylase impurities which could be eliminated by further purification. The action on synthetic polysaccharides could be considerably reduced if amylomaltase preparations were partially purified.

The specificity of amylomaltase induction is high and, moreover, apart from the synthetic polysaccharides and trehalose, maltose and maltodextrins are the only compounds attacked by toluenised preparations of induced cells. Monod and Torriani[5] showed that amylomaltase preparations had no action on sucrose, lactose, melibiose, cellobiose, or methyl α-D-glucoside, and Wiesmeyer and Cohn[7] demonstrated that, of a large number of compounds tested, only glucose, mannose, and methyl β-maltoside could act as glucosyl acceptors with amylose in the reverse reaction catalysed by pure preparations of amylomaltase. Of a number of compounds, including methyl α-D-glucoside, methyl β-maltoside, and 1-thiomaltose, tested for inhibitory action on the activity of toluenised preparations of induced cells, only 1-thiomaltose was an effective inhibitor. These results are in contrast to those of Wiesmeyer and Cohn[7] who showed that methyl α-D-glucoside and methyl β-maltoside competitively inhibited the action of pure preparations of amylomaltase. On the other hand, 1-thiomaltose was without effect on either maltose uptake or induction of growing cells, and it seems likely that with whole cells, and possibly with toluenised preparations, permeability effects are involved.

The results of the studies of the action of purified enzyme preparations on [^{14}C] labelled maltodextrins are difficult to interpret. The experiments with maltotetraose show a different product pattern depending on the concentration of substrate. At high concentration, a typical amylomaltase action, with the production of higher oligosaccharides, is observed, but at low concentration it seems possible that water acts as the chief transglycoside acceptor. In some respects, the action patterns at low substrate concentrations are reminiscent of those reported by Robyt and French[21] for the action of *Bacillus subtilis* amylase on maltodextrins.

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SUMMARY

The effect of a number of compounds structurally related to maltose on the growth, amylomaltase induction, and amylomaltase action of an inducible strain of

Escherichia coli has been investigated. Only maltose and maltodextrins were utilised as sole carbon source for growth, and only maltodextrins had an inducing power comparable to that of maltose. Of a number of compounds tested, only maltose and maltodextrins were attacked at an appreciable rate by induced cells, but induced cells could also produce glucose from synthetic amylopectin. 1-Thiomaltose was the only effective inhibitor of amylomaltase action. The action pattern of purified amylomaltase preparations on [^{14}C] labelled maltodextrins has been examined.

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ARRANGEMENT OF THE L-RHAMNOSE UNITS IN *Diplococcus pneumoniae* TYPE II POLYSACCHARIDE

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INTRODUCTION

Diplococcus pneumoniae Type II capsular polysaccharide contains L-rhamnose[1], together with D-glucose and D-glucuronic acid[2]. Methylation studies[3,4] showed that (a) the L-rhamnose was linked at positions 1 and 3, (b) the D-glucose was involved in 1,4,6-branch points, and (c) the D-glucuronic acid was linked at positions 1 and 4, and also occurred as non-reducing terminal units. Infrared spectra and the optical rotation[3] suggested that some of the glycosidic linkages in the type specific polysaccharide had the α-configuration, but the precise location of these was not known. The present studies were designed to elucidate the anomeric character of the rhamnoside linkages, and to determine the location and arrangement of the rhamnose units in the polysaccharide.

MATERIALS AND METHODS

The sample of *Pneumococcus* Type II specific capsular polysaccharide (SII) used in these studies was kindly purified by Dr. S.M. Amir, employing deproteinisation with trifluorotrichloroethane, followed by fractionation with cetyltrimethylammonium bromide. After ethanol precipitation, and removal of nucleic acid by ribonuclease digestion, a polysaccharide with $[\alpha]_D^{29} + 51.2°$ (*c* 0.81, water) was obtained.

Standard method of induction

Aliquots (25 ml) of enriched medium[5], consisting of KH_2PO_4 (4.7 g), $(NH_4)_2SO_4$ (2.0 g), $MgSO_4.7H_2O$ (0.2 g), $CaCl_2.2H_2O$ (0.01 g), $FeSO_4.7H_2O$ (0.005 g), L-glutamic acid (0.5 g), glycine (0.1 g), succinic acid (6.0 g), and yeast extract (Difco Ltd., 1.0 g) in one litre of aqueous solution adjusted to pH 6.8 with potassium hydroxide, were dispensed in conical flasks (250 ml). After sterilisation, a supplementary vitamin mixture [containing biotin (10 μg), thiamine (1.0 mg), nicotinic acid (1.0 mg), and *p*-aminobenzoic acid (1.0 mg) per ml of solution] (20 ml per litre) was added before inoculation with *Klebsiella aerogenes* (NCIB 9479) from agar slopes. After growth for 24 h at 37°, the organism was again subcultured (5 μl) in the same manner, and

incubated for a further 16 h, by which time maximum growth was obtained. The organism was harvested by centrifugation and washed with sterile distilled water (0.1 × the volume of the previous culture). The washed cells were resuspended in sterile distilled water at a concentration 25 × that of the original culture. Induction units of a minimal mineral medium containing KH_2PO_4 (4.7 g), $(NH_4)_2SO_4$ (2.0 g), $MgSO_4.7H_2O$ (0.2 g), and succinic acid (6.0 g) in one litre of aqueous solution adjusted to pH 6.8 with potassium hydroxide, were inoculated with a sufficient volume of resuspended cells to give a cell concentration of 0.28 mg per ml, determined by the absorption of cell suspensions at 650 mμ. The inducer solution, sterilised by membrane filtration, was added aseptically before commencing incubation at 37°.

Separation of medium constituents

Cell-free samples for analysis were obtained by centrifugation of culture aliquots. A quantitative separation (1.0 ml aliquots) of macromolecular substrate and low molecular-weight cleavage products was obtained by gel filtration on Sephadex G-25 (Pharmacia Ltd., exclusion limit 3-4,000, bed volume 66 ml). Analyses were performed on the material obtained by lyophilisation of the relevant fractions (polysaccharide fraction, 20 to 39 ml; cleavage product fraction, 47 to 64 ml). After characterisation of the cleavage products, a more convenient separation was obtained by ion-exchange chromatography. Fractionation (1.0 ml aliquot) on DEAE Sephadex A-50 (Cl^- form, bed volume 3.0 ml) with linear gradient elution (NaCl, 0–1.0M, in phosphate buffer pH 6.8, 0.05M, 100 ml) afforded a quantitative separation of the neutral cleavage products, induced enzyme, and polysaccharide. The neutral materials were eluted in the first 4.0 ml of eluate, the enzyme in 5.0–30.0 ml, and the polysaccharide in 32–64 ml.

Analysis of cleavage products

Qualitative analyses for carbohydrates were performed by chromatography on Whatman No.1 paper, using descending irrigation with butanol–ethanol–water (4:1:5), and detection by alkaline silver nitrate[6], or aniline hydrogen phthalate[7]. Paper electrophoresis was performed on Whatman No. 3 MM paper in acetate buffer (0.2M, pH 5.0).

Quantitative determinations of carbohydrates were obtained by various spectrophotometric assay techniques. Determinations based on reducing power were obtained by the Nelson colorimetric modification of the Somogyi micro-copper method[8]. 6-Deoxyhexoses and hexoses were determined, essentially as recommended by Dische[9,10], using the cysteine–sulphuric acid reaction. Hexuronic acids were determined by the modified carbazole–sulphuric acid reaction of Dische and Gregory[11]. Analyses for D-glucose in hydrolysates were performed by the selective glucose–oxidase method[12].

Periodate oxidations of oligosaccharides were performed at 2°, at pH 4.5, in the absence of light, for a period of 14–24 h. Sodium metaperiodate (0.01M) corresponding to a five-fold excess of oxidant over the expected consumption was

employed. The reactions were terminated by addition of a two-fold excess of lead dithionate solution (10%, w/v), enabling modifications of the standard spectrophotometric determinations to be performed directly on oxidation products.

Analysis of polysaccharide cores

The three constituent sugars were assayed by the spectrophotometric procedures mentioned above.

Assay of induced enzymes

Glycosidase activity and specificity were determined by incubation of the enzyme with the relevant methyl glycoside. Preliminary qualitative estimations of activity were obtained by chromatography of the digests. Quantitative analysis of methyl rhamnosides in the presence of free rhamnose was initially obtained by preferential adsorption of the free sugar on a column of Dowex 1X2 prepared from an aliquot (1.0 ml) of a 1:1 (v/v) aqueous suspension of the resin. The column was washed with sodium hydroxide (N, 20 ml), and then with carbon dioxide-free distilled water to a neutral pH. Samples (1.0 ml) containing 20–40 μg of mixed methyl rhamnoside and free rhamnose were applied to the column, and the rhamnosides quantitatively eluted in carbon dioxide-free distilled water (10 ml). After concentration, analyses were performed by the cysteine–sulphuric acid reaction on measured aliquots. The free rhamnose was eluted in sodium chloride (10% w/v, 10 ml), but not in a quantitative yield.

Subsequently, a direct method of analysis was employed, based on the reduction of the free sugar with sodium borohydride. Samples containing rhamnoside (0–20 μg) and enzyme in a total volume of 0.45 ml, together with the appropriate standards and controls, were incubated at 37° for 12 h. Aliquots (0.2 ml) were assayed for total rhamnose by the cysteine–sulphuric acid reaction after dilution to 0.5 ml. A second aliquot (0.2 ml) from each tube was transferred to a cooled (iced water) stoppered-tube, and diluted with borate buffer (0.1M, 0.1 ml). Ice-cold sodium borohydride solution (2% w/v, 0.1 ml) was added, the tubes were maintained at 0° for 2 h, and the unreacted borohydride was destroyed by the addition of acetic acid (5% w/v, 0.1 ml). After storage for 30 min at 0°, the contents of each tube were assayed for unreduced rhamnose by the cysteine–sulphuric acid reaction.

Preparation of reference enzymes

Standard preparations of induced enzymes were obtained by induction of *K. aerogenes* with methyl α-L-rhamnopyranoside and methyl β-L-rhamnopyranoside. Cell suspensions of *K. aerogenes*, prepared by the general method, were used to inoculate induction units (25 ml), consisting of medium[5] similar to that used for the initial growth medium but containing no yeast extract, to a cell concentration of 0.28 mg/ml. A sterile solution of inducer was added to give a concentration of 0.25 mg/ml, prior to commencing incubation at 37°. After 24 h, a further addition of inducer was made at the same level of concentration. Cell-free supernatants, obtained

after growth for a further 24 h, were dialysed at 0° against phosphate buffer (0.02M, pH 6.6) and concentrated ten-fold by ultrafiltration.

The specificity and activity of the preparations (0.1 ml aliquots) towards the anomeric methyl rhamnosides were obtained by the methods mentioned above.

Sequential chemical degradation of the polysaccharide

Oxidation

A solution of SII (10.3 mg) was oxidised at 2° with sodium metaperiodate (0.016M, 25 ml). Consumption of periodate was followed by the spectrophotometric method[13] employing the absorbance at 225 mμ. Aliquots (1.0 ml) were analysed for carbohydrate content by modifications of the standard methods, after destruction of excess of periodate with lead dithionate, as described previously. After completion of the oxidation (360 h), residual periodate was removed by the addition of excess of ethylene glycol and dialysis against three daily changes of saline (0.9%) at 0°. After concentration by lyophilisation, the oxidised material was redissolved in water (10 ml).

Reduction

The oxidised material was reduced with sodium borohydride at 2°, using carbon dioxide ebullition to maintain pH 8.0. Sodium borohydride (20 mg) was added at intervals (30 min) over a period of 5 h. After a further hour the reaction mixture was neutralised to pH 7.0 by the addition of acetic acid (5% w/v), and dialysed against three daily changes of phosphate buffer (0.05M, pH 7.0) containing sodium chloride (0.9%).

Mild acid hydrolysis

The reduced material was hydrolysed in 0.1N-hydrochloric acid for 4 h at 20°.

This sequence of periodate oxidation, borohydride reduction, and mild acid hydrolysis was repeated twice on the degraded material.

RESULTS

Cell-free aliquots of an induction of *K. aerogenes* with the capsular polysaccharide SII (25 mg) showed, on chromatographic analysis, a component of R_G 0.25 after incubation for 1 hour, and a component of R_G 1.94 after 6 hours. (*cf.* L-rhamnose, R_G 1.94; D-glucuronic acid, R_G 0.2). Analysis of the polysaccharide cores and cleavage products gave the results shown in Table I.

The isolated component of R_G 0.25, when hydrolysed at 100° for 4 hours with N-sulphuric acid gave 97% rhamnose, and no detectable glucose or glucuronic acid. In the cysteine–sulphuric acid reaction, the original oligosaccharide gave a chromophore identical with that from authentic rhamnose, and with the same sensitivity to water. It behaved as a neutral entity on electrophoresis in acetate buffer and was

not adsorbed on Deacidite FF resin (CO_3^{2-} form), in contrast to reference glucuronic acid. Since the rhamnose content of the periodate-oxidised oligosaccharide was

TABLE I

INDUCTION OF *K. aerogenes* WITH SII

Time (h)	*Core analysis (μg/mg initial SII)*			*Rhamnose liberated*
	Rhamnose[a]	*Glucose*	*Glucuronic acid*	*(μg/mg initial SII)*[a]
0	478(478)	347	162	0.0(0.0)
1	452(393)	346	161	16.0[b](0.0)
2	446(356)	350	163	5.0(0.0)
3	430(342)	346	162	4.5(0.0)
4	425	346	161	4.6
5				4.2
6	414	347	162	5.2
7				4.8
24	267	346	161	

[a]Values in parenthesis were obtained when SII was added in the logarithmic phase of growth.
[b]Present as an oligosaccharide.

33% of that before oxidation, and 34% of the rhamnose was reducible by sodium borohydride, a trisaccharide structure was indicated.

The component of R_G 1.94, isolated after incubation of SII with *K. aerogenes* for 6 hours, behaved as a neutral entity, was stable to acid hydrolysis, and in the cysteine–sulphuric acid reaction gave a chromophore which was identical with that from a 6-deoxyhexose. This chromophore was not formed following periodate oxidation or borohydride reduction of the component, indicating the latter to be unbound rhamnose.

TABLE II

SPECIFICITY OF ENZYMES INDUCED IN CULTURES OF *K. aerogenes*

Inducer	*Substrate % hydrolysis*[a]	
	Methyl α-L-rhamnopyranoside	*Methyl β-L-rhamnopyranoside*
SII (1 h incubation)	36.5	0.0
SII (6 h incubation)	40.9	45.4
Methyl α-L-rhamnopyranoside	39.9(39.0)	0.0(0.0)
Methyl β-L-rhamnopyranoside	0.0(0.0)	43.6(44.1)

[a]Values in parenthesis were obtained by the reduction method.

The specificities of the enzymes present after incubation with SII for 1 and 6 hours, and of the reference enzymes induced with the methyl rhamnosides, are shown in Table II. In the case of the SII-induced enzymes, an aliquot (0.25 ml)

of the ten-fold concentrated fraction from the DEAE Sephadex column was used, whilst an aliquot (0.1 ml) of the concentrated reference enzyme was employed. Prior heat treatment (100°, 5 min) of the digests resulted in complete loss of activity, indicating the enzymatic character of the hydrolysis.

The trisaccharide was not hydrolysed by the reference α-L-rhamnosidase, or by the enzyme present after incubation for 1 hour with SII. Hydrolysis of the trisaccharide was effected by the reference β-L-rhamnosidase. Examination of the ratio of non-reducible to non-oxidisable rhamnose in the digest indicated the sole production of unbound rhamnose.

Incubation of SII with α-L-rhamnosidase, obtained either from a one hour incubation with SII, or from methyl α-L-rhamnopyranoside, resulted in the liberation of a trisaccharide identical with that discussed above. Complete correlation was obtained between the rhamnose content of the trisaccharide and the loss of rhamnose from the polysaccharide recovered by gel filtration of the digest. The cleavage product, obtained by fractionation of the digest of SII with reference β-L-rhamnosidase, contained only unbound rhamnose since it was completely destroyed by periodate oxidation and borohydride reduction.

Periodate oxidation of SII reduced the rhamnose content by 33%, the level of rhamnose remaining constant after 3 hours. Borohydride reduction of the oxidised material did not alter the rhamnose content, demonstrating that the rhamnose was still glycosidically bound. Similarly, mild acid hydrolysis did not significantly alter this value. Repetition of this oxidation, reduction, and hydrolysis sequence lowered the rhamnose content of the initially degraded SII by 49.7%. A final application of the degradative sequence removed entirely the rhamnose portion of the molecule. The action of the reference α-L-rhamnosidase and β-L-rhamnosidase on SII in various states of degradation were determined, the cleaved product being characterised by periodate oxidation and borohydride reduction. Aliquots (0.1 ml) of the enzyme preparations were used, with samples containing 50–100 μg of rhamnose; the assays were performed after incubation for 12 hours at 37°, and the results of these digestions are shown in Table III.

TABLE III

ACTION OF REFERENCE ENZYMES ON CHEMICALLY-DEGRADED SII

-L-*rhamnosidase*	*SII Initial* *Rha–Rha–Rha–X*		*SII 1st degradation* *Rha–Rha–X_1*		*SII 2nd degradation* *Rha–X_2*	
	α	β	α	β	α	β
Rhamnose content of cleavage product (μg/mg initial SII)						
Total	49	53	60	95	42	0
Reducible by borohydride	17	52	29	94	42	0
Oxidisable by periodate	33	53	60	95	42	0

DISCUSSION

A study of the conditions necessary for induced enzymatic degradation of *Pneumococcus* Type II specific capsular polysaccharide (SII), concurrent with the accumulation of cleavage products in the extracellular medium, led to the adoption of the induction conditions formulated above. The critical requirements appear to be an enriched initial-growth medium, followed by an initial high cell-concentration in a minimal mineral induction medium with its associated, prolonged lag-phase of growth, during which the inducer is added. Delay of SII addition, until logarithmic growth was established, resulted in an increased rate of degradation, but gave no detectable accumulation of cleavage products.

Monitoring of cleavage products by measurement of increased reducing power in the culture proved somewhat unreliable by the Nelson method[8]. Although satisfactory in aqueous solutions, or, for example, in the presence of salt from neutralised hydrolysates, the assay was unreliable for the determination of reducing sugars in culture filtrates or dialysates. The presence of succinate ions and other metabolites had a marked depressant effect on chromogen formation, not only in the reagent blanks, but also in the sugar assays. Reasonable results were attainable when calibration was possible in buffer or medium of constant composition, but not in cultures of varying age.

On DEAE Sephadex, quantitative resolution of polysaccharide cores and the cleaved carbohydrate occurred, enabling each of them to be accurately monitored. Analysis of the polysaccharide cores with time in an induction of *K. aerogenes* with SII showed a steady decrease in rhamnose content with incubation time, the glucose and glucuronic acid being unaffected within the limits of experimental error. A rhamnose oligosaccharide, followed by rhamnose, appeared in the extracellular medium. The same DEAE Sephadex column afforded a good separation between the induced enzyme and the carbohydrates. After one hour, the only detectable extracellular carbohydrase was an α-L-rhamnosidase, which degraded methyl α-L-rhamnopyranoside but not methyl β-L-rhamnopyranoside. The fact that the total rhamnose content before and after this enzyme reaction was the same, indicated that the enzyme was purely hydrolytic, and had no fermentative action on rhamnose. The most reliable method for quantitative determination of rhamnose, in the presence of rhamnosides, was by direct analysis of the total rhamnose contents before and after borohydride reduction. Separations on a strongly basic resin gave a quantitative recovery of rhamnosides, but not of free rhamnose which was partially degraded on the column.

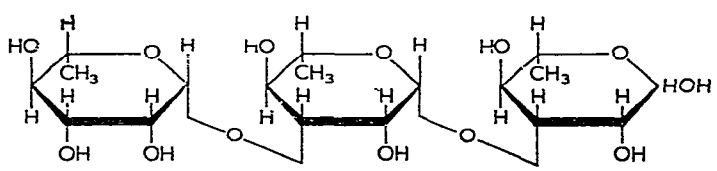
An enzyme was prepared by induction of *K. aerogenes* with methyl α-L-rhamnopyranoside and shown to be stereospecific in its ability to hydrolyse the α- but not the β-L-rhamnopyranoside. It was confirmed that the enzyme was able to hydrolyse the α-anomer in the presence of the β-anomer. When either the enzyme from the induction with SII (after incubation for one hour), or the reference α-L-rhamnosidase, was incubated with SII, the rhamnose oligosaccharide obtained was identical with that obtained from the polysaccharide induction medium (one hour). This indicated that

the rhamnose oligosaccharide and the remainder of the SII molecule were joined by an α-glycosidic link.

The results of the selective spectrophotometric assays combined with ion-exchange chromatography, and electrophoresis, confirmed that the oligosaccharide contained only rhamnose units. If one assumes that all the rhamnose units in SII are linked through positions 1 and 3, as indicated by the detection of only one dimethyl-rhamnose, 2,4-di-*O*-methyl-L-rhamnose, in the methylation studies[3,4], then the periodate oxidation results suggest that the oligosaccharide is a trisaccharide. Oxidation of the reducing and non-reducing terminal units would occur, leaving the central unit intact, and giving a rhamnose ratio of 3 to 1 before and after oxidation. In support of this argument, assay before and after borohydride reduction showed that only one rhamnose unit in three was reducible.

Because only small quantities of the trisaccharide were available, the method of identifying the inter-rhamnose linkages had to be enzymatic. After incubation of the trisaccharide with α-L-rhamnosidase, induced either by SII or by methyl α-L-rhamnopyranoside, no change in the ratio of rhamnose present before and after periodate oxidation was discernible. Any hydrolysis of the oligosaccharide would have resulted in a change in this ratio, due to a lowering of the proportion of rhamnose resistant to oxidation. This technique was judged to be sensitive to 1–2% hydrolysis. The trisaccharide was attacked by β-L-rhamnosidase. The total rhamnose content of the digest remained unchanged after incubation, indicating the absence of non-hydrolytic degradation, but the proportion of rhamnose resistant to periodate oxidation was markedly decreased. At the same time the reducible rhamnose content had increased. Since, after enzymatic digestion, the proportion of rhamnose units resistant to borohydride reduction remained twice that resistant to periodate oxidation, only free rhamnose and unhydrolysed trisaccharide were present. The failure to detect rhamnosyl-rhamnose in the digest indicates that both of the inter-rhamnose linkages in the trisaccharide have the β-configuration. A careful study of the enzymatic hydrolysis of the trisaccharide with time would probably show the transitory appearance of rhamnosyl-rhamnose, since the rates of enzymatic hydrolysis of the two linkages would be expected to be slightly different.

Although colour reactions, in combination with periodate oxidation and borohydride reduction, cannot usually replace the classical tools of structural oligosaccharide chemistry, consideration of the unique combination of products formed in the present case provides a firm basis for assigning structure I to the trisaccharide.

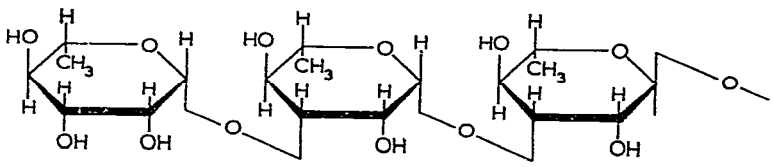


I

The sole product isolated after incubation of *K. aerogenes* with SII for 6 hours is free rhamnose, and this is entirely in agreement with the facts previously discussed. The initially-induced α-L-rhamnosidase liberates the trisaccharide, and the sequentially-induced β-L-rhamnosidase degrades this to rhamnose. The residual level of rhamnose in the medium, although very low, suggests a balance between the hydrolytic release of the sugar and its utilisation by the organism.

The liberation of the trisaccharide from SII by the action of an α-L-rhamnosidase suggested the presence of *O*-β-L-rhamnopyranosyl-(1→3)-*O*-β-L-rhamnopyranosyl-(1→3)-*O*-α-L-rhamnopyranosyl-(1→3)- units as terminal non-reducing chain-ends. To investigate this postulation, the polysaccharide was degraded chemically by sequential application of periodate oxidation, borohydride reduction, mild acid hydrolysis, and repetitions of these processes. On oxidation of SII, only one third of the total rhamnose content was destroyed (*cf.* ref. 14) suggesting that all of the rhamnose was present as Rha 1→3Rha 1→3Rha 1→ terminal units. The oxidised rhamnose units were, presumably, the non-reducing end-groups, and after reduction with sodium borohydride, were removed by mild acid hydrolysis. This did not decrease the rhamnose content, showing that the original resistant rhamnose units were still glycosidically bound; α-L-rhamnosidase liberated only rhamnosyl-rhamnose from the core at this stage. Degradation of the core by β-L-rhamnosidase liberated only rhamnose, thus confirming the existence of a β-linkage between the central and reducing units of the rhamnose trisaccharide. The corresponding evidence for the linkage between the central and non-reducing terminal units is more indirect, no disaccharide being detectable by the action of β-L-rhamnosidase on the trisaccharide.

In further confirmation of the postulated structure, a second oxidation decreased the rhamnose content of the core by one half. Reduction and mild acid hydrolysis of this material caused no alteration in the rhamnose content, and the product showed no reaction with β-L-rhamnosidase, indicating the lack of β-linkages in the core at this stage. α-L-Rhamnosidase released only free rhamnose from this core. A third oxidation finally removed the residual rhamnose units.



II

The sequential degradation of SII by periodate oxidation, borohydride reduction, and mild acid hydrolysis, in combination with use of the reference enzymes, provides good corroborating evidence that the rhamnose units are arranged as in structure II. The gross structure of SII now becomes extremely interesting. If all the D-glucose is present as 1,4,6-branching points, and all the rhamnose units are arranged as repre-

sented in structure II, then it is impossible for an appreciable proportion of the D-glucuronic acid to be present as 1,4-linked intercatenary units. It would, in fact, seem unlikely that all the D-glucose is 1,4,6-linked, since there is some discrepancy between the recovery of the methylated glucose derivative and the total glucose content of SII.

Enzyme induction, coupled with the use of specific enzymes, prepared by induction, has in this instance proved valuable in investigating the structure of a molecule difficult to examine by classical methods.

SUMMARY

Pneumococcus Type II polysaccharide, containing L-rhamnose (47.8%), D-glucose (34.7%), and sodium D-glucuronate (16.2%), induced sequentially in cultures of *Klebsiella aerogenes* an α-L-rhamnosidase, which liberated *O*-β-L-rhamnopyranosyl-(1→3)-*O*-β-L-rhamnopyranosyl-(1→3)-L-rhamnose, and then a β-L-rhamnosidase, which degraded this trisaccharide to L-rhamnose. The arrangement of such rhamnose units in the polysaccharide has been determined by using sequential application of degradation by periodate oxidation, sodium borohydride reduction, and mild acid hydrolysis, and repetitions of this sequence.

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APPLICATIONS OF GAS–LIQUID CHROMATOGRAPHY TO THE DETERMINATION OF POLYSACCHARIDE STRUCTURES

MODEL STUDIES ON A SYNTHETIC D-GALACTAN

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INTRODUCTION

Two common methods for the examination of the structure of a polysaccharide are periodate oxidation and methylation. The former method has recently been greatly enlarged in scope with the development of the Smith degradation procedure[1] by which a polysaccharide may be degraded to a series of glycosylalditols in which the alditol moiety is usually glycerol, erythritol or a threitol. If the polysaccharide contains both α and β linkages and is highly branched, then mixtures of various glycosylalditols with both anomeric configurations are produced. Such mixtures are often inseparable by paper chromatography or electrophoresis, but may be separated as their trimethylsilyl derivatives[2] by using gas–liquid chromatography (g.l.c.)[1]. This paper reports some applications of this technique to a synthetic D-galactan prepared by Mora *et al.*[3]. Although only small amounts of the various galactosylalditols were obtained, their anomeric configurations were readily determined by treatment with β-galactosidase. G.l.c. was also used for the analysis of some of the methylated sugars obtained by hydrolysis of the methylated D-galactan.

DISCUSSION

Smith degradation

When the D-galactan was oxidized with periodate, reduced with borohydride and subjected to mild acid hydrolysis, a mixture of products was obtained which could be partially resolved by paper chromatography into about nine fractions (Table I). The first three (fractions 1, 2, and 3) were identified as glycerol, D-threitol, and L-arabinose respectively. Fraction 4 was a mixture of a non-reducing component and D-galactose. The latter was removed, after bromine oxidation, on an ion-exchange resin. The non-reducing material gave arabinose and glycerol on hydrolysis, and formaldehyde on periodate oxidation. Reduction and hydrolysis gave an equimolar mixture of glycerol and ethylene glycol, showing that the compound was 1-*O*-(L-arabinofuranosyl)glycerol. The isolation of this derivative is evidence for the

TABLE I

SMITH DEGRADATION OF D-GALACTAN

Fraction	*Identity*	*wt.* (g)	R_{Gal}
1	glycerol	1.500	3.05
2	D-threitol	1.200	1.71
3	L-arabinose	0.020	1.35
4a	D-galactose	0.040	1.00
4b	1-*O*-(L-arabinofuranosyl)glycerol	0.020	0.95-1.00
5	*O*-D-galactosylalditols	0.032	0.84-0.90
6		0.056	0.64-0.72
7		0.060	0.49-0.56
8	oligosaccharide	0.078	0.33-0.41
9	oligosaccharide	0.082	0.19-0.26

existence of the D-Gal*f*-(1→6)-D-Gal sequence in the polysaccharide. Fraction 5 was trimethylsilylated[2] and examined by g.l.c. Of the two main components (Fig. 1A), the major product was shown to be the trimethylsilyl derivative of 1-*O*-(α-D-galactopyranosyl)glycerol and the minor product to be that of the β-D anomer, by desilylation[2] and treatment with β-galactosidase. These products demonstrate the existence of D-Gal*p*-(1→6)-D-Gal sequences with both α-D and β-D linkages in the polymer.

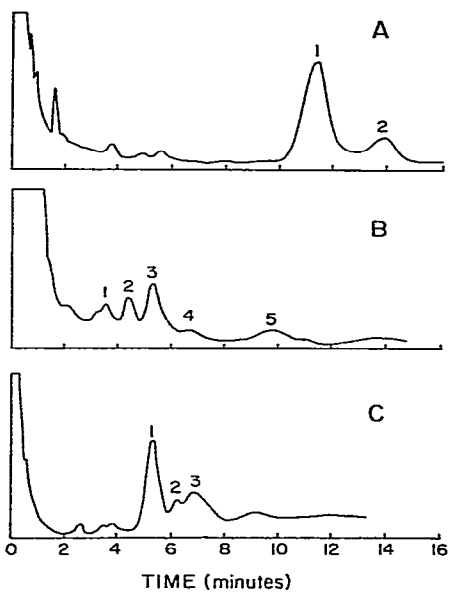

Fig. 1. Gas–liquid chromatography of trimethylsilyl ethers of *O*-D-galactosylalditols from Smith degradation. (A) Column temperature, 225°; He flow 120 ml/min; 1, 1-*O*-(α-D-galactopyranosyl)-glycerol; 2, β-D anomer. (B) Column temperature, 255°; He flow 150 ml/min; 2, 2-*O*-(α-D-galactopyranosyl)-D-threitol; 3, β-D anomer. (C) Column temperature, 255°; He flow 150 ml/min; 1, same as B3; 2, 1-*O*-(α-D-galactopyranosyl)-D-threitol; 3, β-D anomer.

The gas chromatogram of fraction 6 showed several components. Components 2 and 3 (Fig. 1B) could be identified as the trimethylsilyl derivatives of 2-*O*-(α-D-galactopyranosyl)-D-threitol and its β-D anomer respectively, and would result from D-Gal*p*-(1→4)-D-Gal or D-Gal*p*-(1→5)-D-Gal*f* sequences. Peak 1 of fraction 7 corresponded to peak 3 of fraction 6. Components 2 and 3 (Fig. 1C) were not completely separated, but were each shown to be trimethylsilylated 1-*O*-(D-galactopyranosyl)-D-threitols. Since after desilylation the greater part of these two fractions was hydrolyzed by β-galactosidase, component 2 must be derived from 1-*O*-(α-D-galactopyranosyl)-D-threitol, and component 3 from the β-D anomer. These degradation products present further evidence for the existence of (1→6) linkages in the polysaccharide. They may arise from either a D-Gal*p*-(1→6)-D-Gal*f* structure or a D-galactopyranose residue substituted at positions 4 and 6. In each case an insufficient amount of these *O*-glycosylalditols was obtained to prepare derivatives.

The higher fractions contained greater proportions of D-galactose but could not be resolved.

Methylated galactan

The fully methylated D-galactan was hydrolyzed and the mixture of methylated sugars resolved by paper chromatography into the fractions shown in Table II. Fraction 1 was identified chromatographically as 2,3,5,6-tetra-*O*-methyl-D-galactose. Fraction 2 was identified as 2,3,4,6-tetra-*O*-methyl-D-galactose by bromine oxidation and conversion to the crystalline phenylhydrazide. Fraction 3 was believed to contain tri-*O*-methyl-D-galactofuranoses, and 2,3,6-tri-*O*-methyl-D-galactose which has a higher R_F value than the other pyranose isomers.

TABLE II

PAPER CHROMATOGRAPHIC SEPARATION OF D-GALACTOSE METHYL ETHERS

Fraction	R_F	*Components, ethers of D-galactose*	*Weight (mg)*
1	0.87	2,3,5,6-tetra-*O*-methyl-	110
2	0.68	2,3,4,6-tetra-*O*-methyl-	360
3	0.45-0.55	tri-*O*-methyl-, furanose	165
4	0.32-0.40	tri-*O*-methyl-, pyranose	75
5	0.12-0.25	di-*O*-methyl-	205
6	0.01-0.08	mono-*O*-methyl-	70
		Recovery	83%

Paper electrophoresis indicated a minor component (3,5,6-trimethyl ether) which migrated. Glycosidation of the mixture followed by tritylation[4] gave an insoluble trityl compound which on detritylation, hydrolysis, bromine oxidation, and purification by g.l.c. gave crystalline 2,3,5-tri-*O*-methyl-D-galactonolactone. The

remainder of fraction 3 was hydrolyzed and subjected to the sequence borohydride reduction, periodate oxidation and borohydride reduction. The cleavage products were demethylated by boiling with hydrobromic acid[5] and the alditols were separated by paper chromatography. Since each tri-*O*-methyl isomer gives a unique alditol by this procedure the identification of these alditols provides evidence for the presence in fraction 3 of the three tri-*O*-methyl-D-galactoses shown in Table III. Their relative proportions were estimated by a periodate–chromotropic acid procedure[6] and the main components were characterized as crystalline derivatives. Since the quantitative accuracy of the demethylation step is unknown, these figures (Table III) can only indicate approximate proportions. Other work, however, suggests that selective decomposition by hydrobromic acid is not a serious source of error[7].

TABLE III

DEGRADATION OF PART OF FRACTION 3

Tri-O-methyl-D-galactose	*Degradation products*	*Demethylation product*	*% of total*	*Derivative (m.p.)*
2,5,6-	2-*O*-methylglycerol	glycerol	7	*p*-nitrobenzoate (189-192°)
	2,3-di-*O*-methylglycerol	glycerol		
2,3,6-	2,3-di-*O*-methyl-L-threitol	L-threitol		*p*-nitrobenzoate (221-222°)
	2-methoxyethanol	ethylene glycol	85	*p*-nitrobenzoate (142-144°)
3,5,6-	2,4,5-tri-*O*-methyl-D-arabinitol	D-arabinitol	8	

Paper electrophoresis of fraction 4 (2,3,4-, 2,4,6-, and 3,4,6-tri-*O*-methyl-D-galactose) showed that approximately 15% was the 3,4,6-isomer. Conversion into the methyl glycosides and tritylation showed the 2,3,4-isomer to constitute about 75% of the fraction, and hence 10% was the 2,4,6-trimethyl ether. These identifications were confirmed by conversion of the tritylated material into 2,3,4-tri-*O*-methyl-*N*-phenyl-D-galactosylamine. The non-tritylated portion was degraded to an arabinitol (from the 3,4,6-trimethyl ether) and galactitol (from the 2,4,6-trimethyl ether).

Fraction 5, which contained as many as nine di-*O*-methyl ethers, was converted into the methyl glycosides and tritylated. The water-insoluble portion contained those isomers in which the hydroxyl on C-6 was not methylated, *i.e.* the 2,3-, 2,4-, 2,5-, 3,4-, and 3,5-di-*O*-methyl-D-galactoses. The mother liquor contained the 2,6-, 3,6-, 4,6-, and 5,6-isomers. The mixture of tritylated compounds was detritylated, hydrolyzed, and oxidized with dilute periodate. Only the 3,4- and 3,5-ethers may be expected to undergo rapid oxidation. In agreement with this, paper chromatography showed the formation of some fast-moving material (2,3- and 2,4-di-*O*-methyl-D-lyxoses) which was isolated and reduced with borohydride. Periodate degradation and demethylation gave erythritol, from 2,3-di-*O*-methyl-D-lyxose, and a pentitol

presumed to be D-arabinitol (D-lyxitol) from the 2,4-di-*O*-methyl-D-lyxose, thus confirming the presence of the 3,4- and 3,5-di-*O*-methyl-D-galactoses in fraction 5. The material which did not react initially with periodate was reduced, oxidized with periodate, reduced, and demethylated, to give the results given in Table IV. In this instance also, the characterization of the demethylated alditol demonstrated the presence of a specific di-*O*-methyl-D-galactose.

TABLE IV

ANALYSIS OF TRITYLATED DIMETHYL FRACTION

Di-O-methyl-D-galactose	*Derived pentose*	*O-Methylalditol*	*Alditol*	%	*Derivative (m.p.)*
3,4-	2,3-di-*O*-methyl-D-lyxose	2,3-di-*O*-methyl-erythritol	erythritol	7	*p*-nitrobenzoate (251-253°)
3,5-	2,4-di-*O*-methyl-D-lyxose	2,4-di-*O*-methyl-D-lyxitol	arabinitol (lyxitol)	5	
2,3-		2,3-di-*O*-methyl-L-threitol	L-threitol	75	*p*-nitrobenzoate (221-223°)
2,4-		2,4-di-*O*-methyl-L-lyxitol	arabinitol (lyxitol)	8	
2,5-		2-*O*-methyl-glycerol	glycerol	5	*p*-nitrobenzoate (189-192°)

The separation of methylated sugars by g.l.c. has usually been accomplished on the methyl glycosides. This has the disadvantage that anomeric mixtures may be formed for each sugar[8]. In addition, the retention times for mono- and di-*O*-methylhexoses may be inconveniently long. A periodate-degradation scheme as outlined above may degrade a group of isomeric di-*O*-methylhexoses to a mixture of C_2 to C_5 compounds which will have markedly different retention times. Accordingly, the glycosides from fraction 5 which did not form trityl derivatives were hydrolyzed, oxidized with periodate, and reduced with borohydride. The partially methylated alditols were separated by g.l.c. and the parent alditol identified after demethylation.

TABLE V

ANALYSIS OF NON-TRITYLATED DIMETHYL FRACTION BY G.L.C.

Di-O-methyl-D-galactose	*O-Methylalditol*	*Retention time (min)*	*Alditol*	*Derivative (m.p.)*
2,6-	2-*O*-methylglycerol	2.9	glycerol	*p*-nitrobenzoate (189-191°)
	2-methoxyethanol	1.6	ethylene glycol	*p*-nitrobenzoate (142-144°)
3,6-	1,4-di-*O*-methyl-D-arabinitol	18	arabinitol	
4,6-	1,3-di-*O*-methyl-D-threitol	4.1	D-threitol	*p*-nitrobenzoate (221-222°)
5,6-	1,2-di-*O*-methyl-D-threitol	5.0	D-threitol	

The results are shown in Table V. The isomeric 1,2- and 1,3-di-*O*-methyl-D-threitols were readily differentiated by the fact that on further oxidation with periodate only the former gave formaldehyde.

The mono-*O*-methyl-D-galactoses in fraction 6 were treated in a similar manner and the partially methylated alditols were separated by g.l.c. to give the results shown in Table VI.

It is now clear from subsequent work that the *O*-methylalditols listed in Tables III and IV might also have been separated by g.l.c.[7, 9]. Furthermore, if suitable crystalline derivatives of all of these *O*-methylalditols were available, it would be possible to characterize these fragments directly, and hence characterize the original methylated sugars, without the necessity of the demethylation step.

TABLE VI

ANALYSIS OF MONOMETHYL FRACTION BY G.L.C.

Mono-O-methyl-D-galactose	*O-Methylalditol*	*Retention time (min)*	*Alditol*	*Derivative (m.p.)*
2-	2-*O*-methylglycerol	3.0	glycerol	*p*-nitrobenzoate (189-192°)
	+ glycerol	5.1		
3-	2-*O*-methyl-D-lyxitol	23	arabinitol (lyxitol)	
4-	2-*O*-methyl-D-threitol	5.8	D-threitol	*p*-nitrobenzoate (221-222°)
5-				
6-	1-*O*-methyl-D-glycerol	4.4	glycerol	*p*-nitrobenzoate (189-191°)

Conclusions

The results of the Smith degradation and the methylation clearly show that the synthetic D-galactan has a highly ramified structure containing furanose and pyranose residues joined mainly by (1→4) and (1→6) linkages. The synthetic polysaccharide has a structure more complex than those likely to be found in Nature, but the separation of Smith-degradation fragments as their trimethylsilyl derivatives by g.l.c. and the analysis of a group of isomeric methylated sugars by periodate degradation and g.l.c., are methods applicable to galactose-containing polysaccharides in general. Some of these methods have already been applied to the study of an arabinogalactan from centresoma seed[10].

EXPERIMENTAL

General methods

All evaporations were carried out *in vacuo* at a bath temperature of less than 40°. Optical rotations were measured at 22 ± 2°. Sugar analyses were obtained

by the phenol–sulfuric acid method[11] and alditols were analyzed by the periodate–chromotropic acid method[6]. Periodate consumption was measured by the arsenite method[12].

Paper chromatograms were run in solvent A: (8:2:2) ethyl acetate–acetic acid–water, or solvent B: butanone–water azeotrope, and reducing sugars were detected with *p*-anisidine trichloroacetate[5]. Non-reducing substances were detected with silver nitrate[13].

Vapor phase chromatograms were obtained using an Aerograph A90-P2 chromatograph (Wilkens Instrument and Research Inc.), with a 4 ft × 0.25 in O.D. stainless steel column packed with 12% Versamide (F and M Corporation) on Diatoport (Wilkens). For the trimethylsilyl derivatives, all of which were obtained as oils, operating conditions were as shown in Figure 1. For the methylated alditols (Tables V and VI) a 4 ft × 0.25 in O.D. column of 10% Versamide on silanized Chromosorb W was used, with a helium flow rate of 105 ml/min. The temperature was programmed from 110° to 220° at 8°/min and then maintained at 220°.

General properties of the galactan

The galactan used in these experiments was received from Dr. Mora[3].

(a) *Hydrolysis.* Hydrolysis of a sample of the galactan gave D-galactose in almost quantitative yield.

(b) *Partial hydrolysis.* Hydrolysis of the galactan with 0.1N HCl for 8 h at 50° showed the formation of galactose 9%, galactobiose (?) 5%, galactotriose (?) 5%, oligosaccharides 10%, and unchanged polysaccharide 70%.

(c) *Periodate uptake.* A sample of the galactan (1.1 g) was oxidized with 0.1M periodic acid (200 ml) in the dark at 5°. The moles of periodate consumed per mole of hexose, and the moles of formaldehyde formed per mole of hexose were: 5 h, 0.60, 0.085; 12 h, 0.87, 0.108; 24 h, 0.01, 0.124; 48 h, 1.16, 0.128; 60 h, 1.20, 0.134; and 72 h, 1.21, 0.135. The oxidized product was reduced ($NaBH_4$), and hydrolyzed with 1N H_2SO_4. The molar ratio of glycerol, D-threitol, L-arabinose and D-galactose was found to be 9:8:1:2. Each compound was identified as follows: glycerol (224 mg) gave a tri-*p*-nitrobenzoate, m.p. and mixed m.p. 192–193°; D-threitol (255 mg), $[\alpha]_D$ +4.5° (*c* 7.5, in water), gave a tetra-*p*-nitrobenzoate, m.p. and mixed m.p. 220–221° (Found: N, 7.96%. $C_{32}H_{22}N_4O_{16}$ calc.: N, 7.92%); D-galactose (95 mg) crystallized spontaneously, $[\alpha]_D$ +79.5° (*c* 2, in water), m.p. and mixed m.p. 144–145°; L-arabinose (39 mg) crystallized spontaneously, $[\alpha]_D$ +103° (*c* 0.5, in water), m.p. and mixed m.p. 159–160°.

(d) *Determination of degree of polymerization (D.P.).* Two samples (100 mg) of galactan were dissolved in water, and to one was added sodium borohydride (50 mg). After 24 h, acetic acid was added to each solution, and the solutions were then made 0.1M with periodic acid. Formaldehyde production after 72 h was 2.98 mg and 2.22 mg from the reduced and non-reduced samples, respectively. On the assumption that each reducing end group gave two molecules of formaldehyde, this indicated[6] a D.P. of about 50.

Smith degradation

The galactan (6.10 g) was dissolved in water (110 ml), periodic acid (0.5 M, 90 ml) was added, and the oxidation was allowed to proceed in the dark at 5° until the periodate consumption became constant (1.23 moles per hexose residue, about 90 h). The solution was neutralized ($BaCO_3$), the filtrate was reduced (KBH_4, 2.5 g) for 48 h at room temperature, the solution was evaporated, and borate was removed by distillation with methanol containing 1% hydrogen chloride. The residue was dissolved in water, passed through cation and anion exchange resin columns, and the neutral effluent (300 ml) was brought to pH 2–3 by the addition of hydrochloric acid. The solution was left for 7 h at room temperature, neutralized ($PbCO_3$), filtered, and evaporated to give a sirup. Paper chromatographic examination showed the presence of glycerol, threitol, arabinose, galactose (trace), and at least five slower-moving, non-reducing components (Table I).

The mixture was resolved on Whatman 3MM paper using solvent A, and the glycerol, *fraction 1*, was allowed to migrate off the paper. This component was isolated (1.5 g), and a portion was purified by rechromatography and identified by conversion into glycerol tri-*p*-nitrobenzoate, m.p. and mixed m.p. 192–193°. *Fraction 2* (1.2 g) was identified as D-threitol, $[\alpha]_D + 4.5°$ (*c* 7.3, in water), tetra-*p*-nitrobenzoate[10], m.p. and mixed m.p. 220–221°. *Fraction 3* was L-arabinose (20 mg), $[\alpha]_D + 102°$(*c* 0.5, in water), benzylphenylhydrazone, m.p. and mixed m.p. 172–173°.

Fraction 4 appeared to be a mixture containing galactose. The material (60 mg) was dissolved in water (20 ml), and oxidized (Br_2, $CaCO_3$). The solution was resolved into neutral and acidic portions with ion exchange resins. The acidic portion was lactonized and converted into D-galactonamide, m.p. 174–175°, $[\alpha]_D + 31.3°$ (*c* 2, in water). Hydrolysis of a portion (7 mg) of the neutral material (15 mg total) gave arabinose, glycerol, and a trace of galactose. Periodate oxidation of the remainder (8 mg) gave formaldehyde (1.03 mg), while reduction (KBH_4) and hydrolysis gave glycerol and ethylene glycol in a 1:1 molar ratio, indicating that the non-reducing material was 1-*O*-(L-arabinofuranosyl)glycerol.

The fractions having lower chromatographic mobilities were rechromatographed in an attempt to achieve further purification. Considerable trailing was encountered in several solvent systems, and led to poor recoveries giving insufficient material for the preparation of derivatives. A portion (12 mg) of *fraction 5* (32 mg), treated with β-galactosidase gave galactose, glycerol and unchanged material (*ca.* 70%). Complete hydrolysis (0.5N HCl) gave galactose and glycerol (1:1 molar ratio) with traces of arabinose and threitol. Periodate oxidation of a portion (10 mg) gave formaldehyde (1.16 mg) and, after reduction (KBH_4) and hydrolysis, glycerol and ethylene glycol in a 1:1 molar ratio. The remainder (10 mg) was converted to the trimethylsilyl derivative[2] and examined by g.l.c. The results are shown in figure 1A. Component 1 was identified as the trimethylsilyl derivative of 1-*O*-(α-D-galactopyranosyl)-glycerol since the desilylated product (6 mg) was not hydrolyzed by β-galactosidase and gave glycerol and ethylene glycol in a 1:1 molar ratio on periodate degradation.

Component 2 was hydrolyzable by β-galactosidase after desilylation, and was thus the β-D anomer.

In a similar manner, treatment of a portion (12 mg) of *fraction 6* (56 mg) with β-galactosidase gave glycerol, threitol, galactose, and unchanged material. Complete hydrolysis gave glycerol, threitol, arabinose (minor proportion), and galactose. Examination of the trimethylsilyl derivative by g.l.c. gave the complex picture shown in figure 1B. Desilylation of component 3 gave 10 mg of product, a portion (6 mg) of which yielded formaldehyde (0.62 mg) on periodate oxidation. Only glycerol was detected after reduction and hydrolysis of the oxidized product. The remainder (4 mg) underwent hydrolysis by β-galactosidase to give galactose and threitol, and thus the original material must have been 2-*O*-(β-D-galactopyranosyl)-D-threitol. Component 2, after desilylation, gave the same periodate degradation results, but was not hydrolyzed by β-galactosidase and was thus judged to be the α-D anomer. Component 5 was presumed to be the trimethylsilyl derivative of 1-*O*-(L-arabinofuranosyl)-D-threitol, since it gave arabinose and threitol on desilylation followed by hydrolysis.

Fraction 7 (60 mg) was similarly examined and was found to be only partly hydrolyzable by β-galactosidase. Trimethylsilylation and examination by g.l.c. gave the results shown in figure 1C. Component 1 was shown to be the same as component 3 of fraction 6, and was therefore the trimethylsilyl derivative of 2-*O*-(β-D-galactopyranosyl)-D-threitol. Components 2 and 3 were combined and part (10 mg) of the desilylated product (16 mg) was oxidized with periodate to give formaldehyde (1.0 mg) and, after reduction and hydrolysis, glycerol and ethylene glycol in a 1:1 molar ratio. The remainder (6 mg) was hydrolyzed to the extent of about 75% when treated with β-galactosidase. It thus followed that component 3 was the trimethylsilyl derivative of 1-*O*-(β-D-galactopyranosyl)-D-threitol and that component 2 was its α-D anomer. Fractions 8 (78 mg) and 9 (82 mg) were each partially hydrolyzed by β-galactosidase and were shown to be mixtures of di- and tri-saccharide–alditol derivatives, respectively, which were not further examined.

Methylation

Complete methylation was obtained only after repeated methylations by the methods of Haworth, Kuhn, and Purdie as described for a synthetic D-glucan[4]. The methylated galactan (1.2 g, 45.5% methoxyl, no hydroxyl absorption in the infrared) was dissolved in 72% sulfuric acid, and after 45 min the solution was diluted to 2N and boiled for 8 h[14]. The solution was neutralized ($BaCO_3$) and evaporated to a thick sirup which was separated by chromatography on Whatman 3MM paper with solvent B into the fractions listed in Table II.

Fraction 1 corresponded chromatographically to a 2,3,5,6-tetra-*O*-methylgalactose. It was oxidized with bromine, and the resultant lactone was analyzed by g.l.c. and shown to contain only one major (> 90%) component.

Fraction 2, $[\alpha]_D$ +113°, was oxidized with bromine and the resultant lactone, which was essentially pure by g.l.c., was characterized as 2,3,4,6-tetra-*O*-methyl-D-galactonic phenylhydrazide, m.p. 133–136°; Haworth *et al.*[15] gave m.p. 135–137°.

Fraction 3 was shown by paper electrophoresis in borate buffer to contain 7–10% of a compound having M_G 0.25. The mixture was refluxed with 1% methanolic hydrogen chloride, and the resultant mixture of glycosides was tritylated with chlorotriphenylmethane in pyridine to give a water-insoluble, tritylated portion (3a), and a water-soluble portion (3b). Fraction 3a was detritylated and hydrolyzed with acid to give a sugar (20 mg) which was oxidized with bromine. Examination of the resultant lactone by g.l.c. gave one major component, which crystallized in the collection tube and which corresponded to 2,3,5-tri-*O*-methyl-D-galactonolactone, m.p. 88–90°, $[\alpha]_D$ −32° (*c* 1.8, in water); Luckett and Smith[16] gave m.p. 90°, $[\alpha]_D$ −37° → −32° (in water). The portion (3b, 150 mg), which did not form a trityl ether, was refluxed for 8 h with sulfuric acid (N, 20 ml), neutralized ($BaCO_3$), and reduced with potassium borohydride (100 mg) for 48 h at room temperature. The solution was acidified (HOAc), made up to 100 ml, cooled to 5° and periodic acid (0.1M, 10 ml) was added. The solution was kept for 20 h at 5°, after which time barium carbonate was added. The filtrate was reduced (KBH_4, 100 mg, 48 h), evaporated to dryness and borate was removed by several distillations with 1% methanolic hydrogen chloride. The residue was extracted with acetone–ethanol (1:1), and the resulting sirup (115 mg) was demethylated (45% HBr, reflux, 20 min). The solution was diluted, neutralized ($PbCO_3$), and the filtrate was concentrated and examined chromatographically to give the results shown in Table III.

Fraction 4 contained about 15% of electrophoretically mobile material, and the mixture was converted as before into tritylated glycosides and a non-tritylatable portion. The tritylated material amounted to about 75% of the fraction, and from it was obtained 2,3,4-tri-*O*-methyl-*N*-phenyl-D-galactosylamine, m.p. 165–167°, $[\alpha]_D$ +42° (*c* 1.5, in methanol); McCreath and Smith[17] gave m.p. 167–169°, $[\alpha]_D$ −65° → +43° (in methanol). The non-tritylated material was hydrolyzed (N H_2SO_4, 8 h) and degraded as before to give arabinitol and galactitol, identified chromatographically, confirming the presence of the 3,4,6- and 2,4,6-tri-*O*-methyl-D-galactoses.

The dimethyl ethers in fraction 5 were converted into their glycosides (220 mg) and were divided into tritylatable and non-tritylatable parts. The tritylated material was detritylated and the product (138 mg) was hydrolyzed (N H_2SO_4, reflux, 8 h). The free sugars (105 mg) were dissolved in water (100 ml) periodic acid (0.5M, 2 ml) was added, and the oxidation was allowed to proceed in the dark for 15 h at 5°. After neutralization ($BaCO_3$) and concentration, paper chromatography in solvent B showed zones corresponding to the presence of two incompletely separated pentoses (R_F 0.53–0.56), and unchanged hexoses (R_F 0.15–0.21). These two groups were separated by chromatography and gave pentose (17 mg) and hexose (81 mg) derivatives. The pentose portion was dissolved in water (20 ml) and reduced (KBH_4, 48 h). The solution was acidified (H_2SO_4) and oxidized (0.5M HIO_4, 0.5 ml, 24 h at 5°). After neutralization, reduction, and removal of borate as before, the

residue was demethylated (45% HBr, 4 ml, reflux, 20 min), and the solution was diluted and neutralized ($PbCO_3$). The filtrate was evaporated and the residue was extracted with acetone to yield a sirup (11 mg) which was shown chromatographically to consist of erythritol and an arabinitol (or a lyxitol). These were separated, and the erythritol (7 mg) was characterized as the tetra-*p*-nitrobenzoate, m.p. and mixed m.p. 248–250°.

The hexose portion (81 mg) was dissolved in water (20 ml) and reduced (KBH_4, 100 mg, 48 h). The solution was acidified (H_2SO_4), periodic acid (0.5M, 1 ml) was added, and after diluting the solution to 50 ml the oxidation was allowed to proceed for 20 h at 5°. The solution was neutralized ($BaCO_3$), filtered and reduced (KBH_4, 50 mg, 48 h). The borate was removed as before and the residue was demethylated (45% HBr, 3 ml, reflux, 20 min). The solution was diluted, neutralized ($PbCO_3$), filtered, and evaporated. The residue was extracted with acetone to give a sirup (48 mg) which was shown by chromatography to contain glycerol, threitol and an arabinitol (lyxitol), in approximately 5:75:8 proportion (periodate–chromotropic acid). The glycerol (6 mg) and the threitol (25 mg) were separated and characterized as shown in Table IV, and the arabinitol (lyxitol) was identified chromatographically.

The glycosides of fraction 5 which did not react with chlorotriphenylmethane were recovered (80 mg) and hydrolyzed (N H_2SO_4, 10 ml, reflux, 8 h). The solution was neutralized ($BaCO_3$), filtered, diluted to 75 ml, and oxidized (0.5M HIO_4, 1 ml) for 15 h in the dark at 5°. Barium carbonate was added, and the filtrate was reduced (KBH_4, 100 mg) for 48 h. The solution was evaporated, borate was removed, and the residue was extracted with acetone to yield a sirup (47 mg) which was examined by g.l.c. The 1,2- and 1,3-di-*O*-methyl-D-threitols were differentiated by the production of formaldehyde from the former on periodate oxidation. Each component was demethylated and characterized as shown in Table V.

Fraction 6 was similarly oxidized with periodic acid, at a final concentration of 0.001 M, for 15 h in the dark at 5°. The solution was neutralized ($BaCO_3$), reduced (KBH_4), freed from borate, and extracted with acetone to yield a sirup which was examined by g.l.c. Each component was demethylated and characterized as shown in Table VI.

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SUMMARY

When a complex polysaccharide is degraded by the Smith procedure, mixtures of *O*-D-glycosylalditols are produced which are difficult to separate by conventional

methods. Some *O*-D-galactosylalditols, obtained by degradation of a synthetic D-galactan, have been separated as their trimethylsilyl derivatives by gas–liquid chromatography and the following have been characterized: 1-*O*-(α-D-galactopyranosyl)-glycerol and the β-D anomer; 2-*O*-(α-D-galactopyranosyl)-D-threitol and the β-D anomer; 1-*O*-(α-D-galactopyranosyl)-D-threitol and the β-D anomer. The α-D-anomers had shorter retention times than the β-D anomers and they were differentiated by their reaction with β-galactosidase. In addition, 1-*O*-(L-arabinofuranosyl)glycerol was isolated by paper chromatography.

Hydrolysis of the fully methylated D-galactan gave a large number of methylated sugars of which the monomethyl and some of the dimethyl isomers were identified by a combination of periodate degradation and gas–liquid chromatography.

The D-galactan was shown to contain both furanose and pyranose residues, and to be highly branched, with approximately 25% of the end-groups furanose.

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A SYNTHESIS OF CHROMOSE D AND AN IMPROVED SYNTHESIS OF CHROMOSE A

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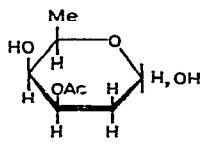
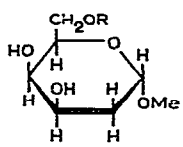
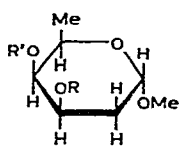
Chromomycin is the name given[1] to a group of cancerostatic and anticancer antibiotics produced by *Streptomyces griseus* No. 7, and is a mixture of several closely-related compounds. The principal antibiotic[2] of this group, chromomycin A_3, on hydrolysis with hot 50 % acetic acid yields the aglycone, chromomycinone, and a water-soluble fraction from which four sugars have been isolated[3] and characterised[4,5] by n.m.r. and chemical evidence as 2,6-dideoxy-4-*O*-methyl-D-*lyxo*-hexose, 4-*O*-acetyl-2,6-dideoxy-3-*C*-methyl-L-*arabino*-hexose, 2,6-dideoxy-D-*arabino*-hexose, and 3-*O*-acetyl-2,6-dideoxy-D-*lyxo*-hexose. These compounds have been designated[3] as chromose A, B, C, and D, respectively, and identical or closely related sugars have also been identified[6] as methanolysis products of olivomycin[7], the principal antibiotic from *Streptomyces olivoreticuli*. The isolation of similar antibiotics has been reported[8]. The structure of chromose A has been established[9] by synthesis,



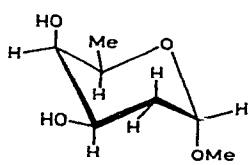
(I)



(II) R = H
(III) R = Ts



(IV) R = R' = H
(V) R = Ac, R' = H
(VI) R = H, R' = Ac
(VII) R = Ac, R' = Me
(VIII) R = CH_2Ph, R' = H



(IV A)

and we now report a synthesis of chromose D which confirms its structure as 3-*O*-acetyl-2,6-dideoxy-D-*lyxo*-hexose (I).

Monotoluene-*p*-sulphonylation of methyl 2-deoxy-α-D-*lyxo*-hexopyranoside[10] (II) gave principally the 6-*O*-toluene-*p*-sulphonate (III) which, on reduction

with lithium aluminium hydride, was smoothly converted into methyl 2,6-dideoxy-α-D-*lyxo*-hexopyranoside (IV). The latter glycoside is of interest since one of the enantiomorphic forms of the free sugar has been identified[11] by chromatography as a constituent of the cardiac glycoside from *Pentopetia androsaemifolia*. On treatment in pyridine with 1.2 mol. of acetic anhydride, compound (IV) gave, *inter alia*, a mixture of two monoacetates which had significantly different mobilities on thin-layer chromatography. The major component of the mixture was separated by chromatography on silica gel and identified as methyl 3-*O*-acetyl-2,6-dideoxy-α-D-*lyxo*-hexopyranoside (V), since, on careful methylation, it gave a product indistinguishable (by thin-layer chromatography) from methyl 3-*O*-acetyl-2,6-dideoxy-4-*O*-methyl-α-D-*lyxo*-hexopyranoside (VII). The latter compound was obtained by acetylation of methyl 2,6-dideoxy-4-*O*-methyl-α-D-*lyxo*-hexopyranoside[9] (methyl α-D-chromoside A). Attempts to ascertain the probable conformation of glycoside (IV) in pyridine solution by n.m.r. spectroscopy were unsuccessful since no clear-cut signal arose from the anomeric proton. However, conformation (IVA) should be adopted by the glycoside, since it has equatorial substituents at positions 3 and 5 and the glycosidic substituent in the preferred axial position[12]. This being so, then preferential acetylation at the C-3 hydroxyl group is to be expected.

Since the assignment of structure to monoacetate (V) was not based on crystalline derivatives, efforts were made to prepare both monoacetates using the ortho-ester exchange method described by Reese and Sulston[13] for the monoacylation of nucleoside *cis*-2,3-diol systems. Treatment of glycoside (IV) with trimethyl orthoacetate in the presence of an acid catalyst, followed by decomposition of the resultant 3,4-*O*-methoxyethylidene derivative, gave a mixture of the 3-*O*- and 4-*O*-acetates in the approximate ratio 1 : 2 (estimated by chromatography). Methyl 4-*O*-acetyl-2,6-dideoxy-α-D-*lyxo*-hexopyranoside (VI) was isolated in crystalline form by chromatography on silica gel, and its structure may be confidently assigned since it was identical with the product obtained by acetylation and catalytic debenzylation[14] of methyl 3-*O*-benzyl-2,6-dideoxy-α-D-*lyxo*-hexopyranoside[9] (VIII). Migration of the acetate group under the neutral conditions used to remove the benzyl group is unlikely, and both monoacetates (V) and (VI) were unaffected by similar treatment.

Support for the above structural assignments was also sought by n.m.r. spectroscopy. It is known[15,16] that the signal for an axial acetoxyl-group attached to the pyranoid ring occurs at lower field than that for the equatorial group. The n.m.r. spectrum* of the 3-*O*-acetate (V) showed characteristic signals at 5.15 (triplet, $J = 3$ c.p.s., equatorial anomeric-proton), 6.61 (OMe), 7.86 (OAc), and 8.68 (doublet, $J = 6$ c.p.s., *sec*-Me) and the spectrum of the 4-*O*-acetate (VI), signals at 5.14 (triplet, $J = 3$ c.p.s., equatorial anomeric-proton), 6.62 (OMe), 7.77 (OAc), and 8.78 (doublet, $J = 6$ c.p.s., *sec*-Me). Thus, the appearance of the signal from the acetoxyl group in

*Determined in chloroform with tetramethylsilane as external reference using a Varian A60 spectrometer. Absorptions are given on the τ scale.

the former compound at higher field is consistent with its equatorial location and is in agreement with the structure assigned by chemical means.

Hydrolysis of compound (V) with hot 50 % acetic acid gave 3-*O*-acetyl-2,6-dideoxy-D-*lyxo*-hexose (I) {m.p. 115–116.5°, $[\alpha]_D^{29}$ +100 (1 min)→+78° (final, *c* 1, water)} which was identical with natural chromose D, which has[17] m.p. 118°, not 128° as reported[5] previously. Although acyl migrations have for most part been studied under alkaline conditions[18], migrations under acidic conditions are not uncommon[19–21] and presumably take place *via* an ortho-ester. The absence of any significant acyl migration under the hydrolytic conditions used was demonstrated when the 3-*O*- and 4-*O*-acetates, (V) and (VI), respectively, gave readily distinguishable products on hydrolysis.

During the course of this work, the need for quantities of chromose A (2,6-dideoxy-4-*O*-methyl-D-*lyxo*-hexose[4,9]) and its methyl glycoside prompted the development of a more convenient synthesis. Since the 6-*O*-toluene-*p*-sulphonate (III) should adopt the *C1* conformation, with equatorial substituents at positions 3 and 5 and the glycosidic substituent in the preferred axial position[12], further toluene-*p*-sulphonylation should lead to selective reaction at the C-3 hydroxyl group[22]. With two mol. of toluene-*p*-sulphonyl chloride, methyl 2-deoxy-α-D-*lyxo*-hexopyranoside (II) gave mainly a single di-*O*-toluene-*p*-sulphonate, together with a little tri-ester. The di-ester was identified as methyl 2-deoxy-3,6-di-*O*-toluene-*p*-sulphonyl-α-D-*lyxo*-hexopyranoside since, on methylation and treatment with lithium aluminium hydride, it was smoothly converted into methyl 2,6-dideoxy-4-*O*-methyl-α-D-*lyxo*-hexopyranoside which was identical with methyl α-D-chromoside A derived from the naturally-occurring[4] and synthetic[9] reducing sugar. Chromose A was obtained from the glycoside by acidic hydrolysis, and the overall yield was better than that obtained by the previously described route[9].

Experimental

Thin-layer chromatography was performed on silica gel (Merck) using ethyl acetate, unless otherwise indicated. Detection was effected with an acidified 3% (w/v) solution of vanillin in ethanol[23] at 115° for 5–10 min. Solvents were usually removed under reduced pressure below 40°.

*Methyl 2-deoxy-6-*O*-toluene-*p*-sulphonyl-α-*D*-lyxo-hexopyranoside (III).*

A cooled solution of toluene-*p*-sulphonyl chloride (12.8 g, 1.1 mol.) in dry pyridine (20 ml) was added to a cooled (−10°) solution of methyl 2-deoxy-α-D-*lyxo*-hexopyranoside[10] (10 g) in dry pyridine (100 ml) and the mixture was set aside at room temperature for 48 h. Water (4 ml) was added and the solvents were removed to give a brown residue which was taken up in chloroform (150 ml). This solution was washed with dilute aqueous solutions of sodium hydrogen sulphite (2×50 ml) and sodium hydrogen carbonate (2×50 ml), water (50 ml), saturated aqueous cadmium chloride (50 ml), and water (2×50 ml), and dried ($MgSO_4$). Evaporation of the

filtered solution gave the product (III) (18.2 g), $[\alpha]_D^{24}+82.5°$ (*c* 7.3, chloroform), as an amorphous solid which was substantially homogeneous on thin-layer chromatograms; the main impurity appeared to be a di-ester compound. The infrared spectrum showed ν_{max} at 3400–3600 (OH), 1180 and 1270 cm^{-1} (sulphonate ester).

*Methyl 2,6-dideoxy-α-*D-lyxo-*hexopyranoside (IV)*

A boiling solution of the foregoing sulphonate (18 g) in benzene–ether (600 ml, 1:2 v/v) was treated with lithium aluminium hydride (2 g) for 4 h, and after addition of a further quantity of hydride (2 g) heating was continued for 14 h. On cooling, ethyl acetate and water were added and insoluble material was collected and washed with ether. The combined filtrate and washings were extracted with water (4 × 100 ml) and the aqueous solution was freeze-dried to give a syrupy residue (4.1 g). Distillation of a sample (0.5 g) of the syrup gave the product (IV) (0.3 g), b.p. 100–110° (bath)/0.05 mm, which crystallised on cooling, and on recrystallisation from benzene–light petroleum (b.p. 80–100°) had m.p. 70–72°, $[\alpha]_D^{16}+122°$ (*c* 2, chloroform). (Found: C, 51.65; H, 8.5. $C_7H_{14}O_4$ calc.: C, 51.8; H, 8.7%). Additional quantities of the product were readily obtained by seeding the original syrup.

*Methyl 3-*O*-acetyl-2,6-dideoxy-α-*D-lyxo-*hexopyranoside (V)*

To a cooled (−10°) solution of the foregoing glycoside (2 g) in dry pyridine (8 ml) was slowly added a cooled solution of acetic anhydride (1.48 g, 1.2 mol.) in dry pyridine (4 ml), and the mixture was set aside at room temperature for 12 h. After the addition of a few drops of water, the solvent was removed, the residue was taken up in chloroform (50 ml), and the organic layer was washed with dilute aqueous sodium hydrogen carbonate (2 × 20 ml), water (20 ml), saturated aqueous cadmium chloride (2 × 20 ml), and water (20 ml), and dried ($MgSO_4$). Evaporation of the filtered solution afforded a syrup (2.1 g) which, on examination by thin-layer chromatography, showed three components, with R_F 0.53 (4-*O*-acetate), 0.59 (3-*O*-acetate, major), and 0.75 (di-*O*-acetate). The syrup was dissolved in dry benzene (10 ml) and chromatographed on silica gel (65 g, 40 × 2 cm, Davison grade 950, 60–200 mesh) by elution with benzene (160 ml), benzene–ether (620 ml, 4:1 v/v), benzene–ether (620 ml, 2:1 v/v), and ether (100 ml). Fractions (40 ml) were collected and examined by thin-layer chromatography. Fractions 7–11 contained the di-*O*-acetate, 15–31 the 3-*O*-acetate, whilst later fractions contained a mixture of both monoacetates. Evaporation of fractions 15–31 gave the syrupy product (V) (0.74 g), $[\alpha]_D^{16}+142°$ (*c* 2.4, chloroform), which could not be induced to crystallise.

Methylation studies

(*a*) *Using methyl iodide and sodium hydride*[24]

A solution of the foregoing acetate (10 mg) in dry tetrahydrofuran (0.4 ml) was treated with redistilled methyl iodide (0.05 ml) and sodium hydride powder (30 mg), and the mixture was set aside at room temperature for 3 h. Examination of the mixture by thin-layer chromatography [using ethyl acetate–chloroform (1:1 v/v)] revealed components with R_F 0.5 (starting material), 0.65, 0.75 (trace, unidentified),

and 0.82 (major). The chromatographic properties of the components at R_F 0.65 and 0.82 were indistinguishable from those of methyl 2,6-dideoxy-3,4-di-*O*-methyl-α-D-*lyxo*-hexopyranoside and methyl 3-*O*-acetyl-2,6-dideoxy-4-*O*-methyl-α-D-*lyxo*-hexopyranoside (see below), respectively. The presence of the component at R_F 0.65 indicates that some saponification of the ester occurred during methylation.

(*b*) *Using diazomethane*

To a solution of the 3-*O*-acetate (V) (15 mg) and boron trifluoride etherate (40 mg) in dry ether (0.2 ml) was added a 5% solution of diazomethane in dry ether (0.1 ml), and the mixture was set aside at room temperature for 2 h. The excess of reagent was destroyed with 50% aqueous acetic acid and the solution taken up in chloroform (5 ml) which was then washed with water and dried ($MgSO_4$). Examination of the concentrated extract by thin-layer chromatography [ethyl acetate–chloroform (1:1 v/v)] showed, in addition to starting material, a component with R_F 0.82 (*cf.* methyl 3-*O*-acetyl-2,6-dideoxy-4-*O*-methyl-α-D-*lyxo*-hexopyranoside).

A sample of methyl 3-*O*-acetyl-2,6-dideoxy-4-*O*-methyl-α-D-*lyxo*-hexopyranoside (VII), $[\alpha]_D^{15}+104°$ (*c* 2.6, chloroform), was prepared by acetylation of methyl 2,6-dideoxy-4-*O*-methyl-α-D-*lyxo*-hexopyranoside[9] (methyl α-D-chromoside A) in the usual manner. Methyl 2,6-dideoxy-3,4-di-*O*-methyl-α-D-*lyxo*-hexopyranoside was prepared from compound (IV) using methyl iodide and sodium hydride[24] by the procedure described above.

Methyl 4-O-acetyl-2,6-dideoxy-α-D-lyxo-hexopyranoside (VI)

(*a*) *By orthoester exchange*[13]

To a stirred solution of methyl 2,6-dideoxy-α-D-*lyxo*-hexopyranoside (0.58 g) in trimethyl orthoacetate (2.2 ml, dried by distillation from calcium hydride) was added mesitylenesulphonic acid (0.36 g) and, after 2 h, the theoretical amount of solid sodium hydrogen carbonate to neutralise the acid. The solution was dispersed in chloroform (30 ml) which was then washed with water (2 × 10 ml) and dried ($MgSO_4$). Concentration of the filtered solution gave a brown syrup (*ca.* 0.7 g) which, on thin-layer chromatography, showed two principal components, with R_F 0.53 (4-*O*-acetate, major) and 0.59 (3-*O*-acetate). The above procedure differs slightly from that described by Reese and Sulston[13] and decomposition of the 3,4-*O*-methoxyethylidene compound occurred during work-up due, probably, to the development of slightly acidic conditions. The syrup was dissolved in benzene (3 ml) and chromatographed on silica gel (15 g), as described previously; 10 ml fractions were collected. Fractions 13–19 contained a mixture of both components, fractions 20–24 contained the product (VI) together with traces of the 3-*O*-acetate. The latter fractions were combined and evaporated to a syrup (0.14 g) which crystallised on seeding (see below) and, on recrystallisation from benzene–light petroleum (b.p. 80–100°), had m.p. 87–90° $[\alpha]_{5461}^{18}+158°$ (*c* 0.95, chloroform) (Found: C, 52.8; H, 7.8. $C_9H_{16}O_5$ calc.: C, 52.9; H, 7.9%).

(*b*) *From methyl 3-O-benzyl-2,6-dideoxy-α-D-lyxo-hexopyranoside (VIII)*

To a cooled (0°) solution of methyl 3-*O*-benzyl-2,6-dideoxy-α-D-*lyxo*-hexo-

pyranoside[9] (0.86 g) in dry pyridine (4 ml) was added acetic anhydride (0.8 ml), and the solution was set aside at room temperature for 24 h. After the addition of water (2 ml), the solvents were removed, the solid residue was dissolved in chloroform (20 ml), and the solution was washed with dilute aqueous sodium hydrogen carbonate (2×10 ml), water (10 ml), saturated aqueous cadmium chloride (20 ml), and water (2×10 ml), and dried ($MgSO_4$). The filtered solution was evaporated to a solid residue which was recrystallised (twice) from light petroleum (b.p. 80–100°) to give methyl 4-*O*-acetyl-3-*O*-benzyl-2,6-dideoxy-α-D-*lyxo*-hexopyranoside (0.4 g), m.p. 87–92°, $[\alpha]_D^{15}+178°$ (*c* 1, chloroform) (Found: C, 65.2; H, 7.4. $C_{16}H_{22}O_5$ calc.: C, 65.3; H, 7.5%).

A solution of the foregoing compound (0.2 g) in ethanol (80 ml) containing palladium–charcoal[14] (0.1 g) was shaken for 20 h at room temperature in the presence of hydrogen at a slight overpressure. The catalyst was removed and the filtrate evaporated under reduced pressure to a crystalline residue (0.14 g) which, on recrystallisation from benzene–light petroleum (b.p. 80–100°), gave pure methyl 4-*O*-acetyl-2,6-dideoxy-α-D-*lyxo*-hexopyranoside (0.1 g), m.p. 90–91°, $[\alpha]_{5461}^{18}+165°$ (*c* 1, chloroform) (Found: C, 53.1; H, 7.8. $C_9H_{16}O_5$ calc.: C, 52.9; H, 7.9%). The infrared spectrum and thin-layer chromatographic properties of this compound were indistinguishable from those of the monoacetate prepared by orthoester exchange.

3-O-Acetyl-2,6-dideoxy-D-lyxo-*hexose (I)*

A solution of compound (V) (0.5 g) was hydrolysed with 50% acetic acid (50 ml) for 20 min at 85–90°. Examination of the hydrolysate by thin-layer chromatography revealed the principal component with R_F 0.40 and a trace of a second component, R_F 0.28. The hydrolysate was neutralised (Ag_2CO_3) and filtered, insoluble material was washed with acetone, and the combined filtrate and washings were evaporated. The resultant syrup was decolourised in methanol with activated charcoal and the solution was concentrated to a syrup (0.48 g) which crystallised on addition of ethyl acetate. The crude product was dissolved in ethyl acetate (5 ml), and the solution was filtered to remove insoluble material, concentrated to *ca.* 1 ml, and allowed to crystallise. Two recrystallisations from ethyl acetate gave the product (I) (0.3 g), m.p. 115–116.5°, $[\alpha]_D^{29}+100$ (1 min)→+78° (final, *c* 1, water); the direction of mutarotation is indicative of the α-configuration (Found: C, 50.2; H, 7.5. $C_8H_{14}O_5$ calc.: C, 50.5; H, 7.4%). No depression of m.p. was observed on admixture with natural chromose D, and the thin-layer chromatographic properties, infrared spectra (Nujol mulls), and X-ray powder photographs of the synthetic and natural sugars were indistinguishable.

Under identical conditions of hydrolysis, methyl 4-*O*-acetyl-2,6-dideoxy-α-D-*lyxo*-hexopyranoside (VI) was shown by thin-layer chromatography to give mainly a component with R_F 0.28 and a trace of a second component with R_F 0.40. Isolation by the method described above gave a syrup which could not be induced to crystallise. Its infrared spectrum differed significantly from that of natural and synthetic chromose D.

*Methyl 2-deoxy-3,6-di-*O*-toluene-*p*-sulphonyl-α-*D*-lyxo-hexopyranoside*

To a cooled (−10°) and stirred solution of methyl 2-deoxy-α-D-*lyxo*-hexopyranoside[10] (2 g) in dry pyridine (18 ml) was slowly added a cooled solution of toluene-*p*-sulphonyl chloride (4.72 g, 2.2 mol.) in dry pyridine (5 ml), and the mixture was set aside at room temperature for 4 days. After the addition of water (1 ml), the solvent was removed and the residue was taken up in chloroform (100 ml) which was washed with dilute aqueous solutions of sodium hydrogen sulphite (2×50 ml) and sodium hydrogen carbonate (50 ml), and water (50 ml). The chloroform extract was freed from pyridine by shaking with a concentrated aqueous solution of cadmium chloride (50 ml), then washed with water (2×100 ml) and dried ($MgSO_4$). Removal of the solvent gave an amorphous residue (5.4 g), $[\alpha]_D^{24}+75°$ (*c* 2, chloroform), which was shown by thin-layer chromatography (using benzene–methanol, 97:3) to contain predominantly methyl 2-deoxy-3,6-di-*O*-toluene-*p*-sulphonyl-α-D-*lyxo*-hexopyranoside, admixed with a little methyl 2-deoxy-3,4,6-tri-*O*-toluene-*p*-sulphonyl-α-D-*lyxo*-hexopyranoside[25] (m.p. 147–148°).

*Methyl 2-deoxy-4-*O*-methyl-3,6-di-*O*-toluene-*p*-sulphonyl-α-*D*-lyxo-hexopyranoside*

To a solution of the foregoing di-ester (5 g) in redistilled methyl iodide (100 ml) was added freshly prepared silver oxide (4 g), and the mixture was heated under reflux for 14 h, with addition of more silver oxide (3 g) after 2 and 4 h. The cooled solution was filtered and the filtrate concentrated to a syrup (4.5 g) which was shown by thin-layer chromatography to be essentially homogeneous. The infrared spectrum of the syrup showed the absence of bands at 3400–3600 cm^{-1} due to hydroxyl absorption. A portion (0.5 g) of the syrup crystallised on storage with a small quantity of methanol. Two recrystallisations from dry methanol gave the product (0.35 g), m.p. 110–111° (dec.), $[\alpha]_{5461}^{21}+135°$ (*c* 1, chloroform) (Found: C, 57.1; H, 5.95. $C_{22}H_{28}O_9S_2$ calc.: C, 52.8; H, 5.6%).

*Methyl 2,6-dideoxy-4-*O*-methyl-α-*D*-lyxo-hexopyranoside*

A solution of the syrupy product (4 g) from the previous experiment in dry benzene–ether (500 ml, 1:1 v/v) was refluxed in the presence of lithium aluminium hydride (3 g) for 48 h. Ethyl acetate and water were added to the cooled mixture to destroy the excess of reagent, insoluble material was collected and washed thoroughly with ether, and the combined filtrate and washings were extracted with water (4×100 ml). The aqueous layer was then continuously extracted with ether for 48 h and the dried ($MgSO_4$) extract was concentrated to a colourless syrup (0.65 g), which rapidly crystallised. Two recrystallisations from *n*-hexane afforded the product (0.35 g), m.p. 95–96°, $[\alpha]_D^{22}+164°$ (*c* 1, chloroform). Miyamoto *et al.*[4] report m.p. 92°, $[\alpha]_D^{22}+122°$ (*c* 1, ethanol) for methyl α-D-chromoside A and Berlin *et al.*[7] give m.p. 98°, $[\alpha]_D^{26}+150°$ (*c* 0.4, ethanol) for methyl α-D-olivomoside A. The infrared spectrum and thin-layer chromatographic properties of the product were indistinguishable from those of natural[4] and synthetic[9] methyl α-D-chromoside A.

2,6-Dideoxy-4-O-methyl-D-lyxo-*hexose*

This compound, m.p. 151–153° (dec.), $[\alpha]_D^{25}+82°$ (final, *c* 1, water), was obtained by hydrolysis of the foregoing glycoside by the procedure previously described[9]. Miyamoto *et al.*[4] report m.p. 151°, $[\alpha]_D^{22}+93\rightarrow+77°$ (final, water) and Berlin *et al.*[7] give m.p. 158–162°, $[\alpha]_D^{23}+98.5\rightarrow+89°$ (final, water) for natural chromose A and olivomose, respectively. The thin-layer chromatographic properties and infrared spectra of the synthetic and natural compounds were identical.

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SUMMARY

The structure of chromose D, a sugar component of the antitumour substance chromomycin A_3, has been confirmed as 3-*O*-acetyl-2,6-dideoxy-D-*lyxo*-hexose by synthesis. The corresponding 4-*O*-acetate has been synthesised for reference purposes. An improved synthesis of chromose A is described.

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THE α-L- AND β-D-PYRANOSIDE LINKAGES IN OLEANDOMYCIN[1]

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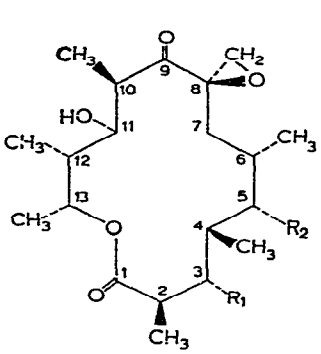
Previous reports from these laboratories[2] have described the isolation, characterization, structure[2e], and total absolute configuration [2f,g] of the macrolide antibiotic oleandomycin[3,4] (I) (see Table I). The structures of the two known sugars liberated from I by acid hydrolysis have been ascertained by other workers: L-oleandrose[5] is 2,6-dideoxy-3-*O*-methyl-L-*arabino*-hexose[6], and D-desosamine[7] is 3,4,6-trideoxy-3-(dimethylamino)-D-*xylo*-hexose[8].

TABLE I
ROTATIONAL DATA ON OLEANDOMYCIN AND DERIVATIVES

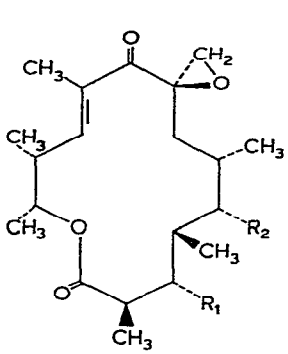
		R_1	R_2	$[M]_D^a$, *degrees*	*Ref.*
Oleandomycin	I	(i)	(ii)	− 447	2b
Desosaminyloleandolide	II	OH	(ii)	− 190	2d
Anhydro-oleandomycin	III	(i)	(ii)	+ 435	2d
Desosaminylanhydrolide	IV	OH	(ii)	+ 610	2e
Anhydrolide	V	OH	OH	+ 690	2e

[a] $[M]_D = [\alpha]_D \times$ M.W./100; all rotations were in methanol (see Experimental part).

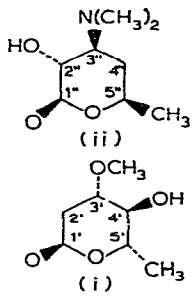
The discovery that α-L-oleandropyranosyloxy and β-D-desosaminopyranosyloxy groups are present in I was briefly described earlier[1]; the present paper furnishes a detailed account of two methods employed for analyses of the anomeric configurations, namely, nuclear magnetic resonance[9] (n.m.r.) and molecular rotational differences[10] (m.r.d.). Individual methyl α- and β-L-oleandropyranosides, as well as *n*-butyl α- and β-D-desosaminopyranosides, were prepared and characterized for their value as models. The molecular rotational and n.m.r. properties of these simple glycosides were found to be within predictable ranges, considering factors of absolute configuration and preferred conformation[11]. These properties, in turn, substantiated the interpretations placed on the n.m.r. and m.r.d. data for I. Examination, by n.m.r., of a preparation of di-*O*-acetyl-L-oleandrose revealed its 1:1 content of the α and β anomers, and provided further characterization of L-oleandrose.



I R_1 = (i), R_2 = (ii)
II R_1 = OH, R_2 = (ii)

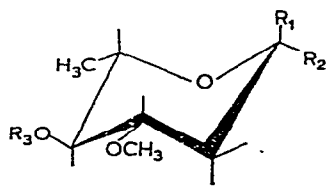


III R_1 = (i), R_2 = (ii)
IV R_1 = OH, R_2 = (ii)
V R_1 = R_2 = OH

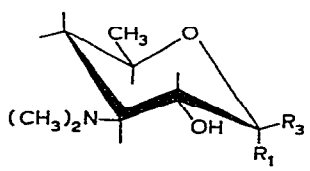


(ii)

(i)



1C (L)



C1 (D)

Oleandrose and its glycosides

Although L-oleandrose has been known for some time as a sugar component of steroid glycosides[12] and [2,4b] of I, descriptions of its individual simple glycosides have not yet been recorded. The pyranoside nature of the methyl α,β-L-oleandrosides obtained from I was proved by further methylation, followed by oxidation to the fully substituted 1,5-lactone[2d]. Since the rates of release of L-oleandrose from I and from the simple glycosides by acid hydrolysis[13] were parallel, it follows that the form present in the antibiotic is pyranoid. This assumption is substantiated by the n.m.r. data presented here and has been further verified[14] in another connection (which involved direct methylation of the L-oleandrosyloxy group in a variant of I).

Separation of the anomers of methyl L-oleandropyranoside, desired for individual inspection, was achieved by column chromatography (on silica gel) of the mixed anomers obtained from I. The α-L form was obtained as an oil, $[M]_D^{25}$ −221° (ethanol), whereas the β-L anomer was crystalline, m.p. 74–78°, $[M]_D^{25}$ +125° (ethanol); see Table II. These anomeric assignments are in accordance with recommended carbohydrate nomenclature[15].

TABLE II

MODEL GLYCOSIDES AND ESTERS OF L-OLEANDROSE AND D-DESOSAMINE

Pyranoside	*Conformation*	R_1	R_2	R_3	$[M]_D^a$, *degrees*	*Solvent*
Methyl α-L-oleandroside	*1C*(L)	CH_3O	H	H	− 221	EtOH
Methyl β-L-oleandroside	*1C*(L)	H	CH_3O	H	+ 125	EtOH
Di-*O*-acetyl-α-L-oleandrose	*1C*(L)	AcO	H	Ac		
Di-*O*-acetyl-β-L-oleandrose	*1C*(L)	H	AcO	Ac		
n-Butyl α-D-desosaminide	*C1*(D)	*n*-BuO	H		+ 330	MeOH
n-Butyl β-D-desosaminide	*C1*(D)	H	*n*-BuO		− 73.6	MeOH

[a] $[M]_D = [\alpha]_D \times$ M.W./100.

The n.m.r. data, listed in Table III, are in harmony with the rotationally based assignments for the anomers in their expected, preferred *1C*(L) conformation[11]. There is no question that methyl β-L-oleandropyranoside exists with all-equatorial substituents (*1C*) rather than all-axial (*C1*); hence, it automatically follows from the data that methyl α-L-oleandropyranoside also has the *1C* conformation. This allows significance to be attached to the chemical shift observed for the methoxyl group at C-3, namely, 6.60 τ in the models and 6.59 τ in I, as an indicator of the *1C* conformation in all these cases. Further characterization of L-oleandrose was achieved by conversion into the diacetate, isolated as a 1:1 mixture of the α and β anomers. Distinctive and common n.m.r. signals of the two anomers were noted (see Table III).

The n.m.r. spectral properties of 2,6-dideoxyglycopyranosides related to the above L-oleandropyranosides have been reported previously, namely, for methyl α-L-mycaroside[16], di-*O*-acetyl-β-L-mycarose[16], di-*O*-acetyl-β-L-cladinose[16], and methyl *O*-acetyl-L-arcanoside[17].

Desosamine and its glycopyranosides

To date, desosamine is only known to occur as the D sugar, peculiar to certain macrolide glycosides[4], including picromycin, methymycin, neomethymycin, narbomycin, the erythromycins, and I. The β-D sugar[8], the β-D-diacetate[8], and anomerically undetermined ethyl and *n*-butyl desosaminides[18] have been described. The preparation of the *n*-butyl desosaminopyranosides was repeated, and the main product proved to be *n*-butyl α-D-desosaminide, m.p. 54–56° (from hexane), $[M]_D^{25}$ +330° (methanol). Processing of the mother liquor afforded crystalline *n*-butyl β-D-desosaminide, m.p. 41–43°, $[M]_D^{25}$ −73.6° (methanol). Glycosides of desosamine can occur only as pyranosides; these have now been unequivocally established, by n.m.r., to exist in the preferred *C1*(D) conformation which is likewise predicted on the basis of available rules[11] (see Tables II and III).

Anomeric configurational analyses of oleandomycin (I)

Nuclear magnetic resonance (n.m.r.)

N.m.r. data on I disclosed downfield chemical shifts for three protons which

TABLE III

PERTINENT N.M.R.[a] DATA ON MODEL GLYCOSIDES AND ESTERS OF L-OLEANDROSE AND D-DESOSAMINE[b]

	C.1′-H		*C.1″-H*		*C.5′-CH₃*		*C.5″-CH₃*		*C.1′-OR*	*C.3′-OMe*	*C.4′-OAc*
Glycoside	τ	*J c.p.s.*	τ	*J c.p.s.*	τ	*J c.p.s.*	τ	*J c.p.s.*	τ	τ	τ
Methyl α-L-oleandroside	5.23	1*e*–2*a* 3.5			8.70	6.0			6.68	6.60	
		1*e*–2*e* 1.5									
Methyl β-L-oleandroside	5.61	1*a*–2*a* 9.5			8.65	5.5			6.51	6.60	
		1*a*–2*e* 2.0									
Di-*O*-acetyl-α-L-oleandrose[c]	3.81	1*e*–2*a* 3.5									
		1*e*–2*e* 1.5			8.86	6.0			7.91	6.64	7.91
Di-*O*-acetyl-β-L-oleandrose[c]	4.28	1*a*–2*a* 10									
		1*a*–2*e* 2.5			8.81	6.0			7.91	6.64	7.91
n-Butyl α-D-desosaminide			5.07	1*e*–2*a* 3.5			8.82	6.2			
n-Butyl β-D-desosaminide			5.73	1*a*–2*a* 7.2			8.72	6.2			

[a] Methods as in Table IV.

[b] Chemical shifts and coupling constants for the remaining protons, and integration values for the entire spectra, were all consistent with the formulations given.

[c] Taken from the n.m.r. spectrum of the mixture.

were distinct and readily analyzed (see Table IV). From its position and splitting pattern, the most deshielded proton (4.37 τ) undoubtedly reflects the lactone terminus[19] *i.e.*, C.13-H.

Proceeding upfield, doubled doublet signals centering at 4.99 τ, *J* 3.5/1.0 c.p.s., correspond to those from the anomeric equatorial proton (C.1′-H) of the α-L-oleandrosyloxy substituent in the *1C*(L) conformation, further substantiated as *1C*(L) by the chemical shift (6.59 τ) observed for the C.3′-*O*-methyl group[20]. The different chemical shifts observed for the anomeric proton in the model (5.23 τ) and in I (4.99 τ) are explicable in terms of the different aglycons in the two.

TABLE IV

N.M.R. DATA ON OLEANDOMYCIN[a] ($C_{35}H_{61}NO_{12}$)

τ	*J* (*c.p.s.*)	*H*	*Assignment* (*see I*)
4.37	6.0[b]	1	C.13-H (lactone terminus)
4.99	3.5/1.0	1	(α-L-) C.1′-H
5.74	7.0	1	(β-D-) C.1″-H
5.9–6.55		6	[c]
6.59	0	3	C.3′-OCH_3
6.65–7.05		7	[c]
7.11		2	C.8a (epoxide) CH_2
7.2–7.6		3	[c]
7.72	0	6	C.3″-$N(CH_3)_2$
7.8–8.55		7	-CH_2- (3) + [c]
8.55–9.25		24	C-CH_3 (8)
	Total	61	

[a] Varian A60 apparatus, $CDCl_3$ solvent, and tetramethylsilane (10.00 τ) as internal reference, were employed.
[b] Doublet emerging from complex fine splitting.
[c] -CH- (14) + -OH (3).

A sharp doublet centering at 5.74 τ, *J* 7.0 c.p.s.[21], could only arise from the axial anomeric proton (C.1″-H) of the β-D-desosaminide substituent in its preferred *C1*(D) conformation.

Persistence of chemical shifts at 4.37 τ (C.13-H) and 5.74 τ (C.1″-H) in desosaminyloleandolide (II), *i.e.*, "desoleandomycin"[2d], is consistent with the above assignments and further indicates that I and II possess related aglycon conformations.

Molecular rotational differences (*m.r.d.*)[9]

Reflection, *i.e.*, $\Delta[M]_D$, of the α-L anomeric configuration in the L-oleandrosyloxy moiety of oleandomycin (I) has been gained by considering I as the glycoside, $[M]_D^g$, = −447°, and known[2d] desosaminyloleandolide (II) as the aglycon, $[M]_D^a$, = −190°, according to $\Delta[M]_D = [M]_D^g - [M]_D^a$, (*cf.*, $\Delta[M]_D$ = −263° and $[M]_D^{25}$ −221° for methyl α-L-oleandroside in Table I). This conclusion, consistent with the n.m.r. analysis, follows from knowledge that the pyranoside in question evidently

possesses the same conformation in I and in the model glycosides, and that the aglycon conformation in I and II is also comparable, as mentioned in the preceding discussion. The same qualitative interpretation is placed on another glycoside–aglycon set of comparisons, namely, the known anhydro-oleandomycin[2d,e] (III), $[M]_D^g = +435°$, and desosaminylanhydrolide[2e] (IV), $[M]_D^a = +610°$, where $\Delta[M]_D = -175°$.

Although the completely sugar-free aglycon "oleandolide" remains a hypothetical compound, its anhydro counterpart, "anhydrolide"[2e] (V) has been prepared. Accordingly, by considering IV as the glycoside, $[M]_D^g = +610°$, and V as the aglycon, $[M]_D^a = +690°$, it follows that $\Delta[M]_D = -80°$. This is in excellent agreement with $[M]_D^{25}$ −73.6° observed for *n*-butyl β-D-desosaminide, and is consistent with the β-D-assignment[22,23] based on n.m.r.

EXPERIMENTAL[24]

Methyl α-L-oleandroside and methyl β-L-oleandroside

Previously described, distilled methyl L-oleandrosides, $[\alpha]_D^{25}$ −75.5° (*c* 5, ethanol), $[\alpha]_D^{25}$ −74.3° (*c* 5.3, methanol), obtained from oleandomycin, were found to consist of a 3:1 mixture of the α and β anomers (gas–liquid chromatography on a column of 5% silicone SE-30 on Haloport F at 173°). Preparative separation of a 0.5-g sample was achieved on a 1.8 × 45 cm column of silica gel H (E. Merck AG) employing a 3:2 benzene–ethyl acetate system and an automatic fraction (*ca.* 2 cc) collector. The degree of separation was judged by thin-layer chromatography (t.l.c.) on silica gel, using a 1:1 benzene–ethyl acetate system followed by a detection spray of sulfuric acid. The first component off the column proved to be the α-L anomer, obtained as an oil; $[\alpha]_D^{22}$ −125.6° (*c* 5.1, ethanol). Calc. for $C_8H_{16}O_4$: C, 54.53; H, 9.15. Found: C, 54.21; H, 8.91.

Later fractions off the column furnished the β-L anomer, as an oil which crystallized, m.p. 74–78°, $[\alpha]_D^{22}$ +71.5° (*c* 2.3, ethanol). Found: C, 55.20; H, 9.31.

A 3:1 weighted average of the rotations of the α-L and β-L anomers gives $[\alpha]_D$ −76.3°, which is very close to the value actually observed for the original mixture of anomers.

n-*Butyl α-D-desosaminide and* n-*butyl β-D-desosaminide*

The *n*-butyl D-desosaminides were prepared by treating D-desosamine hydrochloride with 1-butanol and hydrogen chloride, according to the procedure of Flynn *et al*[18,25]. Most of the product distilled at 98–100°/0.4 mm and crystallized on cooling, m.p. 45–47°. Recrystallization from hexane furnished *n*-butyl α-D-desosaminide, m.p. 54–56°, $[\alpha]_D^{25}$ +143° (*c* 10, methanol). Calc. for $C_{12}H_{25}NO_3$: C, 62.34; H, 10.86. Found: C, 62.07; H, 10.81.

After solvent had been removed from the mother liquor, the resulting oil, showing $[\alpha]_D^{25}$ +21.6° (*c* 5, $CHCl_3$), slowly deposited crystalline *n*-butyl β-D-desosaminide, m.p. 41–43°, $[\alpha]_D^{25}$ −5.3° (*c.* 5, chloroform), $[\alpha]_D^{25}$ −31.9° (*c* 10, methanol). Found: C, 62.00; H, 10.84.

Di-O-acetyl-α- and β-L-oleandrose

L-Oleandrose (20 g) in solution in acetic anhydride (20 cc) and pyridine (20 cc) was kept for 18 h at 25°. After being poured into an ice–water–chloroform mixture, the organic phase was separated and the aqueous layer extracted with a further portion of chloroform. The combined organic extracts were washed with cold water adjusted to pH 7.5 and then with water, dried with anhydrous sodium sulfate, and the solvent removed. Most of the residue distilled at 94–100°/0.2 mm, to afford 23.43 g (77%) of an oil, $[\alpha]_D^{25}$ −32.8 (*c* 6.4, chloroform). Calc. for $C_{11}H_{18}O_6$: C, 53.65; H. 7.37. Found: C, 53.84; H, 7.39.

The mixed anomeric nature of this product was not readily ascertained by t.l.c.; however, an approximately 1:1 mixture of the anomers was evident by n.m.r. analysis (see Table III).

Optical rotations of macrolide compounds (see Table I)

Specific rotations (in methanol) are: oleandomycin[2b] (I), $C_{35}H_{61}NO_{12}$ (M.W., 688): $[\alpha]_D^{25} = -65°$; desosaminyloleandolide (II), $C_{28}H_{49}NO_9$ (M.W., 544), crystallized from ether, m.p. 185–187°: $[\alpha]_D^{25}$ −35° (previously reported[2d], amorphous product, "desoleandomycin": $[\alpha]_D^{25}$ −25.3°); anhydro-oleandomycin[2d,e] (III), $C_{35}H_{59}NO_{11}$ (M.W., 670): $[\alpha]_D^{25}$ +65°; desosaminylanhydrolide (IV), $C_{28}H_{47}NO_8$ (M.W. 526), *i.e.*, "damylanhydrolide"[2e]: $[\alpha]_D^{25}$ +116°; anhydrolide[2e] (V), $C_{20}H_{32}O_6$ (M.W., 368): $[\alpha]_D^{25}$ +187.3°.

ACKNOWLEDGMENTS

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SUMMARY

Polarimetric and nuclear magnetic resonance data are presented as consistent evidence for the α-L and β-D nature of the respective anomeric centers in oleandomycin, thus completing the glycosidic aspects of the stereochemistry of oleandomycin (I); these centers are those of the α-L-oleandrosyloxy and β-D-desosaminyloxy moieties. Individual methyl α- and β-pyranosides of L-oleandrose and *n*-butyl α- and β-pyranosides of D-desosamine were prepared and characterized, to furnish base-line reference data for the analyses of anomeric configuration performed on oleandomycin.

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23 It appears that, when variables are carefully controlled, either m.r.d. or n.m.r. is a reliable means of anomeric configurational analysis. Since both methods are based on principles involving preferred conformation, it is difficult to state categorically which method might theoretically be better. In practice, technical difficulties quite often prevent the application of one or the other method, but less often both. Hence, m.r.d. and n.m.r. are regarded as complementary.

24 Melting points were obtained on a Kofler hot stage, and n.m.r. spectra were measured on a Varian A-60 instrument. The authors are indebted to Dr. R.L. Wagner and his Physical Measurements Staff, as well as to Dr. T. Lees and his Chromatography Laboratory Staff, for analyses.

25 A product of m.p. 50–51°, presumably the α-D anomer, is described in ref. 18.

1,3:2,4-DI-*O*-BENZYLIDENE-L-ARABINITOL AND OBSERVATIONS ON SOME RELATED CYCLIC PHENYLBORONATES

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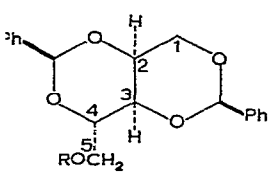
INTRODUCTION

The acid-catalysed benzylidenation of D- or L-arabinitol affords the 1,3-*O*-benzylidene derivative in high yield[1]. No di-*O*-benzylidene derivative has been reported although D-arabinitol affords a 1,3:2,4-di-*O*-methylene compound[2]. The reluctance of arabinitol to form a di-*O*-benzylidene derivative seemed surprising since 2-*O*-benzyl-D-arabinitol gives isomeric 1,3:4,5-di-*O*-benzylidene derivatives, albeit in rather poor yield[3].

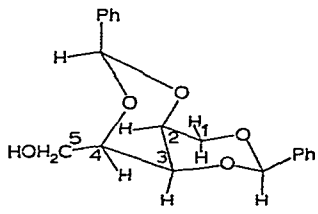
RESULTS AND DISCUSSION

Investigation of the product formed on zinc chloride-catalysed benzylidenation of L-arabinitol revealed, in addition to 1,3-*O*-benzylidene-L-arabinitol, a small amount (< 5%) of a di-*O*-benzylidene derivative. Variation in the conditions of the condensation failed to improve significantly the yield of di-*O*-benzylidene derivative which was also formed in small amount when sulphuric acid was used as catalyst. The di-*O*-benzylidene derivative was dimorphous; forms with melting point 161–162° and 176-178° were encountered.

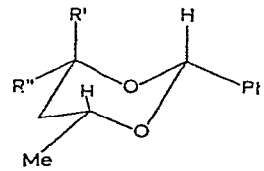
A 1,3:2,4-distribution (I) of the acetal rings in the di-*O*-benzylidene derivative was established by the following reactions. Treatment of the toluene-*p*-sulphonate (II) of compound (I) with sodium benzoate in boiling dimethylformamide[4] gave a benzoate identical with the product obtained on direct benzoylation of the di-*O*-benzylidene derivative (I). Since the "benzoate exchange" proceeded with retention



I R = H
II R = Ts



III



IV R' = H, R" = Me
V R' = Me, R" = H

of configuration, and because of the unique stereochemistry of L- (and D-) arabinitol, the toluene-*p*-sulphonate group in compound (II) must have been attached to C-1, C-3, or C-5. Graded acidic hydrolysis of the di-*O*-benzylidene derivative (I) using toluene-*p*-sulphonic acid in aqueous dioxan afforded 1,3-*O*-benzylidene-L-arabinitol, and therefore the free hydroxyl group was present at C-5 in the parent compound (I) and the hydrolysed acetal group must have spanned the 2,4-positions. Hence, compound (I) is 1,3:2,4-di-*O*-benzylidene-L-arabinitol. The graded hydrolysis of compound (I) could be followed conveniently by observing the change in the pattern of benzyl proton signals in the n.m.r. spectrum of the hydrolysis mixture.

1,3:2,4-Di-*O*-benzylidene-L-arabinitol had benzyl proton signals at τ 4.48 and 4.80 (for a *ca.* 10% solution in dioxan; Varian A60 spectrometer) in the n.m.r. spectrum indicative[5] of the existence of the molecule in the predicted[6] "O"-inside structure (III) with equatorial phenyl groups and an axial hydroxymethyl group. The signal at 4.80 may be assigned to the benzyl proton in the 1,3-acetal ring since the chemical shift is characteristic[7] of an axial benzyl proton in the chair form of a 2-phenyl-1,3-dioxan ring with axial protons at positions 4 and 6. The corresponding proton in the 2,4-acetal is deshielded by the axial hydroxymethyl group and gives the signal at lower field (4.48). An analogy may be drawn both with the results of other workers[8] and with those obtained for the benzylidene derivatives [(IV) and (V), respectively] of *meso*- and DL-pentane-2,4-diol[3]. The former acetal (IV), which has a benzyl proton in an environment similar to that of the corresponding proton in the 1,3-acetal ring of the di-*O*-benzylidene derivative (III), has the relevant signal at 4.91. On the other hand, the benzyl proton in acetal (V) is deshielded by the axial methyl group, and the signal occurs at 4.58. The slight shift to lower field of the benzyl proton signals in the di-*O*-benzylidene derivative (III) compared with those of the monocyclic acetals (IV) and (V) is similar to that observed for other di-*O*-benzylidene derivatives[3]. Other examples of benzylidene acetals containing "O"-inside structures similar to that (III) postulated for 1,3:2,4-di-*O*-benzylidene-L-arabinitol have been considered elsewhere[5], together with the evidence against "H"-inside structures.

In seeking compounds for comparison of physical properties, especially infrared spectra, with those of 1,3:2,4-di-*O*-benzylidene-L-arabinitol, the reaction of 1,3-*O*-benzylidene-L-arabinitol with phenylboronic acid was examined. Although there have been several investigations[9–11] of the reaction of phenylboronic acid with carbohydrate derivatives, apparently there has been no report of its reaction with sugar derivatives which are partially substituted by cyclic acetal groups.

1,3-*O*-Benzylidene-L-arabinitol, which can form an α- (4,5-), βT- (2,4-) or γ- (2,5-) phenylboronate (using Barker and Bourne's terminology[12]), reacted readily with phenylboronic acid in benzene (azeotropic removal of water[11]) to give a monophenylboronate (VI). The cyclic ester (VI) was not readily hydrolysed by boiling water or hot, aqueous, methanolic sodium hydroxide, but was smoothly saponified by passage of an aqueous, methanolic solution over a suitable anion-exchange resin [*e.g.* Amberlite IRA 400 (HO^- form)] with regeneration of 1,3-*O*-benzylidene-L-arabin-

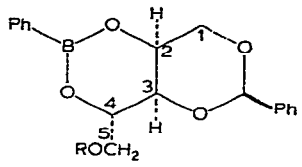
itol (*cf.* the independent observation of Ferrier *et al.*[11b]). Thus, no migration of the benzylidene group occurred during the reaction of 1,3-*O*-benzylidene-L-arabinitol with phenylboronic acid. The phenylboronate group was removed from the benzoate of compound (VI) by passage over neutral alumina. The stability of the phenylboronate group in compound (VI) [and in compound (VIII)] towards saponification contrasts with the lability of many of the cyclic phenylboronates previously described[9–11]. The location of the phenylboronate group in compound (VI) was established as follows.

For dilute solutions (<0.005M) of diols in carbon tetrachloride, intermolecular hydrogen bonding does not usually occur, and absorptions in the hydroxyl stretching region of the infrared can be assigned to free and intramolecularly bonded hydroxyl groups[13]. The absorption pattern [3632 (ε 37), 3624 (ε 39), and 3581 cm^{-1} (ε 50)] shown by the phenylboronate of 1,3-*O*-benzylidene-L-arabinitol (VI) was quite different from that [3582 cm^{-1} (ε 81)] of 1,3-*O*-benzylidene-4,5-*O*-isopropylidene-L-arabinitol indicating that the phenylboronate group in the former compound did not span the 4,5-positions. The band at 3632 cm^{-1} for the phenylboronate (VI) is undoubtedly due to free hydroxyl groups, but the $\Delta\nu$ value[13] ($\nu_{\text{free OH}} - \nu_{\text{bonded OH}}$) of 8 for the absorption at 3624 cm^{-1} is much lower than that (>30) usual[13] for the hydrogen bond OH...O and could be due[14] to an OH...π bond. Such a bond would be sterically possible between the CH_2OH group and the 2,4-phenylboronate residue in structure (VI), but a comparable hydrogen bond involving the hydroxyl group at position 4 would be precluded in any reasonable conformation of the 2,5-analogue. Consequently, the phenylboronate group is assigned to the 2,4-position. The band at 3581 cm^{-1} can be assigned to an OH...O hydrogen bond forming a five-membered ring; although the $\Delta\nu$ value of 51 is somewhat higher than that (30–35) usually observed[13] for the system $CH_2OH.CHOR-$, it is markedly lower than that (>80) for the corresponding hydrogen bond which forms a six-membered or larger ring.

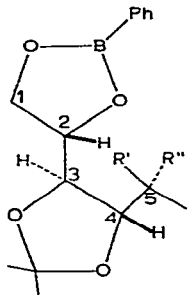
1,3:2,4-Di-*O*-benzylidene-L-arabinitol (I) also had a complex absorption pattern (3633, 3613, and 3581 cm^{-1}) but, because of the poor solubility of the compound, accurate ε values could not be obtained and the frequencies noted should not be regarded as precise. The infrared spectral characteristics of the benzylidene derivatives (I) and (VI), and of related compounds are being further investigated.

1,3-*O*-Benzylidene-4,5-*O*-isopropylidene-L-arabinitol was obtained in high yield by copper sulphate-catalysed acetonation of 1,3-*O*-benzylidene-L-arabinitol, and its structure was established by the following observations: (1) graded acidic hydrolysis gave 1,3-*O*-benzylidene-L-arabinitol indicating the absence of rearrangement during the original acetonation, and (2) the formation of 2-*O*-benzyl-L-arabinitol (characterised as the tetra-*O*-benzoate) on benzylation of the isopropylidene derivative followed by acidic hydrolysis of the acetal and ketal rings. The ease with which 1,3-*O*-benzylidene-L-arabinitol condensed with acetone is in marked contrast to the reluctance of arabinitol to form a di-*O*-benzylidene derivative, and the latter problem is receiving further attention.

Evidence to support the 2,4-assignment of the phenylboronate group in compound (VI) was obtained using the "benzoate-exchange" reaction. The phenyl-



VI R = H
VII R = Ts



VIII R' = OH, R'' = H
IX R' = Ts, R'' = H
X R' = H, R'' = N_3

boronate (VI) readily gave a toluene-*p*-sulphonate (VII) (*cf.* Ferrier's observation[11a]) which on treatment with sodium benzoate in boiling dimethylformamide[4] gave a mixture of products, apparently arising from partial hydrolysis of the phenylboronate group. Although it is likely that a vicinal, free hydroxyl group could interfere during "benzoate exchange" (*cf.* Angyal and Stewart's observations with inositols[15]), the isolation from the product mixture of a benzoate identical with that formed on direct benzoylation of the phenylboronate (VI) indicated that the normal displacement had occurred without change in configuration. Since positions 1 and 3 are blocked by the acetal ring, it follows that in compound (VII) the toluene-*p*-sulphonate group was located at position 5 and the phenylboronate group at the 2,4-positions.

The scope of the phenylboronate group as a blocking agent in the carbohydrate field is further exemplified by a parallel sequence of reactions in the cyclic ketal series using 3,4-*O*-isopropylidene-L-rhamnitol. This readily condensed with phenylboronic acid to give 3,4-*O*-isopropylidene-L-rhamnitol 1,2-phenylboronate (VIII) in which the location of the cyclic ester group was established as follows. Compound (VIII) was not readily hydrolysed by boiling water, hot, aqueous sodium hydroxide, or hot, aqueous, ethanolic D-mannitol[10], but smooth saponification to 3,4-*O*-isopropylidene-L-rhamnitol was effected by the resin method. Thus no migration occurred during the reaction of 3,4-*O*-isopropylidene-L-rhamnitol with phenylboronic acid which must therefore have condensed with two of the hydroxyl groups at C-1, C-2, and C-5. Although it seemed likely that the phenyboronate group spanned the 1,2-positions in compound (VIII), the results of Sugihara and Bowman[10] suggested that a seven-membered cyclic phenylboronate involving the 2,5-positions must also be considered a possibility. The toluene-*p*-sulphonate (IX) of the phenylboronate (VIII) was readily obtained and when treated with sodium azide in boiling dimethylformamide gave an azidodeoxy compound (X) which, on saponification followed by acid-catalysed acetonation, afforded the same product (5-azido-5,6-dideoxy-1,2:3,4-di-*O*-isopropylidene-D-gulitol) as was obtained by application of an "azide-exchange" reac-

tion to 1,2:3,4-di-*O*-isopropylidene-5-*O*-toluene-*p*-sulphonyl-L-rhamnitol[16]. Hence the toluene-*p*-sulphonate group in compound (IX) was located at position 5, and it follows that the phenylboronate group spanned the 1,2-positions.

EXPERIMENTAL

Thin-layer chromatography was performed on Kieselgel, and detection was effected with vanillin–sulphuric acid[17] and/or iodine vapour. Light petroleum refers to the fraction b.p. 60–80°. Where stated, drying of organic solvents was effected with $MgSO_4$. Optical rotations at 5461 Å were determined on 1 or 2 cm layers using a Bendix-Ericsson Type 143A polarimeter. Identification of compounds was based on mixed melting points and comparison of infrared spectra.

Benzylidenation of L-*arabinitol*

(*a*) A mixture of L-arabinitol (6.1 g), benzaldehyde (25 ml), and zinc chloride (12.5 g) was shaken at room temperature for 4 days and then poured into a well-stirred mixture of aqueous sodium hydrogen carbonate and light petroleum. After 1 h the precipitate was collected, washed well with light petroleum, and its solution in chloroform dried and concentrated. Examination of the oily product (3.24 g) by thin-layer chromatography using benzene–ether (1:1) revealed components with R_F values 0.8, 0.65, and 0.25. Two recrystallisations of the product from benzene afforded 1,3:2,4-di-*O*-benzylidene-L-arabinitol (0.36 g, 2.7%), m.p. 160°, R_F 0.25, $[\alpha]_D$ + 40.5° (*c* 0.2, chloroform) (Found: C, 69.7; H, 6.3. $C_{19}H_{20}O_6$ calc.: C, 69.5; H, 6.1%).

The filtered, aqueous sodium hydrogen carbonate–light petroleum mixture was separated and the aqueous layer was extracted with light petroleum (3 × 100 ml). A product (1.45 g) containing components with R_F values 0.95 (benzaldehyde) and 0.65 was present in the combined light petroleum solutions. Subsequent extraction of the aqueous layer with chloroform (3 × 100 ml) removed material (0.39 g), containing components with R_F values 0.65 and 0.25, which, on crystallisation from benzene–light petroleum, afforded a further amount (20 mg) of the di-*O*-benzylidene derivative. Continuous extraction of the aqueous layer with chloroform overnight gave a product (2.27 g) which was essentially 1,3-*O*-benzylidene-L-arabinitol[1], and recrystallisation from ethanol containing a trace of ammonia gave material with m.p. 151° (mixed m.p.). Further continuous extraction for 2 days gave an additional amount (2.8 g) of crude 1,3-*O*-benzylidene derivative.

(*b*) A mixture of L-arabinitol (1 g) and benzaldehyde (2 ml) was shaken and conc. sulphuric acid (0.5 ml) added dropwise during 0.5 h when a homogeneous mixture was obtained. After a further 0.5 h, the solution was poured into a well-stirred mixture of aqueous sodium hydroxide and light petroleum. The precipitate was collected, washed well with light petroleum, and its solution in chloroform was dried and concentrated. Examination of the residue (0.6 g) by thin-layer chromatography as in (*a*) revealed components with R_F values 0.95 (trace), 0.65, and 0.25.

Crystallisation from benzene–light petroleum gave 1,3:2,4-di-*O*-benzylidene-L-arabinitol (60 mg, 2.7%).

The aqueous sodium hydroxide was extracted with light petroleum (3 × 100 ml) and then with chloroform (3 × 100 ml). The combined chloroform extracts were dried and concentrated to yield an oil (0.14 g) from which a further amount (30 mg) of the di-*O*-benzylidene derivative was obtained on crystallisation from benzene–light petroleum.

(*c*) A mixture of L-arabinitol (5 g), benzaldehyde (25 ml), and zinc chloride (10 g) was shaken overnight and then poured into a well-stirred mixture of water and light petroleum. The crystalline precipitate was collected, washed well with light petroleum, and recrystallised from benzene to yield 1,3:2,4-di-*O*-benzylidene-L-arabinitol (0.28 g, 2.5%), m.p. 153°. Further recrystallisation from benzene gave a product (0.18 g), m.p. 161-162°; on addition of light petroleum to the mother liquors a dimorphic form (65 mg), m.p. 176-178°, was obtained. A solution of the latter compound in benzene when seeded with the lower melting form gave the dimorph, m.p. 159–160°. The infrared spectra (Nujol mulls) of the dimorphs were identical.

Benzoylation of the di-*O*-benzylidene derivative (m.p. 161°) by the usual method with benzoyl chloride and pyridine gave 5-*O*-benzoyl-1,3:2,4-di-*O*-benzylidene-L-arabinitol (79%), m.p. 180°, $[\alpha]_D$ + 23° (*c* 0.9, chloroform) (Found: C, 72.2: H, 5.65. $C_{26}H_{24}O_6$ calc.: C, 72.2; H, 5.6%). The benzoate showed no tendency to exist in dimorphic forms.

*Reaction of sodium benzoate with 1,3:2,4-di-*O*-benzylidene-5-*O*-toluene-*p*-sulphonyl-L-arabinitol*

The toluene-*p*-sulphonate of 1,3:2,4-di-*O*-benzylidene-L-arabinitol, prepared in the usual manner with toluene-*p*-sulphonyl chloride and pyridine, had m.p. 75–77° (from ethanol), $[\alpha]_D$ + 18° (*c* 1.1, chloroform) (Found: C, 64.6; H, 5.4; S, 6.7. $C_{26}H_{26}O_7S$ calc.: C, 64.7; H, 5.4; S, 6.6%).

A solution of the foregoing toluene-*p*-sulphonate (30 mg) in dimethylformamide (10 ml) was boiled in the presence of sodium benzoate (0.15 g) for 3 h. The cooled mixture was poured into aqueous sodium hydrogen carbonate, and the precipitate was collected and washed well with water. A solution of the precipitate in chloroform was washed with water, dried, and concentrated. Recrystallisation of the residue (20 mg) from ethanol gave 5-*O*-benzoyl-1,3:2,4-di-*O*-benzylidene-L-arabinitol (17 mg, 63%), m.p. 181° (mixed m.p. 179–180° with the authentic compound described above), $[\alpha]_D$ + 18° (*c* 0.2, chloroform).

*Graded acidic hydrolysis of 1,3:2,4-di-*O*-benzylidene-L-arabinitol*

A solution of the title compound (0.175 g) in a portion (3 ml) of a mixture of toluene-*p*-sulphonic acid (50 mg), dioxan (4.5 ml), and water (0.5 ml) was stored at 25–30°, and the change in the pattern of the benzyl proton signals in the n.m.r. spectrum was observed using a Varian A60 spectrometer. The signals at τ 4.29 and 4.59 for the di-*O*-benzylidene derivative largely disappeared during 4 h and were

replaced by a signal at 4.64. The mixture was then poured into aqueous sodium hydrogen carbonate and extracted with chloroform (4 × 50 ml). Concentration of the combined and dried extracts gave a product which, on examination by thin-layer chromatography using benzene–ether (1:1), was found to contain components with R_F values 0.95 (benzaldehyde), 0.65 (unidentified component), 0.25 (di-*O*-benzylidene derivative), and < 0.01. Using ether–methanol (19:1), components with R_F values 0.94 (di-*O*-benzylidene derivative) and 0.64 (monobenzylidene compound) were detected.

Continuous extraction of the aqueous solution with chloroform containing a trace of ammonia during 12 h gave a product (0.1 g) which, after recrystallisation from benzene, gave 1,3-*O*-benzylidene-L-arabinitol[1] (53 mg), m.p. 146–147° (mixed m.p. 149–150°).

2-O-Benzyl-D-arabinitol

The title compound (m.p. 73–78°, $[\alpha]_D$ −4.5°, methanol) was prepared as previously described[3] and in view of the variation of m.p. according to the conditions of crystallisation, it was further characterised as the tetra-*O*-benzoate, m.p. 104–105° (from ethanol), $[\alpha]_{5461}$ + 34° (*c* 0.65, chloroform) (Found: C, 73.0; H, 5.0. $C_{40}H_{34}O_9$ calcd.: C, 72.9; H, 5.2%), and the tetra-*O*-*p*-phenylazobenzoate, m.p. 196–198° (from benzene–light petroleum) (Found: C, 71.7; H, 4.9. $C_{64}H_{50}N_8O_8$ calc.: C, 71.5; H, 4.7%).

Preparation and proof of structure of 1,3-O-benzylidene-4,5-O-isopropylidene-L-arabinitol

A mixture of 1,3-*O*-benzylidene-L-arabinitol[1] (0.94 g), acetone (150 ml), and anhydrous copper sulphate (4 g, dried at 120° for 4 h) was shaken at room temperature for 3 days. Insoluble material was collected and washed well with acetone, and the combined filtrate and washings were concentrated. Recrystallisation of the residue (1.36 g) from light petroleum gave the product (0.83 g, 76%), m.p. 111–112°, $[\alpha]_D$ −22.5° (*c* 1.6, chloroform) (Found; C, 64.0; H, 7.0. $C_{15}H_{20}O_5$ calc.: C, 64.3; H, 7.2%).

A solution of 1,3-*O*-benzylidene-4,5-*O*-isopropylidene-L-arabinitol (0.41 g) in benzyl chloride (10 ml) was stirred and heated at 115° for 6 h in the presence of crushed potassium hydroxide (2.5 g). The cooled mixture was then partitioned between water and chloroform. The aqueous layer was extracted with chloroform and the combined organic solutions were washed with water, dried, and concentrated. The oily benzyl ether (1.09 g) was hydrolysed at 95–100° with 0.05M-sulphuric acid for 4 h. The hydrolysate was neutralised with sodium hydrogen carbonate and extracted continuously with chloroform overnight. Concentration of the extract and crystallisation of the product (0.25 g) from ethyl acetate–light petroleum gave 2-*O*-benzyl-L-arabinitol (0.12 g), m.p. 75–78°. The product had the same mobility as the D-form described above in paper chromatography using the organic phase of butanol–ethanol–water (4:1:5) and detection with periodate–permanganate[18] and alkaline silver nitrate[19]. Benzoylation of the product in the usual manner with benzoyl

chloride and pyridine gave 1,3,4,5-tetra-*O*-benzoyl-2-*O*-benzyl-L-arabinitol (79%), m.p. 103° (from ethanol), $[\alpha]_{5461}$ −34° (*c* 0.6, chloroform) (Found: C, 72.6; H, 5.5. $C_{40}H_{34}O_9$ calc.: C, 72.9; H, 5.2%). The m.p. was depressed (93–95°) in admixture with the D-form described above, but the infrared spectra (Nujol mulls) of the two compounds were indistinguishable.

A solution of 1,3-*O*-benzylidene-4,5-*O*-isopropylidene-L-arabinitol (0.1 g) in a portion (1 ml) of a mixture of toluene-*p*-sulphonic acid (51 mg), dioxan (4.5 ml), and water (0.5 ml) was stored at 25–30° and the hydrolysis followed by observing the diminution of the Me proton signals for the isopropylidene group in the n.m.r. spectrum. After 19 h, these signals had virtually disappeared, and the mixture was then poured into aqueous sodium hydrogen carbonate and extracted with chloroform (2 × 20 ml), which removed oily material (15 mg) but no benzaldehyde, indicating the stability of the benzylidene acetal under the conditions employed. Subsequent, continuous extraction with chloroform containing a trace of ammonia for 5 h gave 1,3-*O*-benzylidene-L-arabinitol[1] (55 mg) which, after recrystallisation from benzene, had m.p. 146–147° (mixed m.p. 148°).

1,3-O-Benzylidene-L-arabinitol 2,4-phenylboronate

A mixture of benzene (200 ml) and phenylboronic acid (0.5 g) was boiled under reflux with azeotropic removal of water for 3 h, and, after addition of 1,3-*O*-benzylidene-L-arabinitol (1 g), this process was continued for a further 4 h. Concentration of the mixture and recrystallisation of the residue (1.44 g) from benzene–light petroleum yielded the product (1.08 g, 80%), m.p. 120–121°, $[\alpha]_D$ +11° (*c* 1.6, chloroform) (Found: C, 66.1; H, 5.7. $C_{18}H_{19}BO_5$ calc.: C, 66.3; H, 5.8%).

By the usual methods, the phenylboronate was converted into the toluene-*p*-sulphonate, m.p. 141–142° (from ethanol), $[\alpha]_D$ + 16° (*c* 1.0, chloroform) (Found: C, 62.4; H, 5.6; S, 6.3. $C_{25}H_{26}BO_7S$ calc.: C, 62.5; H, 5.2; S, 6.7%), and the benzoate, m.p. 141–142° (from ethanol), $[\alpha]_D$ + 12° (*c* 0.9, chloroform) (Found: C, 70.0; H, 5.6. $C_{25}H_{23}BO_6$ calc.: C, 69.8; H, 5.35%).

Saponification of 1,3-O-benzylidene-L-arabinitol 2,4-phenylboronate and its benzoate

A solution of the phenylboronate (0.33 g) in methanol (2 ml) was added to a column (2.5×35 cm) of Amberlite IRA 400 (HO^- form) which was then eluted with water–methanol (9:1, 1500 ml). Concentration of the eluate gave 1,3-*O*-benzylidene-L-arabinitol[1] (0.19 g, 77%), m.p. 150–151° (mixed m.p.).

When a solution of the phenylboronate (0.67 g) and sodium hydroxide (2 g) in methanol–water (1:1, 40 ml) was heated at 95–100° for 4 h, and then extracted continuously with chloroform overnight, crystalline starting-material was recovered in 52% yield.

A solution of 5-*O*-benzoyl-1,3-*O*-benzylidene-L-arabinitol 2,4-phenylboronate (66 mg) in benzene (1 ml) was added to a column (24 × 1.5 cm) of neutral alumina[20]. Elution with ether (400 ml) gave unidentified material (10 mg), and then with methanol (150 ml) gave a product (46 mg) which, on recrystallisation from benzene–ether,

afforded 5-*O*-benzoyl-1,3-*O*-benzylidene-L-arabinitol (33 mg), m.p. 157–158° (Found: C, 66.3; H, 6.1. $C_{19}H_{20}O_6$ calc.: C, 66.3; H, 5.8%).

Reaction of 1,3-O-benzylidene-5-O-toluene-p-sulphonyl-L-arabinitol 2,4-phenylboronate with sodium benzoate in dimethylformamide

A solution of the title compound (1 g) in dimethylformamide (40 ml) was boiled under reflux for 7 h in the presence of sodium benzoate (1.8 g). The cooled mixture was diluted with aqueous sodium hydrogen carbonate and extracted with chloroform (4 × 100 ml). The combined extracts were washed with water (5 × 150 ml), dried, and concentrated to yield a sulphur-free product (0.7 g) which had infrared bands, *inter alia*, at *ca.* 3500 (OH), 1725 (benzoate C=O), 1670 (dimethylformamide C=O), and 1600 cm^{-1} (aromatic C=C). Crystallisation of the product from ethanol gave material (0.45 g, m.p. 95–100°) which in thin-layer chromatography (benzene–ether, 1:1) showed components with R_F 0.65 and 0.25. After three recrystallisations from ethanol, this afforded slightly impure 5-*O*-benzoyl-1,3-*O*-benzylidene-L-arabinitol 2,4-phenylboronate, m.p. 136° [mixed m.p. 139–140° with the compound (R_F 0.65) described above].

The mother liquors from the above recrystallisations were concentrated and a solution of the residue (0.27 g) in benzene (2 ml) was added to a column (25 × 2.5 cm) of neutral alumina[20]. Elution with ether (250 ml) gave unidentified material (10 mg), and subsequently with methanol (250 ml) gave a product (0.16 g) which, after two recrystallisations from benzene–light petroleum, afforded 5-*O*-benzoyl-1,3-*O*-benzylidene-L-arabinitol (36 mg), m.p. 155–156°, R_F 0.25, identical with the product described above.

3,4-O-Isopropylidene-L-rhamnitol 1,2-phenylboronate

A solution of equimolar proportions of 3,4-*O*-isopropylidene-L-rhamnitol (10 g) and phenylboronic acid (5.8 g) in benzene (1500 ml) was boiled under reflux for 3 h with azeotropic removal of water. Concentration of the solution and recrystallisation of the residue from light petroleum (b.p. 40–60°) gave the product (12.5 g, 75%), m.p. 78–80°, $[\alpha]_D$ −27° (*c* 1.74, chloroform) (Found: C, 62.3; H, 6.9. $C_{15}H_{21}BO_5$ calc.: C, 61.6; H, 7.2%).

The phenylboronate gave a toluene-*p*-sulphonate, m.p. 124–126° (from light petroleum), $[\alpha]_D$ −23° (*c* 1.9, chloroform) (Found: C, 59.7; H, 5.9; S, 7.3. $C_{22}H_{27}BO_7S$ calc.: C, 59.2; H, 6.05; S, 7.2%).

When 3,4-*O*-isopropylidene-L-rhamnitol 1,2-phenylboronate (0.5 g) dissolved in methanol (5 ml) was added to a column of Amberlite IRA 400 (HO^- form) and eluted with aqueous methanol as described above for the L-arabinitol derivative, 3,4-*O*-isopropylidene-L-rhamnitol (0.1 g), m.p. 77–78°, was obtained.

Reaction of 3,4-O-isopropylidene-5-O-toluene-p-sulphonyl-L-rhamnitol 1,2-phenylboronate with sodium azide

A solution of the title compound (10 g) and sodium azide (5 g) in dimethylformamide (130 ml) was boiled under reflux for 2.5 h. The cooled solution was

diluted with water (250 ml) and extracted with chloroform (3 × 75 ml). The combined extracts were washed with water (6 × 100 ml), dried, and concentrated. Distillation of the residue at 180–200° (bath)/0.1 mm gave syrupy 5-azido-5-deoxy-D-*gulo*-derivative (4.8 g, 67%), a solution of which in methanol was added to a column of Amberlite IRA 400 (HO^- form, 200 ml). Elution was effected with aqueous methanol (10%, 1500 ml) and concentration of the eluate gave syrupy 5-azido-5,6-dideoxy-3,4-*O*-isopropylidene-D-gulitol (3.3 g, 85%). A portion (0.2 g) of this material was acetonated by catalysis with anhydrous copper sulphate and conc. sulphuric acid[21] in the standard manner to yield 5-azido-5,6-dideoxy-1,2:3,4-di-*O*-isopropylidene-D-gulitol[16] (0.2 g), m.p. 47–49° (mixed m.p.), $[\alpha]_D$ −100° (*c* 1.5, chloroform).

Infrared Spectra

The dilute solution spectra were measured on 2-cm layers of solutions of the alcohols in CCl_4 using a Unicam S.P. 100 spectrometer equipped with a grating (3000 lines/in).

