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STUDIES ON *tert*-BUTYL DERIVATIVES OF D-GLUCOSE

A. N. DE BELDER, E. J. BOURNE, AND H. WEIGEL
Chemistry Department, Royal Holloway College (University of London), Englefield Green, Surrey (Great Britain)

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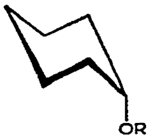
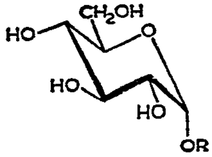
INTRODUCTION

In compounds such as cyclohexanol and its *O*-derivatives, the atom directly attached to the ring, *i.e.* oxygen, determines[1], in large measure, the free energy of the process (I) $\rightleftharpoons$ (II). Other exocyclic atoms attached to the first one are much less important. Thus, the conformational energy of (I), where R = H, Me, Et, MeCO, or p-$CH_3 \cdot C_6H_4 \cdot SO_2$, is 0.6–0.9 kcal. mole^{-1}.



I

II



III R = Me
IV R = Bu^t

However, it was of interest to ascertain whether the replacement of the axially disposed methoxy group in methyl α-D-glucopyranoside (III) by the more bulky *tert*-butoxy group would modify the *C1* conformation (Reeves' nomenclature[2]) of the pyranose ring. For this purpose, several conformationally dependent measurements were made on the *tert*-butyl α- and β-D-glucopyranosides, using the corresponding methyl D-glucosides as references.

RESULTS AND DISCUSSION

Table I shows the molecular rotations of D-glucopyranosides. The calculated values have been obtained by Whiffen's method[3]. The contribution to rotation of the aglycones was calculated empirically from the molecular rotation of the β-D anomer, since, for each pair, this is more likely to adopt the *C1* conformation. In all cases, except *tert*-butyl α-D-glucopyranoside, the observed values are in close agreement with those calculated for the *C1* conformation. The 7% difference between the observed value for the *tert*-butyl α-D-glucopyranoside and that calculated for its *C1* conformation, since this should tend to lower the molecular rotation.

Likewise, the paper-electrophoretic mobility in borate solution (Table II) and the rate of periodate oxidation of *tert*-butyl α-D-glucopyranoside are similar to those of the corresponding methyl derivative. They are thus compatible with the *C1* conformation.

TABLE I

MOLECULAR ROTATIONS, $[M]_D$, OF D-GLUCOPYRANOSIDES

D-*Gluco-pyranoside*	*Contribution from aglycone,°*	*Calculated value,°*		*Observed value,°*
		C1 conformation	*1C conformation*	
Methyl α-	+100	+309	− 69	+309
Methyl β-	−100	− 68	−212	− 66
Cyclohexyl α-	+140	+349	+109	+349
Cyclohexyl β-	−140	−108	−252	−109
tert-Butyl α-	+ 77	+286	+ 46	+308
tert-Butyl β-	− 77	− 45	−156	− 45

TABLE II

PAPER ELECTROPHORETIC MOBILITIES OF D-GLUCOPYRANOSIDES IN BORATE SOLUTION

D-*Glucopyranoside*	Methyl α-	*tert*-Butyl α-	Methyl β-	*tert*-Butyl β-
M_G	0.11	0.10	0.19	0.13

The infrared spectra of the *tert*-butyl D-glucopyranosides were interpreted[4–6] as shown in Table III. However, if the α-D anomer adopts the *C1* conformation, it is not clear why the Type 2a absorption should be a doublet.

Much evidence has been accumulated to demonstrate that the anomeric proton in pyranoses and their derivatives resonates at a lower magnetic field than the other ring protons[7,8], and assignments of conformation have been made from its chemical shift and coupling constant. The anomeric protons of several α-D-gluco-

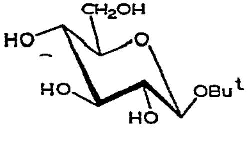
TABLE III

INFRARED SPECTRA OF D-GLUCOPYRANOSIDES

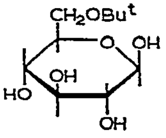
D-*Glucopyranoside*	*Absorption (cm^{-1})*[a]			
	Type 1[4,5]	*Type 2b*[4,5]	O-tert-*Butyl*[6]	*Type 2a*[4,5]
Methyl α-	896 s			840 s
tert-Butyl α-	897 s		865 s	850 m, 840 w
Methyl β-	919 w	884 s		
tert-Butyl β-	918 w	900 s	875 s	

[a] s, m, and w indicate strong, medium, and weak, respectively.

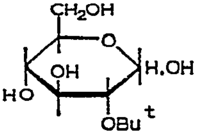
pyranosides exhibit a chemical shift of 4.88 ± 0.33 τ with $J_{1,2}$ 3.2 ± 0.6 c.p.s.[8]. The corresponding figures for the β-D anomers are 5.56 ± 0.06 τ and 7.2 ± 0.2 c.p.s. These values have been interpreted on the basis of the *C1* conformation. The chemical shifts for the anomeric protons in *tert*-butyl α- and β-D-glucopyranosides are 4.9 and 5.6 τ, respectively. The coupling constant for the α-D anomer, $J_{1,2}$ 3.5 c.p.s., is close to an axial–equatorial coupling in cyclohexane systems, while that for the β-D anomer, $J_{1,2}$ 6.0 c.p.s., is in agreement with the corresponding axial–axial coupling. We thus conclude that the *tert*-butyl α-(IV) and β-D-glucopyranoside (V) exist in the *C1* conformation and that the *tert*-butyl group in the α-D anomer is not sufficiently large to modify this conformation.



V



VI



VII

During these investigations, it was found that D-glucose, in the presence of an acid catalyst, reacts with *tert*-butyl alcohol to afford significant proportions of a material which was shown to be 6-*O*-*tert*-butyl-β-D-glucopyranose (VI). The assignment of structure for this ether is based on the following observations: (*a*) the compound was easily hydrolysed to give D-glucose; (*b*) the infrared spectrum of the crystalline material contained absorption bands at 875 (possibly with a shoulder at 870) and 900 cm^{-1}, but not at 844 ± 8 cm^{-1}; (*c*) the direction of mutarotation; (*d*) the p.m.r. spectrum, measured in D_2O, revealed the presence of a *tert*-butyl group and the anomeric protons of α- and β-D-glucopyranoses; (*e*) acetylation afforded the tetra-*O*-acetyl derivative of its β-D anomer; (*d*) when treated with periodate, the ether consumed *ca.* 4 mol. of periodate, but no formaldehyde was produced during the reaction.

Minor products formed by the reaction between D-glucose and *tert*-butyl alcohol were the *tert*-butyl α- and β-D-glucopyranosides and another *tert*-butyl ether. This ether was obtained crystalline, but not in sufficient quantities for a complete structural analysis. However, it was hydrolysed to give D-glucose, and consumed 6.4 mol. of periodate with liberation of 1 mol. of formaldehyde. This, together with the paper-electrophoretic mobilities of the ether and its reduction product in germanate and molybdate solutions, respectively, indicates that the ether is 2-*O*-*tert*-butyl-D-glucose (VII).

By treatment of 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose with *tert*-butyl bromide, and subsequent hydrolysis, Kenner and Richards[9] obtained trace proportions of a material which they assumed to be 3-*O*-*tert*-butyl-D-glucose. The 3-*O*-*tert*-butyl-1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose was, however, synthesised in good yields by the action of 2-methylpropene on 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose[10].

The formation of *tert*-butyl ethers of D-glucose, under the conditions described in this paper, can be understood in terms of the known facility with which *tert*-butyl alcohol reacts with alcohols to give mixed ethers[11]. Good yields are, however, obtained from this reversible reaction only if one of the products is removed from the reaction mixture as it is formed. In our case, this was water, which was removed by barium oxide. This proportion of the 2- and 6-ethers present in the reaction mixture is in agreement with the relative reactivities of simple secondary and primary alcohols[11] in this reaction with *tert*-butyl alcohol. It is also in agreement with the relative reactivities of the hydroxyl groups of D-glucose towards several acylating and alkylating reagents[12].

EXPERIMENTAL

General

The *tert*-butyl α- and β-D-glucopyranosides were prepared as described in the literature[13,14]. Electrophoresis was carried out on Whatman No. 3 paper, using borate[15], germanate[16], or molybdate[17] solutions as electrolytes. Thin-layer chromatography was performed on silica gel, using a benzene–methanol mixture (24:1). The solvent used for paper chromatography was butan-1-ol–ethanol–water (40:11:19).

Spectra. — Potassium bromide discs or Nujol mulls were used for measurements of the infrared spectra of the D-glucopyranosides. Only the range from 750–950 cm^{-1} was examined in detail. The p.m.r. spectra were measured in deuterium oxide, using a Varian A-60 spectrometer with *tert*-butyl alcohol as an internal reference.

Periodate oxidation of methyl and tert-*butyl α-D-glucopyranosides*

The rates of oxidation of the methyl and *tert*-butyl α-D-glucopyranosides in 0.015M potassium periodate (buffered to pH 6.93 with phosphate) at 20° were followed spectrophotometrically[18]. The moles of periodate consumed per mole of the methyl derivative were as follows: 0.25 h, 0.53; 0.5 h, 1.21; 0.75 h, 1.50; 2.0 h, 2.07; 4.5 h, 2.23. The corresponding figures for the *tert*-butyl derivative were: 0.25 h, 0.66; 0.5 h, 1.14; 0.75 h, 1.28; 2.0 h, 1.85; 4.5 h, 2.05; 7.0 h, 2.09.

Reaction between D-glucose and tert-*butyl alcohol*

A suspension of D-glucose (20 g) and Zeo-Carb 225 (H^+ form) (15 g) in *tert*-butyl alcohol (150 ml, dried over calcium oxide and redistilled) was refluxed for 2 h in a Soxhlet apparatus, the thimble of which contained barium oxide. The supernatant liquor was then poured off and replaced by *tert*-butyl alcohol (150 ml), together with additional resin (5 g). The suspension was again refluxed for 2 h. This operation was carried out three times. The combined supernatant liquors were concentrated to a syrup (15 g). This was fractionated (25-ml fractions) on a charcoal–Celite column (5 × 50 cm) using (*a*) water (3 l), (*b*) 0–20% ethanol (4 l), and (*c*) 20–40% ethanol (4 l) as eluants.

Fractions 20–70 contained unreacted D-glucose (*ca.* 10 g of crystallised syrup).

The residue from fractions 157–163 gave, from a small volume of water, 2-*O-tert*-butyl-D-glucose (0.2 g), m.p. 220–223°, $[\alpha]_D^{18}$ + 119° (5 min) → + 103° (equil.) (*c* 3.0, water) (Found: C, 50.1; H, 8.2. $C_{10}H_{20}O_6$ calc.: C, 50.8; H, 8.5%). It showed absorption at 1380, 1365, 1250, 1240, and 860 cm^{-1} and had R_G 3.5, $M_G(Ge)$ 0.03 (consistent with a 2-ether). When it was treated with potassium periodate in a buffered solution [phosphate, pH 6.5 (Ref. 19)] the moles of periodate consumed (determined by the arsenite method[20]) per mole of the ether were as follows: 54 min, 3.9; 18 h, 6.4; 24 h, 6.4. In 24 h, 1 mol. of formaldehyde was produced by this reaction. The reduction product of the ether (obtained by treatment with potassium borohydride) had $M_S(Mo)$ 0.91 (consistent with a 2- or 6-ether).

The 2-*O-tert*-butyl-D-glucose (10 mg) was hydrolysed in 2N sulphuric acid for 1 h at 90°. Paper chromatography of the hydrolysate revealed the presence of a single component corresponding to glucose.

Crystallisation from ethyl acetate of the residue of fractions 164–220 {containing also small amounts of *tert*-butyl α- [R_G 3.6, $M_G(B)$ 0.10] and β-D-glucopyranoside [R_G 3.3, $M_G(B)$ 0.13]} gave 6-*O-tert*-butyl-β-D-glucopyranose (2.0 g), m.p. 149–151°, $[\alpha]_D^{18}$ + 70° (5 min) → + 80° (equil.) (*c* 2.3, water) Found: C, 50.5; H, 8.6. $C_{10}H_{20}O_6$ calc.: C, 50.8; H, 8.5%). It showed absorption at 875 (possibly with a shoulder at 870) and 900 cm^{-1}. Signals in the p.m.r. spectrum were at 8.8 (*tert*-butyl), 5.4 (β, H-1), and 4.8 τ (α, H-1). The moles of periodate consumed per mole of the ether (determined as described above) were as follows: 45 min, 2.5; 3 h, 4.1; 20 h, 4.1. No formaldehyde was produced on treatment of the ether with periodate.

The 6-*O-tert*-butyl derivative (100 mg) was hydrolysed in water (10 ml) in the presence of Zeo-Carb 225 (H^+ form) for 15 min at 90°. Paper chromatography of the hydrolysate revealed the presence of a single component corresponding to glucose. This was converted into *N-p*-nitrophenyl-D-glucosylamine dihydrate[21], m.p. 180–184°.

Acetylation of the 6-*O-tert*-butyl derivative with acetic anhydride in pyridine afforded 1,2,3,4-tetra-*O*-acetyl-6-*O-tert*-butyl-β-D-glucopyranose, m.p. 133–135°, $[\alpha]_D^{20}$ + 21° (*c* 1.0, chloroform) (Found: C, 53.7; H, 6.9. $C_{18}H_{28}O_{10}$ calc.: C, 53.5; H, 7.0%). It showed absorption at 870 and 900 cm^{-1}.