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SUMMARY

Benzylidenation of L-arabinitol using zinc chloride or sulphuric acid affords mainly 1,3-*O*-benzylidene-L-arabinitol, and a small amount (<5%) of 1,3:2,4-di-*O*-benzylidene-L-arabinitol, the structure of which has been established. The conformation of the dibenzylidene derivative is considered on the basis of n.m.r. spectroscopic data. As expected, 1,3-*O*-benzylidene-L-arabinitol affords the 4,5-*O*-isopropylidene derivative, but condensation with phenylboronic acid involves the 2,4-positions. 3,4-*O*-Isopropylidene-L-rhamnitol gives a 1,2-phenylboronate. The phenylboronate group was readily removed from each of the above esters to yield the unchanged parent compound, thus further exemplifying the use of this group as a blocking agent in carbohydrate chemistry.

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SYNTHESIS OF DIAMINO SUGARS FROM 1,2-DIAMINO-1,2-DIDEOXY-ALDITOLS. 4,5-DIACETAMIDO-4,5-DIDEOXY-L-XYLOSE[1]

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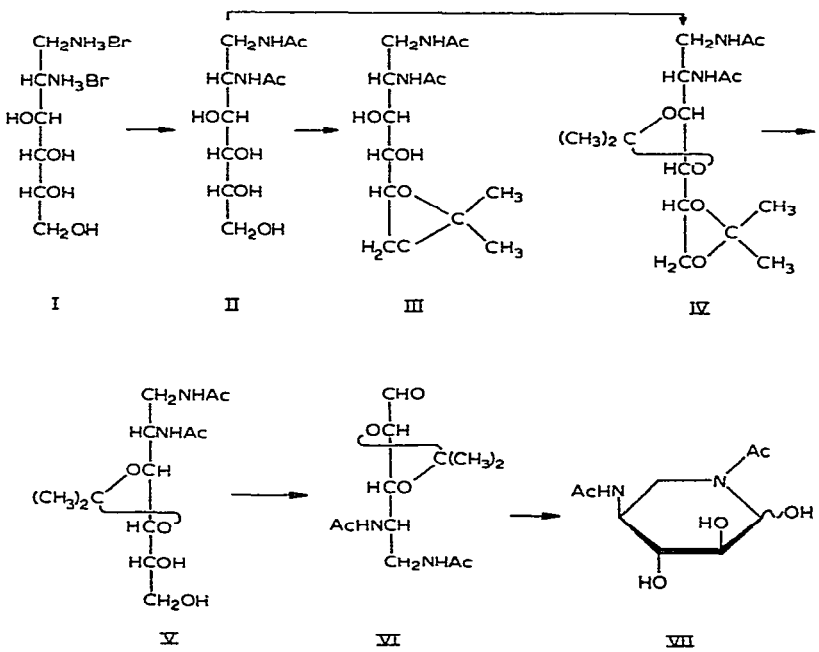
INTRODUCTION

In a recent publication[2] we recorded the synthesis of 1,2-diamino-1,2-dideoxy-alditols by hydrogenation of glycosulose bis(phenylhydrazones) (glycose phenylosazones). We now report an example of the synthesis of a diamino sugar from one such diamino alditol. 1,2-Diamino-1,2-dideoxy-D-glucitol dihydrobromide (I) was converted into 4,5-diacetamido-4,5-dideoxy-L-xylose (VII) by periodate degradation of the appropriately blocked hexitol derivative. The product, existing in either a five- or a six-membered ring form, must have nitrogen as the ring hetero atom.

DISCUSSION

1,2-Diamino-1,2-dideoxy-D-glucitol dihydrobromide (I) was selectively *N*-acetyl-



ated to give 1,2-diacetamido-1,2-dideoxy-D-glucitol (II). This compound could also be obtained from 2-amino-2-deoxy-D-glucose hydrochloride by reduction of its phenylhydrazone, as described previously[3], followed by *N*-acetylation of the reaction mixture.

Acetonation of II using cupric sulfate and sulfuric acid as catalysts gave a crystalline product identified as 1,2-diacetamido-1,2-dideoxy-5,6-*O*-isopropylidene-D-glucitol (III), since the mono-*O*-isopropylidene derivative III consumed one mole of periodate per mole and liberated no formaldehyde. Acetonation of II using zinc chloride, phosphoric acid, and phosphorus pentaoxide gave 1,2-diacetamido-1,2-dideoxy-3,4:5,6-di-*O*-isopropylidene-D-glucitol (IV). By use of thin-layer chromatographic techniques, conditions were established for the partial acid hydrolysis of the di-*O*-isopropylidene derivative IV. The 5,6-*O*-isopropylidene group is removed first. 1,2-Diacetamido-1,2-dideoxy-3,4-*O*-isopropylidene-D-glucitol (V) was readily separated from the completely hydrolyzed material by silica gel column chromatography. The product V consumed one mole of periodate per mole with the liberation of one mole of formaldehyde and the formation of a diaminodideoxy pentose derivative, thus establishing the position of the isopropylidene group.

The 5,6-glycol function of V was cleaved with periodate on a preparative scale to give 4,5-diacetamido-4,5-dideoxy-2,3-*O*-isopropylidene-*aldehydo*-L-xylose (VI). This crystalline compound in concentrated aqueous solution gave a positive Schiff test, a positive Tollens test, and was weakly reducing to Benedict reagent. The aldehyde function was hydrated to the aldehydrol form as indicated by its nuclear magnetic resonance (n.m.r.) spectrum in deuterium oxide. A carbonyl band at 5.8 μ in the infrared spectrum of a sample obtained from a solution containing water increased in intensity from moderate to strong on heating the sample *in vacuo* at 78°. The n.m.r. spectrum in deuteriochloroform revealed a one-proton peak in the region characteristic of aldehyde protons.

The acid-catalyzed hydrolysis of the isopropylidene group of VI was followed by paper chromatography on aliquots withdrawn at selected time intervals. Two products, in addition to starting material, were so indicated; one of these was preponderant. The reaction was complete in three hours at room temperature. After neutralization and filtration, the reaction mixture was concentrated to a sirup. The product which gave the slower-moving, major zone on chromatography, crystallized slowly from the sirup.

The crystalline material (VII) was strongly reducing to Benedict reagent. The infrared spectrum showed an amide I band at 6.2 μ, an amide II band at 6.4 μ, and no aldehydic carbonyl band. It was believed, therefore, that compound VII existed in a cyclic form with nitrogen as the hetero atom. An aqueous solution of VII was dextrorotatory, $[\alpha]_D^{25}$ +39°. No mutarotation was observed during 72 h, and acid catalysis did not affect the rotation over a period of 24 h. Addition of a drop of ammonium hydroxide caused a decrease in specific rotation (+39 → +32° in 29 h). Chromatographic examination of the ammonia-containing solution showed that the minor component, presumably the five-membered ring form, had reappeared.

The relative chromatographic mobilities were consistent with the five- and six-membered ring assignments and compared with the relative mobilities of the five- and six-membered ring forms of 5-acetamido-5-deoxy-D-xylose[4-6].

The n.m.r. spectra of compound VII in deuterium oxide at ambient temperature and at an elevated temperature are presented in Fig. 1. The spectrum determined

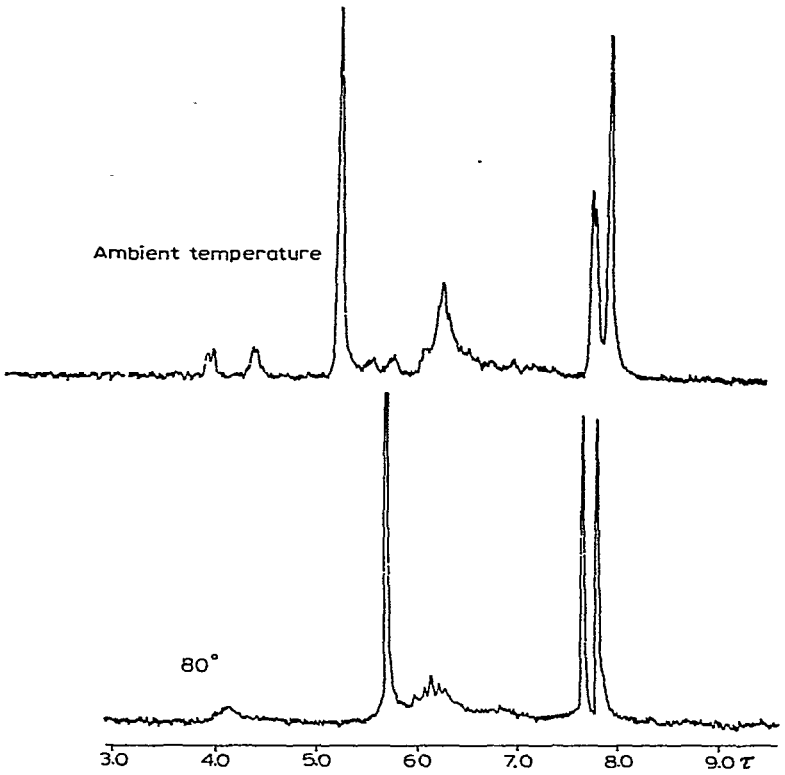


Fig. 1. Nuclear magnetic resonance spectra of 4,5-diacetamido-4,5-dideoxy-L-xylopyranose in deuterium oxide. The upper spectrum was taken at ambient temperature, the lower at 80°. The external standard was a 6% solution of tetramethylsilane in chloroform.

at ambient temperature shows three peaks at τ 7.79, 7.84, and 7.97 of intensity corresponding to six protons characteristic of the protons of *N*-acetyl groups, and two doublets at τ 3.95 and 4.38 which may be assigned to the C-1 protons. At 80° the two C-1 signals have merged and the two *N*-acetyl signals at τ 7.79 and 7.84 attributable to the *N*-acetyl group involved in ring formation have collapsed to a three-proton singlet. On cooling to ambient temperature the original pattern was restored. This behavior is analogous to that of 5-acetamido-5-deoxy-D-xylopyranose reported by Szarek *et al.*[7], and suggests that compound VII exists as two rotational isomers due to restricted rotation about the CO–N bond. Moreover, the striking similarity of the two spectra presented in Fig. 1 to the corresponding spectra of 5-acetamido-5-deoxy-D-xylopyranose[7], of established structure, is a strong indication

that compound VII exists in the pyranose form with nitrogen as the hetero atom in the ring.

EXPERIMENTAL

General

Melting points were taken with a Thomas–Hoover apparatus. X-Ray powder diffraction data refer to interplanar spacing in Å with Cu K_α radiation. Relative intensities were estimated visually: s, strong; m, moderate; w, weak; v, very; b, broad. The first three strongest lines are numbered (1, strongest); double numbers indicate approximately equal intensities. The infrared spectra were taken on potassium bromide discs on a Perkin–Elmer Infracord spectrophotometer. Nuclear magnetic resonance spectra were determined at 60 Mc.p.s. with a Varian A-60 spectrometer. Chemical shifts were measured against an internal tetramethylsilane standard when the solvent was deuteriochloroform. When deuterium oxide was the solvent, a 6% solution of tetramethylsilane in chloroform supplied by Varian Associates (943346-07) was used as an external standard. Thin layer chromatography was performed by the ascending technique on Silica Gel G (E. Merck, Darmstadt, Germany). Davison Silica Gel, Grade 950, 60–200 mesh (W.R. Grace Co., Baltimore, Md.), was used for column chromatography. Amberlite resins, products of the Rohm and Haas Co., Philadelphia, Pa., and Dowex resins, products of the Dow Chemical Co., Midland, Mich., were used for ion-exchange neutralizations and de-ionizations. Paper chromatography was performed by the descending technique on Whatman No. 1 paper. Solvent systems were: A, 1-butanol–ethanol–water (40:12:12 v/v); B, pyridine–ethyl acetate–acetic acid–water (5:5:1:3 v/v)[8]. Zones were detected with silver nitrate–sodium hydroxide (i)[9] or *p*-anisidine hydrochloride (ii)[10] sprays. Mobilities were measured relative to L-rhamnose or the solvent front. Elemental microanalyses were made by W.N. Rond.

1,2-Diacetamido-1,2-dideoxy-D-*glucitol* (*II*)

A 1.77-g quantity of 1,2-diamino-1,2-dideoxy-D-glucitol dihydrobromide[2] was *N*-acetylated according to the procedure of Roseman and Ludowieg[11]. The sample was dissolved in 25 ml of water and treated with 2.5 ml of methanol, 60 ml of hydrated Dowex-1 (CO_3^{2-} form), and 1.4 ml of acetic anhydride. The mixture was stirred at 0° for 90 min, filtered from the resin, and the resin washed with an equal volume of water. The filtrate and washings were then passed through a column containing 10 ml of Amberlite IR 120 (H^+ form), and evaporated to dryness. A crystalline product was obtained; yield 1.01 g (74%). Four recrystallizations from absolute ethanol–ether gave analytically pure material; m.p. 96.0–99.5°; $[\alpha]_D^{22}$ −14° (*c* 5.0, water); λ_{max}^{KBr} 3.0–3.1 μ (OH, NH), 6.1 μ (amide I), and 6.4 μ (amide II); X-ray powder diffraction data: 13.00 w, 11.85 s (3), 9.07 w, 8.16 w, 6.68 vw, 5.47 vw, 4.77 w, 4.54 s (1), 4.27 s (2,2), 4.07 s (2,2).

Once seed crystals had been obtained, the product could be obtained directly from 2-amino-2-deoxy-D-glucose hydrochloride by reduction of its phenylhydrazone

as described previously[3]. A 20-g quantity of 2-amino-2-deoxy-D-glucose hydrochloride was dissolved in 40 ml of warm water and treated with 10 ml of phenylhydrazine and 2 ml of acetic acid in 40 ml of water. The mixture was stirred for 2 h on a water bath at 55°. The temperature was then increased to initial formation of the D-*arabino*-hexosulose bis(phenylhydrazone). The mixture was cooled, filtered, and hydrogenated at room temperature and 3 atm pressure using Raney nickel as catalyst. The reaction mixture was filtered, the catalyst washed with water, and the filtrate and washings were extracted with benzene. The products in the aqueous phase were *N*-acetylated as described above. Quantities were based on the original amount of 2-amino-2-deoxy-D-glucose hydrochloride. The crystalline mass obtained on seeding contained 2-acetamido-2-deoxy-D-glucose in addition to II. The two were separated by dissolving II in absolute ethanol, filtering from the insoluble 2-acetamido-2-deoxy-D-glucose, and crystallizing II by addition of ether to the filtrate to incipient cloudiness; yield 6 g (22%).

Anal. Calc. for $C_{10}H_{20}N_2O_6$: C, 45.43; H, 7.62; N, 10.60. Found: C, 45.47; H, 7.99; N, 10.27.

1,2-Diacetamido-1,2-dideoxy-5,6-O-isopropylidene-D-glucitol (III)

A 3-g quantity of II was shaken for 24 h at room temperature with 8 g of anhydrous cupric sulfate, 50 ml of anhydrous acetone, and 1 drop of concentrated sulfuric acid. The reaction mixture was filtered, neutralized by shaking with calcium hydroxide, filtered, and the acetone was removed under reduced pressure. The resultant sirup crystallized on trituration with a small amount of acetone; yield 0.56 g (17%), m.p. 130–140°. Three recrystallizations from acetone–ether gave a pure product; m.p. 149–150°; $[\alpha]_D^{21}$ −8 (*c* 3, water); X-ray powder diffraction data: 13.19 s (3,3), 10.72 s (3,3), 8.46 s (2), 6.92 vw, 5.68 m, 5.47 m, 5.10 m, 4.90 m, 4.58 m, 4.43 vs (1), 4.22 m, 4.03 m, 3.76 w, 3.60 m, 3.45 m, 3.34 w.

Anal. Calc. for $C_{13}H_{24}N_2O_6$: C, 51.30; H, 7.95; N, 9.21. Found: C, 51.49; H, 8.11; N, 9.38; IO_4^- oxidation[12]: 1.0 mole/mole oxidant; formaldehyde formed: 0.0.

1,2-Diacetamido-1,2-dideoxy-3,4:5,6-di-O-isopropylidene-D-glucitol (IV)

The di-*O*-isopropylidene derivative was synthesized by the general procedure described by Freudenberg *et al*[13]. A mixture of 18.2 g of II, 24 g of fused zinc chloride, 4 g of phosphorus pentaoxide, and 8 g of phosphoric acid (86%) in 400 ml of acetone was shaken mechanically for 22 h. The solution was made basic with 500 ml of an aqueous suspension of sodium carbonate. The precipitated zinc carbonate was removed by filtration and washed with acetone. The major portion of the acetone was removed under reduced pressure. The remaining aqueous solution was thoroughly extracted with chloroform. The chloroform extracts were combined, washed with water, dried, and evaporated to dryness under reduced pressure to give a product which crystallized spontaneously; yield, after one recrystallization from benzene–petroleum ether (b.p. 30–60°), 13.3 g (56%). Two more recrystallizations from benzene–

petroleum ether gave analytically pure material; m.p. 152°; $[\alpha]_D^{20}$ + 33° (*c* 2.0, chloroform); X-ray powder diffraction data : 14.14 s (3,3), 9.16 w, 7.93 sb (2), 6.94 vs (1), 6.30 m, 5.95 w, 5.39 m, 4.72 mb, 4.43 w, 4.18 s (3,3), 3.99 m, 3.74 m, 3.51 w, 3.28 w.

Anal. Calc. for $C_{16}H_{28}N_2O_6$: C, 55.80; H, 8.20; N, 8.13. Found: C, 56.08; H, 8.32; N, 8.27.

*1,2-Diacetamido-1,2-dideoxy-3,4-*O*-isopropylidene-*D*-glucitol (V)*

Conditions for the partial hydrolysis of IV were determined by dissolving 100 mg of IV in 5 ml of an 86% ethanol solution which was 0.24N in hydrochloric acid. Aliquots were removed at timed intervals, neutralized with Dowex-1 (CO_3^{2-} form), and chromatographed by the thin layer technique (ethyl acetate–methanol, 3:1 v/v). After 4 h at 25° only a trace of the fastest moving component IV remained. The intermediate zone corresponding to V was predominant. The fully hydrolyzed material II appeared as a base line zone.

A 3.47-g sample of IV was dissolved in 87 ml of 0.24N hydrochloric acid in 86% ethanol. The solution was maintained at 25° and was neutralized, after 4 h, with Dowex-1 (CO_3^{2-} form). The reaction mixture was concentrated to a sirup and chromatographed on 100 g of silica gel. Compound V was eluted with 3 l of 10% methanol in ethyl acetate. The first 250 ml of eluate was discarded. The remainder was collected in 400-ml fractions which were concentrated to sirups. Crystallization was effected by dissolving the sirups in acetone and treating with ether to incipient cloudiness; total yield 2.21 g (72%), m.p. 98–102°. Four recrystallizations from acetone–ether gave a product, m.p. 100–102°, whose melting point rose to 114–114.5° on drying to constant weight at 56° under reduced pressure; $[\alpha]_D^{21}$ −29° (*c* 4.4, water); X-ray powder diffraction data: 9.51 m, 7.11 s (2), 6. 28 s (3), 5.44 m, 5.14 vw, 4.89 vw, 4.58 vw, 4.20 vs (1), 3.84 mb, 3.57 w, 3.28 m, 3.17 vw, 3.05 vw, 2.95 w, 2.65 w.

Anal. Calc. for $C_{13}H_{24}N_2O_6$: C, 51.30; H, 7.95; N, 9.21. Found: C, 51.39; H, 7.93; N, 9.25; IO_4^- oxidation[12]: 1.0 mole/mole oxidant; formaldehyde formed: 0.9 mole.

*4,5-Diacetamido-4,5-dideoxy-2,3-*O*-isopropylidene-*aldehydo-L*-xylose (VI)*

A 25-ml aqueous solution containing 250 mg of V and 184 mg of sodium metaperiodate was stirred at room temperature for 20 min and then diluted to 100 ml. The diluted solution was passed through a column (10 × 0.8 cm) of Amberlite IRA 410 (OAc^- form) at a flow rate of 1 ml per min. The column was washed with two volumes of water, and the eluate and washings were concentrated to dryness under reduced pressure at a bath temperature below 45°. The residue was extracted with three 50 ml portions of hot, dry acetone. The extracts were evaporated to dryness, and the residual sirup was crystallized from a small amount of dry acetone; yield 135 mg (61%); m.p. 166-169.5° dec. The melting point could be raised to 173° dec. by recrystallization from moist dioxane–ether and moist acetone–ether; λ_{max}^{KBr} 5.8 μ (CHO); n.m.r. data, deuteriochloroform, τ 0.24 (1-proton singlet, CHO);

deuterium oxide, τ 5.03 (1-proton doublet, J 7.0 c.p.s., HCO); $[\alpha]_D^{18}$ −37° (c 1.5, water, no mutarotation in 25 h); addition of dilute ammonium hydroxide failed to affect the rotation value during 48 h; X-ray powder diffraction data: 9.21 m, 6.67 s (3, 3), 6.38 s (3, 3), 6.11 s (3, 3), 5.05 m, 5.02 m, 4.61 w, 4.20 vw, 4.01 vs (1), 3.71 vs (2), 3.61 m, 3.34 w. This compound gave a positive Schiff test[14], a positive Tollens test, and was weakly reducing to Benedict reagent. A sample isolated from aqueous solution showed a band of moderate intensity at 5.8 μ in the infrared spectrum; the band incresaed in intensity when the sample was heated *in vacuo* at 78°.

For larger scale preparations, the excess of periodate ion was reduced with a 10% excess of ethylene glycol and the mixture was lyophilized. The product was extracted from the inorganic salt in a Soxhlet extractor using chloroform as solvent.

Anal. Calc. for $C_{12}H_{20}N_2O_5$: C, 52.93; H, 7.40; N, 10.29. Found: C, 53.06; H, 7.29; N, 9.84.

4,5-Diacetamido-4,5-dideoxy-L-xylose (VII)

A 1.78-g quantity of VI was hydrolyzed at room temperature for 3 h with 76 ml of 1.2N hydrochloric acid. The solution was neutralized with Dowex-1 (CO_3^{2-} form), filtered, and concentrated to a sirup with ethanol at a bath temperature below 35°. The product, which crystallized slowly from the sirup, was triturated with cold ethanol, filtered, and washed; yield 0.65 g (43%). Recrystallization was effected from methanol–ether; m.p. 166–167° dec.; $[\alpha]_D^{19}$ +39° (c 4.3, water, no mutarotation in 72 h, unchanged for 2 h on addition of a trace of hydrochloric acid); +39 → +34 (7 h) → +32° (29 h, constant, c 4.3, 0.25M ammonium hydroxide); n.m.r. data, deuterium oxide, ambient temperature (Fig. 1): τ 7.97 (3-proton singlet, NDAc), τ 7.79, 7.84 (3-proton split peak, ring NAc of rotomers), τ 3.95, 4.38 (1-proton, two doublets, C-1 proton of rotomers); X-ray powder diffraction data: 7.44 vs (2), 7.05 vw, 5.96 s (3), 5.00 w, 4.39 vs (1), 4.08 s, 3.69 w, 3.48 m, 3.34 w, 3.08 vw, 2.97 m, 2.87 m.

Anal. Calc. for $C_9H_{16}N_2O_5$: C, 46.54; H, 6.95; N, 12.07. Found: C, 46.83; H, 6.54; N, 12.18.

The hydrolysis of VI was followed by paper chromatography using solvent A. Two new components with R_F 0.29 (major) and R_F 0.46 (minor) in addition to starting material with R_F 0.88 were indicated by spray (i). The R_F 0.88 zone had disappeared after 3 h at room temperature. The minor component showed marked tailing. Chromatography of the mother liquors of VII in solvent B showed two zones of approximately equal intensity, R_{Rh} 0.8 and R_{Rh} 1.2. Crystalline VII gave a single zone, R_{Rh} 0.8. The tailing of the faster zone was considerably reduced.

The equilibrated acidic, basic, and neutral solutions of VII were chromatographed in solvent systems A and B. All solutions contained the slow-moving zone. In addition, the basic solution contained, as a minor component, the same faster-moving zone noted above. Both components gave a red-brown color with spray (ii).

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SUMMARY

1,2-Diamino-1,2-dideoxy-D-glucitol dihydrobromide (I) was converted into 1,2-diacetamido-1,2-dideoxy-D-glucitol (II). With cupric sulfate and sulfuric acid as catalysts, acetonation of II gave 1,2-diacetamido-1,2-dideoxy-5,6-*O*-isopropylidene-D-glucitol (III), whose structure was established by periodate oxidation. With zinc chloride, phosphorus pentaoxide, and phosphoric acid as catalysts, 1,2-diacetamido-1,2-dideoxy-3,4:5,6-di-*O*-isopropylidene-D-glucitol (IV) was obtained. The 5,6-*O*-isopropylidene group of IV could be selectively removed by acid to give 1,2-diacetamido-1,2-dideoxy-3,4-*O*-isopropylidene-D-glucitol (V), whose structure was likewise established by periodate oxidation. Preparative glycol cleavage of V with periodate afforded 4,5-diacetamido-4,5-dideoxy-2,3-*O*-isopropylidene-*aldehydo*-L-xylose (VI). Paper chromatography of the products of acid hydrolysis of VI indicated a major and a minor component. The major product crystallized from the mixture and was formulated as 4,5-diacetamido-4,5-dideoxy-L-xylopyranose (VII) by nuclear magnetic resonance data. All compounds were obtained in crystalline form.

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4-ACETAMIDO-4,5-DIDEOXY-L-XYLOFURANOSE

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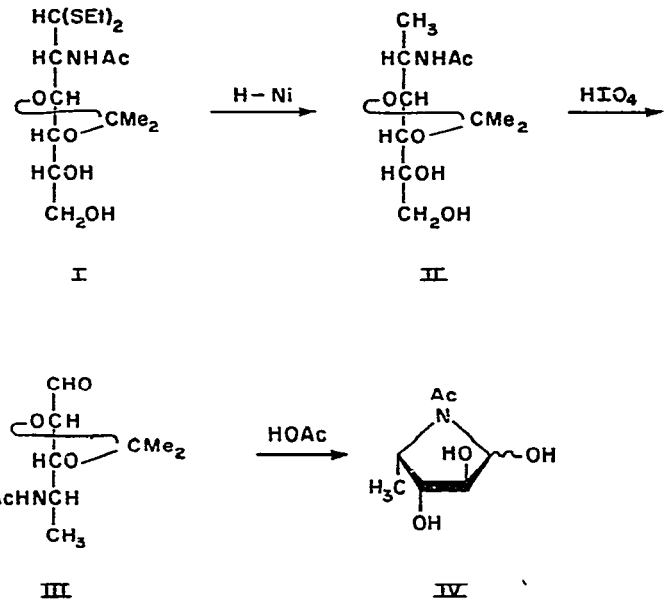
INTRODUCTION

Pyranoid sugars in which the ring oxygen atom is replaced by nitrogen to give a piperidine-type ring system[1–3] are now well known[4]. They are generally obtained, as stable *N*-acetyl derivatives, from 5-acetamido-5-deoxyaldoses, by equilibration of the two possible ring-forms in solution. Furanoid sugars in which the ring oxygen atom is replaced by nitrogen to give a pyrrolidine-type ring system, may be formulated by appropriate cyclization of a 4-acetamido-4-deoxyaldose. Synthesis of this ring system has been achieved in the case of 4-acetamido-4-deoxy-L-erythrofuranose[5], where pyranose formation is not possible, and recently[6] with 4-acetamido-4-deoxy-D-ribose, where conformational factors appear to favor formation of the 5-membered ring in preference to the pyranose ring.

We have prepared 4-acetamido-4,5-dideoxy-L-xylose, a pentose which cannot exist in the pyranoid ring form, and have shown that the pyrrolidine-type cyclic structure is the stable form. Studies on this type of ring system are important in relation to the destruction of 4-amino-4-deoxy sugars and nonulosaminic acids under acid hydrolytic conditions, a factor which complicates studies by fragmentation methods of materials containing these sugars.

DISCUSSION

The starting material for the synthesis, 2-acetamido-2-deoxy-3,4-*O*-isopropylidene-D-glucose diethyl dithioacetal (I) was conveniently prepared in a sequence of high-yielding stages from 2-amino-2-deoxy-D-glucose hydrochloride, by the procedure of Yoshimura and Sato[7]. Compound I was smoothly desulfurized with excess Raney nickel in hot ethanol[8] to give 2-acetamido-1,2-dideoxy-3,4-*O*-isopropylidene-D-glucitol (II) as a chromatographically homogeneous sirup whose infrared and nuclear magnetic resonance (n.m.r.) spectra were in accord with the assigned structure. Acetylation of II gave the 5,6-diacetate, isolated by distillation. The n.m.r. spectrum of the latter showed the C-1 methyl group at τ 8.77 as a doublet, $J_{1,2}$ = 7.0 c.p.s., the isopropylidene methyl signal as a 6-proton singlet at τ 8.59, and the acetyl group signals as 3-proton singlets at τ 8.00, 7.97, and 7.89. The absence of multiplets characteristic of ethyl groups in the n.m.r. spectra of II or its diacetate indicated that the desulfurization reaction had gone to completion.



Oxidation of the free diol II with one equivalent of aqueous periodic acid caused rapid cleavage of the C-5 to C-6 bond to give 4-acetamido-4,5-dideoxy-2,3-*O*-isopropylidene-*aldehydo*-L-xylose (III) as a chromatographically homogeneous sirup, characterized as the crystalline benzylphenylhydrazone. The n.m.r. spectra of III and of its benzylphenylhydrazone were fully consistent with the assigned structures, and showed methyl group signals similar to those observed for II. The free sugar III, as obtained by codistillation with benzene, was formulated as the *aldehydo* form on the basis of infrared absorption at 5.80 μ characteristic of aldehyde carbonyl, a positive Schiff test, and the presence of a one-proton singlet at τ 0.67 in the n.m.r. spectrum due to the aldehyde proton. These data clearly show that III is not intermolecularly combined or intramolecularly cyclized. Intramolecular cyclization of III would lead to a *trans*-fused bicyclo[3.3.0] structure, a strained[9] system which has not yet been observed in carbohydrate structures.

Hydrolysis of 4-acetamido-4,5-dideoxy-2,3-*O*-isopropylidene-*aldehydo*-L-xylose (III) in aqueous acetic acid cleaved the isopropylidene group to give 4-acetamido-4,5-dideoxy-L-xylose as a sirup which was homogeneous by paper chromatography in the Fischer–Nebel[10] solvent system, and well resolved from the starting material (III). The product reduced Fehling solution, but did not restore the color to Schiff reagent. In its infrared spectrum it showed negligible aldehyde group absorption near 5.8 μ; it showed the amide carbonyl absorption at 6.12 μ but negligible amide NH absorption near 6.5 μ. These data fully support formulation of the product as the cyclic structure 4-acetamido-4,5-dideoxy-α,β-L-xylofuranose (IV). Paper chromatography of IV in a 40:11:19 1-butanol–ethanol–water system revealed the product as an incom-

pletely-separated double zone, R_F 0.49 and 0.54. Excision, extraction, and rechromatography of each zone gave the two separate components, but when an aqueous solution of either component was heated for 1 h at 90° chromatography revealed the re-formation of the original double zone. This indicates a slow interconversion between two isomeric forms of IV, and since little of the *aldehydo*-form can be present, the two isomers must be the α-L and β-L anomers of the furanose form. The zones R_F = 0.49 and 0.54 had approximate relative intensities 2:3, and since the zone R_F 0.54 was more dextrorotatory than the equilibrated mixture it was considered to be the β-L anomer. The formation of a single zone when IV was subjected to chromatography in the Fischer–Nebel[10] solvent system may be ascribed to rapid interconversion of anomers in this more-polar solvent system. Paper chromatographic resolution of anomers has been observed[11] for the anomeric 4-acetamido-4-deoxy-L-erythro(and D-threo)furanoses.

The n.m.r. spectrum of IV in deuterium oxide solution showed a three-proton multiplet at τ 8.84 assigned to the C-5 methyl group, a three-proton multiplet at τ 7.85 assigned to the *N*-acetyl methyl group, a three-proton multiplet at τ 5.87 assigned to the ring protons at C-2, C-3, and C-4, and a pair of multiplets, τ 4.82 and 4.68, corresponding to one proton, assigned to the anomeric proton. The fine structure of the spectrum was interpreted to indicate that IV was a mixture of the α-L and β-L anomers, with restricted rotation about the nitrogen–acetyl bond. A small proportion of the acyclic form appeared to be present. The multiplets at τ 4.82 and 4.68 were assigned to the C-1 protons of the β-L and α-L anomeric forms, respectively. These signals show multiplicity greater than simple doublets, attributable [12] to the small difference in chemical shift between the H-2 and H-3 signals, and to rotational isomerism[12] about the nitrogen–acetyl bond. The 5-CH_3 multiplet at τ 8.84 is resolved when the temperature is raised to 86°, into two doublets, τ 8.76, 8.90, $J_{4,5}$ = 7.0 c.p.s., in the approximate intensity ratio 3:2, assigned to the β-L and α-L anomers, respectively. The acetyl methyl signals appeared at τ 7.82, 7.85, 7.88, and 8.01, the last constituting about 5% of the total. The τ 7.85 and 7.88 signals coalesced as the sample was heated, while the intensity of the τ 7.82 signal decreased; the reverse process occurred on cooling. This behavior indicates that rotation about the nitrogen–acetyl bond is restricted[13] at room temperature. The low-intensity singlet, τ 8.01, is unaffected by change of temperature, and it is probably due to a small proportion of the aldehydrol form of IV in equilibrium with the furanose forms.

EXPERIMENTAL

General

Melting points were determined with a Hershberg-type apparatus[14]. Optical rotations were measured with a 2-dm tube. Infrared spectra were measured with a Perkin–Elmer "Infracord" spectrometer. N.m.r. spectra were measured with a Varian A60 n.m.r. spectrometer (Varian Associates, Palo Alto, California) operating

was stirred with ice, and extracted with chloroform. The extract was washed successively with cold dilute hydrochloric acid and aqueous sodium bicarbonate, then dried (magnesium sulfate), and evaporated. The residue was distilled[16], and the distillate crystallized spontaneously, yield, 0.392 g (62%); m.p. 74–76°, $b_{0.1}$ 175° (bath temp.); $[\alpha]_D^{25}$ +36 ± 1° (*c* 0.6, chloroform), λ_{max}^{film} 3.04 μ (NH), 5.74 μ (OAc), 6.09, 6.55 μ (NHAc), 7.30 μ (CMe_2); n.m.r. data (deuteriochloroform): τ 8.77 (3-proton doublet, $J_{1,2}$ = 7.0 c.p.s., 1-CH_3); τ 8.59 (6-proton singlet, CMe_2); τ 8.00 (3-proton singlet, NAc); τ 7.97 (3-proton singlet, OAc); τ 7.89 (3-proton singlet, OAc). The product was homogeneous by thin layer chromatography, R_F 0.75 (methanol), R_F 0.34 (1:1 ether–ethyl acetate).

Anal. Calc. for $C_{15}H_{25}NO_7$: C, 54.38; H, 7.55; N, 4.23. Found: C, 54.52; H, 7.44; N, 4.20.

*4-Acetamido-4,5-dideoxy-2,3-*O*-isopropylidene-*aldehydo-L-*xylose* (*III*)

To a solution of 2-acetamido-1,2-dideoxy-3,4-*O*-isopropylidene-D-glucitol (II, 1.474 g) in ethanol (10 ml) was added 0.9 N aqueous periodic acid (11.1 ml). The mixture was kept for 15 min in the dark, excess barium carbonate was added, the neutralized solution was filtered, and the solution was evaporated. The residue was dissolved in ethanol, the solution was re-evaporated, the sirup was dissolved in benzene, and the solution was centrifuged. The supernatant was evaporated to give the product as a clear sirup, yield 1.05 g (83%); $[\alpha]_D^{20}$ −15 (15 min) → −12.6 ± 1.0° (*c* 1, ethanol); λ_{max}^{film} 3.10 μ (NH), 5.81 μ (CHO), 6.10, 6.50 μ (NHAc), 7.30 μ (CMe_2); n.m.r. data (deuteriochloroform): τ 8.73 (3-proton doublet, $J_{4,5}$ = 6.5 c.p.s., 5-CH_3); τ 8.60 (6-proton singlet, CMe_2); τ 8.00 (3-proton singlet, NAc); τ 0.67 (1-proton singlet, CHO). The product was homogeneous by thin layer chromatography, R_F 0.74 (methanol), R_F 0.24 (1:1 ether–ethyl acetate), and by paper chromatography, R_F 0.85 in solvent A. It recolorized Schiff reagent and gave a positive Fehling test.

Anal. Calc. for $C_{10}H_{17}NO_4$: C, 55.81; H, 7.90; N, 6.51. Found: C, 55.56; H, 8.01; N, 6.03.

*4-Acetamido-4,5-dideoxy-2,3-*O*-isopropylidene-*L*-xylose benzylphenylhydrazone*

A solution of 4-acetamido-4,5-dideoxy-2,3-*O*-isopropylidene-*aldehydo*-L-xylose (III, 315 mg) in 95% ethanol (4 ml) was mixed with a solution of sodium acetate·3H_2O (1.039 g) in water (4 ml), and benzylphenylhydrazine hydrochloride (412 mg) was added. The mixture was refluxed for 2.5 h with addition of a few drops of ethanol to maintain a homogeneous solution. The solvent was evaporated under a stream of nitrogen, water (4 ml) was added to the residue, and the product was extracted with chloroform. The extract was washed with water, dried (sodium sulfate), evaporated, and the residue was crystallized from ether, yield 200 mg (36%); m.p. 151–152°, $[\alpha]_D^{22}$ +32 ± 2° (*c* 0.5, methanol); λ_{max}^{KBr} 3.10 μ (NH), 6.10, 6.42 μ (NHAc), 6.28, 6.70, 6.90 μ (aryl C = C), 7.30 μ (CMe_2), 14.35 μ (substituted benzene); n.m.r. data (deuteriochloroform): τ 8.79 (3-proton doublet, 5-CH_3); τ 8.67, 8.59 (6 protons, CMe_2); τ 8.05 (3 protons, NAc); $\tau \sim 2.75$ (10 protons, aryl); X-ray powder diffrac-

was stirred with ice, and extracted with chloroform. The extract was washed successively with cold dilute hydrochloric acid and aqueous sodium bicarbonate, then dried (magnesium sulfate), and evaporated. The residue was distilled[16], and the distillate crystallized spontaneously, yield, 0.392 g (62%); m.p. 74–76°, $b_{0.1}$ 175° (bath temp.); $[\alpha]_D^{25}$ +36 ± 1° (*c* 0.6, chloroform), λ_{max}^{film} 3.04 μ (NH), 5.74 μ (OAc), 6.09, 6.55 μ (NHAc), 7.30 μ (CMe_2); n.m.r. data (deuteriochloroform): τ 8.77 (3-proton doublet, $J_{1,2}$ = 7.0 c.p.s., 1-CH_3); τ 8.59 (6-proton singlet, CMe_2); τ 8.00 (3-proton singlet, NAc); τ 7.97 (3-proton singlet, OAc); τ 7.89 (3-proton singlet, OAc). The product was homogeneous by thin layer chromatography, R_F 0.75 (methanol), R_F 0.34 (1:1 ether–ethyl acetate).

Anal. Calc. for $C_{15}H_{25}NO_7$: C, 54.38; H, 7.55; N, 4.23. Found: C, 54.52; H, 7.44; N, 4.20.

4-Acetamido-4,5-dideoxy-2,3-O-isopropylidene-aldehydo-L-*xylose* (*III*)

To a solution of 2-acetamido-1,2-dideoxy-3,4-*O*-isopropylidene-D-glucitol (II, 1.474 g) in ethanol (10 ml) was added 0.9 N aqueous periodic acid (11.1 ml). The mixture was kept for 15 min in the dark, excess barium carbonate was added, the neutralized solution was filtered, and the solution was evaporated. The residue was dissolved in ethanol, the solution was re-evaporated, the sirup was dissolved in benzene, and the solution was centrifuged. The supernatant was evaporated to give the product as a clear sirup, yield 1.05 g (83%); $[\alpha]_D^{20}$ −15 (15 min) → −12.6 ± 1.0° (*c* 1, ethanol); λ_{max}^{film} 3.10 μ (NH), 5.81 μ (CHO), 6.10, 6.50 μ (NHAc), 7.30 μ (CMe_2); n.m.r. data (deuteriochloroform): τ 8.73 (3-proton doublet, $J_{4,5}$ = 6.5 c.p.s., 5-CH_3); τ 8.60 (6-proton singlet, CMe_2); τ 8.00 (3-proton singlet, NAc); τ 0.67 (1-proton singlet, CHO). The product was homogeneous by thin layer chromatography, R_F 0.74 (methanol), R_F 0.24 (1:1 ether–ethyl acetate), and by paper chromatography, R_F 0.85 in solvent A. It recolorized Schiff reagent and gave a positive Fehling test.

Anal. Calc. for $C_{10}H_{17}NO_4$: C, 55.81; H, 7.90; N, 6.51. Found: C, 55.56; H, 8.01; N, 6.03.

4-Acetamido-4,5-dideoxy-2,3-O-isopropylidene-L-*xylose benzylphenylhydrazone*

A solution of 4-acetamido-4,5-dideoxy-2,3-*O*-isopropylidene-*aldehydo*-L-xylose (III, 315 mg) in 95% ethanol (4 ml) was mixed with a solution of sodium acetate·$3H_2O$ (1.039 g) in water (4 ml), and benzylphenylhydrazine hydrochloride (412 mg) was added. The mixture was refluxed for 2.5 h with addition of a few drops of ethanol to maintain a homogeneous solution. The solvent was evaporated under a stream of nitrogen, water (4 ml) was added to the residue, and the product was extracted with chloroform. The extract was washed with water, dried (sodium sulfate), evaporated, and the residue was crystallized from ether, yield 200 mg (36%); m.p. 151–152°, $[\alpha]_D^{22}$ +32 ± 2° (*c* 0.5, methanol); λ_{max}^{KBr} 3.10 μ (NH), 6.10, 6.42 μ (NHAc), 6.28, 6.70, 6.90 μ (aryl C = C), 7.30 μ (CMe_2), 14.35 μ (substituted benzene); n.m.r. data (deuteriochloroform): τ 8.79 (3-proton doublet, 5-CH_3); τ 8.67, 8.59 (6 protons, CMe_2); τ 8.05 (3 protons, NAc); $\tau \sim 2.75$ (10 protons, aryl); X-ray powder diffrac-

tion data: 12.72 w, 8.30 m, 7.56 m, 6.83 s (1), 6.19 m, 5.54 w, 5.26 m, 4.74 s (2), 4.46 m, 4.24 w, 4.01 s (3), 3.74 w, 3.59 vw, 3.50 vw, 3.39 vw, 3.28 m.

Anal. Calc. for $C_{23}H_{29}N_3O_3$: C, 69.87; H, 7.34; N, 10.63. Found: C, 69.76; H, 7.68; N, 10.59.

4-Acetamido-4,5-dideoxy-α,β-L-xylofuranose (IV)

A solution of 4-acetamido-4,5-dideoxy-2,3-*O*-isopropylidene-*aldehydo*-L-xylose (III, 0.36 g) in a mixture of acetic acid (1.0 ml) and water (1.0 ml) was heated for 2.5 h at 95°, and the solution was evaporated. Paper chromatography (solvent A) revealed the presence of two coalescent zones, R_F 0.49 and 0.54, together with a small proportion of a component, R_F 0.85, corresponding to starting material (III). The sirupy product was extracted with benzene, and the extract was evaporated to a sirup (20 mg) which was found to be chromatographically homogeneous starting material. The benzene-extracted sirup was found by chromatography to be free from III, it gave a single zone, R_F 0.78 in solvent B, two coalescent zones R_F 0.49 and 0.54, relative proportion 2:3 in solvent A, and was formulated as IV, yield 0.18 g (67%); $[\alpha]_D^{21}$ −10 ± 2° (*c* 0.5, water); λ_{max}^{film} 3.02 μ (OH), 6.12 μ (NAc), slight trace of absorption at 5.80 μ (CHO) and 6.50 μ (NHAc), no absorption at 7.3 μ (CMe_2); n.m.r. data (deuterium oxide): τ 8.84 (3-proton multiplet, 5-CH_3); τ 8.01, 7.88, 7.85, 7.82 (3 protons, singlets, approximate relative intensities 1:3:8:8, NAc); τ 5.87 (3-proton multiplet, H-2, H-3, H-4); τ 4.82, 4.68 (1 proton, two multiplets, H-1, β-L and α-L anomers respectively). The spectrum showed little change when the probe temperature was lowered to 0°, but when it was raised to 86° the τ 8.84 signal was resolved into a pair of doublets, $J_{4,5}$ = 7.0 c.p.s., at τ 8.90 and 8.76, approximate intensity ratios 2:3 (5-CH_3 of α-L and β-L anomers, respectively). At 86° the τ 8.01 signal remained unchanged at about 5% of the total NAc signal intensity, the τ 7.88 and 7.85 signals coalesced, and the τ 7.82 signal was diminished in intensity.

The sirupy IV was resolved by preparative paper chromatography in solvent A. Excised strips from the center of each zone, R_F 0.49 and 0.54 were eluted with the chromatography solvent and the separated components were rechromatographed. The two components migrated as single zones, R_F 0.44 and 0.52. The faster-moving zone was more dextrorotatory than the original mixture. Aqueous solutions of each separated component were heated for 1 h at 90°, evaporated, and rechromatographed. Each component gave a coalescent double zone indistinguishable from that of the original anomeric mixture (IV).

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SUMMARY

4-Acetamido-4,5-dideoxy-L-xylofuranose (IV), a sugar having a ring nitrogen in the five-membered (pyrrolidine type) ring structure, was synthesized. Desulfurization of 2-acetamido-2-deoxy-3,4-*O*-isopropylidene-D-glucose diethyl dithioacetal (I) gave 2-acetamido-1,2-dideoxy-3,4-*O*-isopropylidene-D-glucitol (II). Periodic acid oxidation of II gave 4-acetamido-4,5-dideoxy-2,3-*O*-isopropylidene-L-xylose (III), shown to have the *aldehydo* structure and characterized as the benzylphenylhydrazone. Hydrolysis of III with aqueous acetic acid removed the *O*-isopropylidene group, and the product was shown by n.m.r. and infrared spectral data to exist principally in the cyclic furanoid form.

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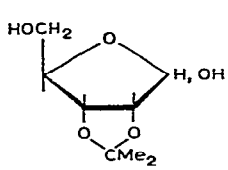
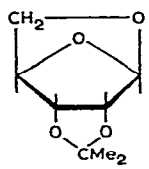
Notes

New isopropylidene derivatives of D-ribose: 1,2:3,4-di-*O*-isopropylidene-D-ribopyranose and 1,2-*O*-isopropylidene-D-ribopyranose

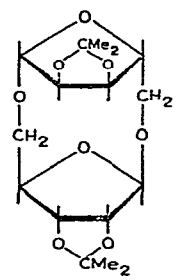
Levene and Stiller first examined the reaction of D-ribose with acetone and observed the formation of 2,3-*O*-isopropylidene-D-ribofuranose (I) together with two anhydro-compounds[1]. The structures of these two latter compounds have been shown to be 1,5-anhydro-2,3-*O*-isopropylidene-D-ribofuranose (II) and di-(2,3-*O*-isopropylidene-*β*-D-ribofuranose) 1,5′:1′,5-dianhydride (III)[2]. Barker and Spoors[3] also observed the formation of the monomeric anhydride (II) and showed that the major product, 2,3-*O*-isopropylidene-D-ribofuranose (I), condensed with benzaldehyde to give a 1,5-*O*-benzylidene-2,3-*O*-isopropylidene-D-ribofuranose (IV). Toluene-*p*-sulphonylation of the crude isopropylidene compound (I) gave a small yield of di-ester which was shown by Mills[4] to be 1,2-*O*-isopropylidene-3,5-di-*O*-toluene-*p*-sulphonyl-D-ribofuranose (V) and to have arisen from a small amount of 1,2-*O*-isopropylidene-D-ribofuranose (VI) present in the crude compound (I). More recently, the 1,2-ketal (VI) has been synthesised from 5-*O*-benzoyl-1,2-*O*-isopropylidene-D-xylofuranose by selective oxidation of the 3-hydroxyl group, followed by reduction and debenzoylation[5].



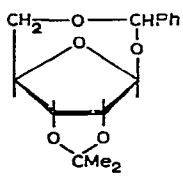
(I)



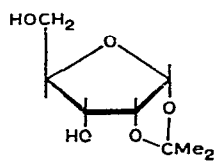
(II)



(III)



(IV)

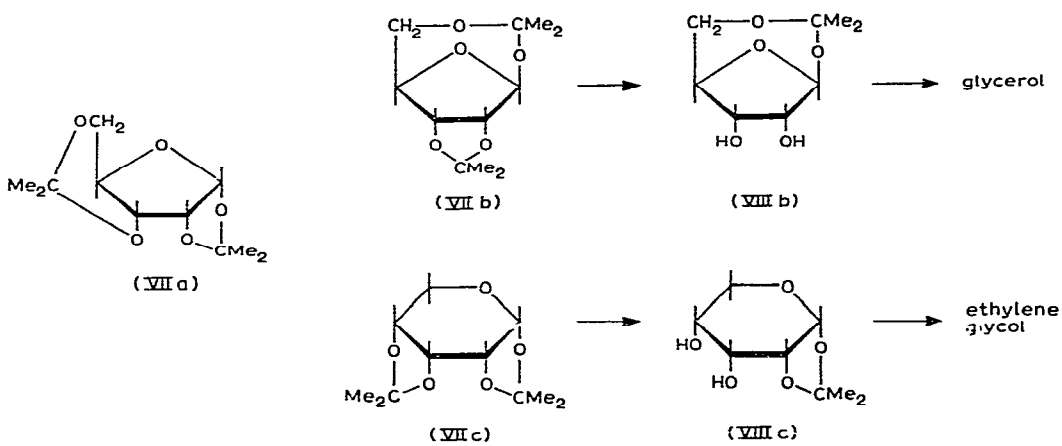


(VI)

In the present work D-ribose was condensed with acetone in the presence of sulphuric acid as catalyst. The crude product was purified by silica chromatography,

when three distinct fractions were obtained. The first fraction was shown by thin-layer chromatography to contain three compounds which could not be separated by further column chromatography but were resolved by repeated micro-sublimation. Two of the compounds were identified as the monomeric and dimeric anhydro-compounds (II) and (III). Only traces of the latter compound were formed. However, when the 2,3-ketal (I) was stored in acetone in the presence of sulphuric acid for longer periods of time the anhydro-compounds (II) and (III) were both formed in greater yield. The second fraction contained only the 2,3-ketal (I), whilst the third fraction contained the 1,2-isomer (VI) and disaccharide compounds. The wide separation of these two isomers on the silica column is probably due to strong intramolecular hydrogen bonding between the 5- and 1-(β)-hydroxyl groups in the 2,3-ketal (I); similar bonding is not possible in the 1,2-ketal (VI).

The third compound in the first fraction gave ribose on acidic hydrolysis and analysed for a di-isopropylideneribose (VII). Three structures are possible for this compound: 1,2:3,5-di-*O*-isopropylidene-D-ribofuranose (VIIa); 1,5:2,3-di-*O*-isopropylidene-D-ribofuranose (VIIb); and 1,2:3,4-di-*O*-isopropylidene-D-ribopyranose (VIIc). The first is unlikely since it contains a five-membered ring *trans*-fused to a six-membered ring. The second is similar to the benzylidene-isopropylidene compound (IV), and the third is remarkable in having a pyranose ring structure. Partial acidic hydrolysis gave a mono-isopropylidene compound (VIII) which was non-reducing, consumed one mol. of periodate, and was not identical with the 1,2-*O*-isopropylidene-D-ribofuranose (VI), thus eliminating structure (VIIa). Borohydride reduction of the periodate-oxidation product followed by acidic hydrolysis gave ethylene glycol and no glycerol. This is in accord with structures (VIIc) and (VIIIc).



(VII a) (VII b) (VIII b) (VII c) (VIII c)

1,2:3,4-Di-*O*-isopropylidene-D-ribopyranose (VIIc) possesses a *cis*, *syn*, *cis*, arrangement of rings. Mills pointed out that in such a structure there would be steric repulsion between the ketal groups[4] and this may account for the preferential hydrolysis of the 3,4-isopropylidene group. In 1,2:3,4-di-*O*-isopropylidene-L-arabino-

pyranose and in 1,2:3,4-di-*O*-isopropylidene-D-galactopyranose, which have a *cis, anti, cis* arrangement of rings, no such preference is observed[6].

In the above acetonation of D-ribose, and in the previous cases mentioned, a strong-acid catalyst was present. With other sugars, variations in the reaction products have been obtained when such non-acidic catalysts as copper sulphate[7] and zinc chloride[8] have been used. When these catalysts were employed with D-ribose, the only marked difference was the absence of the anhydro-compounds (II) and (III) in the products; the yield of the diketal (VIIc) was also reduced.

EXPERIMENTAL

Silica gel, Hopkin and Williams, M.F.C., and Merck, G. grade, respectively, were used for column and thin-layer chromatography. The solvent system, butanol–water (86:14, v/v), was used for paper chromatography.

Condensation of D-*ribose and acetone in the presence of concentrated sulphuric acid*

D-Ribose (12 g) was added with stirring to acetone (240 ml) containing concentrated sulphuric acid (3 ml). Within 5 min the ribose had dissolved, and after 1 h the solution was neutralised with excess of solid sodium carbonate. The solution was filtered and concentrated to a syrup (13 g), which was dissolved in benzene (50 ml) and chromatographed on silica (250 g). Three fractions were obtained:

Fraction A [2.2 g, eluted by benzene–ether (9:1)] was shown by t.l.c. to consist of two major components and traces of a third. The two major components were separated by repeated micro-sublimation. The more volatile 1,5-anhydro-2,3-*O*-isopropylidene-D-ribofuranose (II) (1.3 g, 9 %) sublimed at 25–35°/0.1 mm and had m.p. 61°, $[\alpha]_D^{20}$ −62° (*c* 0.78, methanol) (lit.,[2] m.p. 61°, $[\alpha]_D$ −63°). The second component, 1,2:3,4-di-*O*-isopropylidene-D-ribopyranose (VIIc) (0.6 g, 3 %), sublimed at 35–40°/0.1 mm and had m.p. 68–69°, $[\alpha]_D^{20}$ −51° (*c* 0.6, chloroform) (Found: C, 57.8; H, 7.8. $C_{11}H_{18}O_5$ calc.: C, 57.4; H, 7.8 %). The trace component was not isolated but had the same mobility as di-(2,3-*O*-isopropylidene-*β*-D-ribofuranose) 1,5′:1′,5-dianhydride (III) on thin-layer chromatograms.

Fraction B [9.0 g (59 %), eluted by benzene–ether (1:1)] was shown by t.l.c. to be a single compound, 2,3-*O*-isopropylidene-D-ribose. A sample (0.35 g) was reduced with sodium borohydride and the crude 2,3-*O*-isopropylidene-D-ribitol was benzoylated to give 1,4,5-tri-*O*-benzoyl-2,3-*O*-isopropylidene-D-ribitol (0.55 g), m.p. 94–95° (undepressed on admixture with an authentic sample). Another sample (0.25 g) was oxidised with bromine in aqueous potassium hydrogen carbonate to give 2,3-*O*-isopropylidene-D-ribono-1,4-lactone (0.25 g), m.p. 140–142° (undepressed on admixture with an authentic sample).

Fraction C [1.8 g, eluted by ether–methanol (9:1)] was shown by t.l.c. to consist of several components. Micro-distillation of a sample (105 mg) gave 1,2-*O*-isopropyl-

idene-D-ribofuranose (VI) (50 mg, 6% from D-ribose), b.p. 90°/0.1 mm, which crystallised from benzene–light petroleum as needles, m.p. 86–87° (undepressed on admixture with an authentic sample), $[\alpha]_D^{25}+37°$ (*c* 0.59, chloroform).

1,2-O-Isopropylidene-D-ribopyranose (VIIIc)

(*a*) 1,2:3,4-Di-*O*-isopropylidene-D-ribopyranose (130 mg) was dissolved in methanol (1 ml), 0.15 N-sulphuric acid (1 ml) was added, and the solution was kept at 40° for 3 h. Paper chromatography indicated the presence of ribose (R_F 0.15) and a compound (R_F 0.60) giving a blue-green colour with the periodate–Schiff's reagent[9]. The solution was passed through Dowex-1 (OH^- form) (2 ml) and evaporated to dryness. The residue (20 mg) was purified by micro-sublimation at 70°/0.1 mm to give 1,2-*O*-isopropylidene-D-ribopyranose (VIIIc) (5 mg), m.p. 109–111° (Found: C, 50.6; H, 7.5. $C_8H_{14}O_5$ calc.: C, 50.5; H, 7.4%).

(*b*) A mixture (2.1 g) of 1,2:3,4-di-*O*-isopropylidene-D-ribopyranose and anhydro-compounds (Fraction A above) was dissolved in glacial acetic acid (16 ml) and water (4 ml) was added. After 14 h at room temperature, the solvents were evaporated and the residue (1.84 g) was chromatographed on silica (30 g). Elution with benzene–ether (1:4) gave unchanged anhydro-compounds (II) and (III) (1.41 g) and ether–methanol (9:1) eluted 1,2-*O*-isopropylidene-D-ribopyranose (VIIIc) (0.4 g) which after further purification by sublimation had m.p. 112–114°, $[\alpha]_D^{20}-26°$ (*c* 1.05, water). Periodate uptake: 0.98 mole/mole.

Periodate oxidation and borohydride reduction

1,2-*O*-Isopropylidene-D-ribopyranose (10 mg) was added to water (1 ml) containing sodium periodate (14 mg). The solution was kept for 24 h in the dark at room temperature and then treated with excess of sodium borohydride (20 mg). After a further 40 h the solution was acidified with acetic acid, passed through Dowex-50 (H^+ form), and concentrated to dryness. Borate was removed by repeated distillation of methanol from the residue which was then hydrolysed by 0.1 N-sulphuric acid (1 ml) at 70° for 1 h. Examination of the solution by paper chromatography revealed the presence of ethylene glycol, but not glycerol.

Prolonged treatment of 2,3-O-isopropylidene-D-ribose with acetone and concentrated sulphuric acid

2,3-*O*-Isopropylidene-D-ribose (0.6 g) was dissolved in acetone (10 ml) containing concentrated sulphuric acid (0.1 ml). After 24 h at room temperature, purification as before gave a syrupy residue (0.55 g). Chromatography on silica (20 g) and elution with benzene–ether (9:1) gave a syrup (0.1 g). Further purification of this by micro-sublimation gave, at 30°/0.1 mm, 1,5-anhydro-2,3-*O*-isopropylidene-D-ribofuranose (II) (40 mg), m.p. 61°; and, at 90°/0.1 mm, di-(2,3-*O*-isopropylidene-β-D-ribofuranose) 1,5′:1′,5-dianhydride (III) (30 mg), m.p. 90–93°, $[\alpha]_D^{20}-57°$ (*c* 0.55, chloroform) (lit.,[2] m.p. 86–87° and 97–98°, $[\alpha]_D$ −49°). Between these two fractions, a small amount

of material (25 mg) distilled which appeared to consist mainly of the monomer (II), contaminated with a little of the diketal (VIIc).

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Synthesis and characterization of the anomers of 2,3,4,6-tetra-*O*-acetyl-1-*O*-cinnamoyl-D-glucose

Recently, small amounts of a compound giving a glucose and cinnamic acid on hydrolysis have been isolated from various plant tissues[1]. It thus becomes desirable to have available various cinnamic esters of D-glucose. The only such compound so far known is a 1,2,3,4,6-penta-*O*-cinnamoyl-D-glucose[2]. As D-glucose esters substituted at C-1 are common in Nature, the anomers of 2,3,4,6-tetra-*O*-acetyl-1-*O*-cinnamoyl-D-glucose were prepared and characterized.

Fusion of a mixture of 1,2,3,4,6-penta-*O*-acetyl-α-D-glucose, cinnamic acid and zinc chloride[3,4] gave a crystalline compound showing $[\alpha]_D^{20}$ +154.5°. The method of its synthesis and its high specific rotation indicate that this compound was 2,3,4,6-tetra-*O*-acetyl-1-*O*-cinnamoyl-α-D-glucose. Its infrared spectrogram was very similar to that of 1,2,3,4,6-penta-*O*-acetyl-α-D-glucose[5].

Condensation of silver cinnamate with 2,3,4,6-tetra-*O*-acetyl-α-D-glucosyl bromide yielded a crystalline compound showing $[\alpha]_D^{20}$ −20.1°. The mode of preparation, the low specific rotation, and the infrared spectrogram indicated that the product was 2,3,4,6-tetra-*O*-acetyl-1-*O*-cinnamoyl-β-D-glucose.

EXPERIMENTAL

Evaporations were carried out *in vacuo* at 40–50°. All melting points are corrected.

2,3,4,6-Tetra-O-*acetyl-1*-O-*cinnamoyl-α-D-glucose*

Cinnamic acid (10 g) and α-D-glucopyranose pentaacetate (53 g) were melted at 130° and thoroughly mixed[3,4]. After the temperature had fallen to 120°, powdered, freshly fused zinc chloride (120 g) was added with stirring, and the mixture was kept *in vacuo* for 15 min. The dark-colored product was added to a mixture of 1,2-dichloroethane and water. The organic layer was washed with water and aqueous sodium bicarbonate, dried with anhydrous sodium sulfate, and concentrated. After treatment of a solution of the product in 80% aqueous ethanol with Darco G-60 carbon, filtration, and evaporation, the sirup resulting was dissolved in 50% aqueous ethanol (1 liter). Crystallization occurred immediately, giving 9.0 g of product (73% yield), m.p. 148–149.5°. After six recrystallizations from the same solvent, the compound had m.p. 151.6–152.2° and $[\alpha]_D^{20}$ +154.5° (*c* 1.9, chloroform).

Anal. Calc. for $C_{23}H_{26}O_{11}$: C, 57.74; H. 5.48. Found: C, 57.81; H, 5.64.

The infrared spectrum of the compound in bromoform exhibited absorption maxima at 2950, 2130, 1765, 1650, 1455, 1435, 1370, 1230, 1075, 1040, 980, 930, 905, 860 and 765 cm^{-1}.

X-ray powder diffraction data:* 12.62 vs, 10.82 w, 9.37 vs, 8.57 vw, 7.76 m, 6.29 w, 5.68 m, 5.05 s, 4.74 s, 4.20 s, 3.93 vs, 3.67 m, 3.47 vw, 3.30 w, 3.16 w, 2.97 bd, 2.76 bd. 2.64 vw, 2.53 bd, 2.41 vw, 2.32 bd and 2.23 bd.

2,3,4,6-Tetra-O-acetyl-1-O-cinnamoyl-β-D-glucose

A mixture of silver cinnamate[4] (20 g) and tetra-*O*-acetyl-α-D-glucopyranosyl bromide[6] (20 g, m.p. 86.5–87.5°) was shaken overnight in dry benzene (200 ml) in the dark at room temperature. Insoluble material, mostly silver bromide, was removed by filtration, and the filtrate was concentrated to 50 ml. Crystallization occurred after addition of petroleum ether (10 ml, b.p. 30–60°). The crystals were collected and washed with a 4:1 mixture of benzene and petroleum ether; yield, 8.0 g (34%). After six recrystallizations from hot ethanol, the compound had m.p. 143.6–144.5° and $[\alpha]_D^{20}$ −20.1° (*c* 1.9, chloroform).

Anal. Calc. for $C_{23}H_{26}O_{11}$: C, 57.74; H, 5.48. Found: C, 58.05; H, 5.48.

The infrared spectrum was similar to that of the α-D anomer, except that the band at 930 cm^{-1} was absent. X-ray powder diffraction data: 14.73 s, 10.95 vw, 9.27 vs, 7.20 bd, 5.57 m, 5.08 vs, 4.76 vs, 4.50 s, 4.17 vw, 3.88 s, 3.67 s, 3.27 m, 3.09 w, 2.93 vw, 2.78 bd, 2.63 bd, 2.52 bd, 2.39 bd and 2.24 bd.

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*X-ray powder diffraction data give interplanar spacings, Å, for CuK_α radiation, with relative intensities estimated visually; s, strong; m, moderate, w, weak; bd, broad; v, very.

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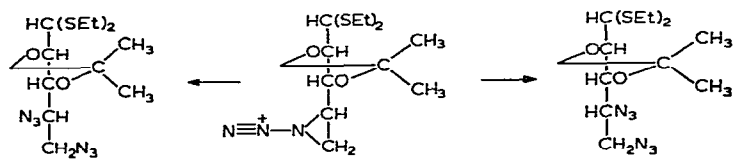
Preliminary communication

Sugars containing nitrogen in the ring

4,5-DIACETAMIDO-4,5-DIDEOXY-L-XYLOPYRANOSE AND THE ANOMERIC 4-ACETAMIDO-4,5-DIDEOXY-D-XYLOFURANOSES

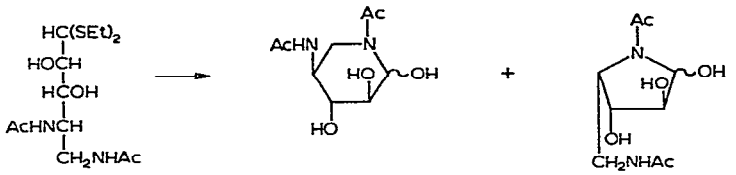
Previous reports from these laboratories described the synthesis of sugars containing nitrogen as a ring atom in six[1]- and five[2]-membered derivatives. Related substances have also been reported by other investigators[3–5].

We now wish to report the synthesis of the title compounds, which can exist as cyclic structures with nitrogen as the hetero atom, or as acyclic modifications. Acetonation of 5-azido-5-deoxy-D-arabinose diethyl dithioacetal[1] afforded the 2,3-*O*-isopropylidene derivative, $[\alpha]_D^{24}$ +61° (*c* 1.16, in chloroform)[6] which was subsequently converted to the 4-*O*-methylsulfonyl derivative, $[\alpha]_D^{24}$ +54° (*c* 0.32, in chloroform). Treatment of the latter with sodium azide in *N*,*N*-dimethylformamide afforded predominantly 4,5-diazido-4,5-dideoxy-2,3-*O*-isopropylidene-L-xylose diethyl dithioacetal (I), isolated as a pure liquid, $[\alpha]_D^{24}$ +110° (*c* 0.145, in chloroform). A second product formed in an approximate ratio of 1:60 with respect to I was also isolated, and assigned structure II by infrared spectral and optical rotational data, $[\alpha]_D^{24}$ + 65°



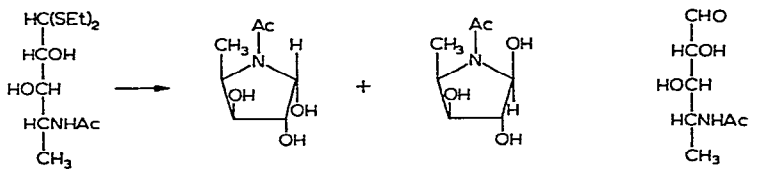
(*c* 0.31, in chloroform). Such a product, corresponding to an overall retention of configuration at C-4, could be formed by neighboring-group participation by the C-5 azide function as in the intermediate "azidonium" ion[7] III. Preferred attack at the primary carbon atom in III would explain the predominance of I. Reduction of I with lithium aluminum hydride, followed by *N*-acetylation, afforded the corresponding 4,5-diacetamido derivative, m.p. 112–114° which, on hydrolysis, gave 4,5-diacetamido-4,5-dideoxy-L-xylose diethyl dithioacetal (IV) as colorless crystals, m.p. 136–137°. The mass spectrum[8,9] of this product was compatible with its structure. Demercaptalation[10] of IV afforded a mixture of the pyranose V and furanose VI products. Compound V, which was the major product (R_F 0.28) was obtained as a colorless homogeneous sirup, $[\alpha]_D^{24}$ +30° (*c* 0.8, in methanol) or a hygroscopic solid. Infrared spectral data: $\lambda_{max.}^{KBr}$ 1640 cm^{-1}, amide I; 1560 cm^{-1}, amide II; n.m.r.

spectral data: (D_2O) τ 7.83, 7.80 (ring acetyl); τ 7.98 (C-4 acetyl); τ 4.43, $J \simeq 3$ c.p.s.; τ 4.00, $J \simeq 3$ c.p.s. (C-1 hydrogen). Isomerization studies in acid and base showed partial conversion of V to VI. The formation of variable proportions of VI and of the acyclic modification (n.m.r.), causing some tailing of the furanose spot, was enhanced by longer reaction times and mode of isolation. Compound VI (R_F 0.48) showed a singlet at τ 8.05 (C-5 acetyl) and a doublet at τ 9.80 (ring acetyl).



(IV) (V) (VI)

In an effort to further validate the participating character of the azido group, the displacement was studied in a derivative where the terminal azide group was replaced by hydrogen. Thus, treatment of 5-deoxy-2,3-*O*-isopropylidene-4-*O*-(*p*-nitrobenzenesulfonyl)-L-arabinose diethyl dithioacetal, $[\alpha]_D^{24}$ −34° (*c* 1.5, chloroform) with sodium azide in *N*,*N*-dimethylformamide gave 4-azido-4,5-dideoxy-2,3-*O*-isopropylidene-D-xylose diethyl dithioacetal as the sole product, $[\alpha]_D^{24}$ −109° (*c* 1.13, in chloroform). Reduction of this product, followed by *N*-acetylation, gave the crystalline 4-acetamido derivative, m.p. 90–91°; $[\alpha]_D^{24}$ −27° (*c* 1, chloroform). Removal of the acetal group afforded crystalline 4-acetamido-4,5-dideoxy-D-xylose diethyl dithioacetal (VII), m.p. 103–105°; $[\alpha]_D^{24}$ +16° (*c* 1, methanol).



(VII) (VIII) (IX) (X)

Demercaptalation[10] of (VII) afforded a sirup (90%), $[\alpha]_D^{24}$ +11.2° (at equil., *c* 4.6, methanol) which formed a double spot on paper chromatograms. Isolation of the respective components afforded the homogeneous products R_F 0.65 (*n*-BuOH–EtOH–H_2O, 3:1:1) and R_F 0.72 as colorless sirups in an approximate ratio of. 1:1[11]. Their identification as anomers was established by chemical, i.r., and n.m.r, studies. For the R_F 0.65 component: τ 4.72 (C-1 hydrogen); τ 7.82, 7.85, 7.88 7.92 (acetyl hydrogens); τ 8.88, 8.97 (C-5 hydrogens, main peaks). A peak at τ 8.03 (acetyl hydrogens) due to the acyclic form X was also detected indicating the presence of X in the original mixture. The ratio was approximately 1:12. The spectrum of the R_F 0.72 component showed peaks at τ 4.84 (C-1 hydrogen); τ 7.82, 7.85, 7.88, 7.92

(acetyl hydrogens); τ 8.86, 8.80, 8.75 (C-5 hydrogens, main peaks). Exposure of the respective deuterium oxide solutions of the anomers to hydrogen chloride vapor afforded identical spectra in each case, indicating rapid equilibration. The same crystalline benzylphenylhydrazone, m.p. 106–107° was obtained from either anomer.

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11 A tentative assignment of α- and β-configurations to VIII and IX respectively could be made based on the presence of a relatively symmetrical multiplet for the C-1 hydrogen signal in IX (higher τ value) compared to an apparent split multiplet ($J \simeq 4$–5 c.p.s.) in the spectrum of VIII (lower τ value). The lack of resolution of the anomeric hydrogen signals at 24° is due to the presence in solution of several rotational isomers[4]. Optical rotational data obtained from VIII and IX are not unambiguously discriminating.

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THE SYNTHESIS OF A 2-AMINO-2-DEOXY-D-GLUCOSYL–MURAMIC ACID DISACCHARIDE: 2-ACETAMIDO-6-*O*-(2-ACETAMIDO-3,4,6-TRI-*O*-ACETYL-2-DEOXY-β-D-GLUCOPYRANOSYL)-1,4-DI-*O*-ACETYL-2-DEOXY-3-*O*-[D-1-(METHYL CARBOXYLATE)ETHYL]-α-D-GLUCOPYRANOSE*

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INTRODUCTION

A disaccharide isolated from the cell wall of *Micrococcus lysodeikticus* by the action of lysozyme[1,2] has been proposed to have the structure *O*-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→6)-*N*-acetylmuramic acid [2-acetamido-6-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucose].

As a model compound, the *O*-acetylated methyl ester methyl α-D-glycoside was at first synthesized[3], followed by synthesis of the disaccharide itself[3,4]. Comparison of the synthetic disaccharide with the natural product showed that the two compounds were not identical[5]. Unfortunately, neither product was crystalline and the comparison was based on color reactions and paper chromatography. The crystalline fully acetylated methyl ester of the natural disaccharide has now been obtained[6]. Since the synthetic disaccharide was obtained only in a very small amount[5,6], it was not possible to prepare from it a peracetylated methyl ester. Consequently a new synthesis of this derivative, namely 2-acetamido-6-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-1,4-di-*O*-acetyl-2-deoxy-3-*O*-[D-1-(methyl carboxylate)ethyl]-α-D-glucopyranose (VIII) was devised.

DISCUSSION

Since it was the α-anomer of the peracetylated methyl ester of the natural disaccharide which had crystallized[6], the synthesis of the peracetylated α-anomer

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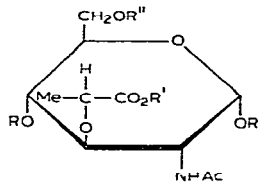
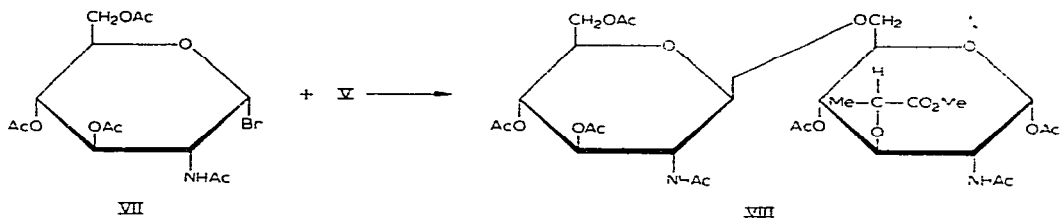
VIII was investigated using a route parallel to that of the synthesis of the methyl α-D-glycoside[3] and of the benzyl α-D-glycoside[4] of the same disaccharide.

2-Acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-α-D-glucose (*N*-acetylmuramic acid) (I)[4,7] was esterified with diazomethane and the resulting amorphous methyl ester II was directly etherified with chlorotriphenylmethane. The resulting crystalline trityl ether III was assumed to be the α-D-anomer on the basis of its high positive optical rotation. When acetylated, it gave the crystalline 1,4-diacetate IV in high yield. The same product IV could be obtained directly from II without isolation of III. Removal of the trityl group of IV with hot 60% acetic acid or by hydrogenation in ethanol solution gave an amorphous product, probably a mixture of compounds resulting from the migration of the *O*-acetyl groups. Removal of the trityl group by exposure for a very short time to hydrogen bromide gave, however, the expected compound V in crystalline form. Evidence that the primary hydroxyl



	R	R'	R''
I	H	H	H
II	H	Me	H
III	H	Me	Tr
IV	Ac	Me	Tr
V	Ac	Me	H
VI	Ac	Me	Ts



VII VIII

group of compound V was free was obtained by formation of the 6-*p*-toluenesulfonate VI, which reacted qualitatively with sodium iodide in acetone[8]. Condensation of 2-acetamido-1,4-di-*O*-acetyl-2-deoxy-3-*O*-[D-1-(methyl carboxylate)ethyl]-α-D-glucopyranose (V) with 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl bromide (VII)[9] was performed according to the method used for the preparation of the methyl[3] and benzyl[4] glycosides. The yield of the resulting disaccharide, 2-acetamido-6-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-1,4-di-*O*-acetyl-2-deoxy-3-*O*-[D-1-(methyl carboxylate)ethyl]-α-D-glucopyranose (VIII), was low (6%) but in the same order of magnitude as that of the preceding syntheses[3,4]. The mode of synthesis is evidence for a (1 → 6) linked disaccharide, since a similar route using benzyl α-D-glycoside derivatives gave a (1 → 6) linked disaccharide as shown by its reaction in the Morgan and Elson reaction[5,6]. Comparison of VIII with the crystalline derivative obtained from the natural product showed that the compounds were not identical; the melting points were nearly identical and both

products showed identical optical rotations and infrared spectra, but in admixture the melting point was depressed, and both X-ray powder analysis and mass spectrometry showed definite differences. A detailed comparison of the properties of both products is reported elsewhere[6].

EXPERIMENTAL

General

Melting points were taken on a hot stage, equipped with a microscope, and correspond to "corrected melting point". Specific rotations were determined in semimicro- or micro- (for amounts smaller than 3 mg) tubes with lengths of 200 or 100 mm, using a Rudolph photoelectric polarimeter attachment, Model 200; the chloroform used was A.R. grade and contained approximately 0.75% of ethanol. X-Ray powder diffraction data refer to interplanar spacing in Å with Cu K_α radiation. Relative intensities were estimated visually: s, strong; m, moderate; w, weak; v, very; b, broad. The first three strongest lines are numbered (1, strongest); double numbers indicate approximately equal intensities. The infrared spectrum was taken as a potassium bromide disc on a Perkin–Elmer spectrophotometer Model 237. Chromatograms were made with the flowing method on "Silica Gel Davison," from the Davison Co., Baltimore 3, Maryland (grade 950, 60–200 mesh), which was used without pretreatment. When deactivation by contact with moist air occurred, reactivation was obtained by heating to 170–200° (manufacturer's instructions). The sequence of eluents was hexane, benzene or 1,2-dichloroethane, ether, ethyl acetate, acetone, and methanol individually or in binary mixtures. The proportion of weight of substance to weight of absorbent was 1 to 50–100. The proportion of weight of substance in grams to volume of fraction of eluent in milliliters was 1 to 100. The ratio of diameter to length of the column was 1 to 20. Evaporations were carried out *in vacuo*, with an outside bath temperature kept below 45°. Amounts of volatile solvent smaller than 20 ml were evaporated under a stream of dry nitrogen. The microanalyses were done by Dr. M. Manser, Zürich, Switzerland.

*2-Acetamido-2-deoxy-3-*O-[D-*1-(methyl carboxylate)ethyl*]-*6*-O-*trityl*-α-D-*glucopyranose* (*III*)

To a solution of 2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-α-D-glucose (*N*-acetyl-D-muramic acid) (I, 0.26 g) in methanol was added a slight excess of diazomethane in ether. The excess of diazomethane and methanol was immediately evaporated. The sirupy residue, the methyl ester II, was not crystallized, but it was dissolved in 10 ml of dry pyridine. After addition of chlorotriphenylmethane (0.26 g), the solution was kept for 24 h at room temperature, heated for 1 h at 100°, and then poured onto cracked ice. The resulting crystals were collected and recrystallized from benzene to give 0.27 g (48%) of prisms, m.p. 126–130°, $[\alpha]_D^{25}$ +69° (*c* 0.52, ethanol). The product contained one mole of benzene of crystallization.

Anal. Calc. for $C_{31}H_{35}NO_8 \cdot C_6H_6$: C, 70.80; H, 6.58; N, 2.23. Found: C, 70.70; H, 6.70; N, 2.33.

2-Acetamido-1,4-di-O-acetyl-2-deoxy-3-O-[D-1-(methyl carboxylate)ethyl]-6-O-trityl-α-D-glucopyranose (IV)

(i) From III. A solution of III (0.20 g) in dry pyridine (3.5 ml) and acetic anhydride (3 ml) was kept for 24 h at room temperature. The solution was then poured into ice-water, and the precipitate was separated by filtration. It was recrystallized from methanol to give 0.22 g (96%) of needles, m.p. 120–122°, $[\alpha]_D^{24} + 118°$ (*c* 0.50, chloroform).

Anal. Calc. for $C_{35}H_{39}NO_{10}$: C, 66.34; H, 6.20; N, 2.21. Found: C, 66.44; H, 6.30; N, 2.31.

(ii) From II. To a solution of II (1.03 g) in dry pyridine (10 ml) was added chlorotriphenylmethane (1.03 g). The solution was heated for 1 h at 100° and then, after addition of acetic anhydride (7 ml) was left for 20 h at room temperature. It was then poured into ice-water, the precipitate was separated, washed thoroughly with water, and dried in a desiccator over calcium chloride. The solid was dissolved in 1,2-dichloroethane and chromatographed on silicic acid. A 9:1 mixture of 1,2-dichloroethane and ether eluted 1.12 g (53%) of crystalline material. It was recrystallized from methanol to give needles, m.p. 120–122°, $[\alpha]_D^{24} + 118°$ (*c* 0.75, chloroform), which showed no depression of the melting point in admixture with the product described above.

2-Acetamido-1,4-di-O-acetyl-2-deoxy-3-O-[D-1-(methyl carboxylate)ethyl]-α-D-glucopyranose (V)

To a solution of IV (100 mg) in acetic acid (1 ml) at 0° was added 30% hydrogen bromide in acetic acid (0.1 ml). The mixture was shaken for 60 seconds with cooling. The precipitated bromotriphenylmethane was removed by filtration, and the filtrate was poured into ice-water and extracted with chloroform. The chloroform extract was dried without washing, and concentrated to a sirup, which was crystallized from a mixture of chloroform, ether, and pentane, to give a product having m.p. 138–140°. Recrystallization from the same solvents gave 45 mg (73%) of prisms, m.p. 142–144°, $[\alpha]_D^{25} -90°$ (*c* 0.62, chloroform).

Anal. Calc. for $C_{16}H_{25}NO_{10}$: C, 49.10; H, 6.44; N, 3.58. Found: C, 49.15; H, 6.36; N, 3.53.

2-Acetamido-1,4-di-O-acetyl-2-deoxy-3-O-[D-1-(methyl carboxylate)ethyl]-6-O-p-tolylsulfonyl-α-D-glucopyranose (VI)

A solution of V (45 mg) and *p*-toluenesulfonyl chloride (26 mg) in dry pyridine (1 ml) was kept for 20 h at room temperature. Evaporation gave a sirupy residue, which was freed from pyridine by codistillation with toluene, and crystallized from aqueous methanol to give 40 mg (63%) of needles. Recrystallization from methanol

gave 25 mg of pure product, m.p. 174–175°, $[\alpha]_D^{25}$ +86° (*c* 0.56, chloroform). The m.p. was not raised by further crystallization.

Anal. Calc. for $C_{23}H_{31}NO_{12}S$: C, 50.63; H, 5.73. Found: C, 50.54; H. 5.60.

A mixture of 3 mg of the above compound with 3 mg of sodium iodide in 0.2 ml of dry acetone was heated in a sealed tube for 2 h at 100°, whereupon characteristic plate-shaped crystals of sodium *p*-toluenesulfonate were deposited.

2-Acetamido-6-O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)-1,4-di-O-acetyl-2-deoxy-3-O-[D-1-(methyl carboxylate)ethyl]-α-D-glucopyranose (VIII)

To a solution of V (135 mg) in dry nitromethane (5 ml) was added under stirring mercuric cyanide (120 mg) and a solution of 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl bromide (VII) in a mixture of dry chloroform (3.5 ml) and dry nitromethane (1.5 ml). The solution of VII had been prepared *in situ* from 2-acetamido-1,3,4,6-tetra-*O*-acetyl-2-deoxy-β-D-glucopyranose (220 mg)[9].

After 4.5 h at room temperature, similar amounts of mercuric cyanide and VII were again added, and the reaction was allowed to proceed for 24 h when a further addition of mercuric cyanide and VII was made. After 24 h the reaction mixture was diluted with chloroform, washed with saturated sodium bicarbonate solution and water, and dried over sodium sulfate. After evaporation, the residue (260 mg) was dissolved in 1,2-dichloroethane and purified by chromatography on silicic acid. A mixture of ethyl acetate and acetone (4:1 and 1:1) eluted 15 mg (6%) of crystalline product. Recrystallization from a mixture of acetone and ether gave needles, m.p. 240–241°, $[\alpha]_D^{24}$ +40° (*c* 0.38, chloroform), X-ray powder diffraction data: 14.73 m, 12.81 m, 10.78 w, 9.31 s (1), 8.04 m, 6.61 m, 5.57 w, 5.04 m, 4.96 m, 4.46 s (2), 4.27 m (3), 3.83 w, 3.71 w, 3.53 w, 3.44 m, 3.30 vw, 2.75 vw, 2.49 vw, 2.27 vw, 2.00 w. The infrared spectrum of the compound exhibited absorption maxima at 3340, 2970, 1745, 1665, 1550, 1440, 1385, 1235, 1130, 1050 and 930 cm^{-1}.

Anal. Calc. for $C_{30}H_{44}N_2O_{10}$: C, 50.00; H, 6.15; N, 3.89. Found: C, 49.83: H, 6.20; N, 3.96.

ACKNOWLEDGMENTS

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SUMMARY

A crystalline disaccharide derivative containing β-(1 → 6)-linked 2-amino-2-deoxy-D-glucose and muramic acid: 2-acetamido-6-*O*-(2-acetamido-3,4,6-tri-*O*-

acetyl-2-deoxy-β-D-glucopyranosyl)-1,4-di-*O*-acetyl-2-deoxy-3-*O*-[D-1-(methyl carboxylate)ethyl]-α-D-glucopyranose (VIII), was synthesized. 2-Acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-α-D-glucose (*N*-acetylmuramic acid) was esterified with diazomethane, tritylated, acetylated, and detritylated, to give 2-acetamido-1,4-di-*O*-acetyl-2-deoxy-3-*O*-[D-1-(methyl carboxylate)ethyl]-α-D-glucopyranose (V). *p*-Toluenesulfonylation of V, foliowed by reaction with sodium iodide showed that position 6 was free. Condensation of V with 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl bromide gave VIII in 6% yield.

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NFRARED SPECTRA OF THE SULFONIC ESTERS OF MONOSACCHARIDES*

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INTRODUCTION

Absorption peaks in the 730–960 cm^{-1} region in the infrared spectra of pyranoid sugar derivatives have been correlated with various stereochemical features in the molecule. Axial and equatorial orientations of substituents at the anomeric carbon atom on the pyranose ring have been studied extensively by infrared spectroscopy[1]. Infrared spectroscopic analysis has also been applied to study of the disposition of sulfate ester groups at positions other than C-1 on the aldopyranose ring. Orr[2] reported infrared spectroscopic studies of some sulfated polysaccharides of biological interest. Lloyd and co-workers[3–5] have reported on various carbohydrate sulfate esters, with particular discussion of the C–O–S vibration. They suggested that the absorption in the 820–850 cm^{-1} region could be assigned to the C–O–S vibration mode of the C–O–SO_3^- group, citing Guthrie and Spedding's work[6] that sulfonic ester groups on the pyranose ring of monosaccharides do not absorb in the corresponding region. However, this work dealt only with compounds which involved both sulfonic and nitric ester groups in the same molecule. The same observation was reported by Anderson *et al.*[7]. As a matter of fact, the C–O–N vibration seems to show absorption similar to that of the C–O–S vibration in the 800–900 cm^{-1} region. These absorption peaks are generally weak and are sometimes absent altogether. Derivatives which show a typical difference of infrared absorption peak depending upon the axial or equatorial orientation of a substituent on the pyranose ring would be of interest.

The present paper is concerned with the orientation of the C–O–SO_2–R group (R = benzyl or alkyl group) at C-2, C-3, C-4, and C-6 of the pyranose ring of monosaccharides as revealed by study of the 800–900 cm^{-1} region in the infrared spectra of the sulfonic esters of carbohydrates. The analysis shows a characteristic difference in this spectral region between sulfonyloxy groups oriented axially and those oriented equatorially.

*Part of this work was presented at the International Symposium on the Chemistry of Natural Products held at Kyoto, Japan, from April 12 to 18, 1964.

EXPERIMENTAL

General

Infrared spectra were measured with a Shimadzu AR-6 spectrophotometer (sodium chloride optics). All compounds were examined as pressed discs in potassium bromide. All melting points are uncorrected.

Methyl 2-acetamido-4,6-O-benzylidene-2-deoxy-3-O-(methylsulfonyl)-α-D-allopyranoside (I)

Methyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-α-D-allopyranoside (I′) was prepared by the method of Jeanloz[8], m.p. 207°, $[\alpha]_D^{20}$ +65° (*c* 1.0, chloroform). The derivative (0.3 g) was dissolved in anhydrous pyridine (10 ml) in a 50-ml flask fitted with a calcium chloride tube. The solution was cooled to 0°, and a solution of methanesulfonyl chloride (0.2 ml) in anhydrous pyridine (5 ml) was added at 0°. After standing for 2 days at 0°, the mixture was poured on to cracked ice. The solution was extracted three times with 20-ml portions of chloroform. The chloroform layer was washed with water, saturated sodium hydrogen carbonate solution, water, 3 N hydrochloric acid, and water, dried (anhydrous sodium sulfate), and evaporated under reduced pressure to dryness. The residue was dissolved in the minimum volume of acetone; addition of petroleum ether (b.p. 40–60°) produced crystals; yield 0.3 g, m.p. 176°, $[\alpha]_D^{20}$ +37° (*c* 1.0, chloroform).

Anal. Calc. for $C_{17}H_{23}NO_8S$: C, 50.86; H, 5.77; N, 3.49; S, 7.98. Found: C, 51.13; H, 5.64; N, 3.22; S, 7.92.

Methyl 2-acetamido-2-deoxy-3-O-(methylsulfonyl)-α-D-allopyranoside (II)

Compound I (0.2 g) was treated by the procedure described by Jeanloz[8] to give methyl 2-acetamido-2-deoxy-3-*O*-(methylsulfonyl)-α-D-allopyranoside (II) as an amorphous powder; yield 0.1 g, $[\alpha]_D^{22}$ +54° (*c* 0.5, water).

Anal. Calc. for $C_{10}H_{19}NO_8S \cdot H_2O$: C, 36.25; H, 6.39; N, 4.23; S, 9.67. Found: C, 36.56; H, 6.52; N, 4.10; S, 9.75.

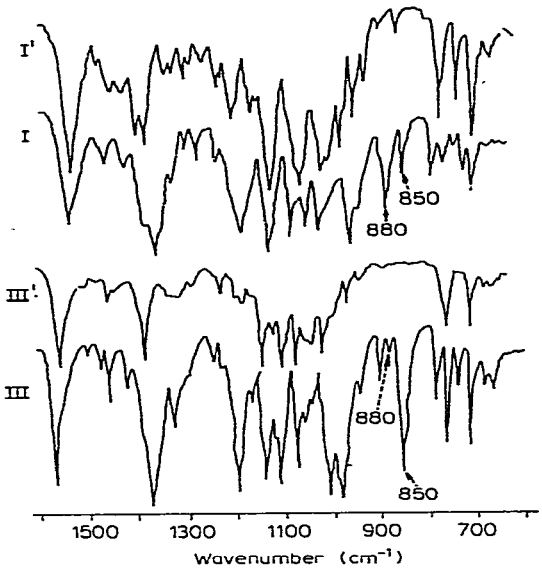
The other sulfonic esters of the hexopyranoses were prepared according to methods given in the references. Details of the spectra of these derivatives are shown in Fig. 1 and Table 1.

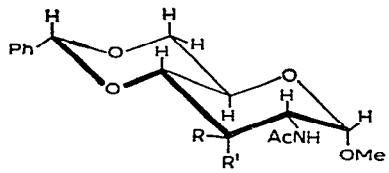
RESULTS AND DISCUSSION

C–O–S Vibration

As shown in Fig. 1 and Table 1, the spectra of all the sulfonic esters of hexopyranoses examined exhibited two strong absorptions at 1,170–1,190 cm^{-1} and at 1,350–1,370 cm^{-1}, due to the symmetrical and asymmetrical stretching vibrations of the $-SO_2-$ group[1,9–11]. They also showed a series of absorptions in the 700–900 cm^{-1} region, where the parent compounds show only slight or no absorption. It seems difficult and unfruitful to analyze the bands at wavenumbers shorter

than 800 cm^{-1}, because of the strong contributions from many kinds of vibration modes, including those due to the pyranose ring of monosaccharides, the phenyl group, and other substituents. However, the appearance in all the spectra of a single band in the 840–890 cm^{-1} region is notable.





Compounds	R	R'
I	H	OSO_2CH_3
I'	H	OH
III	OSO_2CH_3	H
III'	OH	H

Fig. 1. Infrared spectra of the methanesulfonates of methyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-α-D-allopyranoside and -glucopyranoside in the 600–1,600 cm^{-1} region. I, Methyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-3-*O*-(methylsulfonyl)-α-D-allopyranoside (see Experimental); I', methyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-α-D-allopyranoside (see Experimental); III, methyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-3-*O*-(methylsulfonyl)-α-D-glucopyranoside[8], m.p. 208°; III', methyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-α-D-glucopyranoside[8], m.p. 255°. Details of the absorption frequencies of the C–O–S vibration are described in the text.

The assignment of this absorption is considered to be the C–O–SO_2–R group; this is further divided into C–O–S, C–S, C–R, and S=O vibration modes, since the parent compounds show no absorption in the region mentioned above. A study of the published spectra of other organic compounds reveals that the C–S stretching vibration is mainly assigned to absorption at wavenumbers shorter than 800 cm^{-1} and that the C–H out-of-plane vibrations of monosubstituted and 1,4-disubstituted benzene rings are near 720–780 cm^{-1} and 820 cm^{-1}, respectively. The absorption frequency of the band in the 840–890 cm^{-1} region was unchanged with variations in the kind of hexopyranose in the kind and position of the sulfonyloxy group on the pyranose ring, and in the kind of phase used for measurement of the spectra.

These facts exclude the possibility of assigning both the C–H vibration of the pyranose ring and the C–S and CH_3 vibrations of the substituents on the benzene ring to this absorption, but permit assignment of the absorption to the C–O–S vibration. The assignment is supported by the infrared spectroscopic observations

TABLE I

ABSORPTION FREQUENCIES OF THE SULFONIC ESTERS OF SOME HEXOPYRANOSE DERIVATIVES IN THE 800–900 cm^{-1} REGION

Compound[a]	*Absorption peaks*[b] (cm^{-1})					
II Methyl 2-acetamido-2-deoxy-3-*O*-(methylsulfonyl)-α-D-allopyranoside		835 w	855 w		890 vs	
IV Methyl 2-acetamido-2-deoxy-3-*O*-(methylsulfonyl)-α-D-glucopyranoside			860 vs		890[c] vw	
V 1,3,4,6-Tetra-*O*-acetyl-2-*O*-(methylsulfonyl)-α-D-glucopyranose			850 vs		880 w	
VI 1,3,4,6-Tetra-*O*-acetyl-2-*O*-(methylsulfonyl)-α-D-galactopyranose		840[c] m	860 s		890 vw	
VII Methyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-3-*O*-*p*-tolylsulfonyl-α-D-glucopyranoside	820 m		850[c] s	860 vs	880 w	
VIII 6-*O*-*p*-Tolylsulfonyl-β-D-galactopyranose	820 s		855 vs		880 vw	
IX 1,2:3,4-Di-*O*-isopropylidene-6-*O*-*p*-tolylsulfonyl-α-D-galactopyranose	820 s		845 vs		880[c] vw	
X Methyl 6-*O*-*p*-tolylsulfonyl-α-D-glucopyranoside	820 vs		845 s		875 w	
XI 1,2,3,4-Tetra-*O*-acetyl-6-*O*-*p*-tolylsulfonyl-β-D-glucopyranose	820 s		840 s	850 vw	880 w	890[c] w
XII 1,3,4-Tri-*O*-acetyl-2-anisylideneamino-2-deoxy-6-*O*-*p*-tolylsulfonyl-β-D-glucopyranose	813 w		840 vs	855 w	880 w	
XIII Methyl 4,6-*O*-benzylidene-2,3-di-*O*-(methylsulfonyl)-α-D-glucopyranoside			845 vs		880 vw	
XIV 1,3,4-Tri-*O*-acetyl-2-benzamido-2-deoxy-6-*O*-*p*-tolylsulfonyl-β-D-glucopyranose	820 s		840 s	865 w	880 w	895 vw
XV 2-Acetamido-4,6-*O*-benzylidene-3-*O*-benzylsulfonyl-2-deoxy-D-glucopyranose			840 s		880 vw	
XVI 2-Acetamido-1,3-di-*O*-acetyl-2-deoxy-4-*O*-*p*-tolylsulfonyl-6-*O*-trityl-β-D-glucopyranose	820 m		840 s		880 vw	
XVII 2-Acetamido-1,3-di-*O*-acetyl-2-deoxy-4-*O*-(methylsulfonyl)-6-*O*-trityl-D-glucopyranose			840 vs		880 w	

[a] II, see Experimental; IV, m.p. 186°, prepared by removal[8] of the benzylidene group from III; V[13], m.p. 80–84°; VI[13], m.p. 96–98°; VII[14], m.p. 192°; VIII[15], m.p. 135°; IX[16], m.p. 91°; X[17], m.p. 124°; XI[18], m.p. 195°; XII[19], m.p. 200°; XIII[20], m.p. 186°; XIV[21], m.p. 143–146°, prepared by the *p*-toluenesulfonation and acetylation of 2-benzamido-2-deoxy-D-glucopyranose, m.p. 196–199° according to the usual procedure; XV[22], m.p. 90°, prepared by the benzylsulfonation of 2-acetamido-4,6-*O*-benzylidene-2-deoxy-D-glucopyranose m.p. 230°; XVI[23], m.p. 120°, prepared by the *p*-toluenesulfonation of 2-acetamido-1,3-di-*O*-acetyl-2-deoxy-6-*O*-trityl-D-glucopyranose, m.p. 105–107°, which was prepared by the tritylation of 2-acetamido-1,3-di-*O*-acetyl-2-deoxy-D-glucopyranose; XVII[23], m.p. 171°, prepared by the methanesulfonation of 2-acetamido-1,3-di-*O*-acetyl-2-deoxy-6-*O*-trityl-D-glucopyranose.

[b] m = moderate; s = strong; v = very; w = weak.

[c] shoulder.

on sulfates of monosaccharides[3,4], alkylsulfuric esters[5], and some sulfonic esters of cyclohexanol[24].

From these data, the characteristic absorption band in the 840–890 cm^{-1} region is tentatively assigned to the C–O–S vibration mode of the C–O–SO_2–R group in the pyranose ring, but it is open to confirmation by study of types of sulfonic esters other than those of the pyranoses.

On the shift in the absorption frequency of the C–O–S vibration

According to Mills[12], the stable conformation of 4,6-*O*-benzylidene-D-glucopyranose is a rigid, fused-ring structure of the *trans*-decalin type. Therefore, it is believed that compound I, methyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-3-*O*-(methylsulfonyl)-α-D-glucopyranoside (III), and their unbenzylidenated parents (II and IV) have similar stable conformations. The orientation of the sulfonyloxy group of compound I is axial on the pyranose ring, whereas that of compound III is equatorial. There is no gross difference between the n.m.r. spectra of compounds I and III. Especially, no difference was observed in the chemical shift of the proton signal of the S–CH_3 group of the two compounds. This indicates that the n.m.r. spectra are not useful for determining the spatial orientation of methylsulfonyloxy groups on the pyranose ring of monosaccharides. On the other hand, a marked difference was observed in the absorption frequencies of the C–O–S vibration in the infrared spectra of the two compounds, as shown in Fig. 1. Compound I, having an axial sulfonyloxy group, shows a strong absorption band at 880 cm^{-1} and a very weak one at 850 cm^{-1}, whereas compound III, having an equatorial sulfonyloxy group, shows a strong absorption at 850 cm^{-1} and a very weak one at 880 cm^{-1}. This difference remained unchanged with variation in the phase used for the measurement (potassium bromide, chloroform, and Nujol). The same difference in the infrared spectra was observed to exist between the 3-methanesulfonates (II and IV) of methyl 2-acetamido-2-deoxy-α-D-allopyranoside and -glucopyranoside. All other sulfonic esters disposed equatorially on the pyranose ring of monosaccharides showed the characteristic absorption band of the C–O–S vibration at 845 $\pm$ 5 cm^{-1}, as shown in Table 1. These results suggest that the observed shift in frequency is associated only with the difference in the spatial orientation of the sulfonyloxy group on the pyranose ring, as suggested earlier for the sulfates of carbohydrates[2–4]. For confirmation, the axial sulfonyloxy group at C-2 and C-4 of the pyranose ring should be studied.

From these results, it is concluded that axial sulfonyloxy groups on pyranose rings show a strong absorption band at 880–890 cm^{-1} and a weak absorption band at 840–850 cm^{-1}, and that equatorial sulfonyloxy groups on the pyranose ring show a strong absorption band at 840–850 cm^{-1} and a weak one at 880–890 cm^{-1}. When the characteristic absorption bands of the sulfates are compared with those of the sulfonic esters in the 800–900 cm^{-1} region, it is observed that the bands of the former lie at lower wavenumbers (by about 10–40 cm^{-1}).

Infrared spectroscopic analysis of sulfonyloxy groups might be especially

useful for the conformational determination of oligo- and poly-saccharides of biological interest.

ACKNOWLEDGMENT

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SUMMARY

The orientation of sulfonyloxy groups at different carbon atoms of the pyranose ring of monosaccharides has been examined by analysis of infrared spectra in the 800–900 cm^{-1} region, with particular reference to axial and equatorial orientations of the sulfonyloxy groups. Axial sulfonyloxy groups show a strong absorption band at 880–890 cm^{-1}, whereas equatorial ones absorb at 840–850 cm^{-1}. The absorption frequency is not affected by variations in the kind of hexopyranose, by the kind and position of the sulfonyloxy groups on the pyranose ring, or by the kind of phase used for the measurement.

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REACTION OF ARYLHYDRAZINES WITH SOME PERIODATE-OXIDISED GLUCOSIDES AND GALACTOSIDES*

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INTRODUCTION

In Part I[1] of this series, we described the reaction of periodate-oxidised methyl 4,6-*O*-benzylidene-α-D-glucoside (I) with aqueous phenylhydrazine to give methyl 4,6-*O*-benzylidene-3-deoxy-3-phenylazo-α-D-glucoside (IV), a useful intermediate for the synthesis of derivatives of 3-amino-3-deoxy-D-glucose. Subsequently, the reaction was extended[2] to a number of other arylhydrazines. Those with activating substituents, such as *p*-methyl, and moderately deactivating substituents, such as *p*-bromo and *p*-sulphamoyl, gave 3-arylazoglucosides; substituents such as *m*- and *p*-nitro gave only the bisarylhydrazone of the dialdehyde. One feature of the above reactions was that the 3-arylazoglucoside was the only arylazoglycoside formed when 2- or 3-arylazo derivatives of allose, altrose, glucose, or mannose were theoretically possible. We now report on the reaction of arylhydrazines with other periodate-oxidised carbohydrate derivatives.

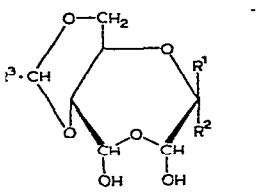
RESULTS AND DISCUSSION

In the first experiments, only minor structural changes were made in the sugar component. Methyl 4,6-*O*-benzylidene-β-D-glucoside, when oxidised by a modification of Honeyman and Shaw's method[3], gave a monoethyl glycoside**, formed from ethanol present in the chloroform used in an extraction step, and not the free hemialdal (II). The structure of the monoethyl glycoside was confirmed by formation of a mono-*p*-nitrobenzoate (*cf.* ref. 4). The hemialdal gave a bis-*p*-nitrophenylhydrazone, showing that it could react either as a hemialdal (II) or a dialdehyde (V), depending on the conditions, as found for the α-anomer[4,5].

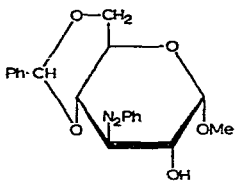
Reaction of the hemialdal (II) with aqueous phenylhydrazine, as for the α-anomer[1], gave an unstable, bright-yellow solid, m.p. 49–60°, which could not be recrystallised. Reduction of the crude product gave aniline (characterised as 2,4-dinitrodiphenylamine) and methyl 3-amino-4,6-*O*-benzylidene-3-deoxy-β-D-

*Nitrogen-containing Carbohydrates. Part VIII [Part VII, R.D. GUTHRIE AND D. MURPHY, *J. Chem. Soc.*, (1965) 3828].
**For an explanation of this nomenclature, see ref. 4.

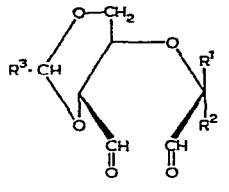
glucoside, characterised as its *N*-acetyl derivative, and by hydrolysis and peracetylation to give known[6] methyl 3-acetamido-3-deoxy-β-D-glucoside 2,4,6-triacetate. Reaction of the hemialdal (II) with *p*-bromophenylhydrazine also gave an unworkable yellow solid, which was reduced, and the reduction product characterised as above.



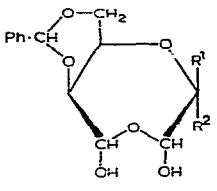
I R^1=H, R^2=OMe, R^3=Ph
II R^1=OMe, R^2=H, R^3=Ph
III R^1=H, R^2=OMe, R^3=Me



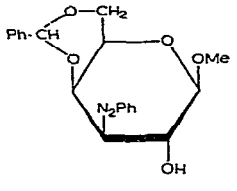
IV



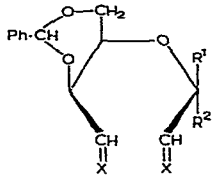
V R^1=OMe, R^2=H, R^3=Ph
VI R^1=H, R^2=OMe, R^3=Me



VII R^1=OMe, R^2=H
VIII R^1=H, R^2=OMe



IX



R^1=OMe, R^2=H
X X=O
XI X=N·HN—⟨⟩—Br
XII X=N·HN—⟨⟩—SO_2NH_2
XIII X=N·HN—⟨⟩(Cl)

R^1=H, R^2=OMe
XIV X=N·HN—⟨⟩—Br
XV X=N·HN—⟨⟩—SO_2NH_2

The next 'dialdehyde' investigated was periodate-oxidised methyl 4,6-*O*-ethylidene-α-D-glucoside[3]. This compound gave a di-*p*-nitrobenzoate [hemialdal (III) derivative] and a bis-*p*-nitrophenylhydrazone [dialdehyde (VI) derivative]. Reaction of the oxidised glucoside with aqueous phenylhydrazine gave another unstable, yellow solid, the infrared spectrum of which suggested that it contained the phenylhydrazone group; thin-layer chromatography revealed four components. Reduction of the crude product gave aniline and, on acetylation of the residue, methyl 3-acetamido-3-deoxy-4,6-*O*-ethylidene-α-D-glucoside (15%, based on the hemialdal used), characterised by hydrolysis and peracetylation to the known[7] methyl 3-acetamido-3-deoxy-α-D-glucoside 2,4,6-triacetate.

Reaction of this hemialdal (III) both with *p*-bromophenylhydrazine and with 2,5-dichlorophenylhydrazine* gave crystalline yellow products having elemental analyses and infrared spectra suggestive of methyl arylazodeoxy-4,6-*O*-ethylidene-

*In previous work[2], this arylhydrazine was not included. It has now been shown that, with periodate-oxidised methyl 4,6-*O*-benzylidene-α-D-glucoside, it forms an azo compound, and not a bisarylhydrazone (see Experimental).

α-D-glycosides. The ultraviolet spectra were very similar to those of the corresponding benzylideneglucosides (see Table I and ref. 8), but reduction gave only intractable material. By analogy with the phenylhydrazine product and with the benzylidene compound, the products are assigned the 3-arylazoglucoside structure.

Attention was next turned to the anomeric methyl 4,6-*O*-benzylidene-D-galactosides. The β-anomer, on oxidation with periodate, affords a crystalline hemialdal (VII) which yields a bis-*m*-nitrophenylhydrazone [dialdehyde (X) derivative] and a di-*p*-nitrobenzoate [hemialdal (VII) derivative].

TABLE I

ULTRAVIOLET MAXIMA FOR ETHANOL SOLUTIONS

	λ_{max}	ε_{max}	λ_{max}	ε_{max}
Methyl 4,6-*O*-benzylidene-3-deoxy-3-(2,5-dichlorophenylazo)-α-D-glucoside	265	8,760	405	182
Methyl 3-arylazo-3-deoxy-4,6-*O*-ethylidene-α-D-glucosides,				
p-bromophenylazo-	272	14,410	392	249
2,5-dichlorophenylazo-	266.5	8,175	400	155
Methyl 4,6-*O*-benzylidene-3-deoxy-3-phenylazo-β-D-galactoside (IX)	266	10,800	385	220
2,4-*O*-Benzylidene-3-*O*-(1β-methoxy-2-oxoethyl)-D-threose bisarylhydrazones,				
p-bromophenylhydrazone (XI)	284	32,470		
p-sulphamoylphenylhydrazone (XII)	287	34,800		
2,5-dichlorophenylhydrazone (XIII)	277	—[a]		
2,4-*O*-Benzylidene-3-*O*-(1α-methoxy-2-oxoethyl)-D-threose bisarylhydrazones,				
p-bromophenylhydrazone (XIV)	284	30,410		
p-sulphamoylhydrazone (XV)	288	29,450		

[a]Too insoluble for accurate determination of ε_{max}.

Reaction of the hemialdal (VII) with aqueous phenylhydrazine in the usual way gave a yellow crystalline product (50% based on converted hemialdal, see below) which had an infrared band for a hydroxyl group, but no band in the 1600–1610 cm^{-1} region, where phenylhydrazones absorb. The ultraviolet spectrum was typical of that for a phenylazo sugar[1,8]. The product did not give a formazan reaction and appeared, therefore, to be a methyl 4,6-*O*-benzylidenedeoxyphenylazoglycoside of the *ido*-, *gulo*-, *talo*-, or *galacto*-series. By analogy with the *gluco*-series, one would expect the product to be methyl 4,6-*O*-benzylidene-3-deoxy-3-phenylazo-β-D-galactoside (IX).

Hydrogenation of the yellow product over Raney nickel gave the corresponding amino compound, characterised as its *N*-acetyl derivative The molecular rotation (−130°) of the amino compound, was close to that (−114°) of methyl 4,6-*O*-benzylidene-β-D-galactoside[9], suggesting that the amino compound has the *galacto*-configuration. (It is an empirical observation that replacement of a hydroxyl group

by an amino group in a sugar has little effect on the rotation[10].) Hydrolysis of the amino compound with aqueous acetic acid gave a pale-brown syrup which formed a crystalline hydrochloride, identical with methyl 3-amino-3-deoxy-β-D-galactoside hydrochloride prepared[11] by another route. Thus, the original yellow product was methyl 4,6-*O*-benzylidene-3-deoxy-3-phenylazo-β-D-galactoside (IX). As in the reaction leading to the corresponding glucoside, this was the only phenylazo compound formed. Ether extraction of the crude azo-compound left a residue of unchanged hemialdal (VII) (23%). Chromatography of the ethereal extract on alumina gave the phenylazogalactoside (IX) (39%), and a brown oil (19%) having a strong phenylhydrazone absorption at 1610 cm^{-1}.

The periodate-oxidised methyl 4,6-*O*-benzylidene-β-D-galactoside was next treated with *p*-bromophenylhydrazine, *p*-sulphamoylphenylhydrazine, and 2,5-dichlorophenylhydrazine to give, in each case, a bisarylhydrazone (XI)–(XIII), and not an arylazo sugar as was found on reaction with the corresponding periodate-oxidised α-D-glucoside[2]. The bisarylhydrazone structures were established by analysis and by ultraviolet spectroscopy (see Table I). Since the electronic factors will presumably be the same for the glucoside and galactoside, the difference in behaviour may be due to a steric effect.

Methyl 4,6-*O*-benzylidene-α-D-galactoside consumes one mol. of periodate[3], and treatment of the product with *p*-nitrophenylhydrazine has now yielded a bis-*p*-nitrophenylhydrazone. Reaction of the crude oxidation product with *p*-nitrobenzoyl chloride gave the hemialdal (VIII) di-*p*-nitrobenzoate, thus showing that the periodate-oxidation product can react in either the dialdehyde or hemialdal form.

Reaction of an aqueous solution of the periodate-oxidation product with phenylhydrazine yielded an unstable, yellow, amorphous product, which did not afford a crystalline acetate. Reaction of the oxidised galactoside with *p*-bromo- and with *p*-sulphamoyl-phenylhydrazine gave the bisarylhydrazones (XIV) and (XV); the elemental analyses, and ultraviolet (see Table I) and infrared spectra were consistent with the assigned structures. These results again differ from those found in the glucose series.

Thus, the reaction of periodate-oxidised carbohydrate derivatives with phenylhydrazine to give phenylazosugars is not a general one. It is interesting to note that some periodate-oxidised nucleosides (adenosine, cytidine, uridine, and guanosine) react with aqueous phenylhydrazine to give unstable, pale-yellow, amorphous products which analyse for bisphenylhydrazones[12].

EXPERIMENTAL

Chromatography was performed on alumina, type 'H', 100–200 mesh, supplied by P. Spence Ltd. Optical rotations are for chloroform solutions, unless otherwise stated. *N,N*-Dimethylformamide will be referred to as DMF. Acetic acid was removed, where necessary, by evaporation and co-distillation, first with water and then with ethanol. Ultraviolet spectra are recorded in Table I.

Periodate oxidation of methyl 4,6-O-benzylidene-β-D-glucoside

A solution of the glucoside (1.99 g) and sodium metaperiodate (4.49 g) in water (1 l) was stored at room temperature in the dark. The course of the oxidation was followed by the Müller–Friedberger[13] method, and, after 75 h, the theoretical uptake was achieved (although metaperiodate was still being consumed, but at a decreased rate). The solution was de-ionised[3] and evaporated, and the residue extracted with boiling chloroform. Evaporation of the extract, and three recrystallisations of the product from acetone–light petroleum gave 7(or 9)-ethoxy-9(or 7)-hydroxy-6β-methoxy-2-phenyl-*trans-m*-dioxano-[5,4-*e*][1:4]-dioxepan* (22%), m.p. 124–125°, $[\alpha]_D^{22}$ −96.5° (*c* 0.64) (Found: C, 58.5; H, 6.8. $C_{16}H_{22}O_7$ calc.: C, 58.9; H, 6.8%). Boiling the product in water for 5 min gave 7,9-dihydroxy-6β-methoxy-2-phenyl-*trans-m*-dioxano-[5,4-*e*][1:4]-dioxepan (II) hydrate, m.p. 108–109°, $[\alpha]_D^{22}$ −72.8° (*c* 0.79, DMF) (Found: C, 53.8; H, 6.5. $C_{14}H_{18}O_7 \cdot H_2O$ calc.: C, 53.2; H, 6.4%). When boiled under reflux with ethanol, followed by evaporation and recrystallisation, the hemialdal gave the monoethyl glycoside, m.p. 122–123°, $[\alpha]_D^{21}$ −96.1° (*c* 0.85).

Reaction of the monoethyl glycoside (0.30 g) in dry pyridine (10 ml) with *p*-nitrobenzoyl chloride (0.46 g) for 18 h at room temperature, and isolation in the usual way gave a white solid (0.37 g) which was recrystallised from propan-1-ol, to give 7(or 9)-ethoxy-6β-methoxy-9(or 7)-*p*-nitrobenzoyloxy-2-phenyl-*trans-m*-dioxano-[5,4-*e*][1:4]-dioxepan, m.p. 194–195°, $[\alpha]_D^{20}$ −158° (*c* 0.98, DMF) (Found: C, 58.35; H, 5.5; N, 2.9. $C_{23}H_{25}NO_{10}$ calc.: C, 58.1; H, 5.3; N, 2.9%).

Reactions of 7,9-dihydroxy-6β-methoxy-2-phenyl-trans-m-*dioxano*-[5,4-e][1:4]-*dioxepan (II)*

(*a*) *With methanol.* The dioxepan (1.0 g) was boiled under reflux with methanol (25 ml) for 2 h. Evaporation of the mother liquors gave a syrupy residue (0.9 g) which solidified on standing. After trituration with a little methanol, a white solid (0.15 g, 14%), m.p. 146–150°, was obtained, which was recrystallised from ethyl acetate to give 7(or 9)-hydroxy-6β,9(or 7)-dimethoxy-2-phenyl-*trans-m*-dioxano-[5,4-*e*][1:4]-dioxepan, m.p. 137–138°, $[\alpha]_D^{21}$ −29.5° (*c* 0.33, methanol) (Found: C, 57.3; H, 6.2. $C_{15}H_{20}O_7$ calc.: C, 57.7; H, 6.4%).

(*b*) *With* p-*nitrophenylhydrazine.* A solution of the dioxepan (0.10 g) and *p*-nitrophenylhydrazine (0.087 g) in ethanol (5 ml) was heated at 60° for 20 min. Concentration of the solution and storage at 0° gave orange crystals which were recrystallised from propan-1-ol to yield 2,4-*O*-benzylidene-3-*O*-(1β-methoxy-2-oxoethyl)-D-erythrose bis-*p*-nitrophenylhydrazone (72%), m.p. 163–166° (dec.), $[\alpha]_D^{23}$ +309° (*c* 0.06, methanol) (Found: C, 56.65; H, 4.9; N, 15.2. $C_{26}H_{26}N_6O_8$ calc.: C, 56.7; H, 4.8; N, 15.3%).

*For an explanation of this nomenclature, see ref. 4.

(*c*) *With phenylhydrazine.* To a solution of the dioxepan (0.10 g) in water (10 ml) at 60° was added phenylhydrazine (0.034 g). A white cloudiness was immediately formed, which turned yellow after 3 min. The mixture was cooled, saturated with sodium chloride, and stored at 0° overnight. A bright-yellow solid (0.12 g), m.p. 49–60°, was obtained which gave only oils and tars on attempted recrystallisation.

Catalytic hydrogenation of this product (0.48 g) in absolute ethanol (150 ml), with Raney nickel for 8 h at 75–85° and 50–60 atm., gave a colourless solution which was filtered and evaporated. Extraction of the residue with light petroleum, evaporation of the extract, and reaction of the yellow oil with 2,4-dinitrochlorobenzene gave 2,4-dinitrodiphenylamine, m.p. 156–157°.

Recrystallisation of the light petroleum-insoluble material gave methyl 3-amino-4,6-*O*-benzylidene-3-deoxy-β-D-glucoside, m.p. 200–201° (sublimed), $[\alpha]_D^{24}$ −69.2° (*c* 0.6) (Found: C, 59.3; H, 6.8. $C_{14}H_{19}NO_5$ calc.: C, 59.8; H, 6.8%). Acetylation of the light petroleum-insoluble fraction, with acetic anhydride in ethanol solution and recrystallisation of the product from ethanol, gave methyl 3-acetamido-4,6-*O*-benzylidene-3-deoxy-β-D-glucoside, m.p. 277–278° (dec., sublimed), $[\alpha]_D^{23}$ −80.8° (*c* 0.5, DMF) (Found: C, 59.5; H, 6.45. $C_{16}H_{21}NO_6$ calc.: C, 59.4; H, 6.55%).

A solution of the acetamidoglucoside (0.35 g) in 50% aqueous acetic acid (10 ml) was boiled under reflux for 30 min. The solution was cooled, and the acetic acid removed. With acetic anhydride–pyridine, the resulting off-white solid gave, after recrystallisation from chloroform–light petroleum (b.p. 40–60°), methyl 3-acetamido-3-deoxy-β-D-glucoside 2,4,6-triacetate, m.p. 156–156.5°, $[\alpha]_D^{22}$ −21.4° (*c* 2.1) {lit.[6], m.p. 160°, $[\alpha]_D$ −21.4°}.

(*d*) *With* p-*bromophenylhydrazine.* To a solution of the dioxepan (1.00 g) in water (50 ml) at 65° was added, with shaking, a filtered solution of *p*-bromophenylhydrazine hydrochloride (0.71 g) and sodium acetate (1.29 g) in water (50 ml). A white cloudiness (which rapidly turned yellow) formed immediately, and, after about 15 sec, the mixture was cooled in ice and then saturated with sodium chloride. Storage overnight gave a yellow solid (1.4 g), m.p. 87–98°. Attempted recrystallisation gave only oily material.

Catalytic hydrogenation of this product (1 g), as described above, gave a brown syrup which was extracted with benzene at room temperature. The extract was evaporated, and the residue treated with 2,4-dinitrochlorobenzene to yield, 4′-bromo-2,4-dinitrodiphenylamine, m.p. 156–157°.

The benzene-insoluble material (which gave a characteristic purple coloration with ninhydrin) was treated with acetic anhydride–pyridine for 5 min to give, after recrystallisation from ethanol, methyl 3-acetamido-4,6-*O*-benzylidene-3-deoxy-β-D-glucoside 2-acetate, m.p. 276–276.5° (dec., sublimed), $[\alpha]_D^{21}$ −96.2° (*c* 1.06) (Found: C, 59.25; H, 6.45; N, 4.0. $C_{18}H_{23}NO_7$ calc.: C, 59.2; H, 6.3; N, 3.8%).

A solution of the acetamidoglucoside acetate (0.10 g) in 50% aqueous acetic acid (3 ml) was boiled under reflux for 30 min. The solution was cooled and the acetic acid removed. With acetic anhydride–pyridine, the resulting straw-coloured

oil gave, after recrystallisation from benzene–light petroleum, methyl 3-acetamido-3-deoxy-β-D-glucoside 2,4,6-triacetate (77%), m.p. 155–156°, $[\alpha]_D^{21}$ −22.1° (*c* 1.3).

Reactions of 7,9-Dihydroxy-6α-methoxy-2-methyl-trans-m-*dioxano*-[*5,4*-e][*1,4*]-*dioxepan (III)*

(*a*) *With* p-*nitrobenzoyl chloride.* A mixture of the dioxepan (0.43 g) and *p*-nitrobenzoyl chloride (1.0 g) in dry pyridine (15 ml) was heated at 70–80° for 20 min. The solution was cooled and poured into saturated aqueous sodium hydrogen carbonate. The precipitate (0.90 g), m.p. 277–280°, was recrystallised from DMF to give 7,9-di-*p*-nitrobenzoyloxy-6α-methoxy-2-methyl-*trans-m*-dioxano-[5,4-*e*][1:4]-dioxepan, m.p. 284–285° (Found: C, 51.9; H, 4.2; N, 5.1. $C_{23}H_{22}N_2O_{13}$ calc.: C, 51.7; H, 4.1; N, 5.2%). The material was too insoluble for determination of $[\alpha]_D$.

(*b*) *With* p-*nitrophenylhydrazine.* A solution of the dioxepan (0.47 g) and *p*-nitrophenylhydrazine (0.58 g) in ethanol (12 ml) containing glacial acetic acid (2 drops) was heated under reflux for 2 h. The solution was evaporated to yield an orange foam which was crystallised from benzene–light petroleum to give 2,4-*O*-ethylidene-3-*O*-(1α-methoxy-2-oxoethyl)-D-erythrose bis-*p*-nitrophenylhydrazone (47%), m.p. 119–120°, $[\alpha]_D^{21}$ +251° (*c* 0.65, ethanol) (Found: C, 51.4; H, 5.1. $C_{21}H_{24}N_6O_8$ calc.: C, 51.6; H, 4.9%).

(*c*) *With phenylhydrazine.* To a solution of the dioxepan (4.72 g) in water (115 ml) at 45–50° was added phenylhydrazine (2.38 g). The mixture was maintained at 45° for about 1 min, then cooled in ice, and stored at 0° for 12 h. The bright-yellow precipitate (3.16 g), m.p. 45–62°, was collected, washed with water, and dried. A second crop (2.28 g) was obtained by ether-extraction of the mother liquors. Attempted recrystallisation gave only syrupy material.

Catalytic hydrogenation of the above material (2 g), as described above, gave a syrup which was extracted with petrol at room temperature. The petrol washings were combined with the ethanol distillate and the solution was boiled under reflux for 30 min with 2,4-dinitrochlorobenzene (1.31 g) and sodium acetate trihydrate (1.33 g). The solution was cooled and concentrated, water was added, and the mixture was ether-extracted; evaporation of the extract, and recrystallisation of the residue from ethanol and then from aqueous acetic acid, gave 2,4-dinitrodiphenylamine, m.p. 156–157°.

The light petroleum-insoluble material from the reduction was dissolved in absolute ethanol (3.5 ml) and acetic anhydride (2 ml) added. An immediate white precipitate (0.38 g) was obtained, which, after a further 5 min at room temperature, was put aside for 1 h at 0°. The solid was collected and recrystallised from ethanol and then from methanol to give methyl 3-acetamido-3-deoxy-4,6-*O*-ethylidene-α-D-glucoside, m.p. 320° (dec., sublimed) (Found: C, 50.2; H, 7.35; $C_{11}H_{19}NO_6$ calc.: C, 50.6; H, 7.3%).

The acetamido sugar (1.90 g) was treated with boiling 50% aqueous acetic acid (40 ml) for 40 min. The solution was cooled and the acetic acid removed. The residue was dissolved in pyridine (25 ml) and acetic anhydride (10 ml) was

added. After 48 h at room temperature, the solution was poured into water, and, after 30 min, extracted with chloroform. Concentration of the extract and recrystallisation of the residue (2.42 g) from ethanol gave methyl 3-acetamido-3-deoxy-α-D-glucoside 2,4,6-triacetate (76%), m.p. 182–184°, $[\alpha]_D^{20}$ +109° (*c* 1.1) {lit.[7], m.p. 179–180°, $[\alpha]_D^{20}$ +110° (*c* 2)}.

(*d*) *With* p-*bromophenylhydrazine.* To a solution of the dioxepan (4.01 g) in DMF–water (10:1, 34 ml) at room temperature was added a solution of *p*-bromophenylhydrazine hydrochloride (3.80 g) and sodium acetate (4.63 g) in the same solvent mixture (68 ml). After storage at room temperature for 36 h, the rotation of the solution became constant. The solution was poured into ice-water (400 ml) to give a flocculent, yellow solid. Sufficient sodium chloride was added to saturate the mixture, which was stored overnight at 0°. The wide-melting product (5.71 g) was recrystallised from light petroleum (46% yield) and then from aqueous alcohol to give methyl 3-*p*-bromophenylazo-3-deoxy-4,6-*O*-ethylidene-α-D-glucoside, m.p. 143–144°, $[\alpha]_D^{20}$ +103.7° (*c* 1.07, ethanol) (Found: C, 46.4; H, 5.0; N, 7.3. $C_{15}H_{19}BrN_2O_5$ calc.: C, 46.5; H, 4.9; N, 7.2%). The product did not form a formazan.

(*e*) *With 2,5-dichlorophenylhydrazine.* To a solution of the dioxepan (0.47 g) in DMF–water (10:1, 5 ml) was added at room temperature a solution of 2,5-dichlorophenylhydrazine hydrochloride (0.43 g) and sodium acetate (0.82 g) in the same solvent mixture (15 ml). The rotation of the solution became constant after 7.5 days. The mixture was worked up as in (*d*) to give a yellow solid (0.48 g), m.p. 76–100°, which was recrystallised from light petroleum and then from ethanol to give methyl 3-(2,5-dichlorophenylazo)-3-deoxy-4,6-*O*-ethylidene-α-D-glucoside, m.p. 152.5–154°, $[\alpha]_D^{22}$ +159° (*c* 0.65, ethanol) (Found: C, 47.95; H, 5.0; N, 7.3. $C_{15}H_{18}Cl_2N_2O_5$ calc.: C, 47.8; H, 4.8; N, 7.4%). The product did not form a formazan.

*Periodate oxidation of methyl 4,6-*O*-benzylidene-β-*D*-galactopyranoside*

Sodium metaperiodate (4.18 g) was added to a solution of methyl 4,6-*O*-benzylidene-β-D-galactopyranoside (5.64 g) in water (125 ml). The solution was stored at room temperature in complete darkness for 3 days. The white, crystalline product was collected, washed, and dried (P_2O_5). Recrystallisation from a small volume of water gave 7,9-dihydroxy-6β-methoxy-2-phenyl-*cis*-*m*-dioxano-[5,4-*e*][1:4]-dioxepan (VII) (96%), m.p. 118–119°, $[\alpha]_D^{22}$ −51° (*c* 0.61, chloroform–DMF 3:1) (lit.[3], m.p. 118–119°).

The product gave a di-*p*-nitrobenzoate, m.p. 310–312° (from DMF) (Found: C, 56.0; H, 4.1. $C_{28}H_{24}N_2O_{13}$ calc.: C, 56.4; H, 4.0%). The compound was too insoluble for determination of $[\alpha]_D$.

*Reaction of 7,9-dihydroxy-6β-methoxy-2-phenyl-*cis*-m-dioxano-[5,4-e][1:4]-dioxepan with arylhydrazines*

(*a*) *With phenylhydrazine.* To a solution of the dioxepan (2.5 g) in water (175 ml) at 75° was added a solution of phenylhydrazine hydrochloride (1.42 g)

and sodium acetate (3.0 g) in water (100 ml) at 70°. Rapid cooling, with shaking, gave a bright-yellow solid (3.0 g) which was collected, washed with water, and dried (P_2O_5). Recrystallisation from propan-2-ol gave methyl 4,6-*O*-benzylidene-3-deoxy-3-phenylazo-β-D-galactoside (IX), (38%), m.p. 190–192°, $[\alpha]_D$ +106° (*c* 0.63) (Found: C, 64.6; H, 6.3; N, 7.6. $C_{20}H_{22}N_2O_5$ calc.: C, 64.9; H, 5.9; N, 7.6%).

The crude 3-phenylazogalactoside (3.0 g) was extracted with ether (200 ml), to give a yellow solution and a white solid (23%), m.p. 112–116°, whose infrared spectrum was similar to that of unchanged dioxepan. Reaction with *p*-nitrobenzoyl chloride in pyridine gave the hemialdal di-*p*-nitrobenzoate, m.p. 308–311° (from DMF).

The yellow solution was chromatographed to give with ether–propan-2-ol (98:2) a yellow component; evaporation of the solvent gave the 3-phenylazogalactoside (1.17 g, 39%; 50% based on used dioxepan), m.p. 189–191°. Further elution with ether–chloroform (1:1) gave a brown, sweet-smelling oil (0.57 g, 19%), ν_{max} 1610 cm^{-1} (strong). Acetylation of the 3-phenylazogalactoside with acetic anhydride–pyridine, in the usual manner, gave material, $[\alpha]_D^{22}$ +119° (*c* 0.98), which could not be crystallised.

Hydrogenation of the 3-phenylazogalactoside was carried out in absolute ethanol, in the presence of Raney nickel, for 8 h at 60–80 atm./70–90°. The colourless solution was evaporated and the residue recrystallised from propan-2-ol or chloroform–light petroleum to give methyl 3-amino-4,6-*O*-benzylidene-3-deoxy-β-D-galactoside (77%), m.p. 190.5–192° (dec.), $[\alpha]_D^{20}$ −36.4° (*c* 0.52) (Found: C, 59.7; H, 6.8; N, 5.2. $C_{14}H_{19}NO_5$ calc.: C, 59.8; H, 6.8; N, 5.0%).

Acetylation of the product, with acetic anhydride–pyridine at room temperature for 5 min, gave methyl 3-acetamido-4,6-*O*-benzylidene-3-deoxy-β-D-galactoside (64%), m.p. 228–230° (dec.) (from propan-2-ol), $[\alpha]_D^{22}$ +23.2° (*c* 0.77, water) (Found: C, 59.0; H, 6.5; N, 4.4. $C_{16}H_{21}NO_6$ calc.: C, 59.4; H, 6.6; N, 4.3%).

Methyl 3-amino-4,6-*O*-benzylidene-3-deoxy-β-D-galactoside was hydrolysed with 50% acetic acid at 100° for 30 min to give methyl 3-amino-3-deoxy-β-D-galactoside $[\alpha]_D^{20}$ −4.2° (*c* 2.73, ethanol). Trituration of the product with dilute ethanolic hydrochloric acid (2%) gave, on subsequent evaporation, methyl 3-amino-3-deoxy-β-D-galactoside hydrochloride, m.p. 218–225° (dec.), $[\alpha]_D^{22}$ −3.5° (*c* 0.73, water), R_{gn} 1.18, [lit.[11], m.p. 227° (dec.), $[\alpha]_D$ −3.1°. An authentic sample supplied by Dr. H.H. Baer had R_{gn} 1.19]. Treatment of an aqueous solution of the hydrochloride with an excess of Amberlite resin IR 4B(HO⁻form), and subsequent evaporation of the elutate *in vacuo*, gave methyl 3-amino-3-deoxy-β-D-galactoside, m.p. 171–173°, $[\alpha]_D$ −3.1° (*c* 0.56), R_{gn} 1.19; an authentic sample supplied by Dr. H.H. Baer had R_{gn} 1.20, 1.21 [lit.[11], m.p. 173–174° (dec.), $[\alpha]_D^{23}$ −3.7°, R_{gn} 1.38].

(*b*) *With substituted phenylhydrazines – general method.* To a solution of the dioxepan (1.0 mol.) in water at 60° was added a solution of the arylhydrazine hydrochloride in water containing an excess of sodium acetate at 70°. Rapid cooling, with shaking, gave a solid product which was purified. Yields quoted are based on arylhydrazine.

m-Nitrophenylhydrazine (1.6 mol.) gave 2,4-*O*-benzylidene-3-*O*-(1β-methoxy-2-oxoethyl)-D-threose bis-*m*-nitrophenylhydrazone, (42%), m.p. 109–111° (from benzene–light petroleum) (Found: C, 57.1; H, 5.2. $C_{26}H_{26}N_6O_8$ calc.: C, 56.7; H, 4.8%).

p-Bromophenylhydrazine (1.1 mol.) gave 2,4-*O*-benzylidene-3-*O*-(1β-methoxy-2-oxoethyl)-D-threose bis-*p*-bromophenylhydrazone (XI) (68%), m.p. 71.5–73° (dec.) (from aqueous DMF) $[\alpha]^{21}$ +52.3° (*c* 0.48, acetone) (Found: C, 50.4; H, 4.4; N, 9.1. $C_{26}H_{26}Br_2N_4O_4$ calc.: C, 50.5; H, 4.2; N, 9.1%).. The product gave a positive formazan reaction.

p-Sulphamoylphenylhydrazine (1.1 mol.) gave 2,4-*O*-benzylidene-3-*O*-(1β-methoxy-2-oxoethyl)-D-threose bis-*p*-sulphamoylphenylhydrazone (XII) (81%), m.p. 184–186° (dec.) [from ethanol:DMF:water (5:1:1)], $[\alpha]_D^{20}$ +20.8° (*c* 0.46, chloroform:DMF 1:1) (Found: C, 50.2; H, 4.95; N, 13.8. $C_{26}H_{30}N_6O_8S_2$ calc.: C, 50.5; H, 4.7; N, 13.6%). The product gave a positive formazan reaction.

2,5-Dichlorophenylhydrazine (1.1 mol.) gave 2,4-*O*-benzylidene-3-*O*-(1β-methoxy-2-oxoethyl)-D-threose bis-2,5-dichlorophenylhydrazone (XIII) (67%), m.p. 162–164° (dec.) (from propan-1-ol), $[\alpha]_D^{21}$ +154° (*c* 0.81, DMF) (Found: C, 52.0; H, 4.4; N, 8.9. $C_{26}H_{24}Cl_4N_4O_4$ calc.: C, 52.2; H, 4.1; N, 9.3%). The product gave a positive formazan reaction.

Periodate oxidation of methyl 4,6-O-benzylidene-α-D-galactopyranoside

Sodium metaperiodate (2.09 g) was added to a solution of methyl 4,6-*O*-benzylidene-α-D-galactopyranoside (2.82 g) in water (70 ml). The solution was kept at room temperature and in complete darkness for 4 days. Barium chloride dihydrate (1.22 g) was added and the precipitate collected. Sodium sulphate was added to the filtrate, barium sulphate was removed, and the filtrate was concentrated at 45–50° to yield a white foam (2.9 g) which was extracted successively with hot chloroform (100 ml) and hot acetone (100 ml). The combined extracts were concentrated to yield a white solid (2.55 g), m.p. 50–55°, for which no suitable recrystallisation solvent could be found. Treatment of the product with *p*-nitrobenzoyl chloride gave 7,9-di-*p*-nitrobenzoyloxy-6-α-methoxy-2-phenyl-*cis*-*m*-dioxano-[5,4-*e*][1:4]-dioxepan m.p. 122–125° (from propan-1-ol), $[\alpha]_D^{25}$ +66.4° (*c* 0.87) (Found: C, 56.1; H, 4.0. $C_{28}H_{24}N_2O_{13}$ calc.: C, 56.4; H, 4.0%).

Reaction of 7,9-dihydroxy-6α-methoxy-2-phenyl-cis-m-*dioxano*-[5,4-e][*1:4*]-*dioxepan with arylhydrazines*

(*a*) *With phenylhydrazine.* Reaction of the dioxepan with phenylhydrazine (1.1 mol.), as described for the β-isomer, gave a yellow solid (m.p. 56–64°) that decomposed rapidly to an orange gum. No suitable solvent for recrystallisation could be found. Reaction of the product with acetic anhydride or *p*-nitrobenzoyl chloride in pyridine gave only intractable gums. Chromatography of the crude product (2 g) in ether on alumina gave two brown tarry fractions (0.4 g, 20%) and

(1.1 g, 55%). Each fraction had a strong infrared absorption at 1610 cm^{-1}, and gave positive formazan reactions.

Catalytic hydrogenation of the crude yellow product in ethanol in the presence of Raney nickel for 8 h at 80–100 atm/100° gave a brown gum, which gave a positive formazan reaction. Acetylation of the gum in acetic anhydride and pyridine at room temperature for 41 h gave, on pouring into ice-water, a sticky brown gum. The gum was extracted with chloroform, and the extracts were washed successively with dilute hydrochloric acid, saturated aqueous sodium hydrogen carbonate, and water. The dried extract was concentrated to give a dark-brown gum which failed to crystallise.

(*b*) *With substituted phenylhydrazines.* The general method was that used for the corresponding β-anomer. The products could not be recrystallised, and were purified by repeated precipitation by water from DMF solutions. All products gave a positive formazan reaction.

p-Nitrophenylhydrazine (excess) gave 2,4-*O*-benzylidene-3-*O*-(1α-methoxy-2-oxoethyl)-D-threose bis-*p*-nitrophenylhydrazone (68%), m.p. 106°. (Found: C, 57.0; H, 5.1. $C_{26}H_{26}N_6O_8$ calc.: C, 56.7; H, 4.8%).

p-Bromophenylhydrazine (1.1 mol.) gave 2,4-*O*-benzylidene-3-*O*-(1α-methoxy-2-oxoethyl)-D-threose bis-*p*-bromophenylhydrazone (XIV) (86%), m.p. 68–70°, $[\alpha]_D^{22}$ +87.3° (*c* 0.63) (Found: C, 50.85; H, 3.9; N, 9.1. $C_{26}H_{26}Br_2N_2O_4$ calc.: C, 50.5; H, 4.2; N, 9.1%).

p-Sulphamoylphenylhydrazine (1.1 mol.) yielded 2,4-*O*-benzylidene-3-*O*-(1α-methoxy-2-oxoethyl)-D-threose bis-*p*-sulphamoylphenylhydrazone (XV) (47%), m.p. 114–116° (dec.), $[\alpha]_D^{25}$ +13.3° (*c* 0.83, DMF) (Found: C, 50.4; H, 4.9; N, 13.6. $C_{26}H_{30}N_6O_8S_2$ calc.: C, 50.5; H, 4.7; N, 13.6%).

Methyl 4,6-O-benzylidene-3-deoxy-3-(2,5-dichlorophenylazo)-α-D-glucoside

Reaction of periodate-oxidised methyl 4,6-*O*-benzylidene-α-D-glucoside (1 mol.) with 2,5-dichlorophenylhydrazine hydrochloride (1.1 mol.) in aqueous sodium acetate, as described for phenylhydrazine[2], gave, on rapid cooling and shaking, a yellow solid (66%). Four recrystallisations from propan-2-ol, and finally from ethanol–acetone, gave methyl 4,6-*O*-benzylidene-3-deoxy-3-(2,5-dichlorophenylazo)-α-D-glucoside, m.p. 192–193°, $[\alpha]_D^{26}$ +6.6° (*c* 0.94) (Found: C, 54.2; H, 4.5; N, 6.5. $C_{20}H_{20}N_2O_5Cl_2$ calc.: C, 54.6; H, 4.6; N, 6.4%).

Catalytic hydrogenation of the product under the conditions described above gave methyl 3-amino-4,6-*O*-benzylidene-3-deoxy-α-D-glucoside (71%), m.p. 186° (dec., sublimed), $[\alpha]_D^{22}$ +102° (*c* 1.02) (lit.[1], m.p. 184.5–186°, $[\alpha]_D^{20}$ +102°).

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SUMMARY

The reaction of several periodate-oxidised methyl 4,6-*O*-ethylidene- and -benzylidene-glycosides of D-glucose and D-galactose with arylhydrazines is described. The only fully characterised arylazo sugar that resulted was methyl 4,6-*O*-benzylidene-3-deoxy-3-phenylazo-β-D-galactoside.

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NFRARED SPECTRA OF THE SULFONIC ESTERS OF MONOSACCHARIDES*

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INTRODUCTION

Absorption peaks in the 730–960 cm^{-1} region in the infrared spectra of pyranoid sugar derivatives have been correlated with various stereochemical features in the molecule. Axial and equatorial orientations of substituents at the anomeric carbon atom on the pyranose ring have been studied extensively by infrared spectroscopy[1]. Infrared spectroscopic analysis has also been applied to study of the disposition of sulfate ester groups at positions other than C-1 on the aldopyranose ring. Orr[2] reported infrared spectroscopic studies of some sulfated polysaccharides of biological interest. Lloyd and co-workers[3–5] have reported on various carbohydrate sulfate esters, with particular discussion of the C–O–S vibration. They suggested that the absorption in the 820–850 cm^{-1} region could be assigned to the C–O–S vibration mode of the C–O–SO_3^- group, citing Guthrie and Spedding's work[6] that sulfonic ester groups on the pyranose ring of monosaccharides do not absorb in the corresponding region. However, this work dealt only with compounds which involved both sulfonic and nitric ester groups in the same molecule. The same observation was reported by Anderson *et al.*[7]. As a matter of fact, the C–O–N vibration seems to show absorption similar to that of the C–O–S vibration in the 800–900 cm^{-1} region. These absorption peaks are generally weak and are sometimes absent altogether. Derivatives which show a typical difference of infrared absorption peak depending upon the axial or equatorial orientation of a substituent on the pyranose ring would be of interest.

The present paper is concerned with the orientation of the C–O–SO_2–R group (R = benzyl or alkyl group) at C-2, C-3, C-4, and C-6 of the pyranose ring of monosaccharides as revealed by study of the 800–900 cm^{-1} region in the infrared spectra of the sulfonic esters of carbohydrates. The analysis shows a characteristic difference in this spectral region between sulfonyloxy groups oriented axially and those oriented equatorially.

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EXPERIMENTAL

General

Infrared spectra were measured with a Shimadzu AR-6 spectrophotometer (sodium chloride optics). All compounds were examined as pressed discs in potassium bromide. All melting points are uncorrected.

Methyl 2-acetamido-4,6-O-benzylidene-2-deoxy-3-O-(methylsulfonyl)-α-D-allopyranoside (I)

Methyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-α-D-allopyranoside (I′) was prepared by the method of Jeanloz[8], m.p. 207°, $[\alpha]_D^{20}$ +65° (*c* 1.0, chloroform). The derivative (0.3 g) was dissolved in anhydrous pyridine (10 ml) in a 50-ml flask fitted with a calcium chloride tube. The solution was cooled to 0°, and a solution of methanesulfonyl chloride (0.2 ml) in anhydrous pyridine (5 ml) was added at 0°. After standing for 2 days at 0°, the mixture was poured on to cracked ice. The solution was extracted three times with 20-ml portions of chloroform. The chloroform layer was washed with water, saturated sodium hydrogen carbonate solution, water, 3 N hydrochloric acid, and water, dried (anhydrous sodium sulfate), and evaporated under reduced pressure to dryness. The residue was dissolved in the minimum volume of acetone; addition of petroleum ether (b.p. 40–60°) produced crystals; yield 0.3 g, m.p. 176°, $[\alpha]_D^{20}$ +37° (*c* 1.0, chloroform).

Anal. Calc. for $C_{17}H_{23}NO_8S$: C, 50.86; H, 5.77; N, 3.49; S, 7.98. Found: C, 51.13; H, 5.64; N, 3.22; S, 7.92.

Methyl 2-acetamido-2-deoxy-3-O-(methylsulfonyl)-α-D-allopyranoside (II)

Compound I (0.2 g) was treated by the procedure described by Jeanloz[8] to give methyl 2-acetamido-2-deoxy-3-*O*-(methylsulfonyl)-α-D-allopyranoside (II) as an amorphous powder; yield 0.1 g, $[\alpha]_D^{22}$ +54° (*c* 0.5, water).

Anal. Calc. for $C_{10}H_{19}NO_8S \cdot H_2O$: C, 36.25; H, 6.39; N, 4.23; S, 9.67. Found: C, 36.56; H, 6.52; N, 4.10; S, 9.75.

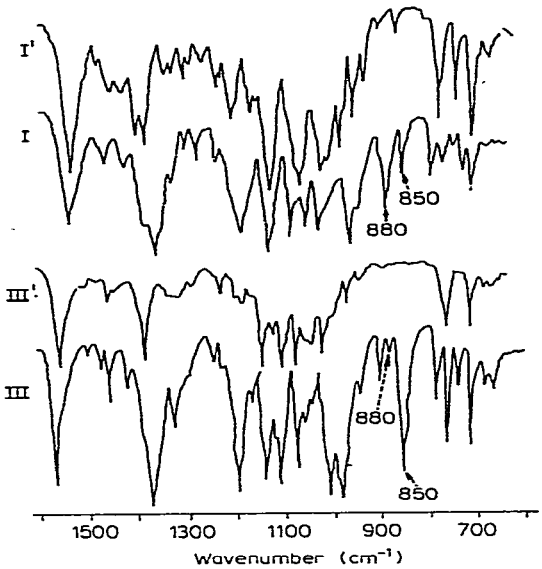
The other sulfonic esters of the hexopyranoses were prepared according to methods given in the references. Details of the spectra of these derivatives are shown in Fig. 1 and Table 1.

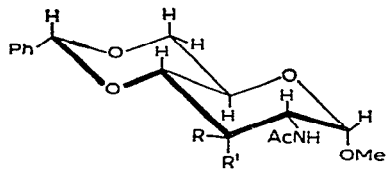
RESULTS AND DISCUSSION

C–O–S Vibration

As shown in Fig. 1 and Table 1, the spectra of all the sulfonic esters of hexopyranoses examined exhibited two strong absorptions at 1,170–1,190 cm^{-1} and at 1,350–1,370 cm^{-1}, due to the symmetrical and asymmetrical stretching vibrations of the $-SO_2-$ group[1,9–11]. They also showed a series of absorptions in the 700–900 cm^{-1} region, where the parent compounds show only slight or no absorption. It seems difficult and unfruitful to analyze the bands at wavenumbers shorter

than 800 cm^{-1}, because of the strong contributions from many kinds of vibration modes, including those due to the pyranose ring of monosaccharides, the phenyl group, and other substituents. However, the appearance in all the spectra of a single band in the 840–890 cm^{-1} region is notable.





Compounds	R	R'
I	H	OSO_2CH_3
I'	H	OH
III	OSO_2CH_3	H
III'	OH	H

Fig. 1. Infrared spectra of the methanesulfonates of methyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-α-D-allopyranoside and -glucopyranoside in the 600–1,600 cm^{-1} region. I, Methyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-3-*O*-(methylsulfonyl)-α-D-allopyranoside (see Experimental); I', methyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-α-D-allopyranoside (see Experimental); III, methyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-3-*O*-(methylsulfonyl)-α-D-glucopyranoside[8], m.p. 208°; III', methyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-α-D-glucopyranoside[8], m.p. 255°. Details of the absorption frequencies of the C–O–S vibration are described in the text.

The assignment of this absorption is considered to be the C–O–SO_2–R group; this is further divided into C–O–S, C–S, C–R, and S=O vibration modes, since the parent compounds show no absorption in the region mentioned above. A study of the published spectra of other organic compounds reveals that the C–S stretching vibration is mainly assigned to absorption at wavenumbers shorter than 800 cm^{-1} and that the C–H out-of-plane vibrations of monosubstituted and 1,4-disubstituted benzene rings are near 720–780 cm^{-1} and 820 cm^{-1}, respectively. The absorption frequency of the band in the 840–890 cm^{-1} region was unchanged with variations in the kind of hexopyranose in the kind and position of the sulfonyloxy group on the pyranose ring, and in the kind of phase used for measurement of the spectra.

These facts exclude the possibility of assigning both the C–H vibration of the pyranose ring and the C–S and CH_3 vibrations of the substituents on the benzene ring to this absorption, but permit assignment of the absorption to the C–O–S vibration. The assignment is supported by the infrared spectroscopic observations

TABLE I

ABSORPTION FREQUENCIES OF THE SULFONIC ESTERS OF SOME HEXOPYRANOSE DERIVATIVES IN THE 800–900 cm^{-1} REGION

Compound[a]	*Absorption peaks*[b] (cm^{-1})					
II Methyl 2-acetamido-2-deoxy-3-*O*-(methylsulfonyl)-α-D-allopyranoside		835 w	855 w		890 vs	
IV Methyl 2-acetamido-2-deoxy-3-*O*-(methylsulfonyl)-α-D-glucopyranoside			860 vs		890[c] vw	
V 1,3,4,6-Tetra-*O*-acetyl-2-*O*-(methylsulfonyl)-α-D-glucopyranose			850 vs		880 w	
VI 1,3,4,6-Tetra-*O*-acetyl-2-*O*-(methylsulfonyl)-α-D-galactopyranose		840[c] m	860 s		890 vw	
VII Methyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-3-*O*-*p*-tolylsulfonyl-α-D-glucopyranoside	820 m		850[c] s	860 vs	880 w	
VIII 6-*O*-*p*-Tolylsulfonyl-β-D-galactopyranose	820 s		855 vs		880 vw	
IX 1,2:3,4-Di-*O*-isopropylidene-6-*O*-*p*-tolylsulfonyl-α-D-galactopyranose	820 s		845 vs		880[c] vw	
X Methyl 6-*O*-*p*-tolylsulfonyl-α-D-glucopyranoside	820 vs		845 s		875 w	
XI 1,2,3,4-Tetra-*O*-acetyl-6-*O*-*p*-tolylsulfonyl-β-D-glucopyranose	820 s		840 s	850 vw	880 w	890[c] w
XII 1,3,4-Tri-*O*-acetyl-2-anisylideneamino-2-deoxy-6-*O*-*p*-tolylsulfonyl-β-D-glucopyranose	813 w		840 vs	855 w	880 w	
XIII Methyl 4,6-*O*-benzylidene-2,3-di-*O*-(methylsulfonyl)-α-D-glucopyranoside			845 vs		880 vw	
XIV 1,3,4-Tri-*O*-acetyl-2-benzamido-2-deoxy-6-*O*-*p*-tolylsulfonyl-β-D-glucopyranose	820 s		840 s	865 w	880 w	895 vw
XV 2-Acetamido-4,6-*O*-benzylidene-3-*O*-benzylsulfonyl-2-deoxy-D-glucopyranose			840 s		880 vw	
XVI 2-Acetamido-1,3-di-*O*-acetyl-2-deoxy-4-*O*-*p*-tolylsulfonyl-6-*O*-trityl-β-D-glucopyranose	820 m		840 s		880 vw	
XVII 2-Acetamido-1,3-di-*O*-acetyl-2-deoxy-4-*O*-(methylsulfonyl)-6-*O*-trityl-D-glucopyranose			840 vs		880 w	

[a] II, see Experimental; IV, m.p. 186°, prepared by removal[8] of the benzylidene group from III; V[13], m.p. 80–84°; VI[13], m.p. 96–98°; VII[14], m.p. 192°; VIII[15], m.p. 135°; IX[16], m.p. 91°; X[17], m.p. 124°; XI[18], m.p. 195°; XII[19], m.p. 200°; XIII[20], m.p. 186°; XIV[21], m.p. 143–146°, prepared by the *p*-toluenesulfonation and acetylation of 2-benzamido-2-deoxy-D-glucopyranose, m.p. 196–199° according to the usual procedure; XV[22], m.p. 90°, prepared by the benzylsulfonation of 2-acetamido-4,6-*O*-benzylidene-2-deoxy-D-glucopyranose m.p. 230°; XVI[23], m.p. 120°, prepared by the *p*-toluenesulfonation of 2-acetamido-1,3-di-*O*-acetyl-2-deoxy-6-*O*-trityl-D-glucopyranose, m.p. 105–107°, which was prepared by the tritylation of 2-acetamido-1,3-di-*O*-acetyl-2-deoxy-D-glucopyranose; XVII[23], m.p. 171°, prepared by the methanesulfonation of 2-acetamido-1,3-di-*O*-acetyl-2-deoxy-6-*O*-trityl-D-glucopyranose.

[b] m = moderate; s = strong; v = very; w = weak.

[c] shoulder.

on sulfates of monosaccharides[3,4], alkylsulfuric esters[5], and some sulfonic esters of cyclohexanol[24].

From these data, the characteristic absorption band in the 840–890 cm^{-1} region is tentatively assigned to the C–O–S vibration mode of the C–O–SO_2–R group in the pyranose ring, but it is open to confirmation by study of types of sulfonic esters other than those of the pyranoses.

On the shift in the absorption frequency of the C–O–S vibration

According to Mills[12], the stable conformation of 4,6-*O*-benzylidene-D-glucopyranose is a rigid, fused-ring structure of the *trans*-decalin type. Therefore, it is believed that compound I, methyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-3-*O*-(methylsulfonyl)-α-D-glucopyranoside (III), and their unbenzylidenated parents (II and IV) have similar stable conformations. The orientation of the sulfonyloxy group of compound I is axial on the pyranose ring, whereas that of compound III is equatorial. There is no gross difference between the n.m.r. spectra of compounds I and III. Especially, no difference was observed in the chemical shift of the proton signal of the S–CH_3 group of the two compounds. This indicates that the n.m.r. spectra are not useful for determining the spatial orientation of methylsulfonyloxy groups on the pyranose ring of monosaccharides. On the other hand, a marked difference was observed in the absorption frequencies of the C–O–S vibration in the infrared spectra of the two compounds, as shown in Fig. 1. Compound I, having an axial sulfonyloxy group, shows a strong absorption band at 880 cm^{-1} and a very weak one at 850 cm^{-1}, whereas compound III, having an equatorial sulfonyloxy group, shows a strong absorption at 850 cm^{-1} and a very weak one at 880 cm^{-1}. This difference remained unchanged with variation in the phase used for the measurement (potassium bromide, chloroform, and Nujol). The same difference in the infrared spectra was observed to exist between the 3-methanesulfonates (II and IV) of methyl 2-acetamido-2-deoxy-α-D-allopyranoside and -glucopyranoside. All other sulfonic esters disposed equatorially on the pyranose ring of monosaccharides showed the characteristic absorption band of the C–O–S vibration at 845 $\pm$ 5 cm^{-1}, as shown in Table 1. These results suggest that the observed shift in frequency is associated only with the difference in the spatial orientation of the sulfonyloxy group on the pyranose ring, as suggested earlier for the sulfates of carbohydrates[2–4]. For confirmation, the axial sulfonyloxy group at C-2 and C-4 of the pyranose ring should be studied.

From these results, it is concluded that axial sulfonyloxy groups on pyranose rings show a strong absorption band at 880–890 cm^{-1} and a weak absorption band at 840–850 cm^{-1}, and that equatorial sulfonyloxy groups on the pyranose ring show a strong absorption band at 840–850 cm^{-1} and a weak one at 880–890 cm^{-1}. When the characteristic absorption bands of the sulfates are compared with those of the sulfonic esters in the 800–900 cm^{-1} region, it is observed that the bands of the former lie at lower wavenumbers (by about 10–40 cm^{-1}).

Infrared spectroscopic analysis of sulfonyloxy groups might be especially

useful for the conformational determination of oligo- and poly-saccharides of biological interest.

ACKNOWLEDGMENT

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SUMMARY

The orientation of sulfonyloxy groups at different carbon atoms of the pyranose ring of monosaccharides has been examined by analysis of infrared spectra in the 800–900 cm^{-1} region, with particular reference to axial and equatorial orientations of the sulfonyloxy groups. Axial sulfonyloxy groups show a strong absorption band at 880–890 cm^{-1}, whereas equatorial ones absorb at 840–850 cm^{-1}. The absorption frequency is not affected by variations in the kind of hexopyranose, by the kind and position of the sulfonyloxy groups on the pyranose ring, or by the kind of phase used for the measurement.

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THE INTRODUCTION OF UNSATURATED LINKAGES INTO SOME CARBOHYDRATES AND ALDITOL DERIVATIVES*

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INTRODUCTION

Corey and Winter[2] reported recently that cyclic thionocarbonates undergo desulphurisation and decarboxylation on treatment with trimethyl phosphite, with the stereospecific introduction of a double bond. This reaction offers a new route to the interesting class of compounds, unsaturated carbohydrates**.

Of the two methods described for the preparation of cyclic thionocarbonates from diols[2], that utilizing bis(imidazol-1-yl)-thione proceeds in high yield, but suffers from the relative inaccessibility of the reagent. The second method, in which the diol is treated successively with equimolar quantities of *n*-butyl-lithium, carbon disulphide, and methyl iodide is facile, but yields are not high. However, recovery of starting material is possible, and it may be recycled.

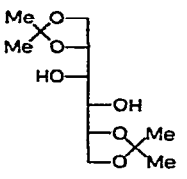
RESULTS AND DISCUSSION

1,2:5,6-Di-*O*-isopropylidene-D-mannitol[4] (I), having a *threo*-vicinal diol grouping, yielded the 3,4-thionocarbonate (II, Y = S) described by Baker and Sachdev[5] as one of the products from the reaction of compound (I) with phenyl isothiocyanate. Treatment of the thionocarbonate (II, Y = S) with refluxing trimethyl phosphite gave crystalline *trans*-3,4-didehydro-3,4-dideoxy-1,2:5,6-di-*O*-isopropylidene-D-*threo*-hexitol (III). The structures of this, and the other unsaturated compounds reported herein, were confirmed by ozonolysis, and reduction of the ozonides to alcohols which were then characterised as crystalline acyl derivatives. These derivatives were compared with authentic materials, prepared by lead tetra-acetate oxidation of the parent diol, followed by reduction, and acylation. Thus, the olefin (III) yielded 1,2-*O*-isopropylidene-D-glycerol (IV, R = H), which was isolated as its crystalline *p*-phenylazobenzoate (IV, R = $CO \cdot C_6H_4 \cdot N{:}NPh$). The reduction of the ozonide was performed in two ways. Firstly, a solution of the ozonide in ethyl acetate was shaken in an atmosphere of hydrogen over Adams'

*A preliminary communication on a part of this work has already been published[1].
**During the course of this work, the application of the reaction to 1,2-*O*-isopropylidene-α-D-glucofuranose was reported[3].

:atalyst, in order to liberate the carbonyl fragment, prior to borohydride reduction. n the second method, the catalytic hydrogenation step was omitted and the ozonide vas reduced directly with borohydride. The yields were 31% and 27%, respectively. The low yields in this and other ozonolysis experiments presumably reflect an ılternative breakdown pathway of the ozonides.

Reaction of 1,2:5,6-di-*O*-isopropylidene-D-altritol (talitol)[6] (V) (*erythro*-vicinal liol) gave the 3,4-thionocarbonate (VI, Y = S). The structures of this and other iew thionocarbonates were confirmed by their conversion (treatment with silver :arbonate in methanol) into the corresponding carbonate, in this case, the 3,4-



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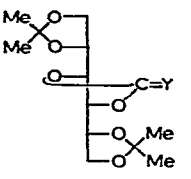

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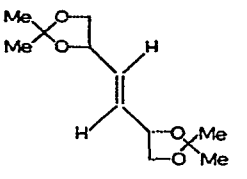

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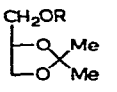

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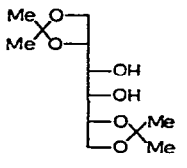

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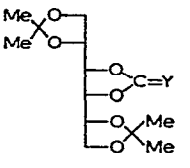

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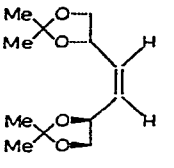

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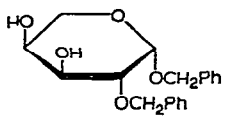

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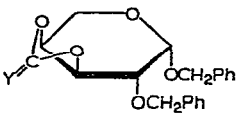

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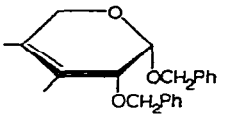

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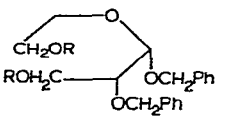

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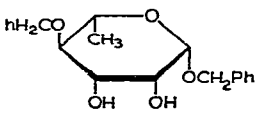

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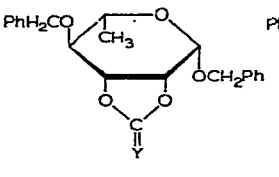

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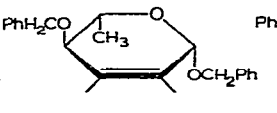

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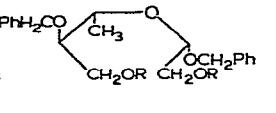

XV

arbonate (VI, Y = O). This product was identical to that obtained from the parent iol by treatment[7] with methyl chloroformate in pyridine–carbon tetrachloride, ollowed by cyclisation of the methyl carbonate with base in *N*,*N*-dimethylformamide.

Conversion of the thionocarbonate (VI, Y = S) into *cis*-3,4-didehydro-3,4-

dideoxy-1,2:5,6-di-*O*-isopropylidene-D-*threo*-hexitol (VII) occurred in trimethyl phosphite. Ozonolysis of this olefin, followed by reduction gave compound (IV, R = H), isolated in 13% yield as its *p*-phenylazobenzoate.

In order to investigate the utility of this reaction for the introduction of double bonds into pyranose and furanose sugars, two suitably blocked members of each of these classes, containing *cis*-vicinal diol groupings were sought. Thus, benzyl 2-*O*-benzyl-β-L-arabinopyranoside[8] (VIII) was converted into the 3,4-thionocarbonate (IX, Y = S), and thence into the low-melting olefin (X), in good yield. Isolation of the product from ozonolysis of olefin (X), followed by reduction, and benzoylation, gave 3-*O*-benzoyl-2-*O*-benzyl-D-glyceraldehyde (2-benzoyloxyethyl) benzyl acetal (XI, R = Bz) in 52% overall yield.

A furanose system which appeared suitable for the introduction of a 2,3-olefinic bond was that reported by Schmidt *et al.*[9]. These authors described the benzylation of 2,3-*O*-isopropylidene-L-rhamnose*, a compound believed to exist mainly in the furanose form, with benzyl chloride at 100° in the presence of potassium hydroxide. They reported that fractional crystallisation of the reaction mixture yielded a compound with m.p. 104°, $[\alpha]_D^{20}$ +30.3° (an isomer, m.p. 84°, $[\alpha]_D^{20}$ −15.4°, was also obtained). The former compound was formulated[9] as benzyl 5-*O*-benzyl-2,3-*O*-isopropylidene-L-rhamnofuranoside, of unspecified anomeric configuration, from which, selective removal of the isopropylidene group yielded benzyl 5-*O*-benzyl-L-rhamnofuranoside, m.p. 78°, $[\alpha]_D^{20}$ +48.2°.

Repetition of this benzylation has now given a product, m.p. 102–104°, $[\alpha]_D^{20}$ +30.1° (compound *A*) which, on partial hydrolysis, gave the 2,3-diol, m.p. 76–78°, $[\alpha]_D^{20}$ +47° (compound *C*). Investigation of the reaction mixture from the benzylation, by thin-layer chromatography in benzene, revealed two main components. One (R_F 0.3) corresponded to compound *A*, and a second component had R_F 0.6. Separation of the two substances on silica gel yielded firstly a compound (*B*, m.p. 95–97°, $[\alpha]_D^{23}$ −67.6°), analysing for a benzyl *O*-benzyl-*O*-isopropylidene-L-rhamnoside, and then compound *A*. Selective removal of the isopropylidene group of compound *B* gave a benzyl *O*-benzyl-L-rhamnoside, m.p. 86–88°, $[\alpha]_D^{25}$ −89° (compound *D*).

The structure and anomeric configuration of both series of compounds were further investigated. That they differed only in the configuration of the anomeric centre was shown when complete hydrolysis of compounds *A* and *B*, followed by reduction, gave the same *O*-benzyl-L-rhamnitol. This was identified as the 4-*O*-benzyl derivative when two mol. of sodium metaperiodate were consumed in a quantitative determination (the benzyl group must be on the 4 or 5 position). The original two compounds must thus have the pyranose structure. Authentic 5-*O*-benzyl-L-rhamnitol, synthesised by benzylation of the known 1,2:3,4-di-*O*-isopropylidene-L-rhamnitol[10], followed by acid hydrolysis, consumed three mol. of periodate per mol.

*See Experimental section for details of this compound.

Since compounds *C* and *D* (derived from compounds *A* and *B*, respectively, by partial hydrolysis) also differ only in their configuration at the anomeric centre, their structures may be assigned by reference to Hudson's rules[11]. Thus, the more laevorotatory isomers in the L-series, *i.e.* compounds *D* and *B*, have the α-configuration; compounds *C* and *A* have the β-configuration. Therefore compound *A* is benzyl 4-*O*-benzyl-2,3-*O*-isopropylidene-β-L-rhamnopyranoside. and compound *B* is the α-isomer. The isomer recorded by Schmidt *et al.*[9] which had m.p. 84°, $[\alpha]_D^{20}$ −15.4°, was presumably a mixture of compounds *A* and *B*, since, in the present work, the α-anomer could not be obtained pure by fractional crystallisation of the reaction product.

Treatment of benzyl 4-*O*-benzyl-β-L-rhamnopyranoside (XII, compound *C*) in the usual manner gave the thionocarbonate (XIII, Y = S), which was converted into benzyl 4-*O*-benzyl-2,3-didehydro-2,3,6-trideoxy-β-L-*erythro*-hexoside (XIV). The acyclic diol (XV, R = H), resulting from ozonolysis and reduction of olefin (XIV), was isolated as its benzoate (XV, R = Bz) in only 6% yield after three crystallisations. (Impure crystalline benzoate was obtained in 10% yield, but was difficult to purify). Thin-layer chromatography of the crude benzoate (before crystallisation) showed other substances to be present, but in smaller amounts; the olefin (XIV) appeared homogenous in two solvent systems. The benzoate (XV, R = Bz) was also obtained from the diol (XII, compound *C*), in the usual manner.

The n.m.r. spectra of the acyclic olefins (III) and (VII) confirmed the stereospecificity of the elimination. Thus, olefin (III) analysed correctly in the olefinic proton region as part of an A_2X_2 system, with a coupling constant between olefinic protons of 15.4 c.p.s. The olefin (VII) analysed in a similar manner and had a coupling constant of 10.95 c.p.s. These values are consistent with the expected arrangements, *i.e.* *trans*- and *cis*-, respectively, about the double bonds[12].

The olefinic protons of the cyclic olefin (X) appeared to be equivalent, since a singlet absorption (τ 4.33) was observed for the two protons. It would appear that $J_{2,3}$ and $J_{4,5}$ are zero, or too small to be resolved. The other cyclic olefin (XIV) showed a quartet (2 protons) at τ 4.15. If the coupling between the olefinic protons and protons on the adjacent carbon atoms is taken as zero [as appears to be true for compound (X)], then the spectrum may be analysed in the olefinic region as a simple AB type (*J*, 10.3 c.p.s.). This coupling constant is in agreement with the *cis*-arrangement about the unsaturated linkage[12].

None of these olefins show absorption in the 1650 cm^{-1} region of the infrared, although this is not to be expected of the acyclic olefins, because of their symmetry. Compounds (III) and (VII) absorbed 1.1 and 1.07 mol. of hydrogen, respectively, over Adams' catalyst; compound (X), in the presence of palladium on charcoal, absorbed 2.5 mol. in 20 min, and 3 mol. after 50 min. In the latter case, uptake continued slowly, presumably due to slow hydrogenation of the aromatic residues. All of the olefins reacted with dilute aqueous potassium permanganate to yield a yellow solution.

Levene and Compton[13] have shown that unimolar toluene-*p*-sulphonylation [at 0° in pyridine–chloroform (5:2; v/v)] of 2,3-*O*-isopropylidene-L-rhamnose (m.p. 93–94°) yields 2,3-*O*-isopropylidene-5-*O*-toluene-*p*-sulphonyl-L-rhamnofuranose in 40% yield. The formation of anomeric aldopyranose derivatives by the benzylation of 2,3-*O*-isopropylidene-L-rhamnose in benzyl chloride at 100° thus warrants further investigation. The formation of different ring isomers may reflect the varying proportions of the two ring forms in equilibrium at 0° and 100°, or may represent a difference in reactivity of ring hydroxyl and side-chain hydroxyl groups to benzylation and toluene-*p*-sulphonylation. Another, perhaps more important, factor is the relative stability of the two ring forms in different solvents. Kuhn and Grassner[14] have carried out investigations which suggest that the position of the furanose $\rightleftharpoons$ pyranose equilibrium may be significantly different in different solvents. Thus, for D-fructose, they found that the furanose form is favoured to a greater extent in dry *N*,*N*-dimethylformamide than in water–*N*,*N*-dimethylformamide mixtures; also, mutarotation of D-fructose occurred much more slowly in dry *N*,*N*-dimethylformamide than in water–*N*,*N*-dimethylformamide mixtures. The isolation of different ring isomers by toluene-*p*-sulphonylation in pyridine–chloroform, and benzylation in benzyl chloride, may be an example of the influence of the solvent on the position of the furanose $\rightleftharpoons$ pyranose equilibrium.

It is pertinent here to consider the work of Freudenburg and Wolf[15], who assigned a furanose structure to 2,3-*O*-isopropylidene-L-rhamnose {m.p. 87–89°, $[\alpha]_{578}^{20}$ +17.8° (water, equilibrium)}. Methylation with methyl iodide–silver oxide, followed by acid hydrolysis, yielded 5-*O*-methyl-L-rhamnose; a pyranose system would have yielded 4-*O*-methyl-L-rhamnose. Presumably, methylation proceeds more quickly than the furanose $\rightleftharpoons$ pyranose ring interconversion, since it is possible that the final position in this equilibrium may be similar in methyl iodide and benzyl chloride.

Data obtained by Perlin[16] from the n.m.r. spectrum of 2,3-*O*-isopropylidene-L-rhamnose in deuterium oxide support the furanose assignment already made on chemical evidence, but suggest the presence of a significant proportion of a different species in aqueous solution.

Therefore, for certain compounds which may exhibit this type of ring isomerism, and whose structures are inferred from reactions in solution, it may be necessary to consider the effect of the solvent on the position of the furanose $\rightleftharpoons$ pyranose equilibrium.

EXPERIMENTAL

Thin-layer chromatography was carried out on Kieselgel G with detection by sulphuric acid–vanillin[17]. Preparative chromatography was done on B.D.H. silica gel. Routine identifications were based on melting points, mixed melting points, and infrared spectra. Solutions were concentrated *in vacuo*, and dried with sodium sulphate, unless stated otherwise. Light petroleum refers to the fraction

of b.p. 60–80°. Proton magnetic resonance spectra were measured at 30° on a Perkin–Elmer R 10 instrument at 60 Mc.p.s. for carbon tetrachloride solutions. An internal reference of tetramethylsilane was used.

1,2: 5,6-Di-O-isopropylidene-D-mannitol 3,4-thionocarbonate (II, Y = S)

To a solution of 1,2:5,6-di-*O*-isopropylidene-D-mannitol[4] (I) (5.2 g) in sodium-dried tetrahydrofuran (50 ml) was added *n*-butyl-lithium in hexane (12.5 ml of a 15% solution). After 5 min, carbon disulphide (1.45 ml) was added, and the reaction mixture stored at room temperature for 0.5 h and then heated under reflux for 0.5 h. The mixture was cooled and methyl iodide (1.2 ml) added, and, after 0.5 h at room temperature, the solution was again heated under reflux for 0.5 h. The solution was concentrated to *ca.* half its original volume, and poured into ice-water (200 ml). The yellow precipitate was collected and recrystallised from ethanol–ethyl acetate to give colourless crystals of the product (1.53 g), m.p. 166–168°, $[\alpha]_D^{26}$ −15° (*c* 1.6, chloroform) {lit.[5], m.p. 160–161°, $[\alpha]_D$ −11° (*c* 0.3, chloroform)} (Found: C, 51.3; H, 7.0; S, 10.6. $C_{13}H_{20}O_6S$ calc.: C, 51.3; H, 6.6; S, 10.5%). A further yield of product (0.52 g), m.p. 163–165°, was obtained from the mother liquors.

The ice-water filtrate was extracted with chloroform (3 × 50 ml) and the combined, dried extracts were concentrated to give a residue which was crystallised from *n*-butyl ether to give the starting diol (1.44 g), m.p. 118–121°.

In a later preparation, the diol (5.2 g) yielded the thionocarbonate (3.45 g), m.p. 162–165°, contaminated with a small amount of the cyclic carbonate (C = O, ν_{max} 1800 cm^{-1}) which was difficult to remove by crystallisation.

trans-*3,4-Didehydro-3,4-dideoxy-1,2: 5,6-di-O-isopropylidene-D-threo-hexitol (III)*:

A solution of 1,2:5,6-di-*O*-isopropylidene-D-mannitol 3,4-thionocarbonate (2.0 g) in trimethyl phosphite (30 ml, redistilled before use) was heated under reflux for 70 h in an atmosphere of nitrogen. To the vigorously stirred solution was added 6N sodium hydroxide solution until, after prolonged stirring, the solution remained alkaline (40 ml). The mixture was extracted with chloroform (4 × 30 ml), and the combined extracts were washed with water (2 × 20 ml) and dried. Removal of the solvent afforded a residue which was crystallised from light petroleum to give the title compound (1.04 g, 67%), m.p. 80–82°, $[\alpha]_D^{26}$ +57.3° (*c* 1.3, chloroform) (Found: C, 62.7; H, 8.7. $C_{12}H_{20}O_4$ calc.: C, 63.1; H, 8.8%).

Ozonolysis of the olefin was carried out as follows. Purified ethyl acetate (40 ml) was cooled to −78° (acetone–Drikold) and ozonised oxygen bubbled through until a permanent blue colour was obtained. The olefin (0.10 g) in ethyl acetate (3 ml) was added, when most of the blue colour was discharged. The solution was removed from the cold bath and, after 10 min, excess of ozone removed by a stream of nitrogen. The solution was shaken in an atmosphere of hydrogen in the presence of Adams' catalyst for 1 h. The filtered solution was concentrated (bath temperature <30°), and the residue taken up in water (15 ml) and ethanol (5 ml). Potassium borohydride (0.1 g) was added and the solution stored overnight

at room temperature, then a further quantity of potassium borohydride (0.1 g) was added. After 0.5 h, the solution was neutralised to pH 7.5 with glacial acetic acid, and the solution extracted continuously with chloroform for 6 h. The dried extract gave, on concentration, a residue (0.079 g) which was dissolved in pyridine (2.5 ml) and treated overnight with *p*-phenylazobenzoyl chloride (0.37 g) in pyridine (5 ml)[18]. Isolation of the product in the usual manner gave, from methanol, 2,3-*O*-isopropylidene-1-*O*-*p*-phenylazobenzoyl-D-glycerol (0.073 g, 24% based on olefin), m.p. 91–92°, mixed m.p. 91–93°, infrared spectrum indistinguishable from that of the authentic compound. A further quantity (0.02 g), m.p. 88–91°, was obtained from the mother liquor.

A second ozonolysis, in which the catalytic hydrogenation step was omitted, gave the same *p*-phenylazobenzoate in 27% yield.

2,3-O-Isopropylidene-1-O-p-phenylazobenzoyl-D-glycerol (IV, R = CO·C$_6$H$_4$N:NPh)

2,3-*O*-Isopropylidene-D-glycerol[19] (0.46 g) was treated with *p*-phenylazobenzoyl chloride (0.97 g) in pyridine (10 ml), and yielded the product, (0.97 g, 77%), m.p. 92–94°, $[\alpha]_D^{21}$ +8.2° (*c* 1.4, acetone) (Found: C, 67.25; H, 5.7; N, 8.3. $C_{19}H_{20}N_2O_4$ calc.: C, 67.0; H, 5.9; N, 8.2%).

1,2 : 5,6-Di-O-isopropylidene-D-altritol 3,4-thionocarbonate (VI, Y = S)

1,2:5,6-Di-*O*-isopropylidene-D-altritol (talitol) (V) was prepared as previously described[6], and in one preparation exhibited anomalous behaviour on determination of the melting point. If heating was commenced below 35°, the compound had m.p. 62–64°, as previously recorded. If placed in the heating block above 45°, melting was immediate. On thin-layer chromatography in each of three different solvent systems, the compound appeared homogeneous. The unusual melting point behaviour was ascribed to dimorphism.

The diol (4.4 g) in tetrahydrofuran (40 ml) was treated successively with *n*-butyl-lithium in hexane (10.5 ml), carbon disulphide (1.22 ml), and methyl iodide (1.01 ml). The isolation procedure was similar to that described above for the mannitol thionocarbonate and yielded, after two crystallisations from light petroleum–benzene, the title compound (1.75 g, 52% on utilised diol), m.p. 120–122°, $[\alpha]_D^{21}$ −53.2° (*c* 2.14, chloroform). (Found: C, 51.4; H, 6.55; S, 10.65. $C_{13}H_{20}O_6S$ calc.: C, 51.3; H, 6.6; S, 10.5%). Extraction of the aqueous filtrate with chloroform gave the starting diol (1.5 g).

cis-*3,4-Didehydro-3,4-dideoxy-di*-O-*isopropylidene*-D-threo-*hexitol (VII)*

Treatment of the altritol thionocarbonate (1.38 g) in trimethyl phosphite (30 ml), as described above for the mannitol derivative, yielded the title compound as a colourless liquid (0.8 g, 77%), b.p. 84–86°/1 mm, $[\alpha]_D^{22}$ −8.1° (*c* 2.2, chloroform), n_D^{26} 1.4499. (Found: C, 63.1; H, 8.7. $C_{12}H_{20}O_4$ calc.: C, 63.1; H, 8.8%).

Ozonolysis of the olefin, as described above, followed by reduction (with catalytic hydrogenation), yielded a residue (0.067 g, 60%) which was *p*-phenyl-

azobenzoylated[18] to give 2,3-*O*-isopropylidene-1-*O*-*p*-phenylazobenzoyl-D-glycerol (0.038 g, 13% based on olefin), showing a double m.p. at 83° and 92–94°. The infrared spectrum and that of the authentic material were identical. The material showing double melting-point behaviour was redissolved in methanol and seeded with authentic compound, m.p. 92–94°. The material obtained had m.p. 92–94°, (without preliminary melting at 83°), and, with the authentic compound, mixed m.p. 92–94°.

1,2:5,6-Di-O-isopropylidene-D-altritol 3,4-carbonate (VI, Y = O)

(*a*) *From the diol.* 1,2:5,6-Di-*O*-isopropylidene-D-altritol[6] (V) (2.02 g) in pyridine (20 ml) was cooled in an ice-salt bath and a solution of methyl chloroformate (0.66 ml) in carbon tetrachloride (25 ml) added dropwise with stirring over 15 min. The mixture was stored overnight at 0° and then poured into a stirred mixture of ice-water (50 ml) and chloroform (50 ml). The organic layer was separated, and the aqueous layer further extracted with chloroform (3 × 20 ml). The combined extracts were washed with aqueous sodium hydrogen carbonate and water. Concentration of the dried solution gave a residue from which last traces of pyridine were removed by the repeated addition and distillation of toluene. Dry *N,N*-dimethylformamide (20 ml) and a catalytic quantity of sodium methoxide (*ca.* 0.025 g) were added, and the mixture heated at 100° for 15 min. The residue obtained on concentration was dissolved in chloroform, washed with dilute acetic acid and water, and then dried. Removal of the solvent and crystallisation of the residue from methanol gave the 3,4-carbonate (0.55 g, 25%), m.p. 175–177°, $[\alpha]_D^{21}$ −78.3° (*c* 1.7, chloroform) (Found: C, 54.3; H, 7.1. $C_{13}H_{20}O_7$ calc.: C, 54.15; H, 7.0%).

(*b*) *From the thionocarbonate.* 1,2:5,6-Di-*O*-isopropylidene-D-altritol 3,4-thionocarbonate (0.1 g) in methanol (20 ml) was stirred vigorously with silver carbonate for 2 h. The filtered solution was concentrated, clarified with charcoal in methanol, and crystallised from the same solvent to give the title compound (0.04 g, 42%), m.p. 175–177° (mixed m.p. 175–177°), infrared spectrum indistinguishable from that of the authentic compound.

Benzyl 2-O-benzyl-β-L-arabinopyranoside (VIII)

The hydrolysis is a modification of that due to Wold[8]. A mixture of benzyl 2-*O*-benzyl-3,4-*O*-isopropylidene-β-L-arabinopyranoside[8] (11.24 g) in water (80 ml) and glacial acetic acid (20 ml) was boiled under reflux for 2 h with vigorous stirring; complete solution was not achieved. The crystalline mass obtained on cooling was collected and dried *in vacuo* over phosphorus pentoxide to give the product (8.0 g, 85%), m.p. 130–131°, $[\alpha]_D^{26}$ +194° (*c* 0.75, chloroform) (Found: C, 69.35; H, 6.65. $C_{19}H_{22}O_5$ calc.: C, 69.1; H, 6.7%).

Benzyl 2-O-benzyl-β-L-arabinopyranoside 3,4-thionocarbonate (IX, Y = S)

The mixture obtained by treatment of benzyl 2-*O*-benzyl-β-L-arabinopyranoside (6.6 g) in tetrahydrofuran (50 ml) successively with *n*-butyl-lithium solution (12.3 ml),

carbon disulphide (1.45 ml), and methyl iodide (1.25 ml) was poured into water to give a yellow solid which was collected and dissolved in chloroform (60 ml). Light petroleum (60 ml) was added and, after storage at 0° for 1 h, the unchanged starting diol (2.2 g) was collected. The filtrate was concentrated, and the residue crystallised from benzene–light petroleum to give a further quantity of diol (0.77 g). The solvent was removed from the mother liquor, and the material crystallised from ethanol to give the title compound (2.95 g, 73% on utilised diol), m.p. 99–101°, $[\alpha]_D^{26}$ +175° (*c* 1.3, chloroform). (Found: C, 64.7; H, 5.7; S, 8.5%. $C_{20}H_{20}O_5S$ calc.: C, 64.5; H, 5.4; S, 8.6%).

*Benzyl 2-O-benzyl-3,4-didehydro-3,4-dideoxy-α-*D-glycero-*pentoside (X)*

Reaction of the above thionocarbonate (2.2 g) in trimethyl phosphite (35 ml) yielded, on crystallisation from ethyl acetate–light petroleum, the product (1.24 g, 73%), m.p. 33–35°, $[\alpha]_D^{26}$ +115° (*c* 1.9, chloroform) (Found: C, 76.55; H, 6.8. $C_{19}H_{20}O_3$ calc.: C, 77.0; H, 6.8%).

Ozonolysis of the olefin in the manner already described (without catalytic hydrogenation), followed by reduction, and benzoylation of the diol (0.097 g) so produced, gave 3-*O*-benzoyl-2-*O*-benzyl-D-glyceraldehyde (2-benzoyloxyethyl) benzyl acetal (XI) (0.094 g, 52% on olefin), m.p. 78–79°, alone or in admixture with the authentic compound. The infrared spectrum was indistinguishable from that of the authentic compound.

*3-O-Benzoyl-2-O-benzyl-*D*-glyceraldehyde (2-benzoyloxyethyl) benzyl acetal (XI, R = Bz)*

To a stirred solution of benzyl 2-*O*-benzyl-β-L-arabinopyranoside (0.66 g) in purified ethyl acetate (20 ml) was added lead tetra-acetate (0.886 g) over 15 min. The precipitated lead acetate was removed and the filtrate concentrated. The residue was dissolved in ethanol (20 ml) and water (10 ml), and potassium borohydride (0.7 g) added. After 12 h at room temperature, the solution was brought to pH 7.5 with glacial acetic acid and, after evaporation of the ethanol, was extracted with chloroform (4 × 50 ml). The combined extracts were washed with water (20 ml), dried, and concentrated to give the syrupy diol (XI, R = H) (0.59 g). Benzoylation in pyridine gave the product (0.35 g, 66%), m.p. 79–80°, $[\alpha]_D^{27}$ +40.3° (*c* 1.3, chloroform) (Found: C, 73.5; H, 5.95. $C_{33}H_{32}O_7$, calc.: C, 73.3; H, 6.0%).

*Benzyl 2-O-benzyl-β-*L*-arabinopyranoside 3,4-carbonate (IX, Y = O)*

(*a*) *From the diol.* Benzyl 2-*O*-benzyl-β-L-arabinopyranoside (1.1 g) was treated with methyl chloroformate (0.29 ml) in pyridine (15 ml) and carbon tetrachloride (10 ml), as previously described for the preparation of the altritol derivative. The product obtained after the cyclisation of the acyclic carbonate in *N,N*-dimethylformamide was crystallised from ethyl acetate–light petroleum to give the starting diol (0.39 g). The mother liquor was concentrated and the residue crystallised from methanol to yield a product (0.37 g, 48% on unrecovered diol), m.p. 83–89°,

which was recrystallised from methanol to give the title compound, m.p. 88–90°*, $[\alpha]_D^{28}$ +166° (*c* 1.0, chloroform) (Found: C, 67.0; H, 5.7. $C_{20}H_{20}O_6$ calc.: C, 67.4; H, 5.7%).

(*b*) *From the thionocarbonate.* Treatment of benzyl 2-*O*-benzyl-β-L-arabinopyranoside 3,4-thionocarbonate (0.25 g) in methanol (10 ml) and benzene 10 ml) with silver carbonate, in the same way as for the altritol thionocarbonate derivative, gave, from methanol, the carbonate (0.054 g, 23%), m.p. 86.5–87° (mixed m.p. 87–89°), infrared spectrum indistinguishable from that of the authentic compound.

Benzylation of 2,3-O-isopropylidene-L-rhamnose

2,3-*O*-Isopropylidene-L-rhamnose was prepared by the method already described[20], except that the residue obtained on concentration of the chloroform solution was not distilled. Instead, it was partitioned between ethyl acetate (100 ml) and saturated aqueous sodium hydrogen carbonate (35 ml). The aqueous layer was extracted with ethyl acetate (2 × 75 ml), and the combined organic solutions were washed with water (2 × 10 ml) and dried. Concentration of the solution gave a residue which was crystallised from ethyl acetate–light petroleum to yield 2,3-*O*-isopropylidene-L-rhamnose (15.4 g), m.p. 84–86°, $[\alpha]_D^{25}$ +17.6° (0.5 h) → +20.7° (15 h) (*c* 2.8, water)**. Further product (9.7 g) was obtained from the mother liquor and had m.p. 82–90°. Material of variable m.p. (83–90°) was obtained on recrystallisation; the m.p. was not consistently raised by further recrystallisations. After storage for several months over phosphorus pentoxide, the material had m.p. 90–93°.

The benzylation of 2,3-*O*-isopropylidene-L-rhamnose*** (m.p. 84–86°) was carried out as described by Schmidt *et al.*[9]. The isomer, m.p. 102–104°, $[\alpha]_D^{20}$ +30.1° (*c* 2.0, acetone) (lit.[9], m.p. 104°, $[\alpha]_D^{20}$ +30.3° in acetone) was obtained by fractional crystallisation of the reaction product. Two components of the crude benzylation product were detected by thin-layer chromatography in benzene. One (R_F 0.3) corresponded to the isomer with m.p. 102–104°, and the other had R_F 0.6, and was present apparently in the lesser amount. A preparative separation of the crude product (13.7 g) was carried out on silica gel (1.1 kg) using benzene–ether (24.7:0.3, v/v) and collecting 500 ml fractions. Fractions 18–32 gave the compound with R_F 0.6 (4.8 g), which on recrystallisation from methanol gave benzyl 4-*O*-benzyl-2,3-*O*-isopropylidene-α-L-rhamnopyranoside (1.9 g), m.p. 95–97°, $[\alpha]_D^{23}$ −67.6°

*The analytical sample showed a double melting point. On first determination, it had m.p. 84–86° and, after resolidification, m.p. 88–90°.

**Freudenberg and Wolf[15] describe two isomers of this compound; one had m.p. 87–89°, $[\alpha]_{578}^{?}$ + 13.5° (5 min) → + 17.8° (equilibrium, water), and the second had m.p. 79–80°, $[\alpha]_{578}^{20}$ + 10.9° (5 min) → + 17.6° (equilibrium, water). Both isomers give 5-*O*-methyl-L-rhamnose on methylation followed by hydrolysis[15,21], and have been formulated as β- and α-rhamnofuranose derivatives, respectively[21].

***Schmidt *et al.*[9] gave no constants for this compound, but gave reference to its method of preparation, from which it may be assumed that it had m.p. 90–91°.

(*c* 2.1, acetone) (Found: C, 71.9; H, 7.3. $C_{23}H_{28}O_5$ calc.: C, 71.85; H, 7.3%).

Further elution with benzene–ether (1:1, v/v) yielded the material with R_F 0.3 (7.0 g), which was crystallised from methanol to give benzyl 4-*O*-benzyl-2,3-*O*-isopropylidene-β-L-rhamnopyranoside (3.7 g), m.p. 102–104°.

Acid hydrolysis of benzyl 4-O-benzyl-2,3-O-isopropylidene-α- and β-L-rhamnopyranoside

(*a*) *Removal of the isopropylidene group.* A mixture of benzyl 4-*O*-benzyl-2,3-*O*-isopropylidene-α-L-rhamnopyranoside (0.86 g) and 0.05N hydrochloric acid (9 ml) was heated under reflux at 100°, and enough ethanol (*ca.* 10 ml) added to achieve solution. After 1 h, the solution was neutralised with sodium hydrogen carbonate and cooled to give colourless crystals which were collected and dried over phosphorus pentoxide *in vacuo*. Recrystallisation of the dried material from ethyl acetate–light petroleum gave benzyl 4-*O*-benzyl-α-L-rhamnopyranoside (0.47 g, 61%), m.p. 86–88°, $[\alpha]_D^{25}$ −88.8° (*c* 3.5, acetone) (Found: C, 69.7; H, 7.1. $C_{20}H_{24}O_5$ calc.: C, 69.7; H, 7.0%).

Similar treatment of benzyl 4-*O*-benzyl-2,3-*O*-isopropylidene-β-L-rhamnopyranoside (3.95 g) gave benzyl 4-*O*-benzyl-β-L-rhamnopyranoside (2.94 g, 83%), m.p. 76–78°, $[\alpha]_D^{20}$ +47° in acetone; lit.[9], m.p. 77.5°, $[\alpha]_D^{20}$ +48.2° in acetone.

(*b*) *Complete hydrolysis followed by reduction.* A solution of benzyl 4-*O*-benzyl-2,3-*O*-isopropylidene-α-L-rhamnopyranoside (1 g) in ethanol (20 ml) and N sulphuric acid (58 ml) was heated at 100°. Aliquots were taken at intervals and their rotations measured. After 6 h, the rotation was constant ($[\alpha]_D$ −24°) and sodium hydrogen carbonate was added until the solution was neutral. After the addition of potassium borohydride (0.5 g), the solution was stored overnight at room temperature. Glacial acetic acid was added until the pH was 7.5, and the solution was then concentrated. After repeated evaporation of methanol from the residue, it was dissolved in water (30 ml) and the solution was extracted continuously with chloroform for 5.5 h. On concentration, the dried extract gave an oil which crystallised from ethyl acetate to give a solid (0.25 g), m.p. 86–89°; two further recrystallisations from the same solvent gave 4-*O*-benzyl-L-rhamnitol, m.p. 90–91°, $[\alpha]_D^{23}$ −7.0° (*c.* 1.4, ethanol) (Found: C, 61.3; H, 8.0. $C_{13}H_{20}O_5$ calc.: C, 60.9; H, 7.9%). The compound reacted with 1.98 mol. of sodium metaperiodate per mol. in a quantitative determination.

Similarly, benzyl 4-*O*-benzyl-2,3-*O*-isopropylidene-β-L-rhamnopyranoside yielded 4-*O*-benzyl-L-rhamnitol, m.p. 90–91°, alone or in admixture with the compound from the above preparation. The infrared spectra of the products from the two preparations were indistinguishable.

5-O-Benzyl-L-rhamnitol

1,2:3,4-Di-*O*-isopropylidene-L-rhamnitol[10] (2.5 g) in benzyl chloride (10 ml) was stirred with powdered potassium hydroxide (5 g) at 100° for 5 h. Water (50 ml) was then added and the reaction mixture was extracted with chloroform (4 × 50 ml). The combined extracts were washed with water (20 ml) and the solution dried

(K_2CO_3) overnight. Concentration, finally at 80°/2 mm, gave a liquid which showed one main component (R_F 0.53) on thin-layer chromatography ($CHCl_3$), a small amount of starting material, and a fast-running compound (R_F 0.86). The liquid was distilled (b.p. 140–160°/0.6 mm) to yield a product containing no starting material, but which was still contaminated with the material with R_F 0.86. Chromatography on silica gel (35 g), using chloroform and collecting 20 ml fractions, gave the fast-running compound (0.05 g) in fractions 4–7. Elution with chloroform–ethanol (4:1, v/v) gave the chromatographically pure product (0.22 g). In a large scale preparation, the crude benzylation product (2.55 g) yielded, after chromatography, the required compound (R_F 0.53) (1.46 g, yield reduced by spillage), which was distilled to give 5-*O*-benzyl-1,2:3,4-di-*O*-isopropylidene-L-rhamnitol (1.1 g), b.p. 154–156°/0.6 mm, n_D 1.4851, $[\alpha]_D^{22}$ 0.0° (*c* 0.96, chloroform) (Found: C, 67.6; H, 8.1. $C_{19}H_{28}O_5$ calc.: C, 67.8; H, 8.4).

The above compound (0.61 g) in aqueous acetic acid (12 ml, 1:1, v/v) was boiled under reflux for 4 h. Concentration yielded a residue from which water was removed by repeated addition and evaporation of absolute alcohol. Alcohol was removed similarly with ethyl acetate. Crystallisation of the material from ethyl acetate gave 5-*O*-benzyl-L-rhamnitol (0.25 g, 54%), m.p. 143–146°, $[\alpha]_D^{22}$ +24.4° (*c* 1.2, ethanol) (Found: C, 60.7; H, 8.1. $C_{13}H_{20}O_5$ calc.: C, 60.9; H, 7.9%). The compound reacted with 2.9 mol. of sodium metaperiodate per mol.

Benzyl 4-O-benzyl-β-L-rhamnopyranoside 2,3-thionocarbonate (XIII, Y = S)

Benzyl 4-*O*-benzyl-β-L-rhamnopyranoside (2.94 g) in tetrahydrofuran (50 ml) was treated successively with *n*-butyl-lithium (5.3 ml), carbon disulphide (0.63 ml), and methyl iodide (0.54 ml), as described for the preparation of the mannitol thionocarbonate. The mixture was poured into ice-water (150 ml) and extracted with chloroform (3 × 50 ml). The extract was washed with water (10 ml), dried, and concentrated. The residue was dissolved in chloroform (10 ml) and light petroleum (40 ml) and, after storage at 0° overnight, the precipitated material was collected. Recrystallisation from absolute ethanol gave the product (0.79 g, 38% based on utilised diol), m.p. 141–142°, $[\alpha]_D^{28}$ +49.7° (*c* 1.4, chloroform) (Found: C, 65.0; H, 6.05; S, 8.4. $C_{21}H_{22}O_5S$ calc.: C, 65.25; H, 5.7; S, 8.3%).

Evaporation of the solvent from the mother liquor, and crystallisation of the residue from ethyl acetate–light petroleum gave the starting diol (1.1 g).

*Benzyl 4-O-benzyl-2,3-didehydro-2,3,6-trideoxy-β-L-*erythro*-hexoside (XIV)*

Treatment of benzyl 4-*O*-benzyl-β-L-rhamnopyranoside 2,3-thionocarbonate (0.76 g) with trimethyl phosphite (15 ml), in the manner previously described, gave a liquid which was distilled to yield the title compound (0.35 g, 57%), b.p. 200–210°/1 mm, $[\alpha]_D^{25}$ −73.5° (*c* 1.7, chloroform) (Found: C, 77.0; H, 7.0. $C_{20}H_{22}O_3$ calc.: C, 77.4; H, 7.2%). The olefin appeared homogeneous by thin-layer chromatography in chloroform and benzene. On storage at −15°, the product solidified.

Ozonolysis of the olefin (0.10 g), as described previously (without catalytic hydrogenation), gave a liquid (0.073 g) which was benzoylated in the usual manner. Thin-layer chromatography of the crude benzoate showed the presence of several components, with the expected product predominating. The crude product was treated in methanol with charcoal and the solution stored at −15°, when crystallisation occurred slowly. The solid (0.018 g) was collected, and two crystallisations from ethyl acetate–light petroleum gave 4-*O*-benzoyl-2-*O*-(2-benzoyloxy-1-benzyloxyethyl)-3-*O*-benzyl-1-deoxy-D-erythritol (XV, R = Bz) (0.010 g, 6% on olefin), m.p. 60–61° (mixed m.p. 60–62°). The infrared spectrum was indistinguishable from that of the authentic sample. No other product separated out on storage of the mother liquor at −15° for several weeks.

4-O-Benzoyl-2-O-(2-benzoyloxy-1-benzyloxyethyl)-3-O-benzyl-1-deoxy-D-erythritol (XV, R = Bz)

Benzyl 4-*O*-benzyl-β-L-rhamnopyranoside (0.24 g) was oxidised with lead tetra-acetate (0.31 g) in purified ethyl acetate (20 ml). Filtration and concentration of the reaction mixture gave a syrup. Toluene (5 ml) was distilled from the residue which was then dissolved in water (20 ml) and ethanol (20 ml), and sufficient sodium hydrogen carbonate added to make the solution alkaline. After the addition of potassium borohydride (0.3 g), the solution was stored at room temperature overnight. Glacial acetic acid was then added to adjust the pH to 7.5, and ethanol was removed by evaporation of the solution to half its original volume. Extraction with chloroform (5 × 20 ml), followed by concentration of the dried extract, gave a syrup (0.16 g) which was benzoylated in the usual manner. Crystallisation of the product twice from ethyl acetate–light petroleum yielded material (0.14 g, 55%), m.p. 59–62°. Two further crystallisations from methanol yielded the title compound, m.p. 60–62°, $[\alpha]_D^{28}$ +2.9° (*c* 1.55, chloroform) (Found: C, 73.7; H, 6.2. $C_{34}H_{34}O_7$ calc.: C, 73.6; H, 5.8%).

Benzyl 4-O-benzyl-β-L-rhamnopyranoside 2,3-carbonate (XIII, Y = O)

(*a*) *From the diol.* Benzyl 4-*O*-benzyl-β-L-rhamnopyranoside (1.24 g) was treated in pyridine (15 ml) and carbon tetrachloride (20 ml) with methyl chloroformate (0.31 ml) in carbon tetrachloride (15 ml), and the cyclic carbonate prepared and isolated as described previously. Attempted preferential crystallisation of the cyclic carbonate from methanol was unsuccessful. The reaction product was therefore chromatographed on silica gel (100 g) using chloroform–ether (9:1, v/v). The cyclic carbonate was eluted first and crystallised from ethyl acetate–light petroleum to yield the title compound (0.08 g, 14% on utilised diol), m.p. 92–93°, $[\alpha]_D^{25}$ +31.7° (*c* 1.9, chloroform) (Found: C, 67.8; H, 5.9. $C_{21}H_{22}O_6$ calc.: C, 68.1; H, 6.00).

Further elution of the column with chloroform–ether (1:1, v/v) gave the starting diol (0.71 g).

(*b*) *From the thionocarbonate.* A solution of benzyl 4-*O*-benzyl-β-L-rhamnopyranoside 2,3-thionocarbonate (0.117 g) in methanol (5 ml) and benzene

(5 ml) was shaken vigorously with silver carbonate (0.36 g) for 5 h. The filtered solution was concentrated, and the material crystallised from ethyl acetate–light petroleum to yield the carbonate (0.024 g, 21%), m.p. 91–93° (mixed m.p.), infrared spectrum indistinguishable from that of the authentic compound.

Test for unsaturation with aqueous potassium permanganate

Each of the four olefinic compounds described herein reacted with aqueous potassium permanganate to yield yellow solutions. Vigorous shaking was necessary due to the low solubility of the compounds in aqueous media.

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SUMMARY

The degradation of thionocarbonates, prepared from vicinal diols in the alditol series, and of *cis*-vicinal diols when part of an aldopyranose, has been investigated as a means of introducing unsaturation into molecules of the carbohydrate type.

The *O*-benzylation of mono-*O*-isopropylidene-L-rhamnose has been shown to give the anomeric benzyl 4-*O*-benzyl-2,3-*O*-isopropylidene-L-rhamnopyranosides. This contrasts with *O*-toluene-*p*-sulphonylation in pyridine, and *O*-methylation by Purdie's method, both of which yield 5-substituted 2,3-*O*-isopropylidene-L-rhamnofuranose derivatives.

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STUDIES ON STARCH-DEGRADING ENZYMES

PART II* THE Z-ENZYME FROM SOYA BEANS; PURIFICATION AND PROPERTIES

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INTRODUCTION

Z-Enzyme was first found to be associated with soya-bean β-amylase when amylose, which was incompletely hydrolysed by crystalline sweet-potato β-amylase, was *completely* degraded into maltose by the "purified" soya-bean enzyme[1,2]. Most samples of amylose are now known to contain some type of barrier to the action of pure β-amylase[3–5]. Peat *et al.*[2,6] initially suggested that Z-enzyme specifically removed this structural feature. However, by preferentially inhibiting the β-amylase, we established that Z-enzyme was α-amylolytic in character, and consequently non-specific with regard to its action on the barrier in amylose[7]. This general conclusion has been confirmed by later workers[8,9].

In this paper, we extend our earlier observations on Z-enzyme, and report a convenient method for isolating and extensively purifying this enzyme from soya beans. This has enabled us to make the first detailed studies of the properties of the purified enzyme.

EXPERIMENTAL

General analytical methods

Routine determinations of protein concentrations were made from absorption measurements at 280 mμ, the method being calibrated by micro-Kjeldahl estimations. The concentration of polysaccharide solutions was estimated by hydrolysis, and titration of the liberated glucose with alkaline ferricyanide[3]. Concentrations of reducing sugar in enzymic digests were estimated by the same reagent. β-Amylolysis limits, [β], were carried out as described earlier[3], except that crystalline sweet-potato β-amylase (Worthington Biochemical Corporation, New Jersey, U.S.A.) was used. (This enzyme was shown to be free from Z-enzyme activity.) The technique for measuring the limiting-viscosity number, [η], has been detailed elsewhere[10]; 0.2 M potassium hydroxide was used as solvent, and measurements were made at 25°.

* For Part I, see ref 31.

Substrates

Soluble starch ('Analar', B.D.H.) was used for estimation of β-amylase activity. Amylose ($[\eta] = 500$, $[\beta] = 80$) and amylopectin ($[\eta] = 180$, $[\beta] = 56$) were prepared[3] from a dispersion of potato starch (var. Redskin). *Linear* amylose ($[\eta] = 260$, $[\beta] = 100$) was obtained by aqueous leaching of potato-starch granules[3]. Glycogen ($[\beta] = 45$) was extracted from rabbit livers with cold trichloroacetic acid[11]. β-Limit dextrins from amylopectin and glycogen were obtained by dialysis and freeze-drying of the appropriate digests.

Digest conditions

Unless otherwise stated, digests were carried out at 35°, and the pH was controlled by acetate buffer (0.2 M, pH 5.5).

Measurement of enzymic activity

β-Amylase. Measurement was made of the amount of maltose produced in a 1 ml portion of a digest containing starch solution (0.6%, 25 ml), buffer (4 ml, pH 3.6), and enzyme (1 ml), after incubation for 30 min. Activities were then expressed as mg of maltose produced per mg of protein.

Z-Enzyme. A modification of the procedure devised by Briggs[12] was employed. The β-limit dextrin from amylopectin was used as substrate, and the "time reference point[12]" was chosen as the time when the corrected absorption value (A.V.) had fallen to 2.00 colorimeter units.

Digests were prepared from buffer (1 ml, pH 5.5), dextrin (amylopectin β-limit) solution (2 ml, 0.6%), enzyme solution, and water to give a total volume of 8 ml. Aliquot portions (2 ml) were removed at intervals and treated with iodine (1 ml, 0.2% in 2% potassium iodide) and hydrochloric acid (0.2 ml, 5 M), in a total volume of 50 ml. The A.V. was then measured at 540 mμ in an EEL-colorimeter (filter No. 625). Activities were expressed[12] as iodine–dextrin-colour units/mg of protein/ml of digest.

Preparation of Z-enzyme

(*a*) *Initial extraction and fractionation.* Dry, defatted, finely-ground, soya-bean flour (300 g) was shaken with calcium chloride solution (0.2%, 1500 ml) for 4 h at 18°. After centrifugation at 900 *g*, the resultant supernatant liquor (*ca.* 40 mg of protein/ml) was cooled to 0°. Acetone at −5° was added to a concentration of 10% (v/v). The temperature of the mixture was then lowered to −5° and cold acetone was added slowly, with continuous stirring. Protein fractions obtained by centrifugation (at −5°, 1100 *g*) were air-dried to remove excess of acetone, and suspended in water at 2°; any insoluble residue was removed by centrifugation. The fractions were characterized, and a typical result is shown below:

Acetone concentration (v/v)	0	20	30	35	40	45	50	55	60
Fraction	0	1	2	3	4	5	6	7	8
Specific activity of Z-enzyme	6.4	5.0	5.3	7.8	15.4	20.0	35.0	36.0	4.6
Specific activity of β-amylase	7.6	6.7	7.0	8.5	13.0	12.0	12.0	9.0	5.0

(*b*) *Removal of β-amylase; preliminary experiments.* Portions of fraction 5 were heated on a water-bath at 70° (i) in the presence of added calcium acetate (2 mg/ml), and (ii) without additional calcium ions. The protein content, β-amylase, and Z-enzyme activities of aliquot portions of the cooled, centrifuged (1500 *g*) digests were then measured at appropriate intervals. The results in Fig. 1 indicate

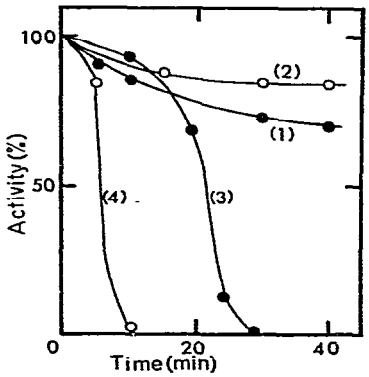

Fig. 1. Effect of heat treatment at 70° and pH 5.5 on the activity of Z-enzyme and β-amylase in soya-bean extract: (1) Z-enzyme in the absence of Ca^{2+}; (2) Z-enzyme in the presence of Ca^{2+} (10^{-2} M); (3) β-amylase in the absence of Ca^{2+}; (4) β-amylase in the presence of Ca^{2+} (10^{-2} M).

that, although the activity of the crude Z-enzyme preparation was remarkably stable at pH 5.5 and 70° (curve 1), the stability was further increased by the addition of calcium ions (curve 2). In the presence of this metal ion, the β-amylase was almost completely deactivated in 10 min under these conditions (curve 4).

Removal of β-amylase; final procedure. Uniform heat-treatment on the water-bath was ensured by heating standard portions (10–15 ml) of the enzyme fractions. To such portions of fraction 7 were added calcium acetate (20 mg), and acetate buffer to bring the pH to 5.5. The mixture was then maintained at 70° for 20 min, cooled, and centrifuged at 1500 *g*. Resultant supernatant liquors were combined. Typical specific activities were as below:

	β-Amylase activity	Z-Enzyme activity
Initially	9	36
After heat treatment	0	51

This heat treatment was shown to irreversibly deactivate the β-amylase.

(*c*) *Fractionation with acetone.* The heat-treated enzyme solution was sub-fractionated with acetone at −5°. Typical results for the protein fractions obtained were as below:

Acetone concentration (v/v)	0	35	42	47	52	57
Fraction	7	7.1	7.2	7.3	7.4	7.5
Specific activity of Z-enzyme	51	6.8	33	90	450	92

(*d*) *Glycogen-complex formation.* The acetone-precipitated fractions were finally purified as the glycogen complex, by the method of Loyter and Schramm[13].

Ethanol to 40% v/v was slowly added with stirring to the enzyme solution at 2°. After 15 min, any precipitated protein was removed at 1100 *g*. Phosphate buffer (0.5 ml/10 ml of enzyme solution, pH 8.0, 0.2 M) and glycogen (2%, 0.2 ml/10 ml of enzyme) were then added to the enzyme in 40% v/v ethanol. The suspension was stirred for 10 min, and then centrifuged at 1100 *g* to yield a precipitate which was suspended in phosphate buffer (pH 6.7, 0.02 M). The suspension was maintained at 35° for 6 h to aid digestion of contaminating glycogen, and centrifuged at 1500 *g*. The resultant solution was cooled to 2° and treated with excess of acetone, and the precipitated material redissolved in water at 2°. Enzyme solutions were found to retain their specific activity (*ca.* 800) at this temperature for several weeks.

Effect of temperature and pH

In experiments where the effect of temperature on activity was studied, digests at pH 5.5 were pre-incubated to temperature equilibrium before enzyme was added. In stability experiments at pH 5.5, the enzyme solution and buffer were maintained at the appropriate temperature for 1 h, and then cooled to 35° before addition of the dextrin. Resultant activities were compared with those obtained at 35°, without prior incubation. The pH dependence of activity at 35° was obtained using McIlvaine's standard buffer solutions. The effect of pH on enzyme stability was determined by maintaining the enzyme and buffer at 20° for 75 min. Digests were then brought to pH 5.5 and incubated with dextrin at 35°, and the activities determined.

Activity at pH 3.6. Digests were prepared as follows:

(*a*) Buffer (2 ml, pH 5.5) + enzyme (0.2 ml) + amylose (20 ml, 3 mg/ml)
(*b*) Buffer (2 ml, pH 5.5) + amylose (20 ml, 3 mg/ml)
(*c*) Buffer (2 ml, pH 3.6) + enzyme (0.2 ml) + amylose (20 ml, 3 mg/ml)
(*d*) Buffer (2 ml, pH 3.6) + enzyme (0.2 ml); incubated for 2 h at 20° before amylose (20 ml, 3 mg/ml) was added.

After incubation at 35° for 24 h, the digests were heated on a boiling water-bath for 5 min, cooled, and filtered, and the amylose product precipitated with excess of butan-1-ol. No butan-1-ol complex was obtained in digest (*a*). For the products from digests (*b*)–(*d*), $[\eta]$ was measured.

Effect on amylose-viscosity of Z-enzyme pretreated with mercuric and calcium chlorides

Digests of enzyme and buffer (pH 5.5) were prepared containing (*e*) 10^{-3} M mercuric chloride, (*f*) 10^{-4} M mercuric chloride, (*g*) 10^{-3} M calcium chloride, and (*h*) no additional salts. After incubation at 20° for 2 h, the solutions were added to equal volumes of the same amylose solution (3 mg/ml) and incubated at 35°.

Butan-1-ol was added to digests (*g*) and (*h*) after 1 h, and to digests (*e*) and (*f*) after 24 h; [η] of the amylose products was then determined.

Effect of ethylenediaminetetra-acetate (EDTA) and trypsin

Digests (*i*)–(*n*) were prepared by adding to 10 ml portions of digests at pH 6.6 (McIlvaine's buffer); for (*i*) and (*j*), EDTA (0.1 ml, 10^{-1} M) and trypsin (0.1 ml, 0.4 mg); for (*k*) and (*l*), EDTA (0.1 ml, 10^{-1} M); for (*m*) and (*n*), trypsin (0.1 ml, 0.4 mg). A control digest (*o*) was also prepared. After incubation at 20° for 12 h, calcium chloride (1 ml, 1 M) was added to digests (*i*), (*k*), and (*m*), and coagulated protein removed by centrifugation. Equal volumes of the same amylose solution (3 mg/ml) were added to all of the digests, which were then incubated at 35° for 24 h. Excess of butan-1-ol was then added; no amylose was precipitated from digests (*m*) and (*o*). For the amylose products from the other digests, [η] was determined.

Modification of Z-enzyme

Coupling with p-diazobenzenesulphonic acid[14]. Phosphate buffer (1 ml, pH 8.2) and *p*-diazobenzenesulphonic acid (0.1 ml, 0.6%) were added to Z-enzyme (2 ml, activity = 2 units/ml). A control digest was set up containing sulphanilic acid (0.1 ml, 0.6%). The mixtures were left at 18° for 1 h, dialysed at 2° against calcium acetate (0.2%, 3 × 200 ml), and centrifuged. Activities were then determined in the usual way.

Acetylation[14]. Sodium acetate (250 mg) was added to Z-enzyme (1 ml, activity = 2 units/ml) at 0°, and then acetic anhydride (0.03 ml) was added. After 1 h, the mixture was dialysed as above. A control was prepared without the anhydride, and the activities of both digests were determined.

RESULTS AND DISCUSSION

Isolation and purification of Z-enzyme

Z-Enzyme was first characterized[1,2] as the enzyme which — associated with, and acting in conjunction with, soya-bean β-amylase — would completely degrade any amylose at pH 4.6, but which was itself inhibited completely at pH 3.6. This latter behaviour, in conjunction with the fact that classical reducing-power tests were negative, led to the suggestion that Z-enzyme was not an α-amylase. The more sensitive, physical techniques of viscosity and light-scattering were necessary to establish the α-amylolytic character of the enzyme[7]. In this work, therefore, we have used procedures applicable to α-amylases to isolate and purify the Z-enzyme in soya beans.

Initially, a method for measuring Z-enzyme activity in the presence of the contaminating β-amylase had to be developed. Methods involving estimation of reducing power[15], fall in viscosity[16], or decrease in starch–iodine stain[17] are all influenced by concurrent β-activity. However, a modification of Briggs' method[12],

in which we used β-limit dextrin from amylopectin as the substrate, was satisfactory. Any fall in iodine-staining power of this substrate must be due to α-amylolysis. (Although in the presence of excess of β-amylase, α-amylolytic activity will be followed by β-amylolysis, the effect of this on the iodine-staining ability of the dextrin was shown to be small). This method, which is based on the assay of Sandstedt *et al.*[18], measures activity as the reciprocal of the time taken to decrease the dextrin–iodine stain by a standard amount. Here, the standard graph, from which subsequent Z-enzyme activities were calculated, was constructed by measuring the effect of salivary α-amylase on the limit dextrin. (It was later found that hydrolyses of the dextrin by salivary α-amylase and the purified Z-enzyme were very similar in the initial stages).

Preliminary experiments showed that fractionation of the soya-bean protein by ammonium sulphate and alcohol was not successful, little separation of β-amylase and Z-enzyme activities being achieved. (All β-amylase activities were determined at pH 3.6 to inhibit the Z-enzyme.) A successful separation was made, however, by the use of acetone at low temperatures. Heat-treatment was attempted to remove the contaminating β-amylase, as it has been shown[19,20] that β-amylase activity can be preferentially removed from malted-barley α-amylase preparations by heating the enzyme mixture at 70°, in the presence of calcium ions. Experiments showed (Fig. 1) that a comparable heat-treatment at 70° and pH 5.5 preferentially, and irreversibly, deactivates the β-amylase in the Z-enzyme/β-amylase fractions. After this removal of the β-amylase, further inert protein was removed by a second fractionation with acetone. Finally, the specific activity of the Z-enzyme was nearly doubled by the formation of a glycogen complex. The latter procedure has been suggested[13,21] as a general method for preparing α-amylases of very high activity.

The purification procedure increased the overall specific activity of Z-enzyme by a factor of *ca.* 150. Maltase, laminarase, and cellobiase were absent, as shown by digestion with the appropriate substrate, followed by paper chromatographic analysis. Similarly, incubation of the enzyme preparation with maltotriose for 72 h showed the presence of trace amounts of glucose and maltose, probably arising from the slow action of Z-enzyme itself; D-enzyme was absent.

Effect of temperature on activity and stability

The temperature of maximal activity of Z-enzyme is *ca.* 55° (see Fig. 2*a*). The purified enzyme lost only *ca.* 10% of its original activity after 1 h at 50°, but there was then a very rapid decrease between 55 and 60°. In the heat-treatment stage of the preparation procedure, the Z-enzyme appears to be stabilized by contaminating protein. An Arrhenius plot of the temperature dependence of the activity is shown in Fig. 2*b*; the apparent heat of activation varies from 14 kcal at 9°, to 6 kcal at 25°, and is zero at 55°.

Effect of pH on activity and stability

The effect of pH on the enzymic activity is shown in Fig. 3*b*, where the ratio

(activity at a given pH, V_{H^+})/(maximum activity at the optimum pH, V_{max}) is plotted against the pH. A variation in substrate concentration did not affect the shape and position of the curve. The results have been analysed, using the scheme shown in

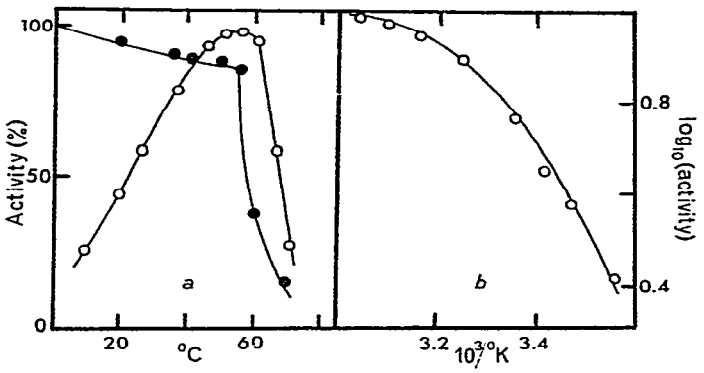

Fig. 2. (*a*) Effect of temperature on the activity (-○-) and stability (-●-) of Z-enzyme; (*b*) Arrhenius plot of temperature dependence of activity.

Fig. 4, for an enzyme with two ionizable groups[22–25]. If (i) the form EHS is assumed to be the only one of the three enzyme–substrate complexes capable of reacting to give the products, and (ii) the ionization of the two groups concerned is considered

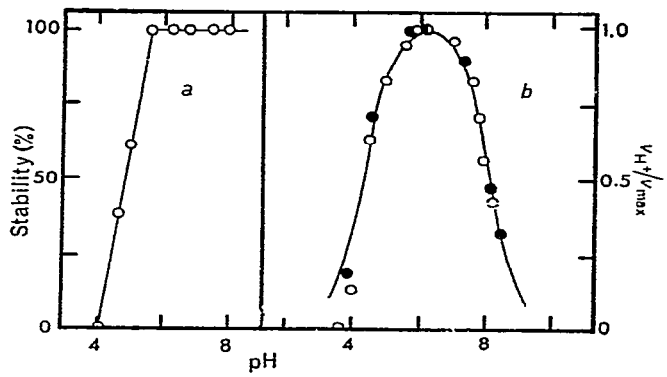

Fig. 3. Effect of pH on the stability (*a*) and activity (*b*) of Z-enzyme: (-○-) substrate concentration = 0.7 mg/ml; (-●-) substrate concentration = 2.8 mg/ml. The solid line represents the theoretical curve discussed in the text.

to be unaffected by substrate binding, *i.e.* $K'_a = K_a$ and $K'_b = K_b$, it can be readily shown[25] that

$$\frac{V_{H^+}}{V_{max}} = \frac{1 + 2\sqrt{K_a/K_b}}{1 + K_a/[H^+] + [H^+]/K_b}$$

i.e. V_{H^+}/V_{max} is a function which is independent of the substrate concentration. As our experimental data showed such independence, the function and experimental

points in Fig. 3*b* were used to calculate K_a and K_b. Values of $pK_a = 8.15$, and $pK_b = 4.3$, were found. The solid line in Fig. 3*b* represents the function V_{H^+}/V_{max} calculated from these values. Essentially, the experimental points lie on this theoretical curve, except at pH 4.0 and below, where irreversible denaturation of the enzyme may be occurring.

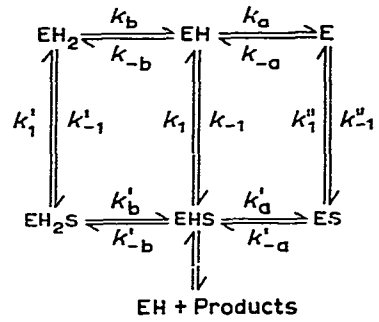

Fig. 4. Hypothetical scheme for the ionization and reaction of the enzyme; E = enzyme, H = proton S = substrate. Velocity constants (k) are as indicated. Ionization constants of the enzyme (K_a, K_b and the enzyme/substrate complex (K'_a, K'_b) are defined as:
$K_a = k_a/k_{-a}$, $K_b = k_b/k_{-b}$
$K'_a = k'_a/k'_{-a}$, $K'_b = k'_b/k'_{-b}$.

The stability of Z-enzyme at various pH values is shown in Fig. 3*a*. Although these results suggest that all experimental values obtained below pH 5.5 in Fig. 3*b* should lie beneath the theoretical curve, the two sets of data are not directly comparable; in the stability experiments, the enzyme was incubated in the absence of substrate before the activity was determined, and hence the stabilizing effect of an enzyme–substrate complex was not present, as it was for the results in Fig. 3*b*.

Nature of the active centres in Z-enzyme

The nature of the ionizing groups under consideration may be inferred from the pK-values; the group with pK 4.3 is probably a carboxylic acid[26], whilst that with pK 8.15 is likely to be an ammonium group[26]. However, there is the possibility that interaction with an anion may have displaced the pH-activity curve[27], in which case the ionizing group might be an imidazolium group (as in histidine)[25,26]. As the behaviour of the enzyme can be explained in terms of the fact that the ionization is unaffected by binding with substrate, these groups may be involved in the breakdown of the enzyme–substrate complex to give the reaction products, rather than in the formation of a complex.

In order to investigate the nature of the group with pK = 8.15, digests were prepared incorporating (i) iodine (3×10^{-6} M), and (ii) sodium *p*-chloromercuribenzoate (3×10^{-5} M). In digest (i), inhibition was complete, whilst in (ii) there was no change in enzyme activity. Since iodine reacts preferentially with histidine, tyrosine, and sulphydryl groups, whilst the sodium salt only reacts with sulphydryl

groups, the results suggest that histidine and/or tyrosine are necessary for activity of Z-enzyme. This was confirmed when coupling of the enzyme with *p*-diazobenzenesulphonic acid — a reagent which combines primarily with tyrosine and histidine residues in proteins — diminished the activity by 95%. Another reagent, which is fairly specific for free amino groups in proteins, although it also reacts with free sulphydryl groups and, in some cases, free phenolic groups, is acetic anhydride. Acetylation reduced the activity of the Z-enzyme by 75%, suggesting that free amino groups are necessary. However, insufficient enzyme was available for an estimation of the degree of acetylation, or of the types of group acetylated. Thus diminished activity of the enzyme may have been caused by partial acylation of tyrosine residues.

TABLE I

ACTIVITY OF Z-ENZYME AT pH 3.6

Digest	(*a*)	(*b*)	(*c*)	(*d*)
Conditions[a]	pH 5.5	Control	pH 3.6; directly	pH 3.6; pre-incubation
$[\eta]$ of amylose, after incubation	10[b]	510	400	500

[a]See Experimental section (p.232).
[b]$[\eta]$ too small to be measured accurately.

TABLE II

EFFECT OF VARIOUS REAGENTS ON ACTIVITY OF Z-ENZYME

% inhibition from iodine-staining measurements is quoted

Molarity	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Mercuric chloride	100	100	42	15
Potassium cyanide	0	0	0	0
Ammonium molybdate	20	0	0	0
Tryptophan	0	0	0	0
Ascorbic acid	100	90	70	42

Activity of Z-enzyme at pH 3.6

As the behaviour of Z-enzyme at pH 3.6 is of critical consequence, we made a careful study of the effect of this pH on the enzyme's activity by following changes in $[\eta]$ of an amylose. This technique yields an extremely sensitive measure of any hydrolytic action. The results of these experiments are shown in Table I, where the negligible $[\eta]$ of digest (*a*) shows the high rate of amylolytic degradation at pH 5.5. The difference in $[\eta]$ between samples (*b*) and (*d*) is within experimental error, and shows that pre-incubation at pH 3.6 for 2 h completely destroys the Z-enzyme activity. There was, however, a significant decrease in the viscosity of sample (*c*), showing that the substrate had been hydrolysed before complete inhibition of the

enzyme had been achieved. Pre-incubation for at least 2 h at pH 3.6 is essential, therefore, before β-amylolysis limits can be obtained using β-amylase preparations which contain Z-enzyme.

Effect of various reagents on activity

The effect of various reagents on the activity of Z-enzyme is shown in Table II. Potassium cyanide and tryptophane do not affect the activity, whilst the negligible effect of ammonium molybdate shows that the activities of Z- and R-enzyme are distinct[28]. Ascorbic acid (10^{-3} M) and mercuric chloride (10^{-4} M) are very efficient inhibitors of Z-enzyme activity. Table II also shows that quite extensive inhibition of Z-enzyme must have occurred at the concentration of mercuric chloride (1.5×10^{-6} M) used in our original work[7]. Complete inhibition by mercuric chloride is shown by the [η]-values for digests (*e*) and (*f*) in Table III. This Table also shows, from a comparison of the [η]-values for digests (*g*) and (*h*), that calcium chloride (10^{-3} M) did not activate the enzyme.

TABLE III

EFFECT ON AMYLOSE-VISCOSITY OF Z-ENZYME PRETREATED WITH MERCURIC AND CALCIUM CHLORIDES

Digest[a]	(*e*)	(*f*)	(*g*)	(*h*)
Pretreatment conditions at pH 5.5[a]	$Hg^{2+} = 10^{-3}$ M	$Hg^{2+} = 10^{-4}$ M	$Ca^{2+} = 10^{-3}$ M	—
[η] of amylose, after incubation[b]	500	500	80	75

[a]See Experimental section (p. 232).
[b][η] of amylose = 510.

The α-amylolytic nature of Z-enzyme suggested that its action might be very dependent on the presence of calcium ions. The importance of this ion to α-amylase activity has been extensively studied by Fischer and his collaborators[29]. Calcium ions may be effectively removed from aqueous solution by the chelating action of ethylenediaminetetra-acetate (EDTA). In our experiments, the effect of the presence of EDTA on Z-enzyme activity was followed by measuring the amylolysis of amylose by changes in [η], as this method again provides the most sensitive measure of assay. The fall in viscosity [45%; Table IV, digest (*l*)] shows that although the enzyme is inhibited to some extent [compare, digest (*o*)], hydrolysis has taken place in the EDTA/Z-enzyme/amylose digest. This inhibition is largely reversible, because on addition of excess of calcium ions, the extent of hydrolysis is greatly increased [digest (*k*)]. The protease, trypsin, has little effect on the enzyme activity as shown by the large decrease in [η] for the amylose in digest (*n*), although this fall is even larger in the presence of calcium ions [digest (*m*)]. However, under the combined action of EDTA and trypsin there was only a 10% fall in [η] for the amylose sample. Thus a mixture of EDTA and trypsin is a more efficient inhibitor than EDTA by itself. This inhibition is not completely reversible as, on the addition of excess of calcium ions, the amylose in digest (*i*) was not degraded to the same

extent as that in (*k*). This effect may be due to protease attack on calcium-deficient protein molecules.

TABLE IV

EFFECT OF EDTA AND TRYPSIN ON Z-ENZYME ACTIVITY

Digest[a]	(*i*)	(*j*)	(*k*)	(*l*)	(*m*)	(*n*)	(*o*)
Initial conditions[a]	EDTA/trypsin	EDTA/trypsin	EDTA	EDTA	trypsin	trypsin	control
Final conditions[a]	$+Ca^{2+}$	—	$+Ca^{2+}$	—	$+Ca^{2+}$	—	—
$[\eta]$ of amylose[b]	75	455	33	280	10[c]	40	10[c]

[a]See Experimental section (p. 233).
[b]$[\eta]$ of amylose = 510.
[c]$[\eta]$ too small to be measured accurately.

Action of Z-enzyme on various substrates

In our earlier work[7], the hydrolysis of linear amylose by Z-enzyme was indicated by viscosity results, whilst attack on amylopectin and amylopectin limit-dextrin was shown by light-scattering measurements. The preparation of purified enzyme has now enabled the action on these substrates to be studied by classical iodine-staining and reducing-power measurements. Fig. 5 shows that all three substrates can be hydrolysed to the "achroic limit". In particular, it should be noted that the action of Z-enzyme on the β-limit dextrin from amylopectin is comparable to that of salivary α-amylase. This justifies the method of estimation of activity, whilst showing the similar nature of the two enzymes.

It is apparent that there are two distinct stages in the hydrolysis of linear amylose by Z-enzyme. There is first a rapid decrease in size of the amylose molecules, as shown by the fall in colour of the iodine stain. This is accompanied by an increase in reducing power of the solution. The second part of the reaction begins at the achroic limit of the amylose solution, and is characterized by a slow increase in reducing power of the solution. The discontinuity occurs at *ca.* 30% apparent conversion into maltose, and the disappearance of the iodine stain indicates that there are only small maltodextrins present at this stage in the reaction. However, it has to be stressed that the achroic point during α-amylolysis of amylose is not invariant, but depends entirely on the amylose–enzyme ratio[30].

The effect of Z-enzyme on the β-limit dextrin from glycogen is not yet known with certainty. Our earlier light-scattering measurements indicated that there is no attack on this substrate, whilst Cunningham *et al.*[9] obtained an increase in reducing power of the digest, using very large quantities of enzyme and prolonged incubation. If limited α-amylolytic attack is occurring, a more sensitive measure of this can be obtained from the concurrent action of β-amylase and the purified Z-enzyme. Under our normal digest conditions, with the addition of β-amylase, we found a

2% apparent conversion into maltose after 24 h. This result indicates that purified Z-enzyme does cause degradation of glycogen limit-dextrin.

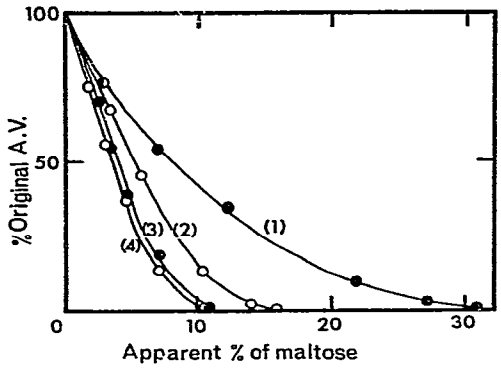

Fig. 5. Graph of % of original A.V. *versus* apparent conversion into maltose. (1) amylose/Z-enzyme; (2) amylopectin/Z-enzyme; (3) amylopectin β-limit dextrin/salivary α-amylase; (4) amylopectin β-limit dextrin/Z-enzyme.

The character of Z-enzyme

The above experiments have extended our original observations regarding the α-amylolytic character of Z-enzyme. The variation of activity and stability with temperature and pH are comparable to those of other α-amylases[25,29]. The enzyme is irreversibly deactivated by pre-incubation at pH 3.6, but we have found that this behaviour is a characteristic of several other plant α-amylases[31]. Inhibition occurs in the presence of mercuric chloride, whilst calcium ions are essential for activity. As with other α-amylases[28], hydrolytic degradation of amylose, amylopectin, and glycogen occurs.

Earlier, we suggested[7] that Z-enzyme might be a dormant form of α-amylase. However, when soya beans were germinated, we found that the increase in Z-enzyme activity was not significant, compared to that which occurs in barley. It would appear, therefore, that Z-enzyme is similar to other plant α-amylases, but is normally present in extremely small quantities in the soya bean.

ACKNOWLEDGEMENTS

The authors are indebted to the Corn Industries Research Foundation (Washington, D.C., U.S.A.) for its generous support of this work, and they also thank the Department of Scientific and Industrial Research for a maintenance grant (to E.A.M.).

SUMMARY

A method for the isolation and purification of the Z-enzyme in soya beans is described. This procedure involves the formation of the glycogen–enzyme complex.

A method for assay of activity is presented. The properties of the purified enzyme have been studied; in particular, the variation of activity and stability with temperature and pH has enabled the nature of the active sites in the enzyme to be investigated. Characterization of the activity of Z-enzyme at pH 3.6, and in the presence of a variety of reagents, has been achieved by viscometric techniques. Both linear and branched glucans are attacked by the purified enzyme. It is concluded that the properties of Z-enzyme are similar to those of other plant α-amylases.

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OXETANS

PART I. 3,5-ANHYDRO-1,2-*O*-ISOPROPYLIDENE-α-D-GLUCOFURANOSE AND -β-L-IDOFURANOSE

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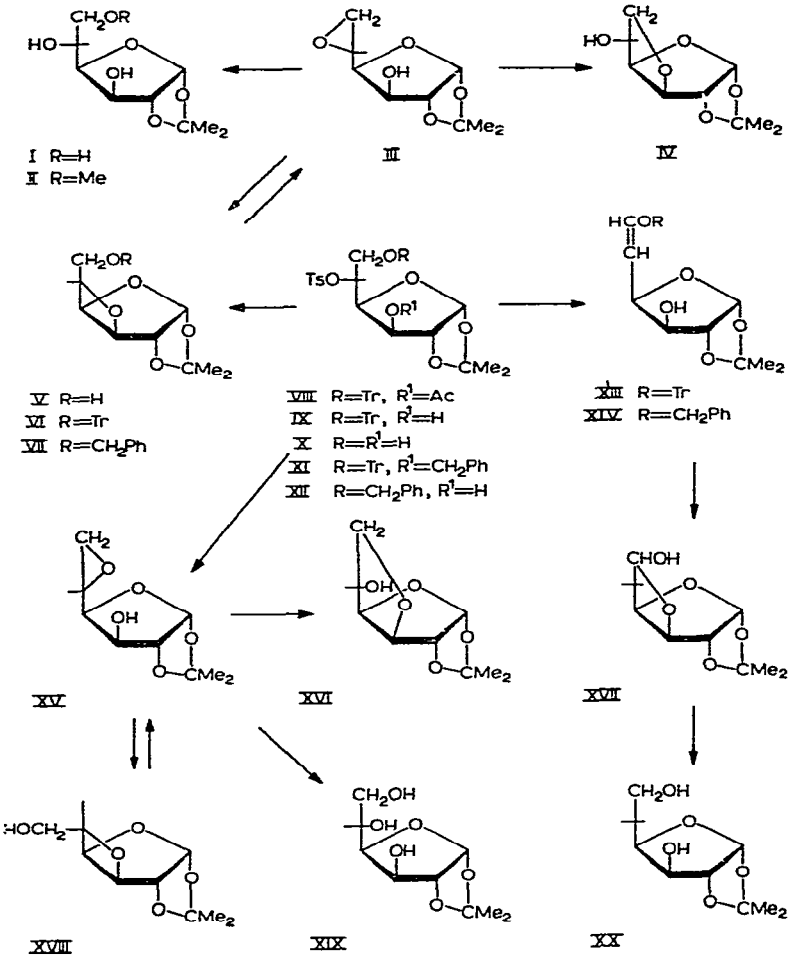
INTRODUCTION

Vicinal epoxides derived from sugars can undergo a number of reactions in which ring opening is caused by intramolecular, nucleophilic attack[1,2]. We have previously studied several of these reactions which occur under either acidic[3–5] or alkaline conditions[5,6]. Seebeck *et al.*[7] have shown that 3,6-anhydro-1,2-*O*-isopropylidene-α-D-glucofuranose (IV) is one of the products when the 5,6-anhydro-compound (III) is treated with aqueous alkali. In this laboratory Dr. J. Conn[8] attempted to carry out the analogous reaction on the L-*ido*-epoxide (XV)[9], in order to obtain 3,6-anhydro-1,2-*O*-isopropylidene-β-L-idofuranose (XVI), required in another study[3]. He concluded that another anhydride was produced, but was unable to pursue the matter at that time. We have examined the reaction in some detail, and a preliminary account has already been published[10].

RESULTS AND DISCUSSION

When the action of aqueous alkali on the 5,6-epoxide (XV) was studied by thin-layer chromatography, several products were observed. Meyer and Reichstein[9] had shown that 1,2-*O*-isopropylidene-β-L-idofuranose (XIX) was the major product, and this was confirmed. In addition, there was a small amount of the 3,6-anhydride (XVI), together with a product of low R_F value, which may be a bimolecular compound[7], and an unknown compound whose R_F value resembled that of the 5,6-anhydride. On a preparative scale, the unknown compound (m.p. 68–69°, $[\alpha]_D$ +38.4°) crystallised in 12% yield after chromatography on silica gel, and gave correct analyses for an isopropylidenehexose anhydride. Since it differed from the two known anhydrides (XV)[9] and (XVI)[11], it probably contained a 3,5-anhydro-ring, such as that existing in 3,5-anhydro-1,2-*O*-isopropylidene-α-D-xylofuranose (XXI)[12]. The behaviour on hydrolysis with N sulphuric acid at 100° was consistent with such a structure. Initially, a reducing sugar of high R_F value was formed (presumably a 3,5-anhydrohexose), and this was further hydrolysed to L-idose (identified chromatographically); 1,6-anhydro-β-L-idopyranose triacetate was isolated after acetylation of the hydrolysis products. When 3,5-anhydro-1,2-*O*-isopropylidene-α-D-xylofuranose (XXI) was hydrolysed under the same conditions, the reaction

followed a similar course, yielding finally xylose; the rate of hydrolysis was greater than in the previous case. The assignment of the D-*gluco*-configuration (XVIII) to the 3,5-anhydride is based on the reasonable assumption that formation and opening of the 3,5-anhydro-ring are each accompanied by inversion at the carbon atom involved. It is interesting that D-allose, which would have arisen by inversion at C-3, was not detected in the acid hydrolysate.



The behaviour of the 3,5-anhydro-compound (XVIII) towards N sodium hydroxide has proved particularly interesting. Thin-layer chromatography of the products showed them to be the same as those formed by similar treatment of the 5,6-epoxide (XV); 1,2-*O*-isopropylidene-β-L-idofuranose (XIX) and its 3,6-anhydride (XVI) were isolated from the mixture, after chromatography. It appears, therefore, that the oxides (XV) and (XVIII) are interconvertible under alkaline conditions, and that it is the more reactive 5,6-epoxide (XV) which undergoes ultimate

irreversible ring scission. In agreement with this idea, it was found that 3,5-anhydro-1,2-*O*-isopropylidene-α-D-xylofuranose (XXI) reacted with N sodium hydroxide more slowly, despite the presence of a primary carbon at C-5, giving 1,2-*O*-isopropylidene-α-D-xylofuranose.

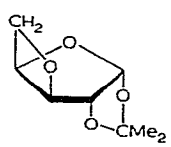
In the light of these observations, we reinvestigated the action of aqueous alkali on 5,6-anhydro-1,2-*O*-isopropylidene-α-D-glucofuranose (III), in the hope of detecting the 3,5-anhydride (V). Under the conditions used by the Swiss workers[7], the sole product having a high R_F value in thin-layer chromatography was the 3,6-anhydride (IV). When shorter reaction times were used, however, a new anhydro-compound could be detected. After extensive chromatography, 3,5-anhydro-1,2-*O*-isopropylidene-β-L-idofuranose (V) (m.p. 49°) was isolated in low yield. It was subsequently prepared in larger amounts by a different route, as described below, and its properties resembled those of the D-*gluco*-anhydride (XVIII). Acid hydrolysis with N sulphuric acid at 100° yielded D-glucose as the sole hexose, again indicating specific attack at C-5. As expected, N sodium hydroxide converted it into the same mixture of products as obtained from the 5,6-epoxide (III). 1,2-*O*-Isopropylidene-α-D-glucofuranose (I) and its 3,6-anhydride (IV) were identified chromatographically. From the reaction of the 3,5-anhydride with sodium methoxide, 1,2-*O*-isopropylidene-6-*O*-methyl-α-D-glucofuranose (II) was isolated.

We believe that these are the first cases to be reported of a reversible "oxide migration" involving a 3- and a 4-membered ring. It is not possible with these compounds to study an equilibration of the two ring-systems, because of the reactivity of the 5,6-epoxides. It is hoped to study such an equilibrated system by suitable choice of compounds.

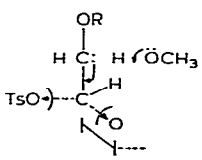
In order to obtain a larger quantity of the 3,5-anhydro-L-*ido*-compound (V) than was available from oxide migration, the action of sodium methoxide on the 5-toluene-*p*-sulphonate (VIII) was investigated. Under the reaction conditions, deacetylation occurs first to give the alcohol (IX), and the method is analogous to that used for the preparation of the 3,5-anhydro-D-xylose derivative (XXI)[12]. The major product when the sulphonate was heated with methanolic sodium methoxide was the enol ether (XIII)[10], which was obtained independently by Gramera *et al.*[13]. The chemistry of this reaction will be discussed below. A product having a higher R_F value in thin-layer chromatography was also noted, and was purified by chromatography on silica gel, after removal of most of the enol ether (XIII) by crystallisation. It was the triphenylmethyl ether (VI), and was characterised when hydrolysis with acetic acid gave 3,5-anhydro-1,2-*O*-isopropylidene-β-L-idofuranose (V), identical to the 3,5-anhydride prepared from the 5,6-anhydro-compound (III) by oxide migration. The overall yield was 6%. The formation of this compound from the 5-sulphonate of a D-glucose derivative gives further support to the configurational assignments at C-5 in (V) and (XVIII).

The enol ether (XIII) crystallises with solvent of crystallisation from chloroform or benzene–light petroleum. Analytical samples were carefully dried to constant weight (see Experimental). Gramera *et al.*[13,14] do not comment on this

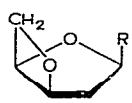
property, but, in one paper[13], their quoted yield is in excess of 100%. The structure of the enol ether was proved by acid hydrolysis to give the syrupy 5-deoxy-1,2-*O*-isopropylidene-α-D-*xylo*-hexodialdo-1,4:6,3-difuranose (XVII), which was characterised as its 2,4-dinitrophenylhydrazone. The same dialdohexofuranose (XVII) resulted as a minor product from the action of sodium methoxide on 1,2-*O*-isopropylidene-5-*O*-toluene-*p*-sulphonyl-α-D-glucofuranose (X), and its structure was proved by borohydride reduction to give 5-deoxy-1,2-*O*-isopropylidene-α-D-*xylo*-hexofuranose (XX)[15,16]. Gramera *et al.*[13] have examined the action of sodium methoxide on several pairs of 5-toluene-*p*-sulphonates, one in each pair having an alkali-stable group on the 3-hydroxyl group, *e.g.* (VIII) and (XI). They conclude, from a study of four such pairs of sulphonates, that a free hydroxyl group at C-3 is essential for the β-elimination reaction leading to an enol ether. These workers[13,17] have been unable to isolate a 3,5-anhydride from any of their reactions, but have postulated that formation of the 3,5-L-*ido*-anhydride (VI) is in some way linked to the formation of the enol ether (XIII). Two mechanisms are discussed, the first of which involves the sequence shown in (XXII). The authors omit the negative charges in the region of C-5 and on the methoxide ion, which would certainly repel each other; we shall not discuss this mechanism further. In the second mechanism, the 3,5-anhydride is the actual intermediate, and we have investigated this possibility. The crystalline 3,5-anhydride (V) was converted into its triphenylmethyl ether (VI), and heated with sodium methoxide under conditions more drastic than those used to prepare the enol ether. No reaction was observed, and the anhydride (V) was recovered in high yield after removal of the triphenylmethyl group by acid hydrolysis. We conclude that the 3,5-anhydride (VI) is not an intermediate in the conversion of the 5-sulphonate (IX) into the enol ether (XIII) by sodium methoxide. Horwitz *et al.*[18] have recently described an elimination reaction of the nucleoside oxetans (XXIII), using potassium *t*-butoxide in dimethyl sulphoxide, to give the olefins (XXIV). An attempted elimination using sodium methoxide was unsuccessful[18].



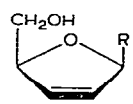
XXI



XXII



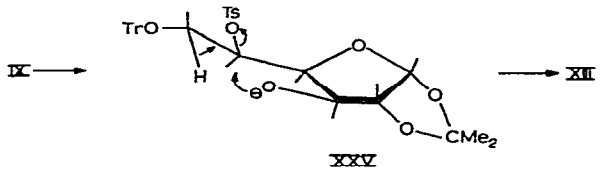
XXIII R=uracil or thymine



XXIV R=uracil or thymine

The observations of Gramera *et al.*[13] can be explained if the alkoxy anion resulting from removal of the proton from the 3-hydroxyl group in the sulphonate (IX) acts as the base for removal of the proton on C-6. If this is so, one can form a six-membered, cyclic transition-state (XXV), in which the large groups are equatorial, leading specifically to the *trans*-enol ether (XIII). It is assumed that the triphenylmethyl enol ether (XIII) has a trans arrangement of hydrogen atoms at C-5 and C-6 by analogy with the benzyl compound (XIV)[17]; the infrared spectrum

of the triphenylmethyl ether is too complex for unambiguous interpretation. The same alkoxy-anion is, of course, the intermediate for the formation of the 3,5-anhydride and it is difficult to see how one could prevent the two reactions



XXV

occurring side by side. Gramera *et al.*[13] quote unpublished work of Gramera and Whistler which indicates that the 3,5-anhydride (VII) is formed during hydrazinolysis of the benzyl ether (XII) and undergoes nucleophilic attack by hydrazine at both C-3 and C-5. The major product is still the enol ether (XIV)[17]. Whistler and his colleagues clearly believe that by using hydrazine they have "trapped" the oxetan (VII), which would otherwise have been converted into the enol ether (XIV). This interpretation is contrary to our findings in the triphenylmethyl ether series.

EXPERIMENTAL

Evaporations were carried out under reduced pressure with a bath temperature below 40°. Melting points are uncorrected. Infrared spectra were measured for potassium bromide discs. Light petroleum refers to the fraction of b.p. 60–80°. Comparison of materials with authentic substances was made, unless stated otherwise, by mixed m.p. determination, infrared spectra, and thin-layer chromatography (t.l.c.).

Chromatographic methods. Adsorption chromatography was carried out on silica gel (Hopkin and Williams) and neutral alumina (Woelm). T.l.c. on Kieselgel G (Merck) was used in preliminary investigations, and also to monitor the fractions from chromatography columns. Carbohydrates were detected by anisaldehyde–sulphuric acid[19]; triphenylmethyl ethers appeared as yellow spots with this reagent on gentle heating. Toluene-*p*-sulphonates were detected by the diphenylamine method[20]; a number of non-sulphonates gave a very weak positive reaction. Paper chromatography was carried out on Whatman No. 1 paper using butan-1-ol–pyridine–water (3:1:1, v/v), and aniline phthalate[21] to detect reducing sugars.

*3-O-Acetyl-1,2-O-isopropylidene-5-O-toluene-*p*-sulphonyl-6-O-triphenylmethyl-α-D-glucose (VIII)*

This was prepared by triphenylmethylation and subsequent toluene-*p*-sulphonylation of 3-*O*-acetyl-1,2-*O*-isopropylidene-α-D-glucofuranose (*cf.* refs. 13, 14). The product contained triphenylmethanol, and in t.l.c. had the same R_F value as the latter (anisaldehyde spray).

1,2-O-Isopropylidene-5-O-toluene-p-sulphonyl-α-D-glucofuranose (X)

(*a*) 3-*O*-Benzyl-1,2-*O*-isopropylidene-5-*O*-toluene-*p*-sulphonyl-6-*O*-triphenylmethyl-α-D-glucofuranose[22] (5.68 g) in glacial acetic acid (50 ml) was hydrogenated over palladium black (1.3 g) at atmospheric pressure for 16 h. After removal of the palladium by filtration, the solution was heated at 100° for 5 min and evaporated to dryness. The product crystallised from benzene–light petroleum to give the 5-sulphonate (2.34 g, 78%), m.p. 122°, $[\alpha]_D^{23}$ +5.2° (*c* 2.4, chloroform). Gramera *et al.*[17] give m.p. 124°, $[\alpha]_D$ +8.0° (chloroform). (Found: C, 51.3; H, 6.1; S, 8.4. $C_{16}H_{22}O_8S$ calc.: C, 51.3; H, 5.9; S, 8.6%).

(*b*) The above 3-acetate (VIII) (0.78 g) was treated with methanol (50 ml) containing sodium methoxide [from sodium (10 mg)], at 20° for 4 h. The solution was neutralised (CO_2) and evaporated to dryness, and the residue extracted with chloroform. After removal of the chloroform, the residue was dissolved in glacial acetic acid (4.8 ml), ethanol (1.5 ml), and water (1.2 ml), and heated for 12 min under reflux. The solution was evaporated to dryness, and extracted with hot benzene. After cooling and addition of light petroleum, the 5-sulphonate (0.28 g, 64%) m.p. 122°, crystallised; the infrared spectrum was identical to that of the compound in (*a*) above.

5,6-Anhydro-1,2-O-isopropylidene-β-L-idofuranose[9] *(XV)*

(*a*) 6-*O*-Benzoyl-1,2-*O*-isopropylidene-5-*O*-toluene-*p*-sulphonyl-α-D-glucofuranose[23] (0.42 g) was dissolved in chloroform (1.5 ml) and cooled to −15°. Sodium methoxide [from Na (0.05 g)] in methanol (0.6 ml) was added, and the mixture kept at −15° for 0.5 h, and 0° for a further 2 h. 5% Aqueous sodium hydrogen carbonate (4 ml) was added and the solvent evaporated at 0°. The residue was extracted with chloroform, dried (sodium sulphate), and evaporated to a syrup (0.17 g). A benzene solution was chromatographed on silica gel. Ether eluted the 5,6-anhydro-compound (0.14 g, 79%), m.p. 69–71° (lit.[9], m.p. 73–75°).

(*b*) 1,2-*O*-Isopropylidene-5-*O*-toluene-*p*-sulphonyl-α-D-glucofuranose (4.2 g) was dissolved in chloroform and cooled to 0°. Sodium methoxide [from sodium (0.3 g)] in cold methanol (5 ml) was added, and the mixture kept for 0.5 h in an ice bath. 25% Aqueous potassium hydrogen carbonate (4 ml) was added and the solution concentrated; the product was extracted and chromatographed as above. Benzene–ether (3:1) eluted 5-deoxy-1,2-*O*-isopropylidene-α-D-*xylo*-hexodialdo-1,4:6,3-difuranose (XVII) (0.13 g, 6%), $[\alpha]_D^{23}$ +34.9° (*c* 1.75, chloroform). Ether eluted the 5,6-anhydrocompound (2.02 g, 89%), m.p. 72–74°.

5-Deoxy-1,2-O-isopropylidene-α-D-xylo-hexodialdo-1,4-furanose 2,4-dinitrophenylhydrazone

(*a*) The above sugar (33 mg) was dissolved in methanol (1 ml) and 2,4-dinitrophenylhydrazine (33 mg) in methanol (1 ml) added, followed by acetic acid (0.2 ml). The mixture was left at room temperature overnight, when yellow crystals were precipitated. Recrystallised from methanol, the hydrazone (42 mg,

64%) had m.p. 184–185°, $[\alpha]_D^{23}$ −17.4° (*c* 1.35, dioxan). (Found: C, 47.1; H, 4.9; N, 14.7. $C_{15}H_{18}N_4O_8$ calc.: C, 47.2; H, 4.7; N, 14.65%).

(*b*) 5-Deoxy-1,2-*O*-isopropylidene-6-*O*-triphenylmethyl-α-D-*xylo*-hexofuran-5-enose (XIII) (see below) (98 mg) was heated with 80% acetic acid (v/v, 1 ml) containing sodium acetate (2 mg) for 5 min at 100°. The cooled solution was evaporated to dryness, dissolved in benzene, and chromatographed on silica gel. Benzene–ether (1:1) eluted the hexodialdo-sugar (22 mg, 48%) which was treated with 2,4-dinitrophenylhydrazine as in (*a*). The hydrazone (24 mg) had m.p. 183°, and was identical to that in (*a*) above.

*5-Deoxy-1,2-*O*-isopropylidene-α-*D*-xylo-hexofuranose (XX)*

5-Deoxy-1,2-*O*-isopropylidene-α-D-*xylo*-hexodialdo-1,4-furanose (18 mg) was dissolved in ethanol (0.5 ml), and sodium borohydride (6 mg) added. The mixture was kept at room temperature for 16 h. Acetic acid (1 drop) was added and sodium ions were removed by passage through Dowex-50 (H^+ form) resin. The eluate was concentrated, and boric acid removed by distillation three times with methanol. The crystalline diol (15 mg, 81%), m.p. 87–88.5° (raised by sublimation to m.p. 93.5–94°), was identical to an authentic sample kindly given to us by Dr. E.J. Hedgley[15].

*3,5-Anhydro-1,2-*O*-isopropylidene-α-*D*-xylose (XXI)*

The anhydro-compound was prepared from D-xylose, according to Levene and Raymond's method[12].

Acid hydrolysis. (*a*) The anhydro-compound (4 mg) was hydrolysed with N sulphuric acid (0.1 ml) at 100° and the reaction followed by paper chromatography. After 2 min, xylose was detected in addition to 3,5-anhydroxylose. After 10 min, only xylose was detected. For R_F values, see Table I.

TABLE I

PAPER CHROMATOGRAPHY OF ACID HYDROLYSIS PRODUCTS OF 3,5-ANHYDRO-COMPOUNDS[a]

Sugar	*Colour of spot*	*R_F value*
D-Xylose	Red-brown	0.22
D-Glucose	Brown	0.12
L-Idose	Brown	0.26
3,5-Anhydro-D-xylose	Red-brown	0.52
3,5-Anhydro-D-glucose	Brown	0.40
3,5-Anhydro-L-idose	Brown	0.38

[a]For conditions, see under Chromatographic Methods.

(*b*) The anhydro-compound (0.16 g) was treated with N sulphuric acid (3 ml) at 100° for 2 h. The solution was neutralised with Dowex-2 (CO_3^{2-} form) resin,

filtered, and concentrated to a syrup. The syrup was dissolved in water (3 ml) and sodium borohydride (0.04 g) in water (3 ml) added, and the solution left at room temperature for 6 h. Excess of borohydride was destroyed with acetic acid, sodium ions were removed by passage through a column of Dowex-50 (H^+ form) resin, and the solution was evaporated to dryness. Boric acid was removed as methyl borate by methanol distillation, and the residue acetylated with acetic anhydride (1 ml) and pyridine (2 ml). The product was isolated using chloroform, and recrystallised from ethanol–light petroleum to give xylitol penta-acetate (0.25 g, 73%), m.p. 61–62°, identical with an authentic sample.

Alkaline hydrolysis. The anhydro-compound (0.19 g) was dissolved in N sodium hydroxide (2.3 ml) and heated at 100° for 16 h. The solution was neutralised (N sulphuric acid) and evaporated to dryness. A benzene extract was chromatographed on silica gel. Benzene–ether (9:1) eluted starting material (34 mg, 18%), $[\alpha]_D$ +9.2° (*c* 1.53, chloroform) [lit.[12], $[\alpha]_D$ +11.7° (chloroform)]; it is probable that some was lost owing to the volatility of this compound. Chloroform–ethanol (9:1) eluted 1,2-*O*-isopropylidene-α-D-xylofuranose (83 mg, 40%), which was converted, in 79% yield, into its 3,5-ditoluene-*p*-sulphonate, m.p. 98°, $[\alpha]_D^{22}$ −33.8° (*c* 1.89, chloroform). This is a new crystalline form, whose infrared spectrum was not identical with that of an authentic sample, m.p. 89–90°, $[\alpha]_D$ −36°, kindly given to us by Mr. P.R.H. Speakman, but the mixed m.p. was 98–99°. Karrer and Boettcher[24] give m.p. 91–92°, $[\alpha]_D$ −37.91° for this compound. When the sample of m.p. 89–90° was recrystallised from ethanol using seeds of the higher-melting form, only the latter, m.p. 100°, crystallised; the infrared spectra were identical.

3,5-Anhydro-1,2-O-isopropylidene-α-D-glucofuranose (XVIII)

5,6-Anhydro-1,2-*O*-isopropylidene-β-L-idofuranose (1.45 g) was dissolved in N sodium hydroxide (15 ml) and heated at 100° for 7 min. The solution was cooled, and passed through a column of Dowex-50 (NH_4^+ form) resin, and the eluate and washings were concentrated to a syrup. The residual syrup was extracted several times with hot benzene, and the benzene solution chromatographed on silica gel. Ether eluted first 3,6-anhydro-1,2-*O*-isopropylidene-β-L-idofuranose (39 mg, 3%), m.p. 101–102°, followed by 3,5-anhydro-1,2-*O*-isopropylidene-α-D-glucofuranose (0.168 g, 12%), m.p. 68–69° (from ether–light petroleum), $[\alpha]_D^{21}$ +38.4° (*c* 1.66, chloroform). (Found: C, 53.5; H, 7.1. $C_9H_{14}O_5$ calc.: C, 53.5; H, 6.9%). Some rechromatography of mixed fractions was necessary.

Acid hydrolysis. (*a*) The anhydro-compound (2 mg) was heated with 0.1 N sulphuric acid (0.1 ml) at 100°, and the hydrolysis followed by paper chromatography (see Table I). The isopropylidene group was removed within 5 min to give 3,5-anhydroglucose; idose was detectable after 15 min. When N sulphuric acid was used, idose was present after 4 min.

(*b*) The 3,5-anhydride (42 mg) was heated with N sulphuric acid (0.8 ml) at 100° for 14 h. The cooled solution was neutralised with Dowex-2 (CO_3^{2-} form) resin, filtered, and concentrated. Idose was removed by passing the solution through

a column of Dowex-1 (HO^- form) resin, and syrupy 1,6-anhydro-β-L-idopyranose (15 mg) was obtained on evaporation of the eluate. The product was acetylated with acetic anhydride and pyridine, and the acetate isolated using chloroform. The resulting syrup crystallised slowly and was purified by sublimation to give 2,3,4-tri-*O*-acetyl-1,6-anhydro-β-L-idopyranose (23 mg, 39%), m.p. 63°, indistinguishable from an authentic sample, m.p. 63.5–64.5°. This compound exists in two crystalline forms, m.p. 66-67° (D-series)[25] and m.p. 85–86° (L-series)[26], 86–87° (D-series)[27]

Alkaline hydrolysis. 3,5-Anhydro-1,2-*O*-isopropylidene-α-D-glucose (0.14 g) was heated with N sodium hydroxide (1.5 ml) at 100° for 16 h. The cooled solution was passed through a column of Dowex-50 (NH_4^+ form) resin, and the eluate and washings evaporated to dryness. The syrup was dissolved in chloroform–ethanol (9:1) and chromatographed on silica gel. The first fractions were evaporated, and the residue was dissolved in ether and rechromatographed on silica gel, when ether eluted 3,6-anhydro-1,2-*O*-isopropylidene-β-L-idofuranose, purified by sublimation (7 mg, 5%), m.p. 101°, identical with an authentic sample. Later fractions from the first column were dissolved in ethyl acetate and rechromatographed on silica gel to give 1,2-*O*-isopropylidene-β-L-idose (eluted with ethyl acetate and purified by sublimation, 34 mg, 22%), m.p. 112–114°, identical with an authentic sample.

3,5-Anhydro-1,2-O-isopropylidene-β-L-idofuranose (V)

(*a*) 5,6-Anhydro-1,2-*O*-isopropylidene-α-D-glucose (0.5 g) was heated with N sodium hydroxide (5 ml) at 100° for 3 min. The solution was immediately cooled and passed through a column of Dowex-50 (NH_4^+ form) resin. The eluate and aqueous washings were evaporated to a syrup which was dissolved in chloroform–ethanol (9:1) and chromatographed on silica gel. The first fractions were evaporated, and the residue was dissolved in benzene and rechromatographed on silica gel. Ether eluted first 3,6-anhydro-1,2-*O*-isopropylidene-α-D-glucofuranose (89 mg, 18%), m.p. 54° (lit.[7], m.p. 55–56°), followed by a mixture (60 mg) of the 3,6- and 3,5-anhydro-compounds. The syrup was dissolved in benzene and chromatographed on neutral alumina. Elution with ether–ethanol (9:1) gave a pure fraction (8 mg), m.p. 48–49°.

(*b*) Crude 3-*O*-acetyl-1,2-*O*-isopropylidene-5-*O*-toluene-*p*-sulphonyl-6-*O*-triphenylmethyl-α-D-glucofuranose (60 g) was heated under reflux in methanol (200 ml) containing sodium methoxide [from sodium (4 g)] for 1 h. Saturated aqueous potassium hydrogen carbonate (10 ml) was added and the solution evaporated. The residue was extracted thrice with chloroform, and the combined extracts were washed with water, dried (sodium sulphate), and evaporated to a syrup. Trituration with chloroform caused crystallisation of 5-deoxy-1,2-*O*-isopropylidene-6-*O*-triphenylmethyl-α-D-*xylo*-hexofuran-5-enose (24 g), m.p. 82–85°, $[\alpha]_D^{26}$ −14.7° (*c* 1.6, benzene). The infrared spectrum showed a strong band at 1667 cm^{-1} (C = C). Recrystallised from benzene–light petroleum, the product had m.p. 85–92°. For analysis, a sample was dried *in vacuo* at 50° to constant weight;

it then had m.p. 85–115°, and was still homogeneous on t.l.c. (Found: C, 76.2; H, 6.6. $C_{28}H_{28}O_5$ calc.: C, 75.7; H, 6.3%).

Concentration of the chloroform mother-liquors gave a syrup which was dissolved in benzene and chromatographed on neutral silica gel[5]. Benzene–ether (49:1) eluted 3,5-anhydro-1,2-*O*-isopropylidene-6-*O*-triphenylmethyl-β-L-idofuranose as a chromatographically homogeneous syrup (4.5 g). Elution with benzene–ether (9:1) yielded further enol ether (1.3 g). The above anhydro-compound (4.5 g) was heated under reflux in ethanol (15 ml), acetic acid (48 ml), and water (12 ml) for 12 min. Triphenylmethanol was filtered off from the cooled solution, and the filtrate evaporated to dryness. The residue was dissolved in benzene and chromatographed on silica gel. Benzene eluted more carbinol, and elution with ether afforded 3,5-anhydro-1,2-*O*-isopropylidene-β-L-idofuranose, further purified by vacuum distillation. Care must be taken to avoid over-heating of the syrup. Yield, 1.04 g (6%), m.p. 49–50°, $[\alpha]_D^{27}$ +53.2° (*c* 1.2, chloroform). (Found: C, 53.3; H, 6.7. $C_9H_{14}O_5$ calc.: C, 53.5; H, 6.9%).

Acid hydrolysis. (*a*) The anhydro-compound (2 mg) was heated with N sulphuric acid (0.1 ml) at 100°, and samples were subjected to paper chromatography (see Table I). Initial hydrolysis gave 3,5-anhydroidose, which was further hydrolysed to glucose, first detectable after 12 min.

(*b*) The anhydro-compound (23 mg) was heated with N sulphuric acid (0.5 ml) for 7 h at 100°. The solution was neutralised with Dowex-2 (CO_3^{2-} form) resin, filtered, and evaporated to a syrup (17 mg). The syrup was dissolved in water (1.0 ml), sodium borohydride (20 mg) added, and the solution kept at 20° for 5 h. Excess of borohydride was destroyed with acetic acid and sodium ions were removed by passage through Dowex-50 (H^+ form) resin. After evaporation of the eluate and washings to dryness, boric acid was removed as methyl borate by distillation with methanol. The resulting syrup was acetylated with acetic anhyride and pyridine overnight, and the product isolated using chloroform. The crude hexa-acetate (32 mg, 62%) crystallised, and was purified by sublimation to give D-glucitol hexa-acetate, m.p. 96–98°, indistinguishable from an authentic sample, m.p. 98°.