The residue of the mother liquor from the crystallisation of the 6-*O-tert*-butyl derivative was acetylated. Fractional crystallisation from ethanol–light petroleum (b.p. 40–60°) yielded *tert*-butyl 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside[14] (0.4 g), m.p. 144°, $[\alpha]_D^{18}$ − 16° (*c* 0.8, chloroform), and 1,2,3,4-tetra-*O*-acetyl-6-*O-tert*-butyl-β-D-glucopyranose (0.4 g), m.p. and mixed m.p. 133–135°, $[\alpha]_D^{20}$ + 21° (*c* 1.0, chloroform).

ACKNOWLEDGMENTS

The authors thank Dr. K. McLaughlan for valuable discussions on the p.m.r. spectra and Mr. B. F. Smethurst for numerous infrared analyses.

SUMMARY

tert-Butyl α- and β-D-glucopyranoside have been shown to adopt the *C1* conformation.

Treatment of D-glucose with *tert*-butyl alcohol, in the presence of an acid catalyst, yields 6-*O*-*tert*-butyl-β-D-glucopyranose and a small proportion of 2-*O*-*tert*-butyl-D-glucose.

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STUDIES ON STARCHES OF HIGH AMYLOSE-CONTENT

PART IV.* THE FRACTIONATION OF AMYLOMAIZE STARCH; A STUDY OF THE BRANCHED COMPONENT

C. T. GREENWOOD AND S. MACKENZIE
Department of Chemistry, The University, Edinburgh 9 (Great Britain)

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INTRODUCTION

Amylomaize, the maize starch having a high amylose-content, has aroused considerable interest in view of its industrial importance, but only a limited number of fundamental studies have been made of the properties of this type of starch and its component fractions. This field has been reviewed[1] .

It is apparent that one of the main features of amylomaize starch is the nature and structure of the branched component. This "amylopectin" apparently possesses a high average-length of unit-chain of about 36 D-glucose residues, and a high β-amylolysis limit of about 65% conversion into maltose[1]. It is not known whether, in fact, these properties are due to real characteristics[2–4], or whether they are the result of an artifact[5].

We have now extended our earlier observations[5] and have fractionated samples of amylomaize having an apparent amylose-content of 50% and 70% by (*1*) our normal procedure involving dispersion after pretreatment with liquid ammonia[6], and (2) the extraction–sedimentation procedure of Montgomery *et al.*[3]. The properties of the resulting starch-fractions, particularly the branched one, have been studied.

EXPERIMENTAL

Samples of amylomaize

Two commercial samples of amylomaize were provided through the courtesy of Dr. J. Goodwin of the Corn Industries Research Foundation. The samples were "Amylon 50" and "Amylon 70", and were reputed to contain 50% and 70% of amylose, respectively. Prior to fractionation, the starches were shaken in saline suspension with toluene to remove protein, and stirred for 3 h in boiling, 85% aqueous methanol to remove fatty contaminants. [Protein found (%): Amylon 50, 0.38; Amylon 70, 0.44.] The iodine affinity of the starches was determined as described earlier[7].

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*For Part III, see Ref. 1.

Fractionation of the starches

(*a*) The samples were pretreated twice with liquid ammonia and dispersed into water by boiling for 1 h under nitrogen, and the "amylose" component was precipitated by addition of thymol. The complex was purified by recrystallization twice as the butan-l-ol complex. The starch fraction in the supernatant liquors, after removal of the thymol complex, was obtained by freeze-drying[6].

(*b*) The starches were subjected to the extraction–sedimentation procedure of Montgomery *et al.*[3]. This method is shown schematically below.

Amylomaize

Pretreated with H_2O/BuOH/glycerol at 98°.
Extracted with EtOH/NaOH(3M)
Neutralized
Centrifuged

Supernatant A1

Refluxed BuOH
Complex formation
Centrifuged

Sediment Ap1

Alkaline extraction
Refluxed AmOH
Complex formation
Centrifuged

Sediment A2

Refluxed BuOH
Complex formation
Centrifuged

Supernatant Ap2

Sediment A4

Supernatant Ap4

Dialysis
Freeze-drying

Ap4

Sediment A3

Supernatant Ap3

Freeze-drying

Ap3

Characterization of the fractionation products

Methods described earlier[6] were used to measure (*a*) iodine affinity, to obtain purity; (*b*) percentage conversion into maltose under (*i*) the action of crystalline β-amylase, and (*ii*) the concurrent action of β-amylase and Z-enzyme; (*c*) limiting-viscosity number $[\eta]$ in M potassium hydroxide at 25°; (*d*) Average length of unit chain by periodate oxidation.

Subfractionation of the branched fraction

(*a*) *Differential ultracentrifugation.* This was carried out by spinning aqueous solutions (0.25%) for (*i*) 1 h at 90,000 *g*, and (*ii*) 2 h at 90,000 *g* twice[5]. The sediments were dissolved in water and freeze-dried; material in the supernatant liquors was also recovered by freeze-drying.

(*b*) *Iodine-complex formation.* Fraction *Ap4* (75 ml; 0.2%) was subfractionated by the addition of iodine (5 ml; 0.2M in 2M potassium iodide) at room temperature. The mixture was maintained at 4° for 48 h before the precipitated iodine-complex was removed by centrifugation. Iodine in the complex and supernatant liquors was destroyed by the addition of thiosulphate, the solutions were then dialysed, and the polysaccharide was obtained by freeze-drying.

RESULTS AND DISCUSSION

Properties of the whole starches

Although the protein content of the amylomaize starches was low, and the samples had been exhaustively defatted, the potentiometric iodine-titration curves obtained by our usual procedure[7] were found to be of an unusual shape. Fig. 1 shows that the binding of iodine occurred more slowly, and terminated at a higher free-iodine concentration, than is generally found for regular maize starch. Again, when the linear portion of the adsorption curve is extrapolated to zero free-iodine concentration, the iodine affinity obtained[7] corresponded to only 38% and 45% of amylose in Amylon 50 and Amylon 70, respectively. This problem will be discussed elsewhere[8].

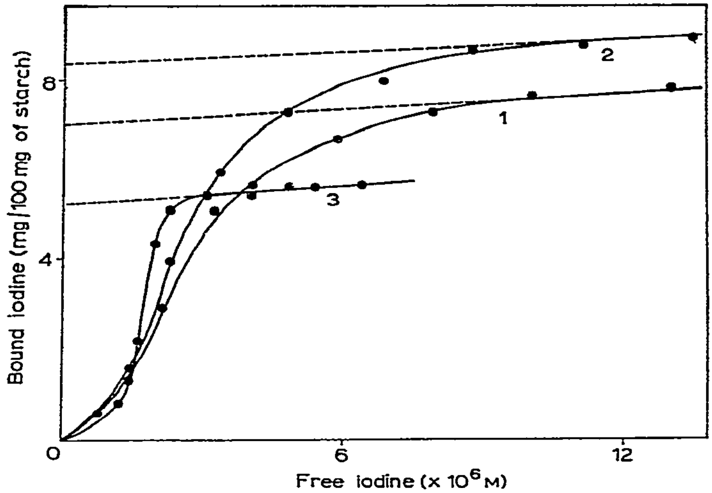

Fig. 1. Iodine-titration curves for the amylomaize starches (extrapolations to obtain the "iodine affinity" are shown by dotted lines): (1) "Amylon 50"; (2) "Amylon 70"; (3) regular maize-starch.

Properties of fractions from the conventional aqueous dispersion

Table I shows the properties of the thymol-fractionation products. The *amylcses*, even after two recrystallizations with butan-1-ol, were impure, as they were incompletely hydrolysed into maltose by the concurrent action of β-amylase and Z-enzyme. The low values for the limiting-viscosity number suggest further that the amyloses are of relatively short chain-length. (*cf.* values of $[\eta]$ given in Ref. 6).

In agreement with earlier reports[2–5], the non-precipitable "amylopectin fraction" was found to have an apparent average-length of unit-chain of 35 or 36 D-glucose residues, and a β-amylolysis limit of 65 or 66% conversion into maltose.

TABLE I

PROPERTIES OF THE FRACTIONS SEPARATED FROM AQUEOUS DISPERSIONS OF THE AMYLOMAIZE STARCHES

	Amyloses				*Amylopectins*			
	β-Limit[a] (*i*)	*β-Limit*[a] (*ii*)	*Purity* (%)[b]	[η]	*Purity* (%)[c]	*β-Limit* (*i*)	*Chain-length*	*Internal chain-length*[d]
Amylon 50	75	94	88	100	93	65	35	10
Amylon 70	70	90	80	100	92	66	36	10

[a]Percentage conversion into maltose under the action of (*i*) pure β-amylase, and (*ii*) β-amylase and Z-enzyme. [b]Calculated from β-limit (*ii*). [c]Calculated from iodine-affinity measurements. [d]Calculated from {chain-length—[(chain-length × β-limit) + 2.5]}, to the nearest whole-number.

The calculated values for the internal chain-length of 10 are only slightly higher than those found for potato amylopectin (8–9)[6].

However, it was observed that the apparent reducing-power of the samples (10^{-3} equiv./g), measured directly with alkaline ferricyanide, was much higher than that for a normal potato-amylopectin (1.6×10^{-4} equiv./g), which suggested that contaminating short-chain material was present. Further, the iodine-titration curves were also of an unusual shape and not like those for normal amylopectins (see Fig. 2). Subfractionation of the "amylopectin" was then attempted.

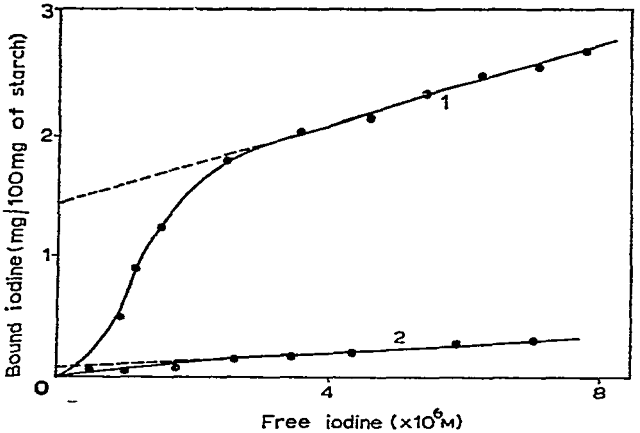

Fig. 2. Iodine-titration curves for the non-precipitable "amylopectin fraction" from amylomaize starch ("Amylon 50"): (1) initial polysaccharide-material; (2) fraction obtained after ultracentrifugation.

Properties of subfractions of the "amylopectins"

By differential ultracentrifugation, the "amylopectins" were separated into sedimentable and non-sedimentable portions. The properties of these are shown in Tables II and III.

TABLE II

PROPERTIES OF THE SEDIMENTABLE SUBFRACTION OF AMYLOPECTIN

Sample	*Centrifugation time (h)*	*Yield (%)*	*β-Limit (i)*[a]	*Chain-length*	*Iodine stain*	*λ_{max} (mμ)*
Amylon 50	1	70	56	27	Red	560
Amylon 70	1	75	57	28	Red	560
Amylon 50	2	80	57	27	Red	560
Amylon 70	2	80	57	28	Red	560

[a] As in Table I.

TABLE III

PROPERTIES OF THE NON-SEDIMENTABLE SUBFRACTION OF AMYLOPECTIN

Sample	*Centrifugation time (h)*	*Yield (%)*	*β-Limit (i)*[a]	*Iodine stain*	*λ_{max} (mμ)*
Amylon 50	1	30	76	Blue	580
Amylon 70	1	25	81	Blue	580
Amylon 50	2	20	79	Blue	580
Amylon 70	2	20	88	Blue	585

[a] As in Table I.

The sedimented material was some 75% of the total and possessed β-amylolysis limits and average lengths of unit-chain comparable to those for a *normal amylopectin*[9]. Furthermore, the colour and wavelength of maximum adsorption of solutions produced on staining of the sedimented materials with iodine, indicated the absence of any normal, long-chain, amylose-type material. The iodine-binding curve was also that for a pure amylopectin as shown in Fig. 2.

The characteristics of the polysaccharide remaining in the supernatant solutions after ultracentrifugation are those of a short-chain amylose. In contrast to our earlier results[5], degradation with β-amylase was incomplete, but this could arise from some natural differences in the starches of higher amylose-content used here. The value of λ_{max} for the iodine complex corresponded to a chain length of *ca.* 55 for a linear molecule[10].

These results are in agreement with those of Greenwood and Thomson[5]. Furthermore, it is to be noted that unpublished experiments by G. K. Adkins, M.Sc., in these laboratories, have shown that an exactly comparable behaviour is found if the amylomaize starch is pretreated with dimethyl sulphoxide and then precipitated with ethanol, prior to aqueous dispersion[11].

The extraction–sedimentation method[3,4] of fractionating amylomaize was then examined.

Properties of fractions obtained by extraction–sedimentation

The properties of the starch fractions obtained by the procedure of Montgomery *et al.*[3,4] are shown in Table IV.

TABLE IV

PROPERTIES OF FRACTIONS OBTAINED BY EXTRACTION–SEDIMENTATION OF THE AMYLOMAIZES

Fraction[a]	*Iodine affinity* (%)	*Amylose* (%)[b]	*β-Limit* (*i*)	(*ii*)[c]	[η]	*Chain-length*
A1	n.d.[d]	n. d.	63	69	50	n.d.
A2	12.0	63	70	78	70	n.d.
A3	17.8	89	83	94	100	n.d.
A4	13.0	69	75	82	75	n.d.
Ap2	0.2	1.0	57	n.d.	n.d.	n.d.
Ap3	0.3	1.5	60	n.d.	n.d.	30
Ap4	1.8	9.5	65	n.d.	n.d.	35

[a] See fractionation scheme in Experimental. [b] Calculated from the iodine affinity. [c] As in Table I. [d] n.d. = not determined.

The "amylose" fractions were impure as shown by the results of iodine titrations and enzymic assay. Amylose-product *A3* was similar to that obtained in the thymol fractionation. Again, recrystallization by butan-1-ol did not successfully purify the amylose products.

It is of interest to note that the *β*-amylolysis limits of the products remaining in solution after recrystallization with butan-1-ol (*i.e.*, *Ap2* and *Ap3*) were more similar to the value of 58% found for normal maize-amylopectin[9] than that for the largest amylopectin product (*Ap4*). Fraction *Ap4* was similar in character to the product obtained using thymol as the amylose precipitant, and had an apparent chain-length of 35 units and a *β*-amylolysis limit of 65%.

However, on subfractionation with iodine, the non-complexing portion (85% of the whole) was found to have a chain-length of 28 units and a *β*-amylolysis limit of 59%, which were again comparable to those for a *normal amylopectin.* Also, the subfraction with iodine-complex forming properties appeared to have the properties of 50-unit amylose[10] (see Table V).

CONCLUSIONS

Notwithstanding the difficulties in separating amylomaize into its component fractions, various fractionation techniques appear to yield a branched fraction having an apparent chain-length of 36 D-glucose residues. The homogeneity of this product is,

TABLE V

PROPERTIES OF SUBFRACTIONS OBTAINED FROM *Ap4*[a] BY FRACTIONATION WITH IODINE

Subfraction	*Yield* (%)	*Iodine affinity* (%)[a]	λ_{max} ($m\mu$)	*β-Limit* (*i*)	(*ii*)[a]	*Chain-length*
Non-complexing	85	0.4	560	59	n.d.[b]	28
Iodine complex	15	n.d.	580	100	100	n.d.

[a] As in Table IV. [b] n.d. = not determined.

however, in doubt, and the evidence suggests that the branched component is contaminated with short-chain, relatively linear, amylose-type material. It is to be noted that we have found a comparable result for the branched component of the high-amylose starch from the wrinkled-seeded pea[12].

The significance of these results with regard to biosynthetic theories will be discussed elsewhere.

ACKNOWLEDGMENTS

We are indebted to the Corn Industries Research Foundation, Inc., Washington, D.C., U.S.A., for their support of these investigations.

SUMMARY

Amylomaize starches of 50% and 70% apparent amylose-content have been investigated. The iodine-binding characteristics of these starches are abnormal. The starches have been fractionated by a conventional dispersion and an extraction–sedimentation procedure. Both methods yielded a branched product having an apparent chain-length of 36 D-glucose residues, and a β-amylolysis limit of 65% conversion into maltose. Subfractionation of these anomalous amylopectins by both differential ultracentrifugation and iodine-complex formation gave fractions having properties comparable to those of normal amylopectin. It is considered, therefore, that 36-unit amylopectins in amylomaize starches are artifacts.

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PREPARATION OF DERIVATIVES LEADING TO 2-AMINO-2-DEOXY-D-GULURONIC ACID

GERALD D. SHRYOCK AND HOWARD K. ZIMMERMAN
Department of Chemistry, University of the Pacific, Stockton, California 95204 (U.S.A.)

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INTRODUCTION

The availability of 2-amino-2-deoxy-D-gulose derivatives by interconversion from 2-amino-2-deoxy-D-glucose derivatives[1], and the importance of the former structure in natural products[2], have led us to begin a systematic study of 2-amino-2-deoxy-D-gulose derivatives. Uronic acids are of value[3,4] as intermediates for a variety of preparative purposes in the amino sugar field. It is possible that 2-amino-2-deoxy-D-guluronic acid may eventually be found in Nature, as have been 2-amino-2-deoxy-D-galacturonic acid[5], 2-amino-2-deoxy-D-glucuronic acid[6], and 2-amino-2-deoxy-D-mannuronic acid[7]. This first report is concerned with possible routes to derivatives of 2-amino-2-deoxy-D-guluronic acid.

RESULTS AND DISCUSSION

Initially, a route through the known 2-amino-2-deoxy-D-glucuronic acid[8] appeared convenient, provided that the 3-*O*-acetyl derivative (**1**) could be oxidized to the acid, to furnish an intermediate which could be subjected to configurational inversions[1,9] at C-3 and C-4 and give the desired material in only four subsequent steps. However, catalytic oxidation of each of the anomeric 3-*O*-acetyl derivatives (**1a** and **1b**)* proved to be accompanied by deacetylation, leading to **2a** and **2b**, respectively. Both products were identified and further characterized by their known[10,11] methyl esters (**3a** and **3b**). The 3,4-dimethanesulfonate of this ester, **4b**, was prepared, with a view to conversion into **21** by treatment with base, by the well-known procedure for preparing an epoxy sugar[12] from a vicinal disulfonate. However, all efforts in that direction were unsuccessful.

Substance **1b** was selected for the remaining work, because of the generally superior crystallizing and handling properties noted with the β-D-glycosides. Substance **1b** was converted into the 4,6-dimethanesulfonate (**6**), and thence into the 3,6-di-*O*-acetyl-4-*O*-(methylsulfonyl) derivative (**9**). The relatively high yields in this direct route to **9** were not consistently reproducible. Moreover, although alkaline conversion

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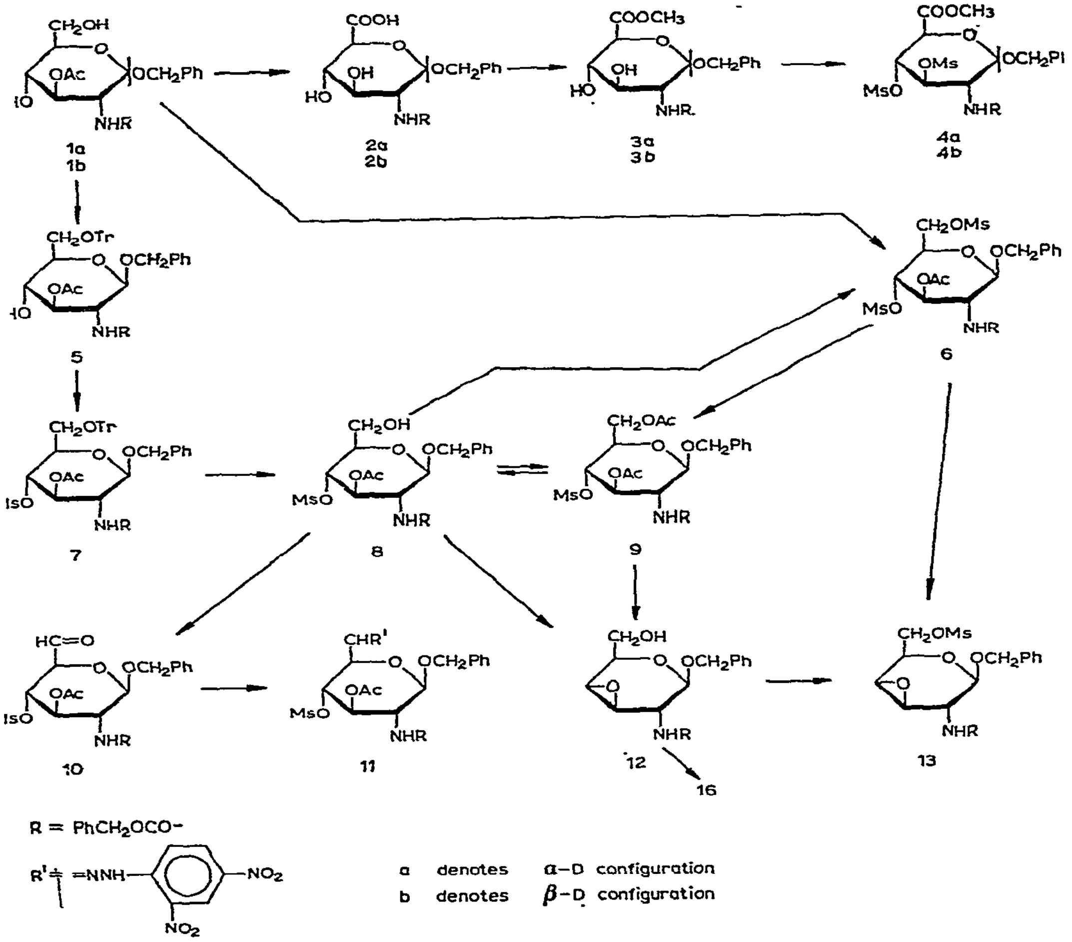
*The anomeric benzyl glycosides are indicated throughout by "a" for the α-D anomer and "b" for the β-D anomer.

of **9** into the 3,4-epoxy intermediate (**12**) gave excellent crude yields, the intensive recrystallization necessary to remove the last traces of sulfur-bearing contaminants from **12**—essential if poisoning of the catalyst was to be avoided in the following oxidation step— diminished the usable yield of epoxide to not greater than 65%.



Accordingly, an alternative route through the 6-trityl ether (**5**) derived from **1b** was examined. Substance **5** could be readily converted into the 4-methanesulfonate (**7**) and then be detritylated with aqueous acetic acid to give **8**. The structure of **8** was confirmed by methanesulfonation, to give the previously known[13] **6**, and by its conversion into **9**. Attempts at catalytic oxidation of **8** resulted in immediate poisoning of the catalyst, with no subsequent oxidation. It may be noted that the selectivity of the deacetylation of **9** at C-6 (pH 10 at 0°) to give **8** is a little surprising in view of the extraordinarily facile deacetylation at C-3 (pH 8 at 80°) observed during catalytic oxidation of **1a** and **1b**. It appears that the solvolysis of the 3-*O*-acetyl group can be facilitated or prevented, simply by a suitable adjustment of the temperature. Not only was the overall yield of **9** from **1b** by the indirect route *via* the trityl ether slightly superior (75% *vs.* 73%) to that by the direct route, but, more important, the direct alkaline conversion of **8** into **12** immediately gave an uncontaminated product, suitable for oxidation to **16**.

A further advantage of the indirect route appeared when it was found that **8** could be specifically oxidized* to the 6-aldehyde (**10**) by methyl sulfoxide in the presence of *N,N'*-dicyclohexylcarbodiimide and phosphoric acid[15]. Substance **10** was not isolated, but was characterized as its (2,4-dinitrophenyl)hydrazone (**11**). The aldehyde **10** and analogous derivatives offer promise as intermediates for future study.

Alkaline conversion of **6** into **13**, as used for the α-D anomer[9] of **13**, provided a reference substance for further characterization of **12**. Methanesulfonation of the latter gave **13**. Treatment with hot, aqueous acetic acid converted **12** into the 2,3-carbamate** (**18**) having the 2-amino-2-deoxy-D-gulose configuration, and the same treatment of **13** gave the 2,3-carbamate **14**. The latter was characterized as the 4,6-dimethanesulfonate (**19**) and also as the 4-*O*-acetyl-6-*O*-(methylsulfonyl) derivative (**15**). Substance **19** also served as an aid in confirming the structure of **18**, which was also characterized as the diacetate (**22**). The 2-amino-2-deoxy-D-gulose structure of **18** was finally verified by alkaline hydrolysis to give benzyl 2-amino-2-deoxy-β-D-guloside (**23**), which could be converted by acid hydrolysis into the known[1] 2-amino-2-deoxy-D-gulose hydrochloride.

When **12** was subjected to catalytic oxidation, it readily provided benzyl 3,4-anhydro-2-[(benzyloxycarbonyl)amino]-2-deoxy-β-D-galactopyranosiduronic acid (**16**) in a yield (60%) comparable to that obtained by catalytic oxidation of otherwise unprotected benzyl 2-[(benzyloxycarbonyl)amino]-2-deoxy-D-glucopyranosides[8,10,11] and -galactopyranosides [17,18]. It is evident from this fact that the 3,4-epoxide bridge is quite stable to the conditions required for this oxidation, in marked contrast to the 3-acetyl group, which is labile. Substance **16** was readily characterized as its methyl ester (**20**), which could be prepared in quantitative yield by treatment with diazomethane. The derivative **16** could also be converted, by aqueous acetic acid, into a hygroscopic carbamate (**17**) having the D-*gulo* configuration. The carbamate (**17**) was also obtained, but in markedly lower yield, by catalytic oxidation of **18**. Substance **18** showed a strong tendency to become adsorbed to the catalyst, hindering the oxidation. The hygroscopic carbamate **17** was characterized as its methyl ester (**21**) and 3-acetate (**24**). Attempts to cleave the carbamate **17** with alkali gave an uncrystallizable gum that was not characterized.

The preferred route from **1b** to **17** is considered to be as follows: **1b**→**6**→**7**→**8** →**12**→**16**→**18**. However, the ester derivative (**21**) obviously affords a more felicitous material for handling and storage than does the acid **17**.