Alkaline hydrolysis. (*a*) The anhydro-compound (2 mg) was heated in N sodium hydroxide (0.1 ml) at 100°. Samples were examined by t.l.c., using ether as solvent. After 30 min starting material was still present, together with a compound whose R_F value and colour reaction with the anisaldehyde spray resembled that of 3,6-anhydro-1,2-*O*-isopropylidene-α-D-glucofuranose; compounds of low R_F value, resembling 1,2-*O*-isopropylidene-α-D-glucofuranose, were also detected. After 3 h, no starting material could be detected.

(*b*) The anhydro-compound (0.12 g) was heated under reflux with sodium methoxide [from sodium (0.06 g)] in methanol (1 ml) until t.l.c. showed that no starting material was present. The solution was neutralised with N sulphuric acid and evaporated to dryness, and a benzene extract of the resulting product subjected to chromatography on silica gel. Ether eluted 1,2-*O*-isopropylidene-6-*O*-methyl-

α-D-glucofuranose (63 mg, 45%) which, after recrystallisation from ether–light petroleum had m.p. 69–70.5°, and was indistinguishable from an authentic sample, m.p. 69–70°[28].

Triphenylmethylation of 3,5-anhydro-1,2-O-isopropylidene-β-L-idofuranose and treatment of the product with sodium methoxide

The 3,5-anhydride (0.12 g) was dissolved in pyridine (2 ml), triphenylmethyl chloride (0.2 g) added, and the mixture kept at 37° for 2 days. Methanol (0.1 ml) was added and, after 1 h, the mixture was poured into water. The product was isolated using chloroform, and the final syrup was dissolved in benzene–light petroleum (1:1) and chromatographed on silica gel. After elution of non-carbohydrate triphenylmethyl compounds with benzene–light petroleum (1:1), benzene eluted 3,5-anhydro-1,2-*O*-isopropylidene-6-*O*-triphenylmethyl-β-L-idofuranose (VI) (0.2 g, 78%), $[\alpha]_D^{24}$ +31.4° (*c* 0.94, chloroform); it was homogeneous on t.l.c.

The above triphenylmethyl ether (91 mg) was heated in a sealed tube with methanol (0.5 ml), containing sodium methoxide [from sodium (10 mg)], at 65° for 2 h. Examination of the products by t.l.c. [benzene–ether (9:1)] showed the presence only of a compound having the same R_F value as starting material [the enol ether (XIII), which has a lower R_F value, was clearly absent]. The solution was neutralised with N sulphuric acid, filtered, and evaporated to dryness. The product was heated with aqueous acetic acid (80% v/v, 1 ml) at 95° for 10 min, and the cooled solution evaporated to dryness. The product was dissolved in benzene and chromatographed on silica gel. Benzene–ether (9:1) eluted triphenylmethanol (41 mg, 78%), and ether eluted a compound (37 mg) indistinguishable from 3,5-anhydro-1,2-*O*-isopropylidene-β-L-idofuranose on t.l.c. (ether). Part of the product, on distillation, gave the 3,5-anhydride, m.p. 47°, identical with an authentic sample.

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SUMMARY

3,5-Anhydro-1,2-*O*-isopropylidene-α-D-glucofuranose and -β-L-idofuranose have been prepared, and their behaviour towards aqueous acid and alkali examined. Dilute sulphuric acid causes removal of the isopropylidene group, followed by specific ring cleavage at C-5, in each case. Under alkaline conditions, the 3,5-anhydro-compounds undergo nucleophilic attack by the oxygen atom on C-6 to give the 5,6-epoxides. This reversible reaction is the first example of an oxide migration involving interconversion of an oxetan and oxiran.

The mechanism of an elimination reaction undergone by certain 5-toluene-*p*-sulphonates of 1,2-*O*-isopropylidene-α-D-glucofuranose is discussed.

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NUCLEOSIDES AND RELATED SUBSTANCES

PART IV. THE SYNTHESIS OF 9-α-D-GLUCOPYRANOSYLADENINE BY THE FUSION REACTION*

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INTRODUCTION

Since Fischer and Helferich[1] synthesised purine nucleosides in 1914, many workers have investigated the synthesis of nucleosides. The naturally occurring nucleosides and nucleic acid-nucleosides generally have a *trans* configuration for the substituents at C-1 and C-2 of the carbohydrate moiety, and the anomers may be of interest from a biochemical viewpoint. Thus, vitamin B_{12} contains a *cis* arrangement of the substituents at C-1 and C-2 of the sugar moiety. A number of recent reports[2–11] have been concerned with the synthesis of nucleosides containing *cis* substituents at these positions.

In our laboratory, a novel procedure for the synthesis of both purine and pyrimidine nucleosides has been developed using 1-*O*-trichloroacetyl sugars[12]; the production of an anomeric mixture occurs in this reaction. The present paper concerns the anomeric mixtures of nucleosides formed in the fusion of 2,3,4,6-tetra-*O*-acetyl-D-glucopyranose, and some of its 1-*O*-substituted derivatives, with 6-benzamidopurine in the presence of certain catalysts, and the isolation of the α-nucleoside from the anomeric mixture obtained by the fusion of 2,3,4,6-tetra-*O*-acetyl-1-*O*-trichloroacetyl-α-D-glucopyranose with 6-benzamidopurine in the presence of toluene-*p*-sulphonic acid.

DISCUSSION

Two main factors influencing the anomeric configuration of nucleosides formed in the fusion reaction have been examined; the specificity of purine and pyrimidine bases was discussed in our previous paper[13]. The first factor is the effect of the catalyst, and the influence of various catalysts is shown in Table I. It is of interest to note that the effect of catalysts on the anomeric configuration of nucleosides produced by the fusion reaction is opposite to that operative in the production of phenolic *O*-glycosides. Zinc sulphate and zinc hydroxide have little catalytic effect in the reactions of 2,3,4,6-tetra-*O*-acetyl-1-*O*-trichloroacetyl-α-D-glucopyranose

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and 1,2,3,4,6-penta-*O*-acetyl-β-D-glucopyranose with 6-benzamidopurine. Mercuric chloride, stannous chloride, and zinc chloride exert similar catalytic effects in these reactions. The experimental results indicate that the mechanism involved in the fusions in the presence of metal halides is different from that in the presence of toluene-*p*-sulphonic acid[6].

TABLE I

THE FORMATION OF 9-α- AND 9-β-D-GLUCOPYRANOSYLADENINES

Catalyst	*2,3,4,6-Tetra-O-acetyl-D-glucopyranose derivatives*				
	1-O-Trichloroacetyl-	*1-O-Acetyl-*	*1-Bromo-1-deoxy-*	*1-Chloro-1-deoxy-*	*Free hydroxyl at C-1*
Zinc chloride	35%[a] (only β)	11% (only β)	(only β)	(only β)	
Zinc sulphate	(α and β)	none			
Zinc hydroxide	(α and β)	none			
Mercuric chloride	(only β)	(only β)			
Stannous chloride	(only β)	(only β)			
Toluene-*p*-sulphonic acid	27.8% (α:β = 28:72)	(α and β)	(only β)	14% (only β)	(only β)
Ethyl polyphosphate	26.9% (α:β = 21:79)	17% (α:β = 19:81)	(only β)	(α and β)	(only β)
No catalyst	9.3% (α:β = 20:80)	none	(only β)	10.5% (only β)	

[a]Numbers show the yields of isolated nucleosides. Ratios of anomers are given in parentheses; otherwise the yields and ratios were not determined.

The second factor is the effect of the substituent at C-1 in derivatives of 2,3,4,6-tetra-*O*-acetyl-D-glucopyranose. 2,3,4,6-Tetra-*O*-acetyl-1-*O*-trichloroacetyl-α-D-glucopyranose and 1,2,3,4,6-penta-*O*-acetyl-β-D-glucopyranose give different proportions of the α- and β-nucleosides, both in the absence of catalyst, and in the presence of zinc sulphate or zinc hydroxide. 2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranose and 2,3,4,6-tetra-*O*-acetyl-D-glucopyranosyl halides give only the β-nucleoside, although an exception is the fusion of 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl chloride with 6-benzamidopurine in the presence of ethyl polyphosphate as catalyst. These results indicate that neither 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranose nor a 2,3,4,6-tetra-*O*-acetyl-D-glucopyranosyl halide is the intermediate in the formation of the α-nucleoside by the fusion reaction. It is difficult to explain the production of an anomeric mixture of nucleosides in the fusion of 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl chloride with 6-benzamidopurine in the presence of ethyl polyphosphate as catalyst.

In all of the experiments, the yields of the nucleosides were in the range of 10–35%.

EXPERIMENTAL*

Anomeric mixtures of 9-D-glucopyranosyladenines

2,3,4,6-Tetra-*O*-acetyl-1-*O*-trichloroacetyl-α-D-glucopyranose, 1,2,3,4,6-penta-*O*-acetyl-β-D-glucopyranose, 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide, and 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl chloride were separately melted with 6-benzamidopurine at 140–160° for 60 min, in a molar ratio of 1:1.2, in the presence of zinc chloride, toluene-*p*-sulphonic acid, or ethyl polyphosphate[14] as catalyst. The reaction product was deacylated with methanolic ammonia, and then examined by paper chromatography in order to detect 9-β-D-glucopyranosyladenine (R_F 0.10) and 9-α-D-glucopyranosyladenine (R_F 0.14). The appropriate zones of the paper chromatogram were cut out and eluted with water, and the proportion of anomers was determined by comparison of the optical densities of the solutions at 259 mμ. Isolation of nucleosides was carried out on the deacylated products, and the results are summarised in Table I.

9-α-D-Glucopyranosyladenine

A mixture of 2,3,4,6-tetra-*O*-acetyl-1-*O*-trichloroacetyl-α-D-glucopyranose (2.6 g) and 6-benzamidopurine (1.2 g) was melted at 150–160°. A trace of toluene-*p*-sulphonic acid was added and the mixture was kept at 140–160° for one h. The reaction product was dissolved in methanol (30 ml) and insoluble materials were removed by filtration. To the solution was added methanol (100 ml) saturated with ammonia. The methanolic solution was stored overnight at *ca.* 0° and then concentrated under reduced pressure. Paper chromatographic examination of the resulting syrup showed the presence of two compounds, with R_F values 0.14 and 0.10, respectively. The n.m.r. spectrum of the syrup showed doublets at τ 3.25 (J 4.5 c.p.s.) and 3.95 (J 9.0 c.p.s.) which were assigned to the C-1 protons of 9-α- and 9-β-D-glucopyranosyladenines, respectively. The syrup was separated into two components by preparative paper chromatography [descending irrigation for 11 days with butan-1-ol–water (86:14, v/v), Toyo Roshi filter paper no. 51]. The compound (0.31 g, 20.0%) with R_F 0.10 was isolated and crystallised, and had m.p. 206°, $[\alpha]_D^{31}$ −6.2° (*c.* 1.0, water). It was identified as 9-β-D-glucopyranosyladenine by n.m.r. and i.r. spectroscopy, and by a mixed m.p. determination with an authentic sample[12]. The zones of the sheets containing the substance with R_F 0.14 were cut out and eluted with water. The eluates were concentrated under reduced pressure to give a thin syrup (0.12 g, 7.8%) which was purified by elution from a column of Dowex-50 (H^+ form) with N ammonium hydroxide (preliminary elution with water). The u.v.-absorbing fractions were collected and concentrated to dryness. Paper chromatographic examination showed a single spot (R_F 0.14), but the syrup, $[\alpha]_D^{14}$ +92° (*c* 0.5, water) failed to crystallise. The n.m.r. spectrum of the syrup

*All melting points are uncorrected. Paper chromatography was carried out with Toyo Roshi filter paper No. 51 by descending irrigation with butan-1-ol–water (86:14, v/v).

showed a doublet at τ 3.25 ($J = 4.5$ c.p.s.). The u.v. absorption spectrum of the syrup showed $\lambda_{max}^{H_2O}$ at 259 mμ and $\lambda_{min}^{H_2O}$ at 233 mμ, at pH 7. The syrup gave a crystalline picrate, m.p. 210–215°.

Anal. Calc. for $C_{17}H_{18}N_8O_{12}$: C, 38.78; H, 3.42; N, 21.29. Found: C, 38.89; H, 3.32; N, 21.21.

Attempted transformation of 9-β-D-glucopyranosyladenine into the 9-α-D-anomer

9-(2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl)-6-benzamidopurine[13] (m.p. 174°, 0.1 g) was heated at 160–180° for 1 h in the presence of toluene-*p*-sulphonic acid (15 mg). Paper chromatographic examination of the deacylated product showed the absence of the α-D anomer. From the deacylated product, only 9-β-D-glucopyranosyladenine was recovered.

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SUMMARY

An anomeric mixture of nucleosides is produced in the fusion of 2,3,4,6-tetra-*O*-acetyl-D-glucopyranose, and various 1-*O*-substituted derivatives thereof, with 6-benzamidopurine in the presence of certain catalysts. 9-α-D-Glucopyranosyladenine was isolated from the anomeric mixture obtained by the fusion of 2,3,4,6-tetra-*O*-acetyl-1-*O*-trichloroacetyl-α-D-glucopyranose with 6-benzamidopurine in the presence of toluene-*p*-sulphonic acid as catalyst.

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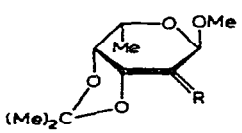
Note

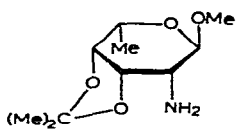
A new synthesis of L-fucosamine (2-amino-2,6-dideoxy-L-galactose)

Acidic hydrolysis of *Pneumococcus* Type V capsular polysaccharide[1,2] yields, *inter alia*, 2-amino-2,6-dideoxy-L-talose[3,4] (pneumosamine) and 2-amino-2,6-dideoxy-L-galactose[2] (L-fucosamine). The latter amino sugar has also been found as a constituent of the mucopolysaccharides of certain enteric bacteria[5] (*e.g.*, *Citrobacter freundii*), whilst the enantiomorph is a constituent of the lipopolysaccharide of *Chromobacterium violaceum*[6], and of the insoluble cell-wall residue of *Bacillus cereus*[7] and *Bacillus licheniformis*[8]. Two constitutional syntheses of D-fucosamine have been described[9] recently which prompt us to report a new synthesis of L-fucosamine, which was required in connection with structural studies on *Pneumococcus* Type V polysaccharide. A synthesis of L-fucosamine from 5-deoxy-L-lyxose has been accomplished by Kuhn *et al.*[10].

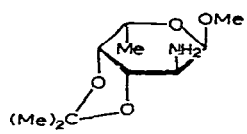
We have shown[4] that catalytic reduction of the oxime (II) derived from methyl 6-deoxy-3,4-*O*-isopropylidene-α-L-*lyxo*-hexopyranosidulose (I) affords predominantly the amino sugar derivative (III) having the L-*talo* configuration. Although chromatographic evidence indicated that a small amount of the L-*galacto* epimer (IV) is also formed, we were unable to isolate workable amounts of L-fucosamine. However,



I R=O
II R=N·OH



III



IV

reduction of the oxime (II) with lithium aluminium hydride gave a product mixture in which the proportion of the L-*galacto* epimer (IV) appeared (as judged by thin-layer chromatography) to be substantially greater than that for the catalytic reduction. The product mixture (III and IV) was *N*-acetylated, the protecting groups were removed by treatment with acid, and the mixture of free amino sugars was fractionated on Dowex-50 (H^+ form). L-Fucosamine was eluted first, and was characterised as its crystalline hydrochloride, which was identical (m.p., rotation, infrared spectrum) with the compound isolated[1,2] from Type V pneumococcal polysaccharide. The infrared spectrum of this derivative was also indistinguishable from those recorded for the amino sugar hydrochlorides recovered from *Chromobacterium*

violaceum[5,7], *Citrobacter freundii*[5], and *Bacillus cereus*[7]. The second amino sugar, 2-amino-2,6-dideoxy-L-talose hydrochloride, was characterised by comparison of its physical properties with those of the D-enantiomorph, synthesised by an unequivocal route[3]. 2-Amino-2,6-dideoxy-L-talose and -galactose hydrochlorides were recovered from the resin in a ratio of 2.5:1; the L-*allo* epimer also predominated in the similar reduction carried out by Collins and Overend[11]. The stereochemistry of the metal hydride reduction of methyl 6-deoxy-3,4-*O*-isopropylidene-α-L-*lyxo*-hexopyranosidulose (I) has been discussed recently by these workers[12], and similar effects are likely to be operating, to some extent, on reduction of the oxime (II).

EXPERIMENTAL

Reduction of methyl 6-deoxy-3,4-O-isopropylidene-α-L-lyxo-hexopyranosidulose oxime (II)

A solution of the oxime[4] {$[\alpha]_D^{26}$ −130° (*c* 1, chloroform), 1.25 g} in dry ether (50 ml) was gradually added to a stirred suspension of lithium aluminium hydride (1.25 g) in dry ether (50 ml), and the mixture was stirred at room temperature for 1 h and then heated under reflux for 3 h. Water was added to the cooled mixture to decompose the excess of reagent, the ethereal layer was separated, and the residue extracted thoroughly with ether (4 × 100 ml). Removal of the solvent from the combined and dried ($MgSO_4$) extracts gave a syrup (1 g) which on thin-layer chromatograms (ethyl acetate) showed (vanillin–ethanol[13]) two major components.

L-*Fucosamine hydrochloride and 2-amino-2,6-dideoxy-L-talose hydrochloride*

A solution of the foregoing syrup (1 g) in water (40 ml) and methanol (2.8 ml), containing acetic anhydride (2.8 ml) and Dowex-1 (CO_3^{2-} form) (70 ml), was stirred at 0° for 90 min. The combined filtrate and aqueous washings were stirred for 10 min with Amberlite IR-120 (H^+ form), the solution was filtered, and the resin washed thoroughly with water. The combined filtrate and washings were evaporated (<40°) to a syrup (*ca.* 0.73 g) which was taken up in 2N hydrochloric acid (40 ml) and heated at 95° for 8 h. The solution was diluted to *ca.* 100 ml with water, applied to a freshly regenerated column of Dowex-50 (H^+ form) (43 × 4 cm, 200–400 mesh), and the column eluted with 0.3N hydrochloric acid. Fractions (25 ml) were collected automatically, and an aliquot portion (0.5 ml) of every fourth fraction was analysed for amino sugars with the Elson–Morgan reagent[14]. L-Fucosamine hydrochloride was eluted between 2.27–2.77 l, and the appropriate fractions were combined and evaporated, with frequent additions of methanol, to give a crystalline residue (0.12 g). Recrystallisation from water–methanol–acetone yielded the pure hydrochloride (60 mg), m.p. 191–192° (dec.), $[\alpha]_D^{29}$ −122° (3 min) → −93° (final, *c* 0.31, water). (Found: C, 36.3; H, 7.3. $C_6H_{14}ClNO_4$ calc.: C, 36.1; H, 7.1%). Kuhn *et al.*[10] give m.p. 192–193° (dec.), $[\alpha]_D^{27}$ −119° (2 min) → −92° (final, *c* 0.89, water) for this compound, and Zehavi and Sharon[9] give m.p. 192° (dec.), $[\alpha]_D^{23}$ +117° (3 min) → +92° ± 1.2° (final, *c* 0.2, water) for the D-enantiomorph.

2-Amino-2,6-dideoxy-L-talose hydrochloride (0.3 g) was obtained by similar treatment of the fractions eluted between 2.97–3.72 l, and the pure hydrochloride (0.18 g, from water–methanol–acetone) had m.p. 162–163° (dec.), $[\alpha]_D^{30}$ −4° (3 min) → +6° (10 min) → +10° (final, *c* 1, water) (lit.[3], D-enantiomorph, m.p. 161.5–163.5° (dec.), $[\alpha]_D^{18.5}$ +8° (4 min) → −10° (final, *c* 1.7, water). The chromatographic properties and infrared spectrum (Nujol mull) of the hydrochloride were indistinguishable from those of pneumosamine hydrochloride[1,2,4] and 2-amino-2,6-dideoxy-D-talose hydrochloride[3].

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REACTION BETWEEN 2-AMINO-2-DEOXY-D-GLUCOSE AND 2,4-PENTANEDIONE

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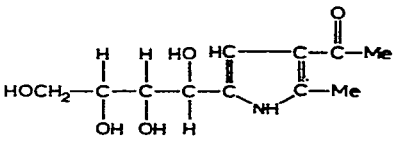
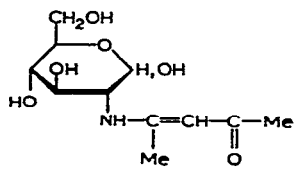
INTRODUCTION

The reaction between 2-amino-2-deoxy-D-glucose and 2,4-pentanedione[1–7] has attracted attention because of its relation to the Elson–Morgan procedure[8,9] for the estimation of aldosamines. Although it is agreed that when the reaction is carried out under neutral conditions the product is 3-acetyl-2-methyl-5-(D-*arabino*-tetrahydroxybutyl)pyrrole (I), there are discrepancies in the physical properties (melting point) ascribed to this substance[1–4,7,10], and in no case has a rigorous proof of the structure been given. It is possible that more than one substance is produced



I



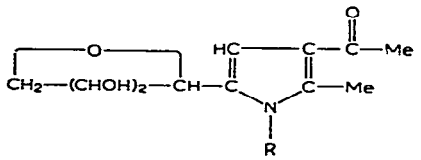
II

and, indeed, when the reaction is performed in a slightly alkaline medium, at least four compounds giving positive pyrrole tests to the Ehrlich reagent (*p*-dimethylaminobenzaldehyde–hydrochloric acid) have been detected[5]; two of these were identified[6] as 2-methylpyrrole and 3-acetyl-2-methylpyrrole. We report now the results of a recent investigation of this reaction; this study is an extension of previous work[3,4].

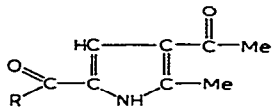
RESULTS AND DISCUSSION

Preparation of 3-acetyl-2-methyl-5-(D-*arabino*-tetrahydroxybutyl)pyrrole (I) is best carried out from 2-amino-2-deoxy-D-glucose hydrochloride, with the equivalent amount of sodium carbonate, and 2,4-pentanedione in aqueous acetone, at room temperature and neutral pH. The almost pure product (I) crystallizes from the reaction medium, as a monohydrate (m.p. 106–107°), in yields of over 80%; crystallization of the monohydrate from ethanol gives the anhydrous form (m.p. 142–142.5°). Other products (detected chromatographically) of the reaction under these conditions

are 3-acetyl-2-methylpyrrole and 2-deoxy-2-[2-(4-oxo-2-pentenyl)amino]-D-glucose (II) (see below). When the reaction is performed at a higher temperature and substituting ethanol for acetone, substantial amounts of 3-acetyl-5-[2-(3,4-dihydroxytetrahydrofuryl)]-2-methylpyrrole (III) are also produced. Compound (III) can be detected in aged reaction mixtures of 2-amino-2-deoxy-D-glucose and 2,4-



III R=H
IV R=Et
V R=n-C_4H_9



VI R=H
VII R=OH

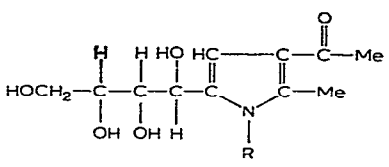
pentanedione, and often in the mother liquor of crystallization of compound (I). If the reaction is carried out using 2-amino-2-deoxy-D-glucose hydrochloride and the equivalent amount of sodium methoxide in ethanol, as indicated by Boyer and Fürth[2], yields of the pyrrole (I) are lower, and the whole procedure is more involved. In this case, the anhydrous form (m.p. 142–142.5°) is directly obtained.

Evidence for the structure of compound (I) is as follows. When treated with Ehrlich reagent, compound (I) gives an unstable red colour having maxima at 530 mμ (strong) and *ca.* 500 mμ (weaker) (Fig. 1). The absorption at 530 mμ decreases with time, and that at 500 mμ increases. Both the hydrated and the anhydrous form of the compound have the ultraviolet absorption pattern (maxima at 210, 246, and 290 mμ) typical of a methyl pyrrol-3-yl ketone[11]. The infrared spectrum shows the NH and C=O bands at the frequencies observed in other methyl pyrrol-3-yl ketones[12]. Acetylation of either form gives the same tetra-acetyl derivative. Oxidation with sodium metaperiodate, or with minium (Pb_3O_4) in acetic acid, furnishes 4-acetyl-5-methyl-2-pyrrolecarboxaldehyde (VI); the preparation using minium can be accomplished with an over-all yield of 50% from 2-amino-2-deoxy-D-glucose hydrochloride. Oxidation of compound (VI) with silver oxide–sodium hydroxide gives 4-acetyl-5-methyl-2-pyrrolecarboxylic acid (VII) which can be decarboxylated to known[6] 3-acetyl-2-methylpyrrole*.

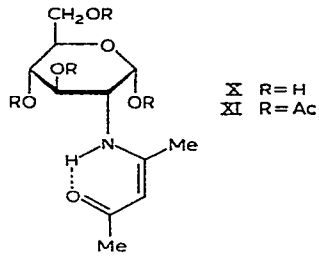
Tetrahydroxybutyl-furans and -pyrroles similar to compound (I) lose a molecule of water in the tetrahydroxybutyl chain giving 1,4-anhydro-derivatives[13]. 3-Acetyl-2-methyl-5-(D-*arabino*-tetrahydroxybutyl)pyrrole (I), upon heating in the dry state *in vacuo*, or in neutral or slightly acid solutions, readily gives compound (III) which affords a diacetate. The results of its periodate oxidation, also in agreement with

*This reaction sequence was described in the paper[4a] of 1950, but the sample of 3-acetyl-2-methylpyrrole (m.p. 75°) then obtained was not completely pure, *cf.* the product (m.p. 94–95°) synthesized[6] afterwards from aminoacetaldehyde and 2,4-pentanedione.

structure (III), have been reported previously[4b]. Similarly, the *N*-alkyl derivatives of the pyrrole (I), substances (VIII) and (IX), give the corresponding anhydroderivatives (IV) and (V).



VIII R = Et
IX R = *n*-C_4H_9



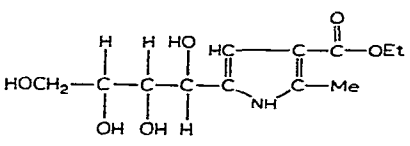
X R = H
XI R = Ac

The reaction between 2-amino-2-deoxy-D-glucose and 2,4-pentanedione in dilute aqueous solution at pH 9.5–10 gives 3-acetyl-2-methyl-5-(D-*arabino*-tetrahydroxybutyl)pyrrole (I) in 30% yield. This substance is therefore a product of the reaction under conditions similar to those prescribed in the analytical method of Elson and Morgan. Other pyrrole compounds previously identified in the reaction mixture obtained under these conditions are 2-methylpyrrole (yield 10%) and 3-acetyl-2-methylpyrrole[6]. As compound (I), when heated with *p*-dimethylaminobenzaldehyde in hydrochloric acid, gives (see above) a red colour absorbing maximally at 530 mμ (*i.e.*, the wavelength used in the analytical method), it can be considered to be a chromogen in the Elson–Morgan reaction. It should be pointed out in this connection that in several modifications[14–18] of the Elson–Morgan method colours having absorption maxima at 530 mμ are obtained. This fact seems to be in conflict with the current view[6,9] that 2-methylpyrrole, which gives with the *p*-dimethylaminobenzaldehyde–hydrochloric acid reagent a colour with maximum at 548 mμ[6], is the main chromogen. To ascertain whether steam-volatile 2-methylpyrrole derives from the more complex pyrrole (I) under the conditions prevailing in the analytical method, this compound was heated in dilute aqueous solution at pH 10. No pyrrole compound could be detected in a steam distillate of the reaction mixture. Also, the absorption curve given by compound (I) (preheated with sodium carbonate at pH 10) and the Ehrlich reagent is practically the same as that obtained with a non-pretreated solution of (I) (Fig. 1). If any conversion of compound (I) into 2-methylpyrrole had occurred, it would have produced a shift of the absorption to 548 mμ.

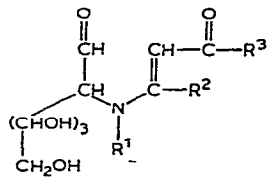
The formation of pyrrole (I) from 2-amino-2-deoxy-D-glucose and 2,4-pentanedione in a mixture of triethylamine and methanol has been reported[7] recently. We have repeated this work and established the identity of the product so obtained with the one described above. Under these conditions, the enamine (II) can be isolated. Cessi and Serafini-Cessi[7], who first obtained this compound, proposed structure (II) on the basis of its ultraviolet absorption, and quantitative hydrolysis to 2-amino-2-deoxy-D-glucose and 2,4-pentanedione. Additional evidence is now

presented with the infrared spectrum of compound (II), which shows a band at 1613 cm^{-1} (C=O group in an intramolecularly bonded β-amino-α,β-unsaturated ketone[19–21]), and a broad, strong band centred at *ca.* 1543 cm^{-1}. Compound (II) gives a tetra-*O*-acetyl derivative, the infrared spectrum (carbon tetrachloride solution) of which has bands at 1616, 1577, and 1495 cm^{-1}, typical of chelated β-amino-α,β-unsaturated ketones[19–21]. As in other compounds of this class[19], no NH stretching absorption was observed either in solution or in the solid state. Both compound (II) and its tetra-*O*-acetyl derivative have high, positive, optical rotations, suggestive of α-D anomeric configurations. Accordingly, these compounds are better represented by formulae (X) and (XI). 2-Deoxy-2-[2-(4-oxo-2-pentenyl)-amino]-α-D-glucose (II or X), when stored in aqueous solution at room temperature, cyclized to a mixture of 3-acetyl-2-methylpyrrole and 3-acetyl-2-methyl-5-(D-*arabino*-tetrahydroxybutyl)pyrrole (I). D-Erythrose could be detected chromatographically in the reaction mixture. When compound (II) was heated at pH 10, the product was 3-acetyl-2-methylpyrrole. 2-Methylpyrrole was detected in only trace amounts (see also ref. 7).

2-Amino-2-deoxy-D-glucose and ethyl acetoacetate react in aqueous solution at pH 9.5–10 to give ethyl 2-methyl-3-pyrrolecarboxylate and ethyl 2-methyl-5-(D-*arabino*-tetrahydroxybutyl)-3-pyrrolecarboxylate (XII), in the approximate ratio



XII



XIII

2:3, and the over-all yield is 50%. No 2-methylpyrrole was found. In the reaction in the presence of triethylamine, the tetrahydroxybutyl ester (XII) was the only product to be detected chromatographically and isolated.

The formation of pyrrole compounds from amino sugars and β-dicarbonyl compounds is probably a stepwise process, the mechanism of which is still uncertain. The isolation of compound (II), and its conversion into 3-acetyl-2-methyl-5-(D-*arabino*-tetrahydroxybutyl)pyrrole (I) and 3-acetyl-2-methylpyrrole, suggests that enamines of the type (XIII) may be the first intermediates in these reactions. A similar point of view has been expressed previously by Cornforth and his co-workers[22], and by Gottschalk[23]. However, the low yield of 2-methylpyrrole obtained from the enamine (II), when heated at pH 9–10, in comparison with that obtained from 2-amino-2-deoxy-D-glucose and 2,4-pentanedione under similar conditions, seems to indicate that some other mechanism(s) may be operative. The mechanism of the reaction between 2-amino-2-deoxy-D-glucose and 2,4-pentanedione will be discussed elsewhere[24], under the more general context of the reaction of monosaccharides and β-dicarbonyl compounds.

EXPERIMENTAL

Melting points are uncorrected. Concentrations and evaporations of solvents were carried out *in vacuo* at temperatures not over 50°. Paper chromatography was carried out on Whatman No. 1 paper by the horizontal technique at room temperature using butan-1-ol–ethanol–water (10:1:2), unless otherwise stated. Detection reagents used were silver nitrate–sodium hydroxide for monosaccharides and polyhydroxylic compounds, and chromatographic Ehrlich reagent (prepared by dissolving 1.0 g of *p*-dimethylaminobenzaldehyde in 30 ml of ethanol, adding 30 ml of concentrated hydrochloric acid, and diluting with butan-1-ol to 100 ml) for pyrrole derivatives. The analytical Ehrlich reagent was prepared by dissolving 0.5 g of *p*-dimethylaminobenzaldehyde in 6 ml of ethanol and 6 ml of conc. hydrochloric acid[6]. The Ehrlich reagent for other purposes was prepared as indicated in the literature[25]. The u.v. spectra were obtained with a Beckman DU spectrophotometer. The i.r. spectra were obtained with a Beckman IR-5A instrument, and those marked (*) with a Perkin–Elmer 237 instrument.

*3-Acetyl-2-methyl-5-(*D-arabino-*tetrahydroxybutyl)pyrrole (I)*

2-Amino-2-deoxy-D-glucose hydrochloride (21.6 g, 0.1 mole) and 5.3 g (0.05 mole) of sodium carbonate were dissolved in 75 ml of water, and 10.0 g (0.1 mole) of 2,4-pentanedione and 12 ml of acetone added. The resulting solution was left at room temperature. Paper chromatography after 20 min showed the presence of the following substances (R_F): (I) (0.53), 2-deoxy-2-[2-(4-oxo-2-pentenyl)amino]-D-glucose (X) (0.62), and 3-acetyl-2-methylpyrrole (0.89). When sprayed with the Ehrlich reagent, compound (I) produces a red colour which turns green, and compound (X) an orange colour that turns yellow. Crystallization of compound (I), as a monohydrate, began after 10–12 h. After three days, the crystalline mass was filtered off and dried in the air; yield, 21.3 g (85%), m.p. 103–105°. Two recrystallizations from water gave the analytical sample, m.p. 106–107°, $[\alpha]_D^{18}$ −34° (*c* 0.9, water); λ_{max} (ethanol) 211, 247, 290 mμ (ε 16785, 7699, 5820); ν_{max} (Nujol) 3279, 1626 cm^{-1}.

Anal. Calc. for $C_{11}H_{17}NO_5 \cdot H_2O$: C, 50.56; H, 7.33; N, 5.36. Found: C, 50.79,, H, 7.58; N, 5.23.

The above monohydrate (2 g) was dissolved in 20 ml of boiling ethanol. On cooling, 1.7 g of the anhydrous form of compound (I) crystallized, m.p. 141–142°. Two additional recrystallizations from the same solvent gave a product having m.p. 142–142.5°, $[\alpha]_D^{18}$ −52° (*c* 0.7, water); λ_{max} 210, 246, 290 mμ (ε 19990, 8278, 6081).

Anal. Calc. for $C_{11}H_{17}NO_5$: C, 54.31; H, 7.04; N, 5.75. Found: C, 54.41; H, 7.01; N, 5.82.

Recrystallization of the anhydrous form, m.p. 142–142.5°, from water regenerated the monohydrate, m.p. 104–105°.

Anal. Found: C, 50.66; H, 7.09; N, 5.43.

The mother liquor of the crystallization of compound (I) (monohydrate) was concentrated and extracted with ether. The ethereal extracts were dried ($MgSO_4$) and concentrated. Thin-layer chromatography [silica gel; light petroleum–ether (1:1)] showed the presence of a substance having the same mobility as 3-acetyl-2-methylpyrrole. Evaporation of the solvent left a dark oily residue that was not further examined.

Preparation of compound (I) according to Boyer and Fürth[2] gave a 60% yield of product, m.p. and mixed m.p. with the sample described above 142–142.5°. Boyer and Fürth give m.p. 137°.

The reaction in the presence of triethylamine[7] was carried out as follows: 2.0 g (9 mmole) of 2-amino-2-deoxy-D-glucose hydrochloride, 25.0 g (0.25 mole) of 2,4-pentanedione, 25 ml of triethylamine, and 1 ml of pyridine in 50 ml of methanol were heated at 55° for 15 h. Evaporation of solvents left a syrup that crystallized on treatment with a little hot water. A portion (0.5 g) of this material (1.3 g) was recrystallized twice from water yielding compound (I) monohydrate, m.p. and mixed m.p. with the sample described above 106–107°. Another sample (0.5 g) was recrystallized from ethanol, as indicated before, yielding compound (I), m.p. and mixed m.p. 142–142.5°.

*3-Acetyl-2-methyl-5-(*D-arabino-*tetra-acetoxybutyl)pyrrole*

A solution of 1.0 g of compound (I) in 6 ml of pyridine was treated with 6 ml of acetic anhydride at 0°. After storage for 48 h in the refrigerator, the product was precipitated by pouring the reaction mixture onto ice. After recrystallization from ethanol–water (1:1), the product (71%) had m.p. 132–133°, ν_{max} (chloroform) 3460, 3300, 1730, 1650, 1585, and 1524 cm^{-1}.

Anal. Calc. for $C_{15}H_{24}NO_5$: C, 55.47; H, 6.13; N, 3.41. Found: C, 55.26; H, 6.38; N, 3.70.

4-Acetyl-5-methyl-2-pyrrolecarboxaldehyde (VI)

(*a*) A solution of compound (I) (1.1 g, 4.5 mmole) in water was treated with a slight excess of a saturated solution of sodium metaperiodate. After 30 min, the crystalline solid was collected, washed with cold water, and recrystallized from acetone–water (1:1) to yield 0.33 g (49%) of compound (VI), m.p. 144–145°, λ_{max} (ethanol) 228, 298 (ε 14960, 17840), ν_{max} (chloroform) 3436, 3279, 3012, 2817, 1650, 1567, and 1504 cm^{-1}.

(*b*) A suspension of the monohydrate of compound (I) (0.65 g, 2.5 mole) in 80 ml of acetic acid–water (1:3) was treated under vigorous stirring with 20.5 g (0.03 mole) of minium (Pb_3O_4), added in small portions over a period of 9 h, and stirring was continued for a further 14 h. The reaction mixture was extracted with ether (7 × 20 ml), and the combined extract washed with water (3 × 20 ml) and dried (Na_2SO_4). Evaporation of the solvent left crude compound (VI) (0.3 g, 59%), m.p. 139–142°. Recrystallization from acetone–water gave the analytical sample, m.p. 144–144.5°.

Anal. Calc. for $C_8H_9NO_2$: C, 63.56; H, 6.00; N, 9.26. Found: C, 63.38; H, 5.98; N, 9.62.

4-Acetyl-5-methyl-2-pyrrolecarboxylic acid (VII)

A suspension of silver oxide in water was prepared by dissolving 7.4 g (44 mmole) of silver nitrate in 15 ml of water, and adding 75 ml of N sodium hydroxide. Compound (VI) (3.0 g, 20 mmole) was added, and the mixture was heated under reflux for 40 min. The cooled reaction mixture was filtered and the solid residue washed several times with water. The filtrate and the washings were mixed and acidified (Congo Red) with dilute nitric acid. The acid (VII), precipitated as a dark solid (2.5 g), was filtered off and washed with water. The mother liquor was extracted with ether (3 × 10 ml), and the combined extracts were dried ($MgSO_4$). Evaporation of the solvent left an additional 0.2 g of compound (VII). Crude compound (VII) (2.7 g) was dissolved in 10 ml ethanol, applied to a column (25 × 3 cm) of alumina, and eluted with a mixture of conc. ammonia (sp. gr. 0.973) and ethanol (1:9). The eluates giving a positive reaction to the Ehrlich reagent were collected and concentrated. Acidification with hydrochloric acid gave 1.2 g (36%) of compound (VII), m.p. 255–256°. Recrystallization from ethanol–water (2:1) gave the analytical sample, m.p. 255–256° (dec.), λ_{max} (ethanol) 252, 284 mμ (ε 9533, 9400); ν_{max} (Nujol) 3279, 2632 (broad), 1653, 1575, 1508 cm^{-1}.

Anal. Calc. for $C_8H_9NO_3$: C, 57.47; H, 5.43; H, 8.38; neutralization equivalent, 167. Found: C, 57.48; H, 5.52; N, 8.40; neutralization equivalent, 170.

3-Acetyl-2-methylpyrrole

4-Acetyl-5-methyl-2-pyrrolecarboxylic acid (VII) (100 mg) was heated at 250–260° (bath temperature) in a tube fitted with a "cold finger". The sublimate (51 mg), m.p. 86–89°, was recrystallized from light petroleum (40–70°) to give pure 3-acetyl-2-methylpyrrole, m.p. 95–97° (lit.[6], 94–95°); λ_{max} (ethanol) 243, 282 mμ (ε 7822, 5822).

Anal. Calc. for C_7H_9NO: C, 68.26; H, 7.44; N, 11.38. Found: C, 68.20; H, 7.12; N, 11.30.

3-Acetyl-5-[2-(3,4-dihydroxytetrahydrofuryl)]-2-methylpyrrole (III)

(*a*) Compound (I) monohydrate (5.0 g) was heated to constant weight over phosphorus pentoxide at 70°/20 mm [wt. loss: 0.7 g (calc. for two mol. of water, 0.8 g)]. The residual material had m.p. 159–163° and, after recrystallization from ethanol, m.p. 165–166°, $[\alpha]_D$ −104° (*c* 1, water).

(*b*) Compound (I) monohydrate (2 g) in water (10 ml) was heated at 100° for 3 h. Evaporation of the solvent left a crystalline residue which, after recrystallization from ethanol, gave compound (III) (1.1 g, 64%), m.p. 163–164°.

(*c*) Compound I monohydrate (5.0 g) dissolved in 25 ml of water containing 0.25 ml of acetic acid was heated at 100° for 3 h. Working up as before gave 2.6 g (60%) of compound (III), m.p. 165–166°.

Anal. Calc. for $C_{11}H_{15}NO_4$; C, 58.66; H, 6.71; N, 6.21. Found: C, 58.67; H, 6.64; N, 6.45.

3-Acetyl-5-[2-(3,4-diacetoxytetrahydrofuryl)]-2-methylpyrrole

Acetylation of compound (III), as indicated before for compound (I), gave the corresponding diacetyl derivative (84%) which, after recrystallization from ethanol, had m.p. 133.5–134.5°.

Anal. Calc. for $C_{15}H_{19}NO_6$: C, 58.28; H, 6.19; N, 4.52. Found: C, 58.53; H, 6.13; N, 4.82.

3-Acetyl-5-[2-(3,4-dihydroxytetrahydrofuryl)]-1-ethyl-2-methylpyrrole (IV)

A solution of compound (VIII) (0.2 g) in 2.5 ml of water was heated at 50° for 7 h. The crystalline material which separated on cooling was collected and recrystallized from ethanol, yielding 0.12 g (64%) of compound (IV), m.p. 189–191°, $[\alpha]_D^{17}$ −142° (*c* 3.5, pyridine); λ_{max} (ethanol) 219, 252 mμ (ε 20982, 7827, 6034).

Anal. Calc. for $C_{13}H_{19}NO_4$: C, 61.66; H, 7.50; N, 5.53. Found: C, 61.83; H, 7.53; N, 5.80.

In an analytical oxidation, there was a consumption of 1.05 mol. of periodic acid (calc., 1.0 mol.).

3-Acetyl-1-butyl-5-[2-(3,4-dihydroxytetrahydrofuryl)]-2-methylpyrrole (V)

A saturated solution of 0.2 g of compound (IX) in water was heated at 50°

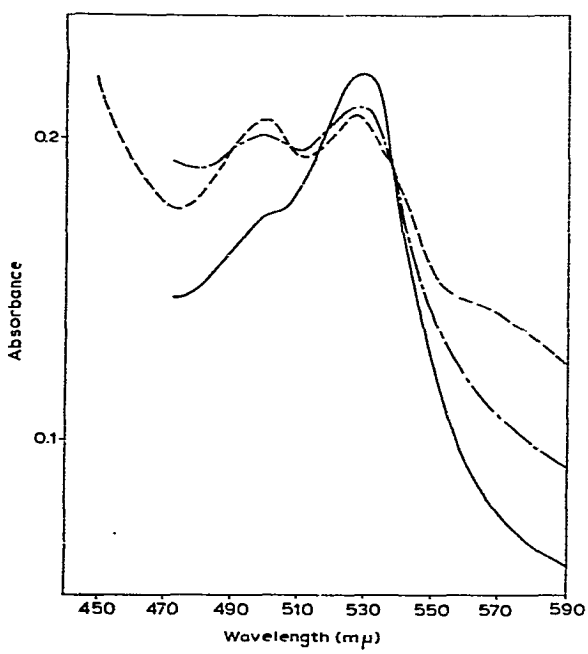


Fig. 1. Colour reaction given by compound (I) and *p*-dimethylaminobenzaldehyde: ——— after 3 h; · – · – · after 7 h; – – – – compound (I) previously heated at pH 9.5.

for 24 h; compound (V) crystallized on cooling. Recrystallization from ethanol yielded 0.1 g (53%) of the analytically pure substance, m.p. 167–168°, $[\alpha]_D^{17}$ −116° (*c* 0.8, pyridine); λ_{max} (ethanol) 220, 251, 283 mμ (ε 18243, 7268, 5463).

Anal. Calc. for $C_{15}H_{23}NO_4$: C, 64.05; H, 8.19; N, 4.98. Found: C, 63.96; H, 8.34; N, 4.87.

The compound consumed 1.0 mol. of periodic acid (calc., 1.0 mol.).

Colour reaction of compound (I) with the Ehrlich reagent

A solution of compound (I) (1.0 mg) in water (1 ml) was diluted with ethanol (5 ml) and the analytical Ehrlich reagent (0.5 ml) was added. The mixture was heated under reflux in a boiling-water bath for 15 min, cooled in an ice-water bath, and the red colour measured against a solution similar to the reaction mixture but for omission of the *p*-dimethylaminobenzaldehyde. The absorption curves at different intervals of time are shown in Fig. 1.

A solution of compound (I) (50.0 mg) in 25 ml of a sodium carbonate–sodium hydrogen carbonate buffer (pH 9.5) was heated at 100° for 20 min. After being cooled, a portion (0.5 ml) of this solution was diluted with water (0.5 ml) and ethanol (5 ml), and treated with the analytical Ehrlich reagent, as indicated before. The absorption curve of the colour developed appears in Fig. 1.

*Treatment of 3-acetyl-2-methyl-5-(*D-arabino-*tetrahydroxybutyl)pyrrole (I) with sodium carbonate at pH 9–10*

A solution of compound (I) (60.0 mg) in 10 ml of a buffer of sodium carbonate–sodium hydrogen carbonate (pH 9.55) was heated at 100° for 30 min. The reaction mixture was distilled at 20 mm pressure until it was reduced to a volume of 2 ml, the distillate being collected in a flask immersed in an ice-salt bath. A portion of this distillate did not develop any colour when treated with the analytical Ehrlich reagent as indicated before.

*Formation of compound (I) from 2-amino-2-deoxy-*D*-glucose and 2,4-pentanedione at pH 9-10*

2-Amino-2-deoxy-D-glucose hydrochloride (5.4 g, 25 mmole) and 2,4-pentanedione (7.5 g, 75 mmole) were dissolved in 175 ml of water containing sodium carbonate (6 g) and sodium hydrogen carbonate (0.5 g), and the pH was adjusted to 9.7 by addition of 5N sodium hydroxide. The solution was diluted to 200 ml, heated at 100° for 30 min, and cooled, and the pH was brought to 7.0 by addition of Zeokarb 225 (H^+ form). The resin was filtered off and the filtrate extracted repeatedly with ether until the extracts no longer gave a reaction with the Ehrlich reagent. The aqueous fraction was concentrated until crystallization of compound (I) started, and then refrigerated. Compound (I) monohydrate, m.p. 101–103°, was collected and air-dried. Concentration of the mother liquor, and treatment of the syrup with a little hot water afforded a second crop, m.p. 103–105°. Total

yield, 2.0 g (30%). After recrystallization from water, it had m.p. and mixed m.p. 106–107°.

Anal. Found: C, 50.77; H, 7.48; N, 5.45.

2-Deoxy-2-[2-(4-oxo-2-pentenyl)amino]-D-glucose (X)

The method of Cessi and Serafini-Cessi[7] is incorporated in the following procedure. A mixture of 10.8 g (0.05 mole) of 2-amino-2-deoxy-D-glucose hydrochloride, 15.0 g (0.15 mole) of 2,4-pentanedione, 15 ml of triethylamine, and 220 ml of methanol was heated until dissolution occurred, and then under reflux for 5 min. The mixture was concentrated to half its volume and refrigerated. The crystal mass of compound (X) (9.2 g, 70%), m.p. 109–111°, was filtered off and washed with ethanol. After recrystallization from ethanol, it had m.p. 111–112°, $[\alpha]_D^{24}$ +215° (*c* 2, water); λ_{max} (ethanol) 312 mμ (ε 18700); ν_{max} (Nujol) 3185, 1613, 1543 cm^{-1}. Cessi and Serafini-Cessi[7] give m.p. 107° (for a monohydrate), $[\alpha]_D^{20}$ +181° (water).

Anal. Calc. for $C_{11}H_{19}NO_6$: C, 50.56; H, 7.33; N, 5.36. Found: C, 50.41; H,7.52; N, 5.24.

Paper chromatography of an analytical sample of compound (X) showed, with the silver nitrate spray, a spot of R_F 0.58, and traces of compound (I) (R_F 0.51) and 2-amino-2-deoxy-D-glucose. The presence of the last two substances is considered to be due to partial cyclization [to give (I)] and hydrolysis of compound (X). The Ehrlich reagent spray showed only the spot of R_F 0.58.

1,3,4,6-Tetra-O-acetyl-2-deoxy-2-[2-(4-oxo-2-pentenyl)amino]-α-D-glucopyranose (XI)

A solution of compound (X) (1.0 g) in 4 ml of pyridine was treated with 4 ml of acetic anhydride at 0°, and stored at room temperature for two days. The reaction mixture was poured onto ice, and the solid precipitate filtered and washed with water. Recrystallization from ethanol gave 1.0 g (62%) of compound (XI), m.p. 174–175°, $[\alpha]_D^{18}$ 194° (*c* 1, pyridine); ν_{max} (KBr) 1754, 1739, 1610, 1569, 1513 cm^{-1} (*); ν_{max} (carbon tetrachloride) 1754, 1616, 1577, 1495 cm^{-1} (*).

Anal. Calc. for $C_{19}H_{27}NO_{10}$: C, 53.14; H, 6.34; N, 3.49. Found: C, 52.89; H, 6.40; N, 3.48.

Transformation of compound (X) into compound (I) and 3-acetyl-2-methylpyrrole

A solution of 15.0 g of compound (X) in 230 ml of water was left at room temperature. After three days, it was extracted with ether (5 × 15 ml), and the combined extracts were dried (Na_2SO_4) and evaporated. The residue (2.1 g), m.p. 88–94°, was recrystallized from ethanol–water (4:1) to yield 3-acetyl-2-methylpyrrole, m.p. and mixed m.p. with the sample described above 95–97°.

Anal. Found: C, 68.42; H, 7.01; N, 11.64.

The aqueous fraction was concentrated to yield 4.6 g (32%) of compound (I) (monohydrate) which, after recrystallization from water, had m.p. and mixed m.p. 106–107°. Paper chromatography (descending, butan-1-ol–acetic acid–water

4:1:5 upper layer) of the mother liquor showed the presence of a compound (R_F 0.42) having the same mobility as D-erythrose.

Treatment of compound (X) at pH 9–10

A solution of compound (X) (60.0 mg) in 10 ml of a sodium carbonate–sodium hydrogen carbonate buffer (pH 9.5), was heated at 100° for 30 min. The cooled reaction mixture was brought to pH 7.0 by addition of Zeokarb 225 (H^+ form), the resin filtered off, and the filtrate concentrated *in vacuo* (50–60° [bath]/20 mm) to a volume of 2 ml. The distillate was collected in a cooled (ice-salt) receiver. A portion (0.5 ml) of the distillate was diluted with water (0.5 ml) and ethanol (5 ml), and treated with 0.5 ml of the analytical Ehrlich reagent, as indicated above. No colour reaction developed in the cold, and only a very weak colour upon heating. Another portion (0.5 ml) of the distillate, treated with water (0.5 ml) and the Ehrlich reagent (0.5 ml) in the same way, developed in the cold a weak pink colour that had maximal absorption at 549 mμ. Thin-layer chromatography [silica gel; light petroleum–ether (1:1)] of the distillation residue showed the presence of 3-acetyl-2-methylpyrrole.

Reaction between 2-amino-2-deoxy-D-glucose and ethyl acetoacetate

(*a*) *At pH 9–10.* 2-Amino-2-deoxy-D-glucose hydrochloride (10.8 g, 0.05 mole) and 13.0 g (0.1 mole) of ethyl acetoacetate were dissolved in 350 ml of a buffer prepared by dissolving 10.6 g of sodium carbonate and 1.5 g of sodium hydrogen carbonate in 500 ml of water. The pH was adjusted to 9.7 by addition of 5N sodium hydroxide, and the resulting solution diluted with water to 400 ml, heated to 100° for 30 min, and cooled. The pH was brought to 7.2 by addition of Amberlite IR-120 (H^+ form), the resin was removed, and the filtrate extracted with ether (4 × 20 ml, and afterwards in a continuous extractor for 9 h). The combined extracts were dried (Na_2SO_4) and evaporated to leave a crystalline residue of ethyl 2-methyl-3-pyrrolecarboxylate (1.4 g, 18%), m.p. 75–78°. After recrystallization from ethanol–water (2:1), it had m.p. 79.5–81.5°; λ_{max} (ethanol) 225, 256 mμ (ε 7555, 6442); ν_{max} (chloroform) 3484, 3436, 3154, 3012, 1689, 1582, 1502 cm^{-1}. Lit.[26], m.p. 78–79°.

Anal. Calc. for $C_8H_{11}NO_2$: C, 62.72; H, 7.23; N, 9.1. Found: C, 62.44; H, 7.38; N, 9.11.

A sample (197 mg) of the above ester was refluxed with 13 ml of 3.25N sodium hydroxide for 2 h. The resulting solution was filtered, ice-cooled, and acidified (to Congo Red) with conc. hydrochloric acid. On storage in the refrigerator, crystallization of 2-methyl-3-pyrrolecarboxylic acid took place. Recrystallization from water yielded 67 mg (42%) of product, m.p. 171–173° (dec.) (lit.[26,27], 169°].

The aqueous solution, after the extraction with ether, was concentrated to half its volume and refrigerated. Ethyl 2-methyl-5-(D-*arabino*-tetrahydroxybutyl)-3-pyrrolecarboxylate (XII)[13] (4.0 g), m.p. 139–140°, was filtered off and dried. Concentration of the mother liquor afforded additional product (0.2 g), m.p. 138–140°

(total yield 39%). After recrystallization from water, the product had m.p. and mixed m.p. 141–142°.

(*b*) *In the presence of triethylamine.* A mixture of 10.8 g (0.05 mole) of 2-amino-2-deoxy-D-glucose hydrochloride, 13.0 g (0.1 mole) of ethyl acetoacetate, 13 ml of triethylamine, and 200 ml of methanol was heated gently until complete dissolution occurred, and then under reflux for 5 min. Paper chromatography [descending, butan-1-ol–ethanol–water–ammonia (40:10:49:1)] showed the presence of D-glucosamine and a substance having the same mobility (R_F 0.73) as compound (XII). Evaporation of the solvents left a residue that crystallized upon treatment with ether. Recrystallization from ethanol afforded pure compound (XII), m.p. and mixed m.p. 141–142°.

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SUMMARY

2-Amino-2-deoxy-D-glucose and 2,4-pentanedione react in neutral solution giving 3-acetyl-2-methyl-5-(D-*arabino*-tetrahydroxybutyl)pyrrole (I), and minor amounts of 3-acetyl-2-methylpyrrole and 2-deoxy-2-[2-(4-oxo-2-pentenyl)amino]-D-glucose (II). The structure of compound (I) is demonstrated and its properties described. The pyrrole (I) is also a product of the reaction at pH 9–10, and its possible role as a chromogen in the Elson–Morgan method for the estimation of 2-amino-2-deoxy-D-glucose is discussed. Directions for the preparation of compound (II) are given, and its structure and properties studied. Reaction between 2-amino-2-deoxy-D-glucose and ethyl acetoacetate at pH 9–10 yields ethyl 2-methyl-5-(D-*arabino*-tetrahydroxybutyl)-3-pyrrolecarboxylate (XII) and ethyl 2-methyl-3-pyrrolecarboxylate.

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THE PARTIAL SYNTHESIS OF TWO 1,2-*cis* CARDENOLIDES*

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Klyne[1] has noted that, with various steroidal glycosides, the molecular rotation is approximately the sum of the molecular rotations of the steroidal alcohol and of the methyl α- or β-(D or L)-glycoside corresponding configurationally to the sugar residue in the glycoside. On this basis, he has shown that cardenolides of natural origin containing D-sugars are β-D anomers, and those which contain L-sugars have the same absolute, or α-L, anomeric configuration. Previously, the syntheses of the α-digitoxoside (2,6-dideoxy-α-D-*ribo*-hexoside) of digitoxigenin[2] and the α-D-rhamnosides (6-deoxy-α-D-mannosides, X and XII)[3] of digitoxigenin and of strophanthidin have been reported. All three contain the "unnatural", α-D-glycosidic linkage and show surprisingly low cardiotonic activities when compared with related glycosides of natural origin[4]. This suggested that the α-D-glycosidic linkage in cardenolides containing D-sugars provides for a molecular configuration that is not optimum for cardiotonic activity[3b]. However, the basis for this postulate rested on the comparison of the activities of cardenolides containing the α-L and β-D forms of the same sugar because anomeric pairs of cardenolides are not available.

The stable form for the *O*-acylglycosyl halides of the enantiomorphic aldohexopyranoses (to include rhamnose) is the α-(D or L) anomer. When there is a 1,2-*cis* relationship between the halogen at C-1 and the acyloxyl group at C-2, replacement occurs at C-1 with inversion. Conversely, 1,2-*trans* halides may undergo replacement with net retention of configuration[5]. The result is that it is difficult to synthesize 1,2-*cis* glycosides; in fact, with all previous syntheses of cardenolides, the 1,2-*trans* glycoside has been the exclusive product. Recently, it was reported by Gorin and Perlin[6] that β-D-glycosides are the preponderant products from the reactions of 4,6-di-*O*-acetyl-2,3-*O*-carbonyl-α-D-mannopyranosyl bromide with 1,2,3,4-tetra-*O*-acetyl-β-D-glucose and with 1,2,3,4-tetra-*O*-acetyl-β-D-mannose. Only trace amounts of the α-D-glycosides were formed during the reaction. Since the inverted products

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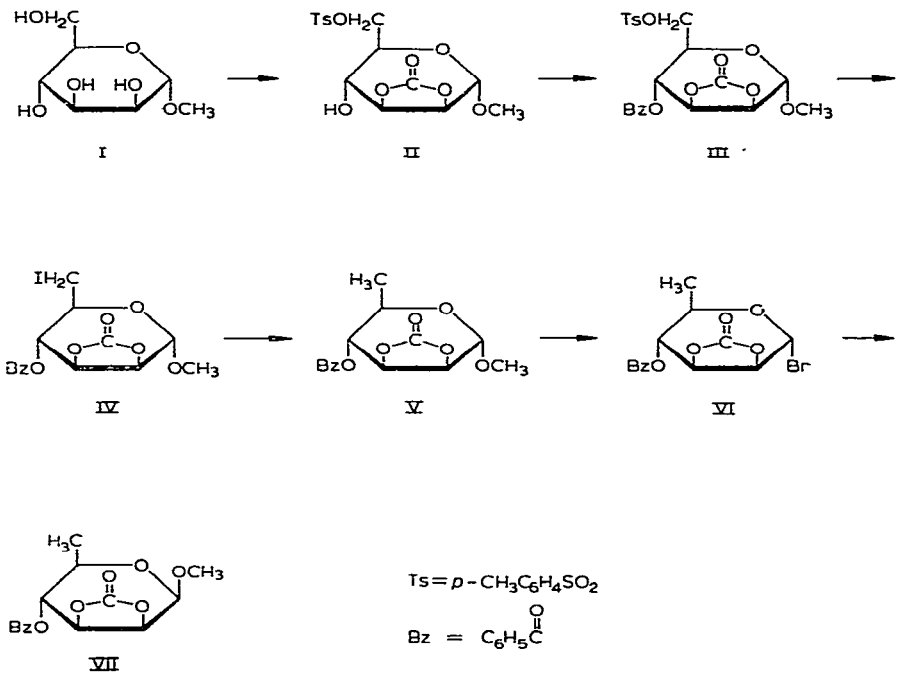
were formed almost exclusively, the use of 2,3-*O*-carbonylglycosyl halides could presumably also lead to the synthesis of 1,2-*cis* cardenolides. On this basis, the synthesis of the β-D-rhamnosides (XI and XIII) of digitoxigenin and of strophanthidin was undertaken. The choice of the β-D-rhamnosides was dictated by the availability of the α-D anomers for comparison purposes.

Two syntheses of the non-naturally occurring D-rhamnose are available[7], but, for the present purpose, both methods are indirect and, furthermore, give low yields. Several alternative approaches to a D-rhamnosyl halide containing the 2,3-*O*-carbonyl group were explored, and the sequence finally adopted is given in Scheme I. Methyl

Scheme 1



α-D-mannopyranoside (I) was *p*-toluenesulfonated and, without isolation of the sirupy *p*-tolylsulfonyl derivative, this was treated with carbonyl chloride, giving crystalline methyl 2,3-*O*-carbonyl-6-*O*-*p*-tolylsulfonyl-α-D-mannopyranoside (II). The reduction of the 6-*O*-tolylsulfonyl group of II with lithium aluminum hydride in ether, or with sodium in liquid ammonia in the presence of alcohols, could not be accomplished without involving the carbonate ring[8]. Since direct reduction failed, the C-4 hydroxyl group of II was protected by benzoylation, to give the crystalline derivative III. In subsequent conversions, *p*-tolylsulfonation, carbonylation, and benzoylation were carried out without isolation of II. In this way, the yield of III, based on starting material I, was 37 %.

The *p*-tolylsulfonyloxyl group of III was next replaced by iodine to give the 6-iodo derivative IV. Attempts to reduce IV in methanol by hydrogenation, using Raney nickel as the catalyst, also involved attack on the carbonate ring[8], but, when the derivative IV was hydrogenated in the presence of a "nickel boride" catalyst[9] under anhydrous conditions, the desired methyl 4-*O*-benzoyl-2,3-*O*-carbonyl-6-deoxy-α-D-mannoside (V) was obtained in excellent yield. Treatment of V with hydrogen bromide in acetic acid gave crystalline 4-*O*-benzoyl-2,3-*O*-carbonyl-6-deoxy-α-D-mannosyl bromide (VI). This derivative was very reactive and decomposed rapidly in moist air; it could, however, be stored in a desiccator away from light at −78°. The bromide VI readily underwent methanolysis in the presence of silver carbonate to give, by inversion, methyl 4-*O*-benzoyl-2,3-*O*-carbonyl-6-deoxy-β-D-mannoside (VII) and these results are consistent with those reported by Gorin and Perlin[6].

However, coupling of digitoxigenin (VIII) with the bromide VI in 1,1,2-trichloroethane in the presence of silver carbonate, followed by saponification of the reaction mixture, gave 48 % of the previously described α-D-rhamnoside (X)[3a], but only 13 % of the desired β-D anomer (XI) (Scheme II). Coupling with strophanthidin (IX) under essentially the same conditions gave 8 % of the known α-D-rhamnoside (XII)[3b] and 6 % of the β-D anomer (XIII). In both cases, failure to yield inverted products exclusively is most likely due to steric factors in which the bulky steroid aglycon attacks the initially formed carbonium ion[10] from below the plane of the ring to give the α-D anomer as the major product.

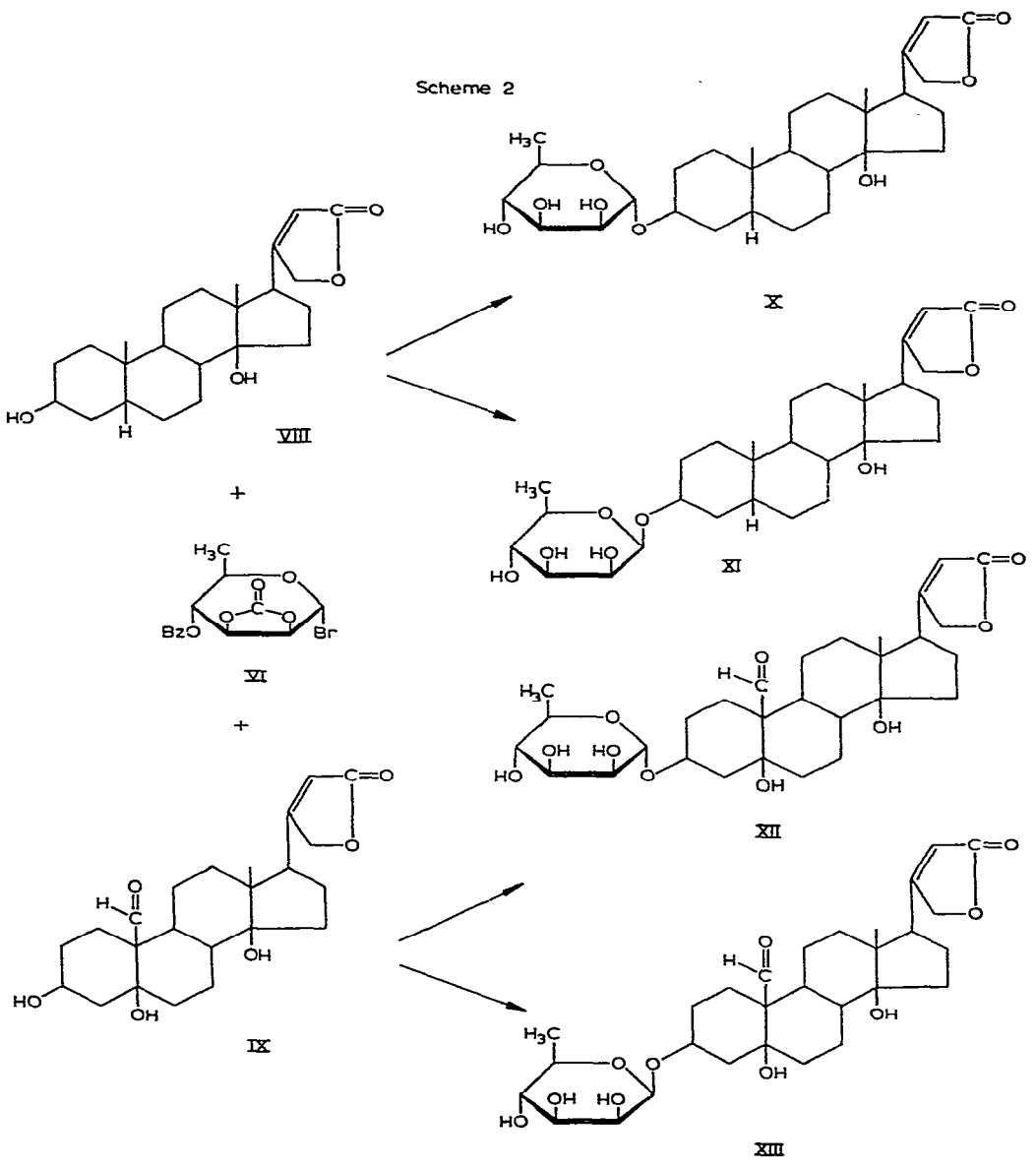
TABLE I

CARDIOTONIC ACTIVITY OF RHAMNOSIDES OF DIGITOXIGENIN AND OF STROPHANTHIDIN AS MEASURED INTRAVENOUSLY IN CATS

Rhamnoside of digitoxigenin	*LD/mg*[11]
α-L (evomonoside)[12a]	3.6
β-D (XI)	2.8
α-D (X)[2a]	1.6
Rhamnoside of strophanthidin	
α-L (convallatoxin)[12b]	12.6
β-D (XIII)	10.1
α-D (XII)[3b]	7.2

Assay results for the two new cardenolides XI and XIII (see Table I) show enhanced activity as compared with the two "unnatural" α-D-rhamnosides. These results, as anticipated, favor the postulate that the α-D-glycosidic linkage in cardenolides containing D-sugars is unfavorable for cardiotonic activity. It is interesting to note, however, that in each case the potency of the two synthetic cardenolides XI and XIII is somewhat less than the potency of the corresponding natural α-L-rhamnoside of digitoxigenin (evomonoside) and of strophanthidin (convallatoxin).

Scheme 2



EXPERIMENTAL

All melting points were determined using a Kofler hot stage.

Methyl 2,3-O-carbonyl-6-O-p-tolylsulfonyl-α-D-mannoside (II)

To a stirred solution of 40.0 g (0.206 mole) of methyl α-D-mannopyranoside (I) in 520 ml of dry pyridine at 0° was added 42.2 g (0.227 mole) of *p*-toluenesulfonyl

chloride. The solution was allowed to warm to room temperature and was stirred overnight. Then the solution was cooled to —20°, and 20.8 g (0.211 mole) of carbonyl chloride in 72 ml of toluene was added dropwise with stirring. After being stirred for 4 h at —20°, the solution was kept at 5° overnight. To the cold solution was added 45 ml of concentrated ammonium hydroxide and, after the mixture had been stirred for 2 h, the solvent was removed under diminished pressure at 30°. The residual sirup was dissolved in 400 ml of methylene chloride and was stirred overnight with 40.0 g of powdered tartaric acid. After the addition of 400 ml of benzene, the salts were removed by filtration, and washed with 100 ml of methylene chloride–benzene (1:1). The combined filtrate and washings was extracted with four 50-ml portions of water, dried with anhydrous sodium sulfate, and filtered, and the filtrate was evaporated to about 100 ml under diminished pressure. The precipitate was filtered off and recrystallized from benzene, to give 26.0 g (33.7 %) of product, melting at 126–127°. From the mother liquor was obtained an additional 2.67 g (3.5 %) melting at 126–127°, giving a total yield of 37.2 %. A second recrystallization from benzene raised the m.p. to 129–130°, $[\alpha]_D^{25}+29.0°$ (*c* 1.103, chloroform). Calc. for $C_{15}H_{18}O_9S$: C, 48.13; H, 4.85; S, 8.56. Found: C, 48.27; H, 5.18; S, 8.72.

Methyl 4-O-benzoyl-2,3-O-carbonyl-6-O-p-tolylsulfonyl-α-D-mannoside (III)

(*a*) To a solution of 49.6 g (0.255 mole) of methyl α-D-mannopyranoside (I) in 600 ml of dry pyridine at 0° was added, with stirring, 30.0 g (0.157 mole) of *p*-toluenesulfonyl chloride. After 1 h, an additional 30.0 g (0.157 mole) of the chloride was added, stirring was continued for 1 h, and the mixture was kept overnight in a refrigerator. A solution of 25.8 g (0.261 mole) of carbonyl chloride in 100 ml of toluene was now added during 1 h at 0°. The solution was stirred for an additional hour and was then kept at 5° overnight. To the solution at 0° was added 36.6 g (0.325 mole) of freshly distilled benzoyl chloride. After the solution had been kept overnight in a refrigerator, 600 ml of ether was added, and the solids were filtered off and successively washed thoroughly with water, 95 % alcohol, and ether, to give 35.4 g (29.0 %) of fine needles melting at 190–193°.

The filtrate was evaporated to about 200 ml under diminished pressure at 40°, 200 ml of water was added, and the solids were filtered off and washed successively with water, alcohol, and ether. After recrystallization from benzene–methylene chloride (2:1), the solid material gave an additional 9.8 g, m.p. 192–194°, for a total yield of 37.0 %. Recrystallization of the combined product from benzene–methylene chloride raised the m.p. to 193–195°, $[\alpha]_D^{27}+47.6°$ (*c* 1.282, chloroform). Calc. for $C_{22}H_{22}O_{10}S$: C, 55.22; H, 4.64; S, 6.70. Found: C, 55.20; H, 4.79; S, 6.95.

(*b*) To a solution of 20.00 g (0.053 mole) of methyl 2,3-*O*-carbonyl-6-*O*-*p*-tolylsulfonyl-α-D-mannoside (II) in 130 ml of methylene chloride–pyridine (12:1) at 0°, was added 8.25 g (0.059 mole) of freshly distilled benzoyl chloride, and the mixture was kept overnight at 5°. An additional 75 ml of methylene chloride was added, and the solution was extracted with 100 ml of 1.3N hydrochloric acid followed by four 25-ml portions of water. The solvent was evaporated, and the solid

residue was washed with 150 ml of absolute alcohol and 100 ml of benzene, and dried at 80°; the yield was 23.1 g (90.5 %), m.p. 193–195, $[\alpha]_D^{27}$ +48.2° (*c* 1.274, chloroform).

Methyl 4-O-benzoyl-2,3-O-carbonyl-6-deoxy-6-iodo-α-D-mannoside (IV)

A solution of 23.0 g (0.049 mole) of methyl 4-*O*-benzoyl-2,3-*O*-carbonyl-6-*O*-*p*-tolylsulfonyl-α-D-mannoside (III) and 40.0 g of sodium iodide in 300 ml of acetone was heated in a sealed container at 100° for 2 h. After the mixture had been cooled, the sodium *p*-toluenesulfonate was filtered off and washed with acetone (wt. 9.58 g, calc. 9.46 g). The filtrate and washings were combined and evaporated, and the solid residue was extracted with water and dried, to give 20.8 g (99.8 %) of crude material. By recrystallization from benzene, 19.3 g (92.4 %) of product melting at 177–179° was obtained. An analytical sample melted at 179° (180–182°), $[\alpha]_D^{25}$ +32.6° (*c* 1.244, methylene chloride). Calc. for $C_{15}H_{15}IO_7$: C, 41.49; H, 3.72; I, 29.23. Found: C, 41.61; H, 3.96; I, 29.37.

Methyl 4-O-benzoyl-2,3-O-carbonyl-6-deoxy-α-D-mannoside (V)

(*a*) *Preparation of the catalyst*

To a solution of 24.0 g of nickelous chloride hexahydrate and 0.54 g of chromic sulfate pentahydrate in 350 ml of water, slurried with 6.0 g of Norit A, was added dropwise, with stirring, a solution of 12.0 g of sodium borohydride in 60 ml of water. The catalyst was filtered off with suction and washed free of chlorides with water. The catalyst was then transferred to a flask and dried by azeotropic distillation with petroleum ether (b.p. 39–46°). After the water had been removed by means of a Stark–Dean trap, the catalyst was filtered off in a dry box, washed with dry tetrahydrofuran, and transferred to the hydrogenation vessel.

(*b*) *Reduction of IV*

A solution of 21.5 g (0.050 mole) of IV in 250 ml of dry tetrahydrofuran and 22 ml of dry triethylamine was slurried with the catalyst, and was hydrogenated under 3.5 atm. of hydrogen at room temperature for 72 h. The catalyst was removed by filtration and washed with dry tetrahydrofuran, and the combined filtrate and washings was evaporated to dryness under diminished pressure at 40°. The solid product was taken up in 200 ml of methylene chloride, the solution washed with 60 ml of water, and dried with sodium sulfate. After filtration, the solvent was removed by evaporation under diminished pressure, and the product crystallized from 350 ml of absolute alcohol–methylene chloride (5:2) to yield 13.5 g (88.3 %) of product melting at 196–197°. By partial evaporation of the mother liquor, an additional 0.46 g (3.0 %), melting at 194–196°, was obtained, to bring the total yield to 91.3 %, $[\alpha]_D^{25}$ −4.8° (*c* 0.898, methylene chloride). Calc. for $C_{15}H_{16}O_7$: C, 58.46; H, 5.23. Found: C, 58.21; H, 5.39.

4-O-Benzoyl-2,3-O-carbonyl-6-deoxy-α-D-mannopyranosyl bromide (VI)

To 1.48 g (4.8 mmole) of methyl 4-*O*-benzoyl-2,3-*O*-carbonyl-6-deoxy-

α-D-mannoside (V) in 30 ml of glacial acetic acid–methylene chloride (1:1) was added 15 ml of a 35 % solution of hydrogen bromide in glacial acetic acid. The solution was kept at room temperature in a well-stoppered flask for 24 h, taken up in 90 ml of cold methylene chloride, and quickly washed in succession with two 90-ml portions of cold water, 90 ml of cold saturated sodium bicarbonate, and 90 ml of cold water. The solution was immediately dried with magnesium sulfate, filtered after 30 min at room temperature, and the filtrate evaporated under diminished pressure. The residue was washed (by decantation) with three 5-ml portions of dry ether, and dried *in vacuo* at room temperature for 1 h to give 1.44 g (83.8 %) of colorless needless melting at 148–156° (dec.), $[\alpha]_D^{25}+82.9°$ (*c* 1.160, methylene chloride). Calc. for $C_{14}H_{13}BrO_6$: C, 47.08; H, 3.67; Br, 22.37. Found: C, 46.92; H, 4.24; Br, 22.18[13].

Methyl 4-O-benzoyl-2,3-O-carbonyl-6-deoxy-β-D-mannoside (VII)

A solution of 0.287 g (0.8 mmole) of 4-*O*-benzoyl-2,3-*O*-carbonyl-6-deoxy-α-D-mannosyl bromide (VI) in 3 ml of dry methylene chloride was added dropwise to 10 ml of methanol slurried with 600 mg of dry, freshly prepared silver carbonate, and the mixture was stirred for 30 min at room temperature. The salts were removed by filtration, and the filtrate was evaporated under diminished pressure. The residue, consisting of a mixture of well-formed needles and elongated prisms, and weighing 0.238 g (96 %), m.p. 174–186°, was repeatedly recrystallized from absolute ethanol to give 0.114 g (46.0 %) of elongated prisms, m.p. 187–187.5°, $[\alpha]_D^{25}-89.4°$ (*c* 0.910, methylene chloride). Calc. for $C_{15}H_{16}O_7$: C, 58.46; H, 5.23. Found: C, 58.34; H, 5.28.

3 β-(6-Deoxy-β-D-mannopyranosyl)-14 β-hydroxy-5 β-card-20(22)-enolide (XI) and α-D anomer (X)

To a magnetically stirred solution of 2.120 g (5.66 mmole) of digitoxigenin (VIII) in 30 ml of 1,1,2-trichloroethane, slurried with a powdered mixture of 4.041 g of dry, freshly prepared silver carbonate and 20.0 g of Drierite, was added, in 0.500-g portions over a period of 8 h, a total of 4.500 g (12.6 mmole) of 4-*O*-benzoyl-2,3-*O*-carbonyl-6-deoxy-α-D-mannosyl bromide (VI). The slurry was stirred overnight, taken up in 200 ml of methylene chloride, filtered, and the filtrate evaporated to a sirup which was dissolved in 1 l of methanol, and treated with 350 ml of water containing 10.0 g of potassium bicarbonate. The resulting clear solution was kept at room temperature for 6 days and then evaporated under diminished pressure to *ca.* 200 ml. The concentrated solution was extracted with four 200-ml portions of chloroform–ethanol (9:1), which were combined, dried with magnesium sulfate, filtered, and the filtrate evaporated under diminished pressure to a crude solid. The solid was boiled briefly with 20 ml of acetone, and filtered after being cooled.

(*a*) *α-Cardenolide X*

The acetone-insoluble material (2.340 g) was repeatedly recrystallized from 2-propanol to yield 1.284 g of the α-D anomer X. The mother liquors were combined and evaporated to dryness. To the residue was added 5 ml of formamide and the

mixture was kept, with occasional swirling, for 30 min at room temperature, and filtered, and the insoluble material washed with three 1-ml portions of formamide. The filtrate (A) was set aside (see *b*), and the insoluble material was washed with a little water, to remove the formamide, and recrystallized twice from 2-propanol to give an additional 0.133 g, for a total yield of 1.417 g (48.1 %), melting at 254–255°, $[\alpha]_D^{23}+51.9°$ (*c* 1.136, methanol), $\lambda_{max}^{CH_3OH}$ 218 mμ (log ε 4.2). Lit. values[3a]: m.p. 253–254.5°, $[\alpha]_D^{23}+53.4°$ (*c* 1.023, methanol), $\lambda_{max}^{CH_3OH}$ 218 mμ (log ε 4.23). Calc. for $C_{29}H_{44}O_8$: C, 66.90; H, 8.52. Found: C, 66.95; H, 8.33.

(*b*) *β-Cardenolide XI*

The formamide filtrate (A) was taken up in 100 ml of water and extracted with four 50-ml portions of chloroform–ethanol (9:1), the combined extracts were dried with magnesium sulfate and filtered, and the filtrate evaporated to a solid which, when recrystallized twice from acetone, gave 0.378 mg (12.8 %) of the crystalline β-D anomer XI, melting at 221.5–223.5°, $[\alpha]_D^{23}-12.4°$ (*c* 0.928, methanol), $\lambda_{max}^{CH_3OH}$ 218 mμ (log ε 4.2). The compound gave a positive Kedde test[14] and a negative tetranitromethane test. Calc. for [M] (digitoxigenin+methyl 6-deoxy-α-D-mannopyranoside)[7a]: +71°+109° = 180°. Calc. for [M] (digitoxigenin+methyl 6-deoxy-β-D-mannopyranoside)[15]: +71°−170° = −99°. Found for [M] (XI): −65°. The glycosidic linkage has, therefore, the β-D configuration. Calc. for $C_{29}H_{44}O_8$: C, 66.90; H, 8.52. Found: C, 66.66; H, 8.69.

3 β-(6-Deoxy-β-D-mannopyranosyl)-5 β, 14 β-dihydroxy-19-oxocard-20(22)-enolide (XIII) and α-D anomer (XII)

To a magnetically stirred solution of 1.230 g (3.0 mmole) of strophanthidin (IX) in 35 ml of dry methylene chloride, slurried with a powdered mixture of 4.14 g of dry, freshly prepared silver carbonate and 20.0 g of Drierite, was added a total of 3.20 g (8.4 mmole) of 4-*O*-benzoyl-2,3-*O*-carbonyl-6-deoxy-α-D-mannosyl bromide (VI) in six equal portions during 5 h. After being stirred for an additional 6 h, the slurry was taken up in 100 ml of methylene chloride, filtered, and the filtrate washed with 25 ml of water. After the solution had been dried (sodium sulfate), the solvent was removed by evaporation under diminished pressure; the sirupy residue was dissolved in 700 ml of methanol and to the solution was added, with stirring, 250 ml of water containing 7.00 g of potassium bicarbonate. The resulting solution was kept at room temperature for 7 days under a nitrogen atmosphere, and then evaporated under diminished pressure at 25–30° to *ca.* 200 ml. The concentrated solution was washed with five 200-ml portions of chloroform (which were discarded), and with seven 200-ml portions of chloroform–ethanol (9:2), which were combined, dried with magnesium sulfate, filtered, and the filtrate evaporated under reduced pressure to a crude, amorphous mass.

The crude material was dissolved in 5 ml of water and then thoroughly mixed with 10.0 g of Celite 545 which was tamped on top of a 3×31 cm column consisting of a mixture of 50 ml of water and 100 g of Celite 545. The column was eluted with

250 ml of chloroform–ethanol (36:1) (which was discarded), and then with 400 ml of chloroform–ethanol (9:1) to remove the α-D anomer XII. An additional, 500-ml eluate of chloroform–ethanol (9:1) contained the β-D anomer XIII contaminated with a trace of the α-D anomer.

(a) α-Cardenolide (XII)

The eluate containing the α-D anomer was evaporated under diminished pressure to give 241 mg (14.5 %) of the crystalline 6-deoxy-α-D-mannopyranoside (XII). Recrystallization from methanol gave 135 mg (8.1 %) of the pure derivative melting at 250–254°, $[\alpha]_D^{25}$ +92.0° (*c* 1.095, methanol), $\lambda_{max}^{CH_3OH}$ 218 mμ (log ε 4.21), $\lambda_{max}^{CH_3OH}$ 301 mμ (log ε 1.53). Lit. values:[3b] m.p. 249–254°, $[\alpha]_D^{15}$ +95.8° (*c* 0.855, methanol), $\lambda_{max}^{CH_3OH}$ 218 mμ (log ε 4.2). Calc. for $C_{29}H_{42}O_{10}$: C, 63.25; H, 7.69. Found: C, 63.06; H, 7.48.

(b) β-Cardenolide (XIII)

After evaporation of the second chloroform–ethanol (9:1) eluate, the β-D anomer weighed 191 mg (11.6 %). Recrystallization from absolute ethanol gave 51 mg of pure material melting at 228–231°.

The mother liquor was evaporated under diminished pressure, and the residue was rechromatographed on a Celite 545–water (50:25) column (see above). The column was eluted with 600 ml of chloroform–ethanol (72:1) (which was discarded), and the β-D anomer was eluted with 700 ml of chloroform–ethanol (36:1). Removal of the solvent by evaporation under diminished pressure, and recrystallization of the residual solid from absolute ethanol afforded an additional 60 mg of pure product melting at 228–231°, for a total yield of 111 mg (6.6 %). The compound had $[\alpha]_D^{25}$ +15.3° (*c* 1.235, methanol), $\lambda_{max}^{CH_3OH}$ 218 mμ (log ε 4.22), and $\lambda_{max}^{CH_3OH}$ 302 mμ (log ε 1.45). The derivative gave a positive Kedde test[14] and a negative tetranitromethane test. Calc. for [M] (strophanthidin+methyl 6-deoxy-α-D-mannopyranoside)[7a]: +174°+109° = +283°. Calc. for [M] (strophanthidin+methyl 6-deoxy-β-D-mannopyranoside)[15]: +174°−170° = +4°. Found for [M] (XIII): +84°. The glycosidic linkage has, therefore, the β-D configuration. Calc. for $C_{29}H_{42}O_{10}$: C, 63.25; H, 7.69. Found: C, 63.29; H, 7.95.

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The potential utility of 2,3-*O*-carbonylglycosyl halides in the synthesis of 1,2-*cis* cardenolides was communicated privately to the authors by Dr. A.S. Perlin, to whom they are most grateful. The authors are also much indebted to Prof. K.K. Chen, Department of Pharmacology, Indiana University Medical School, Indianapolis, Indiana, for carrying out the assays on the β-D-rhamnosides XI and XIII. Our thanks are also due Mrs. P. Wheeler and Miss A. Richardson, Naval Propellant Plant, for the microanalyses.

SUMMARY

Treatment of methyl α-D-mannopyranoside (I) in succession *in situ* with *p*-toluenesulfonyl chloride, carbonyl chloride, and benzoyl chloride gave methyl 4-*O*-benzoyl-2,3-*O*-carbonyl-6-*O*-*p*-tolylsulfonyl-α-D-mannoside (III). Replacement of the *p*-tolylsulfonyloxyl group of III with iodide ion gave the 6-iodo derivative IV, which underwent reduction to give methyl 4-*O*-benzoyl-2,3-*O*-carbonyl-6-deoxy-α-D-mannoside (V). Replacement of the C-1 methoxyl group of V by bromide ion gave 4-*O*-benzoyl-2,3-*O*-carbonyl-6-deoxy-α-D-mannosyl bromide (VI), which underwent methanolysis by inversion, to yield methyl 4-*O*-benzoyl-2,3-*O*-carbonyl-6-deoxy-β-D-mannoside (VII). The bromide VI was coupled with digitoxigenin (VIII) to give, after saponification, 13 % of 3-(β-D-rhamnopyranosyl)digitoxigenin (XI) and 48 % of the corresponding α-D anomer X. Coupling of the bromide VI with strophanthidin (IX), followed by saponification, gave 6 % of 3-(β-D-rhamnopyranosyl)strophanthidin (XIII) and 8 % of the alternative anomeric form XII.

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NON-AQUEOUS SUGAR SOLVENTS: A NEW SERIES

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(Received March 20th, 1965)

INTRODUCTION

The chemistry and technology of carbohydrates is limited by their general insolubility in organic solvents. In recent years, the very meagre list of organic solvents for sugars[1], of which pyridine is the best known, has been supplemented by a number of less-common organic solvents. These include *N*,*N*-dimethylformamide[1], dimethyl sulphoxide[1], sulpholane (tetrahydrothiophen 1,1-dioxide), morpholine and substituted morpholines[1,2], substituted pyrrolidones[1,2], and γ-butyrolactone[3]. Certain of these solvents are now commercially available, but are nevertheless relatively expensive. We have sought new non-aqueous solvents for sugars, to supplement the above compounds.

DISCUSSION

In our search for new non-aqueous solvents, we initially examined glycerol and ethylene glycol, because of their structural similarity to sugars; the solutions obtained were too viscous for our purposes.

An alternative approach involved the examination of all readily-available liquids listed in Lange's Handbook, which boiled above 100° and were infinitely miscible with water. A number of these compounds were found to be very good solvents for sucrose, glucose, and fructose. These included furfuryl alcohol and its reduction product, tetrahydrofurfuryl alcohol, and, more surprisingly, monoalkyl

I II

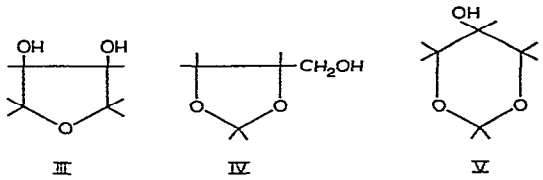
I R = alkyl, for ethylene glycol monoalkyl ethers (alkyl Cellosolves).
R = R′O–CH_2–CH_2– (R′ = alkyl), for diethylene glycol monoalkyl ethers (alkyl carbitols).

II R = –C_3H_3–, for furfuryl alcohol.
R = –C_3H_7–, for tetrahydrofurfuryl alcohol.

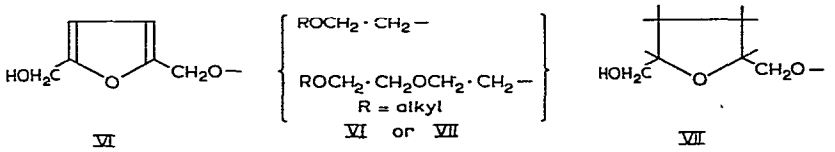
ethers of ethylene glycol and diethylene glycol. A common structural feature was recognised in these compounds, and further work was undertaken to determine whether the grouping had any significance.

The glycol ethers (I) and the furfuryl alcohols (II) contain the structural grouping R–O–Ċ–Ċ–OH. The synthesis of nine further compounds containing this grouping provided strong evidence to support the theory that it conferred good solubilising powers for sugars and many of their derivatives. Whilst 1,4-anhydro-erythritol (III) also contains the ethylene glycol grouping, the 1,2- and 1,3-*O*-methyleneglycerols (IV) and (V), respectively, and the furan (VI) and tetrahydro-furan (VII) derivatives do not. All proved to be excellent solvents for glucose, fructose, and sucrose. The hexoses, in particular, are miscible with the solvents at the boiling point of the solvent, and crystallise readily from the cooled solutions. Due to their instability, the *O*-methyleneglycerols could not be examined above 100°.

2-Formyloxy- and 2-acetoxy-ethanol, on the other hand, are poorer solvents for sucrose, and this suggests that where "R" in (I) is an electron-withdrawing group, the availability of electrons on the ether oxygen of the solvent is reduced, and the ability of this oxygen to form hydrogen bonds is diminished accordingly.



III IV V



VI VI or VII VII

The effect of an extension of chain length on solvent properties was also examined, and the ω-methoxy derivatives of propanol and butanol were found to be better solvents than the corresponding monohydric alcohols (See Fig. 1).

Solvent power is possibly due primarily to the formation of hydrogen bonds between solvent and sugar hydroxyl groups, at the expense of hydrogen bonds between sugar molecules. The higher boiling points of these solvents would assist this process by enabling the necessary energy to be provided to disrupt the latter intermolecular hydrogen bonds. Solvent–sugar hydrogen bonds could be formed in a variety of ways, but the relationship of the ether and the hydroxyl groups in the solvent must be important. Several possibilities are shown in Fig. 2, where only hydrogen bonding with one sugar molecule has been considered. The solvent could equally well be shown as bridging two sugar molecules, but we feel that this is less likely, as each end of the solvent molecule would be functioning more or less independently (especially for ω-methoxybutanol), and the solvent would not be expected to be a better solvent than its simpler, monofunctional relatives.

The properties of solutions of sugars in the β-ether solvents were favourable for their subsequent use in physical and chemical processes[4,5]. The solubility of glucose and fructose in the simple glycol ethers, and in tetrahydrofurfuryl alcohol, was very high at temperatures above the melting points of the sugars, and, as already

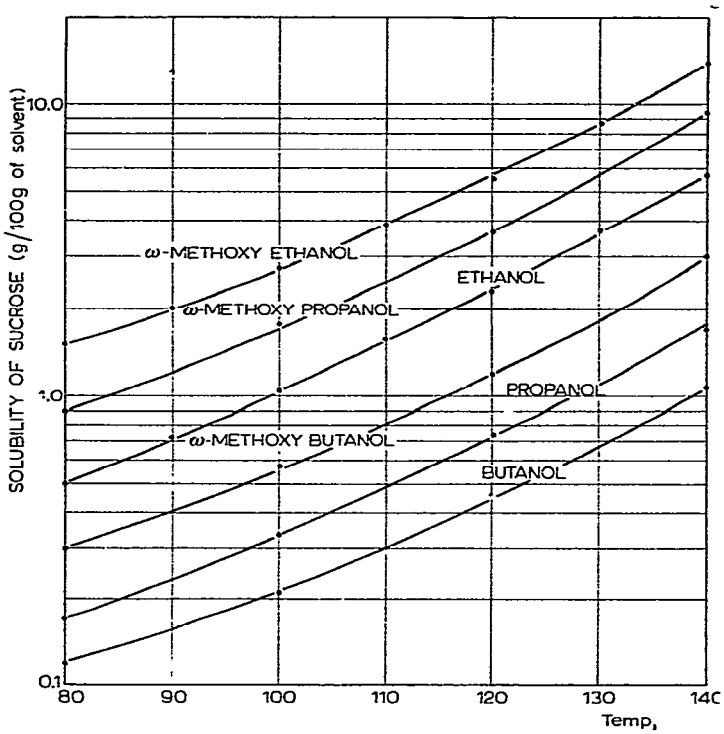


Fig. 1. A comparison of sucrose solubility in alcohols and their ω-alkoxy derivatives.

mentioned, complete miscibility resulted near the boiling point of the solvents. The solutions were not unduly viscous, and the sugars crystallised readily on cooling.

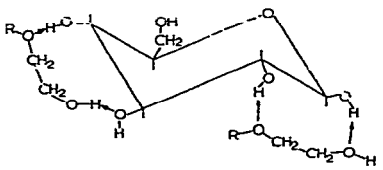


Fig. 2. Depicting the solvation of β-D-glucopyranose in a glycol mono-ether solvent.

Sucrose was not as soluble as the hexoses, and its solubility in a number of these solvents is recorded in Fig. 3.

Due to lack of solvent, it was not possible to determine the solubility of sucrose in solvents of types (VI) and (VII), but approximate solubilities were deter-

mined using small quantities of solvent (250–500 μl). It appeared that the solubility of sucrose ranged between 15–25% at the boiling point of the solvent (where R and R′ in VI and VII = methyl).

The most important feature of these systems was their heat stability. Spectral and chromatographic investigation of a number of the solvents (purified) showed that almost negligible decomposition resulted when a sucrose solution was heated for up to six hours at temperatures between 130–160°C.

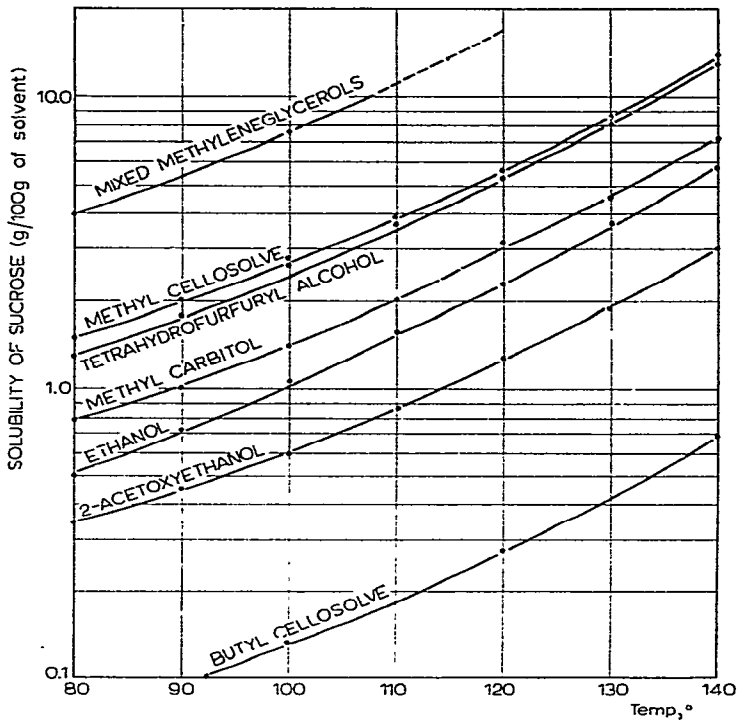


Fig. 3. Solubility of sucrose in the RO–C–C–OH solvent series
---- solvent decomposed.

The sensitivity of fructose and sucrose to autocatalytic decomposition by hydrogen ion is well known, and it is believed that the hydrogen-bonded structures depicted in Fig. 2 help to protect the sugar from such decomposition.

Besides being useful as non-aqueous media for certain sucro-chemical reactions[7], we have found these solvents to have great potential value as purification media for sugars and their derivatives[4]. Their value may be further extended by judicious use of another miscible organic solvent; the glycol monoalkyl ethers are miscible with a wide range of common organic solvents.

As a corollary to the theory that hydrogen-bond formation essentially involves cyclic bonding within single sugar molecules, as depicted in Fig. 2, one might expect a correlation to exist between sugar solubility and stereochemistry. Further, provided

it can be prepared in suitable forms, hydroxyethylated cellulose should prove to have advantages in chromatographic separations of sugars.

EXPERIMENTAL

Materials

All solvents were thoroughly dried and doubly distilled before use. Sucrose, of very high purity, was ground, sieved through a 100-mesh sieve and vacuum dried overnight at 60°. 1,4-Anhydroerythritol was prepared according to the method of Otey and Mehltretter[6], and the monoalkyl ether derivatives of 2,5-dihydroxymethylfuran and tetrahydro-2,5-dihydroxymethylfuran were prepared by acid treatment of fructose in the appropriate alcoholic solvent[4], followed by reduction of the resulting ether of 5-hydroxymethylfurfural. ω-Methoxy alcohols were obtained by the method of Pummerer and Schönamsgruber[7], and the *O*-methyleneglycerols by Hibbert and Carter's method[8].

Solubility experiments

These experiments were conducted in sealed, rotating tubes in a thermostatically controlled oven. The quantity of solvent used per tube depended on its availability, and ranged from 1–10 g. For small quantities, particular care was necessary in sealing the tubes, in order to prevent charring of the contents or condensation of water in the neck.

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SUMMARY

The ω-methoxy derivatives of ethanol (methyl Cellosolve), propanol, and butanol are better solvents for sugars than are the related simple alcohols or ethers. Liquids containing a cyclic or acyclic ether grouping in the β-position to an alcohol function are generally good sugar solvents. It is believed that these solvents are effective because they readily form hydrogen bonds with the hydroxyl groups of the sugars, after sugar intermolecular hydrogen bonds have been disrupted at elevated temperatures.

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A NEW SYNTHESIS OF A 2,3-EPIMINO-α-D-ALLOPYRANOSIDE

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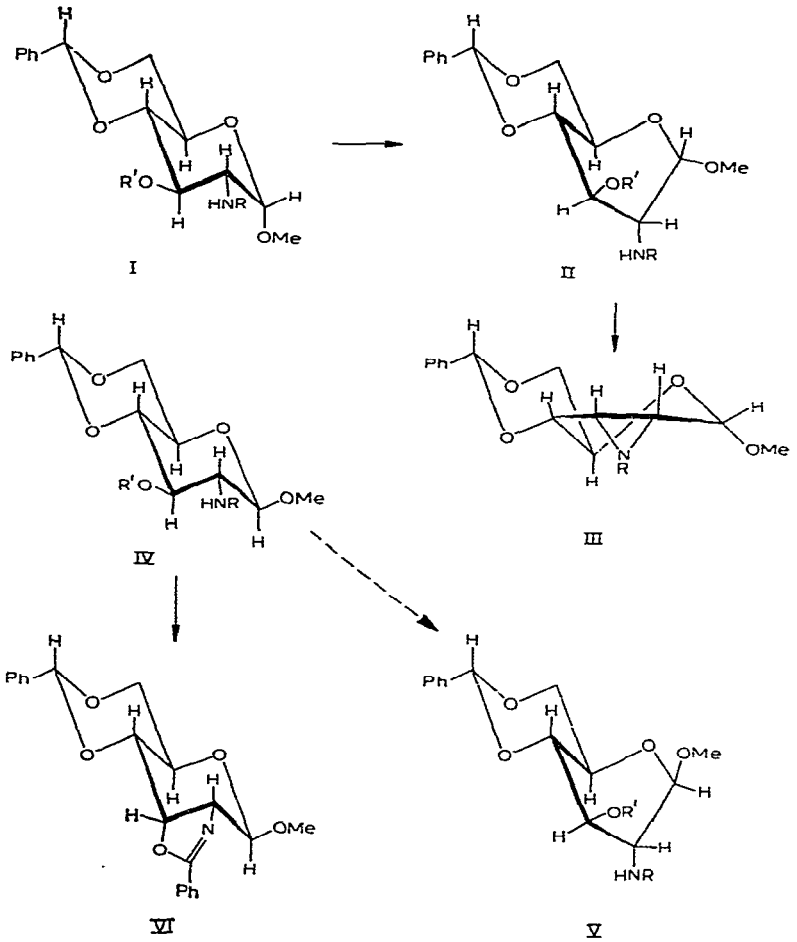
INTRODUCTION

2,3-Epimino derivatives of pyranosides (*e.g.*, III) are valuable synthetic intermediates, undergoing various ring opening reactions to give amino-dideoxy, amino-halogeno, and diamino derivatives of biochemical interest[1,2]. Unfortunately,



the preparation of these epimines requires starting materials that are difficultly accessible. Thus, methyl 4,6-*O*-benzylidene-2,3-dideoxy-2,3-epimino-α-D-allopyranoside (III; R = H) has been prepared from methyl 3-azido-4,6-*O*-benzylidene-3-deoxy-2-*O*-methanesulphonyl-α-D-altropyranoside, by treatment with either hydrazine and Raney nickel[3] or lithium aluminium hydride[4], and from methyl 3-benzamido-4,6-*O*-benzylidene-3-deoxy-2-*O*-methanesulphonyl-α-D-altropyranoside, by treatment with either lithium aluminium hydride in tetrahydrofuran, or ethanolic sodium ethoxide[5]. Both these precursors possess a *trans*-diaxial relationship for the reacting groups in the preferred chair conformation. A *trans*-diaxial relationship is thought to be an essential prerequisite for epimine formation, whereas a *trans*-diequatorial relationship favours the formation of an oxazoline.

We have now examined the possibility of synthesising the *allo*-epimine (III; R = H) from the more readily available methyl 2-benzamido-4,6-*O*-benzylidene-2-deoxy-3-*O*-methanesulphonyl-α-D-glucopyranoside (I; R = Bz, R′ = Ms). Whilst the participating and departing groups are in the *trans*-diequatorial relationship in the preferred chair conformation (I), it seemed likely that a transition state involving a boat (II) or related skew-boat conformation would lead to formation of the *allo*-2,3-epimine (III; R = H), by analogy with the formation of *allo*-2,3-epoxides from similar derivatives having the D-*gluco* configuration[6]. Furthermore, Meyer zu Reckendorf has demonstrated that the β-D-anomer (IV; R = Bz, R′ = Ms) gives a mixture of the *N*-benzoylepimine and the oxazoline (VI) on treatment with potassium cyanide in *N*,*N*-dimethylformamide[2], but treatment with sodium ethoxide in ethanol yields only the oxazoline (VI)[7].

RESULTS AND DISCUSSION

Treatment of methyl 2-benzamido-4,6-*O*-benzylidene-2-deoxy-3-*O*-methanesulphonyl-α-D-glucopyranoside (I; R = Bz, R′ = Ms) with lithium aluminium hydride gave the *allo*-epimine (III; R = H) in 44% yield, and, after treatment of the mother liquors with acetic anhydride in ethanol, an additional 7% was obtained as the *N*-acetyl derivative (III; R = Ac). Consequently, this method does provide a more satisfactory route to the epimine. On one occasion, a second compound, methyl 2-benzylamino-4,6-*O*-benzylidene-α-D-glucopyranoside, was encountered, in addition to the epimine.

In order to compare the participating behaviour of acetamido and benzamido groups in epimine formation, methyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-3-*O*-methanesulphonyl-α-D-glucopyranoside (I; R = Ac, R′ = Ms) was treated with lithium aluminium hydride. Two products resulted, one of which was coincident on thin-layer chromatograms with the *allo*-2,3-epimine, but was not isolated. The major product was shown to be methyl 4,6-*O*-benzylidene-2-deoxy-2-ethylamino-α-D-glucopyranoside (I; R = Et, R′ = H), from its infrared spectrum and that of its diacetyl derivative, from its n.m.r. spectrum, and by synthesis from methyl

2-acetamido-4,6-*O*-benzylidene-α-D-glucopyranoside (I; R = Ac, R′ = H) using reduction with lithium aluminium hydride.

Treatment of methyl 2-benzamido-4,6-*O*-benzylidene-2-deoxy-3-*O*-methanesulphonyl-α-D-glucopyranoside (I; R = Bz, R′ = Ms) with hot, ethanolic sodium ethoxide yielded two products, the principal one being the *allo*-epimine (III; R = H), with a small amount of methyl 2-benzamido-4,6-*O*-benzylidene-2-deoxy-α-D-glucopyranoside (I; R = Bz, R′ = H).

The predominant formation of epimine from the α-glycoside, and oxazoline from the β-glycoside[7], is understandable if epimine formation involves a transition state in the boat conformation (II) having the reacting groups in the *trans*-diaxial relationship. The boat conformation (II) of the α-glycoside is much more favourable than that (V; R = Ms) of the β-glycoside in which there is a 1,3-diaxial interaction between the methoxyl and methanesulphonyl groups. Thus, the α-glycoside can achieve the *trans*-diaxial relationship of reacting groups, and form an epimine whereas, in the β-glycoside, the reacting groups will remain *trans*-diequatorial, leading to the formation of the oxazoline. This interpretation also accounts for the observations of Meyer zu Reckendorf and Bonner[12], and of Baker and Neilson[13], who obtained only thiazolines on anionic ring closure of *N*-(methylthio)thiocarbonyl and *N*-thiocarbamoyl derivatives of 2-amino-2-deoxy-β-D-glucopyranoside (IV; R = CS.SMe and $CS.NH_2$, respectively). In contrast, the related 3-amino-3-deoxy-α-D-altropyranosides gave 2,3-epimines, as expected[5] from *trans*-diaxial neighbouring-group participation.

EXPERIMENTAL

Concentrations were carried out under reduced pressure. Melting points were determined on a Kofler microstage apparatus, and are uncorrected. Optical rotations were measured manually at 20 ±1°. Thin-layer chromatography (t.l.c.) was performed at room temperature on silica gel G (Merck) with chloroform–ether (1:1, v/v). The separated materials were detected with 5% ethanolic sulphuric acid at 110–115° for *ca.* 10 min, or with saturated aqueous potassium permanganate, made alkaline with potassium carbonate. Light petroleum (b.p. 60–80°) was used throughout. Methanol was dried with magnesium and iodine.

Methyl 2-benzamido-2-deoxy-α-D-glucopyranoside

(*a*) A solution of 2-benzamido-2-deoxy-D-glucose[8] (114 g) in dry methanol (1.5 l) was heated under reflux with Amberlite IR-120 (H^+ form) resin (220 g), with mechanical stirring, for 20 h. After filtration, the solution was cooled, when methyl 2-benzamido-2-deoxy-α-D-glucopyranoside crystallised, m.p. 224–227°, $[\alpha]_D$ +108° (*c* 0.5, water); lit.[9], m.p. 225–226°, $[\alpha]_D$ +114°. A further crop was obtained by concentration of the mother liquors (total yield, 74 g; 62%).

(*b*) The benzamido sugar (165 g) was suspended in methanol (1 l) and conc. hydrochloric acid added (22 ml). The mixture was heated under reflux for 22 h,

and the resulting homogeneous solution treated with a little decolourising charcoal. After filtration, addition of ether and then light petroleum caused crystallisation of the α-pyranoside (105 g, 60%), identical with the compound described in (*a*).

Methyl 2-benzamido-4,6-O-benzylidene-2-deoxy-α-D-glucopyranoside (I; R = Bz, R′ = H)

Methyl 2-benzamido-2-deoxy-α-D-glucopyranoside (74 g) was shaken with benzaldehyde (200 ml) and anhydrous zinc chloride (74 g) for 18 h. The resulting viscous mixture was poured into water, with stirring, and the crystals were collected and washed with light petroleum. Recrystallisation from ethanol yielded fine needles (77 g, 80%), m.p. 239–245°. A further recrystallisation from ethanol gave needles, m.p. 247–248°, $[\alpha]_D$ +57° (*c* 2.0, chloroform) (Found: C, 65.3; H, 6.2; N, 3.5. $C_{21}H_{23}NO_6$ calc.: C, 65.5; H, 6.0; N, 3.6%).

Methyl 2-benzamido-4,6-O-benzylidene-2-deoxy-3-O-methanesulphonyl-α-D-glucopyranoside (I: R = Bz, R′ = Ms)

To a cooled solution of methyl 2-benzamido-4,6-*O*-benzylidene-2-deoxy-α-D-glucopyranoside (74 g) in pyridine (1 l), was added methanesulphonyl chloride (35 ml), and the solution was stored for 24 h at 3°. The reaction mixture was decomposed by the addition of ice-water, and, after storage for 18 h at 3°, the 3-*O*-methanesulphonate (62 g, 75%) was collected. Recrystallisation from ethanol gave needles, m.p. 195–195.5°, $[\alpha]_D$ +77.5° (*c* 5.0, chloroform) (Found: C, 57.2; H, 5.5; N, 2.9. $C_{22}H_{25}NO_8S$ calc.: C, 57.0; H, 5.4; N, 3.0%).

Methyl 4,6-O-benzylidene-2,3-dideoxy-2,3-epimino-α-D-allopyranoside (III; R = H)

Lithium aluminium hydride (9 g) was added to methyl 2-benzamido-4,6-*O*-benzylidene-2-deoxy-3-*O*-methanesulphonyl-α-D-glucopyranoside (10 g) in dry tetrahydrofuran (500 ml), and the mixture heated under reflux for 4.5 h. The excess of lithium aluminium hydride in the cooled mixture was decomposed by the addition of a saturated solution of Rochelle salt. The solid was then filtered off and washed well with tetrahydrofuran and chloroform. Concentration of the combined filtrate and washings yielded a syrup which crystallised (2.5 g, 44%) on the addition of ether. Recrystallisation from ethyl acetate–light petroleum yielded methyl 4,6-*O*-benzylidene-2,3-dideoxy-2,3-epimino-α-D-allopyranoside as needles, m.p. and mixed m.p. 154–155°, $[\alpha]_D$ +142° (*c* 0.7, chloroform). The infrared spectrum was identical with that of an authentic sample. Lit.[5], m.p. 150–154°, $[\alpha]_D$ +143°; lit.[3], m.p. 152–153°, $[\alpha]_D$ +150°.

The mother liquors were concentrated to a syrup which was dissolved in ethanol, and acetic anhydride (5 ml) was added. After storage at room temperature for 30 min, the solution was evaporated to a syrup which crystallised (0.5 g, 7.6%) on trituration with fresh ethanol. Recrystallisation from ethanol–light petroleum gave methyl 2,3-acetylepimino-4,6-*O*-benzylidene-2,3-dideoxy-α-D-allopyranoside as

needles, m.p. 183–185°, $[\alpha]_D$ +147°; lit.[5], m.p. 184–185°, $[\alpha]_D$ +147°. The infrared spectrum was identical with that of an authentic sample.

On one occasion, when only half the quantity of lithium aluminium hydride was used, a mixture of two components was formed, one of which was the epimine, as indicated by t.l.c. Separation of the two products on a column of silica gel, using ether as the eluent, afforded methyl 2-benzylamino-4,6-*O*-benzylidene-2-deoxy-α-D-glucopyranoside (275 mg, 34%), m.p. 135–137°, $[\alpha]_D$+70° (*c* 2.3, chloroform). (Found: C, 68.0; H, 6.9; N, 3.75. $C_{21}H_{25}NO_5$ calc.: C, 67.9; H, 6.75; N, 3.75%). The epimine (200 mg, 35%) was eluted with acetone.

Action of lithium aluminium hydride on methyl 2-acetamido-4,6-O-benzylidene-2-deoxy-3-O-methanesulphonyl-α-D-glucopyranoside

To a solution of methyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-3-*O*-methanesulphonyl-α-D-glucopyranoside[10] (I; R = Ac, R′ = Ms) (2 g) in dry tetrahydrofuran (80 ml), was added lithium aluminium hydride (2 g), and the mixture was heated under reflux for 1 h. The reaction mixture, when worked up as above, afforded a partially crystalline residue. Recrystallisation from ethyl acetate–light petroleum yielded methyl 4,6-*O*-benzylidene-2-deoxy-2-ethylamino-α-D-glucopyranoside (I; R = Et, R′ = H) (1 g, 52%). A further recrystallisation from the same solvent mixture gave the analytical sample, m.p. 125–127°, $[\alpha]_D$ +107° (*c* 1.0, chloroform). (Found: C, 62.0; H, 7.5; N, 4.3. $C_{16}H_{23}NO_5$ calc.: C, 62.1; H, 7.5; N, 4.5%). The presence of a hydroxyl group and a secondary amino group was evident from the infrared spectrum of the derived diacetyl derivative, m.p. 145–146°, $[\alpha]_D$ +43.6° (*c* 1.3, chloroform). (Found: N, 3.4. $C_{20}H_{27}NO_7$ calc.: N, 3.55%), obtained by treatment of the above compound with acetic anhydride in pyridine for 4 h. This derivative had ν_{max} at 1730 (ester C=O) and 1640 cm^{-1} (tertiary amide C=O), but no absorption for N–H stretching or deformation. The n.m.r. spectrum of the ethylamino compound (I; R = Et, R′ = H) was in accord with the structure assigned.

T.l.c. of the mother liquors from the crystallisation of the ethylamino compound indicated the presence of the *allo*-epimine (III; R = H). In subsequent experiments, higher proportions of the epimine were formed, making several recrystallisations necessary for purification of the 2-ethylamino compound.

Methyl 4,6-O-benzylidene-2-deoxy-2-ethylamino-α-D-glucopyranoside (I; R = Et, R′ = H)

To methyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-α-D-glucopyranoside[11] (0.1 g) in dry tetrahydrofuran (5 ml), was added lithium aluminium hydride (0.15 g), and the mixture was heated under reflux for 4.5 h. Treatment of the reaction mixture as above yielded a syrup, which was dissolved in chloroform, and the solution washed well with water. The dried ($MgSO_4$) solution was concentrated to a syrup which crystallised on storage. Recrystallisation from ethyl acetate–light petroleum yielded methyl 4,6-*O*-benzylidene-2-deoxy-2-ethylamino-α-D-glucopyranoside (49 mg,

51%), m.p. 126–128°, $[\alpha]_D$ +108°, identical with that obtained as above (i.r. and mixed m.p.).

The action of sodium ethoxide on methyl 2-benzamido-4,6-O-benzylidene-2-deoxy-3-O-methanesulphonyl-α-D-glucopyranoside

Methyl 2-benzamido-4,6-*O*-benzylidene-2-deoxy-3-*O*-methanesulphonyl-α-D-glucopyranoside (1 g) was heated under reflux with 0.27 N ethanolic sodium ethoxide (100 ml). After 4 h, the solution was evaporated to dryness, and partitioned between water and chloroform. The chloroform layer was washed with water, dried (Na_2SO_4), and evaporated. T.l.c. of the crystalline residue indicated the presence of two components, the major one being coincident with the *allo*-epimine (III; R = H), and the minor component coincident with methyl 2-benzamido-4,6-*O*-benzylidene-2-deoxy-α-D-glucopyranoside (I; R = Bz, R′ = H). The crystalline material was extracted with cold ethanol, and a small amount of undissolved material was recrystallised from hot ethanol to yield methyl 2-benzamido-4,6-*O*-benzylidene-2-deoxy-α-D-glucopyranoside, m.p. 248–248.5°, mixed m.p. 247–248°, $[\alpha]_D$ +58°. The ethanolic extract was evaporated, and the crystalline residue was recrystallised twice from ethyl acetate–light petroleum to yield the *allo*-epimine (0.167 g, 31%), still contaminated with a trace of the other product, which could not be removed by further recrystallisation. Extraction with cold acetone, followed by evaporation of the solution and recrystallisation of the residue from ethanol–light petroleum, yielded a sample with m.p. 149–151°, mixed m.p. 149–152°, $[\alpha]_D$ +135° (*c* 0.7, chloroform).

ACKNOWLEDGEMENT

One of us (C.F.G.) thanks the D.S.I.R. for a maintenance award.

SUMMARY

Methyl 4,6-*O*-benzylidene-2,3-dideoxy-2,3-epimino-α-D-allopyranoside has been prepared from methyl 2-benzamido-4,6-*O*-benzylidene-2-deoxy-3-*O*-methanesulphonyl-α-D-glucopyranoside, by the action of lithium aluminium hydride in tetrahydrofuran. On treatment of the same compound with ethanolic sodium ethoxide, the *allo*-imine was again the main product, together with a small amount of methyl 2-benzamido-4,6-*O*-benzylidene-α-D-glucopyranoside. This is in contrast to the predominant formation of oxazolines and similar derivatives from related compounds in the β-D-glucopyranose series, observed by other authors. These reactions are rationalised on the basis of conformational analysis.

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QUANTITATIVE PAPER RADIOCHROMATOGRAPHY USING TOLLENS REAGENT

PART III. OXIDATION OF SUB-MICRO AMOUNTS OF MONOSACCHARIDES AND POLYOLS FOR RADIOMETRIC ESTIMATION ON PAPER

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INTRODUCTION

In a previous paper[1], a method was described for the determination of saccharides and polyols, after chromatography on paper, in amounts greater than 0.1 μmole (approx. 20 μg in the case of hexoses or hexitols). The compounds to be determined were oxidized directly on the paper chromatograms with ammoniacal silver nitrate, the resulting deposit of elementary silver was transformed into $Ag^{131}I$, and the radioactivities of the spots were measured. On the basis of the high spot-activities obtained, the method should be sensitive enough for the determination of much smaller amounts of substances. However, the use of aqueous solutions of Tollens reagent as a spray was found to be the limiting factor.

Other possibilities for increasing the sensitivity of the method followed the introduction of acetone–ammoniacal silver nitrate reagents, containing less than equimolar amounts of ammonia[2]. Studies on the application of these reagents for quantitative oxidation of sub-micro amounts of monosaccharides and polyols on paper will now be described.

EXPERIMENTAL

Reagents

Analytical grade, commercial preparations of D-glucose, D-galactose, D-fructose, D-sorbose, D-glucitol, and *myo*-inositol were used in all experiments.

Chromatography

The compounds were chromatographed on Whatman No. 1 paper by the ascending technique with butan-1-ol–acetic acid–water (6:1:2)[2,3]. Solvents were removed by drying of the chromatograms at room temperature for 72 h. Careful removal of chromatographic solvents is of the greatest importance for reproducibility of results. This is especially important for the solvents containing acetic acid or organic bases which are widely used for separation of saccharides.

Silver reagents

Acetone–silver nitrate solution[4] (SNS) and acetone–ammoniacal silver nitrate

solution (ASNS) were prepared as described previously[2]. The solutions were mixed in different proportions immediately before use to give the seven reagents listed in Table I.

TABLE I

THE COMPOSITION OF AMMONIACAL SILVER NITRATE REAGENTS CONTAINING DIFFERENT AMOUNTS OF AMMONIA.

Reagent	*SNS*	*R17*	*R33*	*R50*	*R67*	*R83*	*ASNS*
ASNS content (%)	0	16.7	33.3	50.0	66.7	83.4	100

Treatment of chromatograms

Oxidation reactions were carried out in the manner described earlier[2]. Dried chromatograms were drawn through the silver reagents three times and heated at 50°. Excess of reagent was removed with the use of 10% sodium thiosulphate.

Conversion of elementary silver into $Ag^{131}I$ was carried out as previously described[1] using aqueous iodine–potassium iodide containing ^{131}I. The specific activity of the radioactive reagents used was *ca.* 2 μc per ml.

Measurements of radioactivity on radiochromatograms were carried out with an end-window counter (mica window, 4 mg/cm^2) in a screening device, equipped with a Perspex screen (4-mm thick) having a 5-mm slit[5].

Determination of specific activity of the radioactive reagent was carried out as follows. Samples of radioactive reagent were placed on Whatman No. 1 paper sheets previously impregnated with 10% aqueous sodium thiosulphate. Samples were taken after dilution of $K^{131}I$ with KI–KIO_3 carrier solution, but before acidification[1]. In each experiment, nine aliquot portions (comprising three series of 4, 8, and 12 μl samples) were taken, and their activities were measured in the same counting apparatus as that used for measuring the activity of the radiochromatograms.

The amount of silver deposited on the paper was determined by comparison of the integrated spot-activities with the specific activity of the radioactive reagent used for conversion of Ag into $Ag^{131}I$.

RESULTS AND DISCUSSION

The oxidative ability of the reagents containing different proportions of silver nitrate and ammoniacal silver nitrate (Table I) were compared in the course of oxidations of D-glucose, D-galactose, D-fructose, D-sorbose, D-glucitol, and *myo*-inositol. For this purpose, the saccharides were chromatographed in amounts of 4×10^{-2} μmole, and oxidized with the reagents listed in Table I. The oxidations were interrupted at time intervals of 0.25, 1, 2, 3, and 4 h, and the amounts of liberated silver were determined radiometrically.

It was found that, of the six compounds used, D-glucose was most easily, and *myo*-inositol least easily oxidized. The results obtained for these two compounds are presented in Fig. 1, where the amounts of silver deposited are expressed as atoms liberated per molecule of saccharide.

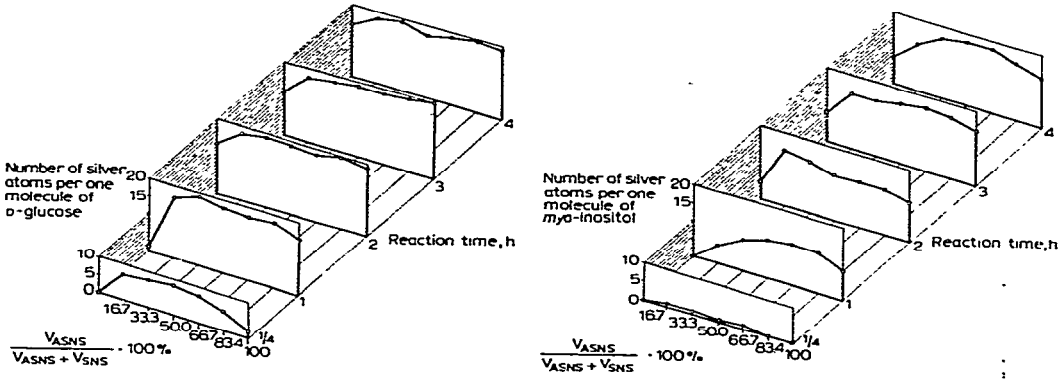

Fig. 1. Oxidation of D-glucose and *myo*-inositol with the reagents containing different proportions of silver nitrate and ammoniacal silver nitrate.

The results of the reaction interrupted at 0.25, 1, and 2 h show clearly that the oxidative abilities of the mixtures of ASNS and the SNS are higher than that of Tollens reagent, which, on the other hand, is a much more potent oxidant of the compounds used than is silver nitrate. The difference between the oxidative abilities of ASNS–SNS mixtures and ASNS decreases, or even disappears, at later stages of reaction in the case of aldoses and ketoses, but not with the less reactive polyols.

The heating times needed to produce quantitative reactions with the reagents tested are presented in Fig. 2. It can be seen that the reagents containing 33–100% of ASNS caused quantitative oxidation of aldoses and ketoses after a reaction time of 2 h. A similar result was achieved in the case of D-glucitol with reagent R67. The same reagent was also the most efficient in the case of *myo*-inositol, but heating for 3 h was necessary.

Reagent R67 was used in experimental estimations of various micro and sub-micro amounts of D-galactose, D-sorbose, and *myo*-inositol. The compounds were applied to paper chromatograms in amounts ranging from 0.1×10^{-2} to 12×10^{-2} μmole, and the radiochromatographic determinations were performed. It was found that, in all cases, even the smallest amounts used could be detected and estimated radiometrically. However, the determinations of the smallest amounts were accompanied by considerable errors, ranging up to 40%.

The integrated spot-activity-amount of substance relationships were analyzed and it was found that, for the regions given in Table II, the best lines drawn through the points obtained and the origin are not significantly different from the spot-activity-amount of substance least-square lines. Therefore, determination of the

slope of the calibration curves from the mean values of the determinations of standard samples is recommended. Such lines were constructed, and the errors

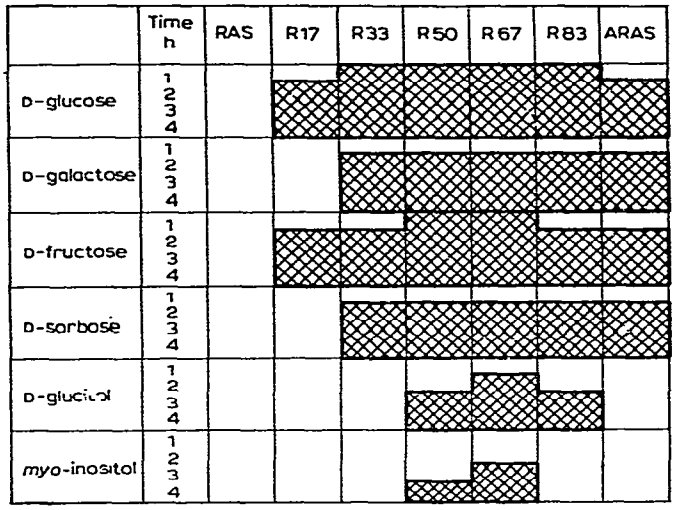

Fig. 2. Heating time needed to produce quantitative oxidation of monosaccharides and polyols with the reagents tested. Shaded area represents reagent composition and time range for which the reaction is completed.

of separate estimations were calculated; the results are presented in Fig. 3 and Table II.

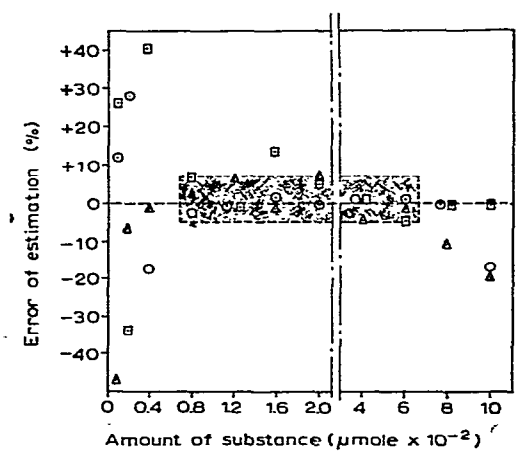

Fig. 3. Errors of the experimental analyses of different amounts of D-galactose (⊙); D-sorbose (△); and *myo*-inositol (⊡).

From these data, it can be concluded that the region of application of reagent R67 in quantitative radiochromatography of monosaccharides and polyols lies in the range between 0.8×10^{-2} and 6×10^{-2} μmole (*i.e.*, 1.5–10 μg of hexoses or hexitols).

The high sensitivity of the method described is due to the extensive, oxidative breakdown of the substances analyzed, which is quite remarkable for reactions carried out on a cellulose matrix. The final, mean consumption of oxidant varied from 17 to 20 oxygen equivalents for all six compounds tested. It should be noted that with copper salts, widely used in gravimetric and volumetric analyses of reducing sugars, consumption of only five to six oxygen equivalents occurs when a hexose is oxidized.

TABLE II

REGIONS OF PROPORTIONALITY AND THE AVERAGE ERRORS FOUND IN THE EXPERIMENTAL ANALYSES

Compound	*Region of proportionality* (μmole $\times 10^{-2}$)	*Average error*, (%)
D-Galactose	0.8–8.0	±1.1
D-Sorbose	0.4–6.0	±3.4
myo-Inositol	0.8–10.0	±4.2

The chemical character of the substances used in these experiments, and the results presented, show that oxidations with ammoniacal silver nitrate reagents, containing less than equimolar proportions of ammonia, can be used in very sensitive analyses of reducing and non-reducing saccharides, unlike the majority of existing analytical procedures. It should be mentioned that the elution methods, the applicability and accuracy of which can be compared with that of the method presented, require not less than 10 μg of substance per spot[6,7], quite often more than 30 μg[8,9], and sometimes more than 100 μg[10].

Densitometric methods, which are relatively simpler from the technical point of view, but not so accurate[11] can be used for the determination of reducing sugars only[12,13]. Similar limitation applies to Jaarma's radiochromatographic technique[14], which can be used for aldoses and ketoses chromatographed in amounts greater than 3 μg.

The fact that ammoniacal silver nitrate solutions have not previously been applied in quantitative analysis of saccharides was due to difficulties in determining the conditions leading to complete reaction. The results presented imply an important role for the cellulose support.

The question still remains as to whether the favourable conditions on paper are due to the increased surface area, which allows better contact between reactants, or whether the surface itself acts catalytically.

ACKNOWLEDGEMENT

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SUMMARY

The properties of reagents containing various proportions of silver nitrate (SNS) and ammoniacal silver nitrate (ASNS) as oxidants of paper-chromatographed aldoses, ketoses, and polyols have been studied. A reagent containing 33.3% of SNS and 66.7% of ASNS was found to be the most effective oxidant. This reagent can be applied in radiometric determinations of monosaccharides and polyols chromatographed in amounts of 1.5 to 10 μg. Average errors of experimental analyses ranged from ±1.1 to 4.2%.

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STUDIES ON STARCH-DEGRADING ENZYMES

PART III. THE ACTION PATTERN OF SOYA-BEAN Z-ENZYME

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INTRODUCTION

In Part II of this series[1], the purification and general properties of the Z-enzyme from soya-bean were described. We now report the first studies of the action pattern of this α-amylolytic enzyme. Linear amylose[2] has been used as the substrate for these investigations, as the production of maltodextrins is uncomplicated by branched products. The initial stage of amylolysis has been studied by viscometry, whilst maltodextrin formation at the "achroic limit" of the reaction has been followed by paper-chromatographic techniques.

EXPERIMENTAL

The preparations of the Z-enzyme and the substrates have been described earlier. Maltodextrins* (G_1, G_2, ... G_9) were isolated, by the paper-chromatographic technique described by Commerford *et al.*[3], from the salivary α-amylolysis of amylose; control samples of maltodextrins were kindly donated by Professor W.J. Whelan. Qualitative separations of maltodextrins from digests were made on Whatman No. 1 chromatography paper by the multiple-descent technique, using ethyl acetate–pyridine–water (10:4:3; v/v) at 20°. Chromatograms were developed using the reagents of Trevelyan *et al.*[4]. For quantitative work on 3MM paper, a multiple-ascent method[5], using 70% aqueous propanol, was found to give more satisfactory separations. Chromatograms using this solvent were also irrigated at 20°.

Kinetics of the initial action of Z-enzyme on amylose

The initial action of Z-enzyme on amylose was followed viscometrically[6] at 25°. Linear amylose ($[\eta] = 400$ in 0.2M KOH; 3 mg/ml; *DP*, *ca.* 3000) was dissolved in buffer at pH 5.5 (0.01M; acetate), and the specific viscosity (η_{sp}) of the solution was determined. After the addition of enzyme (0.05 units of activity[1]), η_{sp} was measured at regular intervals for 24 h. A comparable experiment was carried out at pH 7.8 (McIlvaine's buffer, 0.001M).

The hydrolysis of the amylose in 0.5M hydrochloric acid was studied similarly.

*G_1 = D-glucose; G_2 = maltose; G_3 = maltotriose; etc.

Production of maltodextrins

Amylose solution (80 ml, unbuffered, 1.3 mg/ml) was incubated with Z-enzyme (15 units of activity[1]) at 35°. At intervals, aliquots (2 ml) were removed for measurement of reducing power and iodine staining. Portions (10 ml) of the digest, after being heated on a boiling-water bath for 5 min to deactivate the enzyme, were concentrated on a rotatory evaporator, and analysed by paper chromatography.

In quantitative estimations, sugars were removed from the paper by irrigation with water. Amounts were determined by hydrolysing the oligosaccharides to D-glucose (1.5N HCl for 2 h), and estimating the reducing power by alkaline ferricyanide[2]. Control experiments showed that oligosaccharide recovery was 97–100%.