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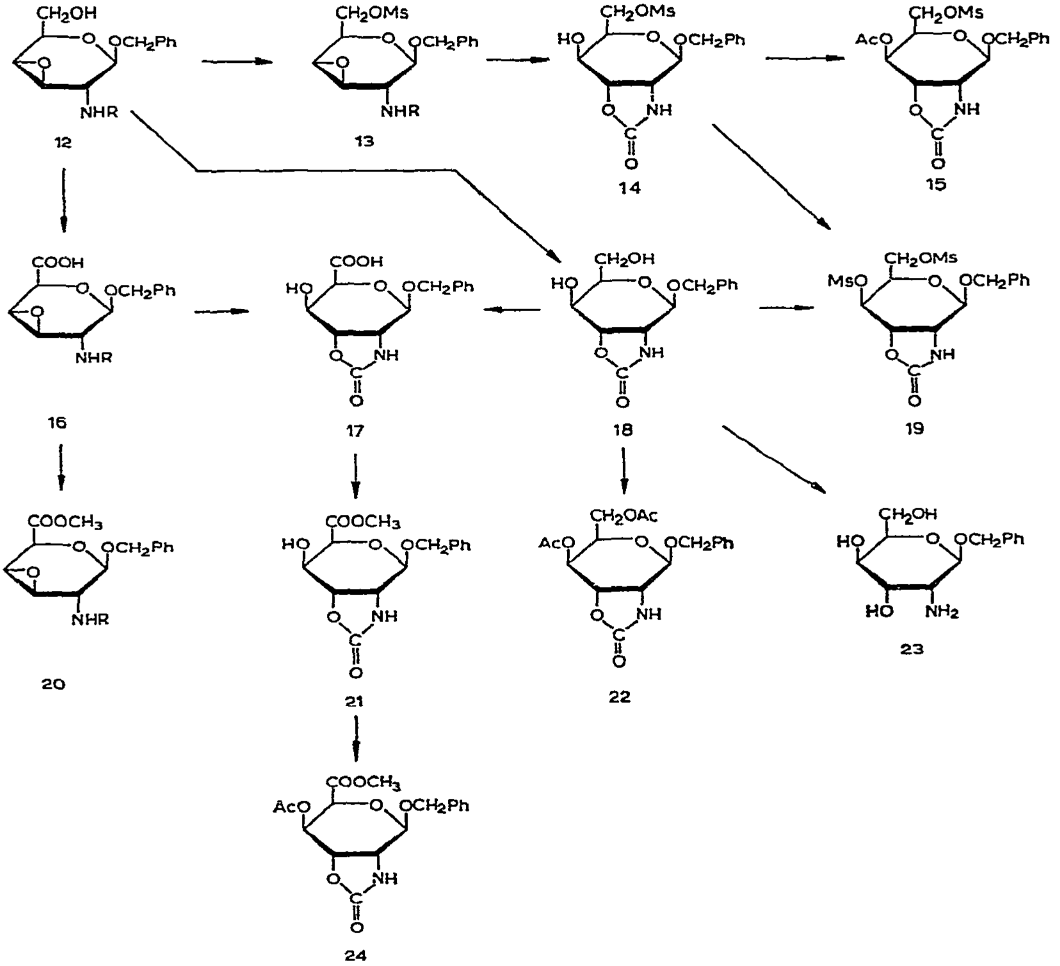
*Using conditions similar to those above, Horton *et al.*[14] have converted the primary alcohol function in 1,2:3,4-di-*O*-isopropylidene-α-D-galactopyranose into an aldehyde function.

**This class of compound has been referred to as oxazolidone in earlier publications[16].

EXPERIMENTAL

All melting points are uncorrected. Thin-layer chromatograms were made on silica gel, with benzene–methanol mixtures, usually in a 19:1 ratio, as developer. In a few cases, slight deviations from these proportions were required in order to assure satisfactory migration of the spots.



Benzyl 2-[(benzyloxycarbonyl)amino]-2-deoxy-α-D-glucopyranosiduronic acid (2a)

A suspension of 3.2 g of **1a**[13] in 700 ml of distilled water was heated to 80° with stirring at 4000 to 5000 rpm, and 1.2 g of pre-hydrogenated platinum dioxide was added. Oxygen was bubbled through the stirred mixture for 4 h, during which time the pH was maintained at 8 by continual addition of sodium hydrogen carbonate. After being cooled to 10°, the suspension was filtered, and the filtrate was concentrated to 50 ml. This solution was cooled to 5°, filtered, and acidified to pH 3 by careful addition of concentrated hydrochloric acid. After storage for 2h at 0°, the product was filtered off, washed at 0° with water, and recrystallized from 60% methanol, to give 1.1 g (30%) of long, white needles having properties identical with those given in the literature[8,10] for **2a**.

Benzyl 2-[(benzyloxycarbonyl)amino]-2-deoxy-β-D-glucopyranosiduronic acid (**2b**)

Treatment of **1b**[13] in a manner identical to that given above gave **2b** (yield 30%) having properties identical with those previously described[11].

Methyl (benzyl 2-[(benzyloxycarbonyl)amino]-2-deoxy-3,4-di-O-(methylsulfonyl)-α-D-glucopyranosid)uronate (**4a**)

Substance **2a** was converted into the methyl ester (**3a**) by treatment with diazomethane, as described in the literature [10]. To 0.5 g of **3a** in pyridine at —5° was added 0.5 ml of methanesulfonyl chloride. After storage at —5° for 2 days, the mixture was poured into ice-water to precipitate a gum, which was separated by decantation and recrystallized from 80% aqueous methanol, to afford 0.37 g (64%) of long, white needles, m.p. 141–2°, $[\alpha]_D^{27}$ +93° (*c* 2.4, pyridine).

Anal. Calc. for $C_{24}H_{29}NO_{12}S_2$: C, 49.06; H, 4.97; N, 2.39. Found: C, 49.43; H, 4.98; N, 2.41.

Methyl (benzyl 2-[(benzyloxycarbonyl)amino]-2-deoxy-3,4-di-O-(methylsulfonyl)-β-D-glucopyranosid)uronate (**4b**)

Substance **2b** was converted into the methyl ester (**3b**) with diazomethane[11]. The ester (0.7 g) was methanesulfonated as described for **4a**. The crystals which formed when the product was poured into ice-water were filtered off and recrystallized from methanol, to provide 0.8 g (85%) of needles, m.p. 180°, $[\alpha]_D^{27}$ —10° (*c* 1.2, pyridine).

Anal. Calc. for $C_{24}H_{29}NO_{12}S_2$: C, 49.06; H, 4.97; N, 2.39; O, 32.67; S, 10.91. Found: C, 49.29; H, 5.11; N, 2.42; O, 32.98; S, 10.66.

Benzyl 3-O-acetyl-2-[(benzyloxycarbonyl)amino]-2-deoxy-6-O-trityl-β-D- glucopyranoside (**5**)

Substance **1b** (13 g), which had been dried for 24 h at 90° and 3 mm over P_2O_5, was dissolved in 30 ml of absolute pyridine, treated with 8.2 g of chlorotriphenylmethane, and shaken for 48 h at room temperature. The bulk of this solution of **5** was used, without isolation of **5**, for the preparation of **7**. A small aliquot was removed, poured into ice-water, and the precipitated syrup dissolved in hot methanol. Crystals of **5** were formed upon cooling, m.p. 130–1°, $[\alpha]_D^{27}$ —32° (*c* 1.8, pyridine).

Anal. Calc. for $C_{42}H_{41}NO_8$: C, 73.34; H, 6.01; N, 2.04; O, 18.61. Found: C, 73.05; H, 6.06; N, 2.33; O, 18.61.

Benzyl 3-O-acetyl-2-[(benzyloxycarbonyl)amino]-2-deoxy-4-O-(methylsulfonyl)-6-O-trityl-β-D-glucopyranoside (**7**)

A further 40 ml of pyridine was added to the solution of **5** described above, the whole was cooled to —5°, and 13 ml of methanesulfonyl chloride was added slowly with stirring. The mixture was kept for 48 h at —5°, poured into ice-water, and filtered, and the precipitate was recrystallized from isopropyl alcohol to give 21.3 g (90%) of **7**, m.p. 135–6°, $[\alpha]_D^{27}$ —11° (*c* 1.0, pyridine).

Anal. Calc. for $C_{43}H_{43}NO_{10}S$: C, 67.44; H, 5.66; N, 1.83; O, 20.89; S, 4.19. Found: C, 66.47; H, 5.77; N, 1.94; O, 21.11; S, 4.74.

Benzyl 3-O-acetyl-2-[(benzyloxycarbonyl)amino]-2-deoxy-4-O-(methylsulfonyl)-β-D-glucopyranoside (**8**)

(a) To a solution of **7** (19.4 g) in glacial acetic acid (200 ml) at 95° was added water (200 ml) during 45 min. After refrigeration overnight, the mixture was filtered. The precipitate was successively extracted with four 100-ml portions of benzene, and the residual product (10.8 g, 84%) was recrystallized from chloroform–isopropyl ether, m.p. 166–7°, $[\alpha]_D^{27}$ —14° (*c* 2.0, pyridine).

Anal. Calc. for $C_{24}H_{29}NO_{10}S$: C, 55.06; H, 5.55; N, 2.68; O, 30.56; S, 6.14 Found: C, 54.33; H, 5.38; N, 2.72; O, 31.55; S, 6.25.

(b) A solution of 3 g of **9** in 50 ml of *p*-dioxane was cooled to 0°, and 0.5N aqueous potassium hydroxide was added during 3 h, at a rate sufficient to maintain the pH at 10. The solution was then neutralized with a few drops of glacial acetic acid, and concentrated to give a gel. This residue was suspended in 100 ml of water, and the suspension was shaken for 12 h, and filtered. The solid was recrystallized from chloroform–isopropyl ether, to provide 2.0 g (80%) of product identical in all respects (m.p., specific rotation, t.l.c., i.r. spectrum) with the product obtained by method (a).

Benzyl 3,6-di-O-acetyl-2-[(benzyloxycarbonyl)amino]-2-deoxy-4-O-(methylsulfonyl)-β-D glucopyranoside (**9**)

(a) A mixture of 5 g of **6**[13], 10 g of potassium acetate, and 32 ml of a 3:1 (v/v) mixture of glacial acetic acid and acetic anhydride was heated for 10 h at 105°, and then evaporated at 12–15 mm pressure.

The residue was successively re-evaporated with two 10-ml portions of glacial acetic acid, and then two 20-ml portions of toluene, to give a solid which was twice extracted with 75-ml portions of tetrahydrofuran. The combined extracts were filtered through a thin layer of silica gel, and the solution was evaporated to a syrup and re-evaporated twice with toluene; the residue was crystallized from ethanol, to afford 3.5 g (76% crude) of **9**, m.p. 158° (dec.), $[\alpha]_D^{21}$ —16° (*c* 1.9, pyridine).

Anal. Calc. for $C_{26}H_{31}NO_{11}S$: C, 55.21; H, 5.53; N, 2.48; O, 31.11; S, 5.68. Found: C, 54.65; H, 5.42; N, 2.52; O, 31.94; S, 5.88.

(b) A solution of **8** (0.5 g) in the minimal volume of pyridine was cooled to 0°, and acetylated at room temperature for 12 h with acetic anhydride (0.5 ml), after which time it was poured into ice-water to give 0.7 g (quantitative yield) of **9**, which was recrystallized from ethanol. This material was identical (m.p., specific rotation, t.l.c., i.r. spectrum) with that prepared by method (a).

*Benzyl 3-O-acetyl-2-[(benzyloxycarbonyl)amino]-2-deoxy-4-O-(methylsulfonyl)-β-*D-gluco-*hexodialdo-1,5-pyranoside 6-(2,4-dinitrophenyl)hydrazone* (**11**)

To a stirred solution of **8** (0.913 g) in 20 ml of methyl sulfoxide at 21°, which had been treated with 1.47 g of *N,N'*-dicyclohexylcarbodiimide, was added dropwise 0.46 ml of anhydrous orthophosphoric acid. Stirring was continued for 21 h at room temperature (25°), after which time the mixture was filtered, and the residue was washed with small portions of methyl sulfoxide and acetone. The filtrate and washings were combined and diluted with an excess of chloroform, followed by water and sufficient 2.4N potassium hydrogen carbonate solution to give a pH of 8. The aqueous layer was extracted repeatedly with chloroform, and the combined organic layers were washed with water until they were neutral. The organic solution was evaporated to a syrup, which was dissolved in methanol; a product was precipitated by addition of water. Thin-layer chromatography of this product showed the presence of starting material (**8**) and one other component (presumably **10**). This mixture was dissolved in the minimal volume of methanol, and treated with an excess of alcoholic (2,4-dinitrophenyl)hydrazine. After 45 min, the product was filtered off and recrystallized from ethyl acetate–methanol to give 0.47 g (27% based on **8**) of yellow crystals, m.p. 219° (dec.).

Anal. Calc. for $C_{30}H_{31}N_5O_{13}$: C, 51.35; H, 4.45; N, 9.98. Found C, 51.44; H, 4.44; N, 9.80.

*Benzyl 3,4-anhydro-2-[(benzyloxycarbonyl)amino]-2-deoxy-β-*D-*galactopyranoside* (**12**)

(a) A solution of **9** (6.5 g) in 30 ml of absolute *p*-dioxane was cooled to 0°, ice-cold 0.51M sodium methoxide (33 ml) was added, and the mixture was stirred for 3 h, and kept overnight at 0°. The mixture was filtered, the residue was washed thoroughly with tetrahydrofuran, and the filtrate and washings were combined, filtered through a thin layer of silica gel, and evaporated to dryness at 12–15 mm. The residual solid was recrystallized by dissolving it in ethyl acetate and adding isopropyl ether, to give 4.1 g (95%) of **12**, m.p. 159–60°, $[\alpha]_D^{22}$ −106° (*c* 1.3, pyridine), ν_{max}^{KBr} 3500 (hydroxyl), 3350, 1700, 1520 (urethan), 1250 (epoxide), 730, 700 (phenyl) cm^{-1}.

Anal. Calc. for $C_{21}H_{23}NO_6$: C, 65.44; H, 6.01; N, 3.64. Found: C, 65.44; H, 5.99 N, 3.82.

(b) Treatment of **8** in the manner described in method (a) above gave **12** (77% yield), having properties (m.p., specific rotation, t.l.c., i.r. spectrum) identical with those of the foregoing product.

*Benzyl 3,4-anhydro-2-[(benzyloxycarbonyl)amino]-2-deoxy-6-O-(methylsulfonyl)-β-*D-*galactopyranoside* (**13**)

(a) To an ice-cold solution of 3 g of **6**[13] in 50 ml of absolute *p*-dioxane was added

17 ml of ice-cold 0.38M sodium isopropoxide. With continued stirring, the partially frozen material dissolved completely. After stirring the mixture for 12 h in the cold, tetrahydrofuran (30 ml) was added, the mixture was filtered, and the filtrate was concentrated to a syrup at 12–15 mm. The residue was dissolved in tetrahydrofuran, the mixture was filtered through a thin layer of silica gel, and the product was precipitated from the filtrate with isopropyl ether containing a little heptane. It was recrystallized from methanol–isopropyl ether to give 1.8 g (78%) of white crystals, m.p. 145–6°, $[\alpha]_D^{25}$ $-101°$ (c 1.4, pyridine), ν_{max}^{KBr} 3350, 1700, 1550 (urethan), 1260 (epoxide), 1185 (sulfonate), 735, 700 (phenyl) cm^{-1}.

Anal. Calc. for $C_{22}H_{25}NO_8S$: C, 57.01; H, 5.44; N, 3.02. Found: C, 57.19; H, 5.44; N, 3.02.

(b) To **12** (0.22 g) in the minimal volume of pyridine at $-5°$ was added methanesulfonyl chloride (0.25 ml), and the mixture was kept for 2 days at $-5°$. When poured over crushed ice, it gave 0.23 g (89%) of product, which was recrystallized as in (a) and was identical in all respects (m.p., specific rotation, t.l.c., i.r. spectrum) with the preceding preparation.

*Benzyl 2-amino-2-deoxy-β-*D*-gulopyranoside 2,3-carbamate* (**18**)

To a solution of **12** (1.5 g) in 60 ml of glacial acetic acid at 105° was added 60 ml of water during 1 h, after which time the solution was evaporated at 12–15 mm. The residue was re-evaporated with ethanol and then with toluene, to give a dry syrup which was dissolved in a small volume of methanol; the product crystallized on addition of isopropyl ether. Recrystallization from the same solvent afforded 0.9 g (82%) of needles, m.p. 115°, $[\alpha]_D^{23}$ $-101°$ (c 2.6, pyridine) ν_{max}^{KBr} 3500 (hydroxyl), 3350, 1750 (carbamate), 740, 700 (phenyl) cm^{-1}; amide-II band at 1500–1550 cm^{-1} absent because of ring formation.

Anal. Calc. for $C_{14}H_{17}NO_6$: C, 56.94; H, 5.80; N, 4.75; O, 32.51. Found: C, 56.63; H, 5.97; N, 4.83; O, 32.63.

*Benzyl 4,6-di-*O*-(methylsulfonyl)-β-*D*-gulopyranoside 2,3-carbamate* (**19**)

(a) Compound **13** (1 g) was treated as described above for **18**, and after evaporation, the product was re-evaporated once with glacial acetic acid and twice with toluene, to give **14** as a syrup. The latter was dissolved in a small volume of pyridine, cooled to $-5°$, and cold methanesulfonyl chloride (1 ml) was added. The mixture was kept for 2 days at $-5°$, and then poured over ice, to give 0.6 g (67%, based on **13**) of crystals which were recrystallized from acetone–isopropyl ether, m.p. 202°, $[\alpha]_D^{25}$ $-69°$ (c 1.5, pyridine).

Anal. Calc. for $C_{16}H_{21}NO_{10}S_2$: C, 42.57; H, 4.69; N, 3.10. Found: C, 42.78; H, 4.73; N, 3.13.

(b) Treatment of 0.14 g of **18** with methanesulfonyl chloride, and recrystallization as in method (a), gave 0.17 g (76%) of product identical (m.p., specific rotation, t.l.c., i.r. spectrum) with that described above.

Benzyl 3-O-acetyl-6-O-(methylsulfonyl)-β-D-gulopyranoside 2,3-carbamate (15)

Substance **13** (7.5 g) was converted into **14** by the method described in the preparation of **19**. To a cold solution of syrupy **14** in pyridine was added acetic anhydride (3 ml), and the solution was kept for 12 h at room temperature. When poured into ice-water, it afforded 6.6 g (98% based on **13**) of product, which was recrystallized from methanol, m.p. 180°, $[\alpha]_D^{27}$ $-55°$ (*c* 2.2, pyridine).

Anal. Calc. for $C_{17}H_{21}NO_9S$: C, 49.15; H, 5.10; N, 3.37; O, 34.66; S, 7.74. Found: C, 49.26; H, 5.39; N, 3.53; O, 34.78; S, 7.63.

Benzyl 4,6-di-O-acetyl-β-D-gulopyranoside 2,3-carbamate (22)

Substance **18** (0.17 g) was acetylated in pyridine–acetic anhydride for 12 h at 0°, and, after being poured onto ice, it gave 0.13 g (60%) of product, which was recrystallized from methanol, m.p. 168–9°, $[\alpha]_D^{27}$ $-71°$ (*c* 1.0, pyridine.)

Anal. Calc. for $C_{18}H_{21}NO_8$: C, 56.99; H, 5.58; N, 3.69; O, 33.74. Found: C, 56.82; H, 5.70; N, 4.04; O, 33.45.

Benzyl 2-amino-2-deoxy-β-D-gulopyranoside (23)

To 12 ml of a 15% aqueous potassium hydroxide solution was added **18** (2.5 g), and the mixture was heated for 4 h at 55°. After the mixture had been cooled, the precipitate was filtered off, thoroughly washed with ice-water, and crystallized from methanol by the addition of isopropyl ether, to afford 2.2 g (96%) of short, white needles, m.p. 146–7°, $[\alpha]_D^{21}$ $-75°$ (*c* 1.0, pyridine).

Anal. Calc. for $C_{13}H_{19}NO_5$: C, 57.98; H, 7.11; N, 5.20; O, 29.71. Found: C, 57.12; H, 7.11; N, 5.01; O, 31.16.

Benzyl 3,4-anhydro-2-[(benzyloxycarbonyl)amino]-2-deoxy-β-D-galactopyranosiduronic acid (15)

Substance **12** (2.2 g) was oxidized by the procedure described for the preparation of **2a**. After the product from the hydrochloric acid solution had been collected, it was dissolved in methanol, and the mixture was filtered through a thin layer of silica gel; the product crystallized from the filtrate on adding water. Recrystallization from 50% aqueous methanol gave 1.6 g (60%) of long, white needles, m.p. 131°, $[\alpha]_D^{26}$ $-151°$ (*c* 1.1, pyridine), $\nu_{\max}^{\text{KBr}}$ 3300, 1690, 1550 (urethan), 1720 (carbonyl), 1250 (epoxide), 730, 700 (phenyl) cm^{-1}.

Anal. Calc. for $C_{21}H_{21}NO_7$: C, 63.15; H, 5.30; N, 3.51; O, 28.04. Found: C, 62.87; H, 5.27; N, 4.07; O, 28.03.

Methyl (benzyl 3,4-anhydro-2-[(benzyloxycarbonyl)amino]-2-deoxy-β-D-galactopyranosid)uronate (20)

To an ice-cold solution of **16** (0.5 g) in anhydrous methanol was added an ethereal solution of diazomethane until the yellow color persisted. The mixture was kept overnight at 0°, and then evaporated at 40°/12–15 mm. The residue was crystallized from hot methanol, to yield 0.6 g (quantitative) of long, white needles, m.p. 177–8°, $[\alpha]_D^{26}$ $-137°$ (*c* 1.3, pyridine).

Anal. Calc. for $C_{22}H_{23}NO_7$: C, 63.91; H, 5.61; N, 3.39; O, 27.09. Found: C, 64.28; H, 5.69; N, 3.50; O, 26.91.

Benzyl β-D-gulopyranosiduronic acid 2,3-carbamate (17)

(a) Substance **18** (0.7 g) was oxidized in the manner described for the preparation of **2a**. After acidification of the cold, concentrated solution, it was saturated with potassium chloride and extracted three times with tetrahydrofuran. The combined organic extracts were evaporated to dryness. The residue was dissolved in a small volume of hot tetrahydrofuran, isopropyl ether was added to incipient opalescence, and the solution was kept for several hours in the cold. The resulting crystals turned to a gum immediately after exposure to the air. The compound was, therefore, characterized as its methyl ester (**21**).

(b) Substance **16** (1.5 g) was treated in hot, aqueous acetic acid as described for the preparation of **18**. Crystallization of the product as in method (a) above gave crystals which turned to a gum, and the product was characterized as its methyl ester.

Methyl (benzyl β-D-gulopyranosid)uronate 2,3-carbamate (21)

The gummy products from (a) and (b) of the preceding experiment were separately dissolved in absolute methanol and cooled to 0°. To each solution was added ethereal diazomethane, after which it was treated as described for the preparation of **20**. Addition of a large excess of isopropyl ether to the methanolic solution gave crystals which were recrystallized in the same way. From **18**, there was obtained 0.1 g (13% for the two steps), and from **16**, 1.0 g (78% for the two steps) of product, m.p. 146–7°, $[\alpha]_D^{21}$ $-125°$ (*c* 1.1, pyridine), ν_{max}^{KBr} 3490 (hydroxyl), 3350, 1750 (carbamate), 1750 (carbonyl), 740, 700 (phenyl) cm^{-1}; amide-II band between 1550 and 1500 cm^{-1} was absent.

Anal. Calc. for $C_{15}H_{16}NO_7$: C, 55.90; H, 5.00; N, 4.35; O, 34.75. Found: C, 55.79; H, 5.25; N, 4.15; O, 34.82.

Methyl (benzyl 4-O-acetyl-β-D-gulopyranosid)uronate 2,3-carbamate (24)

Acetylation of 0.5 g of **21** in pyridine–acetic anhydride for 24 h at 0° gave a crystalline product when the mixture was poured into ice-water. Recrystallization from methanol–isopropyl ether afforded 0.54 g (quantitative) of long needles, m.p. 205–6°, $[\alpha]_D^{21}$ $-87°$ (*c* 1.3, pyridine).

Anal. Calc. for $C_{17}H_{19}NO_8$: C, 55.89; H, 5.24; N, 3.83; C, 35.03. Found: C, 55.96; H, 5.19; N, 3.96; O, 35.14.

ACKNOWLEDGMENTS

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SUMMARY

Inversion of configuration at C-3 and C-4 of 2-amino-2-deoxy-D-glucose, *via* an anchimerically assisted cleavage of a 3,4-epoxide, has been explored as a route for the preparation of derivatives of 2-amino-2-deoxy-D-guluronic acid. It is shown that catalytic oxidation of the benzyl 3-*O*-acetyl-2-[(benzyloxycarbonyl)amino]-2-deoxy-D-glucopyranosides over platinum is accompanied by solvolysis of the 3-*O*-acetyl group in the product. On the other hand, oxidations could be achieved by the action of *N,N'*-dicyclohexylcarbodiimide–phosphoric acid–methyl sulfoxide, without affecting the substituents at C-3 and C-4, on benzyl 3-*O*-acetyl-2-[(benzyloxycarbonyl)-amino]-2-deoxy-4-*O*-(methylsulfonyl)-β-D-glucopyranoside (giving specifically the 6-aldehyde), and also on benzyl 3,4-anhydro-2-[(benzyloxycarbonyl)amino]-2-deoxy-β-D-galactopyranoside over platinum (giving the uronic acid). The latter acid could be transformed readily by aqueous acetic acid into benzyl β-D-gulopyranosiduronic acid 2,3-carbamate.

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STUDIES ON THE PRODUCTS OBTAINED BY THE PERIODATE OXIDATION OF OSAZONES

H. EL KHADEM AND M. M. A. ABDEL RAHMAN
Faculty of Science, Alexandria University, Alexandria (U.A.R.)

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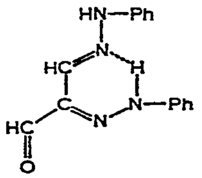
Sugar phenylosazones are known to yield, on oxidation with periodic acid[1], mesoxalaldehyde 1,2-bis(phenylhydrazone) (**1**). Under drastic conditions, the oxidation proceeds further, to give 1-phenyl-4-phenylazo-2-pyrazolin-5-one[2] (**5**), which is also obtained by the action of hydroxylamine hydrochloride[3] on the osazone. We have studied the properties of these two compounds, and have prepared a number of their substituted derivatives.

It was interesting to study the type of chelation in **1**, since the imino proton of the phenylhydrazone residue on C-2 could form a chelated ring, either with the aldimine nitrogen atom on C-1, or with the oxygen atom of the carbonyl group on C-3.

We have compared the chemical shift of this chelated, imino proton with that of the chelated, imino proton of the C-2 phenylhydrazone in mesoxalaldehyde tris(phenylhydrazone) (**2**), which cannot form an oxygen chelate.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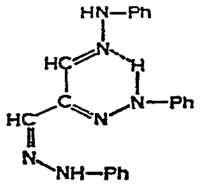
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The n.m.r. spectrum of **1** (see Fig. 1) showed two imino protons, which could be deuterated, at δ 10.83 and 13.15 p.p.m.; the latter was assigned to the chelated proton.

In the case of **2** (see Fig. 2), three deuteratable imino protons were detected, two unchelated imino protons at δ 10.22 and 10.83 p.p.m., and a chelated, imino proton at 12.51 p.p.m. The similarity in the chemical shift of the chelated imino protons in both cases strongly suggests that chelation in **1** is of the same type as in **2**, that is to say, with the imino nitrogen atom of C-1. This conclusion is further supported by our previous experience that hydrogen–oxygen chelates, such as that of Percival's dianhydro-osazone[4], appeared at much lower value.