RESULTS AND DISCUSSION

The hydrolytic effect of soya-bean Z-enzyme on linear amylose was first established by Banks *et al.*[7], when a crude enzyme preparation was found to decrease the limiting-viscosity number ($[\eta]$) of the polysaccharide. Our recent preparation and study of the properties of purified Z-enzyme have confirmed the α-amylolytic character of this enzyme[1]. As with other α-amylases, hydrolysis of amylose occurs in two apparently distinct stages. First, there is a rapid decrease in the size of the amylose molecule, as shown by the fall in iodine stain, and the increase in the reducing power of the solution. This initial stage in the reaction was studied viscometrically. The second stage of the reaction begins when the "achroic limit" of the amylose solution is reached, and is characterised by a slow increase in reducing power. The apparent discontinuity in the hydrolysis reaction occurs at *ca.* 30% conversion into maltose. However, as we have stressed elsewhere[1], the "achroic limit" is an arbitrary concept, since it depends on the amylose:enzyme ratio. The production of maltodextrins at this stage in the reaction has been investigated by qualitative and quantitative paper chromatography.

Kinetics of the initial hydrolysis of amylose

The major problem here is to determine whether the hydrolysis of the α-(1→4)-glycosidic bonds is a random or non-random process. This can be determined by following the rate of substrate degradation. Ideally, number-average methods should be used to follow the change in degree of polymerization (DP) but, experimentally, these techniques are extremely difficult, and, furthermore, they are insensitive to limited degradation. In this work, therefore, we have used the viscometric technique, as this gives a very sensitive measure of the initial rupture of bonds in a polymer. The decrease in DP with time is not a true measure of degradation rate but, for either a zero- or first-order reaction, the rate is proportional[8] to DP^{-1}. Now $[\eta] = K'(DP)^{\alpha}$, but, as it is more convenient to measure the change in η_{sp} with time, we have evaluated DP^{-1} from the relationship[9]

$$DP^{-1} = [K'c(\eta_{sp}^{-1} + k)]^{1/\alpha},$$

where K' is a constant, c is the polymer concentration, α is the exponent in the Staudinger relation above, and k is Huggins' constant. Values of k and α were taken from the results of Banks and Greenwood[10].

Vink[9] has shown that, for a *random* hydrolytic process, the graph of DP^{-1} *versus* time is linear; whilst non-random degradation processes yield non-linear graphs.

The results of treating the data from the experiments involving the action of Z-enzyme on amylose are shown in Figure 1, curves 1 and 2. It can be seen that, for both pH 5.5 and 7.8 (*i.e.*, at optimum and adverse pH-values[1]), the graph of DP^{-1} *versus* time is linear. This suggests that in the initial stages of α-amylolysis the enzymic attack is essentially a random scission of α-(1→4)-glycosidic linkages. This conclusion was substantiated by the analogous results found when hydrolysis of the amylose by 0.5M hydrochloric acid — a known random process — was studied. The linear relation obtained under these conditions is shown by curve 3 in Fig. 1.

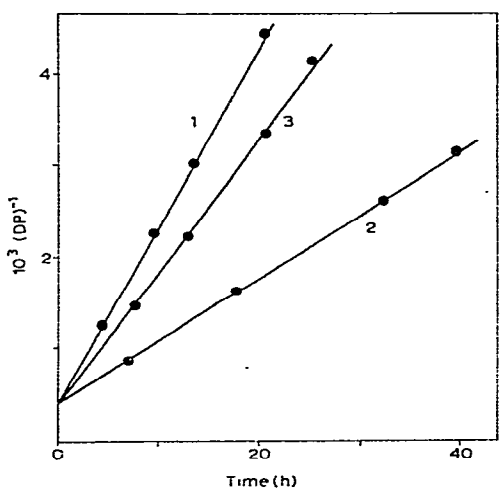

Fig. 1. Graph of DP^{-1} *versus* time for the action of soya-bean α-amylase on linear amylose: 1, pH 5.5; 2, pH 7.8; curve 3 shows the corresponding result for 0.5M hydrochloric acid.

It must be emphasised, however, that, although a large change had taken place in the value of η_{sp} by the end of these measurements, the corresponding average number of bonds broken per initial amylose molecule was only 16, and that the percentage of bonds broken during the period of investigation was therefore about 0.5.

Production of maltodextrins at the achroic point

The results of qualitative studies on the production of various maltodextrins from amylose, by Z-enzyme at the apparent achroic point (in this instance, after 33 h), are shown in Table I. It can be seen that products greater than G_6 predominate at this stage in the hydrolysis, although small quantities of the lower sugars are also present. As the hydrolysis proceeds, all of the saccharides separated (G_1–G_9) increase

in amount, and then the higher ones begin to decrease until only trace amounts of G_5, G_6, and G_7 remain. Saccharide G_4 is hydrolysed extremely slowly, and is always present in the digest.

TABLE I

PRODUCTION OF MALTODEXTRINS BY THE ACTION OF Z-ENZYME ON AMYLOSE

Maltodextrin[a]	*Digest incubation time (h)*				
	33	70	96	120	140
G_1	+[b]	++	+++	++++	+++++
G_2	+	++	+++	++++	+++++
G_3	+	++	+++	++++	+++++
G_4	+	++	+++	++++	++++
G_5	+	++	+++	++	+
G_6	++	++	+++	++	+
G_7	++	++	+++	++	+
G_8	++	++	+++	+	o
G_9	++	++	++	+	o
$>G_9$	++	++	+	o	o
Iodine stain[c]	Blue	Blue	Red	Achroic	Achroic

[a] G_1 = D-glucose; G_2 = maltose; G_3 = maltotriose; etc.
[b] The symbols (+), (++), etc. give an estimate of the relative quantities of each of the different sugars on the chromatogram; quantities can only be compared horizontally, and *not vertically*.
[c] Stain of digest, after 100-fold concentration.

The fact that maltodextrins G_8 and G_9 continue to increase in amount after the "achroic limit" indicates that long-chain substrate molecules are still present at this point. Indeed, it was found that when *concentrated* portions of the digest were stained with iodine—in contrast to the normal diluted aliquots—a blue colour was obtained. A true achroic point was not reached until much later and, at this stage, the chromatographic evidence suggests that maltodextrins greater than G_9 are not present. This observation again stresses the arbitrary nature of the term "achroic limit".

The above results suggested that some of the lower oligosaccharides differed in their susceptibility to degradation by Z-enzyme, and so the effect of the enzyme acting directly, and under comparable conditions, on G_4, G_5, G_6, and G_7 was studied. Results are shown in Table II. It can be seen that G_4 and G_5 are both somewhat resistant to enzymic degradation. Furthermore, although G_6 was hydrolysed faster than G_4 or G_5, the rate of hydrolysis of all these maltodextrins was small compared to that for G_7. This confirms the fact that oligosaccharides differ in their susceptibility to Z-enzyme, and parallels trends we have found for other plant α-amylases[11].

To confirm that, at extensive degrees of enzymic hydrolysis, the process is not completely random as shown by the yields of maltodextrins, a quantitative study of these was carried out. The results are shown in Table III. In Expt. 2, the digest was prepared in the presence of 10^{-6}M sodium *p*-chloromercuribenzoate to ensure

the inhibition of any residual β-amylase molecules that might have remained undetected in our previous tests[1].

TABLE II

PRODUCTS[a] OF THE ACTION OF Z-ENZYME ON MALTODEXTRINS

	Digest incubation time (h)			
Substrate[a]	19	43	90	114
G_4	o	G_2 +	G_2 ++	G_2 +++
G_5	o	G_2, G_3 +, +	G_2, G_3 ++,++	G_2, G_3 ++,++
G_6	G_2, G_3, G_4 +, +, +	G_2, G_3, G_4 2+, 2+, 2+	G_2, G_3, G_4 3+, 3+, 3+	G_2, G_3, G_4 4+, 4+, 4+
G_7	G_1, G_3, G_4, G_6 +, +, +, + G_2, G_5 +, +	G_1, G_3, G_4, G_6 2+, 2+, 2+, 2+ G_2, G_5 +, +	G_1, G_3, G_4, G_6 3+, 3+, 3+, 3+ G_2, G_5 2+, 2+	G_1, G_3, G_4, G_6 4+, 4+, 4+, 4+ G_2, G_5 2+, 2+

[a] Symbols as in Table I.

TABLE III

YIELDS OF MALTODEXTRINS PRODUCED FROM THE ACTION OF Z-ENZYME ON AMYLOSE[a]

	% by weight[b] *of*						
Expt.	G_1	G_2	G_3	G_4	G_5	G_6	*Higher oligomers*
1	4	13	9	7	5	21	41
2a[c]	3	9	10	6	5	17	50
2b[c]	7	13	11	7	6	31	25

[a] For digest conditions, see EXPERIMENTAL.
[b] Values have been quoted to the nearest integer.
[c] Digest contained 10^{-6}M sodium *p*-chloromercuribenzoate; incubation period of digest *b* was twice that of *a*.

Theory for the production of oligomers from the hydrolysis of polymers

The statistical theory of Kuhn[12] and Montroll and Simha[13] shows that the weight fraction of *i*-mer (W_i) produced from the *random* scission of a polymer of x units in length is

$$W_i = \frac{i}{x}[2s(1-s)^{i-1} + (x-i-1)s^2(1-s)^{i-1}] \qquad (1)$$

where s is the degree of scission. Now, the change in this yield with s is given by the differential

$$\frac{dW_i}{ds} = \frac{i}{x}[2(1-s)^{i-1} - 2s(i-1)(1-s)^{i-2} + 2s(x-i-1)(1-s)^{i-1} - (x-i-1)(i-1)s^2(1-s)^{i-2}].$$

Here, positive terms represent the formation of i-mer from larger oligomers, and negative terms give the degradation of i-mer to smaller fragments.

Now, as Painter[14] has pointed out, *if the i-mer is resistant to further attack, but all higher oligomers may be degraded,* the final yield of i-mer is given by the integral of the positive terms in the above differential equation, *i.e.*,

$$W_i = \frac{i}{x}\int_0^1 [2(1-s)^{i-1} + 2s(x-i-1)(1-s)^{i-1}]ds$$
$$= \frac{2}{i+1} \qquad (2)$$

Thus, under these conditions, the yield of i-mer is independent of the size and size distribution of the original polymer, and hence will represent the weight fraction of totally degraded polymer existing as i-mer.

Similarly, if all oligomers smaller than the i-mer are also resistant to hydrolysis, the weight fraction of $(i-j)$-mer is[14]:

$$\frac{2}{(i-j+1)}\prod_{k=0}^{j-1}\frac{(i-k-1)}{(i-k+1)} \qquad \begin{matrix} i = 3,4,5, \text{ etc.} \\ j = 1,2,3, \text{ etc.} \end{matrix}$$

But we have shown that this relation simplifies to

$$W_{i-j} = \frac{2(i-j)}{i(i+1)} \quad \text{when } j < i \qquad (3)$$

This relation gives the theoretical yields of oligomers when a polymer is degraded *completely* to oligomers of size i and smaller. However, in our experiments, the mixture of oligomers was analysed before degradation was complete, and hence, if a fraction x of total polymer has been degraded to oligomers of size i and smaller, fractional yields of oligomers can be calculated from

$$W_{i-j} = \frac{2(i-j)}{i(i+1)} \cdot x \qquad (4)$$

Mechanism of the action of Z-enzyme on amylose

In Table IV, the experimental yields of oligosaccharides are compared to theoretical yields calculated on the assumption that degradation is random, but that (*i*) G_5 and smaller oligomers are effectively resistant, (*ii*) G_6 and smaller oligomers are effectively resistant, and (*iii*) no oligomers are effectively resistant to α-amylolysis.

In the application of Kuhn's theory[12,13] to calculate yields for assumption (*iii*), a value for the degree of scission (s) was arbitrarily chosen so that the total yield of oligomers $> G_6$ approximated to that found experimentally. Now, it can be easily shown that, for any other value of s, there is a correspondingly similar theoretical distribution of the oligomer yields, *i.e.*, there is a *continuous* increase in amount up to a certain oligomer, followed by a *continuous* decrease thereafter; an experimental determination of s to demonstrate this point is therefore unnecessary.

It can be seen that our experimental yields do not follow such a pattern, and so, notwithstanding the evidence from the viscosity experiments, the α-amylolysis cannot be random at the later stages.

TABLE IV

CALCULATED YIELDS OF MALTODEXTRINS

Expt.[a]	*Theoretical assumption*	*% weight of total*						
		G_1	G_2	G_3	G_4	G_5	G_6	*Higher oligomers*
1	—	4	13	9	7	5	21	41
							62.0	
	(*i*) G_5 stable[b]	2.5	5.1	7.6	10.1	12.7		
	(*ii*) G_6 stable[c]	2.8	5.6	8.4	11.2	14.0	16.9	41.0
	(*iii*) Random[d]	7.6	11.0	11.9	11.6	10.5	9.1	38.3
2*a*	—	3	9	10	6	5	17	50
							67.0	
	(*i*) G_5 stable[b]	2.2	4.4	6.6	8.8	11.0		
	(*ii*) G_6 stable[c]	2.4	4.8	7.1	9.5	11.9	14.3	50.0
	(*iii*) Random[d]	5.1	7.8	9.1	9.4	9.1	8.5	49.0
2*b*	—	7	13	11	7	6	31	25
							55	
	(*i*) G_5 stable[b]	3.0	6.0	9.0	12.0	15.0		
	(*ii*) G_6 stable[c]	3.6	7.1	10.7	14.3	17.9	21.4	25
	(*iii*) Random[d]	11.1	14.8	14.8	13.2	11.0	8.8	26.3

[a] See Table III.

[b] Calculated from equation 4 with $i = 5$; $j = 0, 1, \ldots, 4$; $x = 0.38$ for Expt. 1; $x = 0.33$ for Expt. 2*a*; $x = 0.45$ for Expt. 2*b*.

[c] Calculated from equation 4 with $i = 6$; $j = 0, 1, \ldots, 5$; $x = 0.59$ for Expt. 1; $x = 0.50$ for Expt. 2*a*; $x = 0.75$ for Expt. 2*b*.

[d] Calculated from equation 1 with a value of s chosen so that the yield of G_7 and $> G_7$ was comparable to that found experimentally.

Again, when the inherent stability of certain maltodextrins is assumed, better agreement between experiment and theory occurs for (*ii*) than (*i*). But, in both cases, more G_6 and G_2, and less G_4 and G_5 are found experimentally than are predicted, confirming that the α-amylolysis cannot be random.

The presence of excess of G_2 and G_6 may be explained by postulating that, when the enzyme attacks an oligosaccharide, it is unable to attack readily (*a*) the bond nearest the reducing end-group, and (*b*) the five bonds nearest the non-reducing end-group.

Now with G_{12}, for example, if (*a*) holds, the yields of products formed would be $G_1 = 26.4\%$; $G_2 = 73.6\%$. For (*b*), the final yields would be: $G_1 = 8.3\%$; $G_2 = 8.3\%$; $G_3 = 8.3\%$; $G_4 = 8.3\%$; $G_5 = 8.3\%$; $G_6 = 58.5\%$. For (*a*) and (*b*) to hold together, the yields from G_{12} would be: $G_1 = 0$; $G_2 = 10.5\%$; $G_3 = 9.2\%$; $G_4 = 8.8\%$; $G_5 = 8.3\%$; $G_6 = 41.6\%$; $G_7 = 21.7\%$.

Although none of these schemes accounts adequately for the observed yields,

the last one is more satisfactory as it shows the tendency to form more G_2 and G_6, and less G_4 and G_5. It is perhaps reasonable, therefore, to consider that an intermediate scheme may explain the results, *i.e.*, that some bonds in a linear amylose molecule are *more resistant* to α-amylolytic attack than others. It seems likely that these particular bonds are the one adjacent to the reducing group, and the five bonds adjacent to the non-reducing end. This hypothesis is similar to that proposed by Bird and Hopkins[15].

Elsewhere[16], we have developed this hypothesis, and shown that it will explain the preferential non-random degradation of oligosaccharides by α-amylase.

The apparent *random* degradation of amylose in the initial stages is not inconsistent with this scheme in which the enzyme will not hydrolyse the bonds near the ends of molecules as readily as others; in a molecule of degree of polymerization 3000, there will be only 6 bonds which are resistant to attack, leaving 2993 bonds which may be attacked *randomly*. Initially, therefore, the degradation will appear to be a random process but, as α-amylolysis proceeds, the proportion of resistant bonds increases and the rate of enzymic attack will decrease. In the final stage, when only G_8 and $< G_8$ are present, there are few non-resistant bonds in the substrate molecules and the reaction is very slow and non-random.

The two apparent stages in the α-amylolysis of amylose may thus merely be a result of the difference in affinity of the enzyme for large and small substrate molecules.

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SUMMARY

The action pattern of soya-bean Z-enzyme on linear amylose has been investigated. The kinetics of the initial stages of α-amylolysis have been studied by the use of viscometric techniques, and the process is shown to be an apparently random one. Paper-chromatographic studies of the production of maltodextrins at the "achroic limit" indicated, however, that, at this stage, the attack is non-random. This has been confirmed by (*i*) studying the action of the enzyme on individual maltodextrins, and (*ii*) measuring quantitatively the yields of dextrins after the "achroic limit". The latter experimental yields have been compared with those calculated from various theoretical models for the α-amylolytic action. A hypothesis has been developed to account for the observations made at various stages of the α-amylolysis.

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POLYSACCHARIDES OF CARTILAGINOUS TISSUES

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INTRODUCTION

The development of a feasible technique[1] for fractionating the polysaccharides of "ground substance" prompted us to examine a selection of human and animal cartilage since, even in previous studies[2,3] of *nucleus pulposus*, the whole spectrum of polysaccharide constituents had not been examined. Two improvements in the process of isolating the polysaccharides seemed desirable. Firstly, the digestion of protein should be done under aseptic conditions, and secondly, since L-cysteine (and other reducing agents) can degrade hyaluronic acid by a free-radical reaction in the presence of oxygen[4], the enzyme used for such digestion should not require this as an activator. Pronase was therefore investigated as a substitute for ficin in this respect.

METHODS

Sources of the tissues

A pair of normal, interarticular, knee cartilages was removed from a male patient aged 22 years. Human *nucleus pulposus* was obtained from children, aged 10–14 years, suffering from various forms of scoliosis. Ear cartilage was dissected from white guinea-pigs, aged 3–18 months. The tissues were stored at −20° before use.

Preparation of the tissues

The tissues were macerated by grinding in a mortar with liquid nitrogen, and then dissected with a scalpel until a finely divided preparation was obtained. Preparations were lyophilised, and dried to constant weight *in vacuo*, in a toluene drier over phosphorus pentoxide.

Isolation of the mucopolysaccharides

The experimental procedure was based on that used by Barker *et al.*[1] to fractionate the mucopolysaccharides of rat skin. This was altered in method (*a*) to use aseptic conditions, and in method (*b*) to use both aseptic conditions and a different proteolytic enzyme. Method (*b*) was adopted for all separations, except for the first comparison.

(*a*) The acetone-dried *nucleus pulposus* powder (184 mg) was placed in the dialysis bag of a sterile, aseptic-dialysis apparatus[5], containing 0.02M sodium acetate buffer (pH 5.8, 1 l) in its outer compartment. A solution of ficin (20 mg) and L-cysteine hydrochloride (10 mg) in the acetate buffer was Seitz-filtered, and added to the dialysis bag with a sterile pipette. L-Cysteine hydrochloride (200 mg) was added to the buffer in the outer compartment, and the whole apparatus incubated at 37° for 24 h.

(*b*) Pronase-P (Kaken Co., Ltd.) was used, at the same tissue-to-enzyme ratio as ficin (10:1), in 0.1M sodium phosphate buffer (pH 7.0) in the presence of Ca^{2+} ions (0.005M). The aseptic-dialysis apparatus was used as in (*a*).

The extraction procedure[1] was completed with denaturation of the protein, dialysis, and fractionation of the polysaccharides on Deacidite FF (Cl^- form). Fractions were scanned by the orcinol[6] and carbazole[7] assays, and those within the peaks were bulked together and dialysed against tap water, distilled water (2 l), and three changes of deionised water (2 l). Fractions from *nucleus pulposus* were dried and weighed, but those from knee and ear cartilage were concentrated, redissolved in deionised water, and weights assigned from the analysis of constituents.

The incorporation of [^{35}S]*-labelled sulphate groups into the mucopolysaccharides of the guinea-pig ear cartilage*

After removal of skin from the dorsal surface of the ear, the cartilage was dissected out in small pieces (approximately 2×2×1 mm) using a scalpel, and placed on a tissue-culture medium in a sterile petri-dish. The pieces of tissue were divided into sets of four which were placed in sterile bijou bottles containing 0.5 ml of a culture medium consisting of Hanks B.S.S. (6 ml), horse serum (4 ml), and 0.5 mc of carrier-free $Na_2{}^{35}SO_4$ (0.5 ml). The tissues were incubated on a rocker in an atmosphere of oxygen–carbon dioxide (95:5) for 24 h at 37°.

Histological sections and autoradiographs were prepared from one piece of tissue from each incubation, and from the uncultured ear-tissue.

A quantitative determination of the total amount of radioactive sulphate incorporated in the tissue was made. Pieces of tissue (4–6 mg) were dialysed against 0.1M sodium sulphate (20 ml) for 24 h, dried to constant weight, and hydrolysed in conc. hydrochloric acid (1 ml) at 100° for 6 h. The sulphate was precipitated as barium sulphate, washed with acetone, and counted at infinite thickness on a planchette in an end-window counter.

Methods of characterisation

(*1*) *Carbazole:orcinol ratio*

The uronic acid content of the fractions, except those of keratan sulphate, was determined directly by the carbazole[8] and orcinol[6] methods. Chondroitin 4- and 6-sulphates and hyaluronic acid, which contain D-glucuronic acid residues, have ratios of approximately unity, but dermatan sulphate, which contains L-iduronic acid residues, has a low ratio (0.4). Heparin, although containing D-glucuronic acid residues, gives a ratio greater than unity.

(2) *Infrared spectra*

With the *nucleus pulposus* fractions, enough material was obtained to measure infrared spectra (potassium bromide disc) on a Perkin–Elmer Model 21 spectrometer.

(3) *Characterisation of the amino sugars*

A sample of each tissue was hydrolysed at 100° in 3N hydrochloric acid for 4 h, and the liberated amino sugars were chromatographed on Dowex 50 (H^+ form), as in the method of Crumpton[9]. Glucosamine (2-amino-2-deoxy-D-glucose) and galactosamine (2-amino-2-deoxy-D-galactose) were the only amino sugars shown to be present. These were distinguished in the separated fractions by the borate[10] version of the Elson–Morgan reaction[11]. In the presence of borate, glucosamine and galactosamine give 25–30% and 50–65%, respectively, of their original colour intensities.

Heparin, hyaluronic acid, and keratan sulphate contained glucosamine, whilst the chondroitin sulphates and dermatan sulphate contained galactosamine.

(4) *Molar ratio of constituents*

The chondroitin sulphates and dermatan sulphate contain approximately equimolar proportions of uronic acid, hexosamine, and sulphate. Hexosamine was determined by the Tracey[10] method, and expressed in terms of glucosamine or galactosamine, as shown by the borate readings. Sulphate ester was determined, after hydrolysis in N hydrochloric acid at 100° in a sealed tube for 16 h, by the method of Jones and Letham[12].

Keratan sulphate contains equimolar proportions of hexose, hexosamine, and sulphate. The hexose was assayed and characterised as galactose by the primary and secondary L-cysteine–sulphuric acid method of Dische[13]. It was expressed as galactose from a calibration curve of D-galactose.

Hyaluronic acid contains no sulphate, but heparin has a variable sulphate content of between three and five groups per tetrasaccharide unit.

(5) *Susceptibility to testicular hyaluronidase*

Chondroitin 4- and 6-sulphates may be distinguished by incubation with testicular hyaluronidase, and assay[14] of the liberated oligosaccharides for *N*-acetylhexosamine reducing groups. As substitution at C-4 inhibits chromophore formation with Ehrlich's reagent[15], only chondroitin 6-sulphate and hyaluronic acid give a colour (λ_{max} 544, 585 mμ). Dermatan sulphate was used as a control, and gave no colour.

Column chromatography

The elution volumes of the constituent polysaccharides were the same as those found by Barker *et al.*[1].

RESULTS

Identical elution patterns were obtained by assay with orcinol when two aliquots of knee cartilage were submitted to method (*a*) using ficin, or method (*b*)

using pronase P. Method (*b*) was therefore used in all subsequent isolation procedures.

By total hydrolysis of the tissue, radioactive sulphate was shown to be incorporated into guinea-pig ear cartilage. Comparison of the cultured and normal tissue by histology and autoradiography proved that the chondrocytes were unaltered, and that the whole tissue contained labelled sulphate, particularly around the cells and throughout the matrix. The scans of the mucopolysaccharide fractions (Fig. 1) using carbazole (which also reacts non-specifically with keratan sulphate) and radioactivity were coincident, except that fraction 1 contained no radioactivity, as expected for hyaluronic acid.

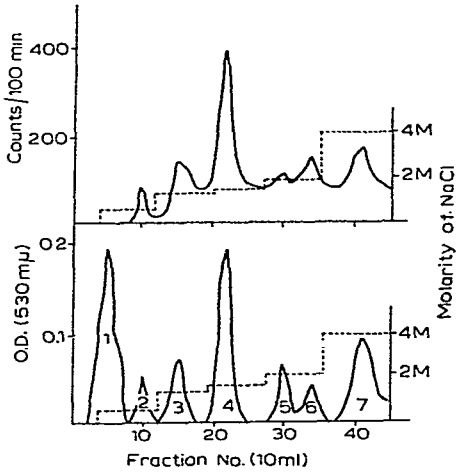

Fig. 1. Fractionation of the mucopolysaccharides of guinea-pig ear cartilage on De-acidite FF (Cl– form), scanned by radioactivity (upper) and carbazole (lower).

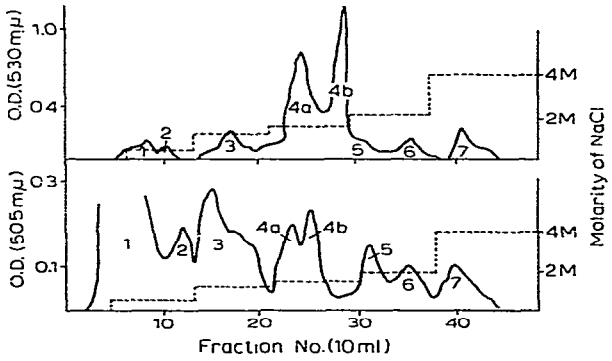

Fig. 2. Fractionation of the mucopolysaccharides of *nucleus pulposus* (upper) and knee cartilage (lower) on De-acidite FF (Cl– form), scanned by carbazole and orcinol, respectively.

The scans for *nucleus pulposus* and knee cartilage (Fig. 2) showed a similar pattern for the first four fractions. In all tissues, fractions 1–3 corresponded in

position to hyaluronic acid, adenosine 3-phosphate 5-phosphosulphate (PAPS), and chondroitin 4-sulphate in the elution pattern of reference mucopolysaccharides[1].

Fraction 1 for *nucleus pulposus* gave an infrared spectrum similar to that of hyaluronic acid. All such tissue fractions contained glucosamine and uronic acid, but the apparent concentration by the carbazole[8] and orcinol[6] assays was higher than the theoretical value for hyaluronic acid. This was probably due to interference by hexoses present in the mucopeptide debris contaminating this fraction. However, little sulphate was found, except in the *nucleus pulposus* fraction, and this was probably due to some overlapping with its neighbouring PAPS fraction. All fractions 1 were susceptible to hyaluronidase treatment. The liberated oligosaccharides contained reducing *N*-acetylhexosamine residues, which reacted in the *N*-acetylhexosamine assay[14]. Each fraction 1 contained a high protein content, as indicated by the ninhydrin assay[16], expressed in terms of D-alanine as a percentage of the uronic acid or hexose component of the polysaccharide fraction. Much less protein contamination was encountered with other fractions.

Fraction 2 was eluted in the position of standard PAPS and was not further investigated.

Fraction 3 from *nucleus pulposus* gave an infrared spectrum with bands for functional groups typical of a chondroitin sulphate, including a band due to *O*-sulphate at 1240 cm^{-1}. For each tissue, this fraction contained a uronic acid (carbazole/orcinol ratio of unity, indicative of glucuronic acid), galactosamine, and sulphate, in equimolar proportions. The fraction was identified as chondroitin 4-sulphate by the negative result of the *N*-acetylhexosamine assay[14] after hyaluronidase treatment. The protein content of the fraction (ninhydrin assay[16]) was low.

Fraction 4a from *nucleus pulposus* gave an infrared spectrum identical to that of chondroitin 6-sulphate[17], with maxima at 775, 820, and 1000 cm^{-1} in the fingerprint region. For all tissues, it contained uronic acid (carbazole/orcinol ratio of unity, indicative of glucuronic acid), galactosamine, and sulphate, in approximately equimolar proportions. The fraction was degraded by hyaluronidase, with liberation of reducing *N*-acetylhexosamine groups which reacted in the assay with Ehrlich's reagent. Its position in the elution pattern of standard mucopolysaccharides corresponded to that of chondroitin 6-sulphate, and it was so designated.

From *nucleus pulposus* and knee cartilage, fraction 4b, which was not present in guinea-pig ear cartilage, appeared to have similar properties to those of chondroitin 6-sulphate. The fraction from *nucleus pulposus* had the same infrared spectrum, with maxima at 775, 820, and 1000 cm^{-1} in the fingerprint region. It was susceptible to hyaluronidase, reacting positively to subsequent assay for *N*-acetylhexosamine. However, although it contained uronic acid (carbazole/orcinol ratio, *ca.* unity) and galactosamine in equimolar proportions, it had a greater proportion of sulphate. It was designated as an "oversulphated" form of chondroitin 6-sulphate.

Fraction 5 from guinea-pig ear and human-knee cartilage contained uronic acid, galactosamine, and sulphate, in equimolar proportions. The ratio of the uronic acid determined by the carbazole[8] and orcinol[6] methods was low (0.4), indicative

of L-iduronic acid. The fractions were not susceptible to hyaluronidase, and were designated as dermatan sulphate.

For all tissues, a fraction 6, with a high sulphate content, was eluted in 2M sodium chloride. This fraction gave a high carbazole reading, and contained equimolar amounts of uronic acid and glucosamine. It was designated as a heparin fraction.

Fraction 7, in all tissues, was eluted in 4M sodium chloride, and that from *nucleus pulposus* gave an infrared spectrum identical with that of keratan sulphate. It contained hexose, glucosamine, and sulphate, in equimolar proportions. The hexose was characterised as galactose by the primary and secondary assays[13] with L-cysteine. The fraction was designated as keratan sulphate and, in each case, contained small amounts of fucose. Assayed as 6-deoxyhexose[13], and expressed as a percentage of the galactose component, this corresponded to 5.2% (*nucleus pulposus*), 11.2% (knee cartilage), and 5.4% (ear cartilage).

DISCUSSION

Previously[2], a partial fractionation of the polysaccharides of human *nucleus pulposus* had been obtained on a cellulose column equilibrated with 80% ethanol containing 0.3% of barium acetate. Polysaccharides were eluted by the use of ethanol, decreasing stepwise in concentration. Two main fractions were distinguished, one containing 21.1% of glucosamine, 16.8% of galactose, 2.3% of fucose, and 5.4% of sulphur (sulphate ester), and the other containing 22.8% of galactosamine and 4.83% of sulphur (sulphate ester), suggesting polysaccharides of the keratan sulphate and chondroitin sulphate type, respectively. A further improvement in separation was effected by gradient elution, when two minor polysaccharide components were distinguished, both containing uronic acid, as did the second major fraction. Later studies[18] suggested that one chondroitin sulphate was the 6-sulphate; small amounts of dermatan sulphate could be detected if the disc was herniated. The total polysaccharide content was reduced from 30% of the dry weight in normal samples to 5% in herniated samples. Our work shows the presence of not more than 19.6% of polysaccharide, and the presence of all the polysaccharides of "ground substance"; the most notable of these were chondroitin 6-sulphate and its novel, oversulphated homologue, which together comprised half of the polysaccharide present. The glucosamine/galactosamine ratio of the sample (age group, 10–14 years) was 0.36, compared with Hallén's[3] finding of 0.5 for subjects aged 15 years.

In knee cartilage, the proportion of chondroitin 4-sulphate to chondroitin 6-sulphate is considerably increased, even when including the "oversulphated" chondroitin 6-sulphate which was again encountered. "Oversulphated" chondroitin 6-sulphate has not been previously reported in mammalian tissue, but has been found in several kinds of elasmobranch cartilage[19].

The elastic cartilage of the outer ear of the guinea-pig contained only the normal type of chondroitin 6-sulphate, but otherwise all the polysaccharides of "ground

TABLE I. FRACTIONATION OF TISSUE CARTILAGE

Fraction	*Polysaccharide*	*Weight (mg)*	*Comparable ninhydrin reaction*	*Constituent ratio*[a]	*Uronic acid, carbazole/ orcinol ratio*	*Hexosamine borate reaction, %*	*Reaction of oligosaccharides*[b] *with Ehrlich's reagent*
Cartilage from guinea-pig ear (160 mg)							
1	Hyaluronic acid	12.1	5.5	1:0.81:0.08	0.9	glucosamine, 28	+
3	Chondroitin 4-sulphate	0.6	3.0	1:0.91:0.91	1.0	galactosamine, 48	−
4	Chondroitin 6-sulphate	2.1	2.1	1:0.92:0.90	0.96	galactosamine, 52	+
5	Dermatan sulphate	1.9	1.5	1:0.87:0.98	0.48	galactosamine, 50	−
6	Heparin	1.0	1.1	1:0.94:1.42	1.54	glucosamine, 34	
7	Keratan sulphate	1.3	2.0	1:0.92:0.91	—	glucosamine, 33	
Human nucleus pulposus (*218 mg*)							
1	Hyaluronic acid	12.0	20.1	1:0.70:0.40	0.9	glucosamine, 35	+
3	Chondroitin 4-sulphate	4.5	7.5	1:0.80:0.98	1.0	galactosamine, 57	−
4a	Chondroitin 6-sulphate	14.0	2.1	1:0.89:0.97	1.0	galactosamine, 51	+
4b	Chondroitin 6-sulphate ("over-sulphated")	7.6	2.4	1:0.95:1.17	0.9	galactosamine, 54	+
6	Heparin	0.6	2.5	1:0.89:1.30	1.19	glucosamine, 30	
7	Keratan sulphate	5.5	0.45	1:1.00:1.03	—	glucosamine, 35	
Human semi-lunar knee cartilage (580 mg)							
1	Hyaluronic acid	22.0	6.0	1:0.80:0.12	0.88	glucosamine, 31	+
3	Chondroitin 4-sulphate	4.5	2.0	1:0.93:0.98	0.97	galactosamine, 49	−
4a	Chondroitin 6-sulphate	2.2	2.0	1:0.97:0.95	0.99	galactosamine, 54	+
4b	Chondroitin 6-sulphate ("over-sulphated")	2.5	2.0	1:0.99:1.18	1.10	galactosamine, 49	+
5	Dermatan sulphate	1.0	3.0	1:1.01:0.95	0.43	galactosamine, 48	−
6	Heparin	0.5	2.5	1:1.05:2.50	1.15	glucosamine, 33	
7	Keratan sulphate	1.3	2.0	1:0.86:0.97	—	glucosamine, 34	

[a] Uronic acid:hexosamine:sulphate; except for keratan sulphate, where it is galactose:hexosamine:sulphate.
[b] After hyaluronidase treatment.

substance" were present. The perichondrium was present in the above cartilages, and is generally considered to be the site of the hyaluronic acid. With ear cartilage, this layer contained very little of the radioactive sulphate, but gave metachromatic stains with toluidine blue and Alcian blue.

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We wish to thank Professor M. Stacey, F.R.S., for his interest in this work, the Nuffield Rheumatism Research Council for financial support, and Professor R. Roaf, F.R.C.S., of the Robert Jones and Agnes Hunt Orthopaedic Hospital, Oswestry, for the provision of certain specimens.

SUMMARY

A technique for the separation of all the polysaccharides of "ground substance" has been applied to three different types of cartilage. Pronase was used, instead of ficin, in the preliminary digestion of protein to avoid oxidative-reductive degradation of hyaluronic acid. In human *nucleus pulposus* and knee cartilage, but not in guinea-pig ear cartilage, an "oversulphated" chondroitin 6-sulphate was encountered, in addition to its normal equivalent.

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STUDIES ON URONIC ACID MATERIALS

PART XI[1]. THE CARBOHYDRATE COMPONENT OF THE OLEORESIN FROM *Boswellia Papyrifera* (DEL.) HOCHST

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INTRODUCTION

The genus *Boswellia* (family *Burseraceae*) contains some 25 species; some of these [*e.g.*, *B. serrata* Roxb. (syn. *B. thurifera*; syn. *B. glabra*)] are indigenous to Central India, and others to Africa [*e.g.*, *B. carteri* Birdw. (syn. *B. sacra*) and *B. papyrifera* (Del.) Hochst.] The oleoresin sold commercially as Frankincense ("gum olibanum") is largely the exudate from *B. carteri*, but this may be adulterated with *B. papyrifera*, which is not so strongly aromatic, and with exudates from species of *Commiphora*. Studies on commercial samples should therefore be avoided; if samples cannot be authoritatively verified during collection, they ought to be backed by suitable botanical specimens of the leaves and inflorescence[2].

The only *Boswellia* species to have been studied chemically to date are *B. serrata* and *B. carteri*. No attention has been given to the exudate from *B. serrata* since Malandkar's preliminary report[3] in 1925, despite the fact that no information was given regarding the identity or amount of the uronic acid present (*cf.* ref. 4). Two investigations of the polysaccharide from *B. carteri* oleoresin have, however, been reported. Unfortunately, these conflict regarding the nature of the uronic acid. Jones and Nunn[5] found D-galactose, L-arabinose, and 4-*O*-methylglucuronic acid, in the ratio 7:1:4, together with traces of L-rhamnose and L-fucose; in contrast, El Khadem and Megahed[6] reported D-galactose, L-arabinose, and D-galacturonic acid, in the ratio 5:1:2.

It is most unusual for the uronic acid to differ markedly from species to species within any botanical genus. Further studies were clearly required to clarify the position, and our current interest[1] in convenient sources of 4-*O*-methylglucuronic acid prompted us to consider *Boswellia* exudates. Our attempts to secure authenticated specimens of *B. carteri* or *B. serrata* have not yet proved successful, but we report here the results of an analytical study of the exudate from *B. papyrifera* (Del.) Hochst, secured for us by the Sudanese Gum Research Officer.

EXPERIMENTAL AND RESULTS

Analytical Methods

Analyses were carried out using the standard methods described previously[7],

except that (*i*) the methoxyl and uronic anhydride contents were determined by infrared methods, after reaction with hydriodic acid[8], and (*ii*) paper chromatography was carried out with the following solvent systems: (*a*) ethyl acetate–acetic acid–formic acid–water (18:3:1:4); (*b*) butan-1-ol–ethanol–water (4:1:5); (*c*) butan-1-ol–pyridine–water–benzene (5:3:3:1); (*d*) acetone–ethanol–propan-2-ol–borate buffer, 0.05M, pH10 (3:1:1:2).

Examination of the crude oleoresin

The small, pale yellow-white "tears" were free from any contaminating adherents. The oleoresin had a pleasant, characteristic odour, and was completely insoluble in water. (Found: moisture, 5.2%. On a dry-weight basis: ash, 0.7%; nitrogen, 0.28%.

Isolation of the polysaccharide

The oleoresin, crushed to a fine powder, was refluxed with ethanol for 24 h. The white polysaccharide residue was collected by centrifugation, and carefully washed with small portions of ethanol until free of waxy and resinous matter; the carbohydrate material was then completely soluble in cold water. A solution was filtered, and electrodialysed in a grease-free perspex cell at 20° until current ceased to flow under an applied potential of 330 v. On being freeze-dried, the purified polysaccharide was obtained in the free-acid form as a white powder; yield, 21% of the crude oleoresin (dry-weight basis).

Determinations on the purified polysaccharide

Found: moisture, 5.0%; ash, nil; nitrogen, 0.78; uronic anhydride, 19; methoxyl, 3.28%; $[\alpha]_D^{25}-14°$ (*c* 1, water); limiting flow-time number, 16.8 (Ubbelohde suspended level dilution viscometer at 25.0°, in M aqueous sodium chloride). The specific infrared method[8] showed that the electrodialysis treatment had removed all traces of the ethanol used in the extraction stage (*cf.* ref. 9). Furthermore, attempted saponification (dilute NaOH) did not decrease the methoxyl content of the polysaccharide, which therefore has no methyl ester groups.

Attempted fractionation

In view of El Khadem and Megahed's claim[6] to have fractionated the carbohydrate from *B. carteri* into acidic ($[\alpha]_D-9.2°$) and neutral ($[\alpha]_D-14.7°$) polysaccharides, fractionation with cetyltrimethylammonium bromide ("Cetavlon") was attempted. Cetavlon (20% solution) was added to an aqueous solution of the electrodialysed material, until precipitation was complete; after removal at the centrifuge, the supernatant contained no polysaccharide (phenol–sulphuric acid test[10]).

Hydrolysis and paper chromatography

Hydrolysis was effected with sulphuric acid (2N) on a boiling-water bath for 8 h. After neutralisation (barium carbonate), filtration, and reduction in volume at 30°

(reduced pressure), the resulting pale-brown syrup was chromatographed against reference sugars on Whatman 3MM paper in each of the solvents (*a*)–(*d*).

Solvents (*a*) and (*d*) showed that the main uronic acid present was 4-*O*-methylglucuronic acid, with a trace of glucuronic acid. There was no galacturonic acid present (*cf.* ref. 6). Development for 48 h in solvent (*a*) gave two aldobiouronic acids having R_{gal} values of 0.28 and 0.56. The faster-moving acid was chromatographically identical to 6-*O*-(4-*O*-methyl-β-D-glucopyranosyluronic acid)D-galactose; hydrolysis of the material eluted from the thick paper gave 4-*O*-methylglucuronic acid and galactose. This aldobiouronic acid was also present in *B. carteri*[5].

The separations achieved in solvents (*a*), (*b*) and (*c*) showed that the neutral sugars present were galactose, arabinose, rhamnose, and fucose; by the standard analytical methods (*cf.* ref. 11), these were present in the ratio 14:2:1:trace. Calculation then gives the essential composition of the polysaccharide to be uronic acid 19%, galactose 66%, arabinose 10%, and rhamnose 5%, with a trace of fucose.

DISCUSSION

The results of these analyses indicate that the acid polysaccharide from *B. papyrifera* contains the same component sugars as that from *B. carteri*[5]. Indeed, the two species have close similarities. In view of this, the results do not support El Khadem and Megahed's suggestion[6] that *Boswellia* species contain galacturonic acid; these authors, unfortunately, investigated a commercial sample of unknown origin, and their claim to have effected a fractionation may also have arisen from this fact. This aspect of their work therefore requires re-investigation.

Jones and Nunn[5] reported a neutralisation equivalent of 545 for *B. carteri*. Assuming that all the titratable acidity arises from the uronic acid groups, calculation shows that the methoxyl content found (5.4%) is sufficient for virtually all of the uronic acid groups to be present in the 4-methoxy form. Our analytical values for *B. papyrifera* lead to a similar conclusion; the traces of free glucuronic acid observed chromatographically may have resulted from some demethylation during hydrolysis.

It is evident that the oleoresins from both *B. carteri* (yield of polysaccharide, 12%; uronic acid content, 32%) and *B. papyrifera* (yield of polysaccharide, 21%; uronic acid content, 19%) are convenient sources of 4-*O*-methylglucuronic acid. Further analytical and structural investigations are, however, necessary to supplement the present work on *B. papyrifera*, and the incomplete study[5] of *B. carteri*. It is hoped that this report will stimulate interest in the chemistry of *Boswellia* species.

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SUMMARY

An acidic polysaccharide is easily extracted from the oleoresin exuded by *B. papyrifera*. The results of an analytical study of the composition of the polysaccharide are given; they support an earlier report of the presence, in *Boswellia* exudates, of 4-*O*-methylglucuronic acid in appreciable quantity.

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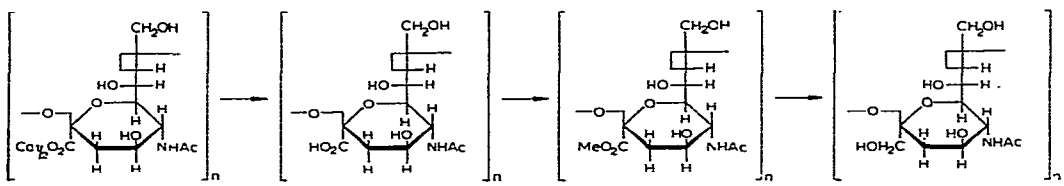
Notes

Sialic acid and related substances

PART III. CARBOXYL-REDUCED COLOMINIC ACID

Colominic acid was first isolated from the liquid culture medium of *Escherichia coli* K235 by Barry and Goebel in 1957[1]. Thereafter, Kimura and Tsurumi[2] reported that better yields of colominic acid were obtained in agar-plate cultures than in liquid cultures. The substance has been identified as a homopolymer of *N*-acetylneuraminic acid[3–5]. The present report concerns the preparation of carboxyl-reduced colominic acid required for a confirmatory study of the glycosidic linkage by methylation.

Colominic acid (II) was isolated as the calcium salt (I) from the culture medium of *E. coli* 016, and converted by way of the methyl ester (III) into the carboxyl-reduced product (IV), according to the following scheme:



I II III IV

On periodate oxidation, carboxyl-reduced colominic acid consumed 0.38 mole of periodate per mole of monosaccharide residue. This consumption is close to that for colominic acid, as reported by McGuire and Binkley[4]. Accordingly, the presence of (2→8)-linkages in the molecule is further substantiated.

EXPERIMENTAL*

Calcium salt of colominic acid (I)

The cells of *E. coli* 016, grown on agar plates[2], were extracted with a mixture (1:1) of 1N acetic acid and 10% aqueous sodium acetate. The extracts were deproteinized by repeated application of Sevag's method, and two volumes of ethanol were added to give a precipitate which was collected by centrifugation and dissolved again in acetic acid–10% aqueous sodium acetate (1:1). This solution was fractionated

* All samples were hygroscopic, and were dried *in vacuo* at 100° for one h over phosphorus pentoxide before analysis.

with ethanol into five fractions. The fractions precipitated at ethanol concentrations from 30 to 50% were relatively rich in sialic acid. These fractions were combined, and reprecipitated by the addition of two volumes of ethanol, as described above. The crude fraction was obtained in a yield of 4.3 g; *ca.* 70 g of the cells (wet weight) were obtained in this culture.

The crude fraction of the calcium salt of colominic acid (3.0 g) was subjected to column chromatography on a Dowex-1x8 (formate form) column (39×2.0 cm) as described by O'Brien and Zilliken[6], and by Kimura and Tsurumi[2]. The combined fractions, which were positive to the thiobarbituric acid reaction, were dialyzed against running water for 42 h, and then concentrated under reduced pressure at 40° to a volume of *ca.* 100 ml. The solution was adjusted to concentrations of 2.5% in calcium acetate and 2N in acetic acid, and two volumes of ethanol were added to give a precipitate (437 mg), $[\alpha]_D^{16}$—45.4° (*c* 1.0, water).

Anal. Calc. for $(C_{11}H_{16}NO_8Ca_{0.5}.3H_2O)_n$: C, 36.26; H, 6.39; N, 3.84. Found: C, 35.49; H, 6.14; N, 3.98.

There was a 13.6% loss in weight when the product was dried *in vacuo* at 100° for 16 h over phosphorus pentoxide.

The substance was found to be a homogeneous polymer using free-boundary electrophoresis (Tiselius cell) in two different systems (*A*; acetic acid–sodium acetate buffer, pH 5.0, $\mu = 0.1$, 10.0 mA, 1,800 sec, 3.5°. *B*; sodium borate buffer, pH 9.2, $\mu = 0.15$, 9.8 mA, 2,100 sec, 4.0°). The ultracentrifuge pattern also confirmed the homogeneity of the sample. The sedimentation constant of the colominic acid was found to be $s_{20,w} = 1.50$. The constant was measured with a 1% solution of colominic acid in 0.15M potassium chloride, at 59,780 rev./min and 19.8°, with the Spinco Model E ultracentrifuge.

Colominic acid (II)

An aqueous solution of the calcium salt (400 mg) of colominic acid was passed through an Amberlite CG 120 (type I, H^+ form) column (7×0.8 cm) and the column was washed with water. The combined effluents were lyophilized to give an amorphous product (350 mg), $[\alpha]_D^{17}$—46° (*c* 1.0, water){lit.[4], $[\alpha]_D^{12}$—47.2° (*c* 0.4, water)}.

Anal. Calc. for $(C_{11}H_{17}NO_8.2H_2O)_n$: C, 40.37; H, 6.47; N, 4.28. Found: C, 40.87; H, 6.01; N, 4.09.

There was a 10.5% loss in weight when the product was dried *in vacuo* at 100° for 16 h over phosphorus pentoxide. The i.r. spectrum of the product was identical with that of authentic colominic acid[2]. A 0.2% solution of the sample was hydrolyzed with 0.1N sulphuric acid at 80° for 90 min. In the hydrolyzate, 79% of sialic acid was found by assay with the resorcinol method[7], and 41% of sialic acid by assay with the thiobarbituric acid method[7].

Methyl ester of colominic acid (III)

Colominic acid (150 mg) was treated according to the procedure of Kantor

and Schubert[9] to give the methyl ester (142 mg). The product was insoluble in water and in organic solvents tested.

Anal. Calc. for $(C_{12}H_{19}NO_8 . 2H_2O)_n$: C, 42.23; H, 6.79; N, 4.10; CH_3O, 9.09. Found: C, 42.38; H, 6.70; N, 3.81; CH_3O, 8.8.

There was a 9.6% loss in weight when the product was dried *in vacuo* at 100° for 16 h over phosphorus pentoxide.

Carboxyl-reduced colominic acid (IV)

The reduction of the methyl ester (200 mg) of colominic acid was performed according to the procedure of Wolfrom and Juliano[9]. The i.r. spectrum of the product showed only a weak absorption at 1.740–1.750 cm^{-1}, in comparison with that of the methyl ester of colominic acid. The esterification and reduction processes were repeated, and the reaction product was passed through columns of Amberlite CG 120 (type I, H^+ form) and Dowex-1x8 (formate form). The effluent was lyophilized, and the product (133 mg) had $[\alpha]_D^{17}$ −58° (*c* 0.6, water). The i.r. spectrum showed no absorption in the 1.740–1.750 cm^{-1} region.

Anal. Calc. for $(C_{11}H_{19}NO_7 . H_2O)_n$: C, 44.78; H, 7.18; N, 4.75. Found: C, 44.40; H, 7.58; N, 4.38.

There was a 5.5% loss in weight when the product was dried *in vacuo* at 100° for 16 h over phosphorus pentoxide.

In hydrolyzates obtained with 0.1N sulphuric acid at 85–90° for 90 min, and with 2N hydrochloric acid at 95–100° for 10 h, no sialic acid was found by assay with thiobarbituric acid. The characteristic absorption of sialic acid at 580 mμ was not observed in the assay with resorcinol, and a new absorption was observed at 455 mμ.

Reducing power of colominic acid

The molecular weight of colominic acid was examined on the basis of the reducing power, which was determined according to the procedure described by Park and Johnson[10], and found to be, on average, 1/31 of that of *N*-acetylneuraminic acid.

Periodate oxidation of carboxyl-reduced colominic acid

Periodate oxidations were performed at 4° in the dark, according to the procedure of Guthrie[11]. The periodate consumption of carboxyl-reduced colominic acid was 0.38 mole per mole of monosaccharide residue during 72 h. The periodate consumption of colominic acid was 0.29 mole per mole of *N*-acetylneuraminic acid residue during 15 h, and the rate of consumption did not change during 72 h.

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The synthesis of *p*-nitrophenyl α-L-arabinofuranoside

Nitrophenyl glycosides are useful chromogenic substrates in the assay of glycosidase enzymes[1]. Following preliminary studies on an α-L-arabinofuranosidase[2], in which phenyl α-L-arabinofuranoside[3] was used as substrate, it seemed desirable to synthesise the corresponding *o*- or *p*-nitrophenyl glycoside.

Attempts to prepare these glycosides by condensation of the nitrophenol with tetra-*O*-acetyl-α-L-arabinofuranose using toluene-*p*-sulphonic acid as catalyst, by a method analogous to that used by Börjeson *et al.*[3] for the phenyl glycoside, were unsuccessful. However, Feier and Westphal[4] had synthesised *p*-nitrophenyl α-L-arabinopyranoside from the pyranosyl acetate using a mercuric cyanide catalyst, and this method was successfully adapted for the corresponding furanosyl acetate. Deacetylation and purification then followed the method of Börjeson *et al.*[3]. The new compound differs in physical properties from the *p*-nitrophenyl α- and β-L-arabinopyranosides prepared by Feier and Westphal[4], and consequently it is the expected furanoside. Comparison of its optical rotation ($[\alpha]_D$ −205°) with those of phenyl α-L-arabinofuranoside[3] ($[\alpha]_D$ −159°) and β-D-galactofuranoside[3] ($[\alpha]_D$ −148°)

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shows quite clearly that the compound is the α-L anomer. The infrared spectra of the *p*-nitrophenyl and phenyl α-L-arabinofuranosides resemble one another, and are readily distinguished from that of *p*-nitrophenyl α-L-arabinopyranoside.

Furthermore, the *p*-nitrophenyl α-L-arabinofuranoside was readily hydrolysed to L-arabinose and *p*-nitrophenol by an α-L-arabinofuranosidase[2], and thus serves as a basis for a colorimetric method of assay[5].

EXPERIMENTAL

Thin-layer chromatography (t.l.c.) was carried out at each stage, using silica gel G (Merck).

p-*Nitrophenyl* α-L-*arabinofuranoside*

L-Arabinose (50 g) was acetylated under conditions which give predominantly L-arabinofuranose tetra-acetate[3]. A mixture of the resulting syrup (35 g) with *p*-nitrophenol (50 g) was heated to 120° (oil bath) under reduced pressure in a rotary-evaporator flask. Finely powdered mercuric cyanide (18 g) was added, and the temperature slowly raised to 130° for 2 h, with constant rotation of the flask to ensure adequate mixing. The temperature was finally raised over a period of 30 min to 150°, and then the reaction mixture was allowed to cool while still under diminished pressure. Benzene–chloroform (1:1, v/v) (500 ml) was added, and the catalyst filtered off. The benzene–chloroform solution was washed with N sodium hydroxide (3×50 ml, to remove unreacted *p*-nitrophenol) and water, dried ($CaCl_2$), and concentrated under diminished pressure at 70° to give a pale-yellow syrup. T.l.c. (ethyl acetate–chloroform; 1:1, v/v) indicated the presence of a main component, assumed to be the acetylated glycofuranoside.

The syrup was dissolved in anhydrous methanol (500 ml), and sodium methoxide (from 0.1 g of sodium) was added. After 3 h at room temperature, the methanol was distilled off, and the residue dissolved in a minimal quantity of water. At this stage, there was a tendency for *p*-nitrophenyl α-L-arabinopyranoside, m.p. 196–197°, $[\alpha]_D^{20}$ −33° (*c* 0.5, methanol), to crystallise out preferentially, necessitating filtration. The resulting solution was passed through columns of Dowex-50 (H^+ form) and Dowex-3 (free base), washed with chloroform (3×25 ml), and then extracted with ethyl acetate (8×100 ml). The dried ($MgSO_4$) ethyl acetate solution was concentrated to a syrup which rapidly crystallised. T.l.c. (ethyl acetate) showed the product to be a mixture of the pyranoside and another compound, believed to be the corresponding furanoside.

The isomers were fractionated on a column (70×4 cm) of silica gel, using ethyl acetate, with t.l.c. (solvent system as before) as monitor. The required isomer was eluted first and, after evaporation of the appropriate fractions under diminished pressure at 40°, *p*-nitrophenyl α-L-arabinofuranoside (1.4 g) crystallised readily in the form of fine, white needles, m.p. 155–156°, $[\alpha]_D^{20}$ −205° (*c* 0.5, water) (Found: C, 48.8; H, 5.15; N, 5.2; $C_{11}H_{13}NO_7$ Calc.: C, 48.7; H, 4.8; N, 5.2%).

The infrared spectrum (NaCl disc. Nujol mull) was compared with that of phenyl α-L-arabinofuranoside, m.p. 48–49°, prepared according to Börjeson *et al.*[3], and with that of *p*-nitrophenyl α-L-arabinopyranoside; the two furanosides showed peaks at 980, 1035, and 3230 cm^{-1}, which were absent for the pyranoside.

ACKNOWLEDGEMENTS

We thank Mr. B. Otter for experimental assistance, Mr. M. J. West for the micro-analyses, Mr. D. R. Clifford for the infrared spectra, and Dr. R. J. W. Byrde for helpful discussion.

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(Received August 10th, 1965)

Isopropylidene derivatives of 2,4-*O*-methylene-D-glucitol

Although much is known[1] of the relative stabilities of the various types of cyclic acetal encountered in carbohydrate chemistry, comparable information on cyclic ketals is relatively sparse. In acidic hydrolysis, the sequence *α-threo* > α holds[2] for cyclic ketal stability, and recent examples[3] support the stability sequences α > β and α ≈ *α-erythro*. We now report an example of another stability sequence involving α- and β-ketals*.

Acetonation of 2,4-*O*-methylene-D-glucitol using copper sulphate–sulphuric acid or zinc chloride as catalyst gave, in good yield, a di-*O*-isopropylidene derivative for which a 1,3:5,6-distribution (I) of the ketal rings is the only reasonable structure. Analogies for the ring system of the *cis*-decalin type formed by the 1,3-ketal and 2,4-

*At the suggestion of Dr. L. Hough, we have used the terms *α-erythro* and *α-threo* to connote vicinal diols with *erythro-* and *threo*-configurations, instead of the equivalent symbols αC and αT of Barker and Bourne's terminology[2]. Likewise, α implies a terminal vicinal diol, and the terms β, *β-erythro*, *β-threo*, γ, *γ-erythro*, and *γ-threo* are equivalent to β, βC, βT, γ, γC, and γT, respectively.

The infrared spectrum (NaCl disc. Nujol mull) was compared with that of phenyl α-L-arabinofuranoside, m.p. 48–49°, prepared according to Börjeson *et al.*[3], and with that of *p*-nitrophenyl α-L-arabinopyranoside; the two furanosides showed peaks at 980, 1035, and 3230 cm^{-1}, which were absent for the pyranoside.

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Acetonation of 2,4-*O*-methylene-D-glucitol using copper sulphate–sulphuric acid or zinc chloride as catalyst gave, in good yield, a di-*O*-isopropylidene derivative for which a 1,3:5,6-distribution (I) of the ketal rings is the only reasonable structure. Analogies for the ring system of the *cis*-decalin type formed by the 1,3-ketal and 2,4-

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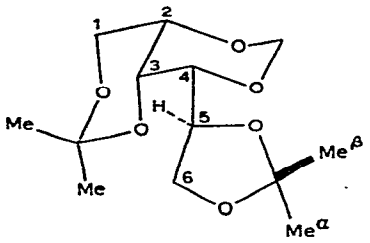
acetal rings in compound (I) are known[3,4]. The n.m.r. spectrum (methanol–water, 3:1) of compound (I) showed, *inter alia*, three Me proton signals at τ 8.70, 8.80,



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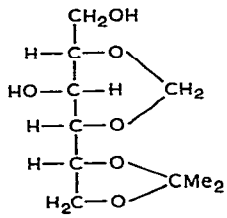

II

and 8.83 (strength ratio, *ca.* 1:2:1). On graded acidic hydrolysis, this pattern simplified to two signals at τ 8.80 and 8.83 (strength ratio, *ca.* 1:1) and, at this stage, the major product was a periodate-resistant mono-*O*-isopropylidene derivative, having Me proton signals (8.80 and 8.83) typical of an α-ketal[3] (*cf.* 1,2-*O*-isopropylideneglycerol, 8.79, 8.84). The structure 5,6-*O*-isopropylidene-2,4-*O*-methylene-D-glucitol (II) is assigned to this product.

By analogy with 1,2-*O*-isopropylideneglycerol and methyl 4,6-*O*-isopropylidene-2,3-di-*O*-methyl-α-D-galactopyranoside[3,4], which contains a ring system of the *cis*-decalin type and has isopropylidene Me proton signals at τ 8.71 and 8.80, the Me proton signal pattern of 1,3:5,6-di-*O*-isopropylidene-2,4-*O*-methylene-D-glucitol (I) may be analysed as follows: 8.70, axial Me group in the 1,3-ketal; 8.80, equatorial Me group in the 1,3-ketal and the Me$^{\beta}$ group in the 5,6-ketal; 8.83, Me$^{\alpha}$ in the 5,6-ketal (see ref. 3 for details of the Me$^{\alpha}$, Me$^{\beta}$ convention).

The above example supports the stability sequence $\alpha > \beta$, where the β-ketal is part of a ring system of the *cis*-decalin type. However, the example cited in the following Communication[5], shows that the sequence may be reversed where the β-ketal is part of a ring system of the *cis*-hydrindanone type.

EXPERIMENTAL

Thin-layer chromatography (t.l.c.) was performed on Kieselgel and detection was effected with vanillin–sulphuric acid[6] and/or iodine vapour. N.m.r. spectra were measured on a Varian A60 spectrometer using a 6% solution of tetramethylsilane in chloroform as external reference.

Acetonation of 2,4-O-methylene-D-glucitol

A mixture of the title compound[7] (5 g, m.p. 161–163°), acetone (150 ml), anhydrous copper sulphate (3 g), and conc. sulphuric acid (0.5 ml) was shaken overnight at room temperature and then poured, with vigorous stirring, into an excess of conc. ammonia. Insoluble material was removed, and the product was isolated from the filtrate in the usual manner[8]. Recrystallisation from ethanol gave 1,3:5,6-di-*O*-isopropylidene-2,4-*O*-methylene-D-glucitol (4.6 g, 65%), m.p. 113–114°,

$[\alpha]_D$ —24° (*c* 1.1, chloroform) (Found: C, 57.3; H, 8.2. $C_{13}H_{22}O_6$ calc.: C, 56.9; H, 8.1%).

The same product was obtained when zinc chloride was used as catalyst.

Graded acidic hydrolysis of 1,3:5,6-di-O-isopropylidene-2,4-O-methylene-D-glucitol

A solution of the title compound (2.01 g) in a methanol–water mixture (20 ml, 3:1, v/v) containing toluene-*p*-sulphonic acid (33.1 mg) was stored at room temperature. The pattern of the Me proton signals in the n.m.r. spectrum changed from a triplet at τ 8.70, 8.80, and 8.83, to a symmetrical doublet at τ 8.80 and 8.83, during 40 min. The mixture was then poured into an excess of saturated aqueous sodium hydrogen carbonate. The methanol was removed by distillation under diminished pressure, and the residual aqueous solution was extracted continuously overnight with chloroform. Evaporation of the dried extract gave a residue (924 mg) which contained three components with R_F values 0.58 (starting material), 0.29, and 0.22 (t.l.c., benzene–methanol, 9:1). Chromatography of the mixture (806 mg) on silica gel (75 g, Davison) gave, with benzene–ether (1:1), starting material (*ca.* 100 mg), and, subsequently with ether, 5,6-*O*-isopropylidene-2,4-*O*-methylene-D-glucitol (0.55 g), m.p. 138–139° (from ethyl acetate), $[\alpha]_D$ —9° (*c* 1.1, ethanol), R_F 0.29 (Found: C, 51.1; H, 7.6. $C_{10}H_{18}O_6$ calc.: C, 51.3; H, 7.8%). The product did not react with periodate under the usual conditions (aqueous solution containing an excess of sodium periodate).

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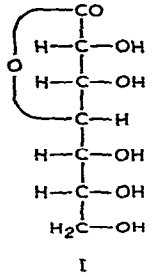
Preliminary communications

Reaction of D-*glycero*-D-*gulo*-heptonolactone with acetone

Whilst the preferential formation of five-membered cyclic ketals in the direct acid-catalysed acetonation of acyclic carbohydrates is well known[1], six-membered cyclic ketals may be formed from certain cyclic polyhydric alcohols[2]. We now report on the acetonation of D-*glycero*-D-*gulo*-heptono-γ-lactone (I).

The main product of acetonation catalysed by sulphuric acid was a di-*O*-isopropylidene derivative *A* [m.p. 153°, $[\alpha]_D$ −76° (*c* 2.0, chloroform), ν_{max} 1788 cm^{-1} characteristic of a γ-lactone[3]], together with a small amount of a mono-*O*-isopropylidene compound *B* [m.p. 167°, $[\alpha]_D$ −30° (*c* 2.0, ethanol), ν_{max} 1758 cm^{-1}]. Reduction of compound *A* with sodium borohydride or lithium aluminium hydride



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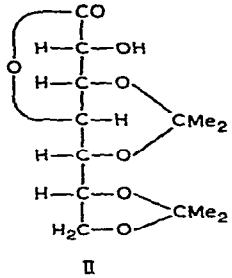

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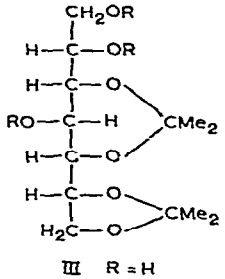
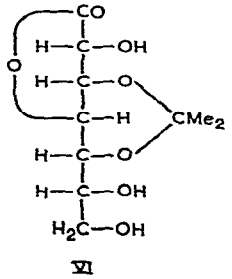


III R = H
IV R = Me



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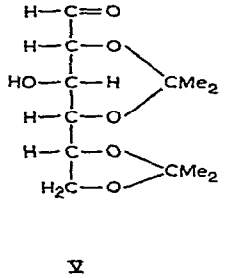

V

gave a di-*O*-isopropylideneheptitol *C* [monohydrate, m.p. 67–68°, $[\alpha]_D$ −6° (*c* 2.0, water)] which consumed 1 mol. of periodate to give a single product *D* [m.p. 88°, $[\alpha]_D$ −12° (*c* 1.0, chloroform)]. Acidic hydrolysis of compound *D* afforded D-glucose (characterised as the penta-acetate). These data established that compound *D* was 2,4:5,6-di-*O*-isopropylidene-D-glucose (V), compound *C* was 1,2:3,5-di-*O*-isopropylidene-L-*glycero*-L-*gulo*-heptitol (III), and compound *A* was 3,5:6,7-di-*O*-isopropyli-

dene-D-*glycero*-D-*gulo*-heptono-γ-lactone (II). The structure of compound *C* was further confirmed when methylation gave a product (IV) [b.p. 120°/0.1 mm, $[\alpha]_D$ −6° (*c* 3.0, chloroform)] which, after acidic hydrolysis, consumed 2 mol. of periodate.

Graded acidic hydrolysis (0.1N hydrochloric acid, 30–40°, 15 min) of compound *A* afforded a mono-*O*-isopropylidene derivative *E* [m.p. 158°, $[\alpha]_D$ −75° (*c* 1.0, ethanol), ν_{max} 1758, 1776 cm^{-1}]. Reduction of compound *E* with sodium borohydride gave a derivative *F* [m.p. 173°, $[\alpha]_D \pm 0°$ (*c* 2.0, methanol)] which consumed 1.87 mol. of periodate. Compounds *E* and *F* were therefore 3,5-*O*-isopropylidene-D-*glycero*-D-*gulo*-heptono-γ-lactone (VI) and 3,5-*O*-isopropylidene-*meso*-*glycero*-*gulo*-heptitol.

The behaviour of compound *A* (II) on graded acidic hydrolysis contrasts with that of 1,3:5,6-di-*O*-isopropylidene-2,4-*O*-methylene-D-glucitol[4] in that the α-ketal is more readily hydrolysed than is the β-*erythro*-ketal. It is also noteworthy that the lactone (I) forms a 3,5-*O*-isopropylidene derivative in preference to a 2,3-ketal. The origin of this apparently unusual selectivity is not clear and may involve steric and/or electronic effects. The problem is being studied further.

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The quantitative analysis of glycopeptides and glycoproteins by gas–liquid chromatography

Recently, we described[1] the application of gas–liquid chromatography (g.l.c.) to the identification of the monosaccharides in glycopeptides and glycoproteins. This method has now been extended to the quantitative analysis of biological materials. The reliability of this quantitative technique was established by the use of materials that had been examined by classical methods[2,4]. Immunoglobulins were supplied by Professor F. W. Putnam, and lacto-*N*-tetraose [β-D-Gal*p*-(1→3)-β-D-GNAc*p*-(1→3)-β-D-Gal*p*-(1→4)-D-G*p*] and lacto-*N*-fucopentaose I [α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→3)-β-D-GNAc*p*-(1→3)-β-D-Gal*p*-(1→4)-D-G*p*] by Professor R. Kuhn. A complete, quanti-

dene-D-*glycero*-D-*gulo*-heptono-γ-lactone (II). The structure of compound *C* was further confirmed when methylation gave a product (IV) [b.p. 120°/0.1 mm, $[\alpha]_D$ −6° (*c* 3.0, chloroform)] which, after acidic hydrolysis, consumed 2 mol. of periodate.

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tative analysis by classical methods involves a sequence of at least six operations, including identification of the monosaccharides present in the glycopeptide or glycoprotein, and the estimation of hexose, 6-deoxy-L-galactose, 2-amino-2-deoxy-D-glucose, *N*-acetylneuraminic acid, and the ratio of D-mannose to D-galactose, whereas the g.l.c. technique gives an analysis for all of these monosaccharides in a single operation.

The apparatus employed was the F and M Biomedical Gas Chromatograph Model 400 equipped with temperature programming, flame-ionization detector, and disc integrator. The preparations of the chromatography column and materials to be analysed were carried out as described[1] previously, apart from the following modifications. A volume containing an accurately known amount of biological material, corresponding to *ca.* 0.25 mg of total carbohydrate, was added to a known volume of D-glucose solution (0.05 mg) in a 10-ml round-bottomed flask. The mixture was concentrated to dryness, placed in a vacuum desiccator over phosphoric oxide for 12 h, and then converted into the methyl glycosides and silylated as before[1]. The trimethylsilyl ethers were injected onto the column, which was maintained at 155° until all of the hexoside peaks had emerged, programmed at 10°/min to 183° for the elution of acetamidohexoside, and finally programmed at 10°/min to 215° for the *N*-acetylneuraminic acid derivative. A series of standard curves was prepared in which the total peak areas for differing concentrations of each glycoside were related to those for the D-glucoside derivative (1.0), with the following results:

TABLE I

CARBOHYDRATE ANALYSES[a] OF GLYCOPROTEIN AND GLYCOPEPTIDE FRACTIONS BY GAS–LIQUID CHROMATOGRAPHY

Glycoprotein preparation, or glycopeptide fraction derived therefrom	*Carbohydrate content (g/100 g)*					
	6-Deoxy-L-galactose	*D-Mannose*	*D-Galactose*	*Total hexose*	*2-Acetamido-2-deoxy-D-glucose*	*N-Acetylneuraminic acid*
IgG globulin fraction	0.35 (0.40)	1.24	0.53	2.12 (1.97)	1.51 (1.57)	0.08
IgA globulin fraction	0.43 (0.47)	2.5	2.85	5.78	2.70 (2.65)	1.08 (1.00)
IgG glycopeptide fraction	7.6 (7.1)	23.6	11.7	42.9 (43.6)	25.9 (32.0)	3.8
IgA glycopeptide fraction	9.9	27.1	13.5	50.5 (49.2)	33.2 (34.8)	
IgA glycopeptide fraction	1.68 (1.60)	29.4 (28.5)	13.3 (12.8)	44.1 (42.8)	38.7 (39.0)	2.2 (3.0)
IgA glycopeptide fraction	3.45 (3.40)	15.6	17.2	36.25 (37.9)	25.1	18.2

[a] Each value is the mean of 3 estimations. The corresponding value obtained by classical methods is given in parentheses.

6-deoxy-L-galactose, 0.4; D-mannose, 1.0; D-galactose, 0.7; 2-acetamido-2-deoxy-D-glucose, 1.0; *N*-acetylneuraminic acid, 0.6.

For the quantitative analysis of glycoproteins and glycopeptides, D-glucose was added prior to methanolysis to act as an internal standard. The ratio of the total peak areas of each monosaccharide to those of D-glucose gave a direct estimate of the monosaccharide content in the original material. For a number of glycoproteins and glycopeptides having a wide range of carbohydrate compositions, the results of such analyses are given in Table I. Furthermore, the method may be used, without added D-glucose as internal standard, to determine the ratio of monosaccharides in biological materials. Thus, ovalbumin was found to contain D-mannose and 2-acetamido-2-deoxy-D-glucose in the ratio of 4.8:3.0 (5:3); lacto-*N*-tetraose had a D-galactose–D-glucose ratio of 1.94:1.00 (2:1), and lacto-*N*-fucopentaose I, a 6-deoxy-L-galactose–D-galactose–D-glucose ratio of 1.06:1.90:1.00 (1:2:1); the values given in parentheses were obtained by classical methods.

Apart from the simplicity of the operations involved, this method permits a complete, quantitative analysis to be performed on micro-amounts of biological materials, and therefore has considerable advantages over classical methods of carbohydrate analysis.

The Medical Research Council Award to C.H.B., and the Science Research Council Award to G.D. are gratefully acknowledged.

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4 J.R. CLAMP, G. DAWSON, AND F.W. PUTNAM, *Biochem. J.*, 95 (1965) 22P.

(Received August 27th, 1965)

Reactivity of acetylated glycosylsulfenyl bromides

A crystalline, unstable compound, formed by treatment of 2,3,4,6-tetra-*O*-acetyl-1-*S*-acetyl-1-thio-β-D-glucopyranose (I) in carbon tetrachloride solution with an excess of bromine, has been formulated as tetra-*O*-acetyl-β-D-glucopyranosylsulfenyl bromide[1] (II). It was anticipated[1] that the sulfur atom in II and its analogs would display electrophilic character, and should attack electron-rich centers. This report describes reactions of II with thiol, amine, and alkene functions, which verify this

6-deoxy-L-galactose, 0.4; D-mannose, 1.0; D-galactose, 0.7; 2-acetamido-2-deoxy-D-glucose, 1.0; *N*-acetylneuraminic acid, 0.6.

For the quantitative analysis of glycoproteins and glycopeptides, D-glucose was added prior to methanolysis to act as an internal standard. The ratio of the total peak areas of each monosaccharide to those of D-glucose gave a direct estimate of the monosaccharide content in the original material. For a number of glycoproteins and glycopeptides having a wide range of carbohydrate compositions, the results of such analyses are given in Table I. Furthermore, the method may be used, without added D-glucose as internal standard, to determine the ratio of monosaccharides in biological materials. Thus, ovalbumin was found to contain D-mannose and 2-acetamido-2-deoxy-D-glucose in the ratio of 4.8:3.0 (5:3); lacto-*N*-tetraose had a D-galactose–D-glucose ratio of 1.94:1.00 (2:1), and lacto-*N*-fucopentaose I, a 6-deoxy-L-galactose–D-galactose–D-glucose ratio of 1.06:1.90:1.00 (1:2:1); the values given in parentheses were obtained by classical methods.

Apart from the simplicity of the operations involved, this method permits a complete, quantitative analysis to be performed on micro-amounts of biological materials, and therefore has considerable advantages over classical methods of carbohydrate analysis.

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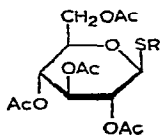
(Received August 27th, 1965)

Reactivity of acetylated glycosylsulfenyl bromides

A crystalline, unstable compound, formed by treatment of 2,3,4,6-tetra-*O*-acetyl-1-*S*-acetyl-1-thio-β-D-glucopyranose (I) in carbon tetrachloride solution with an excess of bromine, has been formulated as tetra-*O*-acetyl-β-D-glucopyranosylsulfenyl bromide[1] (II). It was anticipated[1] that the sulfur atom in II and its analogs would display electrophilic character, and should attack electron-rich centers. This report describes reactions of II with thiol, amine, and alkene functions, which verify this

prediction. It is shown that treatment of acetylated 1-thioaldose derivatives with bromine may lead to (acetylated) glycosylsulfenyl bromides, glycosyl bromides, or diglycosyl disulfides, according to the nature of the *S*-substituent, the solvent, and the duration of the reaction.

The following analogs* of I were prepared from the appropriate poly-*O*-acetylglycosyl bromide and a metal salt of the appropriate thiol: benzyl 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranoside[2] (III), yield 86%, τ 6.07 (benzylic CH_2)**; 2,3,4,6-tetra-*O*-acetyl-1-*S*-benzoyl-1-thio-β-D-glucopyranose[3] (IV), λ_{max}^{EtOH} 206 mμ (ε 13,500), 245 mμ (ε 12,500); 2,3,4,6-tetra-*O*-acetyl-1-*S*-acetyl-1-thio-β-D-galactopyranose[4] (X), yield 88%, m.p. 115–116° (from ethanol), $[\alpha]_D^{22}$ +31.5° (*c* 2, chloroform), λ_{max}^{EtOH} 226 mμ (ε 3500), τ 7.64 (SAc); 2,3,4-tri-*O*-acetyl-1-*S*-acetyl-1-thio-β-D-xylopyranose[4,5] (XI), λ_{max}^{EtOH} 225 mμ (ε 3,000), τ 7.65 (SAc); 2,3,4-tri-*O*-acetyl-1-*S*-acetyl-1-thio-D-ribose[4] (XII), m.p. 94–95° (from ethanol), $[\alpha]_D^{21}$ + 8.7° (chloroform), λ_{max}^{EtOH} 229 mμ (ε 4560). Progress of the reaction of these derivatives with bromine was followed by disappearance of the n.m.r. signal for the *S*-substituent.



I	R = Ac	IV	R = PhCO	VII	R = $PhCH_2S$
II	R = Br	V	R = CMe_3	VIII	R = *o*-chloroanilino
III	R = $PhCH_2$	VI	R = PhS	IX	R = 2-bromocyclohexyl

Conversion of I into II was complete in 1 min at 25° when a 3-molar excess of bromine was used; prolonged treatment with bromine, or the use of chloroform as the solvent, gave tetra-*O*-acetyl-α-D-glucopyranosyl bromide (XIII). Exposure to moisture during the preparation of II gave bis(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl) disulfide (XIV).

Addition of an equivalent of benzenethiol to a suspension of II in carbon tetrachloride at ~25° gave tetra-*O*-acetyl-β-D-glucopyranosyl phenyl disulfide (VI); yield 67%, m.p. 117–118° (from methanol), $[\alpha]_D^{22}$ −228° (*c* 1, chloroform), λ_{max}^{EtOH} 207 mμ (ε 10,400), 232 mμ (ε 10,300); τ ~2.5 (5 protons, Ph), ~7.95 (12 protons, OAc). Treatment of II with α-toluenethiol gave tetra-*O*-acetyl-β-D-glucopyranosyl benzyl disulfide (VII); yield 64%, m.p. 94–95° (from 2-propanol), $[\alpha]_D^{21}$ −200 ± 10° (chloroform); τ 2.60 (5 protons, Ph), 5.92 (2 protons, benzylic CH_2), 7.90, 7.95 (12 protons, OAc).

Addition of *o*-chloroaniline to a suspension of II in carbon tetrachloride gave 2-chloro-1-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosylsulfenamido)benzene (VIII), isolated pure by preparative t.l.c., yield 48%, m.p. 86–87° (from cyclohexane); λ_{max}^{EtOH} 210, 245, 287 mμ; τ 2.7 (4 protons, aryl), 4.12 (1-proton singlet, NH), 7.83, 7.98, 8.08 (12 protons, OAc). The τ 4.12 signal disappeared slowly when the sample was deuterated.

*All stable, crystalline compounds mentioned in this paper were homogeneous by thin-layer chromatography, and gave satisfactory elemental analyses.

**N.m.r. data refer to deuteriochloroform solutions.

A product formed by treatment of II with cyclohexene was purified by preparative t.l.c. to give 2-bromo-1-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosylthio)cyclohexane (IX), yield 49%, m.p. 83–84° (from ether–petroleum ether); τ 7.90, 7.96, 8.02 (12 protons, OAc), 8.43 (8 protons, CH_2 of cyclohexane moiety).

Bromination of 2,3,4-tri-*O*-acetyl-1-*S*-acetyl-1-thio-β-D-xylose (XI) in carbon tetrachloride gave the very unstable tri-*O*-acetyl-β-D-xylopyranosylsulfenyl bromide, m.p. 41–43°, which, with benzenethiol, gave tri-*O*-acetyl-β-D-xylopyranosyl phenyl disulfide, m.p. 122°, $[\alpha]_D^{21}$ −250° (chloroform), τ ~2.5 (5 protons, Ph), ~7.95 (9 protons, OAc). The other acetylated 1-thioaldoses (X and XII) reacted rapidly with bromine in carbon tetrachloride to give sirups which gave stable adducts with benzenethiol (t.l.c.); prolonged treatment with bromine, or reaction in chloroform solution, gave the poly-*O*-acetylaldopyranosyl bromides, revealed by the appearance of a characteristic, low-field doublet[3] (H-1) in the n.m.r. spectrum.

The *S*-benzoyl derivative (IV) was unreactive toward bromine in carbon tetrachloride during 24 h, whereas the *tert*-butyl thioglycoside[7] (V) gave the disulfide[4] (XIV) in 87% yield after 15 min. The benzyl thioglycoside (III) in chloroform solution was converted by bromine into XIII, but, in carbon tetrachloride solution, there were observed additional products, probably II and XIV.

ACKNOWLEDGMENTS

The authors thank C.V. Holland for the preparation of I, X, XI, and XII. N.m.r. measurements were made by W. N. Turner and J. B. Hughes. This work was supported, in part, by the Agricultural Research Service, U.S. Department of Agriculture, Grant No. 12-14-100-7208(71) (O.S.U.R.F. Project 1827) administered by the Northern Utilization Research and Development Division, Peoria, Illinois. The n.m.r. spectrometer was provided through a grant from the National Science Foundation.

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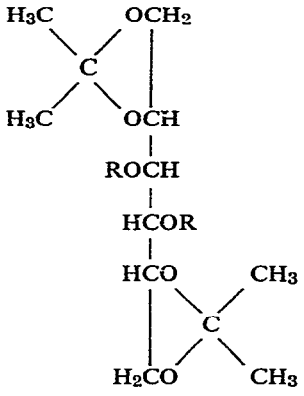
Action of zinc dust and sodium iodide in *N,N*-dimethylformamide on contiguous, secondary sulfonyloxy groups: a simple method for introducing nonterminal unsaturation

Terminal unsaturation is readily introduced[1,2] into an alditol (or an acyclic polyhydroxyalkyl appendage of a cyclic sugar) by the action of sodium iodide (in a suitable solvent) on a derivative having a primary and a secondary sulfonyloxy group contiguous. For acyclic sugar derivatives, there is only one recorded example of a similar reaction involving two contiguous, secondary sulfonyloxy groups. Methyl 4-deoxy-2,3-di-*O*-(methylsulfonyl)-DL-erythronate and -threonate react with sodium iodide[3] to give the corresponding 2,3-unsaturated esters, but the carboxylic ester group has an activating effect[2]. "Unsuccessful attempts were made to prepare an unsaturated compound" from[4] the 3,4-dimethanesulfonate (IIa) and 3,4-di-*p*-toluenesulfonate (IIb) of 1,2:5,6-di-*O*-isopropylidene-D-mannitol (I).

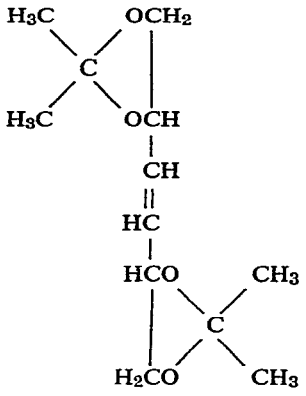
We therefore decided to re-investigate this reaction, avoiding, for convenience, the use of sealed tubes or of an autoclave. We found that IIa is unaffected by treatment with a 10% solution of sodium iodide in acetone during 11 days at room temperature or 8 h at the boiling point. However, reaction occurred when various high-boiling solvents (*e.g.*, 2,5-hexanedione) were used, instead of acetone, at the respective boiling point; free iodine and the sodium methanesulfonate-iodide salt[3,5] were formed, but iodination took place, making isolation of III difficult. We therefore sought (*a*) a solvent which was capable of dissolving sufficient sodium iodide and II (a or b) to be practical and which had a boiling point sufficiently high for the reaction to occur at a reasonable speed, and (*b*) a substance which, when present throughout the reaction, would combine with the iodine as fast as it was formed (thereby precluding iodination and promoting the reaction) and which would prevent the development of acidity.

The reagent sodium iodide–*N,N*-dimethylformamide–zinc dust provides a boiling point high enough for a reasonable rate of reaction and a rapid means for removing iodine. Treatment of IIa or IIb with an excess of sodium iodide (20% solution in dry *N,N*-dimethylformamide), in the presence of an excess of zinc dust, with vigorous boiling and stirring under reflux (drying tube) during 5 h, followed by cooling, filtration, extraction (heptane), evaporation of the extract, retreatment of the residue with heptane, and evaporation, gave crystalline 1,2:5,6-di-*O*-isopropylidene-*trans*-3-hexene-D-*threo*-1,2,5,6-tetrol (III) (64%); it was sublimed at 45°/0.02 mm, and had m.p. 80–82° (from aqueous ethanol); $[\alpha]_D^{24}$ +57.5° (*c* 1.02, chloroform); $J_{3,4}$ 15.5 c.p.s.; it readily decolorized aqueous permanganate solution.

These properties agree with those recorded[6,7] for III prepared by other methods. Compound III showed ν_{max} 1307 and 971 cm^{-1} (*trans* olefinic; not shown[8] by I) and 1156, 1134, and 1053 cm^{-1} (1,3-dioxolane rings; I shows[8] bands at 1160, 1126, and 1044 cm^{-1}).



I (R = H)
IIa (R = Ms)
IIb (R = Ts)



III

IIa + 2.5 NaI→
III + 2 NaOMs · 0.25 NaI + I_2
IIb + 2 NaI→III + 2 NaOTs + I_2

Hydrolysis of III with 80% acetic acid (5 h at 25°), and evaporation to dryness, gave *trans*-3-hexene-D-*threo*-1,2,5,6-tetrol (IV); m.p. 64–65° (from ethanol–ethyl acetate); $[\alpha]_D^{25}$ −13.8° (*c* 2.00, water); $J_{3,4}$ 15.5 c.p.s.; ν_{max} 1653, 1325, and 976 cm^{-1}. [Anet[7] gave * m.p. 114°; $[\alpha]_D$ −2° (water); $J_{3,4}$ 15.5 c.p.s.] Condensation of IV with acetone (anhydrous $CuSO_4$) regenerated III.

The above procedure for introduc ng nonterminal unsaturation should be applicable, not only to alditol derivatives, but also to sulfonic esters of suitable derivatives of certain mono-[9], oligo-, and poly-saccharides, and cyclitols[10]. Moreover, it should constitute an improvement in the production of terminal unsaturation in alditol and other sugar derivatives by use of the appropriate sulfonic esters.

The authors thank Drs. E. D. Becker, L. D. Hall, and R. Schaffer for assistance in recording and interpreting the n.m.r. spectra.

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* *Note added in proof.* For IV, Dr Anet now finds m.p. 64–65°, $[\alpha]_D^{25}$ — 14.6° (*c* 0.5, water), and i.r. spectrum identical with ours (personal communication, Sept. 24th, 1965). Dr Haines has now prepared IV, and finds m.p. 60–62°, $[\alpha]_D^{24}$ — 15.3° (*c* 4.3, water), $J_{3,4}$ 15.9 c.p.s., and i.r. spectrum identical with ours (personal communication, Sept. 28th, 1965).

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Announcement

GORDON RESEARCH CONFERENCE ON CARBOHYDRATES, July 11 — 15th, 1966
Tilton School, Tilton, New Hampshire, U.S.A.

Topics

1 Branched-chain and unsaturated carbohydrates
2 Schardinger dextrins
3 Quantitative conformational analysis
4 Molecular weight of carbohydrates, particularly polyelectrolytes.

Details available from Professor R. MONTGOMERY, Department of Biochemistry, State University of Iowa, Iowa City, Iowa, U.S.A.

BENZOATE DISPLACEMENTS ON SULPHONATE ESTERS OF SOME 1,2-*O*-ISOPROPYLIDENEPENTOFURANOSES

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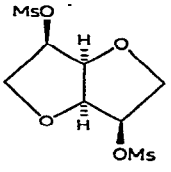
(Received July 6th, 1965)

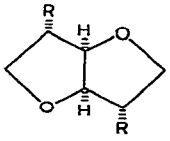
INTRODUCTION

While the nucleophilic displacement of *endo*-sulphonate groups in a system composed of two *cis*-fused five-membered rings is fairly readily achieved, *exo*-sulphonate groups are displaced only with difficulty[1,2]. Thus, 1,4:3,6-dianhydro-2,5-di-*O*-methanesulphonyl-D-mannitol (I) is readily converted into derivatives of L-iditol (II, III, and IV) by acetate ions[2], benzoate ions[3], and ammonia[2], respectively, though the corresponding iditol sulphonate (V) is inert to acetate ions, and gives 1,4:3,6-dianhydro-2,5-imino-D-mannitol only under forcing conditions with ammonia[2]. Similarly, 1,2:5,6-di-*O*-isopropylidene-3-*O*-toluene-*p*-sulphonyl-D-glucofuranose fails to undergo reaction with benzoate or azide ions but, with hydrazine, can be induced to give 3-deoxy-3-hydrazino-1,2:5,6-di-*O*-isopropylidene-D-allofuranose[4].

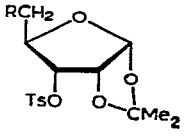
In this work, the reactions of the three 1,2-*O*-isopropylidene-3,5-di-*O*-toluene-*p*-sulphonylpentofuranoses (VI, VII, and VIII), derived from D-ribose, L-arabinose, and D-xylose, respectively, and 5-deoxy-1,2-*O*-isopropylidene-3-*O*-toluene-*p*-sulphonyl-D-ribofuranose (IX) with benzoate ions have been studied to see if neighbouring-group participation facilitates displacement of suitably placed *exo*- and *endo*-sulphonate groups.



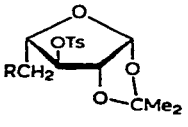
(I)



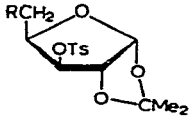
(II) R=OAc (IV) R=NH_2
(III) R=OBz (V) R=OMs



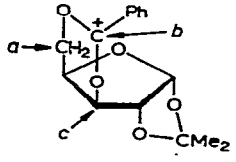
(VI) R=OTs
(IX) R=H
(X) R=OBz



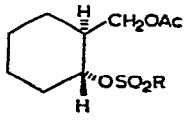
(XVII) R=OTs
(XVIII) R=OBz



(XVIII) R=OTs
(XX) R=OBz



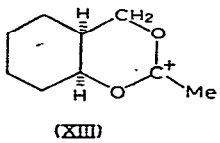
(XII)



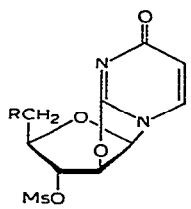
(XIV)

DISCUSSION

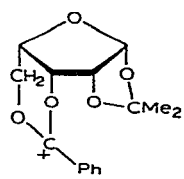
The ditoluene-*p*-sulphonate (VI) was first obtained from the toluene-*p*-sulphonylation of an impure sample of 2,3-*O*-isopropylidene-D-ribofuranose and the wrong structure was assigned to it at the time[5]. Mills[1] deduced the correct structure, and the preparation described herein, from known 1,2-*O*-isopropylidene-D-ribofuranose[6,7], confirms this deduction. Lithium aluminium hydride reduction of the disulphonate (VI) readily gave the 5-deoxy compound (IX). Both the arabinose and xylose compounds (VII and VIII) have been described previously[8,9]. In the present work, the preparation of the arabinose derivative (VII) was shortened by converting 5-*O*-toluene-*p*-sulphonyl-L-arabinose diethyl dithioacetal directly into 1,2-*O*-isopropylidene-5-*O*-toluene-*p*-sulphonyl-L-arabinofuranose, instead of via 5-*O*-toluene-*p*-sulphonyl-L-arabinose[10]. A similar method has already been used for the preparation of 5-*O*-benzoyl-1,2-*O*-isopropylidene-D-arabinofuranose[11].



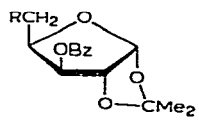
(XIII)



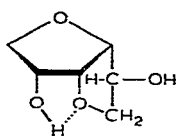
(XV) R=OBz
(XVI) R=H



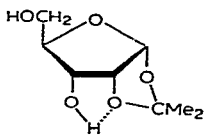
(XIX)



(XI) R=OBz
(XVII) R=H



(XXI)



(XXII)

When the ribose ditoluene-*p*-sulphonate (VI) was heated in *N*-methyl-2-pyrrolidone containing tetra-*n*-butylammonium benzoate[12], the primary sulphonate group was rapidly displaced to give the 5-benzoate (X). The *endo*-sulphonate group of compound (X) then underwent much slower displacement to give 3,5-di-*O*-benzoyl-1,2-*O*-isopropylidene-D-xylofuranose (XI), the structure of which was proved by debenzoylation and monotoluene-*p*-sulphonylation to give the known 1,2-*O*-isopropylidene-5-*O*-toluene-*p*-sulphonyl-D-xylofuranose. Comparison of experimental conditions suggests that displacement of the *endo*-sulphonate group of compound (X) occurs less readily than the corresponding reaction of the *endo*-sulphonate groups of the mannitol derivative (I). Steric hindrance from the *trans*-C-5 group of compound (X) probably accounts for this difference. The slower reaction

also suggests the absence of neighbouring-group participation from the 5-benzoate group assisting the displacement of the *endo*-sulphonate group of compound (X) via an intermediate (XII). A similar intermediate (XIII) has been reported in the acetolysis of *trans*-2-acetoxymethylcyclohexyl sulphonates (XIV) to give a mixture of *cis*- and *trans*-2-acetoxymethylcyclohexyl acetate, by attack of the intermediate (XIII) at the primary and secondary positions, respectively[13]. An intermediate (XII) would be likely to undergo nucleophilic attack at the primary C-5 position (route *a*) or at the carbonium ion centre (route *b*), resulting in a product with the *xylo*-configuration, rather than attack at the secondary C-3 position (route *c*) to result in retention of configuration. The latter possibility was considered in order to account for the benzoate displacement of the methanesulphonyl group in the anhydro nucleoside (XV) with retention of configuration. This possibility was discounted when the 5-deoxy derivative (XVI) also underwent reaction with retention of configuration[14]. Later, it was shown that the pyrimidine residue in these compounds played a participating role in the displacements[15]. In the present work, 5-deoxy-1,2-*O*-isopropylidene-3-*O*-toluene-*p*-sulphonyl-D-ribofuranose (IX) also underwent displacement, to give 3-*O*-benzoyl-5-deoxy-1,2-*O*-isopropylidene-D-xylofuranose (XVII), at a rate similar to that of the compound (X). From these observations, it is concluded that neighbouring-group participation does not assist these displacements.

As expected, the primary sulphonate group of the arabinose ditoluene-*p*-sulphonate (VII) was rapidly replaced. However, the resulting 5-*O*-benzoyl-1,2-*O*-isopropylidene-3-*O*-toluene-*p*-sulphonyl-L-arabinofuranose (XVIII) was resistant to further reaction. Not only is the secondary sulphonate group in the unfavourable *exo* position, but the *trans*-C-5 group also adds to the steric difficulty of a displacement reaction. As in the ribose case, neighbouring-group assistance via an intermediate (XIX) is possible, but evidently does not occur.

The xylose ditoluene-*p*-sulphonate (VIII) smoothly gave the 5-benzoate (XX), and again displacement of the *exo*-sulphonate group did not occur. No neighbouring group assistance is possible in this case. Recently, it was shown that both sulphonate groups of methyl 2-*O*-benzoyl-3,5-di-*O*-toluene-*p*-sulphonyl-$\alpha\beta$-L-xylofuranoside can be readily displaced by benzoate ions[16]. The isolation of a hydroxydibenzoate indicated that the displacement of the 3-sulphonate group had occurred with neighbouring-group assistance from the adjacent 2-benzoate group.

The two new monobenzoates (X) and (XVIII), isolated from the displacement reactions, were synthesised independently to confirm their structures. 5-*O*-Benzoyl-1,2-*O*-isopropylidene-L-arabinofuranose was synthesised by the method described for the D-form[11]; toluene-*p*-sulphonylation then gave the required ester (XVIII). Selective esterification of 1,2-*O*-isopropylidene-D-xylofuranose at the 5-position is readily achieved[17]. However, careful treatment of 1,2-*O*-isopropylidene-D-ribofuranose with one equivalent of benzoyl chloride gave a mixture of products. Examination by thin-layer chromatography suggested the presence of a dibenzoate and two monobenzoates, in addition to unchanged starting material. The monobenzoate

formed in greater yield was isolated by chromatography, and without further purification was toluene-*p*-sulphonylated to give the ester (X).

These results of partial benzoylation of 1,2-*O*-isopropylidene-D-xylofuranose and 1,2-*O*-isopropylidene-D-ribofuranose suggest that the difference in ease of esterification of the primary and secondary hydroxyl groups is greater for the xylose compound, with its *exo*-3-hydroxyl group, than for the ribose compound, with its *endo*-3-hydroxyl group. Esterification of 1,4:3,6-dianhydro-D-glucitol (XXI) with acid chlorides occurs preferentially at the (more hindered) *endo*-5-hydroxyl group[18,19]. The enhancement of the reactivity of this hydroxyl group has been ascribed to the intramolecular hydrogen bond which it can form with the oxygen atom at the 4-position[19]. It is noteworthy that the *endo*-3-hydroxyl group of 1,2-*O*-isopropylidene-D-ribofuranose (XXII) can form a similar intramolecular hydrogen bond to the oxygen atom at the 2-position.

The reactions of these sulphonate esters with amines are now being studied.

EXPERIMENTAL

Silica gel was used for column chromatography (Hopkin and Williams, M.F.C. grade) and for thin-layer chromatography (Merck, G. grade).

1,2-O-Isopropylidene-3,5-di-O-toluene-p-sulphonyl-D-ribofuranose (VI)

1,2-*O*-Isopropylidene-D-ribofuranose (33 mg) was esterified with toluene-*p*-sulphonyl chloride (132 mg) in pyridine (2 ml). Isolation with chloroform in the usual way gave a syrup which from aqueous methanol gave 1,2-*O*-isopropylidene-3,5-di-*O*-toluene-*p*-sulphonyl-D-ribofuranose (65 mg), m.p. 123°, $[\alpha]_D + 71°$ (*c* 1.1, chloroform) (lit.[5], m.p. 122–3°) (Found: S, 12.6. $C_{22}H_{26}O_9S_2$ calc.: S, 12.8%).

5-Deoxy-1,2-O-isopropylidene-3-O-toluene-p-sulphonyl-D-ribofuranose (IX)

A solution of the above disulphonate (1.0 g) in dry ether (20 ml) containing lithium aluminium hydride (0.15 g) was stirred and heated under reflux for 5 h. Excess of hydride was destroyed by ethyl acetate, and the solution was poured into a saturated solution of potassium sodium tartrate. This was extracted with chloroform (50 ml), and the extract was washed with N sulphuric acid and aqueous sodium hydrogen carbonate. Evaporation of the dried chloroform solution yielded crystalline 5-deoxy-1,2-*O*-isopropylidene-3-*O*-toluene-*p*-sulphonyl-D-ribofuranose (0.59 g) which, after recrystallisation from aqueous methanol, had m.p. 127–128°, $[\alpha]_D + 100°$ (*c* 0.85, chloroform). The i.r. spectrum showed no bands in the region of 3000 cm^{-1} indicative of hydroxyl groups. (Found: C, 54.4; H, 6.15; S, 9.8. $C_{15}H_{20}O_6S$ calc.: C, 54.9; H, 6.1; S, 9.8%).

1,2-O-Isopropylidene-5-O-toluene-p-sulphonyl-L-arabinofuranose

Mercuric chloride (3 g) was added to a solution of 5-*O*-toluene-*p*-sulphonyl-L-arabinose diethyl dithioacetal (1.57 g) in acetone (25 ml). The mixture was stirred

at 50–55° for 100 min, neutralised with anhydrous sodium carbonate, and filtered. Evaporation of the solution gave a residue which was extracted with chloroform. The extract was washed with aqueous potassium iodide, and then with water until free of halide ions. Evaporation of the dried extract gave a syrup (1.3 g) which was chromatographed on silica gel (30 g). Elution with benzene–ether (85:15) gave acetone-condensation products (0.41 g), and benzene–ether (1:1) yielded the product (0.66 g). After recrystallisation from ethyl acetate–light petroleum, the 1,2-*O*-isopropylidene-5-*O*-toluene-*p*-sulphonyl-L-arabinofuranose (0.61 g) had m.p. 129°, $[\alpha]_D$ −33.4° (*c* 1.0, chloroform) (lit.[10], m.p. 129–130°, $[\alpha]_D$ −34.8°).

1,2-O-Isopropylidene-3,5-di-O-toluene-p-sulphonyl-L-arabinofuranose (VII)

This compound was prepared from the above monosulphonate, as described by Mitra and Karrer[8], and had m.p. 96°, $[\alpha]_D$ −34.7° (*c* 2.0, chloroform) (lit.[8], m.p. 93°).

1,2-O-Isopropylidene-3,5-di-O-toluene-p-sulphonyl-D-xylofuranose (VIII)

This compound was prepared according to Karrer and Boettcher[9], and had m.p. 89°, $[\alpha]_D$ −36° (*c* 1.0, chloroform) (lit.[9], m.p. 91–92°, $[\alpha]_D$ −37.91°).

Displacement Reactions

(*a*) *On 1,2-O-isopropylidene-3,5-di-O-toluene-p-sulphonyl-D-ribofuranose (VI).* A solution of the above disulphonate (0.59 g) in *N*-methyl-2-pyrrolidone (5 ml) containing tetra-*n*-butylammonium benzoate (1.6 g) was kept at 100°. T.l.c. showed the rapid formation of an initial product, which then slowly diminished in amount as a second product appeared. After 7 days, some of the initial product still remained. The reaction mixture was passed through a column of silica gel (150 g) and eluted with ether (500 ml). The eluate was concentrated to 5 ml, passed through alumina (20 g), and eluted with more ether (60 ml). The residue from evaporation of the ether was finally chromatographed on silica gel (20 g), when the two fractions were obtained.

Fraction *A*, eluted with benzene–ether (96:4), yielded syrupy 3,5-di-*O*-benzoyl-1,2-*O*-isopropylidene-D-xylofuranose (201 mg), $[\alpha]_D$ −50° (*c* 0.7, chloroform) (Found: C, 66.9; H, 5.8; $C_{22}H_{22}O_7$ calc.: C, 66.3; H, 5.5%). A sample (100 mg) was debenzoylated with sodium methoxide in methanol. The resultant 1,2-*O*-isopropylidene-D-xylofuranose (37 mg) was treated with one equivalent of toluene-*p*-sulphonyl chloride (38 mg) in pyridine (0.5 ml) to give 1,2-*O*-isopropylidene-5-*O*-toluene-*p*-sulphonyl-D-xylofuranose (30 mg), which had m.p. 138° (undepressed on admixture with an authentic sample).

Fraction *B*, eluted with benzene–ether (3:1), gave 5-*O*-benzoyl-1,2-*O*-isopropylidene-3-*O*-toluene-*p*-sulphonyl-D-ribofuranose (90 mg) which, after recrystallisation from aqueous ethanol, had m.p. 119°, $[\alpha]_D$ + 96° (*c* 0.5, chloroform) (Found: C, 59.0; H, 5.5; S, 7.1. $C_{22}H_{24}O_8S$ calc.: C, 58.9; H, 5.4; S, 7.15%).

(*b*) *On 5-deoxy-1,2-O-isopropylidene-3-O-toluene-p-sulphonyl-D-ribofuranose (IX).* A solution of the above sulphonate (0.25 g) in *N*-methyl-2-pyrrolidone (3 ml)

containing tetra-*n*-butylammonium benzoate (1.03 g) was held at 104° for 90 h. T.l.c. indicated that some starting material remained. More benzoate (1.0 g) was added, and the solution was held at 125° for a further 160 h. Purification, as before, gave a crude product which was chromatographed on silica gel (20 g). Benzene–ether (95:5) eluted some non-carbohydrate material (0.11 g), followed by the product, 3-*O*-benzoyl-5-deoxy-1,2-*O*-isopropylidene-D-xylofuranose, as a gum (0.16 g), $[\alpha]_D$+6.1° (*c* 0.9, chloroform). (Found: C, 65.2; H, 6.6. $C_{15}H_{18}O_5$ calc.: C, 64.8; H, 6.5%). Debenzoylation of a sample with a catalytic amount of sodium methoxide in methanol, followed by silica-gel chromatography to remove methyl benzoate, gave 5-deoxy-1,2-*O*-isopropylidene-D-xylofuranose, m.p. 68°, $[\alpha]_D$—21° (*c* 0.8, water) (lit.[9], m.p. 68–70°, $[\alpha]_D$—20.4°).

(*c*) *On 1,2-O-isopropylidene-3,5-di-O-toluene-p-sulphonyl-L-arabinofuranose (VII).* In a preliminary experiment, the above sulphonate (10 mg) in *N*-methyl-2-pyrrolidone (0.3 ml) containing tetra-*n*-butylammonium benzoate (35 mg) was kept at 95° for 72 h. T.l.c. of the reaction mixture at intervals indicated the rapid formation of a product still containing a sulphonate group. No further reaction occurred.

A solution of the disulphonate (0.25 g) and tetra-*n*-butylammonium benzoate (0.68 g) in *N*-methyl-2-pyrrolidone (2 ml) was kept at 105° for 20 h. Purification, as before, gave only one product, 5-*O*-benzoyl-1,2-*O*-isopropylidene-3-*O*-toluene-*p*-sulphonyl-L-arabinofuranose (0.20 g), which had m.p. 83°, $[\alpha]_D$—6.0° (*c* 1.2, chloroform), after recrystallisation from aqueous methanol. (Found: C, 58.9; H, 5.7; S, 7.1. $C_{22}H_{24}O_8S$ calc.: C, 58.9; H, 5.4; S, 7.15%).

(*d*) *On 1,2-O-isopropylidene-3,5-di-O-toluene-p-sulphonyl-D-xylofuranose (VIII).* A solution of the above sulphonate (100 mg) and tetra-*n*-butylammonium benzoate (300 mg) in *N*-methyl-2-pyrrolidone (1 ml) was kept at 100°. T.l.c. indicated the rapid formation of a product still containing a sulphonate residue; no further change was observed in 72 h. Purification, as before, and crystallisation from aqueous methanol gave 5-*O*-benzoyl-1,2-*O*-isopropylidene-3-*O*-toluene-*p*-sulphonyl-D-xylofuranose (53 mg), m.p. 94–95° (undepressed on admixture with an authentic sample).

5-O-Benzoyl-1,2-O-isopropylidene-3-O-toluene-p-sulphonyl-D-ribofuranose (X)

Benzoyl chloride (0.054 ml) was added to a solution of 1,2-*O*-isopropylidene-D-ribofuranose (90 mg) in pyridine (1 ml) at 0°. After a further 16 h at 4°, the products were isolated in the usual way. T.l.c. revealed the presence of three products, in addition to unchanged starting material. The fastest-running product was assumed to be dibenzoate, and the two slower-running products to be monobenzoates. By means of preparative t.l.c., the two monobenzoates were separated. The one formed in greater amount (30 mg) was treated with toluene-*p*-sulphonyl chloride (75 mg) in pyridine (1 ml) for 1 day at 25°. Isolation by means of chloroform, and crystallisation from aqueous methanol gave 5-*O*-benzoyl-1,2-*O*-isopropylidene-3-*O*-toluene-*p*-sulphonyl-D-ribofuranose (31 mg), m.p. 119° (undepressed on admixture with the sample obtained from the displacement reaction).

5-O-Benzoyl-1,2-O-isopropylidene-L-arabinofuranose

This compound was synthesised by the method described for the D-form. 5-*O*-Benzoyl-1,2-*O*-isopropylidene-L-arabinofuranose had m.p. 142°, $[\alpha]_D$ −24.4° (*c* 0.8, chloroform) (Found: C, 61.4; H, 6.3%. $C_{15}H_{18}O_6$ calc.: C, 61.2; H, 6.1%). Reist *et al.*[11] give m.p. 148–9°, $[\alpha]_D$ +24° (*c* 1.0, chloroform) for the D-form.

5-O-Benzoyl-1,2-O-isopropylidene-3-O-toluene-p-sulphonyl-L-arabinofuranose (XVIII)

Toluene-*p*-sulphonylation of the above benzoate (18 mg) in pyridine (0.5 ml) with toluene-*p*-sulphonyl chloride (35 mg) for 72 h at 20°, and purification in the usual way gave, after recrystallisation from aqueous methanol, 5-*O*-benzoyl-1,2-*O*-isopropylidene-3-*O*-toluene-*p*-sulphonyl-L-arabinofuranose (15 mg), m.p. 82° (undepressed on admixture with the sample isolated from the displacement reaction).

ACKNOWLEDGEMENTS

We thank Dr. J.G. Buchanan for a sample of 5-*O*-benzoyl-1,2-*O*-isopropylidene-3-*O*-toluene-*p*-sulphonyl-D-xylofuranose, and the D.S.I.R. for a Maintenance Grant (to P.R.H.S.).

SUMMARY

The benzoate displacement of sulphonate derivatives of several 1,2-*O*-isopropylidenepentofuranoses has been studied. Displacement occurs readily with 5-sulphonates, more slowly with *endo*-3-sulphonates, and not at all with *exo*-3-sulphonates. Neighbouring-group participation from 5-benzoate groups does not assist in the displacement of the 3-sulphonates.

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DEGRADATION OF CARBOHYDRATES

PART VII. CONFORMATIONS OF SOME UNSATURATED SUGARS*

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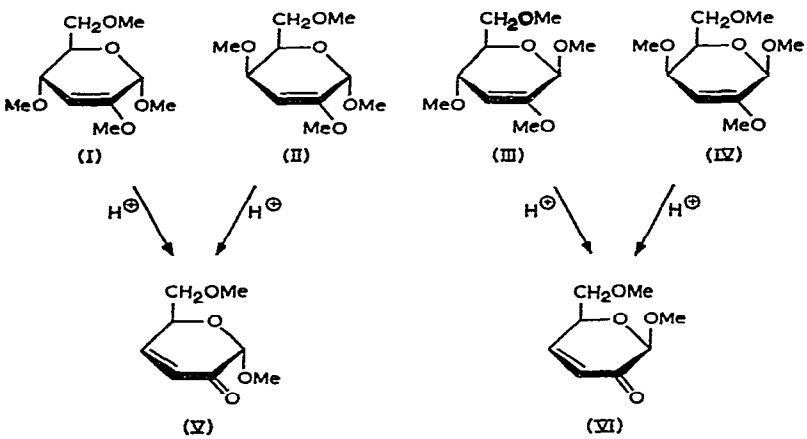
The assignments of the anomeric configurations of hex-2-enopyranoses and hex-3-enopyranosuloses require confirmation, because, in the former series of compounds, the crystalline substances obtained all appeared to be α-D anomers, and, in the latter series, only α,β-mixtures were isolated [1–3].

The fully methylated methyl glycosides of the above compounds should be sufficiently volatile for purification by gas–liquid chromatography, and, if both sets of anomers were thus isolated, their proton magnetic resonance spectra, optical rotations, and other properties could be examined. Furthermore, the conclusions so obtained should stand the test that the hex-3-enosiduloses (V) and (VI) must have the same anomeric configurations as the hex-2-enosides, (I)–(IV), from which they



were derived. These results could be used to confirm the structures and conformations proposed earlier for the free sugars[3].

This paper reports the isolation, anomeric configuration, and conformation of the six unsaturated D-glycosides (I)–(VI).

*For Part VI, see reference 3.

RESULTS AND DISCUSSION

The methyl hexenopyranosides were prepared by treating 3-deoxy-2,4,6-tri-*O*-methylhexenopyranoses with methyl sulfate and alkali. Since the free sugars are relatively stable to alkali, the methylations proceeded smoothly, yielding mixtures of the anomers, which were separated by gas–liquid chromatography. The α-D-*threo* isomer (II) was obtained in the crystalline condition. Each of the four compounds (I)–(IV) was treated with acid to yield a methyl hexenopyranosidulose having the same anomeric configuration as the starting substance (see Table I).

TABLE I

RETENTION VOLUMES AND OPTICAL ROTATIONS OF METHYL 3-DEOXY-2,4,6-TRI-*O*-METHYL-D-HEX-2-ENOPYRANOSIDES (I)–(IV) AND METHYL 3,4-DIDEOXY-6-*O*-METHYL-D-HEX-3-ENOPYRANOSIDULOSES (V) AND (VI)

Glycoside	*Retention volume*[a]	$[\alpha]_D^{20}$, *degrees* (*c*, 0.5)[b]
(I), α-D-*erythro*	88	+115
(II), α-D-*threo*	100	−56
(III), β-D-*erythro*	115	+5
(IV), β-D-*threo*	122	−95
(V), α-D-, from (I)	41	+15
from (III)	41	+18
(VI), β-D-, from (II)	55	−180
from (IV)	55	−180

[a] Gas–liquid chromatography on column (190 cm × 5 mm) of 5 % Carbowax 20M on Chromosorb G, dimethyldichlorosilane treated, acid washed, 60–70 mesh, 120°; 100 ml argon/min; the retention volume of (II) was arbitrarily given the value 100.
[b] (I)–(IV) in acetone, and (V) and (VI) in water.

The anomeric configurations of the compounds in Table I were indicated from their optical rotations. The anomeric assignments made on the basis of Hudson's rule were verified by analysis of the proton magnetic resonance (p.m.r.) spectra.

All the p.m.r. data presented in this paper refer to deuterium oxide solutions, with sodium 2,2-dimethyl-2-silapentane-5-sulfonate as the standard. The spectra of the α,β-hex-2-enosides are also recorded for pyridine and carbon tetrachloride solutions, but, since deuterium oxide gave a greater chemical shift between H-1 and H-3, it was used exclusively as the solvent for the pure anomers. The intensities of all bands were consistent with their assignments. The coupling constants and chemical shifts recorded in this paper are all first-order, but these should approximate to the true values, except for some compounds in Table II where the coupling constants were not small compared to the chemical shifts.

Some details of the p.m.r. spectra of the hex-2-enosides are given in Table II (see also Fig. 1). The absorption due to H-4 and H-5 are not well resolved, except for the α-D-*threo* isomer (II), which gave $J_{3,4}$ 5.9 c.p.s. and $J_{4,5}$ 2.5 c.p.s. The signals

from H-6,6′ overlap those of the vinylic methoxyl groups (δ 3.63 ± 0.02 p.p.m.); those of the glycosidic methoxyl group are at 3.46 ± 0.01 p.p.m.; and those of the other methoxyl groups are at 3.38 ± 0.03 p.p.m. The assignment of the spectra

TABLE II

P.M.R. SPECTRA OF METHYL 3-DEOXY-2,4,6-TRI-*O*-METHYLHEX-2-ENOPYRANOSIDES (I)–(IV) AT 60 MC.P.S. IN DEUTERIUM OXIDE

Hex-2-enoside	*H-1 (singlet)*		*H-3 (doublet)*		
	δ^a	W	δ	$J_{3,4}$	W^b
(I), α-D-*erythro*	4.78	0.9	5.12	2.1[c]	<1.1
(II), α-D-*threo*	4.88	1.1	5.30	5.9	1.1
(III), β-D-*erythro*	4.91	1.9	5.18	5.3[c]	1.8
(IV), β-D-*threo*	5.10	2.2	5.33	5.7[c]	2.0

[a] Chemical shifts (δ) are in p.p.m., coupling constants (J) and widths at half height (W) in c.p.s. (H_2O band gave W = 0.3–0.4 c.p.s.)
[b] Refers to the width, at half-height, of each part of the doublet.
[c] These are splitting values, and may not approximate to the coupling constants, especially for the two β-D anomers (III) and (IV), because H-4, H-5, and H-6,6′ form part of an A_2BC system.

(Table II) is based, firstly, on the chemical shifts with reference to those of 3-deoxy-2,4,6-tri-*O*-methyl-α-D-*erythro*-hex-2-enopyranose (VII)[3]. On glycosidation of (VII),

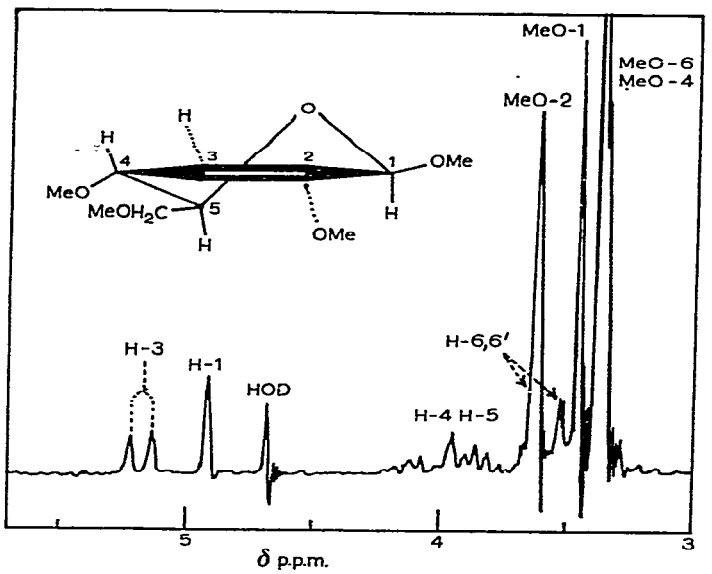
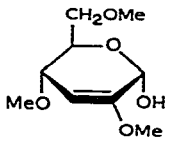


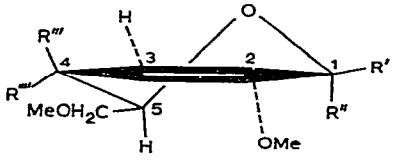
Fig. 1. The p.m.r. spectrum at 60 Mc.p.s. of methyl 3-deoxy-2,4,6-tri-*O*-methyl-β-D-*erythro*-hex-2-enopyranoside (III) in D_2O.

a new absorption of the glycosidic methoxyl group appears, and that of H-1 moves up to a field higher than that of H-3. This assignment of the absorptions from H-1

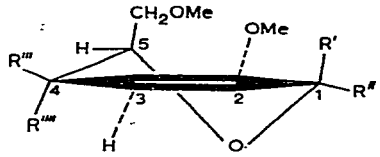
and H-3 was confirmed from an examination of the splitting patterns. As H-1 can couple only by a long-range mechanism, the peaks having the large splittings are due to H-3 (see Table II). This conclusion was further supported for the α-D-*threo* isomer (II) because $J_{3,4}$ could be obtained from the signals of both H-3 and H-4.



(VII)



(VIII)



(IX)

[(I), R' = R''' = H, R'' = R'''' = OMe;
(II), R' = R'''' = H, R'' = R''' = OMe;
(III), R'' = R''' = H, R' = R'''' = OMe;
(IV), R'' = R'''' = H, R' = R''' = OMe]

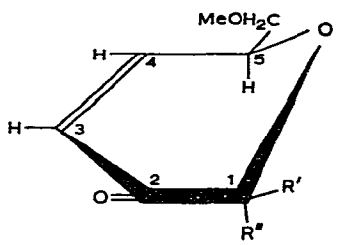
The signals of H-1 and H-3 of the hex-2-enosides (see Table II) are not sharp. Some of this broadening is due to weak, long-range couplings across oxygen atoms. However, the much broader signals from the α-D anomers (I) and (II) result from an allylic coupling of H-1 with H-3. The lack of resolution of these signals into a doublet and quartet, respectively, may arise because H-3 is coupled to an A_2BC system (see Table II)[5]. The value of allylic couplings depends on the dihedral angles between the protons[5], the following relationship having recently been proposed[6]:

$$J = 1.3 \cos^2 \phi - 2.6 \sin^2 \phi \quad (0° \leqslant \phi \leqslant 90°)$$

The hex-2-enosides (I)–(IV) could exist in either of the two half-chair conformations (VIII) and (IX)[3]. Those forms having the H-1 bond axial have $\phi = 80°$ (J — 2.6 c.p.s.) and those with the H-1 bond equatorial have $\phi = 30°$ (J 0.32 c.p.s.). Therefore, the anomers showing no appreciable allylic coupling are either the α-D anomers in conformation (XIII) or the β-D anomers in conformation (IX). The second possibility can be eliminated because, in that conformation, the β-D anomers have the bulky substituents at C-1 and C-5 axial on the same side of the ring in the *meta* position, a most unfavorable situation. It therefore follows that the β-D forms show the allylic couplings, as supported by the optical rotations of (I)–(VI) and the p.m.r. spectra of (V) and (VI). The conformation of all four compounds (I)–(IV) is the half-chair form (VIII).

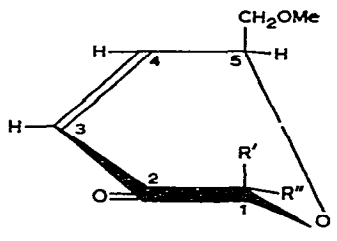
As shown in Table I, the hex-3-enosiduloses were obtained from *erythro*- and *threo*-hex-2-enosides, with retention of the anomeric configuration. Both preparations gave identical p.m.r. spectra, details of which are shown in Tables III and IV. The spectral assignments are straightforward, and follow those of hex-3-enosuloses[3,7], except that glycosidation has caused the signal of H-1 to be moved up-field, as for the hex-2-enosides (I)–(IV). The rigidity of the unsaturated carbonyl system forces the ring carbon atoms into one plane, limiting the hex-3-enopyranosidulose ring to two sofa conformations: (X), with H-5 axial, and (XI), with H-5 equatorial. In both

conformations, H-3 and H-4 are in the plane of the ring, and the geometry at C-1 and C-5 is similar to that of the hex-2-enosides (VIII) and (IX). Now, because, for both anomers, $J_{4,5}$ is small (for a vicinal coupling) and $J_{3,5}$ is large (for an allylic coupling), the dihedral angles H-4–H-5 and H-3–H-5 must[5,6] both be near 90°. Therefore, the hexenopyranosiduloses (V) and (VI) exist in conformation (X), with H-5 perpendicular to the ring. Furthermore, in the case of the β-D anomer, the other sofa conformation is unlikely, because the bulky substituents at C-1 and C-5 would then be axial.



(X)

(V), R'=H, R"=OMe



(XI)

(VI), R"=H, R'=OMe

The main differences in the spectra of the two hex-3-enosiduloses (see Tables III and IV, and Figs. 2 and 3) concern H-1 and H-3. An examination of the signals of

TABLE III

CHEMICAL SHIFTS (δ, P.P.M.) AND MULTIPLICITY (*M*) OF P.M.R. SIGNALS FROM METHYL 3,4-DIDEOXY-6-*O*-METHYLHEX-3-ENOPYRANOSIDULOSES (V) AND (VI) AT 60 MC.P.S. IN DEUTERIUM OXIDE

	H-1	*H-3*	*H-4*	*H-5*	*H-6,6'*	*OMe-1*	*OMe-6*
(V), α-D (δ)	4.92	6.28	7.28	4.8	3.77	3.59	3.42
(*M*)	4[a]	8	4	—[b]	2	1	1
(VI), β-D (δ)	5.07	6.28	7.27	4.8	3.75	3.60	3.42
(*M*)	2	4	4	—[b]	2	1	1

[a] Not fully resolved; width at half height, 1.2 c.p.s.
[b] Complex band H-5 is coupled to H-1, H-3, H-4, and H-6,6'.

TABLE IV

COUPLING CONSTANTS OF METHYL 3,4-DIDEOXY-6-*O*-METHYLHEX-3-ENOPYRANOSIDULOSES (V) AND (VI)

	Coupling constants (c.p.s.)					
	$J_{1,3}$	$J_{1,5}$	$J_{3,4}$	$J_{3,5}$	$J_{4,5}$	$J_{5,6}$
(V), α-D	0.75	0.3	10.7	2.7	1.7	4.5
(VI), β-D	0.2	1.1	10.7	2.6	2.3	5.0

these two protons yields information regarding the anomeric configuration. As long-range couplings H–C–(C=O)–C–H are known to have the maximum value when

the two protons and the 3 carbon atoms are in the same plane[8], the anomer showing this coupling ($J_{1,3}$ 0.75 c.p.s.) is the α-D form (see Fig. 3). The β-D anomer displays a long-range coupling ($J_{1,5}$ 1.1 c.p.s.) which could arise by either of two mechanisms: firstly, a four σ-bond coupling through the oxygen atom, this coupling not being

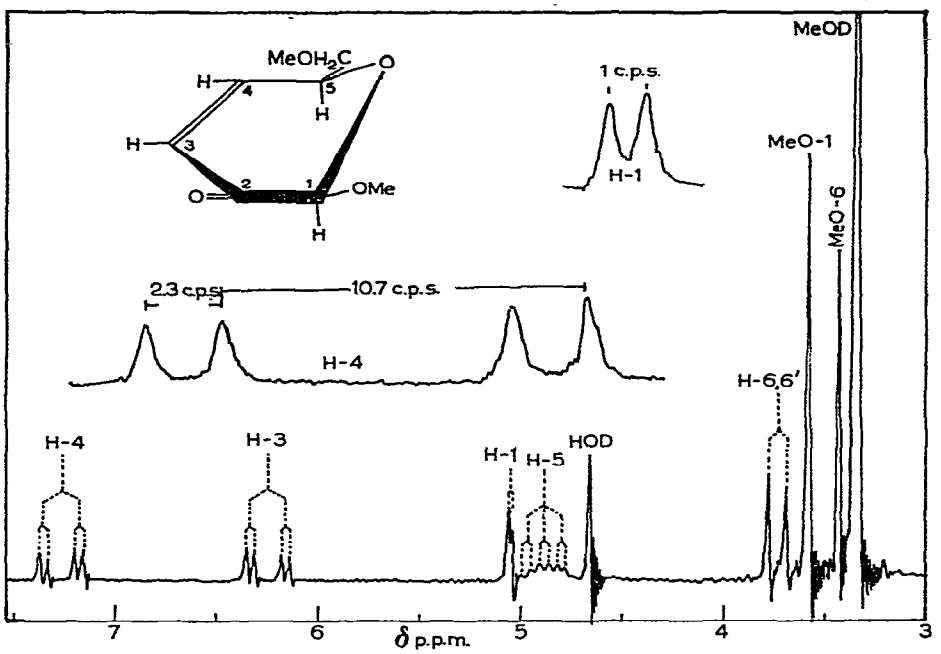


Fig. 2. The p.m.r. spectrum at 60 Mc.p.s. of methyl 3,4-dideoxy-6-*O*-methyl-β-D-*glycero*-hex-3-enopyranosidulose (VI) in D_2O (see also Fig. 3). That each of the lines of the H-5 signal are further split (1 c.p.s.) by H-1 can be seen at a sweep width of 50 c.p.s.

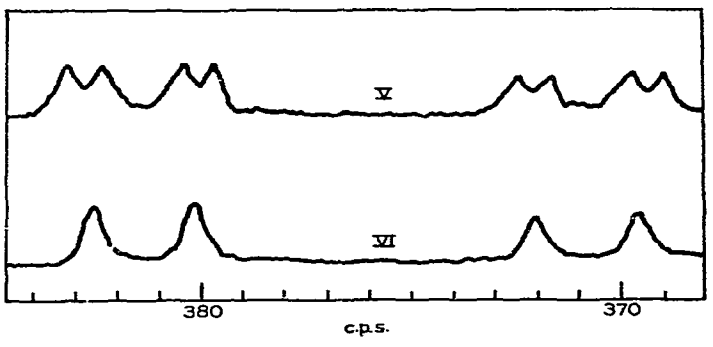


Fig. 3. P.m.r. signals at 60 Mc.p.s. from H-3 of methyl 3,4-dideoxy-6-*O*-methyl-D-*glycero*-hex-3-enopyranosiduloses (V) and (VI).

visible in the β-hexenosides (III) and (IV) where the geometry of these four bonds is identical; and secondly, an unusual six-bond coupling, possibly involving the π-electrons of the unsaturated system. The position here is somewhat similar to that

of 3-deoxypent-2-enofuranose rings, where $J_{1,4}$ is large (up to 4 c.p.s.) for β-D anomers[3,9].

It should be noted that, in each α,β-pair of the six glycosides (I)–(VI), the shielding of H-1 is larger when H-1 is equatorial (α-D anomer), the reverse situation to that normally found with cyclohexane or pyranoid systems. As in the present case, the α-proton of α-halocyclohexanones is more shielded in the equatorial than in the axial position (Ref. 10, and references therein).

Garbish[6] has recently proposed the equation

$$J = 6.6 \cos^2 \phi + 2.6 \sin^2 \phi (0° \leqslant \phi \leqslant 90°)$$

for calculating (vinyl) allylic couplings, the 2.6 $\sin^2 \phi$ term representing the π-electron contribution to the coupling. The minimum value (J 2.6 c.p.s., $\phi = 90°$) obtained from this equation appears to be too large for the compounds studied here (*cf.*, $J_{4,5}$ in Table IV; and $J_{3,4}$ of the α-D-*erythro* isomer in Table II). This is clearer in the previously studied 3-deoxyhex-2-enoses, where the (vinyl) allylic coupling ($J_{3,4}$) ranges from < 0.5 to 2.5 c.p.s. for α-D-*erythro* isomers[3]. It is probable that the parameter of 2.6 used by Garbish is too large.

The interlocking evidence for the two groups of substances here studied establishes their anomeric configurations as given in Table I. The α-D-hex-2-enopyranosides (I) and (II) are characterized by the greater sharpness of the p.m.r. signals of H-1 and H-3, compared with those of the β-D anomers (III) and (IV) (see Table II). Furthermore, for the α-D-*erythro* isomer (I), $J_{3,4}$ is small (2.1 c.p.s.) compared with that of the other isomers (5.3–5.9 c.p.s.). The application of these criteria to the p.m.r. spectra of previously isolated 3-deoxy-2-*O*-methylhex-2-enopyranoses[3] confirms the configuration given to them earlier. The p.m.r. spectra of the hex-3-enosiduloses show that the signal of H-1 of the β-D anomer (VI) appears as a doublet at a field lower than that of the α-D anomer (V). 3,4-Dideoxy-D-*glycero*-hex-3-enopyranosulose and its 6-methyl ether have been found to exist as 2:1 mixtures of two anomers[3,7]. The present work confirms that the minor anomers were the β-D forms.

EXPERIMENTAL

The microanalyses were performed by the Australian Microanalytical Service, Melbourne, and the proton magnetic resonance spectra were obtained on a Varian A60 spectrometer by Mr. P.J. Collins of the Division of Coal Research, C.S.I.R.O.

Analytical gas–liquid chromatography was carried out on a Packard gas chromatograph fitted with an argon ionization detector (see Table I). Preparative separations were achieved with a different instrument, fitted with a stainless-steel column (1 m × 1 cm O.D.) and a thermal conductivity detector. The column, packed with 5% Carbowax* 20M on Chromosorb** G, was operated at 140°.

*Trademark of Union Carbide Corporation.
**Trademark of Johns–Manville Product Corporation.

Repeated injections (10 μl) were made, to obtain sufficient pure material. Each glycoside emerging from the detector was collected in a small, glass tube containing a glass-wool plug. The same tube was used for several experiments. The glycoside was obtained by washing out the tube with acetone, and evaporating the solvent from the washings under diminished pressure.