Mesoxalaldehyde 1,2-bis(phenylhydrazone) (**1**), is known to react with acetic anhydride to give 1-phenyl-4-phenylazopyrazole[5] (**4**). We have effected this conversion with a number of acidic reagents, such as ethanol containing a few drops of concentrated hydrochloric acid, glacial acetic acid, malonic acid, or salts of carbonyl-group reagents, such as hydroxylamine hydrochloride, semicarbazide hydrochloride, and phenylhydrazine hydrochloride. It seems that this acid-catalyzed dehydration occurs through a cyclic intermediate (**3**) after the chelated ring has been broken.

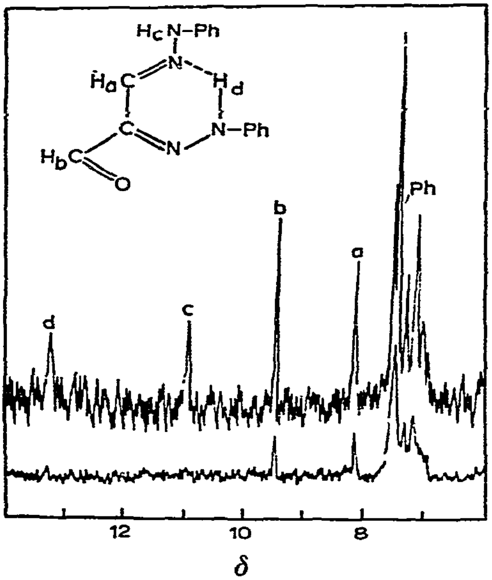


Fig. 1. The n.m.r. spectrum of mesoxalaldehyde 1,2-bis(phenylhydrazone) (**1**) in methyl sulfoxide, before deuteration (upper curve) and after deuteration (lower curve).

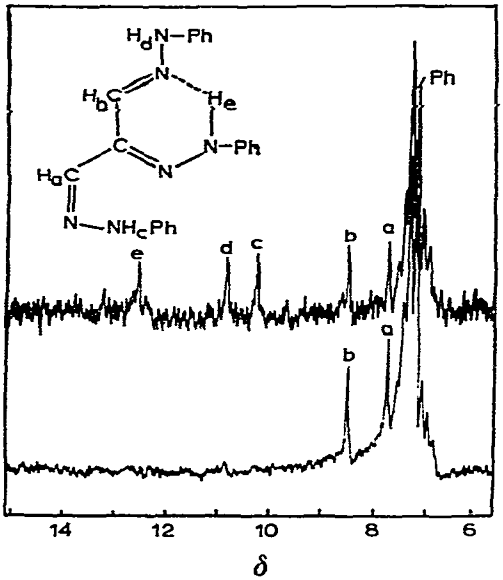
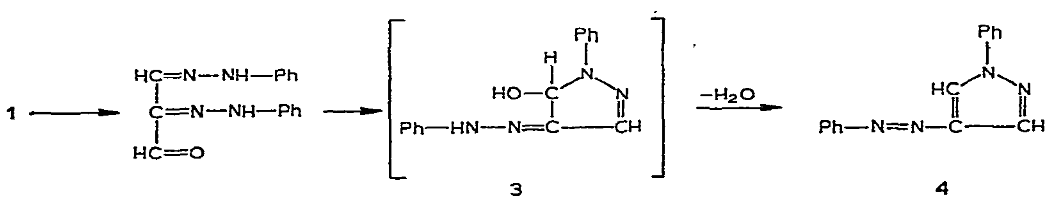


Fig. 2. The n.m.r. spectrum of mesoxalaldehyde tris(phenylhydrazone) (**2**) in methyl sulfoxide, before deuteration (upper curve) and after deuteration (lower curve).



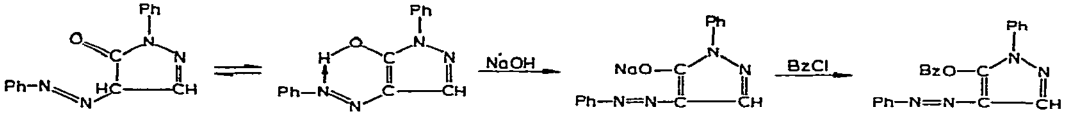
The same product (**4**) was obtained from mesoxalaldehyde tris(phenylhydrazone) (**2**) by the action of the above acidic reagents. In this case, we believe that the phenylhydrazone residue on C-3, not being stabilized by chelation, is first split hydrolytically, giving **1**, which then undergoes cyclization as above.

We have also prepared a number of mesoxalaldehyde 1,2-bis(arylhydrazones) having different groups on the benzene ring, and have converted them with ethanolic hydrochloric acid into the corresponding azopyrazoles. The reaction was successfully applied to mesoxalaldehyde 1,2-bis(arylhydrazones) having *o*-, *m*-, and *p*-Me; *m*- and *p*-Cl; and *p*-Br and 4-Br-2-Me groups on the benzene ring, indicating that this is a general reaction of mesoxalaldehyde 1,2-bis(arylhydrazones).

Mesoxalaldehyde 1,2-bis(phenylhydrazone) (**1**) could also be reduced (with sodium borohydride) to hydroxypyruvaldehyde bis(phenylhydrazone), affording a simple means of obtaining this compound, in high yield, from saccharide osazones.

1-Phenyl-4-phenylazo-2-pyrazolin-5-one (**5**) may be prepared by a method other than vigorous oxidation with periodic acid. This cyclization was first observed by Diels[3], who found that, when hexulose phenylosazones are refluxed in methanol containing hydroxylamine hydrochloride, they undergo fission between C-3 and C-4 to yield **5**. We have found that this reaction may also be effected with phenylhydrazine hydrochloride, phenylhydrazine sulfate, or semicarbazide hydrochloride, acting on hexulose osazones and on osazones from disaccharides having a hexose residue as the reducing moiety.

Pentose and 6-deoxyhexose phenylosazones, however, did not give this reaction, suggesting that the primary hydroxyl group on C-6 in hexoses is, in some way, involved in this reaction, probably through formation of a 3,6-anhydro ring. This conclusion is supported by the observation of Diels that Diels' monoanhydrophenylosazone, which is now recognized[6] to be 3,6-anhydro-D-*ribo*-hexulose phenylosazone, readily gives this reaction. The infrared spectrum of the 2-pyrazolin-5-one showed a weak carbonyl band at 1660 cm^{-1}, suggesting that it exists mainly in the enol form. We could not, however, verify this by n.m.r. spectroscopy because of the extremely low solubility of this compound in deuteriochloroform, and in methyl sulfoxide. Since this compound was not readily acetylated with acetic anhydride in pyridine or with boiling acetic anhydride, or benzoylated with benzoyl chloride in pyridine, we believe that it exists in the chelated form (**6**). We were, however, able to effect the benzoylation with benzoyl chloride and sodium hydroxide, which was used to break down the chelated ring by the formation of the sodium enolate (**7**). The benzoate (**8**) now showed the OBz band at 1770 cm^{-1}.



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EXPERIMENTAL

The n.m.r. spectra were recorded on a Varian A-60 spectrometer in methyl sulfoxide, and tetramethylsilane was used as the internal standard. The imino protons were detected by deuteration. Infrared absorption spectra were recorded on a Unicam S.P. 200 spectrophotometer, and ultraviolet absorption spectra on a Zeiss PMQ II spectrophotometer. Microanalyses were performed by A. Bernhardt, Mülheim, Germany.

Mesoxalaldehyde 1,2-bis(phenylhydrazone) (**1**)

A solution of D-*arabino*-hexulose phenylosazone (5.4 g) in warm 66% ethanol (2 l) was cooled to room temperature and treated with metaperiodic acid (10 g) in water (70 ml). The aldehyde, which separated at once as yellow-orange needles, was filtered off after 15 minutes, and crystallized from ethanol, m.p. 198–200°, lit.[1] m.p. 198°, λ_{max}^{EtOH} 250, 315, 420 mμ; log ε 4.2, 4.4, 4.3; λ_{min}^{EtOH} 270, 365 mμ; log ε 3.9, 3.8; ν_{max}^{KBr} 1665 (C=O), 1605 cm^{-1} (C=N).

Anal. Calc. for $C_{15}H_{14}N_4O$: C, 67.7; H, 5.3. Found: C, 67.6; H, 5.3.

1-Phenyl-4-phenylazopyrazole (**4**)

A solution of **1** (0.3 g) in ethanol (25 ml) was refluxed with 3 drops of concentrated hydrochloric acid (or 3 drops of glacial acetic acid, 0.2 g of malonic acid, 0.2 g of hydroxylamine hydrochloride, 0.2 g of semicarbazide hydrochloride, or 0.2 g of phenylhydrazine hydrochloride) for 20 min. On concentration, substance **4** separated and was crystallized from ethanol as yellow plates, m.p. and mixed m.p. 122–123°, lit.[5] m.p. 126°; λ_{max}^{EtOH} 230, 335 mμ; log ε 4.2, 4.4; λ_{min} 280 mμ; log ε 4.0; ν_{max}^{KBr} 1605 cm^{-1} (C=N).

Anal. Calc. for $C_{15}H_{12}N_4$: C, 72.5; H, 4.9.; N, 22.6. Found: C, 72.5, 72.4, 72.1; H, 5.0, 5.0, 4.8; N, 22.7, 22.8, 22.9.

Hydroxypyruvaldehyde 1,2-bis(phenylhydrazone)

Mesoxalaldehyde 1,2-bis(phenylhydrazone) **1**, (0.2 g) in ethanol (20 ml) was treated with sodium borohydride (0.3 g) in water (10 ml). After 20 min, the reaction mixture was diluted with water, whereupon the title compound separated; it was filtered off and crystallized from benzene, yield 0.12 g, m.p. and mixed m.p. 131°; ν_{max}^{KBr} 3400 (OH), 1605 cm^{-1} (C=N).

Mesoxalaldehyde tris(phenylhydrazone) (**2**)

A suspension of **1** (0.5 g) in ethanol (10 ml) was warmed with phenylhydrazine (0.5 g) until it all dissolved. The solution was diluted with ethanol (20 ml) and refluxed for 10 min. The product **2** separated on addition of hot water, and was crystallized from ethanol–water as yellow plates, m.p. 168°; lit.[1] m.p. 167°; λ_{max}^{EtOH} 240, 300, 345, 410 mμ; log ε 4.2, 4.4, 4.5, 4.5; λ_{min}^{EtOH} 275, 315, 365 mμ; log ε 4.0, 4.3, 4.4; ν_{max}^{KBr} 1605 cm^{-1} (C=N).

Anal. Calc. for $C_{21}H_{20}N_6$: C, 70.8; H, 5.6; N, 23.6. Found: C, 70.8; H, 5.7; N, 23.8.

Mesoxalaldehyde 1,2-bis (o-tolylhydrazone)

A solution of D-*arabino*-hexulose *o*-tolylosazone (1 g) in warm 66% ethanol (400 ml) was cooled to room temperature, and treated with metaperiodic acid (2 g) in water (10 ml). The aldehyde that separated was crystallized from ethanol as orange, prismatic needles, m.p. 202–204°; ν_{max}^{KBr} 1670 (C=O), 1600 cm^{-1} (C=N).

Anal. Calc. for $C_{17}H_{18}N_4O$: C, 69.4; H, 6.1; N, 19.1. Found: C, 69.3; H, 6.3; N, 18.9.

*1-*o*-Tolyl-4-(*o*-tolylazo)pyrazole*

A solution of mesoxalaldehyde 1,2-bis(*o*-tolylhydrazone) (0.2 g) in ethanol (20 ml) was treated with concentrated hydrochloric acid (3 drops) for 20 min. On concentration, the azopyrazole which separated was crystallized from ethanol as yellow, prismatic needles, m.p. 92–94°; $\nu_{\max}^{\mathrm{KBr}}$ 1600 cm^{-1} (C=N).

Anal. Calc. for $C_{17}H_{16}N_4$: C, 73.9; H, 5.8; N, 20.3. Found: C, 73.7; H, 5.9; N, 20.5.

*Mesoxalaldehyde 1,2-bis(*m*-tolylhydrazone)*

A solution of D-*arabino*-hexulose *m*-tolylosazone (1 g) in 66% ethanol (400 ml) was oxidized with metaperiodic acid (2 g) as before. The aldehyde obtained crystallized from ethanol as orange, prismatic needles, m.p. 174–175°; $\nu_{\max}^{\mathrm{KBr}}$ 1670 (C=O), 1600 cm^{-1} (C=N).

Anal. Calc. for $C_{17}H_{18}N_4O$: N, 19.1. Found: N, 19.1.

*1-*m*-Tolyl-4-(*m*-tolylazo)pyrazole*

A solution of mesoxalaldehyde 1,2-bis(*m*-tolylhydrazone) (0.2 g) in ethanol (20 ml) was treated, as before, with concentrated hydrochloric acid (3 drops). The azopyrazole obtained was crystallized from ethanol as yellow, prismatic needles, m.p. 66–68°; $\nu_{\max}^{\mathrm{KBr}}$ 1605 cm^{-1} (C=N).

Anal. Calc. for $C_{17}H_{16}N_4$: C, 73.9; H, 5.8; N, 20.3. Found: C, 74.2; H, 5.9; N, 20.1.

*Mesoxalaldehyde 1,2-bis(*p*-tolylhydrazone)*

A solution of D-*arabino*-hexulose *p*-tolylosazone (1 g) was oxidized as before, with metaperiodic acid (2 g), and the aldehyde which separated was crystallized from ethanol as orange, prismatic needles, m.p. 187–189°; $\nu_{\max}^{\mathrm{KBr}}$ 1670 (C=O), 1610 cm^{-1} (C=N).

Anal. Calc. for $C_{17}H_{18}N_4O$: C, 69.4; H, 6.1; N, 19.1. Found: C, 69.7; H, 6.2; N, 19.2.

*1-*p*-Tolyl-4-(*p*-tolylazo)pyrazole*

A solution of mesoxalaldehyde 1,2-bis(*p*-tolylhydrazone) (0.2 g) was treated with ethanol containing hydrochloric acid, as before, and the azopyrazole obtained on concentration of the alcoholic solution was crystallized from ethanol as yellow, prismatic needles, m.p. 178–180°; $\nu_{\max}^{\mathrm{KBr}}$ 1605 cm^{-1} (C=N).

Anal. Calc. for $C_{17}H_{16}N_4$: C, 73.9; H, 5.8; N, 20.3. Found: C, 73.8; H, 6.1; N, 20.2.

*Mesoxalaldehyde 1,2-bis[(*m*-chlorophenyl)hydrazone]*

D-*arabino*-Hexulose (*m*-chlorophenyl)osazone (1 g) in warm 66% ethanol (400 ml), was oxidized as before with metaperiodic acid (2 g). The aldehyde obtained was crystallized from ethanol as yellow-orange needles, m.p. 204–207°; $\nu_{\max}^{\mathrm{KBr}}$ 1680 (C=O), 1605 cm^{-1} (C=N).

Anal. Calc. for $C_{15}H_{12}Cl_2N_4O$: C, 53.7; H, 3.6; N, 16.7. Found: C, 54.1; H, 3.9; N, 16.8.

1-(m-Chlorophenyl)-4-(m-chlorophenylazo)pyrazole

Mesoxalaldehyde 1,2-bis[(*m*-chlorophenyl)hydrazone] (0.2 g) in ethanol (20 ml) was treated with concentrated hydrochloric acid (3 drops) as before, for 20 min, and the azopyrazole obtained was crystallized from ethanol as yellow, prismatic needles, m.p. 159–161°; ν_{max}^{KBr} 1590 cm^{-1} (C=N).

Anal. Calc. for $C_{15}H_{10}Cl_2N_4$: C, 56.8; H, 3.2; N, 17.7. Found: C, 56.6; H, 3.3; N, 17.7.

1-(p-Chlorophenyl)-4-(p-chlorophenylazo)pyrazole

A solution of D-*arabino*-hexulose (*p*-chlorophenyl)osazone (1 g) in 66% ethanol was oxidized as before, and the aldehyde obtained was dissolved in ethanol (20 ml) and refluxed with 3 drops of concentrated hydrochloric acid for 20 min. 1-(*p*-Chlorophenyl)-4-(*p*-chlorophenylazo)pyrazole separated on concentration of the alcoholic solution, and was crystallized from ethanol as yellow, prismatic needles, m.p. 177–179°.

Anal. Calc. for $C_{15}H_{10}Cl_2N_4$: C, 56.8; H, 3.2. Found: C, 57.0; H, 2.9.

1-(p-Bromophenyl)-4-(p-bromophenylazo)pyrazole

D-*arabino*-Hexulose (*p*-bromophenyl)osazone (1 g) was treated as above, and the azopyrazole obtained was crystallized from ethanol as orange-yellow plates, m.p. 183–185°.

Anal. Calc. for $C_{15}H_{10}Br_2N_4$: C, 44.3; H, 2.5; N, 13.8. Found: C, 44.2; H, 2.6; N, 13.9.

1-(4-Bromo-2-methylphenyl)-4-(4-bromo-2-methylphenylazo)pyrazole

D-*arabino*-Hexulose (4-bromo-2-methylphenyl)osazone (1 g) was oxidized as above, and the aldehyde that separated was similarly treated with ethanolic hydrochloric acid. On concentration of the alcoholic solution, the azopyrazole separated as orange-yellow needles, and was crystallized from ethanol, m.p. 140–142°.

Anal. Calc. for $C_{17}H_{14}Br_2N_4$: C, 47.0; H, 3.2; N, 12.9. Found: C, 46.8; H, 3.3; N, 12.7.

1-Phenyl-4-phenylazo-2-pyrazolin-5-one (5)

A solution of D-*arabino*-hexulose phenylosazone (2 g) in ethanol (100 ml) was efluxed with 2 g of hydroxylamine hydrochloride (or 2 g of semicarbazide hydrochloride, 2 g of phenylhydrazine hydrochloride, or 2 g of phenylhydrazine sulfate) for 7 h; the solution darkened after one h. The 2-pyrazolin-5-one obtained after concentration of the solution was crystallized from ethanol as orange, prismatic needles, m.p. 144–146°, lit.[1] m.p. 150°; λ_{max}^{EtOH} 250, 395 mμ; log ε 4.3, 4.3; λ_{min} 300 mμ; log ε 3.2; ν_{max}^{KBr} 1660 weak (C=O), 1605 cm^{-1} (C=N).

Anal. Calc. for $C_{15}H_{12}N_4O$: C, 68.2; H, 4.6; N, 21.2. Found: C, 68.2; H, 4.6; N, 21.1.

1-Phenyl-4-phenylazo-2-pyrazolin-5-one benzoate (**8**)

1-Phenyl-4-phenylazo-2-pyrazolin-5-one **5**, (0.5 g) in 15% sodium hydroxide solution (70 ml) was treated with benzoyl chloride (12 ml) in portions during 4 h. The reaction mixture was kept overnight at room temperature and filtered, and the crystals were washed repeatedly with water. The benzoate crystallized from ethanol as yellow, prismatic needles, m.p. 109°; $\nu_{\max}^{\mathrm{KBr}}$ 1770 (OBz), 1605 cm^{-1} (C=N).

Anal. Calc. for $C_{22}H_{16}N_4O_2$: C, 71.7; H, 4.4; N, 15.2. Found: C, 71.7; H, 4.5; N, 15.3.

ACKNOWLEDGMENT

The authors thank Professor D. Horton and Mr. J. S. Jewell, The Ohio State University, for making the n.m.r. spectra available.

SUMMARY

The n.m.r. spectra of mesoxalaldehyde 1,2-bis(phenylhydrazone) and tris(phenylhydrazone) suggest chelations as in structures **1** and **2**. Acid reagents convert these two compounds into 1-phenyl-4-phenylazopyrazole (**4**). A number of substituted **4** were prepared from the corresponding **1**. The conversion of saccharide phenylosazones into 1-phenyl-4-phenylazo-2-pyrazolin-5-one (**5**) probably proceeds through the formation of a 3,6-anhydro ring.

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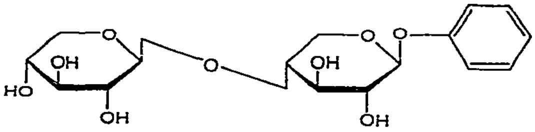
TRANSFER REACTIONS CATALYSED BY A FUNGAL β-D-XYLOSIDASE: ENZYMIC SYNTHESIS OF PHENYL β-D-XYLOBIOSIDE

M. CLAEYSSENS, E. VAN LEEMPUTTEN, F. G. LOONTIENS, AND C. K. DE BRUYNE
Laboratorium voor Anorganische Scheikunde A., H.I.K.W., Rijksuniversiteit Gent, Ghent (Belgium)

(Received April 29th, 1966; in revised form, May 20th, 1966)

As with several other glycosidases, transferase action by β-D-xylosidase (β-D-xyloside xylohydrolase), partially purified from commercial Hemicellulase[1], was an expected side-reaction of the hydrolysis of aromatic β-D-xylopyranosides. Aliphatic alcohols and several polyhydric alcohols were shown to be powerful activators of the hydrolysis reaction, as well as acceptors of the D-xylosyl radical. When glycerol was used as acceptor and *p*-nitrophenyl β-D-xyloside as substrate, activation and transfer were estimated to be 55% and 81%, respectively. With methanol as acceptor, retention of the β-D-configuration in the resulting methyl xyloside could be demonstrated[2]. Of particular interest, was the appearance of several oligosaccharides and their phenyl glycoside derivatives in enzymic digests of phenyl β-D-xylopyranoside solutions (5×10^{-2} to 10^{-2}M). For the main transfer product formed, a bioside, the substrate probably acted, with equal facility, as both donor and acceptor. The structure of this transfer product has been established as phenyl 4-*O*-β-D-xylopyranosyl-β-D-xylopyranoside (**1**). Several other transfer products were formed in minor proportions. Evidence is presented which indicates these products to be lower members of two homologous series consisting of phenyl β-D-glycosides of xylose oligosaccharides containing (1→3)- or (1→4)-linkages.



1

These dismutation reactions or self transfers have been observed with several other glycosidases. Almond emulsin was found to catalyze a similar reaction with arbutin or salicin as substrate[3], and two digalactosyl derivatives of resorcinol have been tentatively identified as transfer products from lactose to resorcinol, using *E. coli* β-galactosidase[4]. Other examples of such transglycosylation reactions can be found in studies dealing with a specific transfructosylase in plants[5], as well as with maltases in rumen protozoa[6], or *Aspergillus niger*, which catalyze the transfer of D-glucose from maltose to resorcinol[7].

EXPERIMENTAL AND RESULTS

General experimental conditions

Paper and thin-layer chromatography (t.l.c.) were carried out on Whatman No. 1 paper and Kieselgel G (Merck), respectively, using the solvent systems: *A*, water-saturated butanol; *B*, propan-2-ol–water–butanol (5:2:3, v/v); *C*, benzene–ethanol (2/1, v/v). Reducing sugars were detected with the *p*-aminohippuric acid [*N*-(*p*-aminobenzoyl)glycine][8] or triphenyltetrazolium salt[9] (TTC) reagents, and both reducing and non-reducing sugars with 5% sulphuric acid in ethanol (10 min at 100°). Solutions were concentrated at 40–50°/20 mm. U.v. spectra were obtained with a Beckman DK-2 spectrophotometer, and i.r. spectra with a Perkin–Elmer 21 instrument, using KBr pellets. A unit of β-D-xylosidase has been defined[1] as causing the hydrolysis of 1 μmole of *p*-nitrophenyl β-D-xyloside (at 2×10^{-3}M) per min, at 40° and pH 3.0 in McIlvain's buffer, in 5 ml of reaction mixture.

Preparation and purification of the transfer product **1**

Phenyl β-D-xylopyranoside (2 g) was dissolved in potassium hydrogen phthalate–hydrochloric acid buffer (pH 3, 100 ml) followed by addition of 1 unit of β-xylosidase, obtained by dissolving commercial "Hemicellulase" (Nutritional Biochemicals Corporation 6013, 60 mg). After 16 h at 40°, the reaction was stopped by heating for 1 min at 100°. The mixture, after extraction with ether, deionisation with Dowex-1 (OH^- form) and Dowex-50W (H^+ form), and concentration, was chromatographed on a Whatman Standard-cellulose column (3×60 cm), by development with solvent *A*. The eluate was continuously analysed with a Uvicord system (LKB) at 257 mμ (phenyl absorption), and each 4-ml fraction was analysed by t.l.c. in solvent *A*. Fractions containing component **1** (R_F 0.45) were collected and rechromatographed (Fig. 1). The procedure was repeated three times until the component was free from any contaminating substance. By concentration (to 10 ml) of the fractions containing the pure product, crystallisation occurred to give a product (40 mg) having m.p. 144–145°, $[\alpha]_D^{20}$ −80° (*c* 1, water). The u.v. spectrum was identical with that of phenyl β-D-xylopyranoside (λ_{max} 265.5 mμ, ε_{max} 0.82×10^6).

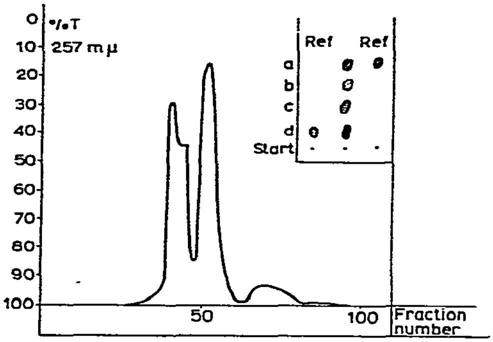
Structure of the transfer product **1**

(*a*) *Total and partial acid hydrolysis.* After complete hydrolysis (3 h in 0.1M hydrochloric acid at 100°), total pentose and phenol concentrations were determined by the Bial[10] and Folin–Ciocalteu[11] procedures, respectively. The molar ratio xylose/phenol was 2.08, indicating the disaccharide character of the transfer product. Partial hydrolysis (0.1M hydrochloric acid for 3 min at 100°) resulted in the formation of xylobiose (4-*O*-β-D-xylopyranosyl-D-xylose), identified (t.l.c., solvent *B*) by comparison with an authentic sample of the disaccharide.

(*b*) *Periodate oxidation.* To the phenyl xylobioside (5.85 mg) in 0.2M acetate buffer (pH 4,10 ml), 0.1M aqueous sodium metaperiodate (12.5 ml) was added, and the mixture was diluted with water to 25 ml. Periodate uptake was determined by the

Fleury–Lange procedure[12]. Formic acid liberation was followed by titration with 0.1M sodium hydroxide, using Methyl Red as indicator.

The consumption of 3 mol. of periodate and the formation of 1 mol. of formic acid (Fig. 2) precluded the presence of a (1→3)-linkage.