(*a*) *Preparation of methyl 3-deoxy-2,4,6-tri-*O*-methylhex-2-enopyranosides* (*I*)–(*IV*)

(*i*) *The* α- *and* β-D-erythro *isomers* (*I*) *and* (*III*). 3-Deoxy-2,4,6-tri-*O*-methyl-α-D-*erythro*-hex-2-enopyranose[3] (VII) (20 g) in carbon tetrachloride (30 ml) was methylated at room temperature by the simultaneous addition, over 10 min, with rapid stirring, of methyl sulfate (20 ml) and a solution of sodium hydroxide (20 g) in water (60 ml). The reaction mixture was stirred for 30 min, and was refluxed for 1 h to destroy excess methyl sulfate. The carbon tetrachloride layer was separated from the cooled mixture, and the aqueous layer was extracted three times with chloroform. The chloroform extracts and the carbon tetrachloride solution were combined, dried with anhydrous sodium sulfate, and the solvents evaporated off. The residue was distilled under diminished pressure (bath temp. 80° and 0.01 mm) to yield a colorless liquid (16 g). The ratio of α-D to β-D anomer varied considerably from batch to batch, but was always greater than unity. The anomers were cleanly separated by preparative gas–liquid chromatography. (Found: C, 54.7, 54.9; H, 8.3, 8.3 for the α-D and β-D anomers, respectively. $C_{10}H_{18}O_5$ requires C, 55.0; H, 8.3%).

(*ii*) *The* α- *and* β-D-threo *isomers* (*II*) *and* (*IV*). 3-Deoxy-2,4,6-tri-*O*-methyl-D-*threo*-hex-2-enopyranose was prepared in a way similar to that used for the *erythro* isomer[3], but was not obtained crystalline. The crude material was methylated by the procedure used for the *erythro* isomer. The distilled product was a colorless liquid which, on gas–liquid chromatography, showed some impurities having lower retention times than those of (II) and (IV). The main bulk of the α-D anomer (II) crystallized from pentane at 0°; needles, m.p. 52–53°. The mother liquors were subjected to preparative, gas chromatography to yield the pure β-D anomer (IV). (Found: C, 55.1, 54.7; H, 8.5, 8.1 for the α-D and β-D anomers, respectively. $C_{10}H_{18}O_5$ requires C, 55.0; H, 8.3%).

(*b*) *Preparation of methyl 3,4-dideoxy-6-*O*-methyl-*α- *and* β-D-glycero-*hex*-2-enopyranosiduloses* (*V*) *and* (*VI*)

(*i*) The α-D anomer (V) was prepared by treating the crystalline hex-2-enoside (II) (500 mg) with 0.01N HCl (10 ml) at 100° for 4 min. The product was obtained by extracting the reaction mixture with chloroform. The washed and dried extract was concentrated and distilled (bath temp. 80° and 0.01 mm) to yield the pure hex-3-enosidulose (V), $[\alpha]_D^{20}$ + 17.5° (*c* 0.5, water) (Found: C, 55.6; H, 7.1; $C_8H_{12}O_4$ requires C, 55.8; H, 7.0%).

(*ii*) The hex-2-enosides (I)–(IV) were heated separately at 100° for 1–2 min in solutions of deuterium oxide (0.5 ml) containing one small drop of trifluoroacetic acid. The

cooled solutions were extracted with chloroform. The extracts were washed once with water, dried with anhydrous sodium sulfate, and evaporated under diminished pressure, to yield the hex-3-enosiduloses (V) and (VI). The four preparations gave the optical rotations recorded in Table I.

(*c*) *Spectroscopy*

(*i*) *P.m.r. spectra.* The p.m.r. spectra of the glycosides (I)–(IV) were determined in deuterium oxide, with sodium 2,2-dimethyl-2-silapentane-5-sulfonate as the internal standard (δ 0.00 p.p.m.). In each instance, after the spectrum had been recorded, one small drop of trifluoroacetic acid was added to the solution in the p.m.r. tube; this solution was heated to 100° for 1–2 min, cooled, and the spectrum re-recorded (Tables II, III and IV).

(*ii*) *Ultraviolet spectra.* The light absorptions of the hex-2-enosides (I)–(IV) were almost indistinguishable, with $\lambda_{max}^{H_2O}$ 198–200 mμ ($\varepsilon \sim 9 \times 10^3$). The anomeric hex-3-enosiduloses, (V) and (VI), gave $\lambda_{max}^{H_2O}$ 228 mμ and 226 mμ (ε 9.5×10^3), respectively.

(*iii*) *Infrared spectra.* The infrared spectra of the hex-2-enosides (I)–(IV) were similar, being characterized by a strong, sharp band at 1678 cm^{-1}, which is assigned to the asymmetrical C=C bond. The hex-3-enosiduloses (V) and (VI) gave an intense band at 1705 cm^{-1} (C=O) and a weak band at 1622 cm^{-1} (C=C).

ACKNOWLEDGMENT

The author is indebted to Mr. D. Gallimore for technical assistance.

SUMMARY

The methyl 3-deoxy-2,4,6-tri-*O*-methyl-D-*erythro*- and -D-*threo*-hex-2-enopyranosides were prepared by glycosidation of the free sugars with methyl sulfate. The methyl α- and β-glycosides were separated by preparative, gas–liquid chromatography, and converted into the methyl 3,4-dideoxy-6-*O*-methyl-α- and -β-D-*glycero*-hex-3-enopyranosiduloses, respectively. The anomeric configurations and the conformations of these compounds were obtained from their proton magnetic resonance spectra and from their optical rotations.

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THE RING CONFORMATION OF PENTA-*O*-ACETYL-α-D-ALTROPYRANOSE

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INTRODUCTION

It has frequently been affirmed that D-altropyranose and its simple derivatives are "conformationally unstable", and tend to exist in solution as equilibrium mixtures of the two possible chair conformations (*e.g.*, II and III)[1]. There is also available some physical[2] and chemical[3] evidence to suggest that crystalline D-altrose is itself not a pyranose form. Examination of the 60 Mc.p.s. proton magnetic resonance spectra of a series of substituted methyl 4,6-*O*-benzylidene-α-D-altropyranosides has recently shown that the presence, in the chair conformations of these pyranose compounds, of a 1,2,3-triaxial system of substituents together with a possible 1,3-diaxial repulsion does not, in solution, cause these conformations to undergo any significant equilibration with non-chair forms[4]. The rather common occurrence of stereospecific long-range coupling of H-1 with H-3 in these conformationally restricted bicyclic derivatives supported the assignment of the chair conformation to the pyranose ring since, in this conformation, these protons possess the 1,3-diequatorial orientation which is favourable[5] for the observation of this type of coupling. It was of interest therefore to re-examine the p.m.r. spectra of some *monocyclic* derivatives of D-altropyranose, in an effort, firstly, to determine the preferred conformation of the pyranose ring, and secondly, to investigate the possible occurrence of long-range coupling in a conformationally mobile system. Using a spectrometer operating at 40 Mc.p.s., Lemieux *et al.*[6] had observed that the signal due to H-1 of penta-*O*-acetyl-α-D-altropyranose (I)[7] was not resolved in chloroform solution, and had reported the half-line width of this signal to be 3 c.p.s. The contribution of both chair conformations to the observed spectrum appeared to be possible[6].

EXPERIMENTAL

The p.m.r. spectra were measured on Varian A-60 and HA-100 spectrometers at 40°, using solutions which contained 195–235 mg of compound in 0.35–0.4 ml of solvent, with tetramethylsilane added as internal reference. The 60 Mc.p.s. spectrometer was calibrated against a 2% v/v solution of benzene in carbon tetrachloride containing tetramethylsilane, the chemical shift of the benzene resonance being

assumed to be 436 c.p.s.[8]. Multiplet spacings were measured at a sweep width of 250 c.p.s.

RESULTS AND DISCUSSION

Determination of the p.m.r. spectrum of a solution of penta-*O*-acetyl-α-D-altropyranose (I) in deuterochloroform at a spectrometer frequency of 60 Mc.p.s. disclosed that the signal assigned[6] to H-1 was an unresolved doublet or broadened singlet at τ 3.98. The positions of the acetoxy methyl resonances at τ 7.84, 7.86, 7.86, 7.92, and 7.98 suggested the presence of three axial and two equatorial substituents in the preferred conformation[6,9]. In an effort to effect better resolution of the H-1 signal, 60 Mc.p.s. spectra of the penta-acetate (I) were measured in a number of other solvents including pyridine, acetone, and acetonitrile. In acetone solution, and especially in acetonitrile (Fig. 1), the signal of H-1 appeared as a more clearly

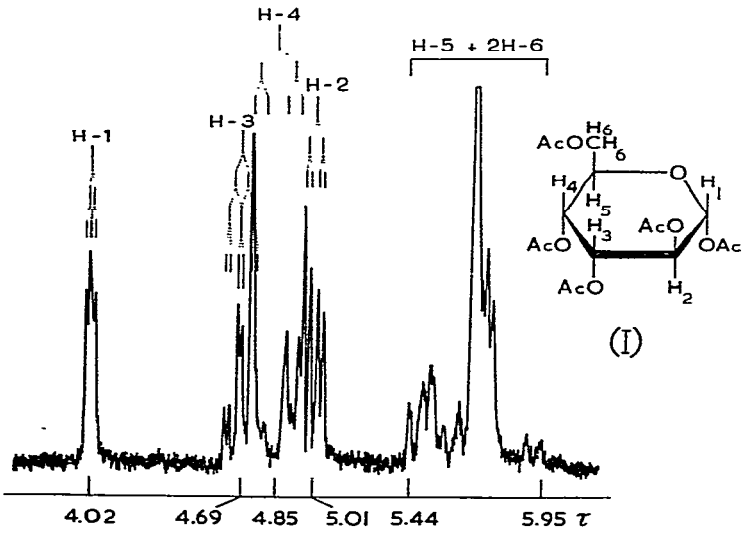


Fig. 1. Partial p.m.r. spectrum of penta-*O*-acetyl-α-D-altropyranose (I) in CH_3CN at 60 Mc.p.s.

defined triplet of small splitting, reminiscent of that observed in the spectra of many methyl 4,6-*O*-benzylidene-α-D-altropyranosides[4], and suggestive of either stereospecific[5], or virtual[10] long-range coupling of H-1 with H-3. Additionally, useful solvent shifts were obtained which allowed some distinction of the spin–spin multiplets due to H-2, H-3 and H-4, which at 60 Mc.p.s. displayed rather similar chemical shifts (Fig. 1). However, since only tentative assignments of these multiplets were possible at 60 Mc.p.s., more substantial evidence for them was obtained from spectra determined at 100 Mc.p.s. with the assistance of frequency-sweep double resonance. The resolution available at 100 Mc.p.s. was not quite as good as that at 60 Mc.p.s., and hence the smaller splittings were more conveniently observed and measured

at the latter frequency (as in Fig. 1). The 100 Mc.p.s. frequency-swept single-resonance spectrum of a solution of the penta-acetate (I) in deuterochloroform is shown in Figure 2*a*, and comparison with the 60 Mc.p.s. spectrum suggested the assignment of the broad singlet (unresolved triplet or quartet) at τ 4.01 to H-1, the quartet at

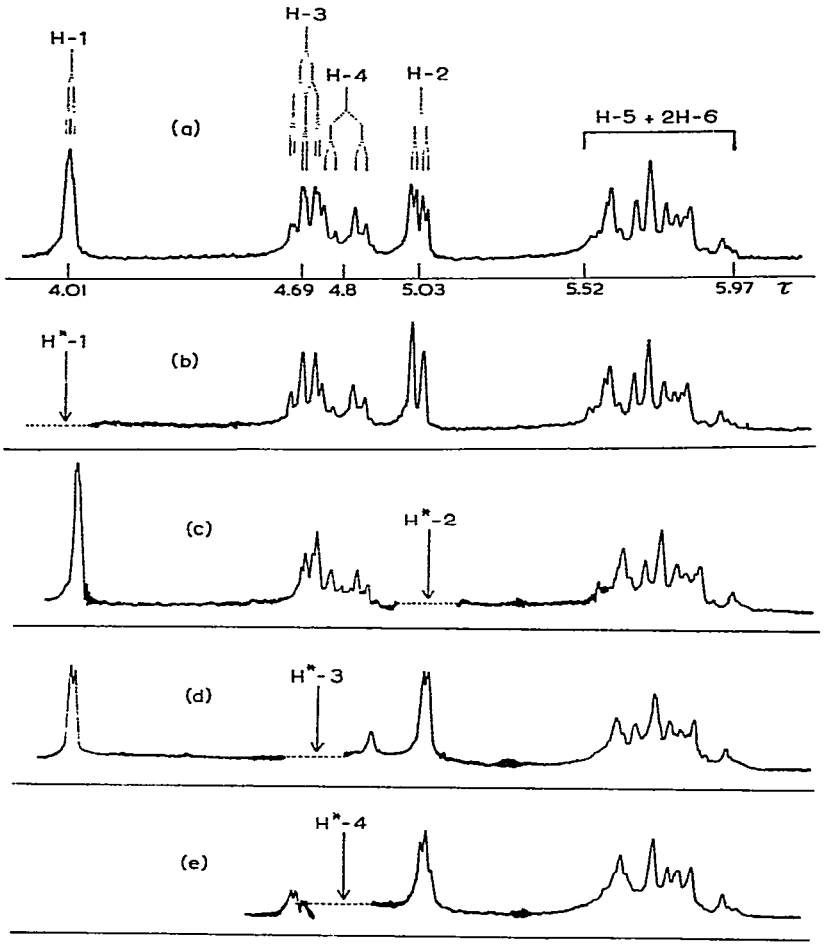


Fig. 2. Partial p.m.r. spectra of penta-*O*-acetyl-α-D-altropyranose (I) in $CDCl_3$ at 100 Mc.p.s. (*a*) Single resonance spectrum, (*b*)–(*e*) frequency-sweep double-resonance experiments. The disturbance due to the presence of the irradiating field is not shown, but its centre and extent are indicated by an arrow and by a broken baseline, respectively. The irradiated proton is starred. (*b*) Decoupling of H-2 and H-3 from H-1. (*c*) Decoupling of H-1 and H-3 from H-2. (*d*) Decoupling of H-1, H-2, and H-4 from H-3. (*e*) Decoupling of H-3 and H-5 from H-4, and also partial decoupling of H-2 from H-3.

τ 5.03 to H-2, the poorly resolved sextet at τ 4.69 to H-3, the quartet at τ 4.8 to H-4, and the complex band in the vicinity of τ 5.52–5.97 to H-5 and 2 H-6. These spectral assignments were confirmed as follows.

Irradiation of H-1 (Fig. 2*b*) resulted in collapse of the H-2 quartet to a doublet,

and of the H-3 sextet to a triplet, thereby removing simultaneously $J_{1,2}$, and the small coupling between H-1 and H-3, respectively. The larger spacings, which were retained in the H-2 and H-3 multiplets when these were decoupled from H-1, were equal in magnitude and were assigned to $J_{2,3}$. When H-2 was irradiated (Fig. 2*c*), the broad singlet due to H-1 sharpened appreciably, giving an unresolved doublet, and the sextet due to H-3 collapsed to a quartet whose major spacing corresponded to the minor splitting in the quartet at τ 4.8, and was therefore assigned as $J_{3,4}$. Irradiation of H-3 (Fig. 2*d*) caused the H-1 and H-2 multiplets both to become doublets, each containing the same small splitting, and confirmed thereby that H-3 is coupled to both H-2 *and* H-1. Irradiation of H-3 also caused the collapse of the H-4 quartet, but because of the proximity of the irradiating frequency to the resonance point of H-4, only one half of its decoupled multiplet could be seen (Fig. 2*d*).

Application of the irradiating field to H-4 (Fig. 2*e*) produced collapse in those parts of the H-3 multiplet which could be observed, and also a partial decoupling of H-2, owing to the spread of the decoupling field over some of the H-3 signal. Changes in the complex band at τ 5.52–5.97 indicated decoupling of H-5 from H-4.

The proton group H-2, H-3, and H-4 corresponds to a weakly coupled ABC sub-system in which the combination lines are weak, and hence the multiplet spacings may be taken as reasonably good approximations of the coupling constants. [Two weak extra lines were observed between the H-2 and H-4 quartets in the spectra of pyridine and acetone solutions of (I) measured at 60 Mc.p.s.]. The chemical shifts and coupling constants derived by first-order analysis of the 60 Mc.p.s. and 100 Mc.p.s. spectra are shown in Table I. The validity of the first-order approach is demonstrated by the fact that, with the exception of $J_{4,5}$ which was difficult to measure accurately at 60 Mc.p.s., the multiplet splittings measured from chloroform solutions at 60 Mc.p.s. were identical with those determined at 100 Mc.p.s.

Since the phenomenon of virtual coupling[10] is often responsible for the extra multiplicity which is occasionally observed in the proton signals of carbohydrates, the theory of Musher and Corey[10] was applied to the penta-acetate (I) to determine if the extra splittings in the signals of H-1 and H-3 were due to this effect. The conditions deduced from the theory may be re-stated for the linear, three-spin, sub-system H-1, H-2, and H-3, as follows. The H-1 quartet (with wings) is reduced to a doublet (splitting of the outside peaks $< 0.1 J_{1,2}$) if (*a*) $|\nu_2-\nu_3| \gtrsim 2\, J_{1,2}$, and $|\nu_2-\nu_3| \gtrsim J_{2,3}$, or if (*b*) $|\nu_2-\nu_3| \approx J_{1,2}$, and $|\nu_2-\nu_3| \gtrsim {}^4/_3 J_{2,3}$. Since the extra splitting was observed to be largest (1.0 c.p.s.), and H-2 and H-3 were most strongly coupled (*i.e.* maximum $J_{2,3}/|\nu_2-\nu_3|$) in the spectrum measured in acetonitrile at 60 Mc.p.s. (Fig. 1), these conditions were examined as being the most likely under which virtual coupling, if any, might have occurred. Application of condition (*a*) to the first-order parameters $|\nu_2-\nu_3| = 19.2$, $J_{1,2} = 1.5$, and $J_{2,3} = 3.6$ c.p.s. obtained in this situation (Table I) shows that the extra splitting of 1.0 c.p.s. cannot be due to virtual coupling, since this would be less than 0.15 c.p.s., and hence must be a true long-range coupling of H-1 with H-3. Similar treatment of the H-3, H-4, H-5 sub-system indicated that the extra multiplicity in the H-3 signal was not due to a large coupling constant

between H-4 and H-5, since, because of their large chemical shift, these protons are still only weakly coupled. That the long range coupling is stereospecific may be inferred from the fact that it was not observed when the orientation of H-1, or of H-3, or of both H-1 and H-3, was changed. Thus, in spectra measured in deuterochloroform at 60 Mc.p.s., the signal due to H-1 in penta-*O*-acetyl-β-D-altropyranose[11] appeared as a sharp doublet (τ_1 3.82, $J_{1,2}$ = 2.0 c.p.s.), as did also the H-1 resonances of penta-*O*-acetyl-α-D-mannopyranose[12] (τ_1 3.93, $J_{1,2}$ = 1.7 c.p.s.) and penta-*O*-acetyl-β-D-mannopyranose[12] (τ_1 4.05, $J_{1,2}$ = 1.0 c.p.s.).

TABLE I

CHEMICAL SHIFTS (τ VALUES) AND 1ST-ORDER COUPLING CONSTANTS (C.P.S.) OF PENTA-*O*-ACETYL-α-D-ALTROPYRANOSE

Solvent	*H-1*	*H-2*	*H-3*	*H-4*	*H-5 and 2H-6*[d]	*OAc*
Pyridine[a]	3.65 t[c] $J_{1,2}$ = 1.5 $J_{1,3}$ = 0.8	4.68 q $J_{2,3}$ = 3.5	4.28 qi (at least)[d] $J_{3,4}$ = 3.4	4.44 q $J_{4,5} \approx$ 8.9	5.18–5.79 m	7.83, 7.88, 7.95, 7.99, 8.01
Acetone[a]	4.03 t $J_{1,2}$ = 1.6 $J_{1,3}$ = 0.9	5.03 q $J_{2,3}$ = 3.6	4.69 qi (at least)[d] $J_{3,4}$ = 3.3	4.83 q $J_{4,5} \approx$ 8.8	5.42–5.97 m	—
CH_3CN[a]	4.02 t $J_{1,2}$ = 1.5 $J_{1,3}$ = 1.0	5.01 q $J_{2,3}$ = 3.6	4.69 qi (at least)[d] $J_{3,4}$ = 3.5	4.85 q $J_{4,5} \approx$ 9.0	5.44–5.95 m	—
$CDCl_3$[a]	3.98 d[d] $J_{1,2}$ = 1.4 $J_{1,3}$ = 0.8	5.01 q $J_{2,3}$ = 3.5	4.65 q (at least)[d] $J_{3,4}$ = 3.4	4.78 q $J_{4,5} \approx$ 7.6	5.41–6.03 m	7.84, 7.86 (×2)[d], 7.92, 7.98
$CDCl_3$[b]	4.01 $J_{1,2}$ = 1.4 $J_{1,3}$ = 0.8	5.03 q $J_{2,3}$ = 3.5	4.69 sx $J_{3,4}$ = 3.4	4.80 q $J_{4,5}$ = 9.3	5.52–5.97 m	7.82 (×3)[d], 7.89, 7.94

[a] At 60 Mc.p.s., [b] At 100 Mc.p.s., the spectrometer was locked on the signal of the tetramethylsilane internal reference; [c] Signal multiplicities are indicated by a symbol following the chemical shift, and are described as d (doublet), t (triplet), q (quartet), qi (quintet), sx (sextet), and m (complex multiplet). The absence of a symbol implies that a singlet was observed; [d] Unresolved.

In view of the suggestion[13] that altroses, lyxoses, and their glycosides do not have a stable chair conformation, and that the *flexible* conformation is a particularly attractive possibility for these configurations, the six flexible forms (IV), (V), (VI), (VII), (VIII), and (IX), which are intermediate between the classical boat conformations, have, for the α-D-altropyranose penta-acetate (I), been considered as being the more likely *possible* alternatives[14] to the chair conformations (II) and (III).

The proton dihedral angles (ϕ) in these conformational possibilities are compared in Table II, with the assumption that the pyranoid ring in compound (I) has geometry similar to that of an idealized cyclohexane ring, *i.e.*, tetrahedral valency angles[15]. Such an assumption is probably not entirely justified[4], but serves as a useful approximation for the comparison of conformations. Owing to geometrical and configurational symmetry, the proton dihedral angles $\phi_{1,2}$ and $\phi_{4,5}$ in any particular skew conformation (IV), (V), (VI), (VII), (VIII), or (IX) are invariably

equal in magnitude (Table II). This is also true for each of the six classical boat conformations (*e.g.* (X), Table II), and for any one of the infinite number of flexible forms which lie between the classical boats and the skew boats (IV)–(IX) listed in Table II. Since such widely differing values of coupling constants as $J_{1,2} = 1.4$ and $J_{4,5} = 9.3$ c.p.s. (*e.g.*, for deuterochloroform solutions of I at 100 Mc.p.s.; Table I) could not possibly represent the couplings of pairs of vicinal protons with approximately the same orientation ($\phi_{1,2} \approx \phi_{4,5}$) in each pair[6,16], the entire cycle of flexible forms may therefore be ruled out as major contributors to the conformational equilibria of compound (I). The ranges of the vicinal coupling constants $J_{1,2} = 1.4$–1.6, $J_{2,3} = 3.5$–3.6, $J_{3,4} = 3.3$–3.5, and $J_{4,5} = 8.8$–9.3 c.p.s. observed in the various solvents do in fact provide a good fit to the *C1* chair conformation (II)

TABLE II

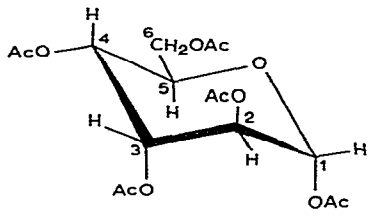
PROTON DIHEDRAL ANGLES FOR THE POSSIBLE CONFORMATIONS OF THE α-D-ALTROPYRANOSE RING ASSUMING IDEALIZED CYCLOHEXANE GEOMETRY

Conformations	$\phi_{1,2}$, °	$\phi_{2,3}$, °	$\phi_{3,4}$, °	$\phi_{4,5}$, °
Chairs				
(II)	60	60	60	180
(III)	180	180	60	60
Skew Boats				
(IV)	169	153	33	169
(V)	153	169	33	153
(VI)	87	153	71	87
(VII)	49	87	33	49
(VIII)	87	49	33	87
(IX)	153	87	71	153
Classical Boat				
(X)	60	60	0	60

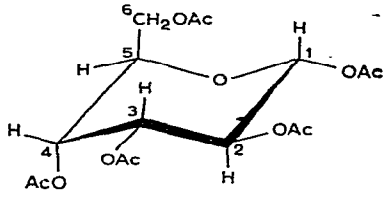
since the small values of $J_{1,2}$, $J_{2,3}$, and $J_{3,4}$ are characteristic of vicinal protons with the *gauche* ($\phi = 60°$) orientation, whereas the larger values of $J_{4,5}$ suggest the *trans*-diaxial orientation for H-4 and H-5, (ref. 6,16). As in the case of the methyl 4,6-*O*-benzylidene-α-D-altropyranosides[4], the observation of the long-range coupling $J_{1,3} = 0.8$–1.0 c.p.s. in compound (I) supports conformations [for instance, (II) and (X)] in which H-1 and H-3 are in the favourable diequatorial orientation. However, the classical boat (X) has already been discounted on the basis of the vicinal coupling constants observed, and is theoretically highly unlikely because of the large "bowsprit–flagpole" interaction. Because the observed values of $J_{1,2}$ and $J_{2,3}$ are small, and that of $J_{4,5}$ is large, the equilibrium concentration of the alternative *1C* chair conformation (III) is apparently insignificant at 40°, since the dihedral angles listed for (III) in Table II would require $J_{1,2} \approx J_{2,3} \approx 8$–$10$ c.p.s. and $J_{4,5} \approx 3$–4 c.p.s. or less.

It is concluded, therefore, that, for the solvents of varying polarity which have been examined, there is no evidence for conformations of compound (I) other than the *C1* chair form (II). Since equilibrated mixtures of penta-*O*-acetyl-α- and -β-D-altropyranoses have been found[11] to contain 65% of the α-anomer, and 35%

of the β-anomer, it appears that conformation (II) of the α-anomer is, in spite of the possible 1,3-diaxial repulsion, still thermodynamically more stable than the preferred



(II)



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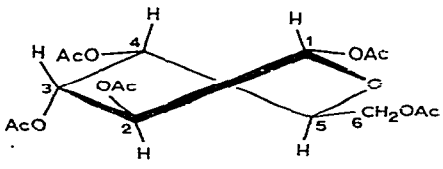
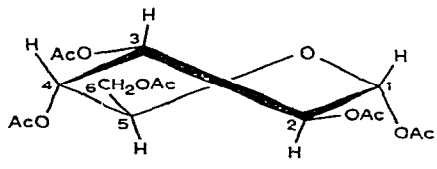


(IV)



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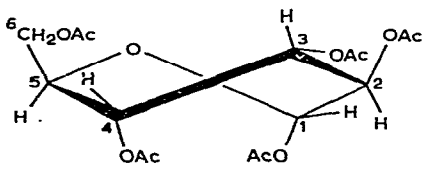
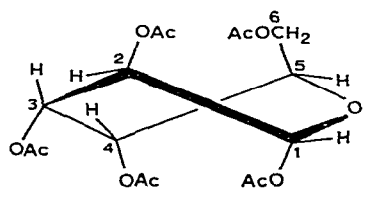


(VI)



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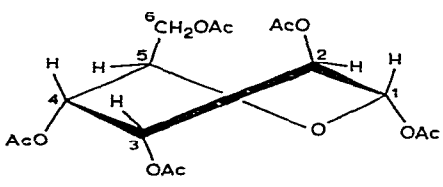
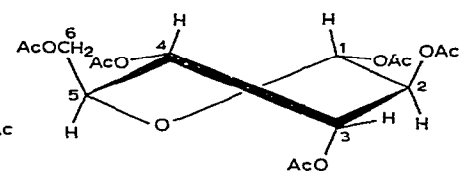


(VIII)



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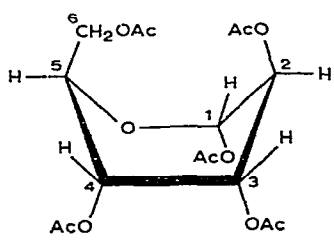

(X)

conformation(s) of the β-anomer. Such stability may be ascribed mainly to the "anomeric effect"[17a], and it has been pointed out[4] that a pyranose ring which is flatter than expected may lead to reduced 1,3-diaxial interactions. It is not clear, however, why relatively poor agreement was obtained between the interaction free energies which were observed and calculated for this particular anomerization equilibrium[17b].

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SUMMARY

P.m.r. spectra of α-D-altropyranose penta-acetate (I) have been measured in several solvents at 60 Mc.p.s., and in deuterochloroform at 100 Mc.p.s. The assignments of the ring proton signals, and of the long-range coupling of H-1 and H-3, were confirmed by frequency-swept double-resonance experiments at 100 Mc.p.s. Comparison with the spectra of three diastereoisomers of compound (I) indicated that the long-range coupling is stereospecific. Considerations of the observed coupling constants of the ring protons, and of symmetry, allow the exclusion of the flexible forms as major contributors to the conformational equilibria of compound (I), and suggest that the *C1* form is the only important conformation in solution at 40°.

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THE INTERACTION ENERGY BETWEEN AN AXIAL METHYL AND AN AXIAL HYDROXYL GROUP IN PYRANOSES

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INTRODUCTION

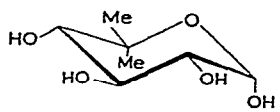
Approximate values for the conformational free energies of pyranoid sugars can be calculated by the use of interaction energies[1]. The magnitudes of the interactions of hydroxyl groups with each other and with hydrogen atoms are known with reasonable accuracy: $(O_a{:}O_a) = 1.9$, $(O_a{:}H_a) = 0.45$, $(O_1{:}O_2) = 0.35$ kcal/mole (where the first two expressions signify interactions between *syn*-axial atoms, and the third gives that between atoms attached to adjacent ring-positions[2]). One other value, namely that of the interaction between an axial hydroxymethyl and an axial hydroxyl group, is required for the calculations. This value can be taken as identical, within the accuracy required, with $(Me_a{:}O_a)$, the interaction between an axial methyl and an axial hydroxyl group (since the additional oxygen atom in the hydroxymethyl group will be oriented "outwards", and will not significantly contribute to the interaction[3]).

Angyal and Klavins[4], from measurements of the equilibrium constants for the formation of borate complexes with some cyclitols, derived the value of 1.6 kcal/mole for $(Me_a{:}O_a)$. This value, which has been used in previous calculations of the free energies of sugars[1], is considerably lower than other reported values[5] (1.9–2.4 kcal/mole), and may not be reliable: the other values, however, were not obtained using aqueous solutions, and it is not certain that the value of interactions of hydroxyl groups is independent of the solvent. It would be desirable to have $(Me_a{:}O_a)$ determined in aqueous solution, preferably by the use of sugars.

This interaction occurs between C-6 and O-1 in the alternative (*1C* in the D-series) chair forms of β-aldohexopyranoses; none of these, however, is stable when compared with the normal (*C1*) chair form[1] (except, possibly, in the case of β-D-idose, a compound which has not yet been isolated). The effect of this interaction may, however, be observed if an additional methyl group is attached to C-5. Each of the chair conformations will then have an axial methyl (or hydroxymethyl) group. The proportion of α- to β-anomers in equilibrium, which can be determined by nuclear magnetic resonance (n.m.r.) spectroscopy, allows the evaluation of the $(Me_a{:}O_a)$ interaction.

DISCUSSION

6-Deoxy-5-*C*-methyl-D-*xylo*-hexopyranose (5,5-di-*C*-methyl-D-xylopyranose)(I) was chosen as a suitable compound. Though previously synthesized[6], it has now been prepared, in a different way, by the action[7] of methylmagnesium iodide on methyl 1,2-*O*-isopropylidene-α-D-xyluronate[8], followed by hydrolysis. The n.m.r. spectrum of this branched-chain sugar in deuterium oxide showed two doublets, due to the anomeric proton of the α-form (Iα) at τ 4.87 ($J_{1,2}$ 3.7 c.p.s.), and that of the β-form (Iβ) at τ 5.24 ($J_{1,2}$ 7.5 c.p.s.). The assignment of signals to the anomers is based on a comparison with glucose, which has the same configuration, except for the lack of the additional methyl group. In α-D-glucose, H-1 appears[9] at τ 4.84 ($J_{1,2}$ 3.0 c.p.s.), and in β-D-glucose at τ 5.42 ($J_{1,2}$ 7.4 c.p.s.). The slightly larger coupling constant of 6-deoxy-5-*C*-methyl-α-D-*xylo*-hexopyranose is accounted for by a rotation of C-1 owing to the repulsion of its hydroxyl group by the axial methyl group; the position of the H-1 signal for the β-anomer, at lower field than that for β-D-glucose, is due to the effect of the *syn*-axial methyl group. The proportion of the α- and β-forms was found to be 8:92 (±1) at 40°, corresponding to a free energy difference of 1.5 (±0.1) kcal/mole.



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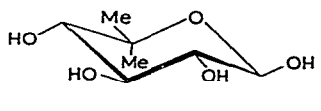
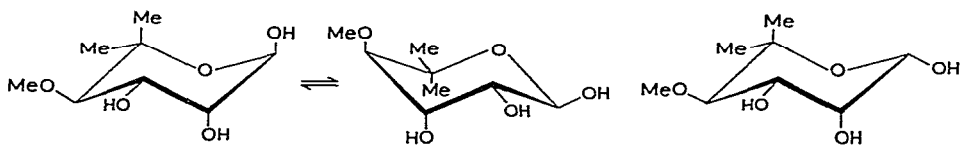


(Iβ)



(IIα) (IIα') (IIβ)

The equilibrium mixture of D-glucose consists of 36% of the α- and 64% of the β-form[9], corresponding to a free-energy difference of 0.35 kcal/mole. This represents the difference between the interaction of the axial hydroxyl group with the axial hydrogen atoms (0.9 kcal/mole) in the α-anomer, and the anomeric effect[10,11] of the equatorial hydroxyl group at C-1 (0.55 kcal/mole) in the β-anomer. All other interactions are the same in both anomers. In the 6-deoxy-5-*C*-methyl-D-*xylo*-hexopyranoses, again neglecting those interactions which are common to both anomers, the α-form (Iα) has a (Me_a:O_a) and an (O_a:H_a) interaction, whereas the β-form (Iβ) has a (Me_a:H_a) interaction and the anomeric effect. Therefore, ΔG° = (Me_a:O_a) + (O_a:H_a) − (Me_a:H_a) − anomeric effect, i.e., 1.5 = (Me_a:O_a) + 0.45 − 0.9 − 0.55, and, therefore, (Me_a:O_a) = 2.5 kcal/mole.

As another example, noviose[12] (6-deoxy-5-*C*-methyl-4-*O*-methyl-L-*lyxo*-hexopyranose, II) was investigated. This sugar is known in one crystalline form

which we have now found to be the β-anomer. Its rotation decreases during mutarotation; in the literature[7,12], only the equilibrium rotation has been reported. In pyridine solution, in which mutarotation is slow, the initial n.m.r. spectrum shows only one anomeric proton (τ 4.74, that of the β-form); another signal appears gradually at τ 4.23, and is assigned to the anomeric proton of the α-anomer. This assignment is confirmed by comparison with the n.m.r. spectrum of methyl α-novioside[13], which shows close similarity to the spectrum appearing during mutarotation, and by the position of the anomeric proton signal at lower field than that of the other anomer[14]. In aqueous solution, the n.m.r. spectrum shows that the α- and β-anomers are present in the ratio 26:74 ($\pm$2). The relevant n.m.r. data are shown in Table I.

TABLE I

CHEMICAL SHIFTS (τ) AND COUPLING CONSTANTS (C.P.S.) IN DEUTERIUM OXIDE.

	H-1	*H-4*	*Me*	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$
Methyl α-novioside	5.35	6.70	8.72, 8.64	2.3	3.4	8.8
α-Noviose	4.94	6.70	8.72, 8.64	3.9	3.4	7.7
β-Noviose	5.04	6.78	8.83, 8.67	1.0	3.3	9.5

The coupling constants indicate that, in aqueous solution, α-noviose represents a conformational mixture: $J_{1,2}$ is considerably larger, and $J_{3,4}$ considerably smaller, than in methyl α-novioside. The n.m.r. spectra of several derivatives of methyl α-novioside in pyridine solution have been published[15] and, in all cases, $J_{1,2} \leqslant 2.3$ and $J_{3,4} \geqslant 9.3$ c.p.s. The large coupling of H-3 and H-4 clearly indicates that they are both axial; both the α- and the β-noviosides are in the normal (*1C* in the L-series) conformation. The coupling constants of α-noviose in aqueous solution, however, must represent the weighted averages of two conformations, and, indeed, summation of the interaction energies indicates that the alternative (*C1*) conformation (IIα′) is only slightly less stable than the normal one (IIα). A calculation of $J_{1,2}$, assuming that in the normal chair form it has the same value (2.3 c.p.s.) as in methyl α-novioside and, in the alternative form, the same value (7.4 c.p.s.) as in 6-deoxy-5-*C*-methyl-β-D-*xylo*-hexopyranose (Iβ), gives 69% as the proportion of the normal chair form. A similar calculation of $J_{3,4}$, assuming that in the normal chair form it has the same value as in the β-anomer (9.5 c.p.s.) and, in the alternative chair form, the value (3.3 c.p.s.) found[16] for $J_{3,4}$ in a conformationally similar compound, diethylsulphonyl(α-D-lyxopyranosyl)methane triacetate, gives 71% as the proportion of the former. Agreement of these two results would seem to justify the method used for the calculations*. α-D-Lyxose, closely related to α-noviose, also consists of a conformational mixture in aqueous solution[9] ($J_{1,2}$ 4.0 c.p.s.).

*It is assumed that the coupling constants of H-1 and H-4 are identical with the splittings of their signals; this appears justifiable[17] because both signals are well-resolved, sharp doublets, and because the difference between the chemical shifts of H-3 and H-2 (τ 5.93 and *ca.* 6.14, respectively) is considerably larger than the coupling constants.

The methyl α-noviosides do not appear as conformational mixtures in pyridine solution[15] because the anomeric effect of a methoxyl group is greater than that of a hydroxyl group, and the effect is greater in pyridine than in water[11]. However, in aqueous solution, methyl α-novioside appears to contain considerable amounts of the alternative chair form, as judged by the value of $J_{3,4}$ (Table I).

The presence of 30% of another conformation in equilibrium decreases the free energy of a compound by 0.2 kcal/mole owing to the entropy of mixing[18], and this has to be taken into account in calculating the conformational free energies of the novioses; the alternative chair form of β-noviose, being of much higher free-energy, makes a negligible contribution to the equilibrium free-energy. The 26:74 (±2) ratio corresponds to a free-energy difference of 0.6 (±0.05) kcal/mole. Again neglecting the common interactions, β-noviose has one (Me_a:H_a) and one (O_1:O_2) interaction, and the anomeric and Δ2 effects, and α-noviose has a (Me_a:O_a) and an (O_a:H_a) interaction, and the entropy-of-mixing. Hence $\Delta G^\circ = 0.6 =$ (Me_a:O_a) + 0.45 − 0.2 − 0.9 − 0.35 − 1.0, and (Me_a:O_a) = 2.6 kcal/mole, a value in good agreement with the previous one.

It might be thought that this value is only applicable to substituents on C-1 and C-5; these two carbon atoms are closer to each other than other non-adjacent carbon atoms in the ring, because they are connected by C–O bonds which are shorter than C–C bonds. It appears, however, that the interaction between substituents on C-3 and C-5 is of approximately the same value. The former interaction occurs in the normal (IIα), and the latter in the alternative (IIα′) chair form of α-noviose; the total interaction energies are (Me_a:O_a) + (Me_a:H_a) + 3(O_a:H_a) + 2(O_1:O_2) + 2(Me_1:O_2) = 6.25 kcal/mole, and (Me_a:O_a) + (Me_a:H_a) + 3(O_a:H_a) + 2(O_1:O_2) + (Me_1:O_2) + anomeric effect = 6.45 kcal/mole, respectively. If the (Me_a:O_a) interaction were substantially less in the second conformation than in the first one, the latter would become the more stable chair form, contrary to the evidence.

EXPERIMENTAL

Methyl 1,2-O-*isopropylidene*-α-D-*xyluronate*[8]

A solution of silver nitrate (2.4 g) in water (20 ml) was added to a solution of potassium 1,2-*O*-isopropylidene-α-D-xyluronate[19] (3.4 g) in water (10 ml). The precipitated silver salt was filtered off, dried *in vacuo*, finely powdered, and then stirred under reflux with a 1:1 mixture of methyl iodide and anhydrous ether (40 ml) for 12 h. The silver iodide was removed by filtration and washed with ether, and the filtrate was evaporated. The residue, on crystallization from benzene–light petroleum, gave the methyl ester (2.4 g, 78%), m.p. 103–104°. Akiya and Watanabe[8] reported m.p. 101–104°.

6-*Deoxy*-1,2-O-*isopropylidene*-5-C-*methyl*-α-D-xylo-*hexofuranose*

A solution of methyl 1,2-*O*-isopropylidene-α-D-xyluronate (1.2 g) in anhydrous

ether (30 ml) was slowly added with stirring to Grignard reagent made from magnesium (0.6 g), methyl iodide (8 ml), and ether (35 ml); a heavy syrup separated. After being heated under reflux for 3 h, the mixture was decomposed with saturated, aqueous ammonium chloride (60 ml). The ethereal layer was washed with aqueous ammonium chloride, dried (Na_2SO_4), and evaporated. The residue, on crystallization from ether–light petroleum, gave the compound (1.0 g, 83%), m.p. 115°, as needles. Wolfrom and Hanessian[6] reproted m.p. 115–116°.

MATERIALS

6-Deoxy-5-*C*-methyl-D-*xylo*-hexopyranose was obtained as a syrup by hydrolysis of the above compound, according to the method of Wolfrom and Hanessian[6]. Methyl α-novioside was prepared by alkaline hydrolysis[13] of methyl 3-*O*-carbamoyl-α-novioside. β-Noviose, obtained by hydrolysis of the novioside[12], had $[\alpha]_D^{23}$ + 46.6 → 17.7° (5 h; *c* 1.2, 95% ethanol); reported values[7,12]: $[\alpha]_D^{25}$ + 22.6° and +19.9° (*c* 1, 50% ethanol).

SPECTRA

The n.m.r. spectra were obtained on a Varian A-60 spectrometer using solutions in deuterium oxide at 40°, with sodium 2,2-dimethyl-2-silapentane-5-sulphonate (τ 10.00) as internal standard. The α:β ratios were determined by repeated integration of the signals for the anomeric protons.

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SUMMARY

From the α:β ratio, observed by n.m.r. spectroscopy, of 6-deoxy-5-*C*-methyl-D-*xylo*-hexopyranose and of noviose in aqueous solution, the interaction energy of an axial methyl group with an axial hydroxyl group is calculated to be 2.5–2.6 kcal/mole. α-Noviose, in aqueous solution, is a mixture of approx. 70% of the normal and 30% of the alternative chair form.

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MASSENSPEKTROMETRISCHE UNTERSUCHUNGEN

IX. MITTEILUNG*. MASSENSPEKTROMETRISCHE UNTERSUCHUNGEN VON PERMETHYLIERTEN ANHYDROZUCKERN

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(Eingegangen den 23. Juli, 1965)

Wegen ihrer Bedeutung für die Strukturanalyse von Kohlenhydraten, der einfachen präparativen Zugänglichkeit und ihrer thermischen Stabilität sind Methyläther mehrfach zur massenspektrometrischen Untersuchung von Zuckern eingesetzt worden[1–4]. Die Massenspektren von spezifisch Deuterium-markierten Derivaten ermöglichen genaue Angaben über Zusammensetzung und Bildungsmechanismen der wichtigen Ionen. Es konnte gezeigt werden, daß die Abbaureaktionen bei permethylierten Pento- bzw. Hexo-siden qualitativ weitgehend übereinstimmen.

In der vorliegenden Arbeit wurden unter Verwendung markierter Verbindungen die Fragmentierungsprozesse der Glykosane eingehend untersucht. Die Untersuchungen wurden ausgedehnt auf Anhydro- bzw. Dianhydro-zucker verschiedener Ringtypen. Der Einfluß der Größe des Halbacetalringes wurde an zwei Beispielen untersucht.

1,6-Anhydrozucker (Tri-O-methylglykosane)

Zur Aufklärung der Abbaureaktionen in der Reihe der Glykosane wurden folgende Verbindungen synthetisiert: Tri-*O*-methylgalaktosan (I), 3,4-Di-*O*-methyl-2-*O*-trideuteromethylgalaktosan (II), 2,4-Di-*O*-Methyl-3-*O*-trideuteromethylglucosan (III), 2,3-Di-*O*-methyl-4-*O*-trideuteromethylmannosan (IV), 2-*O*-Methyl-3,4-di-*O*-trideuteromethylgalaktosan (V), 3-*O*-Methyl-2,4-di-*O*-trideuteromethylglucosan (VI), und 2,3,4-Tri-*O*-trideuteromethylgalaktosan (VII).

Der Einfluß der sterischen Anordnung der Methoxygruppen bzw. der Größe des Halbacetalringes wurde an folgenden Verbindungen untersucht: Trimethyläther vom Galaktosan, Glucosan, Mannosan, Idosan, Altrosan, und 1,6-Anhydro-α-D-galaktofuranose (VIII). Die Massenspektren der Verbindungen I–VIII sind in den Abbildungen 1–8 graphisch dargestellt. Die Intensität der einzelnen Ionen sind in Prozent der Intensität des Haupt-Ions angegeben. Zur Vermeidung von Untergrund-Effekten sind die Ionen mit den Massenzahlen (MZ) 32, 40 und 44 nicht berücksichtigt worden. Tabelle I zeigt zusammenfassend die Massenverschiebungen der typischen Ionen in den Massenspektren der markierten Verbindungen I–VII im Vergleich zu den entsprechenden unmarkierten Derivaten.

*VIII. Mitteilung, Ref. 1.

TABELLE I

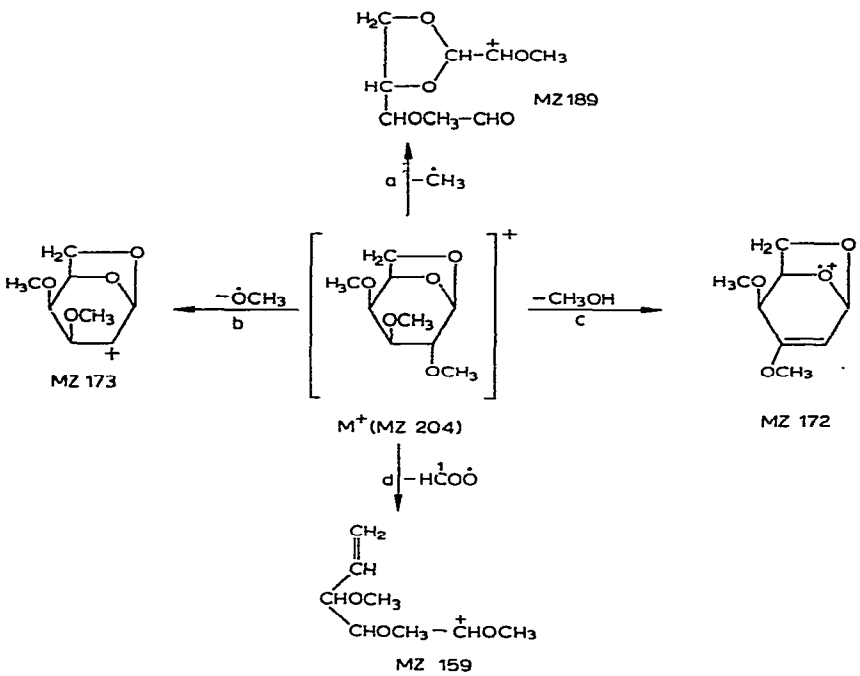
MASSENVERSCHIEBUNGEN IN DEN MASSENSPEKTREN DER SELEKTIV TRIDEUTEROMETHYL-MARKIERTEN TRIMETHYLGLYKOSANE

Intensität = % Haupt-Ion

MZ	*Glu*	*Glu 3-OCD$_3$*	*Glu 2,4-OCD$_3$*	*Glu 2,3,4-OCD$_3$*	*Gal*	*Gal 2-OCD$_3$*	*Gal 3,4-OCD$_3$*	*Man*	*Man 4-OCD$_3$*
45	39,2	22,2	15,6	4,1	27,1	12,5	8,4	38,9	30,6
48	0,1	12,4	19,2	29,7	—	11,8	11,6	0,4	8,5
71	16,1	14,2	3,3	1,6	8,0	7,7	1,8	15,0	—
74	3,5	3,4	14,3	14,8	2,1	2,5	6,2	1,2	14,5
73	38,1	19,2	21,4	2,9	17,5	10,6	8,5	31,2	37,9
76	0,5	19,2	19,6	34,9	0,1	8,9	8,9	0,9	2,2
75	15,0	14,2	3,1	1,7	4,4	1,6	1,2	16,2	2,1
78	—	1,9	2,2	0,7	—	3,1	3,2	0,2	12,2
81	1,2	1,2	12,1	14,4	0,8	1,0	1,0	2,6	1,4
88	100,0	4,6	3,5	5,3	31,6	3,4	1,6	80,0	90,9
91	—	100,0	100,0	0,6	—	31,3	26,6	0,3	10,6
94	0,9	0,8	1,9	100,0	—	—	2,0	1,4	0,9
95	2,1	2,0	0,5	5,7	1,3	1,0	1,1	2,8	1,7
98	0,7	0,5	2,0	1,9	0,3	0,9	1,0	1,0	1,8
101	86,4	94,1	1,3	0,8	100,0	2,3	1,0	100,0	4,6
104	0,3	7,1	9,1	0,7	0,4	100,0	100,0	1,4	100,0
107	—	0,3	80,5	94,8	—	0,1	1,6	0,8	1,2
103	6,4	1,2	6,9	0,3	8,3	8,1	1,0	6,8	7,3
106	—	6,1	1,1	9,2	—	0,6	8,3	0,1	0,7
111	1,09	0,56	0,64	0,33	0,52	0,55	0,21	1,41	0,84
114	0,56	0,94	1,61	1,10	0,24	0,29	0,69	0,60	0,60
117	0,50	0,82	0,27	0,18	0,09	0,29	0,20	0,63	0,49
115	1,27	1,39	0,69	0,58	0,43	0,35	0,25	1,30	0,77
118	0,04	0,49	0,61	0,57	0,01	0,33	0,27	—	0,97
121	—	—	0,68	1,10	—	—	0,19	0,18	0,10
127	5,14	2,30	0,10	0,13	1,45	0,55	0,04	5,00	2,4
130	0,09	4,36	3,95	0,11	0,27	1,51	1,14	0,21	3,6
133	0,14	0,08	1,93	6,10	0,01	0,04	0,40	0,21	0,23
143	2,38	1,91	0,07	0,03	0,25	0,12	0,02	2,05	0,47
146	0,16	1,41	1,23	0,04	—	0,29	0,21	0,18	2,21
149	—	—	1,64	2,91	—	—	0,11	0,42	0,53
144	2,45	0,34	0,31	0,08	0,04	0,06	0,08	2,41	1,72
147	0,01	2,90	3,25	0,12	—	0,05	0,05	0,07	1,63
150	—	—	0,27	3,24	—	—	0,03	0,11	0,06
159	3,28	—	—	—	0,06	—	—	3,30	0,05
162	0,01	4,42	0,09	0,03	—	0,09	—	—	4,01
165	—	—	3,79	0,13	—	—	—	—	—
168	—	—	—	4,64	—	—	0,10	—	—
172	0,18	0,04	0,04	—	0,02	—	0,01	0,18	0,17
175	—	0,21	0,21	0,02	—	0,03	0,04	0,14	0,18
178	—	0,06	0,04	0,33	—	—	0,01	—	0,09
173	0,05	0,01	0,09	—	0,02	0,02	0,02	0,07	0,03
176	—	0,06	0,10	—	—	0,03	0,03	—	0,06
179	—	—	0,07	0,14	—	—	0,04	—	—
189	0,18	0,20	—	0,03	0,01	—	—	0,21	0,04
192	—	—	—	0,04	—	0,01	0,02	—	0,24
195	—	—	0,19	0,25	—	—	—	—	—

Im Massenspektrum des Trimethylgalaktosans (Abb. 1) liegen intensive Peaks bei den MZ 45, 71, 73, 75, 88, und 101. Der Hauptpeak liegt bei der MZ 101. Im Vergleich dazu hat er bei den methylierten Hexopyranosiden die MZ 88. Die Intensität aller Peaks im oberen Massenbereich ist relativ gering.

In der folgenden Übersicht sind am Beispiel des Trimethylgalaktosans die zu den Ionen der MZ 189, 173, 172, und 159 führenden primären Zerfallsschritte zusammengefaßt. Eine detaillierte Beschreibung der Primärprozesse mit ihren Folge-Ionen schließt sich an.



(*a*) *Fragment MZ 189.* Die Abspaltung eines Methylradikals ist bei anderen methylierten Sacchariden bisher nicht beobachtet worden. Die Massenverschiebungen in den Spektren der markierten Verbindungen (Tab. I) beweisen eine ausschließliche Beteiligung der Methylgruppe an C-3. Weitere Abbaureaktionen lassen sich wegen der geringen Intensität dieses Primär-Ions nicht verfolgen.

(*b*) *Fragmente MZ 173, 143, 141, und 111.* Aus der Massenverschiebung im Spektrum der an C-3 markierten Verbindung folgt, daß der Primärangriff nicht an der Methoxygruppe an C-3 erfolgt. Wegen der geringen Intensität läßt sich nur abschätzen, daß die Abspaltung eines Methoxy-Radikals an C-2 und C-4 mit gleicher Wahrscheinlichkeit verläuft.

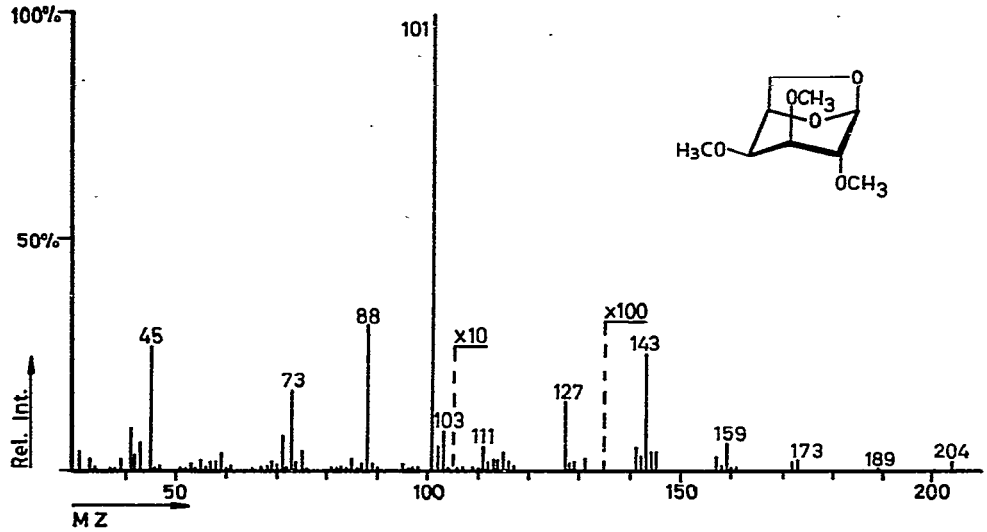
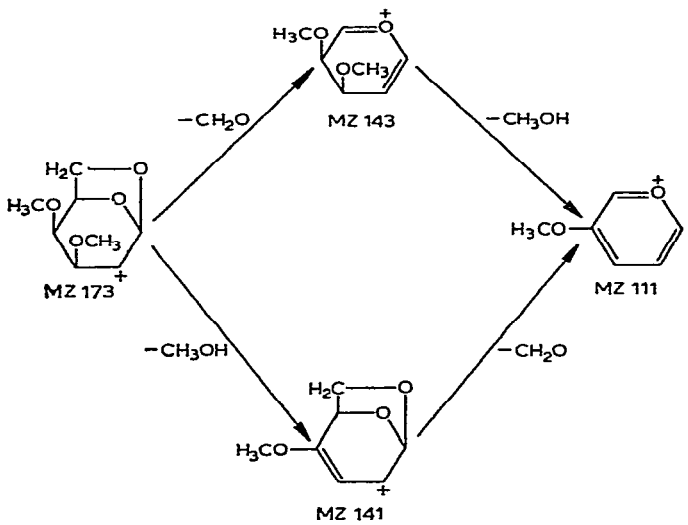


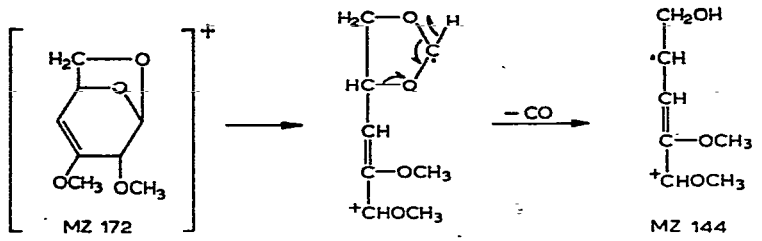
Abb. 1. Tri-*O*-methylgalaktosan.

Die Stabilisierung des Primärfragmentes der MZ 173 erfolgt in zwei Schritten durch Eliminierung von Methanol und Formaldehyd:



Weitere isomere Ionenstrukturen sind nicht im Schema aufgenommen.

(*c*) *Fragmente MZ 172 und 144.* Die Massenverschiebungen zeigen, daß eine primäre Methanol-Eliminierung vorwiegend unter Beteiligung der Methoxygruppen an C-2 bzw. C-4 verläuft. Ein metastabiles Ion bei der MZ 120,6 (III, 123,5) beweist als Folgeschritt die Eliminierung von CO.



Das gebildete Ion MZ 144 enthält die Methoxygruppe an C-3 und mit gleicher Wahrscheinlichkeit die an C-2 bzw. C-4.

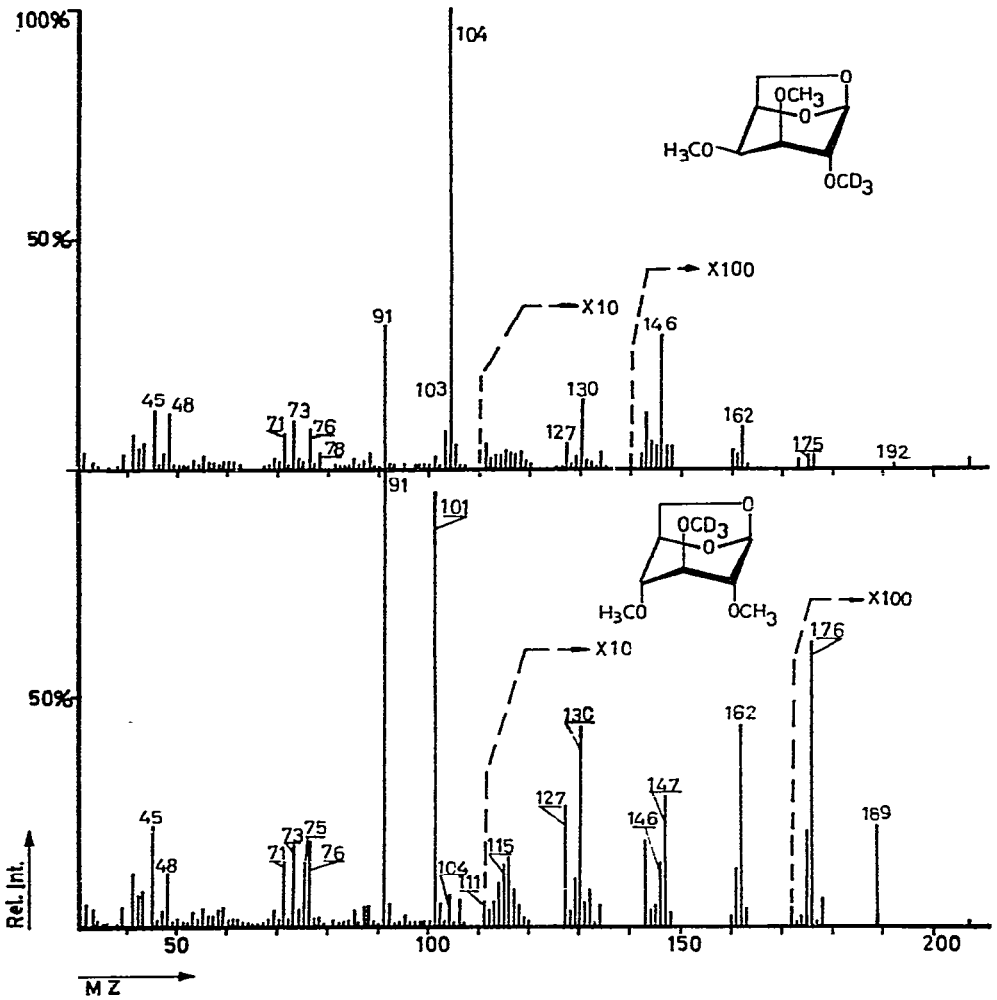
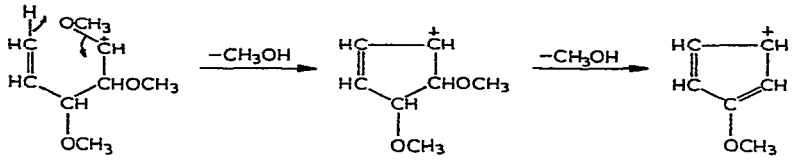


Abb. 2. 3,4-Di-*O*-methyl-2-*O*-trideuteromethylgalaktosan (oben).

Abb. 3. 2,4-Di-*O*-methyl-3-*O*-trideuteromethylglucosan (unten).

(*d*) *Fragmente MZ 159, 127, 95, 88, 73, und 71.* In der an den C-Atomen 2, 3, und 4 markierten Verbindung VII (Abb. 7) verschiebt sich die MZ des Primär-Ions MZ 159 um 9 Masseneinheiten (ME); die an zwei C-Atomen markierten Verbindungen V und VI zeigen eine Verschiebung um 6 ME, die Verbindungen II, III, und IV um 3 ME. Das Ion MZ 159 muß also sämtliche Methoxygruppen enthalten und durch Abspaltung von C-1 als HCOÖ entstanden sein. Als sekundäre Abbaureaktion des Ions MZ 159 erfolgt die Eliminierung von zwei Molekülen Methanol:

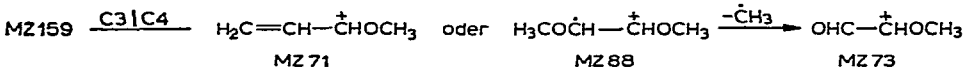


MZ 159 MZ 127 MZ 95

Die Massenverschiebungen in den Spektren der markierten Verbindungen zeigen, daß die Eliminierungsreaktionen sehr komplex verlaufen. Im ersten Eliminierungsschritt sind die drei Methoxygruppen völlig gleichwertig, d.h. es muß für das Ion MZ 127 neben der angegebenen Ringstruktur auch eine Struktur

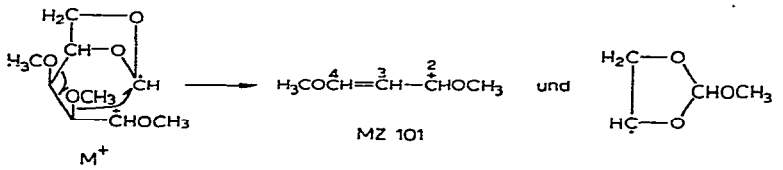
$H_2C{=}CH{-}CH{=}C(OCH_3){-}\overset{+}{C}HOCH_3$ in Betracht gezogen werden.

Neben der Methanol-Eliminierung erfolgt eine Spaltung der Bindung C-3/C-4:



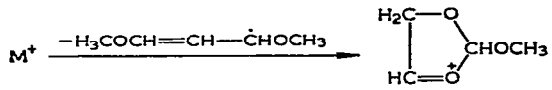
Die quantitative Verschiebung der MZ 71 im Spektrum der an C-4 markierten Verbindung (Abb. 4) um 3 ME zur MZ 74 beweist die angegebene Ionenzusammensetzung. Das Radikal-Ion MZ 88 wird nur zu etwa 90% um 3 ME verschoben, d.h. das C_2-Bruchstück mit den C-Atomen C-3 und C-4 ist mit etwa 10% beteiligt.

Neben den bisher behandelten Primärspaltungen erfolgt die bei den methylierten Pentosiden beobachtete bevorzugte Öffnung der Bindung zwischen den C-Atomen 1 und 2:



Die Massenverschiebungen in den Spektren der markierten Verbindungen beweisen, daß die angegebene Struktur mehr als 90% zur Gesamtintensität beiträgt.

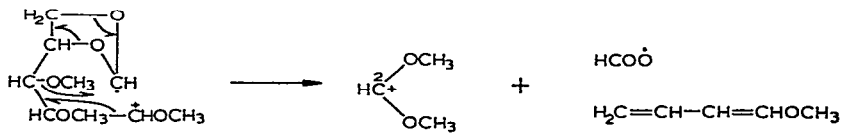
Zu einem geringen Teil kann bei der Spaltung der Bindung C-4/C-5 die Ladung an C-5 verbleiben unter Bildung des Umlagerungs-Ions MZ 103:



MZ 103

Es erfolgt ganz eindeutig nur in den Spektren der Verbindungen, bei denen die Methoxygruppe an C-3 markiert war, eine Verschiebung um 3 ME zur MZ 106.

Das bei den methylierten Glykosiden gefundene Umlagerungs-Ion $HC(OCH_3)_2^+$ MZ 75 tritt bei den Glykosiden nur mit geringer Intensität auf. Die Spektren der markierten Verbindungen zeigen jedoch, daß das Ion durch Wanderung einer Methoxygruppe von C-4 nach C-2 gebildet wird:



MZ 75

Die Massenspektren der isomeren Glykosane

Für die Trimethylglykosane kann wegen des Anhydroringes auch in der Gasphase das Vorliegen der *1C*-D-Konformation angenommen werden. Nach bisherigen Erfahrungen[2] sollten wegen der unterschiedlichen sterischen Anordnung der Methoxygruppen merkliche Intensitätsunterschiede auftreten. Es treten in der Tat relative Intensitätsdifferenzen bis zu 100% auf. Tabelle II gibt für die bisher untersuchten Isomeren die Intensitäten in Prozent des Gesamtionenstromes für die wichtigsten Ionen an.

TABELLE II

TRIMETHYLGLYKOSANE

% Gesamtionenstrom Σ 30

MZ	*Glucosan*	*Galaktosan*	*Mannosan*	*Idosan*	*Altrosan*
45	8,63	9,82	8,12	8,06	8,15
71	3,58	2,91	3,13	3,52	3,53
73	8,49	6,32	6,51	6,99	7,64
75	3,33	1,58	3,28	3,26	2,29
88	22,26	11,45	16,70	18,57	18,45
101	18,99	36,22	20,90	27,51	28,00
103	1,41	1,93	1,42	1,52	1,57

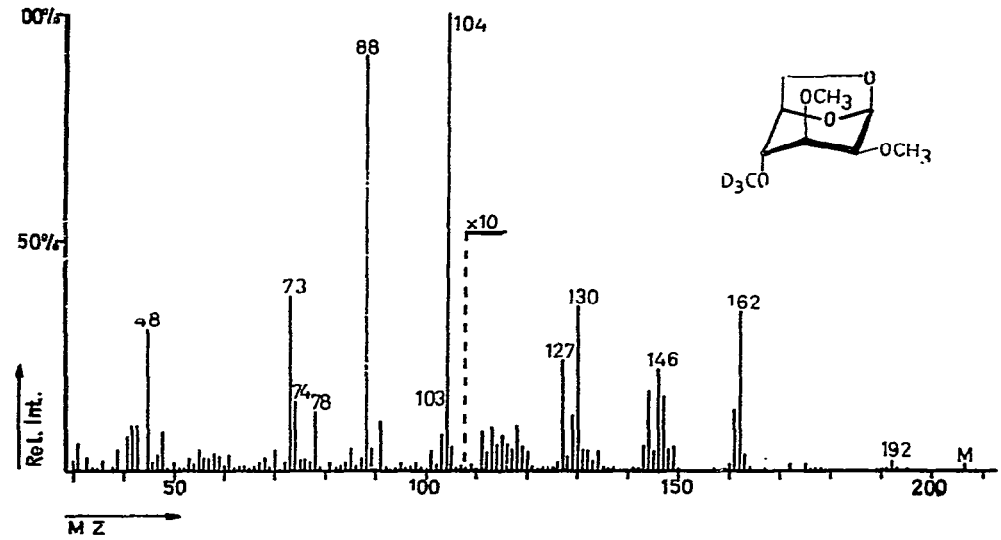


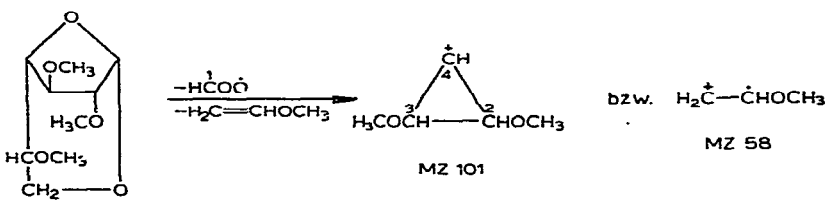
Abb. 4. 2,3-Di-*O*-methyl-4-*O*-trideuteromethylmannosan.

Es soll in diesem Zusammenhang nur auf die starken Intensitätsunterschiede für die Peaks der MZ 88 und 101 im Spektrum von Glucosan und Galaktosan hingewiesen werden. Während beim Glucosan das C_2-Fragment bevorzugt gebildet wird, ist es beim Galaktosan das C_3-Bruchstück. Ähnliche Intensitätsverhältnisse wurden von Kochetkov *et al.*[3] beim Methyl-tetra-*O*-methyl-β-D-gluco- bzw. -galaktopyranosid gefunden. (Die dort angegebenen Werte sind in Prozent-Gesamtionenstrom umzurechnen). Diese Unterschiede treten in gleicher Weise bei allen markierten Verbindungen des Glucosans bzw. Galaktosans auf.

Eine Deutung der Unterschiede kann erst erfolgen, wenn die Spektren aller Isomeren vorliegen.

*1,6-Anhydro-tri-*O*-methyl-α-*D*-galaktofuranose*

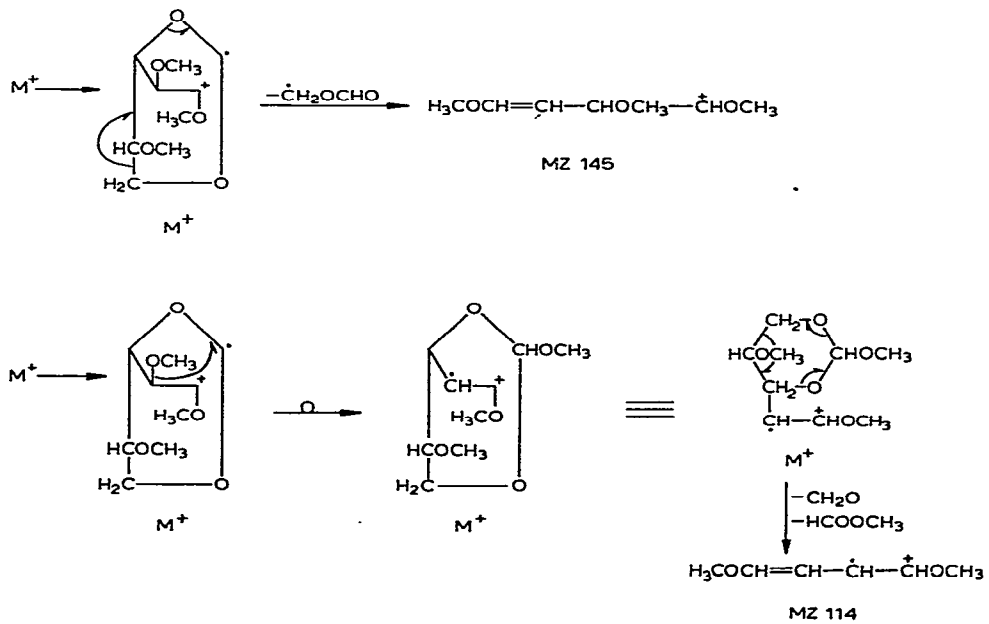
Das Massenspektrum der 1,6-Anhydro-tri-*O*-methyl-α-D-galaktofuranose (Abb. 8) unterscheidet sich auffallend von den Spektren der methylierten 1,6-Anhydrohexopyranosen. Das Haupt-Ion liegt wieder bei der MZ 101; da die Methoxygruppe an C-4 fehlt, muß die Bildung nach einem anderen Mechanismus erfolgen. Die Intensität des Peaks MZ 58 ist im Vergleich zur Pyranose-Form stark angestiegen, daher kann folgende Abbaureaktion angenommen werden:



Das charakteristische C_2-Bruchstück MZ 88 wird aus energetischen Gründen in der Furanose-Form nicht gebildet[2].

Auffallend ist im oberen Massenbereich die größere Stabilität der primär gebildeten Fragmente MZ 189, 173, 172, und 159. Charakteristisch für die Furanose-Form sind die relativ intensiven Peaks bei den MZ 145 und 114, die bei der Pyranose-Form nicht beobachtet werden.

In Analogie zu bisher gefundenen Abbaureaktionen wird folgender Bildungsmechanismus angenommen:



Für die Bildung des Ions MZ 114 ist primär die Spaltung der Bindung C-1/C-2 und Wanderung der Methoxygruppe von C-3 nach C-1 anzunehmen. Daraus resultiert die Möglichkeit der energetisch sehr günstigen Eliminierung von zwei Neutralteilchen unter Bildung des Radikal-Ions MZ 114.

3,6-Anhydrozucker

Zur Untersuchung des massenspektrometrischen Verhaltens von 3,6-Anhydrozuckern wurden die Spektren folgender Verbindungen aufgenommen: Methyl-3,6-anhydro-2,4-di-*O*-methyl-α-D-glucopyranosid (Abb. 9), Methyl-3,6-anhydro-2,4-di-*O*-trideuteromethyl-α-D-glucopyranosid, und Methyl-3,6-anhydro-2,5-di-*O*-methyl-α-D-glucofuranosid (Abb. 10).

3,6-Anhydrohexopyranosid (Abb. 9)

Die im folgenden Abschnitt in Klammern angegebenen MZ sind dem Spektrum der markierten Verbindung entnommen und geben Aufschluß über die Beteiligung der Methoxygruppen an C-2 und C-4 in dem betreffenden Ion.

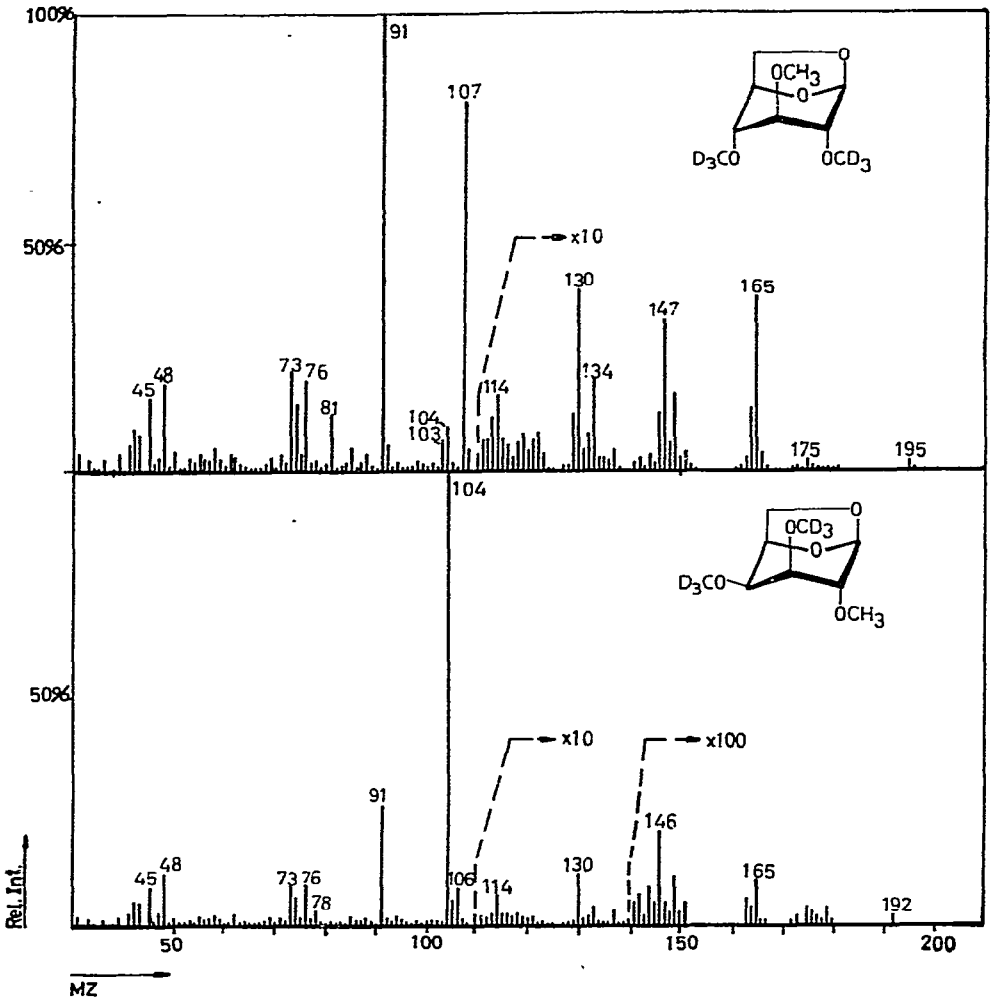
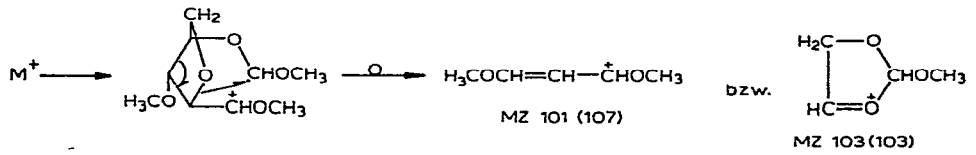


Abb. 5. 2-*O*-Methyl-3,4-di-*O*-trideuteromethylgalaktosan (unten).

Abb. 6. 3-*O*-Methyl-2,4-di-*O*-trideuteromethylglucosan (oben).

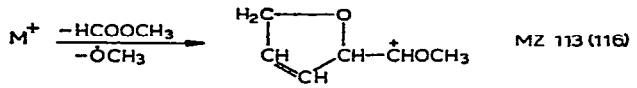
Das Vorliegen des fünfgliedrigen Anhydroringes hat erheblichen Einfluß auf die Fragmentierungsreaktionen bei den methylierten 3,6-Anhydrohexosiden. Im Gegensatz zu den 1,6-Anhydrozuckern führt hier die primäre Abspaltung einer Methoxygruppe, da es sich um die glykosidische Gruppe handelt, zu einem intensiven Peak bei der MZ 173 (179). Eine darauf folgende zweimalige Eliminierung von Methanol führt zu den Ionen MZ 141 (144) und 109 (109).

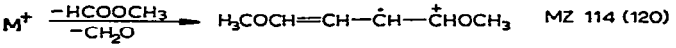
Die intensiven Peaks in den Spektren der methylierten 3,6-Anhydrohexopyranoside liegen bei den MZ 101 (107), 88 (91), 71 (74), und 45 (45, 48). Die Bildung des Ions MZ 101 (107) verläuft unter Umlagerung des Anhydroringes in gleicher Weise wie bei den Glykosanen:



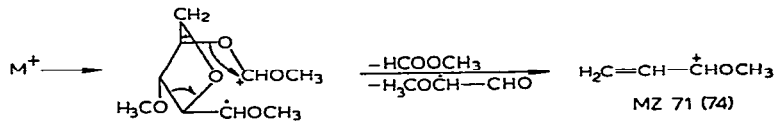
Die Intensität des Radikal-Ions MZ 88 (91) ist entsprechend den Befunden bei den methylierten Hexosiden[3] wegen der geringen Beteiligung von C-1 und C-2 an diesem Bruchstück nur relativ gering.

Simultan mit der Eliminierung von Methylformiat kann auch die Abspaltung eines Methoxy-Radikals bzw. von Formaldehyd verlaufen:





Für die Entstehung des Haupt-Ions MZ 71 (74) wird folgender Mechanismus angenommen:

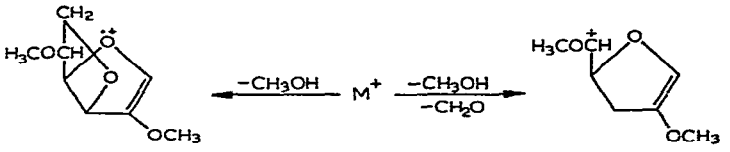


3,6-Anhydrohexofuranosid (Abb. 10)

Während im 3,6-Anhydrohexopyranosid ein fünf- und sechs-gliedriges Ringsystem miteinander verbunden sind, liegen im entsprechenden Furanosid zwei Fünfringe vor. Im Furanosid erfolgt der Primärangriff im wesentlichen am Halbacetalring und der Anhydroring bleibt bei einer Reihe von wichtigen Ionen erhalten.

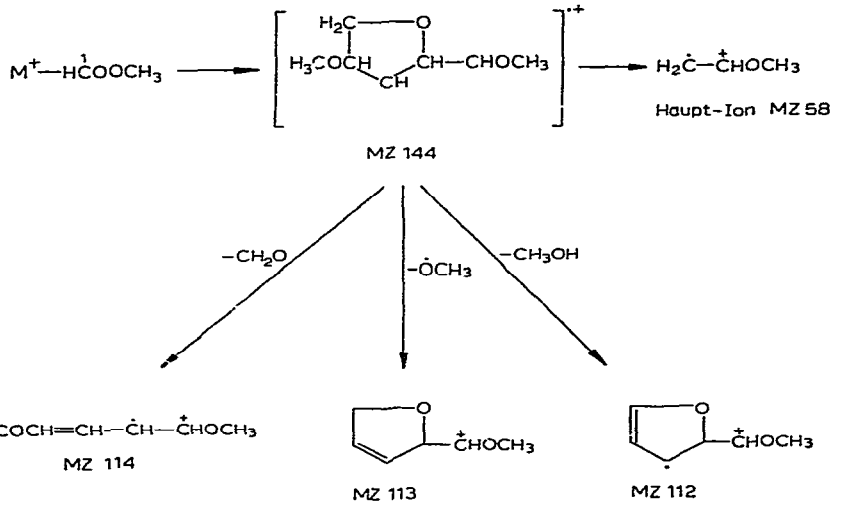
Besonders auffallend im Spektrum des Methyl-3,6-anhydro-2,5-di-*O*-methyl-α-D-glucofuranosids (Abb. 10) ist das Fehlen des für alle bisher untersuchten Methylsaccharide typischen C_3-Bruchstücks MZ 101. Das ist begründet durch das Fehlen 1,3-ständiger Methoxygruppen im Molekül.

Neben der Primärspaltung der glykosidischen Methoxygruppe mit folgender Methanol-Eliminierung (MZ 173 und 141) gewinnt beim Furanosid die primäre Methanol-Eliminierung an Bedeutung.



MZ 172 MZ 142

Eine Reihe wichtiger Ionen wird ausgehend von dem durch Eliminierung von Methylformiat gebildeten primären Radikal-Ion MZ 144 gebildet.



Da bisher keine Spektren von spezifisch deuterierten Derivaten vorliegen, kann auf die Bildung weiterer Ionen in diesem Zusammenhang nicht eingegangen werden.

Anhydrozucker mit Epoxid-Ringen

Als Beispiele für Anhydrozucker mit Epoxid-Ringen wurden die Massenspektren von Methyl-2,3-anhydro-4,6-di-*O*-methyl-β-D-allopyranosid (Abb. 11) und Methyl-3,4-anhydro-2,6-di-*O*-methyl-β-D-allopyranosid (Abb. 12) aufgenommen.

Da C-1 und C-6 nicht am Anhydroring beteiligt sind, lassen sich einige der bei den permethylierten Hexopyranosiden beobachteten Abbaureaktionen auf diesen Anhydrozucker-Typen übertragen. Die Fragmentierungsfolge M $-\dot{O}CH_3$ $-CH_3OH$ $-CH_3OH$ führt zu Peaks sehr geringer Intensität bei den MZ 173, 141, und 109.

Der Abbaufolge M $-\dot{C}H_2OCH_3$ $-CH_3OH$ entsprechen die Ionen MZ 159 bzw. 45 und 127. Das Vorliegen eines Anhydroringes wird angezeigt durch eine Verschiebung dieser Ionen um 46 ME zu niedrigeren MZ gegenüber den permethylierten Hexopyranosiden.

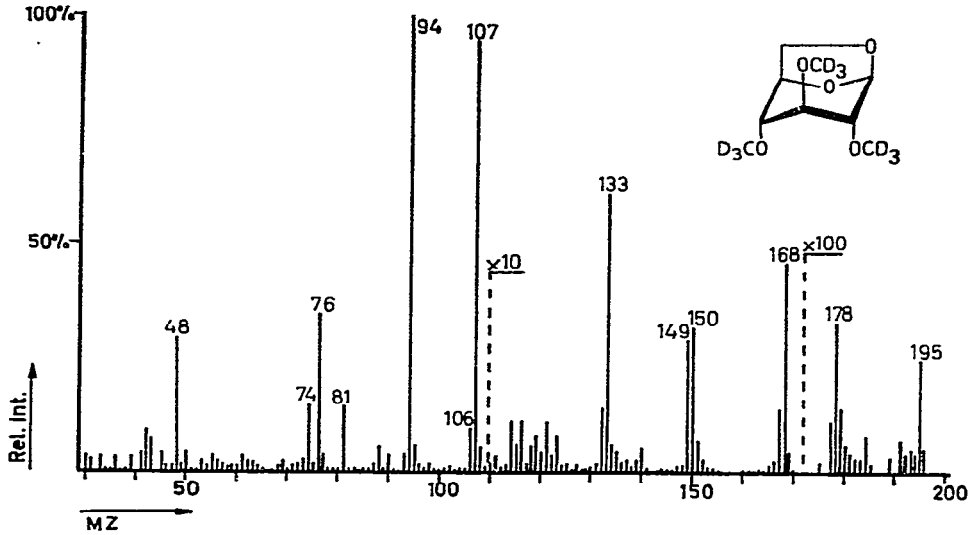


Abb. 7. 2,3,4-Tri-*O*-trideuteromethylglucosan.

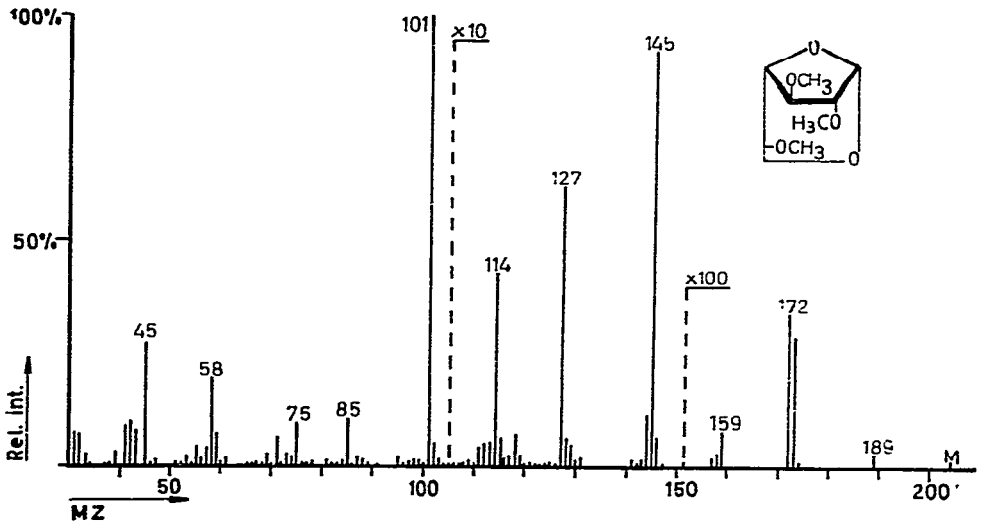
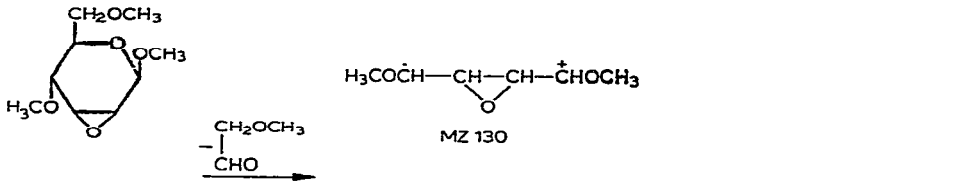
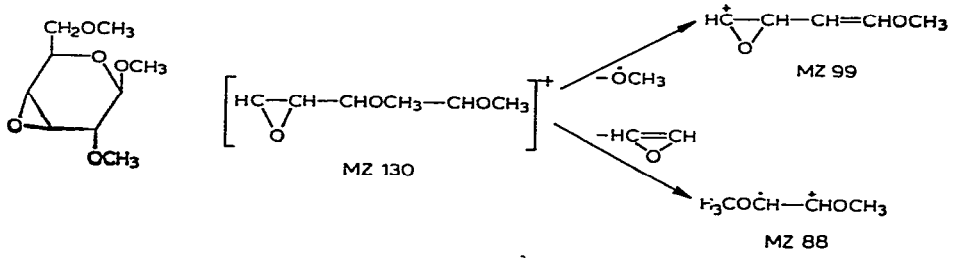


Abb. 8. 1,6-Anhydro-2,3,5-tri-*O*-methyl-α-D-galaktofuranose.

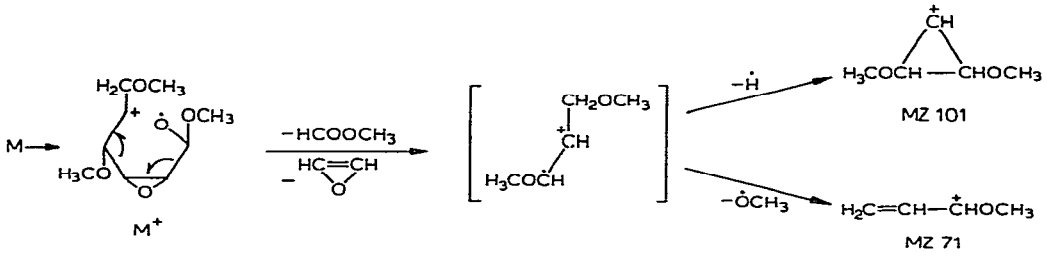
Die primäre Eliminierung von Methoxyacetaldehyd führt zu dem Radikal-Ion MZ 130:





Die Bildung des Radikal-Ions MZ 88 ist nur beim 3,4-Epoxid möglich; denn beim 2,3-Epoxid liegen keine Methoxygruppen an benachbarten C-Atomen vor.

Typisch für das Vorliegen eines 2,3-Anhydroringes ist die Bildung des Fragmentes MZ 101, das beim 3,4-Epoxid nicht gebildet werden kann:



Der energetisch günstigen Eliminierung von zwei Neutralteilchen folgt eine Stabilisierung des entstandenen labilen Radikal-Ions durch Abspaltung eines H-Radikals zum Cyclopropyl-Ion MZ 101 oder eines Methoxyradikals zum Allyl-Ion MZ 71.

Typisch für die Massenspektren der 2,3- bzw. 3,4-Anhydrozucker ist die hohe Intensität des Ions $H_2C = \overset{+}{O}CH_3$, MZ 45. Haupt-Ion im Spektrum des 3,4-Anhydroallosids ist das Umlagerungs-Ion $\overset{+}{H}C(OCH_3)_2$, MZ 75. Da bei den methylierten Hexopyranosiden der Anteil der Ionen der MZ 75, die durch eine 1,2-Wanderung

entstehen, nur gering ist, muß in dem gespannten bicyclischen System der 3,4-Anhydroverbindung eine die Wanderung begünstigende sterische Anordnung vorliegen.

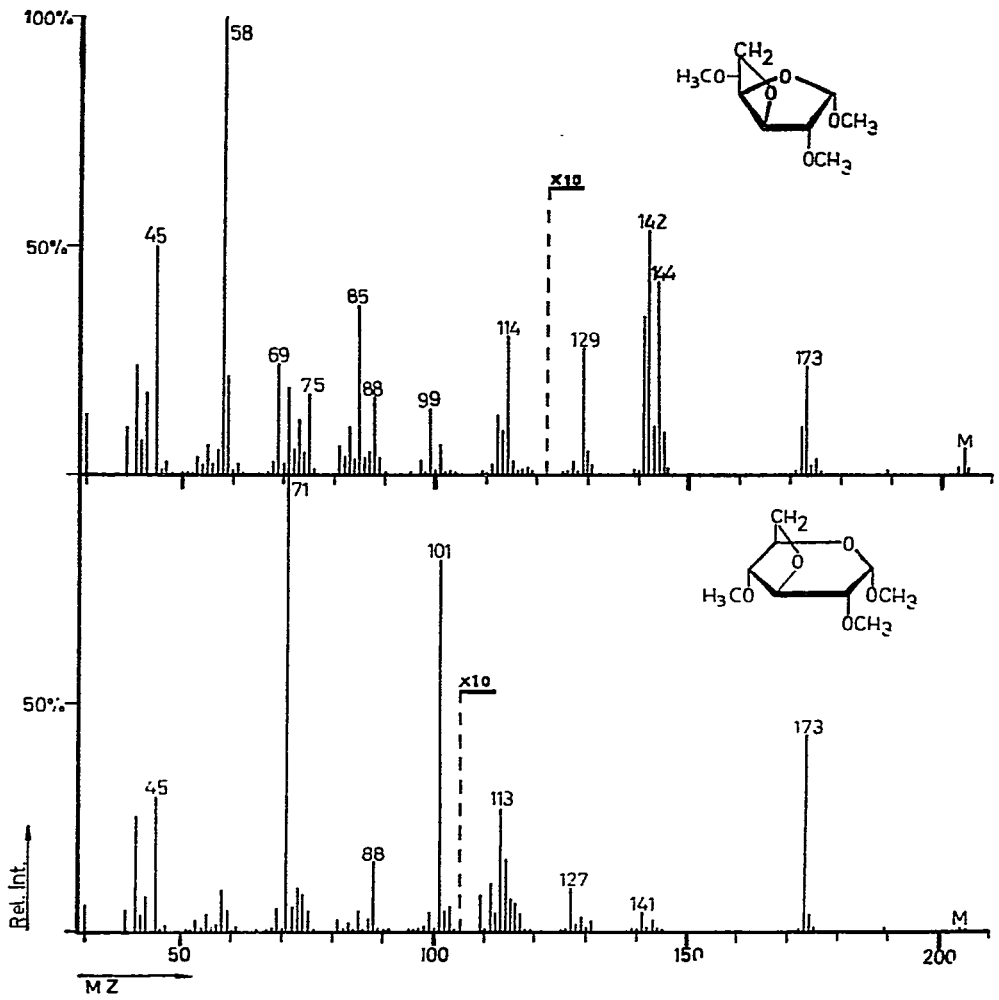


Abb. 9. Methyl 3,6-anhydro-2,4-di-*O*-methyl-α-D-glucopyranosid (unten).
Abb. 10. Methyl 3,6-anhydro-2,5-di-*O*-methyl-α-D-glucofuranosid (oben).

Dianhydrozucker

Als Beispiele für das massenspektrometrische Verhalten von Dianhydrozuckern wurden die Spektren folgender Verbindungen aufgenommen: 1,6:2,3-Dianhydro-β-D-talose (Abb. 13), 1,6:2,3-Dianhydro-4-*O*-methyl-β-D-talose (Abb. 14), 1,4:3,6-Dianhydro-β-D-glucose (Abb. 15), und 1,4:3,6-Dianhydro-2-*O*-methyl-β-D-glucose (Abb. 16).

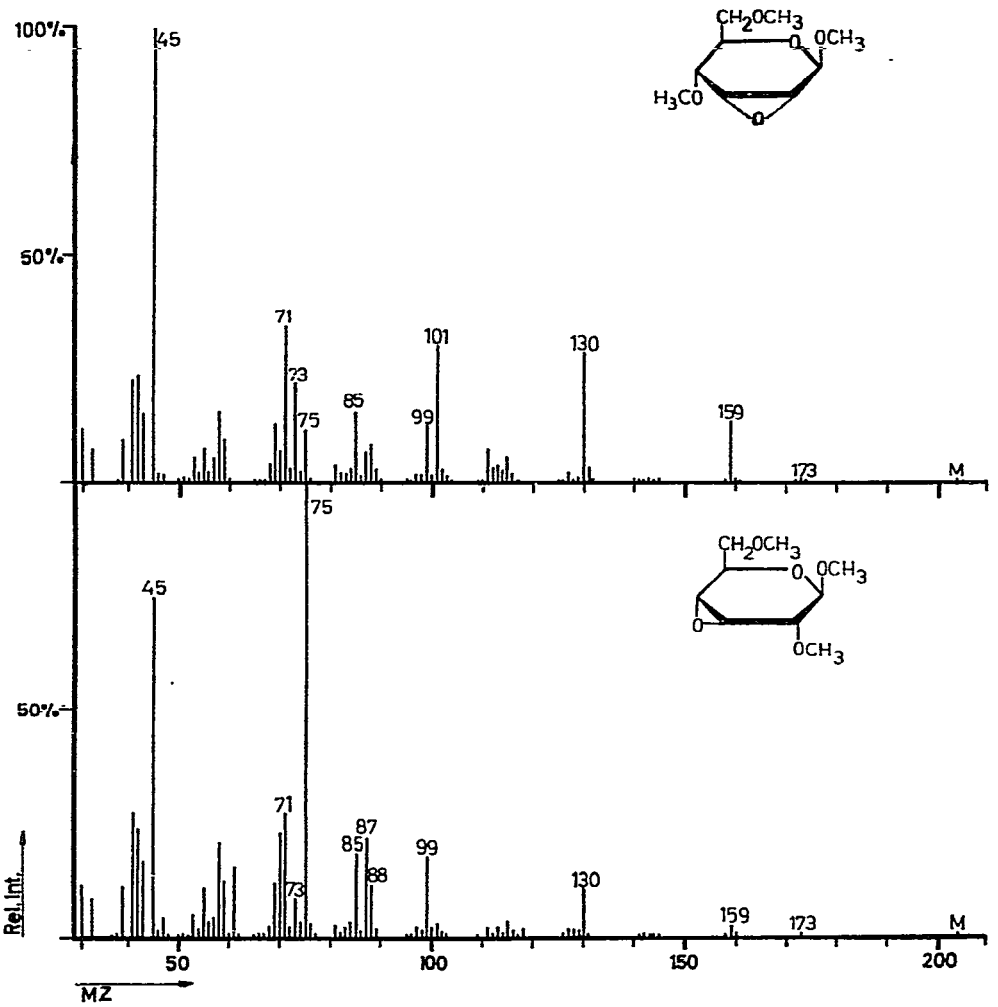


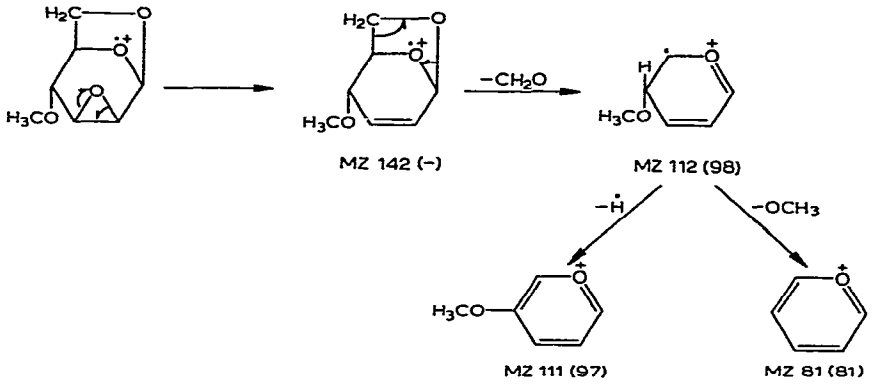
Abb. 11. Methyl 2,3-anhydro-4,6-di-*O*-methyl-β-D-allopyranosid (oben).

Abb. 12. Methyl 3,4-anhydro-2,6-di-*O*-methyl-β-D-allopyranosid (unten).

Die Dianhydrozucker haben genügende thermische Stabilität und ausreichende Flüchtigkeit, so daß die Massenspektren der freien Verbindungen erhalten werden können. Im folgenden werden die Spektren der Methyläther diskutiert. Die MZ der entsprechenden Ionen in den Spektren der freien Zucker sind in Klammern angeführt. Einzelheiten des Abbaumechanismus können nicht diskutiert werden, da diese tricyclischen Systeme sehr komplex fragmentieren. Die präparativ nur schwierig durchzuführende selektive C-D-Markierung wäre dazu notwendig.

*1,6 : 2,3-Dianhydro-4-*O*-methyl-β-*D*-talose (Abb. 14)*

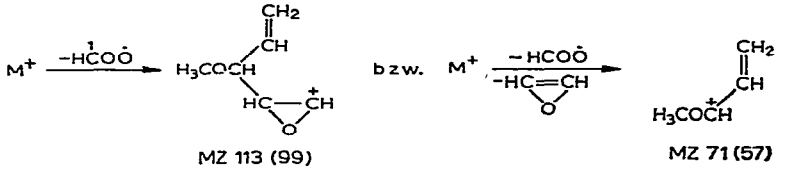
Unerwartet ist die primäre Eliminierung von Sauerstoff, die beim methylierten 2,3-Anhydroallosid nicht beobachtet wurde :



Die Sauerstoff-Eliminierung führt zu einem wesentlichen Nachlassen der Spannung des tricyclischen Ringsystems. Die Stabilisierung durch Verlust eines H-Radikals wird durch ein metastabiles Ion bei der MZ 110,0 belegt. Bei der Bildung des Ions MZ 81 zeigt sich ein deutlicher Einfluß des Substituenten an C-4. Wegen der energetisch günstigeren Abspaltung eines Methoxyradikals gegenüber einem OH-Radikal hat dieser Peak im Spektrum des 4-*O*-Methylderivates wesentlich größere Intensität.

Weitere Primärspaltungen sind die Abspaltung von $\dot{O}CH_3$ zur MZ 127 (beim freien Dianhydrozucker Verlust von $\dot{H}_2O$ zur MZ 126) und Abspaltung eines CHO-Radikals zur MZ 129 (115). Der Verlust von CHO wird durch ein metastabiles Ion bei der MZ 105,4 belegt.

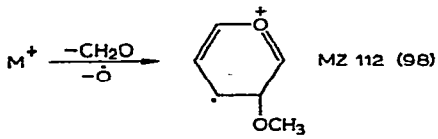
Die bei den 1,6-Anhydrozuckern beobachtete Abspaltung von $\overset{1}{H}C\dot{O}\dot{O}$ wird hier ebenfalls beobachtet:



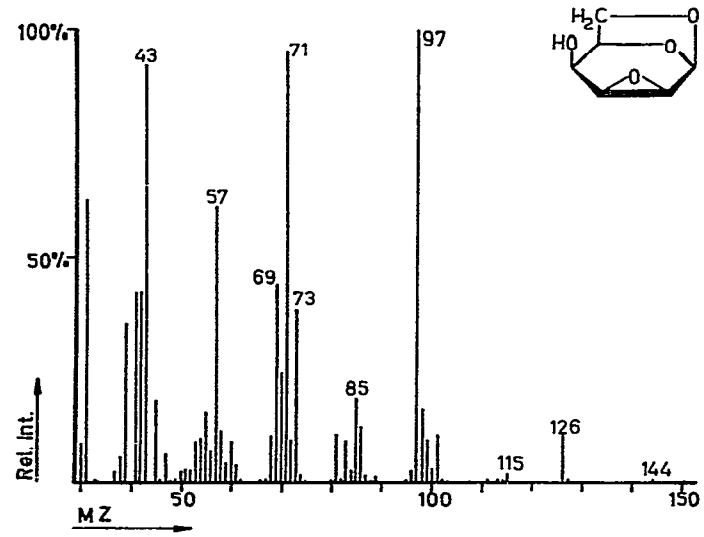
*1,4:3,6-Dianhydro-2-*O*-methyl-β-*D*-glucose (Abb. 16)*

Der primäre Abbau der 1,4:3,6-Dianhydro-2-*O*-methyl-β-D-glucose beginnt an der Methoxygruppe: M—$\dot{C}H_3$ (MZ 143) und M—$\dot{O}CH_3$ (MZ 127). Daneben wird die Eliminierung von Formaldehyd beobachtet: M—CH_2O → MZ 128 (114), eine Fragmentierungsreaktion, die bei den 3,6-Anhydrozuckern nicht beobachtet wurde.

Als Folgereaktion muß auch hier die Eliminierung von Sauerstoff angenommen werden, die zu dem intensiven Peak bei der MZ 112 (98) führt:



Eine primäre Sauerstoff-Eliminierung wie bei der 1,6:2,3-Dianhydro-4-*O*-methyl-*β*-D-talose erfolgt nicht.



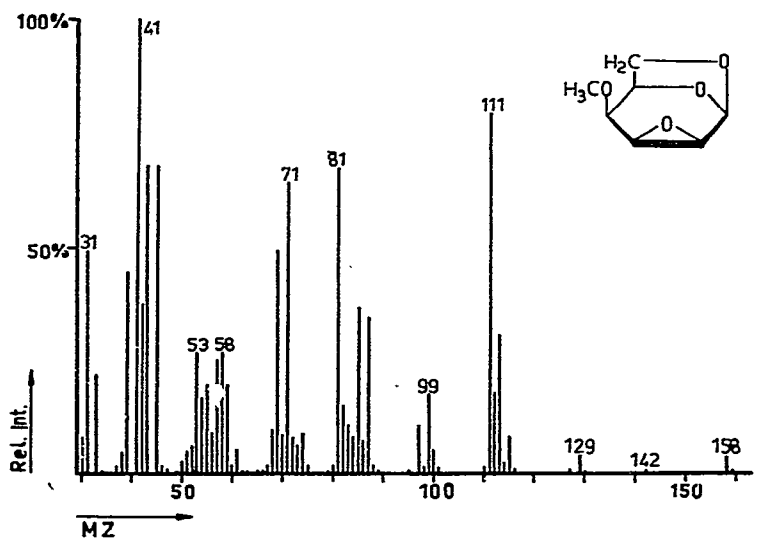
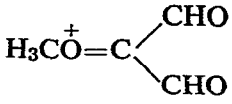


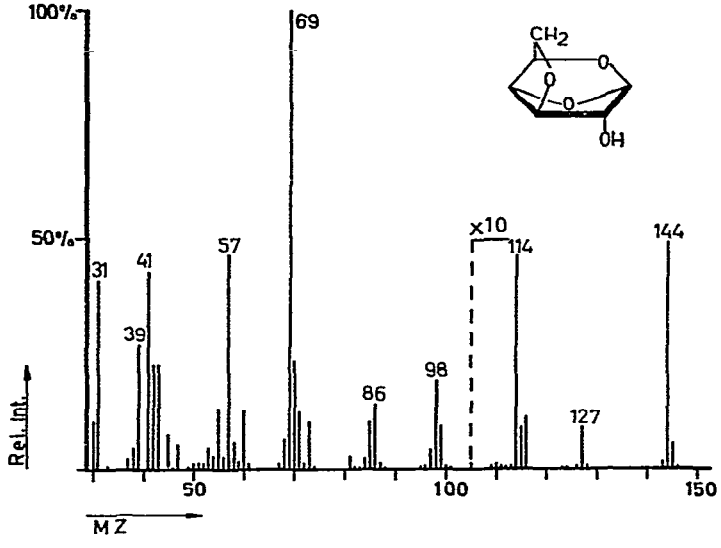
Abb. 13. 1,6:2,3-Dianhydro-β-D-talose (oben).

Abb. 14. 1,6:2,3-Dianhydro-4-*O*-methyl-β-D-talose (unten).

Überraschend war das Auftreten eines Peaks bei der MZ 101, da im Molekül nur eine Methoxygruppe vorhanden ist. Für dieses Ion wird die Struktur



angenommen.



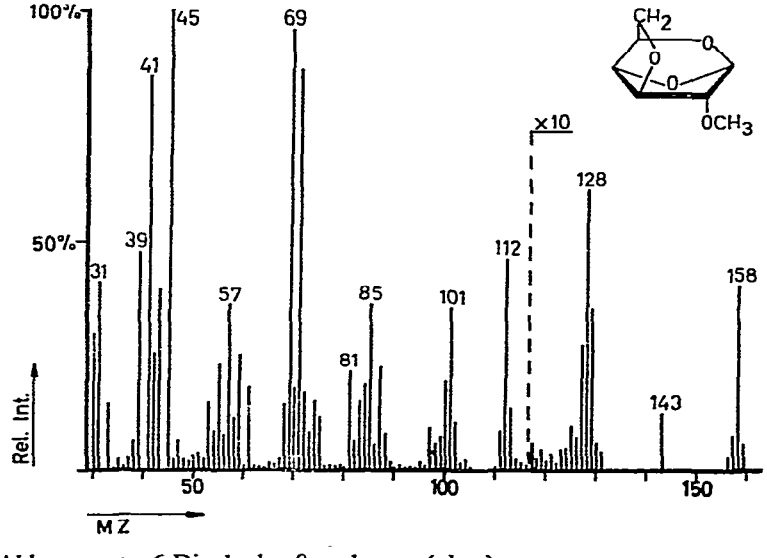
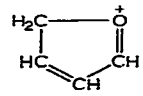


Abb. 15. 1,4:3,6-Dianhydro-β-D-glucose (oben).

Abb. 16. 1,4:3,6-Dianhydro-2-*O*-methyl-β-D-glucose (unten).

Der 3,6-Anhydroring stellt im Spektrum der Dianhydro-D-glucose ein stabiles Ion dar:



MZ 69 (69)

Experimenteller Teil

Die Massenspektren wurden bei einer Elektronenenergie von 70 eV mit einem Massenspektrometer Typ CH4 der ATLAS Mess- und Analysen-technik GmbH Bremen aufgenommen. Der Einlaß erfolgte über den Hochtemperatureinlaßteil bei 150°.

Die Darstellung der Anhydrozucker erfolgte nach in der Literatur beschriebenen Verfahren. Die Methylierung wurde nach Kuhn und Trischmann[5] durchgeführt. Sämtliche Verbindungen wurden molekulardestilliert und dünnschicht- bzw. gaschromatographisch auf Reinheit geprüft.

Zur Synthese der markierten Verbindungen wurde Trideuteromethyljodid der Firma K. Roth, Karlsruhe, verwendet.

Das 3,4-Di-*O*-methyl-2-*O*-trideuteromethylgalaktosan wurde durch Trideuteromethylierung von 3,4-*O*-Isopropylidengalaktosan, Abspaltung der Isopropylidengruppe und nachfolgende Methylierung dargestellt. Zur Synthese von 2-*O*-Methyl-3,4-di-*O*-trideuteromethylgalaktosan wurde zunächst methyliert und nach Entfernung des Isopropylidenrestes trideuteromethyliert.

Das 2,3-Di-*O*-methyl-4-*O*-trideuteromethylmannosan wurde nach dem gleichen Prinzip aus 2,3-*O*-Isopropylidenmannosan erhalten. Zur Synthese von 2,4-Di-*O*-methyl-3-*O*-trideuteromethylglucosan wurde 2,4-Di-*O*-benzylglucosan trideuteromethyliert, die Benzylreste entfernt und methyliert. Durch umgekehrte Anwendung der Methylierungsmittel wurde 3-*O*-Methyl-2,4-di-*O*-trideuteromethylglucosan erhalten.

Tri-*O*-trideuteromethylgalaktosan wurde durch Trideuteromethylierung von Galaktosan dargestellt.

DANK

Wir danken dem Bundesministerium für wissenschaftliche Forschung für die finanzielle Unterstützung dieser Arbeit.

ZUSAMMENFASSUNG

Es wurden die Massenspektren der Methyläther folgender Anhydrozucker-Typen aufgenommen: 1,6-Anhydro-, 3,6-Anhydro-, 2,3-Anhydro-, 3,4-Anhydro-, 1,6:2,3-Dianhydro-, und 1,4:3,6-Dianhydro-Zucker. Aus den Massenverschiebungen in den Spektren der selektiv mit Trideuteromethoxy-Gruppen markierten Verbindungen konnte bei den Glykosanen die Struktur und der Bildungsmechanismus

der charakteristischen Ionen angegeben werden. Die Massenspektren der isomeren Glykosane zeigen deutliche Intensitätsunterschiede und erlauben durch Vergleich mit Eichspektren eine sterische Zuordnung. Die für den Zerfall der permethylierten Pento- bzw. Hexo-side und Glykosane abgeleiteten Gesetzmäßigkeiten lassen eine weitgehende Deutung der Massenspektren der anderen bisher untersuchten Anhydrozucker zu.

Die Massenspektren der methylierten Anhydrozucker machen deutlich, daß die massenspektrometrische Untersuchung eine wertvolle Methode zur Bestimmung der Größe des Anhydroringes und des Halbacetalringes ist. Die folgende Tabelle zeigt, daß mit Ausnahme der 1,6-Anhydrozucker beide Ringgrößen allein durch die Bestimmung des Haupt-Ions eindeutig festgelegt werden können:

Anhydrozucker-Typ	*MZ des Haupt-Ions (im Massenbereich MZ 45–MZ 101)*
2,3	45
3,4	75
1,6 Furanose-Form	101 (—)
1,6 Pyranose-Form	101 (88)
3,6 Furanosid	58
3,6 Pyranosid	71
1,6:2,3	111
1,4:3,6	69

Die Tabelle vermittelt einen Eindruck der Leistungsfähigkeit der Massenspektrometrie bei Strukturuntersuchungen auf diesem Sektor der Kohlenhydratchemie.

SUMMARY

The mass spectra of methyl ethers of 1,6-, 3,6-, 2,3-, 3,4-, 1,6:2,3-di-, and 1,4:3,6-di-anhydro sugars have been measured. The fragmentation patterns and the structures of the characteristic ions can be deduced from the spectra of partially trideuteromethylated derivatives. The mass spectra of isomeric tri-*O*-methyl-1,6-anhydrohexoses show significant differences in the intensities of certain peaks, and comparison with standard spectra enables the stereochemistry to be determined.

The major fragmentation patterns outlined for pyranosides, furanosides, and 1,6-anhydrohexoses can be applied to a detailed interpretation of the mass spectra of other types of anhydro sugar. The mass spectra of permethylated anhydrosugars demonstrate the successful application of mass spectrometry in the determination of the size of anhydro and hemiacetal rings. The ring size may be determined by reference to the base peak (as shown below); for 1,6-anhydro sugars, reference is made to the peak at m/e 88.

Type of anhydro sugar	*m/e of the base peak (mass range, m/e 45–m/e 101)*
2,3-	45
3,4-	75
1,6- (furanose)	101 (—)
1,6- (pyranose)	101 (88)
3,6- (furanoside)	58
3,6- (pyranoside)	71
1,6:2,3-di-	111
1,4:3,6-di-	69

LITERATUR

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PURIFICATION OF *Pneumococcus* TYPES II AND V POLYSACCHARIDES

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INTRODUCTION

Type V *Pneumococcus* polysaccharide (SV) contains[1] residues of *N*-acetylated L-fucosamine (2-amino-2,6-dideoxy-L-galactose) and pneumosamine (2-amino-2,6-dideoxy-L-talose), D-glucose, and D-glucuronic acid. The presence of a high proportion of lipophilic 6-deoxy groups in the polysaccharide renders it especially difficult to purify by conventional methods. Type II *Pneumococcus* polysaccharide (SII) contains[2] 6-deoxy-L-mannose as the major constituent, together with D-glucose and D-glucuronic acid. The carbohydrate-containing entities likely to be encountered in the purification of type-specific *Pneumococcus* polysaccharides are glycolipids[3,4], the species-specific C-substance[5], F-carbohydrate[6] and its lipid complex, and glycogen[7]. Persistent impurities which tend to follow the type-specific polysaccharides during purification are a polyglutamic acid[8] and ribonucleic acid. Purification studies of the type-specific polysaccharide of *Pneumococcus* Types V and II have revealed the answer to some of these problems, particularly as they concern C-substance and ribonucleic acid.

EXPERIMENTAL

Analysis of Polysaccharides

Ultraviolet spectra were obtained using a Unicam SP 500 spectrophotometer and path lengths of 10 and 40 mm. Absorption at 260 mμ was used to assess maximal nucleic acid (NA) impurities, on the basis that 0.05 mg NA/ml had an optical density of 1.0 at 260 mμ in a 1-cm cell. 6-Deoxyhexoses and hexoses were determined essentially by Dische's cysteine–sulphuric acid reaction[9]. Amino sugars were analysed by the method of Svennerholm[10]. Hexuronic acids were determined by a modification of the carbazole reaction of Dische and Gregory[11]. Ester phosphorus was assayed by the method of Jones *et al.*[12]. Optical rotations were performed on aqueous solutions, at polysaccharide concentrations of 0.3 to 1 g/100 ml.

Amino acid analysis, on the fractions hydrolysed with 6N hydrochloric acid at 110° for 12 h under nitrogen, was effected using a Technicon Autoanalyser, which also revealed amino sugars.

Fractionation of Type V Pneumococcus *components*

(*a*) *Fractional precipitation with alcohol.* A crude mixture of the macromolecules obtained from Type V *Pneumococcus* (16.9 g, in 0.2M sodium chloride) was repeatedly deproteinised by being shaken seven times with 1,1,2-trichloro-1,2,2-trifluoroethane, and five times with chloroform–pentyl alcohol (9:1), and the solid protein gels were discarded. Most (665 ml) of the remaining aqueous phase (700 ml) was fractionally precipitated by the addition of ethanol, and the polysaccharide fractions were recovered at ethanol concentrations of 65% (7.96 g; $[\alpha]_D^{23}$ −32.6°; 1.3% NA), 70% (1.32 g, SV; $[\alpha]_D^{26}$ −87.6°; 0.6% NA), 75% (0.37 g, SV; $[\alpha]_D^{25}$ −100.3°; 0.6% NA), and from the supernatant (0.68 g. SV; $[\alpha]_D^{24}$ −106.3°; 1.7% NA). Analysis of the polysaccharide hydrolysates indicated the presence of a polyglutamate in the 65% and 70% fractions. After being redissolved in 0.2M sodium chloride, most (7.09 g; $[\alpha]_D^{24}$ −24.9°; 1.1% NA) of the 65% ethanol fraction was reprecipitated at the same alcohol concentration, but further SV (0.7 g; $[\alpha]_D^{24}$ −80°) was recovered from the supernatant.

(*b*) *Fractional precipitation with detergent.* The major unresolved fraction (7 g) was dissolved in water (700 ml), and cetyltrimethylammonium bromide added to a concentration of 2% w/v. The precipitated complex was dissolved in M sodium chloride, and polysaccharide recovered by precipitation with three volumes of ethanol; after treatment with Amberlite IR 120 (H^+ form), the product (3.96 g; 1.5% NA) had $[\alpha]_D^{25.5}$−35.2°. Detergent was removed from the supernatant by chloroform extraction, and the polysaccharide precipitated in the presence of 0.2M sodium chloride by adding three volumes of ethanol; after treatment with Amberlite IR 120 (H^+ form), the product (1.63 g; 0.4% NA) had $[\alpha]_D^{25}$−11.4°. The polysaccharide having $[\alpha]_D$−35.2° was again reprecipitated with detergent. The supernatant yielded mainly SV (0.38 g; $[\alpha]_D^{23.5}$ −70.1°; 0.8% NA), whilst the precipitated detergent complex was fractionated on the basis of its solubility in 0.25M sodium chloride to give fractions having $[\alpha]_D^{24.5}$ −38.9° (soluble; 2.27 g; 2% NA) and $[\alpha]_D^{24}$ +4.3° (0.07 g; 2.5% NA). Only a trace (0.02 g; $[\alpha]_D^{24}$ −15°) of the material having $[\alpha]_D$ −11.4° was again precipitable with detergent; some of it (0.76 g; $[\alpha]_D^{25}$ +11°; 0.5% NA) was recovered from the supernatant.

(*c*) *Fractionation on DEAE Sephadex A50.* Part (100 mg) of the polysaccharide mixture having $[\alpha]_D^{25}$ +6.7° was applied to a column of the ion exchanger (36.5 × 3.9 cm) equilibrated with phosphate buffer (pH 6; KH_2PO_4 0.0371M, Na_2HPO_4 0.0043M). After passage of buffer alone (116 ml), a sodium chloride gradient of 0→1M was applied, and the collected fractions (10 ml) were analysed with phenol–sulphuric acid to reveal a separation (Fig. 1) into several components. Tubes 9–16, 17–32, 33–65, and 76–108 were bulked and, after dialysis and freeze-drying, yielded components *A* (8.8 mg), *B* (12.7 mg), *C* (41.2 mg; $[\alpha]_D^{26}$ +37°), and *D* (34.6 mg; $[\alpha]_D^{20}$ −60°), respectively, with the properties shown in Table I. Passage of the SV fraction having $[\alpha]_D^{26}$−87.6° (100 mg) down the same column showed only a single peak (89 mg), eluted in the vicinity of component D, and having $[\alpha]_D^{23}$−81°. Only components *B* and *C* gave positive reactions with C-reactive protein in the presence of excess of calcium ions.

The C-reactive protein (kindly provided by Dr. Walton) contained no detectable hexose (< 0.03%), 0.63% of 6-deoxyhexose (estimated[9] as fucose), 0.15% of pentose (estimated[9] as xylose), and 1.83% of hexosamine (estimated[10] as glucosamine).

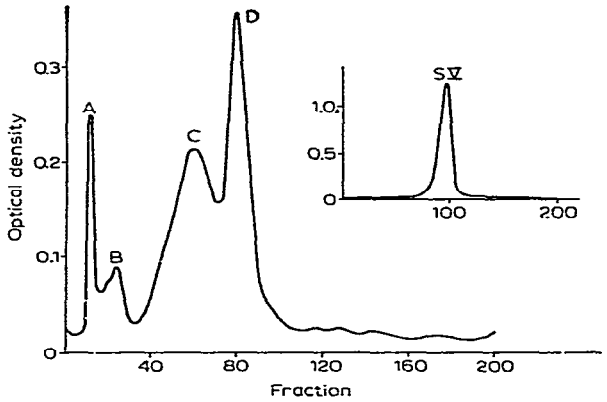

Fig. 1. Fractionation of *Pneumococcus* Type V components on DEAE Sephadex. ———Optical density at 490 mμ in the phenol–sulphuric acid reaction. Inset shows fractionation of SV fraction having $[\alpha]_D^{26}$ −87.6°.

Removal of Nucleic Acid from Type II Pneumococcus *Polysaccharide*

(*a*) *Fractionation on DEAE Sephadex.* Type II polysaccharide (1 mg), purified by deproteinisation, precipitation with cetyltrimethylammonium bromide, and

TABLE I

PROPERTIES OF TYPE V *Pneumococcus* FRACTIONS[a]

Component	A	B	C	SV[b]
Muramic acid	—	—	+	—
Organic P, %	1.87	1.76	3.62	1.90
Uronic acid, %	1.3	1.1	1.5	13.7
Amino sugars	Glucosamine, galactosamine	Glucosamine, galactosamine 6-phosphate	Glucosamine, galactosamine 6-phosphate	Pneumosamine, fucosamine
Glucose, %	none	2	2	10.9
Galactose, %	10.8	3.7	4.8	1.4
Total amino acids, %	8.26	3.55	2.60	1.31
Major constituent amino acids[c], %	Thr. 2.01	Glu. 0.51	Glu. 0.44	Glu. 0.27
	Ser. 1.33	Thr. 0.46	Thr. 0.41	Prol. 0.21
	Prol. 1.21	Ser. 0.43	Ala. 0.33	Ser. 0.17
	Glu. 0.79	Ala. 0.34	Lys. 0.29	Ala. 0.16
	Ala. 0.58	Prol. 0.30	Ser. 0.27	Gly. 0.14

[a] Liu and Gotschlich[13] report that crude C-substance contains lysine 1.29, serine 0.27, glutamic acid 1.6, alanine 1.63, D-glucosamine 11.32, D-galactosamine 6-phosphate 34.82, muramic acid 11.3, and muramic acid phosphate 4.35%.

[b] SV was obtained from the fractionation in Fig. 1.

[c] Thr., threonine; Ser., serine; Prol., proline; Glu., glutamic acid; Ala., alanine; Lys., lysine; Gly., glycine.

treatment with ribonuclease, as previously described[14], was passed down a DEAE Sephadex A-50 column (Cl^- form, bed volume 2.5 ml), and eluted with a linear gradient [NaCl, 0–1.0M, in phosphate buffer (pH 6.5, 0.005M)]. Scanning for 6-deoxyhexose[9] revealed that the polysaccharide was eluted as one peak at a volume of 70–86 ml.

Polysaccharide (13.6 mg) from the same batch, which had not been submitted to ribonuclease treatment, was passed down a similar column (17×1.2 cm; 20 ml bed volume), and a salt gradient applied, as above, over 1600 ml. Figure 2 shows that three peaks could be detected in the 3-ml fractions as follows: 1, at an elution

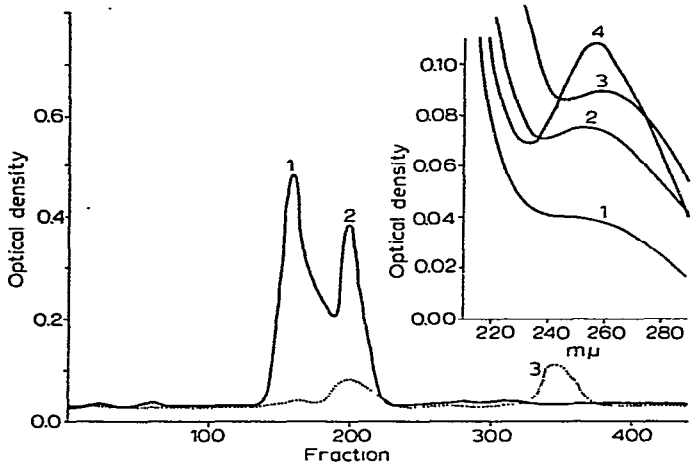

Fig. 2. Fractionation of *Pneumococcus* Type II polysaccharide on DEAE Sephadex A-50. ———Optical density at 400 mμ in the cysteine–sulphuric acid reaction. .....Optical density at 260 mμ. Inset shows the absorption spectra of the resultant peaks: 1, fraction 160; 2, fraction 200; 3, fraction 340; 4, original polysaccharide (680 μg/ml).

volume of 420–510 ml; 2, at 570–660 ml; and 3, at 990–1110 ml. Analyses of the bulked fractions, after dialysis and freeze-drying, are shown in Table II.

An aliquot of peak 2 was passed down the same column from which it had been obtained, and eluted under identical conditions. Two peaks (2.1 and 2.2) could be distinguished, eluted at volumes 435–510 ml and 615–660 ml, respectively, with the properties shown in Table II.

(*b*) *Fractionation on Sephadex G-200.* Type II polysaccharide (7.75 mg), before ribonuclease treatment, was passed down a Sephadex G-200 column (36.5×2 cm) which had been equilibrated with M sodium chloride, and eluted with the same solution. Analysis of fractions (2.6 ml) for hexuronic acids[11] and u.v. absorption (260 mμ) showed (Fig. 3) that the polysaccharide (peak 1) was completely excluded from the gel in fractions 14–21, and most of the nucleic acid (peak 2) in fractions 41–49. Properties of the bulked, dialysed fractions are shown in Table II.

DISCUSSION

In a previous study[15] of SV, it was found that, despite its acidic character, the polysaccharide was incompletely precipitated by commercial cetyltrimethylammonium bromide. Furthermore, a significant amount of SV dissolved during subsequent alcohol washing of the polysaccharide–detergent complex to remove detergent, or was not precipitated in attempts to recover the polysaccharide from

TABLE II

ANALYSIS OF TYPE II *Pneumococcus* FRACTIONS

Type II Specific Polysaccharide	*Glucose, %*	*Sodium glucuronate, %*	*Rhamnose, %*
Before ribonuclease treatment[a]	35	16.45	48.5
DEAE Sephadex. Peak 1	35.1	16.5	48.3
2	34.9	16.45	48.6
3	0	0	0
Peak 2 refractionated. Peak 2.1	35.1	16.39	48.4
2.2	35.1	16.41	48.4
Sephadex G-200. Peak 1	35.2	16.4	48.4
2	0	0	0
After ribonuclease treatment[b]	35.1	16.4	48.5

[a] Optical density at 260 mμ, using 40-mm path length, for a 1 mg/ml solution of the fraction, was 0.470; [b] 0.030.

the supernatant remaining after detergent precipitation. As the first step in the present purification, the crude deproteinised mixture, in aqueous salt solution, was fractionally precipitated with increasing volumes of ethanol, to afford *ca.* 30% of the mixture

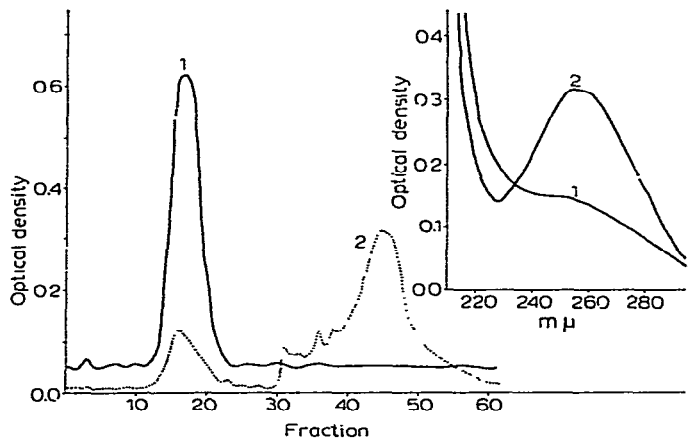

Fig. 3. Fractionation of *Pneumococcus* Type II polysaccharide on G-200 Sephadex. ———Optical density at 530 mμ in the carbazole reaction. .....Optical density at 260 mμ. Inset shows the absorption spectra of the resultant peaks: 1, fraction 16; 2, fraction 45.

as relatively pure SV ($[\alpha]_D$ −87.6→ −106.3°). Subsequent attempts to fractionate the remainder with detergent were only partially successful, since two such refractionations yielded only a further 5% of SV ($[\alpha]_D$ −70°). Losses were again encountered, despite the fact that the detergent–polysaccharide precipitate was dissociated in molar saline before alcohol precipitation and, in recoveries from the supernatant after detergent addition, most of the latter was removed by chloroform extraction before alcohol precipitation of the aqueous phase.

The most rewarding technique for separating the components in one operation, with near quantitative recoveries, was gradient elution from a DEAE Sephadex column, followed by dialysis and freeze-drying. With one fraction, which could not be appreciably further fractionated by either alcohol or detergent, four major components could be clearly distinguished. The first was largely protein in nature, the second and third gave reactions for C-substance, and the fourth was SV. A fraction previously thought to be SV, obtained as above by alcohol fractionation, was homogeneous on this column. The two fractions showing a positive reaction with C-reactive protein differed mainly in that muramic acid was present in one, and not in the other, suggesting complex formation between a cell-wall mucopeptide and C-substance. A preliminary report[16], without experimental details, has claimed that C-substance can be separated into several chemically differentiable, although serologically similar, components.

In attempts to remove the last persistent nucleic acid impurity in Type II *Pneumococcus* specific polysaccharide, it was shown that DEAE Sephadex is able to dissociate complexes. When a salt gradient was applied, polysaccharide, polysaccharide–nucleic acid complex, and nucleic acid were eluted in that order. Subsequent passage of the polysaccharide–nucleic acid complex down the same column caused further dissociation into more polysaccharide and polysaccharide–nucleic acid complex. For a larger-scale preparation (400 mg), a Sephadex G-200 column has been used, since, while the polysaccharide, largely freed from nucleic acid, was completely excluded from the gel, the nucleic acid which had dissociated in molar saline was eluted subsequently.

ACKNOWLEDGEMENTS

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SUMMARY

DEAE Sephadex has been shown to afford separation of Type V *Pneumococcus* specific polysaccharide, C-substance, and other components, when alcohol fractionation or detergent precipitation were only partially effective. Dissociable complex formation between Type II polysaccharide and nucleic acid has been demonstrated

on the same column. Sephadex G-200 can be used to remove nucleic acid impurities from Type II *Pneumococcus* polysaccharide.

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STRUCTURE OF A POLYSACCHARIDE FOUND IN THE CORM-SACS OF *Watsonia pyramidata* (ANDR.) STAPF.

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INTRODUCTION

Polysaccharide exudates found on the stems of plants representative of a large number of different species have been investigated in recent years[1]. These substances are usually acidic by virtue of hexuronic acid residues bound to neutral sugars in highly complex, branched, molecular structures. The majority are based on a branched galactopyranose framework, some upon chains of galactopyranuronic acid residues interspersed with rhamnopyranose residues, and a small group upon a xylan framework, to which are attached short chains of arabinose, galactose, and glucuronic acid residues[2].

This paper describes a structural investigation of the polysaccharide found during the late summer months in the corm-sacs of *Watsonia pyramidata* (Andr.) Stapf. (Order, Liliales; family, Iridaceae). The polysaccharide occurs internally within the corm, which is technically a stem, and, to judge by its disappearance during growth, appears to be used by the plant as a food reserve[3]. Whilst it is not a gum exudate in the strict sense, there seemed to be sufficient link with plant-gum exudates in general to make a structural comparison worth while.

EXPERIMENTAL

General experimental conditions

Paper chromatography was carried out on Whatman No. 1 paper with the following solvent systems (all v/v): (*a*) butan-1-ol–ethanol–water (4:1:5, upper layer), (*b*) ethyl acetate–acetic acid–formic acid–water (18:3:1:4), (*c*) ethyl acetate–pyridine–water (10:4:3), (*d*) butan-1-ol–acetic acid–water (2:1:1), (*e*) propan-2-ol–acetic acid–water (7:1:2), (*f*) butan-2-one–water azeotrope, (*g*) butan-2-one–acetic acid–saturated, aqueous boric acid (9:1:1). Paper ionophoresis was carried out for 4 h at 10 v/cm in 0.2M-borate buffer[4] at pH 9.2. R_{gal}, R_G, and M_g refer to rates of movement relative to galactose, 2,3,4,6-tetra-*O*-methylglucose, and glucose, respectively. Gas–liquid chromatography (g.l.c.) was carried out on a Beckman GC-2A instrument, using helium as carrier gas, with a 4-foot column of ethylene glycol succinate (14%

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on 80–100 mesh Gas Chromosorb W)[5] at 155°, and a flame ionization detector. Retention times[6] were measured relative to methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucoside. Unless otherwise stated, solutions were concentrated at 40°/20 mm in a rotary evaporator, specific rotations were measured on aqueous solutions at *ca.* 20°, melting points are uncorrected, and sugars were revealed on paper chromatograms by spraying with the *p*-anisidine hydrochloride reagent[7] and assayed by the phenol–sulphuric acid procedure[8].

Purification of Watsonia *corm-sac polysaccharide*

The jelly-like polysaccharide (air-dried, 216 g), excised from the exposed sacs of sliced *Watsonia* corms (790, collected in March of 1963 and 1964), was homogenised with water (25 l) and, by the slow addition of ethanol (75 l), the bulk of the material was precipitated as a colourless, stringy mass. Reprecipitation gave fraction *A* (142 g), which, after being washed with ethanol and ether, and dried (for 4 h at 56°/1 mm), had $[\alpha]_D$ −80° (*c* 0.3), equiv. 19,800 (Found: sulphated ash, zero; Ac, zero; OMe, 0.4; N, 0.4%). These constants were unchanged upon further precipitation from aqueous solutions with ethanol. The aqueous-ethanolic mother liquors were evaporated, and the residue was extracted with acetone to yield soluble fraction *B* (0.3 g), $[\alpha]_D$ −17° (*c* 1.03); and insoluble fraction *C* (3.6 g), $[\alpha]_D$ −88° (*c* 1.14), with other constants not significantly different from those of fraction *A*. Portions of each fraction were hydrolysed, and examined by paper chromatography. Fractions *A* and *C* gave similar proportions (visually estimated) of arabinose (3), xylose (1), and galactose (1). Fraction *B* gave glucose (6), galactose (2), and arabinose (1).

Sub-fractionation of fraction *A* [1.36% aqueous solution, passed first through a column of Amberlite IR-120 (H^+ form)] was attempted as follows: (*a*) Ethanol was added to a portion of the solution; this precipitated all of the polysaccharide therein between ethanol concentrations of 65 and 70%. The precipitate so formed was indistinguishable from fraction *A*. (*b*) A solution of polyethyleneglycol in water, when added to the gum solution, precipitated all of the polysaccharide within narrow limites. (*c*) Cetyltrimethylammonium bromide gave no precipitate from an aqueous solution of the polysaccharide (containing 340 mg of gum) at[9] pH 7.0, but a fibrous precipitate (320 mg) was formed at[10] pH 8.5. The supernatant from this fraction afforded a further precipitate (10.3 mg) between pH 11.0 and 12.0. The two precipitates were not significantly different from each other, nor from the original fraction *A*. (*d*) A portion of the polysaccharide solution (containing 180 mg of polysaccharide) was made 0.005M with respect to phosphate buffer (pH 6.0), and added to a column of diethylaminoethylcellulose powder[11] (equilibrated at pH 6.0 with 0.005M phosphate buffer). Elution with the same buffer gave a single carbohydrate component (98 mg) which was indistinguishable from fraction *A*. Stepwise elution up to 0.25M phosphate buffer (pH 6.0) gave no further carbohydrate, but gradient elution with sodium hydroxide gave a second fraction (38 mg), eluted with 0.5N sodium hydroxide). This fraction, after removal of sodium ions, had $[\alpha]_D$ −7° (*c* 1.2), equiv. 600 (Found: carbohydrate, 5%). Hydrolysis of these two fractions afforded the same sugars as

fraction *A*, in the same molar proportions. (*e*) Chromatography of a portion of the polysaccharide solution (containing 2.7 g of polysaccharide) on Duolite A4 (OH^- form) afforded two fractions, one eluted with water (2.58 g) and identical with fraction *A*, and the other eluted with 0.5N sodium hydroxide. The second fraction, after removal of sodium ions, weighed 104 mg and had $[\alpha]_D -7°$ (*c* 1.1), equiv. 610 (Found: sulphated ash, 32.2; carbohydrate, 19%). The hydrolysis products of the second fraction were identical with, and in the same molar proportions as, those from an hydrolysate of fraction *A*. (*f*) Chromatography of the polysaccharide (34 mg) on Sephadex G.50 (medium grade) afforded two fractions, containing 30 mg and 4 mg of carbohydrate, respectively. Neither fraction was substantially different from fraction *A*, though separation on Sephadex does indicate a difference in molecular weight. Chromatography on Sephadex G.50 of the water-eluted fraction from Duolite A4 afforded only one component. Fraction *A*, and the water-eluted fraction from Duolite A4, each gave a single peak on percolation through Sephadex G.25 (medium grade), indicating that the second component of fraction *A*, eluted from Sephadex G.50, had a molecular weight between 5,000 and 10,000.

Partial acid hydrolysis of Watsonia *corm-sac polysaccharide (See the flow-sheet Table IV)*

The polysaccharide (30 g) was homogenised in 0.01N sulphuric acid, and hydrolysed (each step for 7 h at 96°). During this initial hydrolysis, $[\alpha]_D$ changed from −80 (initial) to +53° (final). The neutralised hydrolysate was concentrated to a small volume, and when made 80% with respect to methanol, afforded a precipitate and a syrup. This precipitate was further hydrolysed, in a similar manner, yielding again fractions soluble and insoluble in 80% methanol. This procedure was repeated on the precipitated material twice more, yielding finally syrups 1,2,3, and 4, and precipitate 4.

Syrup 1 (21.7 g), from which crystalline L-arabinose (1.83 g) had been removed after trituration with methanol, was separated into the following components on a column of charcoal–Celite[12] (85 × 4 cm), using water and ethanol for elution.

Fraction I. Crystalline L-arabinose (4.699 g), m.p. and mixed m.p. 159.5° (after recrystallisation from ethanol), $[\alpha]_D +174$ (6 min)→+106° (60 min, constant; *c* 0.72). The derived benzoylhydrazone[13] had m.p. and mixed m.p. 199–200° (dec.).

Fraction II. A syrup (396 mg) containing arabinose, xylose, and galactose in the ratio 7:4:2 (visual estimation of paper chromatograms).

Fraction III. A syrup (618 mg, eluted with water), $[\alpha]_D +80°$ (*c* 0.84), which crystallised from aqueous methanol to give α-D-galactose, m.p. and mixed m.p. 165°, $[\alpha]_D +124$ (15 min)→+78° (equil.; *c* 1.09); derived 2-methyl-2-phenylhydrazone[13], m.p. and mixed m.p. 186°.

Fraction IV. A syrup (3.694 g, eluted with 4% ethanol), $[\alpha]_D +182°$ (*c* 5.78), M_g 0.75, R_{gal} 0.52, 0.78, 0.76 (in solvents *a*, *b*, and *c*, respectively), identified as described earlier[14] as 3-*O*-α-D-galactopyranosyl-L-arabinose.

Fraction V. A syrup (2.615 g, eluted with 9% ethanol), $[\alpha]_D +69°$ (equil.;

c 5.23), R_{gal} 0.32, 0.65, 0.63 (in solvents *a*, *b*, and *c*). Hydrolysis of a portion (2 mg) of this fraction gave first galactose, arabinose, and the disaccharide present in fraction IV, and finally galactose and arabinose in the molar ratio 1:2.1. Sodium borohydride reduction of the syrupy trisaccharide (14 mg) gave the non-reducing glycitol (10 mg), hydrolysis of a portion (3.3 mg) of which gave galactose, arabinose, arabinitol, and the disaccharide present in fraction IV (paper chromatography in solvents *a* and *g*). Oxidation of a further portion (6.68 mg) of the glycitol with periodate yielded, after 20 h, formaldehyde (1.06 mol.) and formic acid (1.9 mol.); reduction of the periodate-oxidised material, followed by hydrolysis, gave arabinose and glycerol.

The syrup (270 mg), on being treated by the Haworth and Purdie procedures, gave a fully methylated product (285 mg, after being dried to constant weight at 40°/1.6 cm), $[\alpha]_D$ +41° (*c* 5.21, chloroform). Hydrolysis of the methylated trisaccharide gave: 2,3,4,6-tetra-*O*-methylgalactose, 2,5-di-*O*-methylarabinose, 3,5-di-*O*-methylarabinofuranose, and 3,4-di-*O*-methylarabinopyranose (all identical with standards in solvents *a* and *f*). G.l.c. of the methanolysed, methylated trisaccharide confirmed the above identifications, giving retention times (of the methyl glycosides) as follows: 2,3,4,6-tetra-*O*-methylgalactose, 2.02; 2,5-di-*O*-methylarabinose, 2.33s, 4.63w; 3,5-di-*O*-methylarabinofuranose, 1.28, 3.21; and 3,4-di-*O*-methylarabinopyranose, 2.82.

As would be expected with a 2-linked, reducing end-group, attempted alkaline degradation, using the method developed by Painter[15], was unsuccessful; no degradation occurred when the trisaccharide (4.7 mg) was heated in 0.005N sodium hydroxide (20 ml) for 90 min at 74°. All of this evidence is consistent with the trisaccharide's being *O*-α-D-galactopyranosyl-(1→3)-*O*-L-arabinofuranosyl-(1→2)-L-arabinose. Hudson's rules[3] indicate that the link between the two arabinose residues is α.

Fraction VI. The syrup (632 mg, eluted with 70% methanol), $[\alpha]_D$ −40° (*c* 0.50), contained components all chromatographically slower than fraction V, and having R_{gal} values of 0.00, 0.02, 0.05, 0.08, 0.13, and 0.40 in solvent *e*. Total hydrolysis of the syrup, and chromatography of the hydrolysate, showed xylose to be the only sugar residue present in these oligosaccharides. Paper chromatography against standards showed them to be identical with (all R_{gal} values in solvent *e*): xyloheptaose, 0.02; xylohexaose, 0.05; xylopentaose, 0.08; xylotetraose, 0.13; and xylotriose, 0.40. These identities were further supported by paper chromatography in solvents *a*, *c*, and *d*, and by plotting a graph of [log $1/R_{gal}$] − 1 against suspected degree of polymerisation. (To give a sufficient number of points, xylose and xylobiose were chromatographed together with fraction VI in each of the four solvents). Straight lines were obtained[16] using R_{gal} values (Table I) in solvents *a*, *c*, *d*, and *e*.

In addition to Fractions I–VI, there were five overlapping fractions which consisted only of mixtures of these fractions, except for a small amount of material (*ca.* 30 mg) heavily contaminated with fraction IV (*ca.* 200 mg) and having R_{gal} 0.60, 1.09, and 0.75 in solvents *a*, *c*, and *d*, respectively. This was later shown to be chromatographically identical with 4-*O*-β-D-xylopyranosyl-D-xylose.

The fraction containing the xylose oligomers (fraction VI) was combined with syrups 2,3, and 4 (total weight 4.2 g), and evaporated to a small bulk. This solution was placed on a charcoal–Celite column (85×4 cm), and eluted, first with water, and then stepwise with water containing increasing concentrations of ethanol. Seven major and a number of overlapping fractions were obtained.

TABLE I

PAPER CHROMATOGRAPHIC DATA ON XYLOSE OLIGOSACCHARIDES

Suspected degree of polymerisation	R_{gal}			
	Solvent a	*Solvent c*	*Solvent d*	*Solvent e*
1	1.67	1.78	1.64	1.33
2	0.60	1.09	0.75	0.84
3	0.18	0.53	0.32	0.40
4	0.07	0.24	0.12	0.13
5	—	0.11	0.05	0.08
6	—	0.03	—	0.05
7	—	—	—	0.02

Fraction 1. A syrup (442 mg, eluted with water) which showed three components on paper chromatography. Cellulose column chromatography of the syrup (elution with solvent *c*) afforded: *Fraction 1a.* A syrup (68 mg) which crystallised and, on recrystallisation from 80% methanol, gave D-xylose, m.p. and mixed m.p. 150°, $[\alpha]_D$ +90 (2 min)→+21° (50 min, constant; *c* 1.35). The derived di-*O*-benzylidene dimethyl acetal had m.p. and mixed m.p. 210°, and $[\alpha]_D$ −8° (*c* 0.7, chloroform). *Fraction 1b.* A syrup (330 mg), $[\alpha]_D$ +107° (*c* 1.2), which was chromatographically identical with arabinose. *Fraction 1c.* A syrup (45 mg), $[\alpha]_D$ +80° (*c* 1.1), which was chromatographically identical with galactose.

Fraction 2. A syrup (54 mg, eluted with water), $[\alpha]_D$ +78° (*c* 0.6), which was chromatographically homogeneous in solvents *a*, *b*, and *c*, and identical with galactose. Crystallisation and recrystallisation from glacial acetic acid yielded α-D-galactose, m.p. and mixed m.p. 166°.

Fraction 3. A syrup (422 mg, eluted with 5% ethanol), $[\alpha]_D$ +184° (*c* 2.3), which was chromatographically identical with 3-*O*-α-D-galactopyranosyl-L-arabinose in solvents *a*, *b*, and *c*.

Fraction 4. A syrup (114 mg, eluted with 6% ethanol) which showed two components on paper chromatography in solvents *a*, *b*, and *c*, one identical with fraction 3, and the other identical with an authentic sample of 4-*O*-β-D-xylopyranosyl-D-xylose. The syrup was fractionated on a cellulose column (solvent *c*) into chromatographically homogeneous (solvents *a* and *b*) *Fractions 4a* (identical with fraction 3) and *4b*.

Fraction 4b was a syrup (92 mg), $[\alpha]_D$ −27° (*c* 0.67), and had R_{gal} 0.60, 0.75, and 0.84 in solvents *a*, *d*, and *e*, respectively. Hydrolysis of a portion of the syrup (5 mg) in N sulphuric acid for 5 h at 96° yielded only xylose. The phenylosazone of

the syrup had m.p. 208.6° (dec.) (lit.[17], xylobiose phenylosazone, m.p. 195–196°). Periodate oxidation of the osazone (1.41 mg) for 2 h yielded no formaldehyde and no precipitate of the 1,2-bisphenylhydrazone of mesoxalaldehyde, whilst 1.06 mol. of formic acid were liberated. These results are consistent only if the disaccharide is 4-*O*-β-D-xylopyranosyl-D-xylose (xylobiose). Acetylation of the syrup (33 mg) yielded a product (29 mg) which, on crystallisation and recrystallisation from equal volumes of ethanol and light petroleum (b.p. 60–80°), gave 4-*O*-β-D-xylopyranosyl-D-xylose hexa-acetate, m.p. and mixed m.p. 155–156°, $[\alpha]_D$ −70° (*c* 0.66, chloroform) (lit.[18] −74°). T.l.c.[19] of the acetate on silica gel G showed it to be identical with the authentic sample.

Fraction 5. A syrup (332 mg, eluted with 13% ethanol), $[\alpha]_D$ +70° (*c* 2.8), which was chromatographically homogeneous and identical with *O*-α-D-galactopyranosyl-(1→3)-*O*-L-arabinofuranosyl-(1→2)-L-arabinose in solvents *a*, *b*, and *c*.

Fraction 6. A syrup (246 mg, eluted with 18% ethanol), $[\alpha]_D$ −38° (*c* 2.0), which was chromatographically homogeneous in three solvents, and on hydrolysis (0.01N sulphuric acid for 5 h at 96°) gave only xylobiose and xylose. Crystallisation and recrystallisation of a portion of the syrup from 85% ethanol afforded *O*-β-D-xylopyranosyl-(1→4)-*O*-β-D-xylopyranosyl-(1→4)-D-xylose (xylotriose), $[\alpha]_D$ −45° (*c* 0.5), m.p. 203° (softening at 154°), mixed m.p. 204° (softening at 156°). The phenylosazone had m.p. 218–219° (dec.) (lit.[17], xylotriosazone, m.p. 214–215°). A portion of the mother liquors from the crystallisation of the xylotriose (46 mg) was acetylated, and gave xylotriose octa-acetate, m.p. and mixed m.p. 111°, $[\alpha]_D$ −90° (*c* 0.4, chloroform). T.l.c. on silica gel G showed the acetate to be identical with the standard.

Fraction 7. A syrup (114 mg, eluted with 80% methanol) which was chromatographically similar to fraction VI, and contained a series of xylose oligomers with degree of polymerisation from four to six. The graph of $[\log 1/R_{gal}]-1$ against suspected degree of polymerisation was again linear for solvents *d* and *e*.

Precipitate 4. This degraded polysaccharide (670 mg), $[\alpha]_D$ −42° (*c* 0.90, 0.5N sodium hydroxide), was sparingly soluble in water, but soluble in dilute alkali. Hydrolysis gave xylose, arabinose, and glucose, in the ratio 11:trace:1. Starch appeared to be present judging by the blue colouration given by the polysaccharide with iodine. Methylation of the degraded polysaccharide (308 mg) gave a derivative (192 mg

TABLE II

G.L.C. DATA ON PRODUCTS FROM METHYLATED, RESIDUAL XYLAN

Methyl glycoside of	T^a	*Approx. molar proportion*
2,3,4-Tri-*O*-methylxylose	0.467, 0.607	20
2,3-Di-*O*-methylxylose	1.78, 1.99, 2.23	160
2,3,4,6-Tetra-*O*-methylglucose	1.00, 1.54	3
2,3,6-Tri-*O*-methylglucose	4.22, 6.16	15

[a] Retention times relative to methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucoside.

after being dried at 60°/0.4 mm for 2 h), $[\alpha]_D$ −38° (*c* 1.48, chloroform) (Found: OMe, 38.0%), which on hydrolysis (N sulphuric acid at 96° for 5 h) yielded 2,3,4,6-tetra-*O*-methylglucose (R_G 1.00), 2,3,4-tri-*O*-methylxylose (R_G 0.94), 2,3,6-tri-*O*-methylglucose (R_G 0.85), and 2,3-di-*O*-methylxylose (R_G 0.78) (solvent *a*). These identities were confirmed by g.l.c. (Table II).

2,3-Di-*O*-methylxylose was obtained from a portion of the above hydrolysate (150 mg) by preparative paper chromatography in solvent *a* and had m.p. and mixed m.p. 77–79°; the "anilide" had m.p. 123–123.5°.

The quantities of the various mono-, di-, tri-, and oligo-saccharides recovered from the columns (including the contributions from overlapping fractions) are presented in Table III (Fractions I→VI and 1→7, and combined totals from the first and second columns). The flow-sheet (Table IV) gives details of the mild acid hydrolytic procedure.

TABLE III

SUGARS ISOLATED FROM PARTIALLY HYDROLYSED *Watsonia* POLYSACCHARIDE

Sugar	*Weight recovered (mg)*		*Total weight recovered (mg)*
	Fractions I→VI	*Fractions 1→7*	
D-Xylose	39	68	107
L-Arabinose	4699	330	5029
D-Galactose	618	100	718
3-*O*-α-D-Galactopyranosyl-L-arabinose	5559	422	5981
Xylobiose	30	92	122
O-α-D-Galactopyranosyl-(1→3)-*O*-α-L-arabinofuranosyl-(1→2)-L-arabinose	2615	332	2947
Xylotriose	—	246	246
Xylose oligomers	632	114	114

Methylation and hydrolysis of Watsonia *corm-sac polysaccharide*

Although methylation of the polysaccharide proved difficult when an aqueous solution (necessarily very dilute) was treated by the Haworth method[20], and then by the Kuhn procedures (silver oxide and methyl iodide in *N,N*-dimethylformamide[21], and barium oxide and methyl iodide in dimethyl sulphoxide[22]), success was attained as follows. *Watsonia* polysaccharide fraction *A* (30 g) was homogenised with N sodium hydroxide (200 ml) at 4°, methylated (for the first 8 h at 0° in an atmosphere of nitrogen, and then during 10 days at room temperature and 4 days at 35°) with dimethyl sulphate (700 ml) and sodium hydroxide (350 g), and dialysed against running water until free of inorganic ions. Evaporation of the solution yielded a clear amber-coloured glass (25 g), $[\alpha]_D$ −80° (*c* 1.4, chloroform) (Found: OMe, 36.8; sulphated ash, 1.4%). A solution of this partially methylated material (12.5 g) in equal volumes of dry *N,N*-dimethylformamide and chloroform was allowed to evaporate until most of the chloroform had been removed. Silver oxide (50 g) and methyl iodide (100 ml) were added, and the mixture was stirred vigorously for two weeks. The glassy

TABLE IV

PARTIAL ACID HYDROLYSIS OF *Watsonia* POLYSACCHARIDE

Watsonia polysaccharide
fraction A (30 g), $[\alpha]_D$ −80°

↓

precipitate 1. (5.251 g), $[\alpha]_D$ −59° + *syrup 1.* (21.7 g), $[\alpha]_D$ +83°

↓ charcoal/Celite column chromatography

FRACTIONS I→VI (Table III)

↓

precipitate 2. (2.404 g), $[\alpha]_D$ −53° + *syrup 2.* (2.323 g), $[\alpha]_D$ +32°

↓

precipitate 3. (1.036 g), $[\alpha]_D$ −39° + *syrup 3.* (1.10 g), $[\alpha]_D$ −8°

↓

precipitate 4. (670 mg), $[\alpha]_D$ −42° + *syrup 4.* (202 mg), $[\alpha]_D$ −17°

↓ methylation and hydrolysis

METHYLATED SUGARS (Table II)

+syrup 2

+syrup 3

+fraction VI
then charcoal/Celite column chromatography

↓

FRACTIONS 1→7 (Table III)

product (12.6 g), obtained by filtration and evaporation, followed by repeated extractions with chloroform and evaporation (to remove as much silver iodide–*N*,*N*-dimethylformamide complex as possible), was taken up in chloroform (50 ml) and extracted three times with 5% aqueous potassium cyanide. The methylated material (7.85 g) obtained from the dried chloroform extract had $[\alpha]_D$ −84° (*c* 0.74, chloroform) (Found: OMe, 37.1; sulphated ash, zero %). Further methylations in boiling methyl iodide (100 ml) in the presence of silver oxide (20 g), until methoxyl and ash contents remained unchanged, gave methylated *Watsonia* polysaccharide as a clear glass (6.3 g), $[\alpha]_D$ −86° (*c* 4.15, chloroform) (Found: OMe, 37.5%). Addition of light petroleum (b.p. 60–80°) to a concentration of 90% did not precipitate the product from chloroform solution. The i.r. spectrum revealed no ester groupings, and showed methylation to be essentially complete.

The methylated polysaccharide (5 g) in a mixture of N sulphuric acid (135 ml) and 98% formic acid (15 ml) was hydrolysed for 5 h at 96°, neutralised with barium carbonate, and filtered. The filtrate was treated with Amberlite IR-120 (H^+ form) and Duolite A4 (OH^- form) to remove barium formate. (Paper chromatography of the filtrate had revealed no methylated uronic acid). Concentration of the solution of methylated monosaccharides (at 60°/2 cm to constant weight) yielded a syrup (4 g) which was added to a cellulose column (98×4.5 cm), and eluted with water-saturated mixtures of light petroleum (b.p. 100–120°) and butan-1-ol, with stepwise increase in the butan-1-ol concentration, the final eluant being butan-1-ol saturated with water. The following thirteen fractions were obtained:

Fraction i. A syrup (635 mg, eluted with light petroleum–butanol, 7:2), $[\alpha]_D$ −35° (*c* 1.2), R_G 0.95 (solvent *a*) (Found: OMe, 46.9. $C_8H_{16}O_5$ calc.: OMe, 48.4%), which was chromatographically homogeneous. Demethylation with 48% hydrobromic acid[23] gave arabinose and a series of arabinose methyl ethers, which were identified by paper chromatography against standards. The derived lactone had $[\alpha]_D$ −59° (*c* 0.75), and the derived amide had m.p. 134.6° and mixed m.p. 135° (with 2,3,5-tri-*O*-methyl-L-arabinonamide).

Fraction ii. A syrup (169 mg, eluted with light petroleum–butanol, 7:3) which gave two spots on chromatography, one (*a*) identical with fraction *i*, and the other (*b*) with fraction *iii*. G.l.c. showed the approximate molar ratio of *a*:*b* to be 2:1.

Fraction iii. A chromatographically homogeneous syrup (397 mg, eluted with light petroleum–butanol, 7:4), $[\alpha]_D$ +107° (*c* 3.32), R_G 0.87 (solvent *a*). Demethylation and paper chromatography gave a series of galactose methyl ethers and galactose. The derived "anilide" had m.p. 196–197° and mixed m.p. 197° (with 2,3,4,6-tetra-*O*-methyl-*N*-phenyl-D-galactosylamine).

Fraction iv. A syrup (312 mg, eluted with light petroleum–butanol, 7:5), $[\alpha]_D$ −57° (*c* 1.12), M_g 0.72, R_G 0.81 (solvent *a*) (Found: OMe, 34.1. $C_7H_{14}O_5$ calc.: OMe 34.8%). Demethylation gave 3- and 5-*O*-methylarabinose, and arabinose. The derived lactone, after recrystallisation from ethyl acetate, had m.p. 73° (lit. values for 3,5-di-*O*-methylarabinonolactone: 75°[24], 73°[25], 78°[26]). On paper chromatography, the lactone had R_F 0.78 and 0.88 in solvents *a* and *f*, respectively. The derived amide had m.p. 145°, $[\alpha]_D$ +15° (*c* 0.93).

Fraction v. A syrup (339 mg, eluted with light petroleum–butanol, 7:5) which, on paper chromatography and electrophoresis, gave two spots identical (*a*) with fraction *iv*, and (*b*) with fraction *vi*. G.l.c. showed the molar ratio of *a*:*b* to be approximately 1:2.

Fraction vi. A syrup (192 mg, eluted with light petroleum–butanol, 7:5), $[\alpha]_D$ −43° (*c* 0.6), M_g 0.05, R_G 0.84 (solvent *a*), which gave 2- and 5-*O*-methylarabinose, and arabinose, on demethylation. The derived acid amide had m.p. 131.6–132.3° (lit. values for 2,5-di-*O*-methyl-L-arabinonamide: 131°[27], 131–132°[28]).

Fraction vii. A syrup (14 mg, eluted with light petroleum–butanol, 7:7) which showed two spots on chromatography, in equal quantities and identical with fractions *vi* and *viii*.

Fraction viii. A syrup (215 mg, eluted with light petroleum–butanol, 7:10), $[\alpha]_D$ +87° (*c* 3.59), R_G 0.74 (solvent *a*), which was chromatographically homogeneous, and gave a series of methyl ethers of galactose, and galactose, on demethylation. The derived lactone had m.p. and mixed m.p. 98° (with 2,3,6-tri-*O*-methyl-D-galactonolactone).

Fraction ix. A syrup (24 mg, eluted with light petroleum–butanol, 7:10), $[\alpha]_D$ +97° (*c* 0.46), which showed two components (having very similar mobilities) on paper chromatography (solvent *a*). G.l.c. showed 2,3,6-tri-*O*-methylgalactose (1 part) and (probably) 2,4,6-tri-*O*-methylgalactose (6 parts).

Fraction x. A syrup (10 mg, eluted with light petroleum–butanol, 7:20) which was shown by g.l.c. to be probably 2,4,6-tri-*O*-methylgalactose (1 part) and fraction *xi* (1 part).

Fraction xi. A syrup (40 mg), eluted with light petroleum–butanol, 7:50), $[\alpha]_D$ +27° (*c* 0.78), M_g 0.84, R_G 0.52 (solvent *a*), which was chromatographically homogeneous. Demethylation gave only xylose, and, of the monomethyl ethers of xylose, only 3-*O*-methylxylose had M_g 0.84.

Fraction xii. A syrup (27 mg, eluted with butanol half-saturated with water), $[\alpha]_D$ +85° (*c* 0.42), R_G 0.37 (solvent *a*), which gave arabinose only on demethylation, and was chromatographically identical with 3-*O*-methylarabinose; the 5- and 2-methyl ethers of arabinose have R_G 0.59 and 0.46, respectively.

Fraction xiii. A syrup (594 mg, eluted with water-saturated butanol), $[\alpha]_D$ +18° (*c* 5.94), R_G 0.26 (solvent *a*), which was chromatographically identical with xylose in solvents *a*, *b*, and *c*. Crystallisation from 85% methanol afforded D-xylose, m.p. and mixed m.p. 148°, $[\alpha]_D$ +18° (*c* 1.2). The derived di-*O*-benzylidene dimethyl acetal had m.p. and mixed m.p. 210°, $[\alpha]_D$ −10° (*c* 1.40, chloroform).

TABLE V

HYDROLYSIS PRODUCTS OF METHYLATED POLYSACCHARIDE

Compound	*Weight (mg)*	*Molar proportion*
2,3,5-Tri-*O*-methylarabinose	747	10.0
2,3,4,6-Tetra-*O*-methylgalactose	453	4.9
3,5-Di-*O*-methylarabinose	425	6.2
2,5-Di-*O*-methylarabinose	418	6.1
2,3,6-Tri-*O*-methylgalactose	218	2.5
2,4,6-Tri-*O*-methylgalactose (probably)	26	0.3
3-*O*-Methylxylose	45	0.7
3-*O*-Methylarabinose	27	0.4
Xylose	594	10.0

Final elution of the column with ethanol–water (1:1) gave no further fractions. The weights and molar proportions of the sugars eluted from the column are presented in Table V.

Methanolysis of the fully methylated gum (in 2.4% methanolic hydrogen

chloride for 18 h at 100°) and subsequent g.l.c. gave the retention times and approximate molar proportions (in all cases, compared with authentic standards) shown in Table VI.

TABLE VI

G.L.C. ANALYSIS OF PRODUCTS FROM METHANOLYSIS OF METHYLATED POLYSACCHARIDE

Methyl glycoside of	*T*[a]	*Molar proportion*	*Molar proportion, %*
2,3,5-Tri-*O*-methylarabinose	0.59 s, 0.79 w	16	24.6
2,3,4,6-Tetra-*O*-methylgalactose	2.02	10	15.4
2,5-Di-*O*-methylarabinose	2.33 s, 4.63 w	18	27.7
3,5-Di-*O*-methylarabinose	1.28, 3.21	16	24.6
2,3,6-Tri-*O*-methylgalactose	3.90 s, 4.77 w, 5.48 w, 6.20 m	5	7.7

[a] Retention times relative to methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucoside.

RESULTS AND DISCUSSION

Analysis of the *Watsonia* corm-sac polysaccharide by identification of the products of partial acid hydrolysis, together with the hydrolysis and methanolysis products of the methylated polysaccharide, enable the main structural features to be discerned. The material, to judge by electron micrographs and the results of chemical examination, consists of elongated particles based upon a linear xylan chain which is highly substituted by short chains, themselves probably unbranched, containing galactose and arabinose residues. Attempted fractionation of the polysaccharide, obtained by dissolving the raw material in water and precipitating with ethanol, indicated a high degree of chemical homogeneity and, in the absence of evidence to the contrary, the results of chemical degradation may be regarded as applying to a single type of complex molecule.

The molar sugar ratios (galactose, arabinose, and xylose 1:3.5:1.42), determined after total acid hydrolysis of the polysaccharide, revealed a lower xylose content than is usual amongst xylans, some resemblance being noted to that of wheat bran[29]. By frequent interruption of the hydrolysis of the polysaccharide by hot dilute acid, a number of oligosaccharides were protected from further degradation after their initial release from the main structure, and a linear xylan (partly degraded) was finally recovered after removal of the arabinose and galactose residues located in the polymer in the form of side chains.

The oligosaccharides recovered from a 30-g sample of polysaccharide (yields in parentheses) were as follows: 3-*O*-α-D-galactopyranosyl-L-arabinose (5.98 g), identified as described in an earlier paper[14]; *O*-α-D-galactopyranosyl-(1→3)-*O*-L-arabinofuranosyl-(1→2)-L-arabinose (2.95 g), whose structure is deduced by degradation studies, an α-linkage to C-2 of the reducing arabinose unit being indicated

by application of Hudson's rules[30]; 4-*O*-β-D-xylopyranosyl-D-xylose (122 mg), characterised finally by direct comparison of the acetate with that of an authentic specimen; and *O*-β-D-xylopyranosyl-(1→4)-*O*-β-D-xylopyranosyl-(1→4)-D-xylose (246 mg), characterised by direct comparison of the crystalline sugar and its octa-acetate with authentic specimens. In addition, mixtures of xylose oligomers containing from three to seven monosaccharide units were isolated, and shown by their relative rates of movement on paper chromatography in four solvent systems to be members of the linear β-(1→4)-linked D-xylopyranose series. Some loss of the xylose oligomers was experienced due to their not being desorbed from charcoal with 70 or 80% methanol. Traces of arabinose oligomers, of dubious structural significance, were also isolated. The ready release of the arabinose-containing oligosaccharides indicates that they are linked as furanosides in the polysaccharide. Methylation of the residual xylan and hydrolysis gave mainly 2,3-di-*O*-methyl-D-xylose, proving the nature of the linear chain, together with some 2,3,4-tri-*O*-methylxylose from end groups produced by scission of the xylan chain. The starch-like character of a contaminating polysaccharide was likewise revealed by the separation at this stage of a trace of 2,3,6-tri-*O*-methylglucose.

The way in which the main oligosaccharide units fit into the structural pattern of the polysaccharide becomes clear on analyzing the products of hydrolysis of the fully methylated polysaccharide. On hydrolysis, the methylated product (OMe, 37.5%) yielded the following sugars (all characterised through the formation of suitable crystalline derivatives), in the molar proportions shown in parenthesis: 2,3,5-tri-*O*-methylarabinose (10); 2,3,4,6-tetra-*O*-methylgalactose and 2,3,6-tri-*O*-methylgalactose (7.35, combined); 2,5-di-*O*-methylarabinose (6.1); 3,5-di-*O*-methylarabinose (6.2); and D-xylose (10). Sugars, concluded to be 3-*O*-methylxylose and 3-*O*-methylarabinose, were found in trace amounts (0.7 and 0.4, respectively). Qualitative and semi-quantitative agreement with these results was obtained by methanolysis of the methylated polysaccharide, and separation of the methyl glycosides of the resulting methylated sugars by gas–liquid chromatography. De-methylation of the 2,3,5-tri-*O*-methylarabinose was observed under the conditions used. The appearance of 2,3,6-tri-*O*-methylgalactose may be due to undermethylation of the axial C-4 hydroxyl group, and to partial de-methylation (observed to occur when the tetra-*O*-methyl sugar is submitted to vigorous acid hydrolysis); for these reasons it is preferred to regard the galactose as wholly non-reducing end-group at this stage of the investigation.

Preliminary experiments involving periodate oxidation, borohydride reduction, and mild acid treatment of the polysaccharide have shown that all of the galactose non-reducing end-groups, together with those of arabinose, are removed completely, and recovered as glycerol; no threitol, which would result from 4-linked galactose present in the polysaccharide, has thus far been detected. The residual arabino-xylan contains arabinose and xylose in the proportions expected from the foregoing data.

The pattern thus revealed, when somewhat simplified, is that of a β-(1→4)-

linked D-xylose chain substituted on nine residues out of ten at C-2 and C-3. The side chains linked to xylose consist of the following types; the approximate number per ten xylose units is given in parentheses:

L-Ara*f*-(1→ (12)
α-D-Gal*p*-(1→3) -L-Ara*f*-(1→ (2)
α-D-Gal*p*-(1→3) -α-L-Ara*f*-(1→2) -L-Ara*f*-(1→ (5)

In addition, there are three remaining L-arabinofuranose units within the chains, two of them 2-linked and one 3-linked.

An outstanding feature of the structure of this polysaccharide is the very high degree of substitution, by sugars, of the hydroxyl groups in the xylan chain. This is greatly in excess of that encountered either in hemicellulosic material or in the plant-gum exudates of this type. The absence of uronic acid groups may in some way be related to the function of the polysaccharide as a food reserve. A somewhat similarly constituted polysaccharide, found on the outside of seed-boxes of a related *Watsonia*, has been shown recently in this laboratory to contain D-glucuronic acid residues attached as non-reducing end-groups, and to resemble rather more closely the typical acidic xylans.

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SUMMARY

A polysaccharide found in corms of *Watsonia pyramidata*, studied by acid fragmentation, and by methylation followed by hydrolysis and methanolysis, consists of a linear chain of β-(1→4)-linked D-xylopyranose residues, to which are attached short side-chains containing D-galactopyranose and L-arabinofuranose residues. The degree of substitution of the xylose residues is unusually high. The release, on mild acid hydrolysis, of two oligosaccharides, 3-*O*-α-D-galactopyranosyl-L-arabinose and *O*-α-D-galactopyranosyl-(1→3)-*O*-L-arabinofuranosyl-(1→2)-L-arabinose, in high yield, is discussed in relation to the occurrence of these sugar sequences in the polysaccharide structure.

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Notes

A polysaccharide found on the seed boxes of *Watsonia versveldii*

Subsequent to our study of the structure of a polysaccharide found in the corm sacs of *Watsonia pyramidata* (Andr.) Stapf.[1,2], we have investigated a polysaccharide exuded from the seed boxes of *Watsonia versveldii*. Described here are the results of experiments designed to reveal any similarities in the structures of the two polysaccharides, whose functions in the plant are manifestly different.

It would appear that the polysaccharides from *Watsonia versveldii* and *Watsonia pyramidata* show marked similarities in structure; the backbone of β-(1→4)-linked D-xylose residues is common to each, as is the presence of 3-*O*-α-D-galactopyranosyl-L-arabinose as a component of the side chains. In the present study, the structure of the latter disaccharide is substantiated by the methylation data, although the possibility of a β-D glycosidic linkage is not ruled out. The short side-chains have residues of D-galactopyranose and L-arabinofuranose as non-reducing end-groups. The absence of *O*-α-D-galactopyranosyl-(1→3)-*O*-L-arabinofuranosyl-(1→2)-L-arabinose from the products of partial hydrolysis by acid of the *Watsonia versveldii* exudate, coupled with the virtual absence of 3,5-di-*O*-methylarabinose after hydrolysis of the methylated polysaccharide, is, however, a major point of difference.

Under the hydrolytic conditions used, no biouronic acids were detected, so that the exact mode of combination of the uronic acid with the rest of the molecule remains obscure; methylation studies do show, however, that it occurs as a non-reducing end-group. Apparently, the extent of substitution of the D-xylose backbone by side-chains is less in the *Watsonia versveldii* exudate than in the *Watsonia pyramidata* polysaccharide. The presence of a significant proportion of a uronic acid is consistent with this material's being of the same general type (D-xylose residues in chains with short branches that may contain residues of L-arabinose, D-xylose, and D-glucuronic acid) as that exuded from certain other plants which are widely scattered through the plant kingdom[9]. Some resemblance is to be seen, for example, to gums of plants in the orders Ebenales (*Sapota achras*) and Bromeliales (*Puya chilensis*), as well as Liliales.

EXPERIMENTAL

General experimental conditions

Paper chromatography was carried out on Whatman No. 1 paper with the

following solvent systems (all v/v): (*a*) butan-1-ol–ethanol–water (4:1:5, upper layer), (*b*) ethyl acetate–acetic acid–formic acid–water (18:3:1:4), (*c*) ethyl acetate–pyridine–water (10:4:3). Retention times[3] (*T* values) were measured relative to that of methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucoside on a 4-foot column of ethylene glycol succinate (14% on 80–100 mesh Gas Chromosorb W)[4] at 155°, using a flame-ionisation detector.

Purification of Watsonia versveldii *polysaccharide*

The polysaccharide (air-dried, 700 mg; collected at the Kirstenbosch Botanical Gardens, Cape Town, in December 1963) was dissolved in water (20 ml) and recovered, as a fine, white precipitate, by the addition of three volumes of ethanol. The product was reprecipitated with ethanol, washed with ethanol and ether, and dried (for 2 h at 76°/1 mm), to afford a white powder (500 mg), $[\alpha]_D^{25}$ −105° (*c* 0.91, water), equiv. wt 2,050. Prior to measurement of equivalent weight, the precipitated gum was freed from any residual cations, by passage through Amberlite IR-120 (H^+ form), and freeze-dried.

Total hydrolysis (N sulphuric acid for 6 h at 96°) of a portion of this powder, with paper chromatography of the hydrolysate, indicated that the polysaccharide was composed of residues of arabinose, galactose, uronic acid, and xylose, in the approximate ratio 3:1:2:2. Neutral sugars were determined (in molar proportions) by the phenol–sulphuric acid assay; the uronic acid was estimated visually.

Partial hydrolysis of the polysaccharide by acid

The ethanol-precipitated material (50 mg) was hydrolysed in 0.01N sulphuric acid for a total of 24 h at 96°, samples being removed from the reaction mixture

TABLE I

G.L.C. ANALYSIS OF PRODUCTS FROM METHANOLYSIS OF THE METHYLATED POLYSACCHARIDE

Methyl glycosides of	*T*[a]	*Approximate molar proportion*[b]
2,3,4-Tri-*O*-methylxylose	0.46	trace
2,3,5-Tri-*O*-methylarabinose	0.59, 0.78	16
3,5-Di-*O*-methylarabinose	1.26, 3.26	2
2,5-Di-*O*-methylarabinose	2.36, 4.74	15
2,3-Di-*O*-methylxylose	1.81, 2.05	3.5
2,3,4,6-Tetra-*O*-methylgalactose	2.05	7
2,3,4-Tri-*O*-methylglucuronic acid	2.84, 3.84	17
2,3,6-Tri-*O*-methylgalactose	3.84, 4.74	5.5
2-*O*-Methylxylose 3-*O*-Methylxylose	6.26 5.40	10

[a]Retention time relative to that of methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucoside.
[b]Xylose is present to the extent of *ca.* 10 molar proportions.

at 1, 2, 4, and 6 h. The following saccharides were identified against standards by paper chromatography in solvents *a*, *b*, and *c*: (*i*) 3-*O*-α-D-galactopyranosyl-

L-arabinose, steadily increasing from 1 to 24 h, (*ii*) galactose, increasing from 2 to 24 h, (*iii*) arabinose, increasing from 1 to 24 h, (*iv*) what appeared to be the same series of xylose oligomers as was reported for the corm-sac polysaccharide of *Watsonia pyramidata*[2]; these oligomers were released only on hydrolysis for 24 h.

Methylation of the polysaccharide

The polysaccharide (326 mg) was methylated by the procedures of Haworth[5], Kuhn[6], and Purdie[7] to yield a clear, yellow glass (197 mg) (Found: OMe, 34.9%). The methoxyl content was not increased by repetition of the Purdie method. Methanolysis of the methylated polysaccharide (in 4% methanolic hydrogen chloride for 16 h at 100° in a sealed tube) and subsequent, qualitative and semi-quantitative[8], gas–liquid chromatography of the methanolysate afforded the results shown in Table 1. These results were substantiated by paper chromatography, each of the components and unmethylated xylose being identified against standards in solvent *a*.

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Separations of Sugars on "Chromagrams"

Recently, "Chromagram" sheets for thin-layer chromatography have become available commercially (Messrs. Kodak Ltd., Kirkby, Liverpool). We have examined the polycarbonate and silica-gel forms of these, and have obtained very rapid, good separations of many sugars, without the tedium of plate preparation. The uniformity of coating allows reproducible R_F values to be obtained without difficulty; moreover, the solvent front runs evenly, and the absence of "edge-effect" facilitates comparison of R_F values of unknown sugars with those of standards on the same chromagram.

EXPERIMENTAL

Chromagrams were developed by the ascending technique in a small tank (Shandon, for thin-layer chromatography) lined with filter sheet to assist vapour-phase equilibration.

The polycarbonate coating was pre-treated by immersion in phosphate buffer (0.2 M, pH 6.8). After the chromagram had been air-dried, 0.5 μl of a 5% aqueous solution of sample was applied. The solvent system, propan-1-ol–ethyl acetate–water (10:3:1), recommended by Messrs. Kodak, Harrow Division, was used; development for 5 h was required. The chromagrams were sprayed with (*a*) saturated ethanolic aniline oxalate (followed by heating in an oven at 140° for 2–3 min), to detect reducing sugars, or (*b*) an aqueous solution of periodate and alkaline permanganate[1] to detect sugar alcohols. Table I gives the R_F values obtained.

TABLE I

R_F VALUES OF SOME SUGARS ON POLYCARBONATE (0.2 M PHOSPHATE BUFFER, pH 6.8) CHROMAGRAMS USING PROPAN-1-OL–ETHYL ACETATE–WATER (10:3:1)

D-Galactose	0.13	D-Xylose	0.44
D-Mannose	0.31	D-Ribose	0.49
D-Glucose	0.18	L-Arabinose	0.29
L-Rhamnose	0.58	Maltose	0.10
L-Fucose	0.49	Lactose	0.05
D-Glucuronic acid	0.03	D-Arabinitol	0.37
D-Galacturonic acid	0.03	Erythritol	0.48
D-Fructose	0.28		

The behaviour of some sugars on the silica-gel chromagrams, pre-treated with 0.1 N boric acid, was examined in two different solvent-systems[2]: solvent 1, butan-1-ol–acetone–water (4:5:1); solvent 2, butanone–acetic acid–water (3:1:1). For these solvents, development of 10 cm required only 1 h. The chromagrams were sprayed with the same reagents as before. The R_F values observed are given in Table II.

TABLE II

R_F VALUES OF SOME SUGARS ON SILICA-GEL (0.1 N BORIC ACID) CHROMAGRAMS

	Solvent 1[a]	*Solvent 2*[a]
D-Galactose	0.32	0.36
D-Mannose	0.43	0.46
D-Glucose	0.41	0.41
L-Rhamnose	0.61	0.58
L-Fucose	0.50	0.48
D-Glucuronic acid	0.05	0.34
D-Galacturonic acid	0.06	0.42
D-Fructose	0.31	0.43
D-Xylose	0.47	0.54
D-Ribose	0.45	0.57
L-Arabinose	0.43	0.48
Maltose	0.29	0.30
Lactose	0.20	0.25
Galactitol	0.14	0.43
D-Arabinitol	0.28	0.47
Erythritol	0.44	0.52
Glycerol	0.49	0.56

[a]Solvent 1: Butan-1-ol–acetone–water (4:5:1). Solvent 2: Butanone–acetic acid–water (3:1:1).

The speed and ease with which good separations of sugars can be achieved, especially on the silica-gel chromagrams, make these analytical aids attractive to the carbohydrate investigator.

ACKNOWLEDGEMENT

We are grateful to Messrs. Kodak Ltd. for gifts of the different forms of "Chromagram" sheet.

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Preliminary communication

Asymmetrischer Galaktosyltransfer auf Glycerin mit β-Galaktosidase aus *E. coli*

Es ist bekannt, daß disaccharidspaltende Enzyme auch transferierende Eigenschaften besitzen[1]. Als Acceptoren kommen ganz allgemein niedermolekulare Verbindungen mit alkoholischer Funktion in Frage. Werden Monosaccharide als Acceptoren verwendet, erhält man auf Grund der sterisch verschiedenen Anordnung der Hydroxylgruppen verschieden verknüpfte Disaccharide in unterschiedlichen Ausbeuten[2].

Die Übertragung eines β-D-Galaktosylrestes auf Glycerin, das symmetrisch gebaut ist, erfolgt bei 1,1-Verknüpfung unter Bildung eines neuen Asymmetriezentrums. Es wurde nun festgestellt, daß von den drei Möglichkeiten des Transfers eines β-D-Galaktosylrestes auf Glycerin, nämlich 1-*O*-β-D-Galaktosyl-D-glycerin, 1-*O*-β-D-Galaktosyl-L-glycerin, und 2-*O*-β-D-Galaktosylglycerin, fast ausschließlich das 1-*O*-β-D-Galaktosyl-D-glycerin gebildet wird.

Dieser Befund entspricht der asymmetrischen Synthese des 1-L-Glycerophosphates bei der enzymatischen Phosphorylierung mit Glycerokinase[3].

Es ist anzunehmen, daß es sich bei der asymmetrischen Reaktion des Glycerins um einen weiteren Fall von unterschiedlichem Verhalten zweier identischer Atomgruppen x in einem Molekül der Formel $y\text{-}\overset{\displaystyle x}{\underset{\displaystyle x}{C}}\text{-}z$ handelt, wenn dieses Molekül mit einem asymmetrischen Reaktionszentrum eines Enzyms diastereomere Übergangszustände verschiedenen Energieniveaus bilden kann[4,5].

Eine Lösung von 200 mg Glycerin und 20–30 mg β-Galaktosid (*o*-Nitrophenyl-β-D-galaktosid, Phenyl-β-D-galaktosid, Lactose) in 2 ml M/16 Natrium-Kalium-Phosphat-Puffer (pH 6,8) wird mit 0,01–0,05 ml Enzymlösung (250 000 Einheiten/ml)[6] inkubiert. Der Reaktionslösung werden nach verschiedenen Zeiten 0,1–0,2 ml entnommen, das Protein durch Aufkochen denaturiert und die Probe zur Trockene eingedampft. Die gaschromatographische Analyse der trimethylsilylierten[7] Proben ergeben neben den Hydrolyseprodukten des Galaktosids stets ein Haupt-transferprodukt (85%) und ein Nebenprodukt (15%).

Zur Identifizierung des Haupt-transferproduktes wird der Galaktosyltransfer mit ^{14}C-Glycerin als Acceptor durchgeführt. Die gesamten, papierchromatographisch [Whatman Nr. 1, 1-Butanol–Propionsäure–Wasser (142:71:100, v/v/v)] von den Hydrolyseprodukten abgetrennten Transferprodukte werden mit authentischem

1-*O*-β-D-Galaktosyl-D-glycerin cokristallisiert. Es zeigt sich, daß 85% der gesamten ^{14}C-Aktivität in das 1-*O*-β-D-Galaktosyl-D-glycerin aufgenommen wird. Das Verhältnis ^{14}C-Aktivität zu mg Substanz bleibt nach mehrmaligem Umkristallisieren aus Äthanol konstant.

Das 1-*O*-β-D-Galaktosyl-D-glycerin konnte von uns auf einfache Weise durch enzymatischen Galaktosyltransfer mit β-Galaktosidase auf 2,3-*O*-Isopropyliden-D-glycerin[8] und Abspaltung der Isopropylidengruppe mit verdünnter Essigsäure in 42%iger Ausbeute erhalten werden. Schmelzpunkt, Drehwert und IR-Spektrum sind mit den Literaturdaten identisch[9].

Das zweite Transferprodukt konnte nach Trimethylsilylierung gaschromatographisch von 2-*O*-β-D-Galaktosylglycerin nicht unterschieden werden. Eine eindeutige Identifizierung des Nebenproduktes soll noch nach der Isotopenverdünnungsmethode vorgenommen werden. Mit dieser Methode soll ebenfalls noch geprüft werden, ob bei der Transferreaktion nicht doch eine Spur des 1-*O*-β-D-Galaktosyl-L-glycerins gebildet wird.

Vor kurzem wurde festgestellt, daß Galaktosylglycerin, wie es beim Transfer auf Glycerin entsteht, die Synthese der Enzyme des Lac-Operons bei *E. coli* noch in einer Verdünnung von 10^{-7} — 10^{-8}M induziert[10]. Dieses Syntheseprodukt ist auf Grund der beschriebenen Versuche 1-*O*-β-D-Galaktosyl-D-glycerin.

Für eine Probe 2-*O*-β-D-Galaktosylglycerin danken wir Herrn Dr. J. G. Buchanan.

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REACTION OF GLYCOSYLAMINES WITH ETHYL ACETOACETATE

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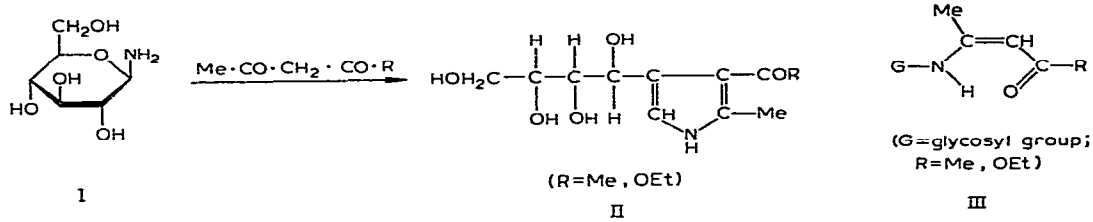
INTRODUCTION

Previous work[1] has shown that glycosylamines and their *N*-alkyl and *N*-aryl derivatives react with β-dicarbonyl compounds (2,4-pentanedione, ethyl acetoacetate) to give substituted (tetrahydroxybutyl)pyrroles, for example, compound (II) from β-D-glucopyranosylamine (I). The yields of pyrroles parallel the facility of the parent



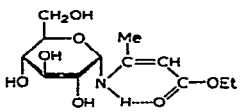
I II III

glycosylamines to undergo the Amadori rearrangement and, on this basis, it was postulated that the reaction proceeds stepwise, one of the steps involving an Amadori rearrangement. We have found now that when this reaction is carried out under mild conditions, *N*-glycosyl derivatives of α,β-unsaturated β-amino ketones or esters (III) are formed, and that these substances can be transformed into pyrroles similar to compound (II). This paper deals with the compounds of type (III) derived from aldohexosylamines and ethyl acetoacetate, and attempts to prepare similar compounds from D-fructosylamine and L-sorbosylamine.

RESULTS AND DISCUSSION

Ethyl 3-(aldohexosylamino)crotonates (IV)–(VII) were obtained by allowing the corresponding aldohexosylamines to stand with a molar excess of ethyl acetoacetate, in the presence of catalytic amounts of piperidine, in methanol at room temperature. Yields of pure products were low (31% of VII; 3–5% of IV–VI) as a result of heavy losses during purification, probably because other isomeric forms were present. Compounds (VI) and (VII) were identical with the compounds obtained directly in the reactions of D-mannose and L-rhamnose with ethyl acetoacetate and ammonia[2,3].

Compounds (IV)–(VII) have high optical rotations (negative in the case of the L-rhamnose derivative) suggestive of α anomeric configurations. They give positive Fehling's and ferric chloride tests, presumably because of their hydrolysis to ethyl acetoacetate and the parent glycoses. Paper chromatography of analytically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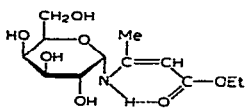
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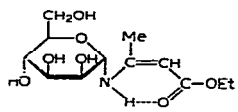
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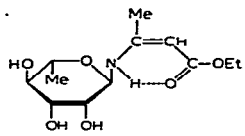

VII

pure samples, using basic (butan-1-ol–ethanol–water–ammonia, 40:10:49:1) or neutral (butan-1-ol–ethanol–water, 10:1:2) solvents, is accompanied by partial hydrolysis, since spots appear corresponding to the parent glycoses and glycosylamines, in addition to those of the ethyl 3-(aldohexosylamino)crotonates (Table I). When chromatography was effected with an acidic solvent (butan-1-ol–acetic acid–water, 4:1:5), only the spots corresponding to the parent glycoses were detected. Similar observations on simpler glycosylamines are found in the literature[4].

TABLE I

CHROMATOGRAPHIC MOBILITIES (R_F)[a] OF ETHYL 3-(ALDOHEXOSYLAMINO)CROTONATES AND THE PARENT ALDOHEXOSYLAMINES AND ALDOHEXOSES

Substance	R_F
Ethyl 3-(D-glucosylamino)crotonate (IV)	0.07, 0.13, 0.17, 0.70
β-D-Glucopyranosylamine	0.08, 0.14, 0.18
D-Glucose	0.18
Ethyl 3-(D-galactosylamino)crotonate (V)	0.12, 0.23, 0.73
β-D-Galactopyranosylamine	0.12, 0.23
D-Galactose	0.23
Ethyl 3-(D-mannosylamino)crotonate (VI)	0.07, 0.17, 0.21, 0.66
β-D-Mannopyranosylamine	0.07, 0.17, 0.21
D-Mannose	0.21
Ethyl 3-(L-rhamnosylamino)crotonate (VII)	0.16, 0.30, 0.67
L-Rhamnosylamine	0.11, 0.16, 0.30
L-Rhamnose	0.30

[a] On No. 1 Whatman paper using butan-1-ol–ethanol–water–ammonia (40:10:49:1) as solvent. R_F measured at 20°.

Hydrolysis of compounds (IV)–(VII) can be followed polarimetrically. The optical rotations of freshly prepared, aqueous solutions of these substances are of the same order as those obtained in anhydrous solvents and, on storage, decrease to constant values. Paper chromatography of these solutions showed the presence of the parent sugar and, in some cases, trace amounts of the corresponding glycosylamines and diglycosylamines. Figure 1 shows the change of $[\alpha]_D$ with time for ethyl 3-(D-mannosylamino)crotonate (VI) in different media.

Further evidence of the formation of the free glycoses in the hydrolysis of ethyl 3-(aldohexosylamino)crotonates was obtained for the D-glucose derivative (IV). Hydrolysis of this substance with 0.1N hydrochloric acid gave a syrup which when analyzed chromatographically and ionophoretically showed only the presence

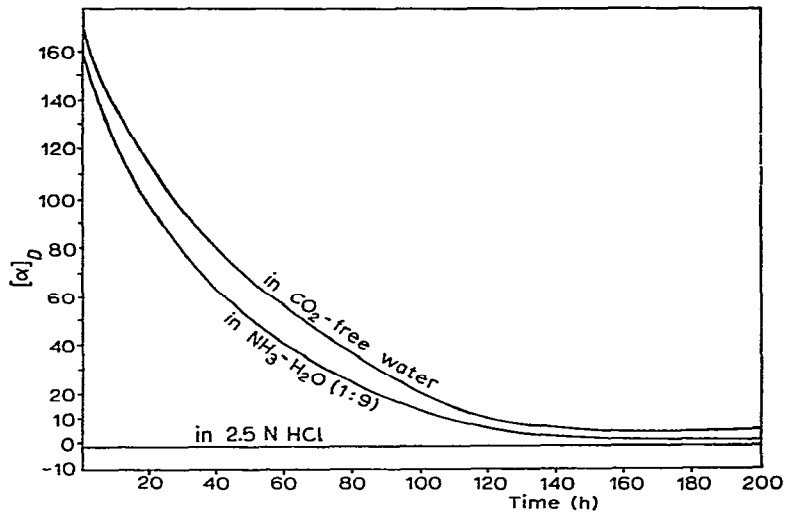


Fig. 1. Change of $[\alpha]_D$ with time for ethyl 3-(D-mannosylamino)crotonate in different media.

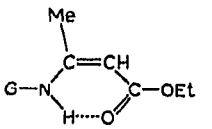
of D-glucose. This syrup, on treatment with *p*-nitroaniline, gave crystalline *N*-*p*-nitrophenyl-β-D-glucopyranosylamine. The hydrolysis of compound (VI) to D-mannose and di-D-mannosylamine has been previously reported[3].

Treatment of compounds (IV)–(VII) with acetic anhydride in pyridine at 0° gave tetra-*O*-acetyl derivatives in the cases of compounds (IV)–(VI), and a tri-*O*-acetyl derivative in the case of compound (VII). Catalytic (sodium ethoxide) deacetylation of these derivatives regenerated the parent compounds (IV)–(VII) and, in the hydrolysis of the tetra-acetate of compound (IV) with dilute formic acid[5], 2,3,4,6-tetra-*O*-acetyl-D-glucose was obtained. The hydrolysis of the acetyl derivative of compound (VI) to 2,3,4,6-tetra-*O*-acetyl-D-mannose has already been reported[3].

The absorption spectra (Table II) of compounds (IV)–(VII) are very similar to those of ethyl 3-aminocrotonate and ethyl 3-(alkylamino)crotonates[6–8]. The ultraviolet spectra show single maxima at 278 mμ (log ε 4.2–4.5). The infrared spectra of compounds (IV)–(VII) and their *O*-acetyl derivatives show the carbonyl bands at frequencies (1650–1670 cm^{-1}) lower than usual for α,β-unsaturated esters. This shift is attributed to the intramolecular hydrogen bonds indicated in formulae (IV)–(VII), and to the contribution of ionic forms (VIII) to the ground state of these molecules. In addition, two bands appear in the double-bond region, a strong one at *ca.* 1600 cm^{-1} (1620 cm^{-1} in the spectra of the *O*-acetyl derivatives in solution) and a second one at *ca.* 1500 cm^{-1}. This set of bands seems to be characteristic of

TABLE II

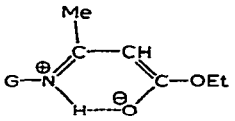
U.V. AND I.R. ABSORPTION[a] OF COMPOUNDS HAVING GENERAL FORMULA



Glycosyl moiety, G	*Formula*	*U.v. absorption*[b]		*I.r. absorption* (cm^{-1})		
		λ_{max} (mμ)	*log* ε	*Phase*	*N–H and O–H stretching region*	*1800–1480* cm^{-1} *region*
D-Glucosyl	IV	278	4.22	N	3448s, 3300s	1650s, 1605s, 1495m
2,3,4,6-Tetra-*O*-acetyl-D-glucopyranosyl				Cf	3279w	1757s, 1667s, 1629s, 1493m
D-Galactosyl	V	278	4.11	N	3448s, 3333s	1667s, 1613s, 1502m
2,3,4,6-Tetra-*O*-acetyl-D-galactopyranosyl				C	3279w	1757s, 1669s, 1629s, 1495m
D-Mannosyl	VI	278	4.36	N	3472s, 3322s	1656s, 1590s, 1508m
2,3,4,6-Tetra-*O*-acetyl-D-mannopyranosyl				C	3279w	1761s, 1669s, 1626s, 1497m
L-Rhamnosyl	VII	278	4.53	N	3333s	1650s, 1597s, 1502m
2,3,4-Tri-*O*-acetyl-L-rhamnopyranosyl				C	3289w	1757s, 1667s, 1621s, 1499m

[a] Abbreviations: N, solid in Nujol mull; Cf, chloroform solution; C, carbon tetrachloride solution; s, strong; m, medium; w, weak.
[b] In ethanol.

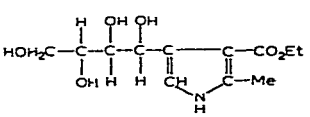
α,β-unsaturated β-amino esters[7,8]. The spectra of the *O*-acetyl derivatives, either in the solid state or in solution, show weak bands at *ca.* 3280 cm^{-1} (position independent of concentration) attributable to the intramolecularly-bonded NH group.



(G = glycosyl group)

VIII

When compounds (IV)–(VII) were heated with acetic acid and potassium acetate in methanol (a treatment that promotes the Amadori rearrangement of glycosylamines[9]), solutions showing reducing properties to the alkaline 2,6-dichloroindophenol reagent (positive reaction of 1-amino-1-deoxyketoses) and positive pyrrole–Ehrlich reactions were obtained. Crystalline ethyl 2-methyl-4-(D-*arabino*-tetrahydroxybutyl)-pyrrole-3-carboxylate (II) was obtained in this way from compounds (IV) and (VI). In the reactions with the D-galactose and L-rhamnose derivatives (V) and (VII), syrupy materials giving intense Ehrlich and polyol reactions, and having chromatographic properties similar to those of compound (II) were obtained. They contained, presumably, compounds (IX) and (X). The formation of these pyrroles can be



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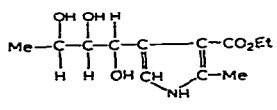
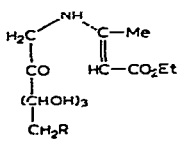
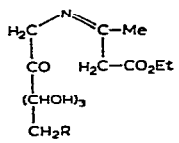


X



(R=H, OH)

XI



(R=H, OH)

XIa

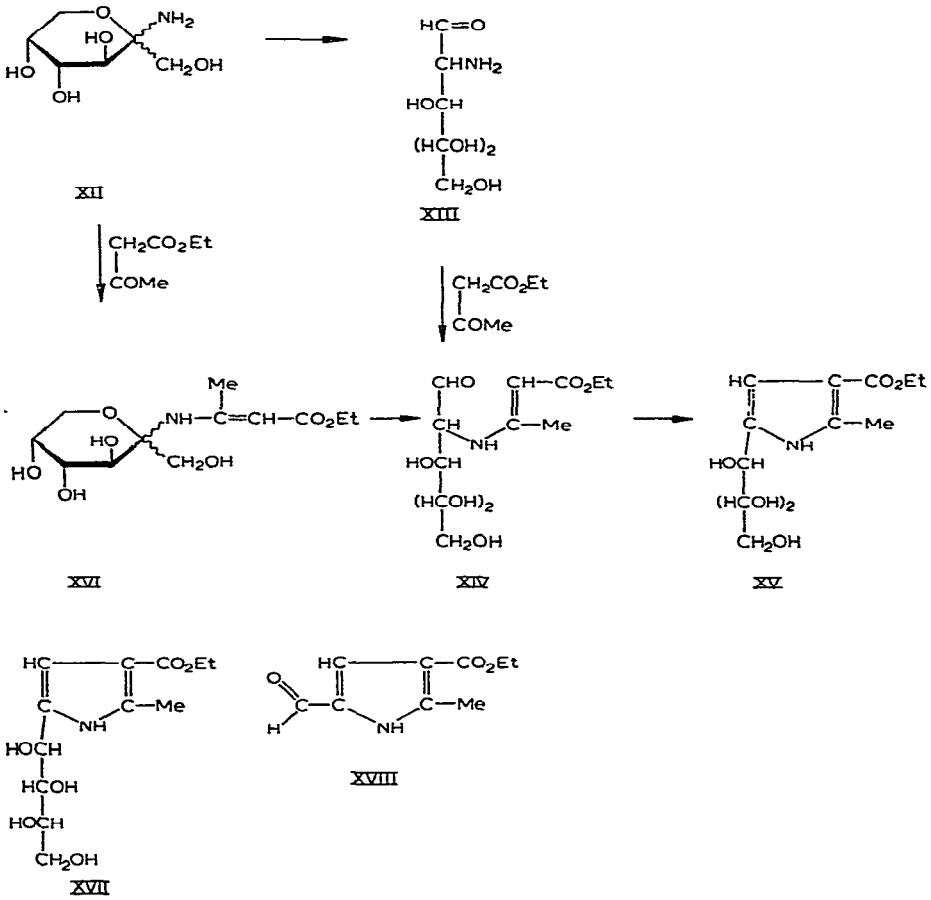
rationalized by assuming the occurrence of an Amadori rearrangement of compounds (IV)–(VII) which would give intermediates (XI) (or their prototropic equivalents XIa), followed by internal aldol condensations.

Treatment of compound (VI) with a carbonate–hydrogen carbonate buffer (pH 9.5) produced a mixture of pyrroles in which ethyl 2-methylpyrrole-3-carboxylate and compound (II) were chromatographically detected.

The reaction of ketohexosylamines with ethyl acetoacetate in methanol at room temperature proceeds readily to yield ethyl 2-methyl-5-(tetrahydroxybutyl)-pyrrole-3-carboxylates instead of *N*-ketohexosyl derivatives of type (III). Pyrrole (XV) was obtained from D-fructosylamine (XII), in accordance with a previous report[1a]. In the reaction with L-sorbosylamine, a chromatographically pure, Ehrlich-positive syrup was obtained, which was presumably compound (XVII) because, on periodate oxidation, it gave 4-ethoxycarbonyl-5-methylpyrrole-2-carboxaldehyde (XVIII).

The results obtained with ketohexosylamines can be explained by taking into account the readiness of these substances to undergo the Amadori rearrangement[10]; the β-keto ester reacts[11] with the rearrangement products (2-amino-2-deoxyaldo-

hexoses). D-Fructosylamine (XII) yields intermediates (XIII) and (XIV), and cyclization of the latter gives compound (XV)[11]. An alternative explanation is that the reaction proceeds through the ethyl 3-(ketohexosylamino)crotonate (XVI),



which then undergoes the Amadori rearrangement very easily, also giving intermediate (XIV).

EXPERIMENTAL

Melting points are uncorrected. Concentrations and evaporations of solvents were carried out *in vacuo* at temperatures not over 50°. Paper chromatography was carried out on Whatman No. 1 paper by the descending technique using (*i*) butan-1-ol–ethanol–water (10:1:2) or (*ii*) butan-1-ol–ethanol–water–ammonia (40:10:49:1, organic phase). Spray reagents used were: (*a*) silver nitrate–sodium hydroxide[12] for monosaccharides and polyhydroxylic compounds; (*b*) aniline hydrogen phthalate[13] for reducing sugars and compounds (IV)–(VII), and (*c*) chromatographic Ehrlich

reagent[11] for pyrrole derivatives. Paper ionophoresis was carried out on Whatman paper No. 3 in 0.2M borate buffer (pH 10), or, for amino sugars, 0.2M acetate buffer (pH 5.0), using the technique and apparatus described by Foster[14]. Thin-layer chromatography was performed on silica gel (G, Merck), and detection was effected with a mixture of sulfuric acid–water (1:1) or, in the case of pyrrole compounds, with reagent (*c*). The u.v. spectra were obtained with a Beckman DU spectrophotometer, and the i.r. spectra with a Beckman IR-5A instrument.

Preparation of glycosylamines

β-D-Glucopyranosylamine was prepared according to Ling and Nanji[15]. After recrystallization from 95% methanol, it had m.p. 127–128°, $[\alpha]_D^{25}$ +20.1° (*c* 1, water) [lit.[15], m.p. 128–129°, $[\alpha]_D^{20}$ +20.3° (*c* 1, water)].

β-D-Galactopyranosylamine was obtained by dissolving the α-anomer (ammonia complex)[16] (20.0 g) in 3% ammonia (30 ml) and adding methanol saturated with ammonia (150 ml). After 48 h, the crystals of product were collected, washed with ethanol, and dried over calcium chloride. The product had m.p. 137–138° (dec.), $[\alpha]_D^{25}$ +62° (*c* 2, water) [lit.[16,17], m.p. 134–136°, $[\alpha]_D^{22}$ +62.2° (*c* 2, water)].

β-D-Mannopyranosylamine monohydrate, obtained according to the method of Isbell and Frush[17], and purified as indicated for β-D-galactopyranosylamine, had m.p. 92–93°, $[\alpha]_D^{25}$ −10.8° (*c* 1, water) [lit.[17], m.p. 93–94°, $[\alpha]_D^{20}$ −11.6° (*c* 2, water)].

L-Rhamnosylamine was prepared according to Lobry de Bruyn and van Leent[18] and, after recrystallization from methanol, it had m.p. 114–116°, $[\alpha]_D^{25}$ +35.1° (*c* 1, water) [lit.[18], m.p. 116°, $[\alpha]_D$ +38° (*c* 1, water)].

L-Sorbosylamine was prepared according to Heyns *et al.*[19], and, after recrystallization from methanol, had m.p. 120–121° (lit.[19], m.p. 119.5°). Ring sizes and anomeric configurations of L-rhamnosylamine and L-sorbosylamine are not known.

Syrupy materials obtained as follows were used as D-fructosylamine: (1) A suspension of D-fructose (20.0 g) in methanol (100 ml) was treated with dry ammonia at 0° until the sugar had dissolved. After the solution had stood for 4 h at 0°, the solvent and excess of ammonia were removed. A solution of the residual syrup in methanol (100 ml) was used in the reactions with ethyl acetoacetate. (2) D-Fructose (1.8 g) was dissolved in dry liquid ammonia (80 ml), with the exclusion of water. The solution was kept in an acetone–solid carbon dioxide bath for 45 min, and then allowed to attain room remperature. The resulting syrup was freed of ammonia by keeping it at 1 mm for 2 h.

Ethyl 3-(D-glucosylamino)crotonate (IV)

β-D-Glucopyranosylamine (5.4 g, 0.03 mole) was suspended in a mixture of dry methanol (50 ml), ethyl acetoacetate (13.0 g, 0.1 mole) and piperidine (0.1 ml), and allowed to stand at room temperature for complete dissolution (*ca.* 15 days). Evaporation of solvent left a crystalline residue which was separated, and washed with ethanol and ether. This material was extracted with boiling ethyl acetate,

and the extract was kept at 0° for 24 h. The crude product (3.0 g, 34%, m.p. 134–137°) was collected and washed with ethanol. After several recrystallizations from ethyl acetate, pure product (0.4 g, 5%) was obtained; m.p. 146–148° (dec.), $[\alpha]_D^{19}+95°$ (*c* 1, ethanol).

Anal. Calc. for $C_{12}H_{21}NO_7$: C, 49.5; H, 7.3; N, 4.8. Found: C, 49.8; H, 7.2; N, 5.0.

Hydrolysis of ethyl 3-(D-glucosylamino)crotonate (IV)

A solution of compound (IV) (2.0 g) in 0.1N hydrochloric acid (100 ml) was allowed to stand at room temperature for 24 h. The reaction mixture was concentrated to *ca.* 10 ml, and extracted with ether (2×10 ml). The aqueous layer was evaporated to a syrup (1.4 g) which, when analyzed chromatographically [solvent (*ii*)] and ionophoretically (borate buffer, pH 9), showed only the presence of D-glucose when sprayed with reagents (*a*) and (*b*). A part (0.5 g) of this syrup was treated with *p*-nitroaniline (0.9 g) and conc. hydrochloric acid (0.05 ml) in boiling methanol (20 ml) for 15 min. Refrigeration of the reaction mixture gave *N*-*p*-nitrophenyl-β-D-glucopyranosylamine dihydrate (0.4 g)[20], m.p. and mixed m.p. 186–188°.

Ethyl 3-(2,3,4,6-tetra-O-acetyl-D-glucopyranosylamino)crotonate

A solution of compound (IV) (0.5 g) in dry pyridine (1.5 ml) was treated with acetic anhydride (2 ml) at 0° for 48 h. The mixture was poured into ice, and the crystalline solid was collected and washed thoroughly with water. Recrystallization from propan-2-ol gave the product (0.4 g), m.p. 131–132°, $[\alpha]_D^{18}-2°$ (*c* 0.5, ethanol). T.l.c. of this preparation (light petroleum–ether, 1:4) showed a single spot.

Anal. Calc. for $C_{20}H_{29}NO_{11}$: C, 52.3; H, 6.3; N, 3.1. Found: C, 52.4; H, 6.6; N, 3.0.

A sample (1.0 g) of this tetra-acetyl derivative was deacetylated by treatment with 0.5% ethanolic sodium ethoxide (3.5 ml) for 24 h. The mixture was neutralized with dil. hydrochloric acid and evaporated. The residual syrup was recrystallized from ethyl acetate, giving compound (IV) (0.4 g), m.p. 133–135°. After several recrystallizations from the same solvent, the product (0.1 g) had m.p. and mixed m.p. 145–147°.

Hydrolysis of ethyl 3-(2,3,4,6-tetra-O-acetyl-D-glucopyranosylamino)crotonate

A suspension of the tetra-acetyl derivative (2.3 g) was treated for 7 h in boiling ethanol (25 ml) with 0.5% aqueous formic acid (90 ml). The solid dissolved in the first few minutes. The ethanol was removed by evaporation, and the residual solution was extracted with chloroform (5×10 ml). The combined extracts were washed succesively with 0.2N hydrochloric acid, 5% sodium hydrogen carbonate, 20% sodium bisulfite, and water (10 ml of each), and dried (calcium chloride). The solvent was removed, and the residual syrup was dissolved in hot ethanol and refrigerated. Crystallization of 2,3,4,6-tetra-*O*-acetyl-D-glucose started after several days, and was completed by dropwise addition of dry ether and scratching. Recrystallization from

dry ether gave the product (0.25 g), m.p. and mixed m.p. 122–123°. Its i.r. spectrum was identical to that of an authentic specimen.

Transformation of compound (IV) into ethyl 2-methyl-4-(D-arabino-*tetrahydroxybutyl)-pyrrole-3-carboxylate (II)*

Compound (IV) (6.0 g, 0.02 mole) was treated in boiling methanol for 2 h with acetic acid (1.8 g, 0.03 mole) and potassium acetate (3.0 g, 0.03 mole). The resulting solution gave strongly positive reactions with the Ehrlich reagent in the cold, and with the Tilmans (2,6-dichloroindophenol–sodium hydroxide) reagent[21]. Paper chromatography in solvent (*ii*) showed the presence of substances with the following R_F values: 0.06, 0.15 (D-glucose), 0.30, and 0.65 (pyrrole II), when sprayed with reagent (*a*), and 0.61 (pyrrole II), when sprayed with reagent (*c*). The reaction mixture was neutralized with N sodium hydroxide, and the solvents were removed by evaporation. The residual syrup was dissolved in water (15 ml), and the solution extracted with ether (3 × 10 ml) and then with ethyl acetate in a continuous extractor for 65 h. The ethyl acetate extract was dried (sodium sulfate), and evaporated to give compound (II) as a syrup which crystallized on treatment with ethyl acetate–ethanol (1:1). After recrystallization from the same mixture, the product (0.2 g) had m.p. 141–142° undepressed when mixed with an authentic sample of compound (II)[22]. The i.r. spectra of the two compounds were identical.

Chromatographic [solvent (*ii*)] and ionophoretic (acetate buffer, pH 5.0) tests for 1-amino-1-deoxy-D-fructose in the aqueous solution from which compound (II) had been extracted gave a negative result.

Ethyl 3-(D-*galactosylamino)crotonate (V)*

A suspension of β-D-galactopyranosylamine (18.0 g, 0.1 mole) was treated for 20 days at room temperature in methanol (200 ml) with ethyl acetoacetate (50.0 g, 0.46 mole) and piperidine (0.1 ml). The reaction mixture was filtered to remove a small portion of undissolved material, and evaporated. The residual, crystalline mass was extracted with ether. From the filtered extract, the crude product (17.5 g, 60%), m.p. 126–129°, was obtained and, after several recrystallizations from ethanol, the product (V) (0.75 g, 2.5% was obtained, m.p. 159–160° (dec.), $[\alpha]_D^{26} + 128°$ (*c* 0.9, ethanol).

Anal. Calc. for $C_{12}H_{21}NO_7$: C, 49.5; H, 7.3; N, 4.8. Found: C, 49.7; H, 7.0; N, 5.3.

*Ethyl 3-(2,3,4,6-tetra-*O-*acetyl-*D-*galactopyranosylamino)crotonate*

Compound (V) (0.5 g) was acetylated with acetic anhydride (2 ml) and pyridine (1.5 ml), as indicated for compound (IV). After recrystallization from ethanol–water (2:1), the product had m.p. 100–101°, $[\alpha]_D^{26} + 28°$ (*c* 1, ethanol). T.l.c. (light petroleum–ether, 1:4) of this preparation showed a single spot.

Anal. Calc. for $C_{20}H_{29}NO_{11}$: C, 52.3; H, 6.3; N, 3.1. Found: C, 52.4; H, 6.6; N, 3.1.

A sample (0.58 g) of the tetra-acetate, when deacetylated with sodium ethoxide in ethanol, yielded compound (V) which, after recrystallization from ethanol, had m.p. and mixed m.p. 160–161°; yield, 0.17 g.

Treatment of compound (V) with acetic acid and sodium acetate

Compound (V) (50 mg, 0.15 mmole) was treated in boiling methanol (0.5 ml) with acetic acid (15 mg, 0.25 mmole) and potassium acetate (25 mg, 0.25 mmole), as indicated for compound (IV). Paper chromatography using solvent (*ii*) showed the presence of substances with the following R_F values: 0.11, 0.22 (D-galactose), 0.30, 0.42, 0.70, and 0.83 (trace), when sprayed with reagent (*a*), and 0.70 and 0.84 (trace), when sprayed with reagent (*c*). The reaction mixture was diluted with water (2 ml), neutralized with N sodium hydroxide, and extracted successively with ether (3×2 ml) and ethyl acetate (15×2 ml). The combined extracts were dried (sodium sulfate) and evaporated to leave a syrup (8 mg) which, when examined chromatographically [solvent (*ii*)], showed only the presence of the compounds having R_F 0.70 and 0.84. The aqueous layer of the extraction still contained the compound having R_F 0.70.

Ethyl 3-(D-mannosylamino)crotonate monohydrate (VI)

β-D-Mannopyranosylamine monohydrate (9.8 g, 0.05 mole), suspended in dry methanol (100 ml), was treated with ethyl acetoacetate (25.0 g, 0.2 mole) and piperidine (0.1 ml), as indicated above. The glycosylamine dissolved in *ca.* 12 h. The reaction mixture was stored for 5 days, when dimannosylamine[17] (3.5 g) was precipitated, m.p. and mixed m.p. (160–162°), $[\alpha]_D^{19}$ −34° (*c* 1, water), R_F 0.07 in solvent (*ii*) [lit.[17], m.p. 157–158°, $[\alpha]_D^{20}$ −36.8° (*c* 5, water)]. Evaporation of the filtrate afforded crude product (VI) (1.55 g, 16%), m.p. 177–179°. After several recrystallizations from 95% ethanol, the product (0.5 g, 5%) had m.p. 187–189° (dec.), $[\alpha]_D^{19}$ +153° (*c* 0.5, ethanol). The m.p. with a sample (m.p. 188–189°) obtained directly from D-mannose, ethyl acetoacetate, and ammonia[3], showed no depression.

Anal. Calc. for $C_{12}H_{21}NO_7 \cdot H_2O$: C, 46.6; H, 7.5; N, 4.6. Found: C, 46.8; H, 7.7; N, 4.3.

Ethyl 3-(2,3,4,6-tetra-O-acetyl-D-mannopyranosylamino)crotonate

Compound (VI) (0.3 g) was acetylated with acetic anhydride (2 ml) and pyridine (1.5 ml), as indicated for compound (IV). After recrystallization from ethanol–water (2:1), the product (0.26 g) had m.p. 149.5–150°, $[\alpha]_D^{16}$ 0° (*c* 0.5, ethanol). T.l.c. (light petroleum–ether, 1:4) of this preparation showed a single spot.

Anal. Calc. for $C_{20}H_{29}NO_{11}$: C, 52.3; H, 6.3; N, 3.1. Found: C, 52.5; H, 6.0; N, 3.2.

A sample (0.35 g) of this tetra-acetate was deacetylated with sodium ethoxide in ethanol, as indicated above, yielding compound (VI). After recrystallization from 95% ethanol, the product (0.15 g) had m.p. and mixed m.p. 187–189° (dec.).

Transformation of compound (VI) into ethyl 2-methyl-4-(D-arabino-*tetrahydroxybutyl)-pyrrole-3-carboxylate (II)*

Compound (VI) (2.5 g, 80 mmole) was treated in boiling methanol (25 ml), with acetic acid (0.75 g, 12 mmole) and potassium acetate (1.25 g, 12 mmole), as indicated above for compound (IV). Chromatographic analysis, using solvent system (*i*) and detection with reagents (*a*) and (*c*), showed the presence of compound (II) (R_F 0.63). The reaction mixture was neutralized with N sodium hydroxide, and evaporated to a pale-yellow syrup which was dissolved in water (15 ml). This solution was extracted successively with ether (3×10 ml) and with ethyl acetate (15×5 ml). The combined ethyl acetate extracts were dried (sodium sulfate) and evaporated. The residual syrup crystallized on treatment with ethyl acetate–ethanol (1:1). After recrystallization from water, the product (II)[22] (0.15 g) had m.p. and mixed m.p. 140–141°.

Paper chromatography, using solvent (*ii*), of the aqueous solution, from which compound (II) had been extracted, showed the presence of compound (II), in addition to D-mannose and an unknown substance of R_F 0.44. Chromatographic and ionophoretic (acetate buffer, pH 5.0) tests for 1-amino-1-deoxy-D-fructose were negative.

Treatment of compound (VI) with carbonate–hydrogen carbonate buffer (pH 9.5)

A solution of compound (VI) (50 mg) in a sodium carbonate–sodium hydrogen carbonate buffer (pH 9.5, 5 ml) was heated at 100° for 30 min. The cooled reaction mixture, which gave a strong pyrrole–Ehrlich reaction, was brought to pH 6.5 by addition of Amberlite IR-120 (H^+ form). The resin was removed, and the filtrate concentrated *in vacuo* (50–60° (bath)/20 mm) to a volume of *ca.* 1 ml, the distillate being collected in a cooled (ice–salt) receiver. Tests for 2-methylpyrrole in this distillate, as described previously[11], gave a negative result. The distillation residue was diluted with water (5 ml), and extracted with ether (3×5 ml). T.l.c. (light petroleum–ether, 1:1) of the extracts showed [reagent (*c*)] a red spot having the same mobility as ethyl 2-methylpyrrole-3-carboxylate[11]. Paper chromatography in solvent (*ii*) of the aqueous layer showed the presence of a compound having the same mobility (R_F 0.64) as that of compound (II).

Ethyl 3-(L-*rhamnosylamino)crotonate monohydrate (VII)*

A suspension of L-rhamnosylamine (14.0 g, 0.08 mole) was treated in methanol (180 ml) at room temperature with ethyl acetoacetate (44.6 g, 0.3 mole) and piperidine (0.1 ml). The glycosylamine dissolved in *ca.* 4 days and, shortly afterwards, the crystallization of compound (VII) began. After 10 days, the product [8.0 g, 39%, m.p. 187–193° (dec.)] was collected and washed with ethanol. After recrystallization from 95% ethanol, the product (7.2 g, 31%) had m.p. 201–202°, $[\alpha]_D^{16} -119°$. The mixed m.p. with a sample (m.p. 203–204°) obtained directly from L-rhamnose, ethyl acetoacetate, and ammonia[3] showed no depression.

Anal. Calc. for $C_{12}H_{21}NO_6$ H_2O: C, 49.0; H, 7.9; N, 4.8. Found: C, 49.2; H, 8.0; N, 4.6.

Ethyl 3-(2,3,4-tri-O-acetyl-L-rhamnopyranosylamino)crotonate

Compound (VII) (0.1 g) was acetylated with acetic anhydride (0.75 ml) and pyridine (1 ml), as indicated for compound (IV). After recrystallization from ethanol–water (2:1), the product (0.11 g) had m.p. 145–146°, $[\alpha]_D^{16}+23°$ (*c* 1, ethanol). T.l.c. (light petroleum–ether, 1:4) of this preparation showed a single spot.

Anal. Calc. for $C_{18}H_{27}NO_9$: C, 53.9; H, 6.8; N, 3.5. Found: C, 54.0; H, 6.5; N, 3.4.

A sample (1.6 g) of this tri-acetate was deacetylated with sodium ethoxide in ethanol, as indicated previously. After recrystallization from 95% ethanol, the product (VII) (0.85 g) had m.p. and mixed m.p. 198–200° (dec.).

Treatment of compound (VII) with acetic acid and potassium acetate

A suspension of compound (VII) (1.45 g, 5 mmole) was treated in boiling methanol (30 ml) with acetic acid (0.5 g, 8 mmole) and potassium acetate (0.6 g, 6 mmole), as indicated for compound (IV). Chromatographic analysis in solvent system (*i*), with reagents (*a*) and (*c*), showed the presence of a substance of R_F 0.94. The reaction mixture was evaporated to a yellow syrup which was dissolved in water (7 ml). The solution was neutralized with N sodium hydroxide, and extracted successively with ether (4×5 ml) and ethyl acetate (20×5 ml). The combined ethyl acetate extracts were dried (sodium sulfate) and concentrated to a syrup (0.19 g). Paper chromatography in solvent (*i*) showed only the compound of R_F 0.94.

Reaction of D-fructosylamine (XII) with ethyl acetoacetate

Ethyl acetoacetate (50.0 g, 0.38 mole) and piperidine (0.1 ml) were added to a methanolic solution (55 ml) of D-fructosylamine [*ca.* 9 g, 0.05 mole, obtained by method (1)]. The mixture was allowed to stand at room temperature. After 4 days, t.l.c. (methanol–ether, 1:1) showed the presence of ethyl 2-methyl-5-(D-*arabino*-tetrahydroxybutyl)pyrrole-3-carboxylate (XV) (R_F 0.71), D-fructose (R_F 0.56), and an unknown substance of R_F 0.83. The last compound disappeared on longer standing. After 20 days, the solvent was removed, and the residue treated with ether. The crystalline mass was separated and recrystallized from ethanol, yielding compound (XV)[23] (0.3 g), m.p. and mixed m.p. 141–142°.

Experiments made with syrupy D-fructosylamine, obtained by method (2), gave the same result, compound (XV) being isolated in 3–5% yield.

Reaction of L-sorbosylamine with ethyl acetoacetate

A suspension of L-sorbosylamine (1.8 g, 0.01 mole) was treated in methanol at room temperature with ethyl acetoacetate (5.2 g, 0.04 mole) and piperidine (0.05 mole). The glycosylamine dissolved during 12 days. Chromatographic analysis of the reaction mixture in solvent system (*i*) showed the presence of substances having R_F 0.04, 0.14 (L-sorbosylamine), and 0.67 when sprayed with reagent (*c*). Removal of solvent left a syrup which was dissolved in water (10 ml). This solution was extracted with ether (5 ml) and then with ethyl acetate (10×5 ml). The combined

ethyl acetate extracts were dried (sodium sulfate), and evaporated to a syrup (0.4 g) which, when examined chromatographically in solvent (*i*), showed only the compound having R_F 0.67. A sample (0.1 g), dissolved in water, was treated at 0° with an excess of sodium metaperiodate solution for 0.5 h. The oxidation mixture was extracted with ether (3 × 10 ml), and the combined extracts were dried (sodium sulfate) and evaporated. The solid residue gave, on recrystallization from acetone–water, 4-ethoxycarbonyl-5-methylpyrrole-2-carboxaldehyde[24] (XVIII) (11 mg), m.p. and mixed m.p. 130–132°.

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SUMMARY

The glycosylamines of the common aldohexoses react with ethyl acetoacetate in methanol at room temperature yielding the corresponding ethyl 3-(glycosylamino)-crotonates. The structures of these compounds are discussed on the basis of their chemical and spectroscopic properties. These substances can be transformed into ethyl 2-methyl-4-(polyhydroxyalkyl)pyrrole-3-carboxylates under conditions that promote the Amadori rearrangement of glycosylamines.

The reaction of ketohexosylamines (D-fructosylamine, L-sorbosylamine) with ethyl acetoacetate proceeds to give ethyl 2-methyl-5-(tetrahydroxybutyl)pyrrole-3-carboxylates.

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LYSOZYME SUBSTRATES. SYNTHESIS OF *p*-NITROPHENYL 2-ACETAMIDO-4-*O*-(2-ACETAMIDO-2-DEOXY-β-D-GLUCOPYRANOSYL)-2-DEOXY-β-D-GLUCOPYRANOSIDE AND ITS β-D-(1 → 6) ISOMER*

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INTRODUCTION

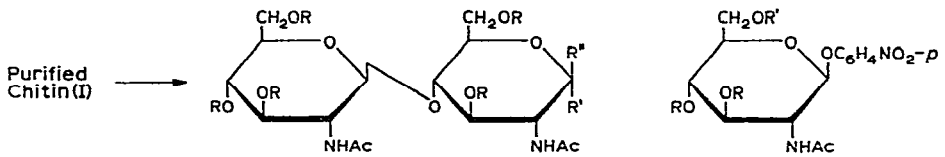
Detailed information is available[1] on the amino acid sequence and physicochemical properties of the enzyme lysozyme, obtained from egg-white. Relatively little is known, however, about the substrate specificity of lysozyme. It was shown by Salton *et al.*[2] that the β-D-(1 → 4) glycosidic bonds between 2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-α-D-glucose (*N*-acetylmuramic acid) and 2-acetamido-2-deoxy-α-D-glucose units of the bacterial cell-wall mucopeptide are hydrolyzed by lysozyme. Berger *et al.*[3] observed that lysozyme could degrade chitin, a β-(1 → 4)-linked 2-acetamido-2-deoxy-D-glucose polymer, but these investigators found that the enzyme does not catalyze the hydrolysis of phenyl 2-acetamido-2-deoxy-β-D-glucopyranoside. More recently, Yamamoto *et al.*[4] reported that phenyl 2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-α-(and -β)-D-glucopyranoside are not susceptible to the enzyme. Meanwhile, the β-*N*-acetylglucosaminidase activity of lysozyme was further confirmed by the finding[5,6] that lysozyme can exert its hydrolytic action on *O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1 → 4)-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-acetamido-2-deoxy-α-D-glucopyranose (*N*,*N*′,*N*″-triacetylchitotriose), which is the simplest lysozyme substrate to have been reported. The evidence summarized above seems to indicate that substrates for lysozyme must contain at least three saccharide units. Uncertainties remain regarding steric requirements in the structure of the terminal non-reducing residue, and in the mode of linkage between the latter and the sugar residue whose glycosidic bond undergoes attack.

This paper describes the synthesis of *p*-nitrophenyl 2-acetamido-4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-β-D-glucopyranoside (V) and its β-D-(1 → 6)-linked isomer (XI), and the reactions of these products with lysozyme. The results obtained are discussed in relation to the structure of a disaccharide isolated from the cell wall of *Micrococcus lysodeikticus* by the action of lysozyme, and to the mechanism of resistance of certain bacteria to egg-white lysozyme.

*This is publication X of a series dealing with nitrogen-containing sugars.

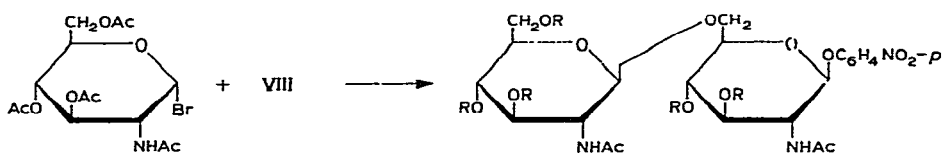
RESULTS

Chitobiose octaacetate[7,8] (II), prepared by acetolysis of purified chitin (I), was treated with acetic anhydride saturated with hydrochloric acid, and the resulting 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 chloride (III) was condensed in aqueous acetone with the sodium salt of *p*-nitrophenol, to give *p*-nitrophenyl 2-acetamido-4-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranoside (IV). This product was *O*-deacetylated in the presence of sodium methoxide to give the disaccharide glycoside (V) in an over-all yield of 13% from II.



	R	R'	R"		R	R'
II	Ac	OAc	H	VI	H	H
III	Ac	Cl	H	VII	Ac	Tr
IV	Ac	H	$OC_6H_4NO_2$-*p*	VIII	Ac	H
V	H	H	$OC_6H_4NO_2$-*p*			

The β-D-(1 → 6)-linked isomer of V, *p*-nitrophenyl 2-acetamido-6-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-β-D-glucopyranoside (XI), was prepared by the Koenigs–Knorr reaction. *p*-Nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside[9] (VI) was tritylated and acetylated to give *p*-nitrophenyl 2-acetamido-3,4-di-*O*-acetyl-2-deoxy-6-*O*-trityl-β-D-glucopyranoside (VII). Detritylation of VII with hot 60% acetic acid gave *p*-nitrophenyl 2-acetamido-3,4-di-*O*-acetyl-2-deoxy-β-D-glucopyranoside (VIII), in an over-all yield of 42% from VI. Condensation of



IX

X R = Ac

XI R = H

VIII with 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl bromide[10] (IX) in the presence of mercuric cyanide, in a mixture of chloroform and nitromethane, gave *p*-nitrophenyl 2-acetamido-6-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-3,4-di-*O*-acetyl-2-deoxy-β-D-glucopyranoside (X). The yield of

blocked disaccharide was low (2.4%), comparable with previously reported syntheses of other 2-amino-(2-amino-2-deoxyglucosyl)-2-deoxyglucose disaccharides [11–13]. *O*-Deacetylation of X with sodium methoxide gave the desired disaccharide glycoside (XI) in 63% yield.

The lysozyme-catalyzed hydrolysis of V, VI, and XI was performed at 37° in a solution containing 0.001M sugar, 5 mg/ml of lysozyme, and 0.04M sodium citrate buffer at the optimum pH (5.1), with a drop of toluene to inhibit bacterial growth. The data are presented in Fig. 1. Under these conditions, compound V was hydrolyzed slowly, and 2-acetamido-4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-D-glucose (*N,N'*-diacetylchitobiose) was liberated, whereas both VI and XI were found to be not susceptible to the enzyme.

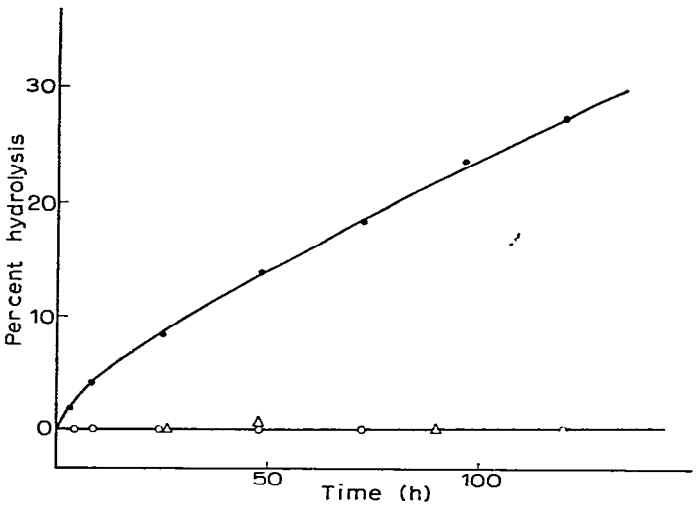


Fig. 1. The lysozyme-catalyzed hydrolysis of V (•), VI (△), and XI (○). Experimental details are given in the text.

EXPERIMENTAL

General

Melting points were taken on a hot stage, equipped with a microscope, and are not corrected. Specific rotations were determined in semi-micro polarimeter tubes having lengths of 2 dm or 1 dm, with a Zeiss polarimeter having a scale reading to 0.01°. The silicic acid used for chromatography was "Silica Gel Davison" (grade 950 60-200 mesh), used without pretreatment. The eluents were used in the following sequence, individually or in binary mixtures: hexane, benzene or 1,2-dichloroethane, ether, ethyl acetate, acetone, and methanol. Evaporations were performed *in vacuo* at 35–40° (bath temperature). Small amounts of volatile solvents (less than 20 ml) were evaporated under a stream of dry nitrogen. Microanalyses were performed by Misses Yamanouchi and Izumizawa, Institute of Applied Microbiology, University of Tokyo.

Preparation of 2-acetamido-4-O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)-1,3,6-tri-O-acetyl-2-deoxy-α-D-glucopyranose (Chitobiose octaacetate)[7,8] *(II)*

Purified chitin (I) was prepared from lobster shells according to the procedure of Barker *et al*[8]. To a cooled mixture of acetic anhydride (100 ml) and conc. sulfuric acid (13 ml) was added I (20 g). The mixture was kept overnight at room temperature and then heated for 8 h at 55°. After being poured into a cooled solution of sodium acetate (80 g) in water (520 ml), the mixture was centrifuged, and the supernatant was extracted with chloroform (3 × 300 ml). The combined extracts were washed successively with water, cold saturated sodium bicarbonate solution, and water. Evaporation of the chloroform solution after drying over sodium sulfate gave a crystalline residue (14 g). It was dissolved in ethyl acetate and chromatographed on silica gel. A 4:1 mixture of ethyl acetate and acetone eluted 5.4 g of crystalline fractions, which, after two recrystallizations from methanol, gave white needles, yield 1.8 g (5.5%); m.p. 301–303° (dec.); $[\alpha]_D^{30}$ +56° (*c* 0.52, acetic acid). [Zechmeister *et al.*[7] reported m.p. 305°, $[\alpha]_D$ +55° (*c* 0.38, acetic acid)].

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 chloride (III)

A solution of II (0.50 g), in glacial acetic acid (6 ml) saturated at 0° with hydrochloric acid, was kept at room temperature for 48 h. After dilution with chloroform (50 ml), the solution was washed once with water, twice with an ice-cold saturated solution of sodium bicarbonate, and finally with water. The solution was dried over sodium sulfate, and the crystalline residue obtained after evaporation of the solvent was recrystallized from a mixture of ethyl acetate, chloroform, and pentane to give white needles, yield 0.32 g (66%); m.p. 208–209° (dec.); $[\alpha]_D^{20}$ +41° (*c* 0.41, chloroform).

Anal. Calc. for $C_{26}H_{38}ClN_2O_{15}$:C, 47.74; H, 5.86; N, 4.28. Found: C, 47.72; H, 5.72; N, 4.38.

p-*Nitrophenyl 2-acetamido-4-O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)-3,6-di-O-acetyl-2-deoxy-β-D-glucopyranoside (IV)*

To a solution of III (289 mg) and *p*-nitrophenol (128 mg) in acetone (10 ml) was added 0.5N sodium hydroxide (1.99 ml). The mixture was kept for 16 h at 5°. After evaporation of acetone, the separated crystals were collected, washed with ice-cold water until the washings were colorless, and dried to give white needles, yield 110 mg (33%); m.p. 245–246° (dec.).

Recrystallization from a mixture of methanol and chloroform raised the m.p. to 256–257° (dec.); $[\alpha]_D^{24}$ −41° (*c* 0.22, pyridine); λ_{max}^{KBr} 2.95 (NH), 5.78 (OAc), 6.06, 6.49 (NHAc), 6.29, 6.70 (aryl C=C), 7.40 (NO_2), 11.17 (β-glucoside).

Anal. Calc. for $C_{32}H_{41}N_3O_{18}$: C, 50.86; H, 5.47; N, 5.57. Found: C, 50.54; H, 5.48; N, 5.60.

p-*Nitrophenyl 2-acetamido-4-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-β-D-glucopyranoside (V)*

A suspension of IV (100 mg) in dry methanol (2.5 ml) was warmed to 40°, 1.0N sodium methoxide (0.1 ml) was added, and the mixture was shaken until solution was complete. After a further 5 min, precipitation of the *O*-deacetylated product began, and the mixture was kept overnight at 5°. The solid was collected, the filtrate was evaporated, and the combined solids were recrystallized from aqueous methanol to give white needles, yield 41 mg (57%); m.p. 226–227° (dec.); $[\alpha]_D^{27}$ −21° (*c* 0.24, 70% methanol in water); λ_{max}^{KBr} 3.00 (OH, NH), 6.08, 6.47 (NHAc), 6.28, 6.67 (aryl C=C), 7.40 (NO_2), 11.20 (β-glucoside). The substance was pure by t.l.c. on silica gel with a 4:5:1 mixture of methanol, acetic acid, and water as developing solvent, and *p*-anisaldehyde–sulfuric acid as detecting reagent; $R_{lactose}$ 1.4.

Anal. Calc. for $C_{22}H_{31}N_3O_{13} \cdot H_2O$: C, 46.89; H, 5.90; N, 7.46. Found: C, 46.94, H, 5.94; N, 7.32.

p-*Nitrophenyl 2-acetamido-3,4-di-O-acetyl-2-deoxy-6-O-trityl-β-D-glucopyranoside (VII)*

To *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside[9] (VI, m.p. 210°, 2.0 g) in dry pyridine (15 ml) was added chlorotriphenylmethane (1.8 g). The solution was heated for 30 min at 100° and then, after addition of acetic anhydride (10 ml), was kept for 24 h at room temperature. The solution then was poured into ice-water and the gummy precipitate was dissolved in chloroform. The chloroform solution was washed three times with ice-cold 10% potassium bisulfate solution, three times with water, dried over sodium sulfate and evaporated. The crystalline residue was recrystallized from a mixture of chloroform and methanol to give white needles, yield 2.0 g (51%); m.p. 256° (dec.); $[\alpha]_D^{20}$ +7° (*c* 0.57, chloroform).

Anal. Calc. for $C_{37}H_{36}N_2O_{10}$: C, 66.46; H, 5.43; N, 4.21. Found: C, 66.37; H, 5.40; N, 4.53.

p-*Nitrophenyl 2-acetamido-3,4-di-O-acetyl-2-deoxy-β-D-glucopyranoside (VIII)*

A solution of VII (1.4 g) in 60% aqueous acetic acid (35 ml) was heated for 30 min at 100°. The solution was evaporated, and the last trace of acetic acid was removed by codistillation with toluene. The crystalline residue was extracted with 50 ml of boiling benzene, and the insoluble part was collected by filtration, and recrystallized from methanol to give white needles, yield 0.75 g (83%); m.p. 241–242° (dec.); $[\alpha]_D^{20}$ −10° (*c* 0.69, 1:1 chloroform–acetone).

Anal. Calc. for $C_{18}H_{22}N_2O_{10}$: C, 50.70; H, 5.20; N, 6.57. Found: C, 50.67; H, 5.24; N, 6.84.

p-*Nitrophenyl 2-acetamido-6-O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)-3,4-di-O-acetyl-2-deoxy-β-D-glucopyranoside (X)*

To a stirred solution of VIII (1.00 g) in dry nitromethane (30 ml) was added mercuric cyanide (660 mg) and a solution of 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-

α-D-glucopyranosyl bromide[10] (IX) in a mixture of dry chloroform (5 ml) and dry nitromethane (5 ml). The bromide IX was prepared *in situ* from 2-acetamido-1,3,4,6-tetra-*O*-acetyl-2-deoxy-β-D-glucopyranose (1.10 g). After 24 h at room temperature, the preceding quantities of mercuric cyanide and IX were again added, and the reaction was allowed to proceed for an additional 24 h. The reaction mixture was then diluted with chloroform, washed with saturated sodium bicarbonate solution, and water, and evaporated. The residue was dissolved in 1,2-dichloroethane and chromatographed on silica gel. The crystalline fractions eluted by ethyl acetate and a 4:1 mixture of ethyl acetate and acetone were recrystallized from acetone–ether to give white needles, yield 43 mg (2.4%); m.p. 251–254° (dec.). A further recrystallization from the same solvent raised the m.p. to 257–258° (dec.); $[\alpha]_D^{23}$ −43° (*c* 0.21, chloroform); the infrared spectrum showed no substantial difference from that of IV: λ_{max}^{KBr} 3.01 (NH), 5.80 (OAc), 6.06, 6.49 (NHAc), 6.30, 6.66 (aryl C=C), 7.40 (NO_2), 11.17 (β-glucoside).

Anal. Calc. for $C_{32}H_{41}N_3O_{18} \cdot H_2O$: C, 49.67; H, 5.60; N, 5.43. Found: C, 50.06, 49.74; H, 5.43, 5.44; N, 5.46.

The analyses were performed on samples from two separate preparations which had been dried at 70° *in vacuo* for 24 h.

p-Nitrophenyl 2-acetamido-6-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-β-D-glucopyranoside (XI)

To a suspension of X (78 mg) in dry methanol (2.0 ml) was added N sodium methoxide at 40°. The mixture was shaken until solution was complete. After a further 5 min, precipitation of the *O*-deacetylated product began, and the mixture was kept overnight at 5°. The solid was collected, the filtrate was evaporated, and the combined solids were recrystallized from aqueous methanol to give white needles, yield 35 mg (63%); m.p. 220–221° (dec.), after softening at 210°; $[\alpha]_D^{25}$ −55° (*c* 0.20, 70% methanol in water); the infrared spectrum showed no substantial difference from that of V: λ_{max}^{KBr} 3.00 (OH, NH), 6.10, 6.47 (NHAc), 6.28, 6.67 (aryl C=C), 7.40 (NO_2), 11.13 (β-glucoside). The substance was pure by t.l.c. on silica gel with a 4:5:1 mixture of methanol, acetic acid, and water as developing solvent, and *p*-anisaldehyde–sulfuric acid as detecting reagent; $R_{lactose}$ 1.4.

Anal. Calc. for $C_{22}H_{31}N_3O_{13} \cdot 2H_2O$: C, 45.44; H, 6.07; N, 7.23. Found: C, 45.05; H, 6.05; N, 6.83.

On being dried at 120° *in vacuo* for 48 h, the substance lost 6.4% of its weight (calc. for dihydrate: 6.6%).

Enzymic hydrolysis of V, XI, and VI

Crystalline lysozyme was prepared from egg-white according to the procedure of Fevold *et al.*[14]. Samples of a 0.001 M solution of the sugar derivative (1.0 ml) in 0.04 M sodium citrate buffer (pH 5.1), containing 5 mg of lysozyme, were incubated at 37°. After an appropiate time, the reaction was terminated by addition of 2.0 ml of 0.2 M borate buffer (pH 9.8) according to Woollen *et al.*[15]. The chromophore

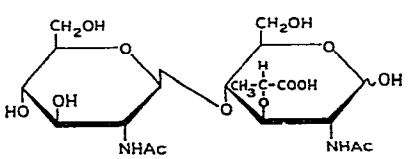
was measured at 400 mμ and the amount of *p*-nitrophenol liberated by the enzyme was determined from the molar extinction coefficient (1.8×10^{-2}) of the chromophore at this wavelength.

The release of 2-acetamido-2-deoxy-D-glucose from V and XI during the hydrolysis was checked by the Morgan–Elson test[16] and was found to be zero in each case.

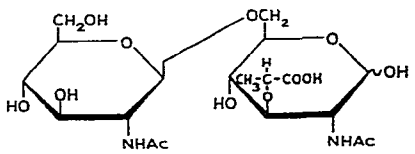
The enzymic hydrolyzate of V was dialyzed thoroughly against distilled water and the dialyzate was concentrated. Descending paper chromatography of the residue, with Toyo Roshi No. 51 paper and a 5:5:1:3 mixture of ethyl acetate, pyridine, acetic acid, and water as solvent[17], revealed the presence of 2-acetamido-4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-D-glucose (*N,N'*-diacetylchitobiose, R_{GNac} 0.71).

DISCUSSION

The structure 2-acetamido-6-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucose (XIII) has been proposed[2,18] for a disaccharide isolated from the cell wall of *Micrococcus lysodeikticus* by the action of egg-white lysozyme. Recently, a crystalline fully acetylated methyl ester derivative of the disaccharide, from the natural source, was compared with synthetic 2-acetamido-6-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-1,4-di-*O*-acetyl-2-deoxy-3-*O*-[D-1-(methyl carboxylate)ethyl]-α-D-glucopyranose[13]. It was shown that the two compounds are not identical[19]. The only possible structure of the natural disaccharide is thus 2-acetamido-4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucopyranose (XII). Although final proof of the structure of the disaccharide from bacterial cell-wall will have to await the unequivocal synthesis of XII, the data obtained from the action of lysozyme on V and XI provide further evidence for the structure XII, because they suggest that lysozyme exerts its hydrolytic action only on the substrate having an unsubstituted hydroxyl group at C-6 of the 2-acetamido-2-deoxy-D-glucose or *N*-acetylmuramic acid residue whose glycosidic bond is attacked.



XII



XIII

It is well known that certain bacterial cell-walls are resistant to lysozyme. Brumfitt *et al.*[20] have suggested that the presence of *O*-acetyl groups in the cell wall renders the bacteria resistant to lysozyme. Recently, Ghuysen *et al.*[21,22] isolated

from a lysozyme-resistant strain of *Staphylococcus aureus* an *O*-acetylated disaccharide, namely 2-acetamido-4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-6-*O*-acetyl-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucose. Although the complete solution of the question of the mechanism of resistance to lysozyme in the bacterial cell-wall is obviously a very complicated task, the fact that XI is not susceptible to lysozyme is in line with the mechanism of resistance suggested by Brumfitt *et al.* The fact supports the theory that the acetoxy groups at C-6 of the *N*-acetylmuramic acid moieties contribute, at least in part, to the resistance of the cell wall to egg-white lysozyme.

Substance V is the first synthetic compound which can be hydrolyzed appreciably by lysozyme. However, its hydrolysis is slow, and requires relatively massive proportions of enzyme. Current investigations on synthetic analogs may provide a compound more suitable for measurement of lysozyme activity.

ACKNOWLEDGMENT

The author thanks Prof. Y. Nakazawa for his interest in this work.

SUMMARY

In order to study the substrate specificities of egg-white lysozyme, *p*-nitrophenyl 2-acetamido-4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-β-D-glucopyranoside (V) and *p*-nitrophenyl 2-acetamido-6-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-β-D-glucopyranoside (XI) were synthesized. Compound V was hydrolyzed by lysozyme, even though its hydrolysis was slow and required relatively massive proportions of the enzyme. On the other hand, both XI and *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (VI) were found to be unaffected by the enzyme. These observations are discussed in relation to the structure of a disaccharide isolated from the cell wall of *Micrococcus lysodeikticus* by the action of lysozyme, and to the mechanism of resistance of certain bacteria to egg-white lysozyme.

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SYNTHESIS AND REACTIONS OF UNSATURATED SUGARS

UNSATURATED SUGARS THROUGH THIONOCARBONATE INTERMEDIATES AND SYNTHESIS OF A 5-DEOXY-6-THIOHEXOSE SYSTEM*

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The objective of this investigation was the synthesis of a 5-deoxy-6-thiohexose system. Such a structure, in which the pyranoid ring cannot be formed, should provide an interesting model for ring-closure reactions under conditions of thermodynamic or kinetic control. Possible ring-forms include the furanoid, the septanoid having sulfur in the ring, and the bicyclic [3:2:1], glycosan type of structure. The present report describes the use of the recently described Corey–Winter olefin synthesis[3] for preparation of 5,6-dideoxy-1,2-*O*-isopropylidene-α-D-*xylo*-hex-5-enofuranose (V) from 1,2-*O*-isopropylidene-α-D-glucofuranose (I). A 5-deoxy-6-thiohexose system was synthesized by the photocatalyzed addition of thiolacetic acid to the unsaturated sugar V, which gives 6-*S*-acetyl-5-deoxy-1,2-*O*-isopropylidene-6-thio-α-D-*xylo*-hexofuranose (IV).

1,2-*O*-Isopropylidene-α-D-glucofuranose (I) was treated with a slight excess of bis(imidazol-1-yl)thione[4] in refluxing acetone to give, in 85% yield, a crystalline product formulated as 1,2-*O*-isopropylidene-α-D-glucofuranose 5,6-thionocarbonate (II). The latter was characterized by elemental analysis, by physical data, and by conversion into the crystalline 3-*p*-phenylazobenzoate (III). The thionocarbonate II was refluxed in trimethyl phosphite for 60 h to give, in 75% yield, a product identified as 5,6-dideoxy-1,2-*O*-isopropylidene-α-D-*xylo*-hex-5-enofuranose (V) by direct comparison with an authentic sample which had been prepared[5] by treatment of 1,2-*O*-isopropylidene-5,6-di-*O*-*p*-tolylsulfonyl-α-D-glucofuranose[5], with sodium iodide in acetone. In our hands, the overall yield, from I, of the unsaturated sugar V was 2–3 times as great by the route through the thionocarbonate II as by the route through the 5,6-di-*p*-toluenesulfonate of I.

The 3-*p*-phenylazobenzoate (III) of the thionocarbonate II underwent conversion in boiling trimethyl phosphite into a crystalline product, isolated in 68% yield, formulated as 5,6-dideoxy-1,2-*O*-isopropylidene-3-*O*-*p*-phenylazobenzoyl-α-D-*xylo*-hex-5-enofuranose (VI). The structure assigned to VI was supported by physical data, and verified by its identity with the compound prepared by *p*-phenylazobenzoylation of 5,6-dideoxy-1,2-*O*-isopropylidene-α-D-*xylo*-hex-5-enofuranose (V).

The n.m.r. spectrum of 5,6-dideoxy-1,2-*O*-isopropylidene-α-D-*xylo*-hex-5-enofuranose (V) permits a straightforward analysis[6], as shown in Figure 1. The

*For preliminary communications, see refs. 1 and 2.

spectrum of the 3-*p*-phenylazobenzoate (VI) is similar, but the H-3 signal is observed at lower field, presumably due to deshielding by the acyl substituent, and there is no hydroxyl proton signal present. Crystallization of compound V was sometimes difficult, but the analytically pure oil obtained by distillation, in the preparation from II, gave an n.m.r. spectrum essentially identical to that of the crystalline material.

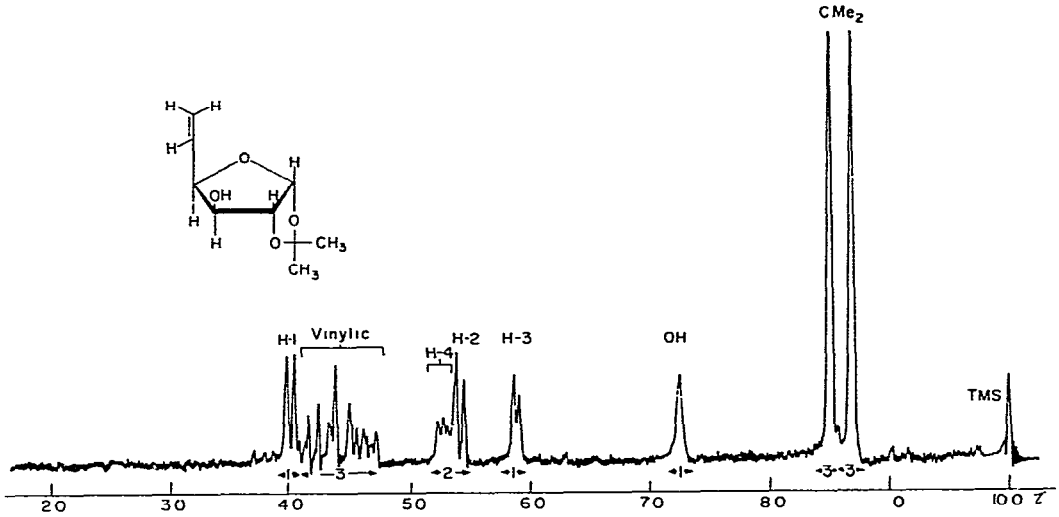
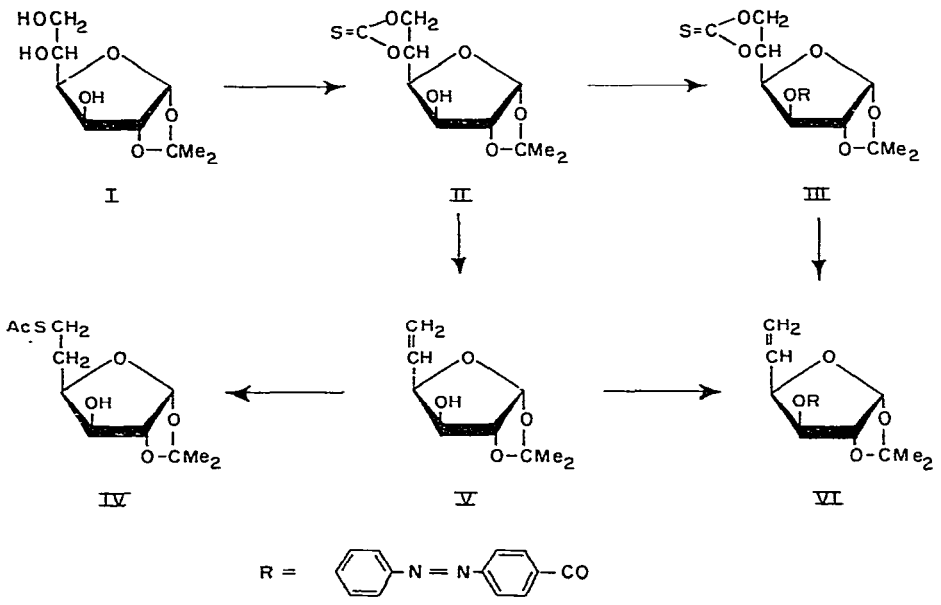


Fig. 1. The n.m.r. spectrum of 5,6-dideoxy-1,2-*O*-isopropylidene-α-D-*xylo*-hex-5-enofuranose (V) at 60 Mc.p.s. in deuteriochloroform. Numbers below the peaks give integrated peak intensities.

The reaction of the unsaturated sugar V with thiolacetic acid, under illumination, was followed by thin-layer chromatography. It is known[7] that thiolacetic acid readily undergoes addition to simple olefins, under illumination from a tungsten lamp, and that anti-Markownikoff addition takes place, presumably by a free-radical mechanism. Conversion of V was very slow under irradiation with visible light, but proceeded smoothly when a source[8] of ultraviolet light at 2537 Å was used. The reaction mixture was processed when all of the starting material had been converted, and a crystalline adduct, formulated as 6-*S*-acetyl-5-deoxy-1,2-*O*-isopropylidene-6-thio-α-D-*xylo*-hexofuranose (IV), was isolated in 54% yield. The structure of this product is firmly established by analytical and physical data, and is further supported by data on its 3-acetate. The infrared spectrum of IV showed absorptions characteristic of the hydroxyl (2.95 μ), *S*-acetyl carbonyl[9] (5.95 μ), and isopropylidene (7.26 μ) groups; the 3-acetate showed an additional carbonyl absorption (5.80 μ, *O*-acetyl carbonyl) and no hydroxyl absorption. These data, together with the microanalytical data, show that IV is an adduct of V and thiolacetic acid. The n.m.r. spectrum of IV (Fig. 2) shows unambiguously that the product has the acetylthio group at C-6, formed by anti-Markownikoff addition, and excludes the alternative structure having the acetylthio group at C-5. The H-1 and H-2

signals[6] appear as narrow doublets at τ 4.08 and 5.43, respectively, and the signals for H-3 and H-4 form an overlapping multiplet at τ 5.81. The two-proton triplet at τ 7.03 is assigned to the C-6 methylene protons; the splitting of the signal is due to spin-coupling with the C-5 methylene protons ($J_{5,6}$ 7.8 c.p.s.). The two-proton multiplet at τ 7.98 may be assigned to the C-5 protons; the signal is split by spin-coupling with protons on C-6 and C-4. The broad, one-proton signal at τ 7.40 is assigned to the hydroxyl proton; its chemical shift varies with the concentration of the sample, and the signal disappears when the sample is deuterated. The methyl protons of the



acetylthio group give rise to the three-proton singlet at τ 7.63, and the three-proton singlets at τ 8.48 and 8.69 are assigned to the isopropylidene methyl groups. The n.m.r. spectrum of the 3-acetate of IV was quite similar to that of IV, but showed an additional, three-proton singlet at τ 7.92 due to the *O*-acetyl group. No hydroxyl signal was observed, and the H-3 signal was observed, at lower field, as a narrow doublet, τ 4.80; the shift is attributable to the deshielding effect of the acetoxy group. The spectra of IV and its 3-acetate showed no signal in the τ 8.9 region, where a doublet for a terminal methyl group would have been expected[10] had the product 6-deoxy-5-thio structure.

S-Acetyl signals are characteristically observed 0.3–0.4 p.p.m. downfield from *O*-acetyl signals, for spectra determined in deuteriochloroform. This has been noted in the case of several acetylated 1-thioaldoses[11] and acetylated thioinositols[12]. A clear example is provided herein by the spectrum of 2,3-di-*O*-acetyl-1-*S*-acetyl-1-thio-DL-glycerol[9], which shows a three-proton singlet, τ 7.67, for the acetylthio group, and a six-proton singlet, τ 8.01, for the acetoxy groups. Bis(DL-2,3-

diacetoxypropyl) disulfide, prepared from 1-thio-DL-glycerol by oxidation and acetylation[9], gives a very similar spectrum, except for the absence of a signal at about τ 7.7.

The thionocarbonate II showed infrared absorptions indicative of the thiocarbonyl group[13], the hydroxyl group, and the isopropylidene group. It showed ultraviolet absorptions at 234 and 306 mμ; and its optical rotatory dispersion spectrum (Fig. 3) exhibited a positive Cotton effect, with a peak at 326 mμ, an inflection at 310 mμ, and a trough at 288 mμ. Derivatives of this type may be of value for configurational correlations[14]. The substance was sparingly soluble in most solvents, but moderately soluble in acetone. Analysis of the n.m.r. spectrum, measured

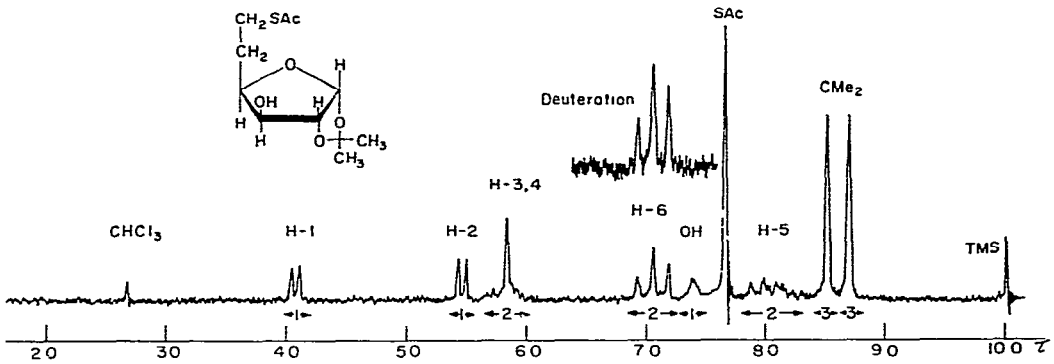


Fig. 2. The n.m.r. spectrum of 6-*S*-acetyl-5-deoxy-1,2-*O*-isopropylidene-6-thio-α-D-*xylo*-hexofuranose (IV) at 60 Mc.p.s. in deuteriochloroform. Numbers below the peaks give integrated peak intensities.

in acetone-d_6 as solvent, was straightforward, and details are recorded in the Experimental section. The 3-*p*-phenylazobenzoate (III) of II gave infrared and n.m.r. spectra (see Experimental) fully consistent with the assigned structure. These data, together with the chemical evidence of the conversion of II and III into the 5,6-unsaturated derivatives V and VI, respectively, provide firm proof of the structure of the thionocarbonates II and III.

1,2-*O*-Isopropylidene-α-D-glucofuranose 5,6-thionocarbonate[2] (II) had been serendipitously encountered[15] among the products of fragmentation of bis(1,2-*O*-isopropylidene-3-*O*-thiocarbonyl-α-D-glucofuranose) disulfide in pyridine solution, and was subsequently prepared, in 48% yield, by treatment of 1,2-*O*-isopropylidene-α-D-glucofuranose with thiophosgene. The recorded[15] melting point and specific rotation are in essential agreement with those reported by us[2]. In our hands, preparation of II by the thiophosgene method gave yields much lower than those attainable by the use of bis(imidazol-1-yl)thione.

Bis(imidazol-1-yl)thione appears to be a useful, mild, specific reagent for the preparation of cyclic thionocarbonates from terminal vicinal glycols, as in the conversion of I into II, and from vicinal *cis*-glycols in cyclic systems, as in the

preparation of a 2,3-thionocarbonate from methyl 4,6-*O*-benzylidene-α-D-mannopyranoside[16]. Intramolecular factors may, however, modify the course of the reaction, as has been observed[17] in the conversion of 5′-*O*-trityluridine by bis(imidazol-1-yl)thione into a 2,2′-anhydride with concomitant inversion at C-2′. Other routes to cyclic thionocarbonates have been reported; the 3,4-thionocarbonate of 1,2:5,6-di-*O*-isopropylidene-D-mannitol, encountered[18] as a side-product from the reaction of phenyl isothiocyanate with 1,2:5,6-di-*O*-isopropylidene-D-mannitol, was subsequently prepared[19] from the latter in 47% yield by successive treatment with *n*-butyllithium, carbon disulfide, and methyl iodide.

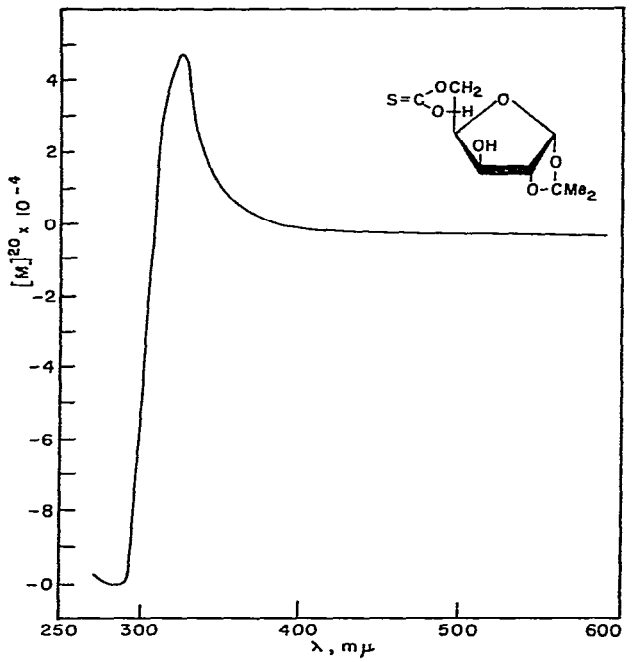


Fig. 3. The optical rotatory dispersion spectrum of 1,2-*O*-isopropylidene-α-D-glucofuranose 5,6-thionocarbonate (II) in tetrahydrofuran (*c* 1.1, 20°).

Triethyl phosphite has been used[20] for synthesis of an unsaturated sugar from an episulfide precursor. In addition to the conversion[2] II→V, trimethyl phosphite has been used for synthesis of unsaturated sugar derivatives from the cyclic thionocarbonates of methyl 4,6-*O*-benzylidene-α-D-mannopyranoside[16], 1,2:5,6-di-*O*-isopropylidene-D-mannitol[19], and benzyl 2-*O*-benzyl-β-L-arabinopyranoside[19].

The photocatalyzed addition of thiolacetic acid to an unsaturated sugar[21] provides a facile route, in the case of the conversion V→IV, to a structure[1] otherwise accessible only by a multi-step procedure[22]. The results of ring-closure reactions of derivatives of IV, under conditions of thermodynamic and kinetic control, will be reported at a later date.

EXPERIMENTAL

General methods

Evaporations were conducted on a rotary evaporator under diminished pressure. Melting points were determined with a Hershberg type of apparatus and are uncorrected. Optical rotations were measured with a 2-dm tube. Optical rotatory dispersion spectra were determined with a Rudolph Model 260/655/850/810-614 photoelectric spectropolarimeter. Infrared spectra were determined with potassium bromide pellets or as liquid films, with a Perkin–Elmer Model 137 Infracord infrared spectrometer. Ultraviolet spectra were measured, in 1-cm cells, with a Bausch and Lomb Spectronic 505 spectrometer. The n.m.r. spectra were measured at 60 Mc.p.s. with a Varian A-60 spectrometer; unless otherwise stated, the samples were used as 5–20% solutions in deuteriochloroform, with tetramethylsilane ($\tau = 10.00$) as the internal standard, and at a probe temperature of approximately 30°. Deuteration was effected by shaking the prepared sample for 30 min with one drop of deuterium oxide. Spectra were analyzed on a first-order basis, and the coupling constants recorded are the observed line-spacings. Chemical-shift values are given on the τ scale, and correspond to the mid-point of each singlet or symmetrical multiplet. For unsymmetrical multiplets, the chemical shifts are given as weighted mean values. Approximate ($\sim$) values are given for chemical shifts and line splittings in those cases where a signal formed part of a complex multiplet, or where satellite peaks were difficult to observe. X-Ray powder diffraction data gave interplanar spacings, in Å, with CuK_α radiation; relative intensities, estimated visually, are given as: s, strong; m, moderate; w, weak; v, very. The first few lines are numbered (1, strongest); double numbers indicate approximately equal intensities. Decolorizations were performed with Darco G-60 activated charcoal (Matheson, Coleman, and Bell, East Rutherford, N.J.). Thin-layer chromatography was performed with Desaga equipment and Silica Gel G (E. Merck, Darmstadt, Germany) with activation of the plates for 1 h at 110°. Indication was effected with sulfuric acid or with iodine vapor. Column chromatography was performed with Silica Gel Davison, grade 950, 60-200 mesh (Davison Division of the W. R. Grace Co., Inc., Baltimore, Maryland). Elemental microanalyses were determined by W.N. Rond.

Preparation of bis(imidazol-1-yl)thione

The following procedure is adapted from that of Staab and Walther[1]. A mixture of dry imidazole (91 g) and anhydrous dichloromethane (500 ml) was stirred under dry nitrogen, and a solution of thiophosgene* (39 g) in anhydrous benzene (150 ml) was added dropwise during 20 min. The mixture was stirred under nitrogen for 1–18 h, and the solution was filtered from imidazole hydrochloride. The filtrate was evaporated to about 150 ml, dry benzene (200 ml) was added, and the solution was refrigerated, to give the crude product, yield 59 g, m.p. 93–96°. Recrystallization from

*Rapter Laboratories, Barrington, Illinois.

anhydrous tetrahydrofuran gave the product, m.p. 100°, whose infrared spectrum was identical with that published[4]. No change in the infrared spectrum was detected in a sample which had been stored in a desiccator for 2 months.

1,2-O-Isopropylidene-α-D-glucofuranose 5,6-thionocarbonate (II)

To a solution of 1,2-*O*-isopropylidene-α-D-glucofuranose[23] (I, 13.5 g) in warm acetone (250 ml), in a flask equipped with a magnetic stirrer and a nitrogen inlet, was added recrystallized bis(imidazol-1-yl)thione (13.1 g). The mixture was boiled under reflux for 1 h, with passage of a slow stream of nitrogen. Activated charcoal (0.5 g) was added and, after 5 min, the solution was filtered and evaporated, to give a light-brown solid, yield 26.8 g. The solid was extracted with warm (45°) methanol (60 ml), and the brown, methanolic extract was decanted from the white solid product, yield 14.45 g (90%). Refrigeration of the methanol extract gave more of the crystalline product, yield 2.17 g. The combined solids were recrystallized from methanol to give fine needles, yield 13.50 g (85%), m.p. 205–206°, $[\alpha]_D^{18}$ −17° (*c* 1, acetone); optical rotatory dispersion spectrum[14], see Figure 3; R_F 0.20 (3:1 chloroform–ether); λ_{max}^{KBr} 2.97 (OH), 7.28 (CMe_2), 8.14 μ (C = S)[13]; λ_{max}^{EtOH} 234 mμ (ε 12,300), 306 mμ (ε 34); n.m.r. data (acetone-d_6): τ 3.98 (1-proton doublet, $J_{1,2}$ 3.4 c.p.s., H-1), τ 4.62 (1-proton, symmetrical 7-peak multiplet, $J_{5,6}$ 8 c.p.s., $J_{5,6'}$ 7.4 c.p.s., $J_{4,5}$ 2.6 c.p.s., H-5), τ 5.16 (1-proton singlet, OH, disappears on deuteration), τ 5.36 (1-proton quartet, H-4), τ 5.45 (1-proton doublet, $J_{3,4}$ 3.7 c.p.s., H-3), τ 5.62 (1-proton doublet, $J_{2,3}$ 0 c.p.s., H-2), τ 6.37 (2 protons, H-6,6'), τ 8.57, 8.70 (3-proton singlets, CMe_2); X-ray powder diffraction data: 11.33 w, 8.59 m, 7.18 m, 5.55 vw, 5.09 s (2,2), 4.83 s (2,2), 4.44 s (1), 4.28 vw, 4.07 s (2,2), 3.67 m, 3.54 m, 3.43 vw, 3.29 m, 3.23 m.

Anal. Calc. for $C_{10}H_{14}O_6S$: C, 45.78; H, 5.34; S, 12.22; mol. wt. 262. Found: C, 45.70; H, 5.15; S, 12.08; mol. wt. (Rast) 235.

For this compound, prepared by rearrangement–fragmentation of bis(1,2-*O*-isopropylidene-3-*O*-thiocarbonyl-α-D-glucofuranose) disulfide, the following constants have been reported[15]: m.p. 206–208°, $[\alpha]_D^{20}$ −16° (acetone). Preparation of II from I in pyridine with thiophosgene, under conditions essentially similar to those recently reported[15], gave the product in only fair yield.

1,2-O-Isopropylidene-3-O-p-phenylazobenzoyl-α-D-glucofuranose 5,6-thionocarbonate (III)

To a solution of 1,2-*O*-isopropylidene-α-D-glucofuranose 5,6-thionocarbonate (II, 2.34 g) in anhydrous pyridine (20 ml) was added *p*-phenylazobenzoyl chloride (3.00 g), and the mixture was shaken for 2 days. The solution was poured into ice-water and the mixture stirred vigorously for 1 h. The product was extracted with dichloromethane (50 ml), and the extract was dried (magnesium sulfate), concentrated, and diluted with ethanol (70 ml) to give red needles, yield 4.93 g, homogeneous by chromatography, R_F 0.55 (3:1 dichloromethane–ether). Recrystallization from ethanol gave pure product, yield 3.41 g (72%), m.p. 178–179°, $[\alpha]_D^{21}$ −109° (*c* 1, chloroform); λ_{max}^{film} 5.80 (C=O), 7.30 (CMe_2), 8.19 μ (C = S)[13]; λ_{max}^{EtOH} 239 mμ (ε 33,400), 325 mμ

(ε 33,900), 442 mμ (ε 751); n.m.r. data: τ 1.7–2.7 (9 protons, aryl), τ 3.91 (1-proton doublet, $J_{1,2}$ 4 c.p.s., H-1), τ 4.37 (1-proton doublet, $J_{3,4}$ 3 c.p.s., $J_{2,3}$ ~0 c.p.s., H-3), τ 4.60–4.96 (2-proton multiplet, H-4,5), τ 5.18 (3-proton multiplet, H-2,6,6'), τ 8.43, 8.67 (3-proton singlets, CMe_2); X-ray powder diffraction data: 14.48 s (2), 7.79 s (3), 5.94 m, 5.73 w, 4.79 s (1), 4.47 m, 4.24 m, 4.08 w, 3.72 w, 3.63 w, 3.58 w, 3.26 w, 3.17 w, 2.93 w.

Anal. Calc. for $C_{23}H_{22}N_2O_7S$: C, 58.69; H, 4.68; N, 5.96; S, 6.80. Found: C, 59.02; H, 4.65; N, 6.51; S, 6.61.

5,6-Dideoxy-1,2-O-isopropylidene-α-D-xylo-hex-5-enofuranose (V)

A solution of 1,2-*O*-isopropylidene-α-D-glucofuranose 5,6-thionocarbonate (II, 5.07 g) in dry trimethyl phosphite (25 ml) was refluxed under nitrogen for 60 h. The solution was concentrated to 15 ml, ethylene glycol (15 ml) was added, and the solution was extracted three times with ether (50 ml). The ether layer was dried (magnesium sulfate), and evaporated to give sirupy 5,6-dideoxy-1,2-*O*-isopropylidene-α-D-*xylo*-hex-5-enofuranose (V), sufficiently pure for subsequent conversions. Purer material was obtained by chromatography on a 4.5×48-cm column of silica gel, with ether as eluant. The ninth 500-ml fraction crystallized upon evaporation; yield 1.82 g (51%), further crops from other fractions raised the total yield to 75%; m.p. 58–60°. Sublimation of this product (twice) at 110° (1 mm) gave the purest preparation, m.p. 70–71°, $[\alpha]_D^{22}$—57.3° (*c* 2.8, chloroform); R_F 0.35 (3:1 chloroform–ether); λ_{max}^{KBr} 2.95 (OH), 7.25, 7.28 μ (CMe_2); n.m.r. data (see Fig. 1): τ 4.05 (1-proton doublet, $J_{1,2}$ 3.8 c.p.s., H-1), τ 4.10–4.75 (3-proton multiplet, H-5,6,6'), τ 5.28 (1-proton quartet, $J_{3,4}$ 3.0 c.p.s., H-4), τ 5.44 (1-proton doublet, $J_{2,3}$ <0.5 c.p.s., H-2), τ 5.91 (1-proton doublet, $J_{3,4}$ 3.5 c.p.s., H-3), τ 7.27 (1-proton singlet, OH, shifts with change in concentration, and observed when at high field, τ 8.1, as a doublet with accompanying change in appearance of the H-3 signal), τ 8.50, 8.67 (3-proton singlets, CMe_2); X-ray powder diffraction data: 8.89 s (3), 6.65 m, 5.29 m, 5.04 s (1), 4.65 s (2), 4.38 m, 4.04 m, 3.75 w, 3.63 m, 3.21 w.

Anal. Calc. for $C_9H_{14}O_4$: C, 58.05; H, 7.57. Found: C, 57.82; H, 7.30.

The X-ray powder diffraction data are in good agreement with reported values[24]. The product was identical, by mixed melting point, and by n.m.r. and i.r. spectra, with a sample of V which had been prepared in 57% yield by treatment of 1,2-*O*-isopropylidene-5,6-di-*O*-*p*-tolylsulfonyl-α-D-glucofuranose with sodium iodide in acetone[5,6]. The following constants for V, prepared by the latter method, have been recorded[5]: m.p. 61–65°, $[\alpha]_D$—51.5° (*c* 1.1, chloroform); and, by another route[25], m.p. 64°, $[\alpha]_D^{25}$—60.5° (*c* 2.0, water).

5,6-Dideoxy-1,2-O-isopropylidene-3-O-p-phenylazobenzoyl-α-D-xylo-hex-5-enofuranose (VI)

(*A*) *From 1,2-O-isopropylidene-3-O-p-phenylazobenzoyl-α-D-glucofuranose 5,6-thionocarbonate (III).* A solution of 1,2-*O*-isopropylidene-3-*O*-*p*-phenylazobenzoyl-α-D-glucofuranose 5,6-thionocarbonate (III, 1.5 g) in dry trimethyl phosphite (20 ml)

was refluxed for 4.5 h under nitrogen. The solution was concentrated to 10 ml, ethylene glycol (6 ml) was added, and the solution was extracted with ether. The extract was evaporated, and the product was crystallized from hot methanol, yield 0.83 g (68%), m.p. 119.5–121.5°, $[\alpha]_D^{22}$ —87.5° (*c* 1.1, chloroform); R_F 0.88 (3:1 chloroform–ether); λ_{max}^{KBr} 5.80 μ (C=O); λ_{max}^{EtOH} 207 mμ (ε 13,500), 227 mμ (ε 13,600) 322 mμ (ε 28,100), 445 mμ (ε 800); n.m.r. data: τ 1.78–2.58 (9-proton multiplet, aryl), τ 3.90 (1-proton doublet, $J_{1,2}$ 3.8 c.p.s., H-1), τ 4.08–4.85 (4-proton multiplet, H-3, H-5, H-6, H-6'), τ 5.07 (1-proton multiplet, H-4), τ 5.26 (1-proton doublet, $J_{2,3}$ <0.5 c.p.s., H-2), τ 8.41, 8.65 (3-proton singlets, CMe_2); X-ray powder diffraction data: 8.13 vw, 7.25 s (3), 6.50 s (2), 5.32 m, 4.64 s (1), 4.33 m, 4.10 m, 3.77 s (2), 3.62 vw, 3.30 m, 3.13 m.

Anal. Calc. for $C_{22}H_{22}N_2O_5$: C, 66.99; H, 5.62; N, 7.10. Found: C, 67.36; H, 5.89; N, 7.53.

(*B*) *From 5,6-dideoxy-1,2-*O*-isopropylidene-α-*D*-xylo-hex-5-enofuranose* (*V*). To a solution of 5,6-dideoxy-1,2-*O*-isopropylidene-α-D-*xylo*-hex-5-enofuranose (V, 303 mg) in dry pyridine (10 ml) was added *p*-phenylazobenzoyl chloride (468 mg), and the mixture was shaken for 18 h. The solution was poured into ice and water (20 ml), the mixture was stirred for 30 min, and the product was extracted with dichloromethane. The organic phase was dried (magnesium sulfate) and evaporated, and the residue was crystallized from methanol to give VI, yield 373 mg (58%), m.p. 120.5–122.5°, identical by mixed melting point, X-ray powder diffraction pattern, and n.m.r. and i.r. spectra, with VI prepared by method A.

*6-*S*-Acetyl-5-deoxy-1,2-*O*-isopropylidene-6-thio-*α*-*D*-xylo-hexofuranose* (*IV*)

A solution of 5,6-dideoxy-1,2-*O*-isopropylidene-α-D-*xylo*-hex-5-enofuranose (V, 250 mg) in redistilled thiolacetic acid (1.0 ml) contained in a Vycor* tube was irradiated for 75 h with an ultraviolet hand-lamp**. The solution was evaporated, codistilled four times with carbon tetrachloride to remove thiolacetic acid, and the residue was recrystallized from Skellysolve B***; yield 190 mg (54%), m.p. 93–94°, $[\alpha]_D^{21}$ —14.5° (*c* 1.8, chloroform), R_F 0.30 (3:1 chloroform–ether); λ_{max}^{KBr} 2.95 (OH), 5.95 (SAc), 7.26 μ (CMe_2); n.m.r. data (see Fig. 2): τ 4.08 (1-proton doublet, $J_{1,2}$ 4.0 c.p.s., H-1), τ 5.43 (1-proton doublet, $J_{2,3}$ <0.5 c.p.s., H-2), τ 5.81 (2-proton multiplet, H-3,4), τ 7.03 (2-proton triplet, $J_{5,6}$ 7.8 c.p.s., H-6), τ 7.40 (1-proton singlet, OH, disappears on deuteration), τ 7.63 (3-proton singlet, SAc), τ 7.98 (2-proton multiplet, H-5), τ 8.48, 8.69 (3-proton singlets, CMe_2), X-ray powder diffraction data: 8.26 s (3), 5.57 m, 5.27 vw, 4.95 s (2), 4.79 m, 4.43 s (1), 4.27 m, 4.09 vw, 3.95 w, 3.64 w.

Anal. Calc. for $C_{11}H_{18}O_5S$: C, 50.38; H, 6.87; S, 12.21. Found: C, 50.54; H, 6.88; S, 12.27.

*Corning Glass Co., Corning, New York.
**Mineralight, model 2537, Ultraviolet Products, Inc., South Pasadena, California.
***Petroleum ether, b.p. 30–60°, Skelly Oil Company, Kansas City, Missouri.

Preparations on a larger scale, performed in a glass flask with irradiation from an immersion type of ultraviolet lamp*, gave comparable yields of IV after shorter periods of irradiation.

*3-O-Acetyl-6-S-acetyl-5-deoxy-1,2-O-isopropylidene-6-thio-α-D-xylo-hexofuranose***

A solution of 6-*S*-acetyl-5-deoxy-1,2-*O*-isopropylidene-6-thio-α-D-*xylo*-hexofuranose (IV, 0.4 g) in pyridine (8 ml) was treated with acetic anhydride (8 ml) and, after 18 h, the mixture was poured into water. The mixture was stirred for 30 min, and then extracted with dichloromethane. The dried (magnesium sulfate) extract was evaporated, and the product was purified by distillation[26], $b_{0.15}$ 130° (bath); R_F 0.83 (4:1 dichloromethane–ether), λ_{max}^{KBr} 5.80 (OAc), 5.90 (SAc), 7.25 μ (CMe_2); n.m.r. data: τ 4.12 (1-proton doublet, $J_{1,2}$ 3.8 c.p.s., H-1), τ 4.80 (1-proton doublet, $J_{3,4}$ 3 c.p.s., H-3); τ 5.52 (1-proton doublet, $J_{2,3}$ <0.5 c.p.s., H-2), τ 5.77 (1-proton sextet, $J_{3,4}$ 3 c.p.s., H-4), τ 7.08 (2-proton triplet, $J_{5,6}$ 7 c.p.s., H-6), τ 7.70 (3-proton singlet, SAc), τ 7.92 (3-proton singlet, OAc), τ 8.18 (2-proton multiplet, H-5), τ 8.51, 8.71 (3-proton singlets, CMe_2).

N.m.r. data on 1-thioglycerol derivatives

An undiluted, liquid sample of 2,3-di-*O*-acetyl-1-thio-DL-glycerol[9] gave the following n.m.r. data: τ 4.91 (1-proton multiplet, H-2), τ 5.85 (2-proton multiplet, H-3), τ 6.86 (2-proton multiplet, H-1), τ 7.67 (3-proton singlet, SAc), τ 8.01 (6-proton singlet, OAc).

An undiluted, liquid sample of bis(2,3-diacetoxypropyl) disulfide[9] gave the following n.m.r. data: τ 4.72 (2-proton multiplet, H-2), τ 5.74 (4-proton multiplet, H-3), τ 7.03 (4-proton doublet, $J_{1,2}$ 6 c.p.s., H-1), τ 7.96 (12-proton singlet, OAc).

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SUMMARY

1,2-*O*-Isopropylidene-α-D-glucofuranose (I) was converted by treatment with bis(imidazol-1-yl)thione into the 5,6-thionocarbonate (II), characterized by physical data and as the 3-*p*-phenylazobenzoate (III). Treatment of II and III with trimethyl

*Pen-Ray Model 11 SC, Ultraviolet Products, Inc., San Gabriel, California.
**Part of this experiment was performed by C.G. Tindall.

phosphite gave the corresponding 5,6-unsaturated derivatives V and VI; substance VI could also be prepared by *p*-phenylazobenzoylation of V. Photocatalyzed addition of thiolacetic acid to V gave 6-*S*-acetyl-5-deoxy-1,2-*O*-isopropylidene-6-thio-α-D-*xylo*-hexofuranose (IV), characterized by n.m.r. and i.r. spectroscopy and also as the 3-acetate.

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NUCLEOSIDES

XXX. SYNTHESIS OF 2-DEOXY-2-FLUORO-D-RIBOSE

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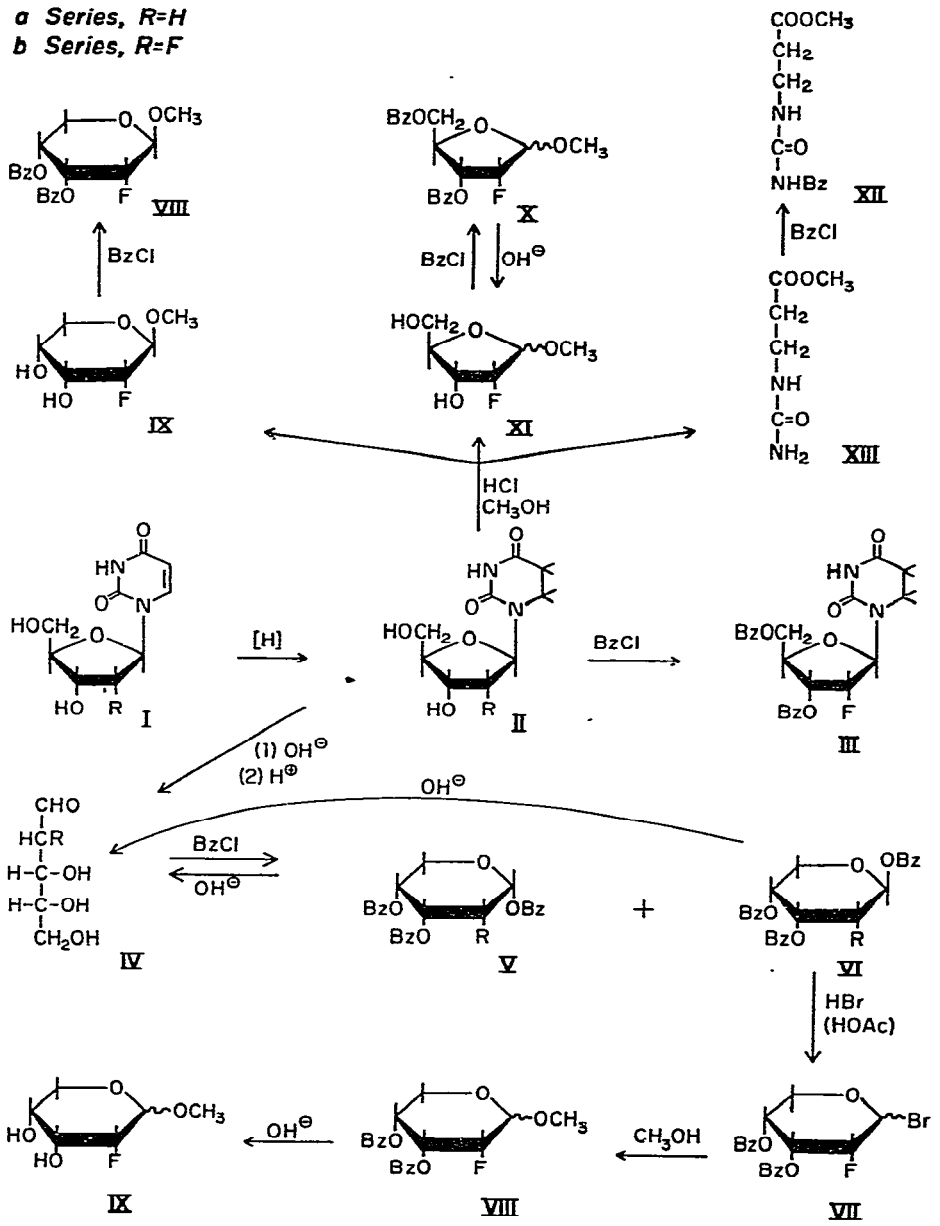
With the successful introduction of fluorine into the ribosyl moiety of nucleosides[1], as in 1-(2-deoxy-2-fluoro-β-D-ribofuranosyl)uracil (2'-deoxy-2'-fluorouridine) (Ib), a route was made available for the synthesis of 2-deoxy-2-fluoro sugars of the *ribo* configuration. Although fluorine had been introduced into the terminal position of several sugars[2,3], it was not until recently that pentoses having a fluorine atom in a nonterminal position were reported with the synthesis of 3-deoxy-3-fluoro derivatives in both the *xylo*[4,5] and *arabino*[5] series. This paper reports the synthesis of 2-deoxy-2-fluoro-D-ribose (IVb). It was anticipated that IVb, as an analog of the biologically important 2-deoxy-D-*erythro*-pentose ("2-deoxy-D-ribose") (IVa), might, either as the free sugar or as an appropriate derivative, possess interesting biological properties*.

As a model compound in this synthesis, 1-(2-deoxy-β-D-*erythro*-pentofuranosyl)-uracil (2'-deoxyuridine) (Ia) was reduced using rhodium-on-alumina catalyst, according to the method of Cohn and Doherty[6], to give the known 1-(2-deoxy-β-D-*erythro*-pentofuranosyl)-5,6-dihydrouracil (IIa)[7] as colorless crystals (see flow chart). Alkaline treatment which would be expected to give a ureidopropionic acid[7,8], followed by heating in dilute acid[9], gave a solution which gave a positive test with Fehling solution. Thin-layer chromatography (t.l.c. in solvent B) revealed a single spot[10] migrating at the same rate as an authentic sample of IVa. Benzoylation of the dried residue, followed by fractional recrystallization, gave both anomers of 1,3,4-tri-*O*-benzoyl-2-deoxy-D-*erythro*-pentose (α, Va; β, VIa) in approximately equal yields (combined yield 25%). The physical properties agreed closely with those of compounds prepared by Pedersen, Diehl, and Fletcher[11]. The infrared spectra of authentic samples, kindly supplied by Dr. Fletcher, were identical with those of Va and VIa, and mixtures of samples from both sources gave no depression of melting points. These data establish the identity of Va and VIa.

In a similar manner, 1-(2-deoxy-2-fluoro-β-D-ribofuranosyl)uracil (Ib) was reduced to 1-(2-deoxy-2-fluoro-β-D-ribofuranosyl)-5,6-dihydrouracil (IIb), which, although analytically pure, was isolated only as a gum. The di-*O*-benzoyl derivative III, however, was obtained from IIb in high yield, as colorless needles. In

*A study of the biological properties of IVb and certain of its derivatives is under way in this Institute.

anticipation that cleavage of the dihydronucleoside IIb would occur in a manner similar to that found for its deoxy analog IIa. compound IIb was subjected to the alkaline and acid hydrolytic treatments which had been successfully applied to IIa.



Positive Fehling and aniline acid phthalate[10] tests suggested the presence of the desired 2-deoxy-2-fluoro-D-ribose (IVb) in the crude mixture. Attempts to isolate IVb in pure form from this mixture were however, unsuccessful.

After removal of the solvent, the residue containing IVb was benzoylated in pyridine solution. The resulting mixture showed two major components (t.l.c. in solvent A). A third compound was also present, but in small proportion. The major components were separated by chromatography on an alumina column, with chloroform as the eluant. One of these components (VIb) was isolated as colorless needles, $[\alpha]_D$ −146°, in 13–21% yield. The elementary analysis corresponded to that of a tribenzoyldeoxyfluoropentose. Two crystalline forms of VIb, having different melting points and infrared spectra (KBr discs) were isolated. Heating of the KBr disc of the lower-melting isomorph at 110–115° readily changed its infrared spectrum to that of the higher-melting form. The other major component isolated from the alumina column was present in about 6% yield. It was obtained only as an amorphous material, which was contaminated with a small proportion of the third product as shown by t.l.c. chromatography. Attempts to crystallize this material failed. Like VIb, this amorphous material, $[\alpha]_D$ −10°, showed three benzoyl groups on titration. Since only the α-D- and β-D-pyranoses, Va and VIa, were isolated in the 2-deoxy series, it seems probable that the second major component present after benzoylation of IVb is 1,3,4-tri-*O*-benzoyl-2-deoxy-2-fluoro-α-D-ribose (Vb). Strict conformity to Hudson's rule[12,13] (based upon known values in the 2-deoxy and 2-deoxy-2-fluoro series: Va +47°, VIa −192° and VIb −146°) would lead to prediction of a positive rotation (+92°) for the α-D anomer Vb. The value of −10°, however, suggests the probable presence of a contaminant having a high, negative rotation.

The relatively small yield of benzoylated sugars obtained by this method in both the 2-deoxy and 2-deoxy-2-fluoro series may possibly be attributable to ring closure of the β-ureidopropionic acid intermediate under acid conditions. Such a reaction was noted by Batt *et al.*[8] and, later, by others[14].

Compound VIb was shown to have a pyranoid structure as follows: Conversion of VIb to the methyl glycoside VIII through the 1-bromo derivative VII was accomplished by treatment with hydrogen bromide–acetic acid (32%) in dichloromethane followed by treatment of VII with methanol in the presence of a little pyridine as the acid acceptor. These reactions were followed polarimetrically. The methyl dibenzoyl glycoside VIII was obtained as a gum, $[\alpha]_D$ −133°. Titration data indicated two benzoyl groups. The unprotected methyl glycoside IX (Fehling negative) was treated with sodium metaperiodate. After 2 h, exactly one mole of periodate had been consumed per mole of compound. This value remained constant during the next several hours, and then rose slowly. After seven days, 2.7 mole of periodate per mole had been consumed. The continued slow oxidation of the molecule may be related to the observation by Huebner and co-workers[15] of the periodate oxidation of active methylene groups. The primary oxidation product, a monoacetal of α-fluoro malonaldehyde, would probably be expected to consume periodate, in view of the uptake reported for malonaldehyde[16] and α-ethylmalonic acid[15]. The uptake of one mole per mole of periodate at 2–6 h proves conclusively the pyranoid ring-structure of IX and establishes VIb as being 1,3,4-tri-*O*-benzoyl-2-deoxy-2-fluoro-D-ribose.

Although the anomeric form of VIb has not yet been definitely determined, its high negative rotation (−146°) indicates that it is probably of the β-D configuration[12]. The corresponding deoxypyranosides[11] are characterized by a high negative rotation (−195°) for the β-D anomer and a positive rotation (+42°) for the α-D anomer.

In view of their high, negative rotations, it seems probable that both 3,4-di-*O*-benzoyl-2-deoxy-2-fluoro-D-ribosyl bromide (VII) (−225°) and methyl 3,4-di-*O*-benzoyl-2-deoxy-2-fluoro-D-riboside (VIII) (−133°) are mainly of the β-D configuration[13,17]. A β-D configuration for the di-*O*-benzoylpyranosyl bromide VII conforms to the rule, formulated by Haynes and Newth[17] and further discussed by Bhattacharya *et al.*[13], that, in poly-*O*-acylglycopyranosyl halides, the halogen atom at C-1 is *trans* to the acyloxy group at C-3.

Crystalline 2-deoxy-2-fluoro-D-ribose (IVb)* was obtained in almost quantitative yield by debenzoylation of VIb. Reaction of VIb with dilute, ethanolic sodium hydroxide, removal of the contaminating material, and evaporation of water *in vacuo* gave a colorless gum which crystallized as fernlike structures. The compound showed a melting point of 106–112° and an optical rotation of −37°. No mutarotation of IVb was observed in either dilute acid or base. The infrared spectrum of IVb, shown in the figure, is characterized by the absence of carbonyl absorption. Compound IVb migrates (t.l.c. in solvent B) as a single spot (R_F 0.50) at a higher rate than 2-deoxy-D-*erythro*-pentose (IVa) (R_{IVa}, 1.25). Good yields of crystalline IVb were obtained by the same method from the amorphous tribenzoylated material (probably, mainly Vb) isolated from the reaction mixture producing VIb (see above).

Methanolysis[18,19] of IIb was performed in the hope of obtaining IVb from the resulting methyl glycosides in an overall yield higher than had been obtained by the method described above (IIb to VIb and Vb, then to IVb). The amorphous product contained three components as shown by t.l.c. in solvent B. It was benzoylated in pyridine solution. The crude mixture gave six spots on t.l.c. developed with solvent A. The mixture was partially separated by chromatography on an alumina column using chloroform as the eluant. Three components were isolated, and their structures elucidated.

The first component to be eluted was isolated as colorless prisms in 8% yield. Debenzoylation of this compound with dilute, ethanolic sodium hydroxide gave a product which crystallized as colorless needles. The results of elementary analysis were consistent either with the methyl pyranoside IX or the (isomeric) methyl furanoside XI. The furanoid structure was established by the fact that the compound consumed essentially no periodate during 60 min, whereas the pyranoside IX consumed 0.8 mole of oxidant per mole during the same period. Thus, the debenzoylated glycoside is established as being methyl 2-deoxy-2-fluoro-D-ribofuranoside (XI) and the benzoylated derivative as methyl 3,5-di-*O*-benzoyl-2-deoxy-2-fluoro-D-riboside (X).

*For convenience, compounds IVa and IVb are represented on the flow chart in the aldehydo form, although it is improbable that they exist mainly in this form either as crystals or in solution.

The second component eluted from the column was isolated only as a gum, in 22% yield (based on IIb). It traveled as a single spot, R_F 0.64 (t.l.c. in solvent A) at a lower rate than XI, R_F 0.75. The elementary analysis of it was consistent with structure VIII, and a benzoyl determination indicated two benzoyl groups per molecule. An optical rotation of −156° was observed. This is somewhat more negative than that of the corresponding amorphous material obtained from the pyranosyl bromide VII (see above). The infrared spectra of the two gums were identical, although the bands were not sharp. On the basis of the optical rotation[12], it may be concluded that this sample of VIII is most probably the β-D-anomer. The debenzoylated product IX consumed approximately one mole of periodate per mole in two h, consistent with a pyranoid structure. These data strongly suggest that the second component is the β-D anomer of methyl 3,4-di-*O*-benzoyl-2-deoxy-2-fluoro-D-riboside (VIII). It is noteworthy that, in the methanolysis of IIb, methyl glycosides of both the pyranoid (IX) and furanoid structure (XI) are formed.

An effort was made to obtain 2-deoxy-2-fluoro-D-ribose (IVb) from VIII. A product giving positive Fehling and aniline acid phthalate tests[10], and migrating at the same rate as IVb, was obtained, but the yields were poor, and analytically pure material was not obtained.

A considerable proportion of the benzoylated material was obtained from the column as a third fraction. Trituration of the residual gum with a small amount of ethanol produced a third component as colorless prisms, in 8% yield. This compound contained nitrogen, but no fluorine. Its elementary analysis was consistent with that calculated for XII, methyl 3-(*N*-benzoylureido)propionate. This component was probably formed during the methanolysis reaction as the methyl ester of ureidopropionic acid (XIII), which had presumably resulted from cleavage between C-3 and C-4 of the dihydrouracil moiety[7,8]. In a second methanolysis experiment, dihydrouracil was, in fact, isolated from the reaction mixture prior to separation on an alumina column.

EXPERIMENTAL

Solvent A for t.l.c. was prepared by the addition of *n*-heptane (108 ml) and ethyl acetate (208 ml) to the upper layer (340 ml) obtained from a mixture of *n*-heptane (400 ml) and methanol (200 ml). Solvent B is 1-butanol–water (86:14). All t.l.c. plates were prepared with Silica Gel G, according to Stahl (Brinkmann Instruments, Inc., Westbury, New York, U.S.A.). After the developed plate had been completely freed of solvent, it was sprayed with sulfuric acid–water (1:1) and heated at 100°. Reducing-sugar spots were identified by spraying with aniline acid phthalate reagent, followed by heating[10].

Melting points were determined on a Thomas–Hoover apparatus (capillary method) and are corrected. Elementary analyses were made by Spang Microanalytical Laboratory, Ann Arbor, Michigan. Infrared spectra were made on a Perkin–Elmer spectrophotometer, Model 221.

Benzoyl determinations were made by stirring a weighed sample at 25–30° with a measured amount of 0.1 N sodium hydroxide in dilute aqueous ethanol. Unreacted base was determined by titrating with 0.1 N hydrochloric acid (phenolphthalein).

*1-(2-Deoxy-β-D-*erythro*-pentofuranosyl)-5,6-dihydrouracil (IIa)*

In accordance with the method of Cohn and Doherty[6], a mixture of 2′-deoxyuridine (Ia) (1.0 g, 4.4 mmole) and rhodium on alumina (5%) (0.70 g) in ethanol (60 ml) absorbed 113 ml of hydrogen at atmospheric pressure within 106 min (theoret., 106 ml). The product, m.p. 133–138° (reported[7] 136–138°), weighed 1.0 g (99%), $[\alpha]_D^{24}$ −7° (*c* 1.1 in water).

1-(2-Deoxy-2-fluoro-β-D-ribofuranosyl)-5,6-dihydrouracil (IIb)

A mixture of 2′-deoxy-2′-fluorouridine (Ib) (0.20 g, 0.82 mmole) and rhodium on alumina (5%) (0.15 g) in ethanol (25 ml) was shaken under one atmosphere of hydrogen for 41 min. An uptake of 23 ml of hydrogen (theoret., 19.7 ml) was recorded. After removal of the catalyst, the filtrate was evaporated to dryness *in vacuo*, leaving a colorless gum, $[\alpha]_D^{24}$ −17° (*c* 0.4 in water). U.v. absorption data (H_2O): shoulder, 205–210 mμ, ratio 220/260 mμ, 87.

Anal. Calc. for $C_9H_{13}FN_2O_5$: C, 43.57; H, 5.28; F, 7.66; N, 11.29. Found: C, 43.56; H, 5.74; F, 7.59; N, 11.15.

1-(3,5-Di-O-benzoyl-2-deoxy-2-fluoro-β-D-ribosyl)-5,6-dihydrouracil (III)

A solution of IIb (0.30 g, 1.22 mmole) in pyridine (8 ml) was cooled to 0–5°, and benzoyl chloride (0.53 g, 3.8 mmole) was added dropwise with stirring. The solution was kept 23 h at about 5° and a small piece of ice was added. Removal of solvent *in vacuo*, followed by addition of ice-water (100 ml) with stirring, gave a colorless solid. This was collected and crystallized from ethanol, yielding colorless needles, 0.40 g (73%), m.p. 160–162°. Additional material (0.05 g), m.p. 155–160°, was obtained from the mother liquor. Recrystallization from ethanol gave colorless needles, m.p. 162–163°, $[\alpha]_D^{24}$ −1° (*c* 0.7 in chloroform).

Anal. Calc. for $C_{23}H_{21}FN_2O_7$: C, 60.55: H, 4.62; F, 4.17; N, 6.13. Found: C, 60.41; H, 4.63; F, 4.21; N, 6.33.

*2-Deoxy-D-*erythro*-pentose (IVa)*

A solution of IIa (0.23 g, 1.0 mmole) in 0.2 N sodium hydroxide (10 ml) was allowed to stand for 20 min at room temperature, and then treated with Dowex 50 (H^+). After removal of the resin, 1 N sulfuric acid was added (3 ml, total volume 12 ml), and the resulting solution was heated at 85° for 20 min. Anions were removed with Dowex 1 (OH^-). The filtrate was evaporated to dryness *in vacuo*. The residue was co-distilled several times with anhydrous benzene, and dried well *in vacuo*. The product gave positive Fehling and diphenylamine[20] tests and migrated on t.l.c.

in solvent B at the same rate as an authentic sample of 2-deoxy-D-*erythro*-pentose, R_F 0.40.

1,3,4-Tri-O-benzoyl-2-deoxy-α-D-erythro-pentose (Va) and 1,3,4-tri-O-benzoyl-2-deoxy-β-D-erythro-pentose (VIa)

Benzoylation was performed in a manner similar to that described by Pedersen, Diehl, and Fletcher[11]. The dried product (IVa) described above was dissolved in pyridine (11 ml) and treated with benzoyl chloride (0.50 g, 3.6 mmole) at 0–5° with stirring, then kept refrigerated for an additional 17 h. After hydrolysis of the remaining benzoyl chloride with a small piece of ice, and removal of solvent, ice-water was added to the residue. As the product did not crystallize, the mixture was extracted with ether, and the ethereal solution dried. Colorless needles, 0.06 g, m.p. 140–150°, were filtered from 2–3 ml of ether. Recrystallization from ethanol gave 0.05 g (11%) of colorless needles, m.p. 159–160°, $[\alpha]_D^{24}$ −192° (*c* 0.2 in chloroform). Pedersen *et al.*[11] report m.p. 159–161° and $[\alpha]_D^{20}$ −195° (chloroform) for the β-D pyranose VIa. A mixture of an authentic sample[11], m.p. 158.5–159.5°, with the above sample, m.p. 159–160°, melted at 159–160°. Infrared spectra (KBr discs) of the two samples showed significant differences in the 10–15 μ region. Upon heating the KBr disc (sample prepared as described above and recrystallized from ethanol) at 110–115° for 60 min, its infrared spectrum was identical with that of a sample prepared by Pedersen *et al.*, which had been crystallized from methanol.

The ether-soluble fraction was crystallized from ethanol to give 0.06 g (14%) of long, colorless needles melting at 147–149°. Recrystallization from methyl Cellosolve gave colorless needles, m.p. 150.5–151.5°, $[\alpha]_D^{23}$ +47° (*c* 0.2 in chloroform). Reported[11] constants for the α-D anomer were m.p. 151–152° and $[\alpha]_D^{20}$ +42° (chloroform). A mixture of a sample prepared as above, m.p. 150.5–151.5°, with an authentic sample[11], m.p. 150.5–151.5°, melted at 150.5–151.5°. Infrared spectra (KBr discs) of the two samples showed significant differences. The KBr disc of the sample prepared as described above (crystallized from methyl Cellosolve) was heated at 110–115° for 60 min. Its infrared spectrum was then identical with that of an authentic sample (crystallized from methanol)[11].

1,3,4-Tri-O-benzoyl-2-deoxy-2-fluoro-β-D-ribose (VIb)

An alkaline solution (40 ml, 0.2 N NaOH) of IIb (0.76 g, 3.07 mmole) was allowed to stand at 23–25° for 40 min. Neutralization with 2 N sulfuric acid (4 ml) was followed by a further addition of 2 N sulfuric acid (4.9 ml). The solution was heated at 85–90° (internal temp.) for 35 min and then treated, while hot, with solid barium carbonate until neutral. Filtration gave a clear solution which was treated with Dowex 50 (H^+), and the filtrate was evaporated to dryness *in vacuo*. The gummy residue gave positive tests with Fehling and aniline acid phthalate[10] reagents. Addition of pyridine to the residue, followed by its removal *in vacuo*, was repeated twice.

The residue was dissolved in dry pyridine (50 ml) and treated dropwise with benzoyl chloride (2.4 g, 17.1 mmole) at 0–5° with stirring. After 16 h at 5–7°, the

mixture was warmed at 35–40° for 60 min. A small piece of ice was added with stirring. Later, the pyridine was removed *in vacuo*, and the residue dissolved in chloroform. The solution was extracted with dilute sodium bicarbonate solution and water, and dried over sodium sulfate.

A chloroform solution (10 ml) of the above material was placed on an alumina (acid washed) column (3 × 42 cm), and the column eluted with chloroform. Fractions containing benzoylated material were eluted in the 484–724 ml portion of effluent. The 484–596 ml fraction was evaporated to dryness *in vacuo*, and the residue crystallized as colorless needles which were triturated with water and filtered, 0.21 g (15%), m.p. 113–115°. An additional 0.05 g (total yield 18%) of VIb was isolated from the second fraction (see below). Recrystallization (0.21 g) from ethanol gave elongated rods, 0.17 g, m.p. 146–147°, $[\alpha]_D^{25}$ −146° (*c* 0.1 in chloroform). Certain samples from other runs exhibited two melting points: initial melting at 112–116°, followed by resolidification and then remelting at 144–145°. The infrared spectra (KBr discs) of the two isomorphic forms were markedly different. Heating the KBr disc of the lower-melting form at 110–115° for 30 min changed its infrared spectrum to that of the higher-melting isomorph.

Anal. Calc. for $C_{26}H_{21}FO_7$: C, 67.25; H, 4.55; F, 4.09. Found: C, 67.49; H, 4.62; F, 4.77.

The 612–724 ml fraction was evaporated to dryness *in vacuo*, giving a yellow gum. This exhibited, on t.l.c., three spots of R_F values 0.73 (corresponding to VIb), 0.66, and 0.25, a very weak spot. The gum was dissolved in chloroform and passed through an alumina column (1.7 × 32 cm) as above. Fraction I (86–116 ml) yielded colorless crystals which, on recrystallization from ethanol, gave 0.05 g of VIb, m.p. 144.5–145.5°. Fraction IV (161–176 ml) yielded a gum (30 mg), $[\alpha]_D^{24}$ −10° (*c* 0.2 in chloroform). This separated into two spots on t.l.c. plates, one corresponding to that of the second major component (R_F 0.66), and the other, a weak spot at R_F 0.25. Compound VIb was absent. The two intermediate fractions, II and III, 70 and 72 mg, respectively, contained both VIb and the second major component, R_F 0.66.

Anal. of Fraction IV: Calc. for $C_{26}H_{21}FO_7$: benzoyl groups, 3.0. Found: benzoyl groups, 2.93.

*2-Deoxy-2-fluoro-*D*-ribose (IVb)*

The debenzoylation of VIb was observed in a polarimeter cell. A mixture of VIb (0.088 g, 0.19 mmole), ethanol (1.5 ml) and 1 N sodium hydroxide (1.5 ml) was shaken for 60 min before dissolution was complete. At that time, the specific rotation had diminished to −11°, based upon IVb (from −146°). The specific rotation increased during the next two h and became constant at −30° (based upon IVb). Ethanol was removed from the solution under reduced pressure, water (6 ml) was added, and the solution was treated with Dowex 50 (H^+). After filtration, benzoic acid was removed by repeated extraction with ether. The solution was adjusted to 3 ml and its rotation was found to be −36°, based on a theoretical yield of IVb. On removal

of the water *in vacuo*, a colorless gum remained which, on standing, crystallized as a fern-like structure, yield 27 mg (96%), m.p. 106–112°, $[\alpha]_D^{24}$ −37°, R_F 0.50, R_{IVa} 1.25 (t.l.c. in solvent B).

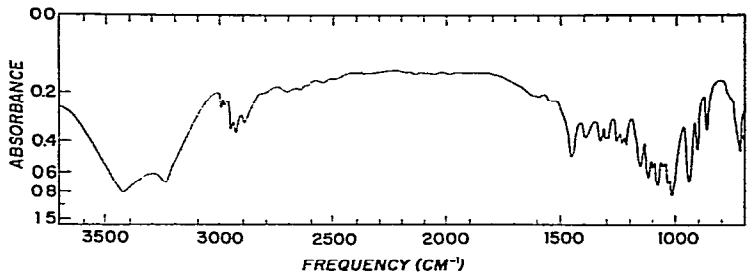


Fig. 1. Infrared spectrum (KBr disc) of 2-deoxy-2-fluoro-D-ribose (IVb).

Fraction III (72 mg) which was obtained as a gum after chromatography of the benzoylation products of crude IVb on an alumina column (see above) was treated with base under the conditions just described. A good yield of crystalline IVb was obtained.

The product isolated in this fashion exhibited no change in optical rotation on being kept in solution in 0.1 N HCl for 17 h or in 0.1 N NaOH for 3 h.

Anal. Calc. for $C_5H_9FO_4$: C, 39.47; H, 5.96; F, 12.49. Found: C, 39.51; H, 6.03; F, 12.55.

Conversion of VIb into methyl 3,4-di-O-benzoyl-2-deoxy-2-fluoro-D-riboside (VIII)

To a solution of VIb (0.035 g, 0.075 mmole) in dichloromethane (0.3 ml) were added 5 drops of hydrogen bromide–acetic acid (32%). After being kept at 20–25° for 19 h, the solution was diluted with dichloromethane (5 ml) and rapidly extracted in the cold with water, sodium bicarbonate solution, and water. The solution was dried with sodium sulfate, and, after filtration, the solvent was removed *in vacuo*. The residue was dissolved in dry methanol (3 ml). The optical rotation, $[\alpha]_D^{24}$, after 15 min based upon VII, was −225°. One drop of pyridine was added to the solution. After 5 h at 20–25°, the rotation was −148°, based upon VIII, and after 22 h, −133°.

The solution was evaporated to dryness, leaving a pale-yellow gum which was triturated well with water, and the water decanted. Moisture was removed by co-distillation with ethanol *in vacuo* several times. The residue was dissolved in ether, and filtered to remove a small amount of insoluble material. Upon removal of the ether, a colorless gum remained, $[\alpha]_D^{24}$ −133° (*c* 0.6 in ethanol). The material gave a negative Fehling test. The chromatographic behavior and infrared spectrum of this material were identical with those found for compound VIII, isolated after methanolysis of IIb, followed by benzoylation (see below).

Anal. Calc. for $C_{20}H_{19}FO_6$: benzoyl groups, 2.00. Found: benzoyl groups, 2.08.

Oxidation of methyl 2-deoxy-2-fluoro-D-riboside (XI) with sodium periodate

To the solution resulting from the determination of the benzoyl groups was added an excess of sodium metaperiodate. Aliquots were buffered at pH 7 and titrated with standard arsenite. The results are given in the table.

Time	*Moles IO_4^- consumed per mole of compound*
0.1	0.21
0.9	0.85
2.1	1.00
3.4	0.98
5.3	1.00
24.0	1.24
71.0	1.80
7 days	2.70

Methanolysis of 1-(2-deoxy-2-fluoro-β-D-ribofuranosyl)-5,6-dihydrouracil (IIb)

Anhydrous methanol saturated with hydrogen chloride (100 ml) was added to IIb (0.74 g, 2.98 mmole). The solution was heated at reflux for 10 h, then neutralized by the addition of solid silver carbonate. After filtration of silver salts, the solvent was removed *in vacuo*, leaving an amorphous residue. T.l.c. in solvent B showed three spots of R_F values 0.34, 0.52, 0.62.

Benzoylation was carried out in pyridine (25 ml) at 0° with the addition of benzoyl chloride (1.82 g, 13.0 mmoles) with stirring. After 41 h at 0–5° a small piece of ice was added. Pyridine was removed *in vacuo*, and a chloroform solution of the residue was extracted in the cold in turn with water, sodium bicarbonate solution and water. After drying, the chloroform was removed *in vacuo*, leaving a yellow gum. T.l.c. in solvent A revealed six spots of R_F values 0.12, 0.21, 0.26, 0.34, 0.48, 0.60. A chloroform solution of the mixture was placed on a column of alumina, 2.4 × 42 cm, and eluted with chloroform. Fractions of 8 ml each were collected. The benzoylated material was eluted from the column in the 290–464 ml portion of effluent. The effluent was studied in four parts: (A) 290–320 ml, (B) 328–360 ml, (C) 367–410 ml, and (D) 420–460 ml.

The solvent was removed from each fraction, leaving a gum residue from fractions (C) and (D), crystals from fraction (A) and a mixture of crystals and gum from fraction (B). Qualitative analysis revealed nitrogen present only in fraction (D).

Methyl 3,5-di-O-benzoyl-2-deoxy-2-fluoro-D-riboside (X)

Trituration of fraction (A) with water, followed by filtration gave 0.055 g of colorless needles, m.p. 80–88°. Trituration of fraction (B) in a small amount of cold ethanol gave an additional 0.026 g of the same product, m.p. 86–88° (total yield 8%). The compound migrated as a single spot, R_F 0.60 (t.l.c. solvent A).,

Methyl 2-deoxy-2-fluoro-D-ribofuranoside (XI)

To a solution of X (0.076 g, 0.20 mmole) in ethanol (3 ml) was added 0.50N sodium hydroxide (3 ml), and the solution was stirred for 4.5 h at 20–25°. Treatment

with Dowex 50 (H^+), filtration and removal of solvent left an amorphous residue. Upon trituration with petroleum ether (30–60°) colorless crystals formed, 0.021 g (62%), m.p. 74–78°. Crystallization from ethyl acetate–petroleum ether (30–60°) gave colorless needles, m.p. 81–83°. Within 60 min XI consumed 0.083 mole of sodium metaperiodate per mole of XI.

Anal. Calc. for $C_6H_{11}FO_4$: C, 43.35; H, 6.67; F, 11.45. Found: C, 43.46; H, 6.76; F, 11.40.

Methyl 3,4-di-O-benzoyl-2-deoxy-2-fluoro-D-riboside (VIII)

The amorphous residue from fraction (C) weighed 0.232 g (20%) and traveled as a single spot of R_F 0.63 (t.l.c., solvent A), $[\alpha]_D^{24}$ −156° (*c* 0.4 in chloroform).

Anal. Calc. for $C_{20}H_{19}FO_6$: C, 64.16; H, 5.12; F, 5.08; benzoyl groups, 2.0. Found: C, 63.05; H, 5.08; F, 5.07; benzoyl groups, 2.0.

Periodate oxidation of VIII after debenzoylation

Consumption of periodate per mole of compound within: 0.1 h, 0.13 mole; 0.6 h, 0.64 mole; 2.0 h, 0.84 mole; 5 h, 0.89 mole; 22 h, 0.94 mole; 48 h, 1.1 mole.

The lower rate of periodate uptake in this experiment, as compared to that for compound IX above, is probably due to the fact that the pH of the reaction mixture was slightly lower in this experiment.

*Methyl 3-(N-benzoylureido)propionate (XII)**

The fraction (D) residue (0.38 g) was triturated in ethanol (2 ml) at 0–5°. Colorless crystals, 0.056 g (8%), m.p. 117–118°, were collected. Crystallization from ethanol gave micaceous plates, m.p. 118–119°. The infrared spectrum (KBr disc) of XII was consistent with an *N*-benzoylureido-propionate. Carbonyl bands: $COOCH_3$, 5.75 μ; $NHCOC_6H_5$, 5.91 μ; NHCONH, 5.98 μ.

Anal. Calc. for $C_{12}H_{14}N_2O_4$: C, 57.59; H, 5.64; N, 11.20. Found: C, 57.33; H, 5.71; N, 11.15.

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*For convenience, the benzoyl group is attached to the terminal nitrogen atom in structure XII, although its exact position has not yet been determined.

SUMMARY

Crystalline 2-deoxy-2-fluoro-D-ribose (IVb) was prepared by debenzoylation of 1,3,4-tri-*O*-benzoyl-2-deoxy-2-fluoro-β-D-ribose (VIb). Reduction of 2'-deoxy-2'-fluorouridine (Ib) gave amorphous 1-(2-deoxy-2-fluoro-β-D-ribofuranosyl)-5,6-dihydrouracil (IIb), which was converted into crystalline 1-(3,5-di-*O*-benzoyl-2-deoxy-2-fluoro-β-D-ribosyl)-5,6-dihydrouracil (III). Glycosylic cleavage of IIb with dilute alkali, followed by heating with dilute acid, gave a mixture containing IVb. Benzoylation of impure IVb produced crystalline VIb. A second product, in amorphous form, probably 1,3,4-tri-*O*-benzoyl-2-deoxy-2-fluoro-α-D-ribose, was isolated; it also gave crystalline IVb upon debenzoylation. The pyranoid structure of VIb was proved as follows: replacement of the benzoyloxy group on C-1 by a methoxyl group gave methyl 3,4-di-*O*-benzoyl-2-deoxy-2-fluoro-D-riboside (VIII). Debenzoylation gave the unsubstituted glycoside IX, which was shown to be a pyranoside, thus establishing the ring structure of VIb.

Methanolysis of IIb, followed by benzoylation, gave crystalline methyl 3,5-di-*O*-benzoyl-2-deoxy-2-fluoro-D-riboside (X) and amorphous VIII, as well as crystalline methyl 3-(*N*-benzoylureido)propionate (XII). Debenzoylation of X produced crystalline methyl 2-deoxy-2-fluoro-D-ribofuranoside (XI).

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DEOXYFLUORO SUGARS

PART I. SYNTHESIS OF 2-DEOXY-2-FLUORO-D-ALTROSE, 2-DEOXY-2-FLUORO-D-ALLOSE, AND 3-DEOXY-3-FLUORO-D-GLUCOSE

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INTRODUCTION

Sugars in which one or several hydroxyl groups are replaced by fluorine are of interest as potential antimetabolites. Those sugars in which a primary hydroxyl group is replaced by fluorine have been prepared from the corresponding sulphonates, but this reaction cannot be used for the introduction of fluorine at secondary positions. In some nucleosides, fluorine has been introduced at C-2 in the D-ribofuranose residue[1]. Recently, two groups have prepared deoxyfluoropentoses by the opening of sugar epoxides, either with hydrogen fluoride in dioxan[2] at 120°, or with potassium fluoride in acetamide[3] at 200°.

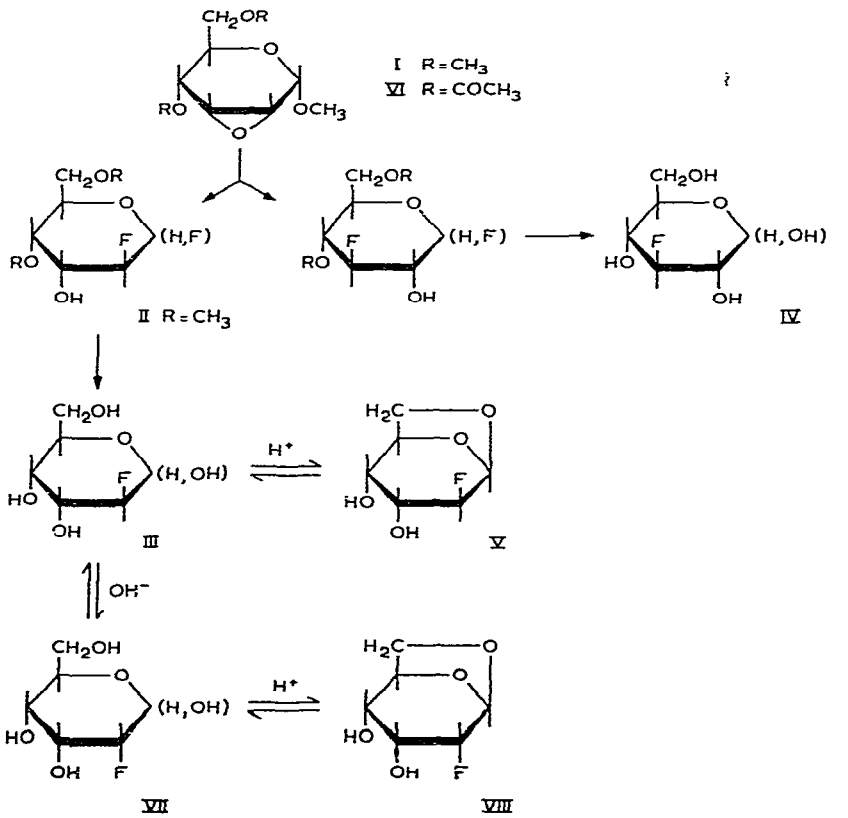
RESULTS AND DISCUSSION

In our approach to the synthesis of deoxyfluoro sugars, we also start from sugar epoxides, but open the epoxide ring by treatment with hydrogen tetrafluoridoborate in hydrogen fluoride at −70°. On such treatment, methyl 2,3-anhydro-4,6-di-*O*-methyl-α-D-allopyranoside (I) yielded a mixture containing two main components, one (II) of which was obtained crystalline. The substance gave correct analytical values for a monodeoxymonofluoro-di-*O*-methylhexosyl fluoride. N.m.r. studies[4] indicated the structure of compound (II) to be 2-deoxy-2-fluoro-4,6-di-*O*-methyl-D-altropyranosyl fluoride, and the α-D configuration is suggested by the high value of the optical rotation.

The crude reaction product was treated with hydrogen chloride in aqueous acetone, in order to replace the fluorine at C-1 by a hydroxyl group, and then demethylated by treatment with boron tribromide[5] to yield a mixture of two reducing sugars (III and IV). One (III) of these was also obtained on similar treatment of compound (II). The sugars were separated by cellulose-column chromatography, and one (III) was obtained crystalline.

The sugars expected from the 2,3-anhydro-D-alloside, assuming *trans* opening of the epoxide ring, are 2-deoxy-2-fluoro-D-altrose and 3-deoxy-3-fluoro-D-glucose, of which the former should be preponderant. The paper-electrophoretic mobilities of compounds (III) and (IV), in borate and germanate[6] buffers, were therefore compared with the mobilities of 2-*O*-methyl-D-altrose [prepared from epoxide (I)]

and 3-*O*-methyl-D-glucose. The values given in Table I show that compound (III) and 2-*O*-methyl-D-altrose have similar mobilities in borate, and that compound (IV) and 3-*O*-methyl-D-glucose have similar mobilities in both systems. The discrepancy between the mobilities of compound (III) and 2-*O*-methyl-D-altrose in germanate buffer is considerable and cannot be explained. The rates of consumption of lead tetra-acetate by compound (III) and 2-*O*-methyl-D-altrose, under the conditions used by Charlson and Perlin[7], followed similar courses. Similarly, compound (IV) and 3-*O*-methyl-D-glucose both rapidly consumed 1 mol. of oxidant, further consumption being insignificant. Finally, treatment of compound (IV) with lime water yielded a mixture of the "α"- and "β"-D-glucometasaccharinic acids, indistinguishable by paper and gas–liquid chromatography[8] from authentic specimens. These results are in agreement with the postulated structures of compounds (III) and (IV) as 2-deoxy-2-fluoro-D-altrose and 3-deoxy-3-fluoro-D-glucose, respectively. N.m.r. studies (^{19}F and ^{1}H)[4] on derivatives of the above deoxyfluoro sugars also support the structures given.



When compound (III) was heated in acidic, aqueous solution, the 1,6-anhydride (V) was formed. The good yield (62%) of this substance, and its strongly negative optical

rotation ($[\alpha]_{5780}^{22}$ −270° in water) further support the D-altrose configuration assigned to compound (III). 1,6-Anhydro-β-D-altrose shows[9] $[\alpha]_{D}^{22}$ −215°, a value which is considerably lower than those reported for the other 1,6-anhydro-β-D-hexopyranoses.

A somewhat simpler route to the sugars (III) and (IV) proceeds from methyl 4,6-di-*O*-acetyl-2,3-anhydro-α-D-allopyranoside (VI). However, during the last step, in which the *O*-acetyl groups are saponified with trimethylamine in aqueous methanol, partial epimerisation of compound (III) into 2-deoxy-2-fluoro-D-allose (VII) took place. Under more drastic conditions, compound (VII) could be obtained from the sugar (III) in 68% yield. As expected (Table I), the paper-electrophoretic mobilities of compounds (III) and (VII) are similar. Acid treatment of compound (VII) gives 1,6-anhydro-2-deoxy-2-fluoro-β-D-allopyranose (VIII), in 15% yield.

TABLE I

MG VALUES FOR THE DEOXYFLUORO SUGARS AND SOME RELATED SUBSTANCES

Sugar	MG *in borate*	MG *in germanate*
2-Deoxy-2-fluoro-D-altrose	0.65	2.58
2-Deoxy-2-fluoro-D-allose	0.63	2.17
3-Deoxy-3-fluoro-D-glucose	0.90	1.79
2-*O*-Methyl-D-altrose	0.55	1.3
3-*O*-Methyl-D-glucose	0.78	1.94

The sugars (III) and (VII) are exceptionally stable and form insignificant amounts of coloured products when they are treated with acids or bases. They give only weak reactions on paper chromatograms with either anisidine hydrogen chloride or silver nitrate–sodium hydroxide, but are preferably detected by the periodate–benzidine reagent. Compound (IV), on the other hand, is easily detected with the above reagents.

In order to obtain anomerically pure acetates of compounds (III) and (IV), they were transformed into the fully acetylated glycosyl bromides, which were then treated with mercuric acetate in acetic acid. Both acetates, which were amorphous, appear to have the β-D configuration. A crystalline acetate of compound (III), presumably the α-D-pyranose acetate, was obtained by treatment of the crude acetate with zinc chloride in acetic anhydride.

EXPERIMENTAL

Melting points are corrected. Concentrations were done under diminished pressure at a bath temperature not exceeding 40°.

*Methyl 2,3-anhydro-4,6-di-*O*-methyl-α-D-allopyranoside (I)*

To a solution of methyl 2,3-anhydro-α-D-allopyranoside[10] (3.0 g) in *N,N*-dimethylformamide (90 ml), kept under nitrogen and cooled to 0°, was added barium

oxide (10 g) and barium hydroxide octahydrate (6 g)[11]. Dimethyl sulphate (20 ml) was then added dropwise with stirring and cooling during 1 h. The temperature was allowed to rise to room temperature, and stirring was continued overnight. Conc. ammonia (20 ml) was then added and, after 30 min, the reaction mixture was extracted with chloroform (5 × 100 ml). The chloroform solution was washed with water, dried over anhydrous sodium sulphate, and concentrated. The remaining syrup was crystallised from light petroleum to yield the title compound (3.0 g). The physical constants, m.p. 63–64°, $[\alpha]_{5780}^{22}$ +193° (*c* 1.0, chloroform), are in good agreement with previously reported values[13].

Treatment of epoxide (I) with hydrogen tetrafluoridoborate in hydrogen fluoride

A solution of the epoxide (I, 5.2 g) in anhydrous, ethanol-free chloroform (22 ml) was added to a solution of boron trifluoride (8.5 g) in hydrogen fluoride (75 g), kept at −70° in a polyethylene flask. The reaction was followed by t.l.c. (alumina, ethyl acetate) and, after 4 h, when no starting material remained, the mixture was poured into ice-water and chloroform. The acids were neutralised with solid sodium hydrogen carbonate. The chloroform phase was washed with water, dried ($CaCl_2$), and concentrated to a syrup (5.0 g). T.l.c. (silicic acid, ethyl acetate) revealed the presence of two main components, one (II) of which crystallised spontaneously from the syrup and was recrystallised from ethyl ether. The substance (1.8 g) had m.p. 88–90°, $[\alpha]_{5780}^{22}$ +99° (*c* 1.0, chloroform). (Found: F, 18.2; OCH_3, 28.7. $C_8H_{14}F_2O_4$ calc.: F, 17.9; OCH_3, 29.3%). Part of the remaining syrup (1.0 g) was fractionated by column chromatography (silicic acid, chloroform). The separation was incomplete; some of the second, main component (80 mg) was obtained chromatographically pure, but did not crystallise. It had $[\alpha]_{5780}^{22}$ −37° (*c* 1.0, chloroform).

Preparation of the free sugars (III) and (IV)

The above, crude reaction-mixture (5.0 g) was dissolved in acetone (100 ml), N hydrochloric acid (100 ml) was added, and the solution was refluxed for 90 min and then neutralised with solid sodium hydrogen carbonate. Salts were removed by filtration, the acetone was distilled off, and the remaining aqueous solution was extracted with chloroform (10 × 50 ml). The chloroform solution was dried ($CaCl_2$) and concentrated. The resulting syrup (3.57 g) was dissolved in dichloromethane (40 ml) and cooled to −70°, and boron tribromide[5] (50 g) was added. The mixture was kept at this temperature for 30 min and then at room temperature overnight, and concentrated to a syrup which was repeatedly (three times) dissolved in methanol (50 ml) and concentrated. Paper chromatography (butanone, saturated with water) of the remaining syrup revealed the presence of two components, the R_F values of which were of the same order of magnitude as that of 6-deoxy-6-fluoro-D-glucose. They were separated on a cellulose column, using the same solvent system, yielding compounds (III, 1.1 g) and (IV, 0.29 g). Compound (III) crystallised spontaneously and was recrystallised from ethanol to give the pure material melting at 149–151°, $[\alpha]_{5780}^{22}$ +42 → 53° (*c* 1.0, water) (Found: C, 39.7; H, 6.19; F, 10.7. $C_6H_{11}FO_5$ calc.:

C, 39.6; H, 6.09; F, 10.4%). The other sugar (IV), $[\alpha]_{5780}^{22}$ +36° (*c* 0.5, water), has not crystallised. Oxidations of (III), (IV), 2-*O*-methyl-D-altrose, and 3-*O*-methyl-D-glucose with lead tetra-acetate were performed as described by Charlson and Perlin[7]. Treatment of 3-deoxy-3-fluoro-D-glucose with lime water was performed as described for the similar treatment of 3-*O*-methyl-D-glucose[12].

β-D-Tetraacetates of 2-deoxy-2-fluoro-D-altrose and 3-deoxy-3-fluoro-D-glucose

The sugar (0.5 g) was acetylated with acetic anhydride in pyridine, and the syrupy acetate was treated with hydrogen bromide in acetic acid. The resulting glycosyl bromide was then treated with mercuric acetate in acetic acid. The yields of the amorphous β-D-acetates were about 0.5 g. Tetra-*O*-acetyl-2-deoxy-2-fluoro-β-D-altrose had $[\alpha]_{5780}^{22}$ −15°, (*c* 1.0, chloroform), and tetra-*O*-acetyl-3-deoxy-3-fluoro-β-D-glucose, $[\alpha]_{5780}^{22}$ +15° (*c* 2.0, chloroform). Both acetates were chromatographically pure (t.l.c., silicic acid, chloroform).

2-Deoxy-2-fluoro-α-D-altropyranose tetraacetate

Compound (III) (0.48 g) was acetylated with acetic anhydride and pyridine, and the syrupy reaction product (0.92 g) was dissolved in a solution of zinc chloride (0.3 g) in acetic anhydride (4.5 ml) and kept for 15 min at 100°. The solution was then poured into ice-water (100 ml), neutralised with sodium hydrogen carbonate, and extracted with chloroform (3×50 ml). Concentration of the chloroform solution yielded a syrup (0.89 g) which crystallised spontaneously. Recrystallisations from isopropyl ether yielded the α-D-acetate (0.23 g), m.p. 112–114°, $[\alpha]_{5780}^{20}$ +100° (*c* 1.0, chloroform) (Found: CH_3CO, 48.4. $C_{14}H_{19}FO_9$ calc.: CH_3CO, 49.2%).

Methyl 4,6-di-O-acetyl-2,3-anhydro-α-D-allopyranoside (VI)

Methyl 2,3-anhydro-α-D-allopyranoside (15 g) was acetylated with acetic anhydride (75 ml) in pyridine (100 ml) at room temperature overnight to yield the title compound (22 g). Part of the product was purified by column chromatography (silicic acid, chloroform) to give a chromatographically pure syrup, $[\alpha]_{5780}^{22}$ +162° (*c* 1.0, chloroform) (Found: CH_3CO, 31.9; OCH_3, 11.3. $C_{11}H_{16}O_7$ calc.: CH_3CO, 31.3; OCH_3, 11.9%).

Preparation of the free sugars (III, IV, and VII) from the epoxide (VI)

The epoxide (VI, 22 g) was dissolved in hydrogen fluoride (120 ml) at −70°. The solution was rapidly saturated with boron trifluoride (about 10% was dissolved), kept for 50 min at −70°, poured into ice and chloroform (500 ml), and neutralised with solid sodium hydrogen carbonate. The aqueous phase (2 l) was extracted with chloroform (5×200 ml). The combined extracts were washed with water (300 ml), dried ($CaCl_2$), and concentrated. The resulting syrup (23 g) was dissolved in a mixture of sulphuric acid (15 ml), acetic acid (100 ml), and acetic anhydride (250 ml), kept at 0°. When the syrup had dissolved, the temperature was allowed to rise to room temperature and the solution was stored overnight. It was then poured into ice-

water (2 l) and extracted with chloroform (5×200 ml). The chloroform solution was washed with water, aqueous sodium hydrogen carbonate, and water, dried ($CaCl_2$), and concentrated. The resulting syrup was dissolved in a mixture of methanol (200 ml), water (75 ml), and 33% aqueous trimethylamine (300 ml), and kept overnight. The solution was concentrated to a syrup, which was resolved by cellulose-column chromatography (butanone saturated with water) into III (5.7 g), IV (0.55 g), and VII (0.40 g), eluted in the order III, VII, IV.

The 2-deoxy-2-fluoro-D-allose (VII) crystallised spontaneously and was recrystallised from ethanol to give the pure substance, m.p. 165–167°, $[\alpha]_{5780}^{22}$ +18 → 28° (*c* 0.5, water) (Found: C, 39.5; H, 6.25; F, 10.2. $C_6H_{11}FO_5$ calc.: C, 39.6; H, 6.09; F, 10.4%). In a separate experiment, III (0.50 g) was dissolved in 33% aqueous trimethylamine (20 ml) and kept for 5 h at 60°. The product obtained after concentration was fractionated on a cellulose column to yield unchanged starting material (0.15 g) and 2-deoxy-2-fluoro-D-allose (0.34 g).

1,6-Anhydro-2-deoxy-2-fluoro-β-D-altropyranose (V)

Compound (III) (0.52 g) was dissolved in N hydrochloric acid (20 ml) and heated under reflux for 13 h, when the optical rotation had reached a constant value. After neutralisation by ion-exchange and concentration, the resulting syrup was fractionated on a cellulose column (water-saturated butanone) to yield III (0.16 g) and V (0.31 g). The 1,6-anhydride crystallised spontaneously and, after recrystallisation from ethyl acetate–light petroleum, had m.p. 85–87°, $[\alpha]_{5780}^{22}$ −270° (*c* 1.0, water) (Found: C, 44.0; H, 5.48; F, 11.8. $C_6H_9FO_4$ calc.: C, 43.9; H, 5.53; F, 11.6%).

1,6-Anhydro-2-deoxy-2-fluoro-β-D-allopyranose (VIII)

This compound was prepared analogously to V. After 43 h, the yield of recovered substance was 15%. The 1,6-anhydride crystallised spontaneously and, after recrystallisation from ethyl acetate, had m.p. 137–139°, $[\alpha]_{5780}^{22}$ −79° (*c* 1.0, water) (Found: C, 44.0; H, 5.78; F, 11.8. $C_6H_9FO_4$ calc.: C, 43.9; H, 5.53; F, 11.6%).

2-O-Methyl-D-altrose

Methyl 2,3-anhydro-4,6-*O*-benzylidene-α-D-allopyranoside (1.0 g) was dissolved in methanol (20 ml), in which sodium (0.5 g) had been dissolved, and the solution was heated under reflux[14] for 24 h. 0.5N Sulphuric acid (20 ml) was then added and refluxing continued for 1 h. The solution was neutralised with barium carbonate, concentrated to dryness, and extracted with boiling acetone. The acetone solution was concentrated, and the remaining syrup was dissolved in a solution of acetic anhydride (20 ml) and conc. sulphuric acid (0.2 ml), kept for 48 h at room temperature, poured into ice-water, neutralised with sodium hydrogen carbonate, and extracted with chloroform (3×100 ml). The crystalline tetra-*O*-acetyl-2-*O*-methyl-α-D-altropyranose (0.75 g) obtained on concentration of the chloroform solution was recrystallised from isopropyl ether to yield the product, m.p. 93–95°, $[\alpha]_{5780}^{22}$ +91° (*c* 1.0, chloroform) (Found: OCH_3, 8.36; $C_{15}H_{22}O_{10}$ calc.: OCH_3, 8.60).

Deacetylation with trimethylamine in aqueous methanol yielded the chromatographically pure free sugar, which did not crystallise.

ACKNOWLEDGEMENT

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SUMMARY

2-Deoxy-2-fluoro-D-altrose and 3-deoxy-3-fluoro-D-glucose have been prepared by treatment of methylated or acetylated methyl 2,3-anhydro-α-D-allopyranoside with hydrogen tetrafluoridoborate in hydrogen fluoride. Epimerisation of 2-deoxy-2-fluoro-D-altrose yielded the corresponding D-allose derivative.

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ACTION OF BORON TRICHLORIDE ON 1,4:3,6-DIANHYDRO-D-GLUCITOL

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INTRODUCTION

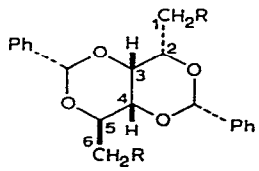
The efficient cleavage of methyl ethers of carbohydrates under mild conditions by boron trichloride[1] prompted an examination of the effect of this reagent on cyclic ethers of carbohydrates, especially those containing tetrahydrofuran rings of which many examples are known[2]. Specific and complete cleavage of tetrahydrofuran rings could endow them with potential value in carbohydrate chemistry as a novel type of blocking group which, in certain cases, would hold a sugar molecule in an unusual conformation. The action of boron trichloride on ethers and related compounds has been well reviewed by Gerrard and Lappert[3]; ethylene oxide, propylene oxide, tetrahydrofuran, tetrahydropyran, and 1,4-dioxan all undergo ring cleavage. We now report on the reaction of boron trichloride with the model compound 1,4:3,6-dianhydro-D-glucitol.

RESULTS AND DISCUSSION

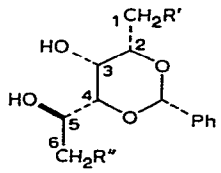
When a mixture of 1,4:3,6-dianhydro-D-glucitol, dichloromethane, and excess of boron trichloride was constituted at *ca.* −80° and allowed to attain room temperature, a substantial change in optical rotation occurred (−45° → *ca.* −11°, water). Examination of the product (*A*) by thin-layer chromatography revealed several components, but fractionation of the mixture (before and after acetylation) on silica gel failed to afford any crystalline material, and chromatography on alumina, after *p*-phenylazobenzoylation[4], did not give any identifiable product. The n.m.r. spectrum of the acetylated material contained a variety of acetyl proton signals indicative of anhydro-ring cleavage and the formation of new hydroxyl groups.

Benzylidenation of product *A* (using toluene-*p*-sulphonic acid as catalyst, and azeotropic removal of water) gave mainly a mono-*O*-benzylidene derivative (*B*, m.p. 184–187°, $[\alpha]_D$ +14° in chloroform) of a dichlorodideoxyhexitol, and a small amount of a di-*O*-benzylidene derivative (*C*, m.p. 209–210°, $[\alpha]_D$ +15° in chloroform). Other, unidentified, benzylidene derivatives were also formed, since the n.m.r. spectrum of the benzylidenated mixture had benzyl proton signals in addition to those associated with compounds *B* and *C*. Compound *C* was readily identified as 2,4:3,5-di-*O*-benzylidene-1,6-dichloro-1,6-dideoxy-D-glucitol (I) by comparison with authentic material obtained from 2,4:3,5-di-*O*-benzylidene-D-

glucitol[5] (II) by methanesulphonylation, followed by treatment with lithium chloride in *N,N*-dimethylformamide. 2,4:3,5-Di-*O*-benzylidene-1,6-dichloro-1,6-dideoxy-D-glucitol was first described by Montgomery and Wiggins[6], but the physical constants recorded (m.p. 223–224°, $[\alpha]_D$ −16.4° in chloroform) were significantly different from those noted for compound *C*. However, repetition of the earlier work (treatment of 1,4:3,6-dianhydro-D-glucitol with fuming hydrochloric acid at 110°, followed by benzylidenation) gave a product having physical constants closely similar to those of compound *C*.



I R = Cl
II R = OH



III R' = R'' = Cl
IV R' = Cl, R'' = OMe

Benzylidenation of compound *B* (which gave a crystalline dibenzoate) gave the di-*O*-benzylidene derivative *C*, in poor yield, indicating that the compounds are both derivatives of 1,6-dichloro-1,6-dideoxy-D-glucitol. The isolation of compound *B* in good yield and its single benzyl proton signal are strongly indicative[7] that it is not a 2-phenyl-1,3-dioxolan derivative, since these compounds are usually formed as nearly equimolar mixtures of diastereoisomers under conditions of vigorous acid-catalysis. 1,6-Di-*O*-benzoyl-2,4:3,5-di-*O*-benzylidene-D-glucitol has benzyl proton signals at τ 4.71 and 4.37 (dioxan), which have been assigned[7] to the 2,4- and 3,5-benzylidene groups, respectively. Since compound *C* was synthesised from the above dibenzoate and must, therefore, have the same configuration, the benzyl proton signals at τ 4.56 and 4.38 may be assigned to the 2,4- (*β-erythro*[8]) and 3,5-benzylidene acetals (*β-threo*), respectively. Compound *B* has a single benzyl proton signal at τ 4.60 which is therefore consistent with a 2,4-benzylidene ring, but, since *β-erythro* and *γ*-(seven-membered) benzylidene acetals give benzyl proton signals of similar chemical shift[7], the possibility of a 2,5-acetal (*γ-erythro*) must be considered. Moreover, the conversion $B \rightarrow C$, noted earlier, does not prove the location of the benzylidene ring in compound *B*, since further benzylidenation may have been accompanied by rearrangement (*cf.* Baggett *et al.*[7]). A *β-erythro* ring in compound *B* seemed to be the more likely structure, since there is no recorded example of the formation of an isolated *γ-erythro* ring in the acid-catalysed condensation of aldehydes with acyclic polyhydric alcohols, whereas the *β-erythro* ring is a sterically very favourable arrangement[9]. Compound *B* was resistant to periodate oxidation under conditions where 2,4-*O*-benzylidene-D-glucitol consumed 1 mol. of oxidant; a 2,5-, but not a 2,4-, *O*-benzylidene derivative of 1,6-dichloro-1,6-dideoxy-D-glucitol would be expected to react with periodate.

Compound *B* was not affected by cold, methanolic sodium methoxide, but, at reflux temperature, compound *D* was formed in which one chlorine atom had been replaced by a methoxyl group. This behaviour would be expected for 2,4-*O*-benzylidene-1,6-dichloro-1,6-dideoxy-D-glucitol (III), since a ready release of chloride ion from position 6 should occur by the formation of an epoxide, which would then afford 2,4-*O*-benzylidene-1-chloro-1-deoxy-6-*O*-methyl-D-glucitol (IV) by further reaction with methoxide ion. An analogy is provided by the conversion[10] of 2,4-*O*-benzylidene-1,6-di-*O*-toluene-*p*-sulphonyl-D-glucitol into 5,6-anhydro-2,4-*O*-benzylidene-1-*O*-toluene-*p*-sulphonyl-D-glucitol by sodium methoxide under mild conditions. There can be little doubt that compounds *B* and *D* have the structures (III) and (IV), respectively.

On reduction of compound *B* with lithium aluminium hydride, both chlorine atoms were removed to give, presumably, 2,4-*O*-benzylidene-1,6-dideoxy-D-glucitol.

The above results establish that the main product arising from the action of boron trichloride on 1,4:3,6-dianhydro-D-glucitol is 1,6-dichloro-1,6-dideoxy-D-glucitol, and that little of the dianhydro compound survives the reaction. An interesting comparison may be made with the observations of Montgomery and Wiggins[6]. Treatment of 1,4:3,6-dianhydro-D-glucitol with fuming hydrochloric acid at 110° for 2 h, followed by benzylidenation (catalysed by zinc chloride) of the product, gave mainly 1,4-anhydro-3,5-*O*-benzylidene-6-chloro-6-deoxy-D-glucitol, together with a small proportion of 2,4:3,5-di-*O*-benzylidene-1,6-dichloro-1,6-dideoxy-D-glucitol. Compound *B*, apparently, was not encountered, and substantial quantities of unreacted dianhydro compound were recovered. Thus, under these drastic conditions, the 3,6-anhydro ring is selectively cleaved, but the 1,4-anhydro ring is relatively stable, whereas, in the boron trichloride reaction, both rings are extensively, if not completely, opened under very mild conditions, and the potential value of the latter reagent for cleaving tetrahydrofuran rings is thereby emphasised.

The nature of the product arising from the action of boron trichloride on tetrahydrofuran is, apparently, markedly dependent on the molar proportion of the Lewis acid[11]. Thus, decomposition of the 1:1 complex of boron trichloride and tetrahydrofuran affords 1,4-dichlorobutane, whereas if a deficiency of Lewis acid is used, the main product is 4-(4-chlorobutoxy)butan-1-ol. The results with 1,4:3,6-dianhydro-D-glucitol show that the use of excess of boron trichloride results in opening of the tetrahydrofuran ring with net addition of hydrogen chloride. Additionally, the direction of ring opening accords with the findings for unsymmetrical acyclic ethers[3], in that the chlorine atom becomes attached to the ether substituent which has the greater electron-releasing character. It is noteworthy that, for acyclic ethers, cleavage of an unsymmetrical ether is specific, in that only one of the two possible alkyl chlorides is formed. Certainly, for 1,4:3,6-dianhydro-D-glucitol, the only chlorine-containing products isolated had the halogen in terminal positions, but, since a quantitative analysis of the reaction mixture was not effected, the formation of non-terminal chloro compounds cannot be precluded.

In an attempt to obtain pure 1,6-dichloro-1,6-dideoxy-D-glucitol, the acid

hydrolysis of compound *B* was examined. With hot, dilute, aqueous-ethanolic hydrochloric acid, a mixture of products was obtained from which 1,4-anhydro-6-chloro-6-deoxy-D-gluctiol[6] (32%) was isolated directly. After benzoylation of the residue, and chromatography on alumina, 1,4:3,6-dianhydro-2,5-di-*O*-benzoyl-D-glucitol and 2,3,4,5-tetra-*O*-benzoyl-1,6-dichloro-1,6-dideoxy-D-glucitol were isolated. The sensitivity of the dichlorodideoxyhexitol to acid and base precluded isolation of a pure sample.

The reaction of boron trichloride with other types of sugar anhydride is being investigated.

EXPERIMENTAL

Thin-layer chromatography (t.l.c.) was performed on Kieselgel, with detection by iodine vapour or vanillin–sulphuric acid[12]. N.m.r. spectra were obtained on *ca.* 20% solutions, with a 6% solution of tetramethylsilane in chloroform as external reference, by using a Varian A60 spectrometer under normal working conditions. Light petroleum refers to the fraction of b.p. 60–80°.

Action of boron trichloride on 1,4:3,6-dianhydro-D-glucitol

A mixture of the dianhydro compound (15 g) and dichloromethane (80 ml) at *ca.* −80° was treated with boron trichloride (25 g) and allowed to attain room temperature during 30 h. The mixture was then cooled and treated with a further amount (25 g) of boron trichloride. After the mixture had again reached room temperature, it was concentrated under diminished pressure, also at room temperature, and the non-volatile borate esters were decomposed by repeated distillation of methanol from the residue. The glassy product (24.3 g), which, presumably, was mainly 1,6-dichloro-1,6-dideoxy-D-glucitol, had $[\alpha]_D$ −11° (*c* 1.0, water), and it was treated with a boiling mixture of benzene (200 ml), benzaldehyde (30 ml), and toluene-*p*-sulphonic acid (20 mg) for 24 h with azeotropic removal of water (*ca.* 2 ml). Benzene was then removed by distillation, and the residue was poured into a mixture of aqueous sodium hydrogen carbonate and light petroleum at 0°. Insoluble material was collected, washed well with light petroleum and water, and dried to yield a product *X* (17 g), m.p. 174–176°, $[\alpha]_D$ +14° (*c* 0.4, chloroform), which had a single broad signal for benzyl protons at τ 4.60 (dioxan). Examination by t.l.c. [benzene–ethyl acetate (7:3)] revealed components having approximate R_F values of 0.94, 0.56, and 0.44.

The aqueous solution and washings were combined and extracted with light petroleum, and then with chloroform (5 × 200 ml). Evaporation of the combined and dried ($MgSO_4$) chloroform extracts gave a syrup *Y* (4.5 g), having $[\alpha]_D$ +1° (*c* 1.05, chloroform) and benzyl proton signals at τ 4.61, 4.34, and 4.21 (dioxan). Examination by t.l.c. (as for *X*) revealed components having approximate R_F values of 0.94, 0.81, 0.75, 0.56, 0.44, and 0.00.

Recrystallisation of product *X* from chloroform gave 2,4-*O*-benzylidene-1,6-

dichloro-1,6-dideoxy-D-glucitol (10.2 g), m.p. 184–187°, $[\alpha]_D$ +14° (*c* 0.4, chloroform), τ 4.60 (benzyl proton, dioxan) (Found: C, 50.9; H, 5.2; Cl, 22.9. $C_{13}H_{16}Cl_2O_4$ calc.: C, 50.8; H, 5.25; Cl, 23.1%). This product afforded a dibenzoate, m.p. 148–149° (from methanol), $[\alpha]_D$ −127° (*c* 0.2, chloroform) (Found: C, 63.2; H, 4.8. $C_{27}H_{24}Cl_2O_6$ calc.: C, 62.9; H, 4.7%).

A further amount (1.52 g) of the mono-*O*-benzylidene derivative was obtained from the mother liquors, and the residual material (τ 4.57, 4.39, and 4.23 for benzyl protons) was combined with the mixture *Y*, stirred with a mixture of aqueous sodium hydrogen carbonate and light petroleum, and then dissolved in chloroform. Concentration of the dried ($MgSO_4$) solution afforded a crystalline product (1.2 g) which was eluted from a column (8×2.5 cm) of alumina with benzene (500 ml) and chloroform (200 ml) to give 2,4:3,5-di-*O*-benzylidene-1,6-dichloro-1,6-dideoxy-D-glucitol (0.63 g), m.p. 209–210°, $[\alpha]_D$ +15° (*c* 1.0, chloroform). Similar fractionation of the product from the chloroform mother liquors gave a further amount (0.83 g) of the di-*O*-benzylidene derivative, together with unidentified benzylidene compounds.

2,4:3,5-Di-O-benzylidene-1,6-dichloro-1,6-dideoxy-D-glucitol

(*a*) Treatment of 2,4:3,5-di-*O*-benzylidene-D-glucitol[5] with methanesulphonyl chloride in pyridine, in the usual manner, gave the 1,6-dimethanesulphonate, m.p. 193–194° (from acetone containing a trace of ammonia), $[\alpha]_D$ −9° (*c* 0.7, pyridine) (Found: C, 51.6; H, 5.2; S, 12.3. $C_{22}H_{26}O_{10}S_2$ calc.: C, 51.4; H, 5.1; S, 12.5%).

A solution of the foregoing dimethanesulphonate (1 g) in *N*,*N*-dimethylformamide (40 ml) containing lithium chloride (2 g) was boiled under reflux for 1 h. The cooled mixture was poured into ice-water (200 ml), and stored overnight at room temperature. The precipitate was collected, washed well with water, and dried to give a product (0.58 g, m.p. *ca.* 140°) which was eluted with benzene from a column (9×2.5 cm) of alumina. The first fraction (50 ml) was discarded; from the second fraction (100 ml), 2.4:3,5-di-*O*-benzylidene-1,6-dichloro-1,6-dideoxy-D-glucitol (0.3 g), m.p. 205–207°, $[\alpha]_D$ +15° (*c* 1.0, chloroform) was obtained, and this was identical with the product obtained in the benzylidenation reaction described above (Found: C, 60.9; H, 5.1; Cl, 18.0. $C_{20}H_{20}Cl_2O_4$ calc.: C, 60.75; H, 5.1; Cl, 18.0%). Montgomery and Wiggins[6] recorded m.p. 223–224°, $[\alpha]_D$ −16.4° in chloroform, for this compound (see discussion).

(*b*) A solution of 2,4-*O*-benzylidene-1,6-dichloro-1,6-dideoxy-D-glucitol (1 g) in benzene (50 ml) containing benzaldehyde (3 ml) and toluene-*p*-sulphonic acid (10 mg) was boiled for 48 h, with azeotropic removal of water. Benzene was then removed by distillation, and the residue was shaken with a mixture of aqueous sodium hydrogen carbonate and light petroleum, and dried to give the title compound (0.47 g, 37%), m.p. 206–208° alone or in admixture with the product described in (*a*).

Acid hydrolysis of 2,4-O-benzylidene-1,6-dichloro-1,6-dideoxy-D-glucitol

A mixture of the title compound (1.2 g), 0.01 N hydrochloric acid, and 50%

aqueous ethanol (110 ml) was boiled under reflux for 2 h, and the ethanol was then removed under diminished pressure at room temperature. The aqueous layer was extracted with light petroleum and then evaporated in a desiccator over sodium hydroxide. Crystallisation of the residue from ethyl acetate gave 1,4-anhydro-6-chloro-6-deoxy-D-glucitol (0.22 g, 32%), m.p. 108–110°, $[\alpha]_D$ −10° (*c* 1.4, acetone) (lit.[6], m.p. 108–109°, $[\alpha]_D$ −14° in acetone), which was further characterised[4] as the tri-*p*-phenylazobenzoate, m.p. 182–185° (from benzene–light petroleum) (Found: C, 67.1; H, 4.6; $C_{45}H_{35}ClN_6O_7$ calc.: C, 67.0; H, 4.3%).

The residue (0.37 g) from the ethyl acetate mother-liquors was benzoylated in the usual manner, and the product (0.57 g), which contained components having approximate R_F values of 0.94 and 0.72 [t.l.c., benzene–ether (9:1)], was fractionated on a column (12×25 cm) of alumina. The following fractions were obtained: 1 (benzene, 125 ml), an unidentified syrup (33 mg); 2 (benzene–ether, 9:1, 100 ml), slightly impure (?) 2,3,4,5-tetra-*O*-benzoyl-1,6-dichloro-1,6-dideoxy-D-glucitol (0.26 g), m.p. *ca.* 40°, R_F *ca.* 0.92 with trace components at *ca.* 0.40, 0.24, and 0.04 (Found: Cl, 11.6. $C_{24}H_{28}Cl_2O_8$ calc.: Cl, 11.2%); 3 (benzene–ether, 9:1, 100 ml), unidentified material (32 mg); 4 (ether, 200 ml), crude 1,4:3,6-dianhydro-2,5-di-*O*-benzoyl-D-glucitol (0.11 g) which, after recrystallisation from ethanol, had m.p. 102–103°, $[\alpha]_D$ +24° (*c* 0.5, chloroform); lit.[13], m.p. 102–103°, $[\alpha]_D$ +24.5° in chloroform.

Reactions of 2,4-O-benzylidene-1,6-dichloro-1,6-dideoxy-D-glucitol

(*a*) *With sodium methoxide*

A solution of the title compound (0.6 g) and sodium methoxide (from 0.19 g of sodium) in methanol (70 ml) was boiled under reflux overnight, cooled, neutralised with dilute sulphuric acid, and concentrated. The residue was extracted with hot ethyl acetate to yield a product (0.6 g), m.p. 142–143°, $[\alpha]_D$ +10° (*c* 0.5, chloroform), which, on recrystallisation from chloroform–light petroleum and then from chloroform, gave 2,4-*O*-benzylidene-1-chloro-1-deoxy-6-*O*-methyl-D-glucitol, m.p. 144–145°, $[\alpha]_D$ +13° (*c* 0.4, methanol) (Found: C, 55.2; H, 6.3; Cl, 12.2. $C_{14}H_{19}ClO_5$ calc.: C, 55.4; H, 6.3; Cl, 11.7%).

(*b*) *With sodium metaperiodate*

A solution of 2,4-*O*-benzylidene-D-glucitol[10] (5 mg) in *N,N*-dimethylformamide (10 ml), phosphate buffer[14] (pH 6.98, 5 ml), and 0.02 M sodium metaperiodate (5 ml) was made up to 25 ml with *N,N*-dimethylformamide[15]. Aliquot portions (1 ml) were withdrawn at intervals, diluted with 20% aqueous potassium iodide (1 ml), and titrated with 0.01 N sodium thiosulphate using Thyodene indicator. After 48 h, a periodate consumption of 1.01 mol. had occurred, whereas 2,4-*O*-benzylidene-1,6-dichloro-1,6-dideoxy-D-glucitol (6.5 mg) consumed no periodate under the same reaction conditions.

(*c*) *With lithium aluminium hydride*

To a slurry of lithium aluminium hydride (0.27 g) in tetrahydrofuran (20 ml)

was added a solution of the title compound (1 g) in tetrahydrofuran (20 ml) during 10 min. The mixture was then stirred and boiled under reflux for 60 h. Excess of reductant was destroyed with ethyl acetate (10 ml), and alkoxides with water (2 ml). Insoluble material was collected and washed with ethyl acetate. Concentration of the combined filtrate and washings gave a product (0.94 g), m.p. 137–142°, $[\alpha]_D$ +9° (*c* 0.44, chloroform). Examination by t.l.c. (benzene–methanol, 9:1) revealed three main components having approximate R_F values of 0.50, 0.44, and 0.38. Recrystallisation from benzene–light petroleum gave a product, m.p. 143–146°, with benzyl proton signals at τ 4.59, 4.61, and 4.65 (tetrahydrofuran), a portion of which (347 mg) was eluted from a column (30×2.5 cm) of silica gel (Davison, 950). The following fractions (50 ml) were obtained: 1–14 (benzene–ether, 9:1), unidentified material (17 mg); 15 (benzene–ether, 1:1), 2,4-*O*-benzylidene-1,6-dichloro-1,6-dideoxy-D-glucitol (40 mg), m.p. 183–185°; 17–18 (benzene–ether, 1:1), a mixture of chlorodideoxy derivatives of 2,4-*O*-benzylidene-D-glucitol (90 mg), m.p. 147–148°, R_F *ca.* 0.44 and 0.41 (t.l.c., carbon tetrachloride–ethyl acetate, 1:1), $[\alpha]_D$ +14° (*c* 1.1, chloroform), τ 4.54 and 4.58 (tetrahydrofuran, benzyl protons) (Found: C, 57.5; H, 6.5. $C_{13}H_{17}ClO_4$ calc.: C, 57.2; H, 6.2%); 21–25 (benzene–ether, 1:1), 2,4-*O*-benzylidene-1,6-dideoxy-D-glucitol (88 mg), m.p. 166–167°, $[\alpha]_D$ +10° (*c* 0.8, chloroform), τ 4.52 (tetrahydrofuran, benzyl proton), 9.90 and 9.87 (doublets, *J* 10 c.p.s., *C*-methyl protons) (Found: C, 65.4; H, 7.8. $C_{13}H_{18}O_4$ calc.: C, 65.5; H, 7.6%).

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SUMMARY

Under mild conditions, 1,4:3,6-dianhydro-D-glucitol is converted by boron trichloride mainly into 1,6-dichloro-1,6-dideoxy-D-glucitol, isolated as the 2,4-*O*-benzylidene and 2,4:3,5-di-*O*-benzylidene derivatives. 2,4-*O*-Benzylidene-1,6-dichloro-1,6-dideoxy-D-glucitol is converted by sodium methoxide into 2,4-*O*-benzylidene-1-chloro-1-deoxy-6-*O*-methyl-D-glucitol, and by lithium aluminium hydride into 2,4-*O*-benzylidene-1,6-dideoxy-D-glucitol. The ready cleavage of tetrahydrofuran rings by boron trichloride endows them with potential value as novel blocking groups in carbohydrate chemistry.

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Notes

The infrared spectroscopy of ketoses

Infrared spectroscopy is useful in organic chemistry for group diagnosis by means of characteristic frequencies[1,2] and many interesting assignments have been made in the field of carbohydrates during the past decade[3]. In a previous publication[4], it was suggested that some kind of skeletal vibration associated with the keto grouping at C-2 causes the regular absorptions observed in the range 1 000 cm^{-1} to 700 cm^{-1} for ketoses, both pyranoid and furanoid. Investigation of the spectra of known disaccharides[5], including those in which both moieties are aldose residues, tabulated earlier by Barker[6], indicated that the ketose assignment[4] could be extended to oligosaccharides. The data presented in Tables I through III confirm this conclusion and suggest the use of these absorption bands for identification of a ketose residue in disaccharides and, even, in trisaccharides.

EXPERIMENTAL

The sugars used in this study were all of high purity, and were kindly provided by Dr. N.K. Richtmyer, National Institutes of Health, Bethesda, Md., except for the leucrose sample, which was provided by Dr. F.H. Stodola, Northern Utilization Research and Development Division, Peoria, Ill.

The spectra of the crystalline sugars (in potassium bromide disks) were measured with a Grubb & Parsons "Spectromaster", double-beam spectrophotometer.

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TABLE I

BANDS (cm^{-1}) OBSERVED IN THE INFRARED SPECTRA OF SOME 2-KETOSES

Sugar	*Type*					
	1	*I*	*2*	*IIA*	*IIB*	*3*
D-*xylo*-Hexulose	902	885		821		
L-*gluco*-Heptulose	947	873		828		
D-*allo*-Heptulose	937	891	860	823		784
D-*glycero*-D-*gulo*-Octulose	940	881/870	858	819		793
2,7-Anhydro-β-D-*ido*-heptulose	966	881/870		813	826	
2,7-Anhydro-β-L-*gulo*-heptulose	896	878	844	800		
2,7-Anhydro-β-D-*altro*-heptulose	920	873	858	829	835	
Turanose	933	866	840	808		
Leucrose	917	871		817		789

TABLE II

BANDS (cm^{-1}) RECORDED IN THE LITERATURE FOR SUGARS CONTAINING A 2-KETOSE RESIDUE

Oligosaccharide	*Type*						*References*
	1	*I*	*2*	*IIA*	*IIB*	*3*	
Reducing disaccharides							
Inulobiose	925	870		825		780	5
Turanose	933	866	840	808		780	5
Maltulose	927	867	842	820		795	5
Leucrose	914	875		820		792	5
Nonreducing oligosaccharides							
Sucrose	913		857		838		6
1-Kestose	930	870	855	810	835		6
6-Kestose	925	875	855	805	835		6
Neokestose	925	870	860	810	830		6

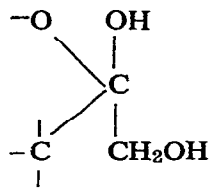
TABLE III

BANDS (cm^{-1}) RECORDED IN THE LITERATURE[6] FOR DISACCHARIDES CONTAINING AN ALDOSE RESIDUE

Sugar	*Type* 1	*Type 2* α	*Type 2* β	*Type* 3
β-Maltose	907	846	894	778
β(?)-Isomaltose	919	838		768
Nigerose	919	840		783
α,α-Trehalose	924/909	850/840		802
α,β-Trehalose	923	843	884	780
β,β-Trehalose	919		896	
β-Cellobiose	925		892	773
β(?)-Gentiobiose	917		894	770
Luteose	919		888	
Laminaribiose	919		888/872	778
Sophorose	919	843	892	765

DISCUSSION

The specificity of absorptions I (at 875 cm^{-1}; standard deviation = ±9) and II (at 817 cm^{-1}; standard deviation = ±9) was confirmed by the spectrograms, so that their presence may be taken as sufficient proof of structure. Definite assignment to the 2-ketose residue seems valid, for their appearance is always caused by the structure shown, whereas substitution or linkage with other molecules by



means of C-1 or the anomeric group are followed by shifting of type IIA to type IIB absorption. Although no theoretical background is yet available to explain this phenomenon, a similar skeletal vibration has been suggested for the absorptions displayed in the aldose series[7]. The only restriction is that a free, anomeric hydroxyl group must be present; that is, such nonreducing disaccharides as sucrose are excluded, because disaccharides having the aldosyl ketoside structure merely display noncharacteristic absorbances, mostly influenced by the aldose moiety. On the other hand, reducing disaccharides show absorption of both types I and II, so the presence of the 2-ketose residue can be simply, rapidly, and exactly ascertained.

The original statement[4] that type IIB absorption (at 836 cm^{-1}; standard

TABLE IV

RELATION BETWEEN THE STABLE CONFORMATION AND TYPE 3 ABSORPTION OF VARIOUS 2-KETOSES

Sugar	*Stable pyranose conformation*	*Number of equatorial hydrogen atoms*[a]	*Bands* (cm^{-1})
xylo *Configuration*			
D-*xylo*-Hexulose	CA	—	—
L-*gluco*-Heptulose	CA	—	—
2,7-Anhydro-β-D-*ido*-heptulose	CE	—	—
lyxo *Configuration*			
D-*glycero*-D-*gulo*-Octulose	CE	1	793
2,7-Anhydro-β-L-*gulo*-heptulose	CA	—	—
arabino *Configuration*			
2,7-Anhydro-β-D-*altro*-heptulose	CE	—	—
ribo *Configuration*			
D-*allo*-Heptulose	CA	1	784

[a]Equatorial hydrogen atoms on carbon atoms other than carbon atom 5.

deviation = ±6) could be explained by the fusion of pyranose and furanose rings is indirectly confirmed by the spectra of inulobiose and leucrose. However, a slight modification would be better, in that any linkage with another compound by means of C-1 and C-2 causes appearance of these bands, so that a definite assignment for these bands is very difficult.

Type 2 absorption (at 850 cm^{-1}; standard deviation = ±10) is most probably restricted to the furanose form, but the anomalous results shown by D-*allo*-heptulose and D-*glycero*-D-*gulo*-octulose (absorption bands at 860 cm^{-1} and 858 cm^{-1}, respectively) may possibly be explained by their mode of preparation. Their crystallization may have been effected after some equilibration, so that, for both sugars, a certain percentage may have been present in the furanose form. This phenomenon was mentioned for the first time in the study of Tipson and Isbell[8]; D-*manno*-heptulose (having a distinct absorption at 816 cm^{-1}) displayed two weak bands at 822 and 840 cm^{-1} after equilibrium had been attained.

Type 3 absorption (at 789 cm^{-1}; standard deviation = ±5) is caused by the ring-breathing frequency of the pyranoid ring[2,6,7] and has been assigned to factors causing the conformational instability[8] of some rather specific configurations[4]. This conclusion is now confirmed (see Table IV). The nomenclature used for the conformations listed in Table IV is the improved one of Isbell and Tipson[9].

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Reaction of 6-*O*-benzoyl-1,2:4,5-di-*O*-isopropylidene-3-*O*-methanesulphonyl-D-mannitol with sodium benzoate in dimethylformamide. A novel elimination reaction

Treatment of secondary sulphonates of acyclic carbohydrates with sodium benzoate in dimethylformamide[1] affords, in the absence of neighbouring-group reactions (such as arise from vicinal acyl groups[2]), benzoates having inverted configurations. With the exception of 1,2:5,6-di-*O*-isopropylidene-3,4-di-*O*-toluene-*p*-sulphonyl-D-mannitol, which afforded a substantial amount of an enol sulphonate[3] (*cf.* Angyal and Stewart's results[4]), elimination of sulphonic acid with concomitant formation of unsaturated compounds was not detected in the examples studied, although its occurrence cannot be ruled out since quantitative yields of products were not obtained.

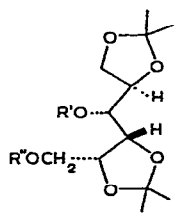
We now report on an apparent exception to this general pattern. Treatment of 1,2:4,5-di-*O*-isopropylidene-3,6-di-*O*-methanesulphonyl-D-mannitol (I) with sodium benzoate in boiling dimethylformamide resulted in the replacement of one methanesulphonyloxy group by a benzoyloxy group, and elimination of one mol. of methanesulphonic acid, to give a single crystalline product (*A*) in good yield. That elimination involved the secondary methanesulphonate group was established when 6-*O*-benzoyl-1,2:4,5-di-*O*-isopropylidene-3-*O*-methanesulphonyl-D-mannitol (II) was also converted into compound *A* under similar reaction conditions. The methanesulphonate (II) was obtained by selective benzoylation of 1,2:4,5-di-*O*-isopropylidene-D-mannitol (III) followed by methanesulphonylation, and its structure was confirmed when alkaline treatment afforded known[5] 1,4-anhydro-2,3:5,6-di-*O*-isopropylidene-D-talitol (IV).

Two lines of evidence indicated that elimination had occurred at C-3–C-4 in the methanesulphonates (I) and (II), and that compound *A* was the enol ether, 6-*O*-benzoyl-3-deoxy-3,4-didehydro-1,2:4,5-di-*O*-isopropylidene-D-*threo*-hexitol (V). Firstly, the n.m.r. spectrum of compound *A* in carbon tetrachloride showed, *inter alia*, a triplet (corresponding to one proton) at τ 6.68 (J 8 c.p.s.) which can be assigned to H-5 (signal split by coupling to the two equivalent protons at position 6) and a quartet [corresponding to one proton, and each peak of which was further split (J *ca.* 0.5 c.p.s.)] at τ 5.95–6.22, which may be assigned to H-2 (splitting due to strong coupling with the two non-equivalent protons at position 1). A significantly different coupling pattern would be expected for the alternative structure, formed by elimination of methanesulphonic acid from compounds (I) or (II) at C-2–C-3.

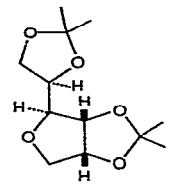
Secondly, application in sequence of ozonolysis, borohydride reduction, and acidic hydrolysis to compound *A* gave glycerol (characterised as the tris-*p*-phenylazobenzoate).

Although no direct experimental evidence could be obtained to reveal the

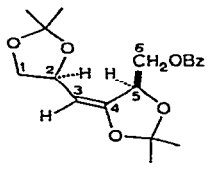
configuration about the double bond in compound *A*, a tentative assignment of structure (V) is made on the basis of the following argument. The formation of the enol ether in high yield from the methanesulphonates (I) and (II) contrasts with the behaviour of the sulphonates of the structurally related compounds 1,2:4,5-di-*O*-isopropylideneribitol[6], 1,2:5,6-di-*O*-isopropylidene-3-*O*-methyl-D-glucitol[2], and 1,2:5,6-di-*O*-isopropylidene-3-*O*-methyl-D-mannitol[7], which undergo normal dis-



(I) R' = R" = Ms
(II) R' = Ms, R" = Bz
(III) R' = R" = H



(IV)



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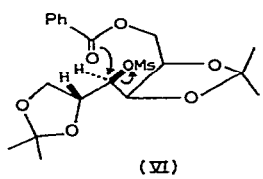
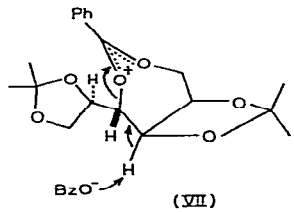


(VI)



(VII)

placement reactions with sodium benzoate or sodium acetate in dimethylformamide. Moreover, examination of molecular models reveals no obvious steric situation which would account for elimination at C-3–C-4 in the methanesulphonates (I) and (II), rather than at C-2–C-3. This behaviour may, however, be rationalised in terms of participation by the terminal benzoate group of compound (II) in the displacement of the sulphonate group. Because the 4,5-ketal involves an *erythro*-diol, C-4 and C-6 are *cis*-disposed, and the benzoate group in compound (II) [which is a reasonable first product in the reaction of the dimethanesulphonate (I) with benzoate ions[8]] is well situated for anchimeric assistance (VI) of the methanesulphonate solvolysis. The resultant carbonium ion can assume a conformation (VII) with the geometry necessary to permit elimination to give the enol ether (V). Direct elimination of methanesulphonic acid by attack of, for example, benzoate ions at the C-4 hydrogen atom is not precluded by the experimental data, and would give an enol ether having the configuration of the substituents about the double bond different from that in the enol ether (V).

EXPERIMENTAL

Thin-layer chromatography (t.l.c.) was performed on Kieselgel and detection effected with vanillin–sulphuric acid[9] or iodine vapour. Organic solutions were dried over $MgSO_4$.

Benzoylation of 1,2:4,5-di-O-isopropylidene-D-mannitol

The di-*O*-isopropylidene derivative[10] (1 g) was treated with benzoyl chloride (0.5 ml) and pyridine (3 ml) for 12 h at room temperature. Isolation of the product in the usual manner gave a crude benzoate (0.7 g), m.p. 95°, which was fractionally crystallised from ethanol to yield, as the first crop, the 3,6-dibenzoate (0.25 g), m.p. 135–137°, $[\alpha]_D$ −36° (*c* 1.0, $CHCl_3$) (Found: C, 66.1; H, 6.6. $C_{26}H_{30}O_8$ calc.: C, 66.4; H, 6.4 %). The second crop (0.18 g) was mainly the 6-benzoate which, after recrystallisation, had m.p. 108–110°, $[\alpha]_D$ +6° (*c* 0.8, $CHCl_3$) (Found: C, 62.4; H, 7.1. $C_{19}H_{26}O_7$ calc.: C, 62.3; H, 7.1 %). In t.l.c., the R_F values [for benzene–ether (9:1)] were: 3,6-dibenzoate, 0.49; 6-benzoate, 0.17.

Reaction of methanesulphonates with sodium benzoate in dimethylformamide

(*a*) A solution of 6-*O*-benzoyl-1,2:4,5-di-*O*-isopropylidene-D-mannitol (15.2 g) in pyridine (80 ml) was treated with methanesulphonyl chloride (15 ml) at room temperature overnight. The mixture was poured into ice-water (1 l) and extracted with chloroform (total vol. 1 l). The chloroform solution was washed with water, dried, and passed through a column (10 × 3.5 cm) of neutral alumina. Evaporation of the eluate and repeated distillation of benzene from the residue gave the syrupy 3-methanesulphonate (21.8 g), $[\alpha]_D$ +34° (*c* 1.0, $CHCl_3$), which appeared homogeneous in t.l.c. and had R_F values 0.20 [carbon tetrachloride–ether (4:1)] and 0.94 [benzene–methanol (19:1)]. The i.r. spectrum showed negligible hydroxyl absorption.

A solution of the foregoing methanesulphonate (21.7 g) in dimethylformamide (1.2 l) was boiled under reflux in the presence of sodium benzoate (44 g) for *ca.* 12 h. The cooled mixture was diluted with water (1.2 l) containing sodium hydrogen carbonate, and stored at 0° for 2 days. The crystalline precipitate was collected and well washed with water to yield the crude product (12.1 g, 71 %), m.p. 53–56°, $[\alpha]_D$ +55° (*c* 1.1, $CHCl_3$). Recrystallisation from methanol gave 6-*O*-benzoyl-3-deoxy-3,4-didehydro-1,2:4,5-di-*O*-isopropylidene-D-*threo*-hexitol (V), m.p. 62–64°, $[\alpha]_D$ +57° (*c* 0.4, $CHCl_3$), which was homogeneous in t.l.c. [R_F 0.48, using carbon tetrachloride–ether (4:1)] (Found: C, 66.1; H, 6.8. $C_{19}H_{24}O_6$ calc.: C, 65.5; H, 6.9 %).

(*b*) Methanesulphonylation, as in (*a*), of 1,2:4,5-di-*O*-isopropylidene-D-mannitol (47.6 g) with pyridine (700 ml) and methanesulphonyl chloride (70 ml) gave syrupy 3,6-dimethanesulphonate (64.9 g, 85 %), $[\alpha]_D$ +28° (*c* 0.9, $CHCl_3$). Examination by t.l.c. [benzene–methanol (9:1)] revealed a major component with R_F 0.63, and traces of contaminants (R_F 0.79 and 0.32).

The 3,6-dimethanesulphonate (6.3 g) was treated with dimethylformamide (315 ml) and sodium benzoate (36 g) as in (*a*) to give crude enol ether (V) (4.44 g) which, after recrystallisation from methanol, had m.p. 60–62°, alone or in admixture with the product described in (*a*).

The n.m.r. spectrum of the enol ether (V) (10 % solution in carbon tetrachloride with a 6 % solution of tetramethylsilane in chloroform as external reference; Varian A60 spectrometer) had the following signals: τ 1.85–2.10 (multiplet) and 2.40–2.80 (multiplet), aromatic protons; 4.90–5.40 (2-proton multiplet); 5.40–5.90

(3-proton multiplet); 5.95–6.22 (1-proton quartet); 6.68 (1-proton triplet, *J ca.* 8 c.p.s.); 8.30–8.90 (triplet), isopropylidene Me protons.

Degradation of the enol ether (V)

Ozonised oxygen was bubbled through a solution of the enol ether (V) (0.17 g) in chloroform (30 ml) at −60° for 1 h. The resultant acidic solution (pH 2) was treated dropwise with a solution of sodium borohydride (0.25 g) in ethanol (18 ml) and water (3 ml), and then boiled under reflux for 3 h. More sodium borohydride (100 mg) was added to the cooled solution which was then stored at room temperature overnight. The mixture was concentrated and a solution of the residue in water (25 ml) was deionized using Amberlite resins IR-120 (H^+ form) and IRA-400 (HO^- form). Concentration of the solution gave a syrup which was hydrolysed with 5N hydrochloric acid at *ca.* 100° for 1.5 h. Evaporation of the hydrolysate gave a residue (70 mg) which, on examination by paper chromatography using butanol–ethanol–water (40:11:19) and detection with silver nitrate[11], appeared to contain a single component having a mobility identical to that of glycerol (R_F 0.48).

The foregoing product (35 mg) was treated with pyridine (1.5 ml) and *p*-phenylazobenzoyl chloride (0.35 g) in the usual manner[12] to give a crude ester (214 mg), which was recrystallised from chloroform–light petroleum (b.p. 60–80°) to yield a product having m.p. 212–214°, alone or in admixture with authentic 1,2,3-tri-*O*-*p*-phenylazobenzoylglycerol.

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Preparation of inososes from their phenylhydrazones by use of a cation-exchange resin; separation of certain phenylhydrazones from phenylosazones

Inososes are usually purified through their phenylhydrazones, from which the phenylhydrazine residue is removed either by acid hydrolysis or by an exchange reaction[1]. Benzaldehyde, a reagent well known in sugar chemistry for removing the phenylhydrazine moiety by an exchange reaction, has been successfully applied to inosose phenylhydrazones by Posternak[2] and by Carter and co-workers[3]. We have sought a more direct and convenient method for preparing inososes from their phenylhydrazones.

We have now found that a cation-exchange resin of the sulfonic-acid type removes the phenylhydrazine moiety at 80 to 90°. The liberated phenylhydrazine combines with the resin, and therefore no subsequent extraction process is necessary. The cleavage reaction may be conducted in aqueous isopropyl alcohol or aqueous *p*-dioxane, but the best results have been obtained with water. The method has proved successful with compounds having a labile phenylhydrazono group. Compounds in which the phenylhydrazono group is either chelated or resonance-stabilized by the presence of an additional aromatic ring cannot be cleaved by the resin*.

We have also found that *phenylosazones* of inososes are unaffected by cation-exchange resins; consequently, they can be readily separated from phenylhydrazones. Use of this property has been found helpful in separating complex mixtures of phenylhydrazine derivatives.

Some applications of cation-exchange resin in removing the phenylhydrazine moiety are summarized in Table I, and the yields of the recovered products are compared with those obtained by the benzaldehyde method.

EXPERIMENTAL

Preparation of inososes from their phenylhydrazones

(*a*) A cation-exchange resin** in the hydrogen form (330 ml, wet volume) was added to 600 ml of water in a 2-liter beaker, and the suspension was stirred and heated to 85–90°. Crude, finely powdered DL-*epi*-inosose-2 phenylhydrazone[5] (66 g) was added portionwise during 2–4 min, and the suspension was stirred and kept at

*Use of a cation-exchange resin for the cleavage of phenylhydrazones of sugars has been found to be less effective than other methods because considerable degradation accompanies the removal of the phenylhydrazine residue. Treatment of cyclohexanone phenylhydrazone with a cation-exchange resin gives 1,2,3,4-tetrahydrocarbazole in high yield[4].

**Amberlite IR-120, a product of Rohm and Haas Company, Philadelphia, Pa., U.S.A. Mention of a commercial product in this article does not constitute endorsement by the National Bureau of Standards, nor does it imply that the product identified is necessarily the best for the purpose.

TABLE I

YIELDS OF PRODUCTS OBTAINED BY CLEAVAGE OF PHENYLHYDRAZINE DERIVATIVES

Starting material	*Product recovered*	*Yield*	
		Reported by benzaldehyde method, %	*Found by resin method, %*
DL-*epi*-Inosose-2 phenylhydrazone	DL-*epi*-inosose-2	63[a]	64–68
myo-Inosose-2 phenylhydrazone	*myo*-inosose-2	78[a] 88[b]	70–75
L-*epi*-Inosose-2 phenylhydrazone	L-*epi*-inosose-2	96[c]	82
Oxalic acid bis(phenylhydrazide)	oxalic acid		75–80
Benzaldehyde phenylhydrazone	starting material		
An inosose phenylosazone	starting material		
1,2,3-Cyclohexanetrione tris(phenylhydrazone)	starting material		

[a]See ref. 5. [b]See ref. 3. [c]See ref. 6.

85–90° until the solid phenylhydrazone had disappeared and the resin had turned black (10–15 min). Decolorizing carbon (10 g) was then added, and the stirred mixture was heated for 5 min and immediately filtered. The resin (which had acquired a strong, aromatic odor) was washed with hot water and discarded. The filtrate and washings were combined and evaporated at 40° under diminished pressure to about 300 ml, at which point crystallization began. The suspension was transferred to a beaker, refrigerated for several hours, and filtered. The colorless crystals were washed with 50% aqueous methanol and dried in a vacuum desiccator; yield, 28–30 g.

A sample, recrystallized from water, melted at 206–208° (decomp.); mixed m.p. with authentic[5] DL-*epi*-inosose-2, 205–207°. The product was further identified by its pentaacetate, m.p. 138–140°; lit. m.p.[7] 130–131°; and[8] 139–140°.

(*b*) A suspension of 4.2 g of L-*epi*-inosose-2 phenylhydrazone[6] and 30 ml of the cation-exchange resin in 100 ml of water, treated as described above, yielded 2.3 g of L-*epi*-inosose-2.

Separation and determination of components in a mixture of myo-*inosose-2 phenylhydrazone and* DL-myo-*inosose-1 phenylosazone*

A suspension of 54 mg (0.2 mmole) of *myo*-inosose-2 phenylhydrazone[5], 36 mg (0.1 mmole) of DL-*myo*-inosose-1 phenylosazone [DL-1,2-dideoxy-1,2-dioxo-*myo*-inositol bis(phenylhydrazone)][3], and 3 ml of the cation-exchange resin in 20 ml of distilled water was stirred and heated for 10 min at 85–90°. The suspension was filtered while hot, and the residue on the filter was thoroughly washed with warm water. The filtrate (which reduced Benedict solution in the cold) was titrated by

the method of Heyns and Paulsen[9]; it showed the presence of a reducing component equivalent to 96% of the original *myo*-inosose-2 phenylhydrazone.

The mixture of unchanged phenylosazone and resin on the filter was extracted with warm ethyl alcohol, and the extracts and washings (about 65 ml) were transferred to a 100-ml volumetric flask. The solution was diluted to volume with water, and aliquots were titrated with periodic acid according to the procedure of Magasanik and Chargaff[10]. The material in the extract reacted with 0.31 mmole of periodic acid, indicating quantitative recovery of the unchanged phenylosazone from the mixture.

It was found that the inosose phenylosazone may be titrated with periodate as successfully in an aqueous solution of *N,N*-dimethylformamide as in aqueous ethyl alcohol. The solution should be cooled to 15° before addition of the oxidant.

For the recovery of an inosose phenylosazone after treatment of a phenylhydrazone–phenylosazone mixture with the cation-exchange resin, the resin may be extracted with *N,N*-dimethylformamide. Usually, addition of water to the extract precipitates the product in almost pure condition.

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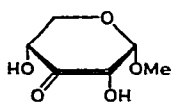
Preliminary communications

The anomeric methyl D-*erythro*-pentopyranosid-3-uloses

Previous syntheses of unsubstituted pento- and hexo-pyranosiduloses have given low yields of products contaminated with starting material and further oxidation products[1]. We now report the use of phenylboronic esters as convenient blocking groups in the synthesis of pentopyranosiduloses in good yield.

Oxidation of methyl α- and β-D-xylopyranoside 2,4-phenylboronates[2] with a dimethyl sulphoxide–acetic anhydride mixture[3] for 75 min. at 40° gave both anomers of methyl D-*erythro*-pentopyranosid-3-ulose in good yield. After partition of the reaction mixture between chloroform and water, the keto sugars were isolated by elution of the aqueous phase through Dowex-IX (HSO_3^--form) ion-exchange resin which allowed separation of the keto sugar from the other products and reactants[4]. Subsequent elution with warm acetone–water (1:9) afforded the desired product.



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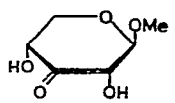

II

The α-D anomer (I), m.p. 79–82°, was obtained in 50–60% yield. This material was sufficiently pure for most purposes; vacuum sublimation at 68–70° yielded pure methyl α-D-*erythro*-pentopyranosid-3-ulose, m.p. 80–82°, $[\alpha]_{5780}^{20}$ + 191° (*c* 1.17, water), $M_{Vanillin}$ 0.26 (pH 4.7, bisulphite electrophoresis)[5]. Reduction of compound (I) with sodium borohydride followed by acid hydrolysis gave only ribose and a trace of xylose, as shown by paper chromatography.

In the same way, the crude, crystalline β-D anomer (II) was obtained in a yield of 70–80%, and sublimation at 68–70° yielded pure methyl β-D-*erythro*-pentopyranosid-3-ulose, m.p. 85–88°, $[\alpha]_{5780}^{20}$ −77.3° (*c* 1.05, water), $M_{Vanillin}$ 0.99 (Ref 5). Borohydride reduction of compound (II) followed by acid hydrolysis yielded an approximately equimolar mixture of ribose and xylose. Compounds (I) and (II) both had a strong infrared absorption band at 1720 cm^{-1} and gave satisfactory elemental analyses.

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Bis(2-amino-2-deoxy-D-glucopyranosyl)amine derivatives

In studies on the synthesis of *N*-L-*β*-aspartyl-2-acetamido-2-deoxy-*β*-D-glucopyranosylamine, Yamamoto *et al.*[1] isolated a by-product, during their preparation of 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-*β*-D-glucopyranosylamine (II, R=H), which they tentatively identified as a bis(2-acetamido-tri-*O*-acetyl-2-deoxy-D-glucosyl)-amine [m.p. 222–224°, $[\alpha]_D$ −25° (chloroform)] and upon which we have made further observations.

Our initial attempts to reduce the azide (I) to the amine (II, R=H) by catalytic hydrogenation in ethyl acetate, dioxan, or tetrahydrofuran led, in each case, to the formation of at least two by-products as shown by t.l.c. on silica gel. Their formation occurred during the evaporation of the solvent, but was obviated by hydrogenation in ethanol and subsequent evaporation at < 30° to give the pure amine (II, R=H) [m.p. 150°, $[\alpha]_D$ −25.5° (chloroform)]. The by-products were isolated in high yield by heating the amine (II, R=H) in ethyl acetate when they crystallized out as the reaction proceeded with the evolution of ammonia. After fractional crystallization followed by purification on a column of silica gel, two isomeric bis(2-acetamido-2-deoxy-D-glucopyranosyl)amines (III, R=Ac) were isolated [*A*, m.p. 260°, $[\alpha]_D$ −32° (chloroform), mol. wt. (v.p. osmometer) 680 ± 5; *B*, m.p. 245–246°, $[\alpha]_D$ +44° (chloroform), mol. wt. 680 ± 5]. At room temperature, compounds *A* and *B* showed mutarotation in chloroform containing a trace of hydrochloric acid due to interconversion and

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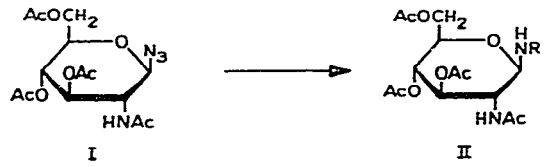

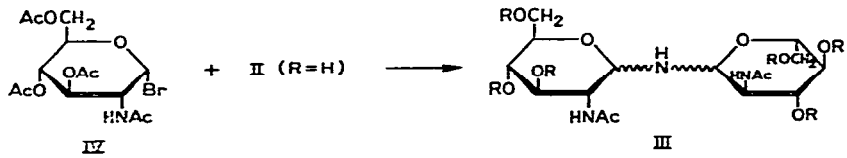

equilibration. De-*O*-acetylation of *A* and of *B* with sodium methoxide or methanolic ammonia apparently gave only one product (III, R=H) [*C*, m.p. 169–170° $[\alpha]_D$ +71° (methanol)]. Reacetylation of compound *C* (III, R=H) with acetic anhydride and pyridine gave both derivatives *A* and *B* (III, R=Ac), and, similarly, benzoylation of compound *C* (III, R=H) gave two hexabenzoates [m.p. 178-180°, $[\alpha]_D$ −10° (chloroform); m.p. 122–124°, $[\alpha]_D$ +10° (chloroform), respectively].

The same bis(2-acetamido-2-deoxy-D-glucopyranosyl)amines (*A* and *B*) were obtained when 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl bromide (IV) was treated with the amine (II, R=H) in nitromethane with mercuric cyanide as catalyst[2]. Attempts to acetylate, benzoylate, and formylate the amines *A* and *B* (III, R=Ac) were unsuccessful due to the very weak basicity of the –NH– group, presumably as a result of the electron-withdrawing effects of the neighbouring ring-oxygens and/or the crowded stereochemistry about the –NH– group. Inspection of molecular models indicates that the α,β and the β,β anomeric configurations are favoured, and a study of the molecular rotations[3] suggests that compounds *A* and *B* are the β,β- and α,β-isomers, respectively.

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The identity of γ-D-galactose

In 1928, Smith and Lowry[1] deduced the occurrence of a third isomer to explain mathematically the complex mutarotation of D-galactose. They calculated that, in addition to α- and β-D-galactose, the third component (γ) should be present at a concentration of about 12% at equilibrium. Later, Isbell and Pigman[2] reasoned that the initial rapid phase of mutarotation of D-galactose was due to pyranose–furanose interconversion, and the slow phase to the interconversion of the known pyranose anomers. By applying conformational principles, Angyal[3] concluded that γ-D-galactose is probably β-D-galactofuranose, since all-*trans* bulky substituents on a five-membered ring are favored.

In view of the significance of D-galactose in biochemical and carbohydrate studies, it is important that the identity of the presumed γ-D-isomer be more firmly established. In this communication, direct isolation of a derivative of γ-D-galactose is reported. The formation of the compound during mutarotation in pyridine was followed by separation of trimethylsilyl tetra-*O*-(trimethylsilyl)-D-galactosides using gas–liquid chromatography; the derivative was isolated in sufficient quantity, using preparative g.l.c., for characterization by polarimetry, n.m.r., and i.r. spectroscopy as trimethylsilyl 2,3,5,6-tetra-*O*-(trimethylsilyl)-β-D-galactofuranoside (I).

α-D-Galactopyranose (0.5%) was allowed to mutarotate in pyridine. Aliquots (1 ml) were taken at intervals during 48 h, and were trimethylsilylated[4]. With D-galactose, this reaction is so rapid that further interconversion is arrested. The volatile derivatives were then separated by g.l.c. with a Barber–Coleman Model No. 10 apparatus, using a β-ray ionization detector and a 1.8 m by 0.5 mm ID diethylene glycol succinate column[5] at 145°, with an argon stream (62 ml/min).

During mutarotation of α-D-galactopyranose, two additional components formed. After trimethylsilylation, one gave a product which had the same retention time (41 min) as authentic trimethylsilyl tetra-*O*-(trimethylsilyl)-β-D-galactopyranoside (II) prepared from β-D-galactose. When the mole % (g%) of the three components, calculated from the chart records by triangulation, was plotted against mutarotation time, two distinct phases of mutarotation were evident. The first was an initial, rapid interconversion of the three isomers, lasting 8 h, during which the third component reached a steady-state concentration. The second phase was characterized by slow interconversion of the pyranose anomers. The third component is, therefore, "γ-D-galactose". The final equilibrium mixture contained α-D-galactopyranose 32.5%,

β-D-galactopyranose 53.4%, and γ-D-galactose 14.1% (Retention time 31, 41 and 28 min, respectively).

About 25 mg of I was isolated from the equilibrium mixture by preparative g.l.c. with a Beckman Megachrom unit, equipped with a 1.8 m by 0.94 mm ID silicone column and helium carrier. Larger quantities of II and trimethylsilyl tetra-*O*-(trimethylsilyl)-α-D-galactopyranoside (III) were prepared by direct trimethylsilylation[4,6] of the sugars.

Pyridine was removed from these reaction mixtures by evaporating to dryness *in vacuo*. Salts were removed by extracting with anhydrous ether and again evaporating to dryness.

An i.r. analysis (film), using a Beckman Model IR-7 instrument, showed that I, II, and III lacked free carbonyl absorption, indicating that I is not the acyclic form and must be a furanose.

N.m.r. spectra of I, II, and III, determined at 60 Mc.p.s. in chloroform (Varian Model A60 spectrometer) with tetramethylsilane as an internal standard, gave the following data: III, τ 5.1 (hydrogen doublet $J_{1,2}$ 2.5 c.p.s.); τ 6.1–7.0 (complex six-hydrogen multiplet); II, τ 5.65 (hydrogen doublet, $J_{1,2}$ 6 c.p.s.); τ 6.1–7.0 (complex six-hydrogen multiplet). The doublets for the pyranoside derivatives are assigned to the poorly shielded, anomeric hydrogen atoms[6,7], and, for the β-D anomer, the signal is shifted up-field. The coupling constants and chemical-shift data for II and III accord with the anticipated *C1*(D) conformation[7].

The n.m.r. spectrum of I showed a hydrogen doublet, τ 4.95, again assigned to H-1, since it is the only possible methine group attached to two oxygen atoms. The *J* value for this doublet, 2.8 c.p.s., suggests[6,9] (by reference to the Karplus equation), that the probable H–1—H–2 dihedral angle is between 75° and 165°, and H–1 and H–2 therefore have the *trans* configuration.

Optical rotatory data obtained on the three isolated compounds were as follows; III, $[\alpha]_D^{23}$ +60° (*c* 0.9, chloroform); II, $[\alpha]_D^{23}$ +12° (*c* 0.8, chloroform); I, $[\alpha]_D^{23}$ −27° (*c* 0.6, chloroform).

Molecular rotations for the α- and β-D-pyranosides II and III are in good agreement with those reported for other D-galactopyranosides[10]. The molecular rotation of I, −14,500, is in good accord with that of known 1,2,3,5,6-penta-*O*-acetyl-β-D-galactofuranose (−16,400)[11].

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Book review

Mucopolysaccharides. Chemical structure, distribution and isolation. by J.S. BRIMACOMBE AND J.M. WEBBER, Elsevier, Amsterdam, 1964, x + 184 pages, 55 s (B.B.A. Library, Vol. 6).

The monograph on "Mucopolysaccharides" by J.S. Brimacombe and J.M. Webber covers an ever-expanding and important area of biochemistry, the hexosamine-containing polysaccharides occurring in animal tissues. The book deals mainly with the polyanionic polysaccharides, hyaluronic acid, chondroitin, the chondroitin sulfates, heparin, heparitin sulfate, and keratosulfate. Included also is a chapter on chitin, which of course does not occur in higher animals and is not anionic, and on the blood-group substances, which may or may not be anionic, depending upon whether or not they contain sialic acids. Also included is a polyanionic bacterial polysaccharide, teichuronic acid. All these substances contain hexosamine and are exclusively or predominantly carbohydrate in nature. The authors, like all their predecessors, are faced with the impossible task of justifying the scope of the subject by providing systematic and noncontradictory definitions.

The book deals concisely and expertly with the major data known up to 1963, and in part in 1964, on chemical structure, distribution, and isolation. The book has the advantage over texts in similar areas in that it is written by carbohydrate chemists, who in the past appear not to have been deeply involved in the specific subject area.

The book ought to be of considerable merit as a readable monograph in a field of increasing importance to biology, chemistry, and medicine.

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