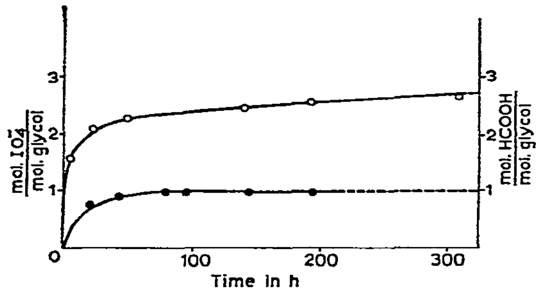


Fig. 1. Cellulose-column chromatography of an enzymic digest of phenyl β-D-xylopyranoside. Shoulder, transfer product **2**; second peak, transfer product **1**; first peak, phenyl β-D-xylopyranoside. Inset, t.l.c. of the digest: (*a*) phenyl β-D-xylopyranoside, (*b*) transfer product **2**, (*c*) transfer product **1** (*d*) D-xylose.

Fig. 2. Periodate oxidation of phenyl β-D-xylobioside (1→4). —o—, periodate uptake; —•—, formic acid production.

(*c*) *Methylation*. Complete methylation of the phenyl xylobioside (10 mg) by the method of Kuhn[13], subsequent hydrolysis (12% sulphuric acid for 4 h at 100°), and analysis of the reaction products on t.l.c. (solvent *C*), indicated the formation of a 2,3,4-tri-*O*-methylxylose and a 2,3-di-*O*-methylxylose. The possibility of the presence of a 3,4-di-*O*-methylxylose was excluded, as the reaction with the TTC reagent was negative[14].

The results of both periodate oxidation and methylation studies produced evidence in favour of a (1→4)-position of the disaccharide linkage.

(*d*) *I.r. spectroscopy*. Comparison of the spectra of phenyl α- and β-D-xylopyranoside with the spectrum of the transfer product revealed no bands characteristic of the α-D-configuration[15] (*cf.* the retention of configuration in the transfer reaction with methanol as acceptor).

(*e*) *Enzymic breakdown*. The enzymic hydrolysis of a 5×10^{-3}M solution of the phenyl xylobioside (PXX) by a purified β-xylosidase sample[1] was followed at room temperature in a DK2 Beckman continuously recording spectrophotometer at 280 mμ[16] (phenol formation) (Fig. 3). The most likely explanation for the observed non-linearity is that the phenol (P) is not liberated directly from the substrate, but from the intermediately formed phenyl xyloside (PX), as depicted in the following two-step reaction scheme:

$$\mathrm{PXX} \rightarrow \mathrm{PX} + \mathrm{X} \qquad (1)$$
$$\mathrm{PX} \rightarrow \mathrm{P} + \mathrm{X} \qquad (2)$$

This situation is similar to that encountered in the enzymic hydrolysis of phenolphthalein diphosphate to phenolphthalein and orthophosphate by a phosphatase[17]. The rate of phenol formation will increase with time, since the enzymic attack initially involves the "holoside" bond (Fig. 3). Examination of the hydrolysis products on t.l.c. (solvent *B*) confirmed this reaction sequence, since the intermediate phenyl xyloside could easily be detected. On the other hand, no xylobiose formation could be observed, and the enzyme was therefore devoid of any xylobiosidase activity. The apparent xylobiase (reaction 1) and xylosidase (reaction 2) activities were tentatively assigned to the same enzyme. Further purification of the β-xylosidase on DEAE-Sephadex A-50 ion-exchanger did not produce any separation of the two activities. The column was equilibrated with KH_2PO_4/Na_2HPO_4 buffer (pH 7.1, $\mu = 0.05$), and the concave sodium chloride-gradient (0→0.2M) was obtained by using the Varigrad system (Fig. 4). The β-xylosidase activity was tested against *p*-nitrophenyl β-D-xylopyranoside.

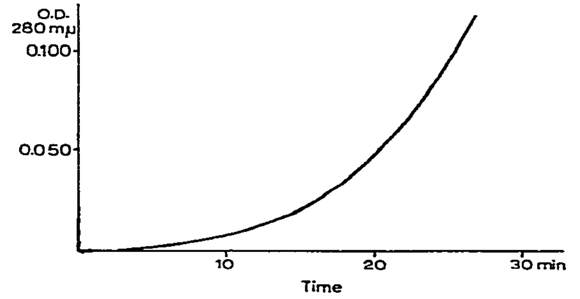


Fig. 3. Enzymic hydrolysis of phenyl β-D-xylobioside (1→4).

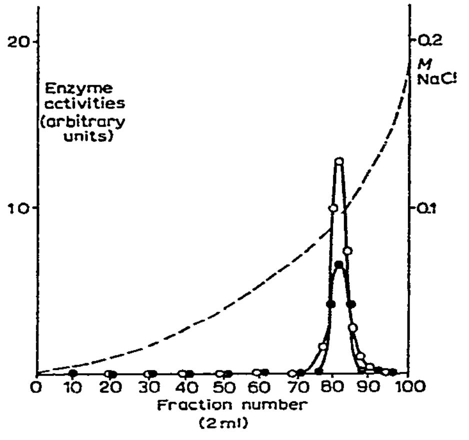


Fig. 4. DEAE Sephadex A-50 chromatography of β-xylosidase. —o—, activity against *p*-nitrophenyl β-D-xylopyranoside; —•—, activity against phenyl β-xylobioside.

Xylobiase activity was estimated by following the D-xylose liberation from the bioside, using the Somogyi–Nelson[18] colorimetric procedure.

Nature of the other transfer products

On paper chromatograms and t.l.c. (solvent *B*), the presence of xylobiose and xylotriose in the enzymic digests could be demonstrated by comparison with authentic samples of the two oligosaccharides.

Component 2, having R_F 0.54 on t.l.c. (solvent *A*) (Fig. 1), was obtained in pure form (5 mg) and methylated by the Kuhn method. After acid hydrolysis (12% sulphuric acid for 4 h at 100°), only the formation of a 2,3,4-tri-*O*-methylxylose and a 2,4-di-*O*-methylxylose was observed on t.l.c. (solvent *C*), indicating the presence of a (1→3)-linkage in the transfer product. Examination of the R_F values [on t.l.c. and paper chromatography (solvent *A*)] of other phenyl derivatives, found in further fractions from the cellulose column, revealed a linear relationship between R_M[log $(1/R_F - 1)$] and the hypothetical degree of polymerisation of the oligosaccharides. Two series, each fitting a line, were obtained, indicating the presence of two homologous series of oligosaccharides containing respectively (1→4)- and (1→3)-linkages (Fig. 5). Products having R_M values not fitting these plots presumably possess mixed (1→3)- and (1→4)-linkages.

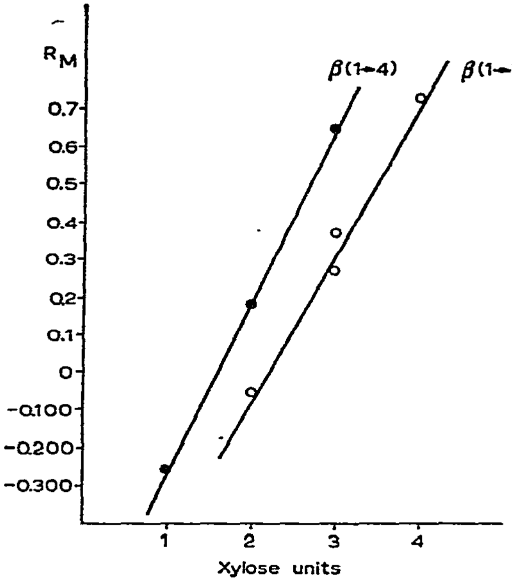


Fig. 5. R_M-plot against hypothetical number of D-xylose units in phenyl β-D-glycosides of D-xylose oligosaccharides.

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We thank Prof. Dr. L. Massart for his interest in this work and Prof. R. L. Whistler for a gift of xylobiose and xylotriose. We are indebted to Miss M. Cambier for technical assistance.

SUMMARY

Because of the transferase activity of partially purified β-D-xyloside xylohydrolase, enzymic breakdown of phenyl β-D-xylopyranoside results in the formation of by-products of particular interest. The lower members of two homologous series of phenyl β-D-glycosides of D-xylose oligosaccharides, respectively containing (1→3)- and (1→4)-linkages, were tentatively identified by t.l.c. and paper chromatography. Two phenyl β-D-xylobiosides were isolated, and the main transfer product was charac-

terised as phenyl 4-*O*-β-D-xylopyranosyl-β-D-xylopyranoside by total and partial acid and enzymic hydrolysis, periodate oxidation, and i.r. spectroscopy. Chemical evidence for the (1→3)-linkage in the second phenyl β-D-xylobioside is also given.

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SYNTHESIS OF NEW SUGAR DERIVATIVES HAVING POTENTIAL ANTITUMOUR ACTIVITY

PART IX*. CYCLIC PHOSPHORIC ACID DERIVATIVES

J. KUSZMANN AND L. VARGHA
Research Institute for Pharmaceutical Chemistry, Budapest (Hungary)

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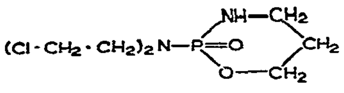
Phosphoric acid derivatives containing phosphorus attached to a nitrogen mustard group were first described by Friedman and Seligman[1] who assumed that the nitrogen–phosphorus bond would be split preferentially by tumour cells, owing to their relatively higher phosphoramidase content[2], and that the nitrogen mustard thus formed *in situ* might exert its cytostatic effect. These authors prepared a highly reactive phosphamide, bis(2-chloroethyl)phosphoramidic dichloride (**1**), which was used to synthesize a number of esters and amides.

$(Cl\cdot CH_2\cdot CH_2)_2N\cdot POCl_2$

1



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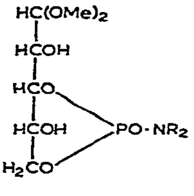
On further examination, the dichloride (**1**) appeared to react with certain diols, amino alcohols, or diamines, to furnish, principally, cyclic esters or amides. One of these cyclic compounds is the amido-ester, tetrahydro-2-[bis(2-chloroethyl)amino]-1,3,2-oxazaphosphorine 2-oxide (**2**), prepared by Arnold *et al.*[3] and used therapeutically, under the name Endoxan or Cytoxan, in the treatment of malignant diseases.

Recently, experiments were also carried out to obtain cyclic phosphamides of polyhydroxy compounds. Serfontein and Jordaan[4] treated 2,4-*O*-benzylidene-D-ribose dimethyl acetal with the dichloride (**1**), and subsequent debenzylidenation gave impure D-ribose dimethyl acetal cyclic 3,5-[bis(2-chloroethyl)phosphoramidate] (**3**)



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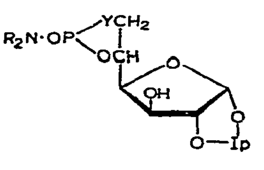
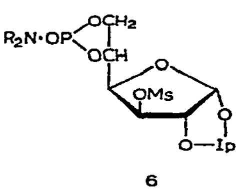


4 Y = O
5 Y = NH



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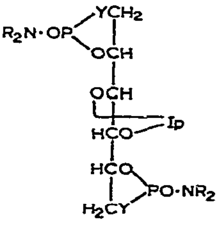

7 Y = O
8 Y = NH

R = $CH_2\cdot CH_2\cdot Cl$, Ip = Me_2C
Ms = $MeSO_2$

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* Part VIII: *Ann.*, 684 (1965) 231.

In our own experiments, we wished to investigate, in detail, the reaction of polyhydroxy compounds, such as sugars and sugar alcohols, with dichloride **1** and also the ring size of the resulting esters. We were also interested in the biological effects of such compounds since, according to our previous experience[5], polyhydroxy compounds seemed to be suitable carriers for cytotoxic groups.

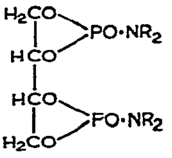
The first experiments were carried out with 1,2-*O*-isopropylidene-α-D-glucofuranose. This substance could give cyclic esters having the phosphorus atom attached to the 5,6-, 3,5-, and 3,6-oxygen atoms, respectively. Moreover, the formation of stereoisomers would be expected (as for the analogous benzylidene derivatives), owing to the asymmetry of the phosphorus atom. Acylation with dichloride **1** in pyridine gave a crystalline substance (isolated in 20% yield) in which the phosphoric acid group must span the 5,6-positions (**4**), since the *O*-methanesulphonyl derivative (**6**) was identical with the product obtained from the reaction of 1,2-*O*-isopropylidene-3-*O*-methanesulphonyl-α-D-glucofuranose[6] with dichloride **1**. Compound **4** was formed in the reaction of 1,2-*O*-isopropylidene-α-D-glucofuranose with the dichloride (**1**) under various conditions and with various ratios of the components, thus showing that, under favourable sterical conditions, the dichloride reacts with polyhydroxy compounds principally to give cyclic derivatives. The P–N bond of the D-glucose derivative (**4**) is readily split in acidic media (*cf.* the results of Stock *et al.*[7]). Thus, selective hydrolysis of the isopropylidene group could not be achieved.

6-Amino-6-deoxy-1,2-*O*-isopropylidene-α-D-glucofuranose (hitherto known[8], but not in the crystalline state) was expected to give a 5,6-(amido-ester) (**5**) on treatment with the dichloride (**1**). However the product failed to crystallise and, presumably, was a mixture of isomers.

From 3,4-*O*-isopropylidene-D-mannitol and 2 mol. of dichloride **1**, a cyclic diphosphoric acid ester (**7**), probably having a structure analogous to that of 1,2:3,4:5,6-tri-*O*-isopropylidene-D-mannitol, was formed. Two crystalline substances were isolated from the reaction product, and these compounds are, presumably, stereoisomers arising from the asymmetry of the cyclic phosphorus atom; this inference is supported by their nearly identical i.r. spectra. With 2 mol. of dichloride **1**, 1,6-diamino-1,6-dideoxy-3,4-*O*-isopropylidene-D-mannitol[9] gave a 1,2:5,6-di(amido-ester) (**8**), from which isomeric products could not be separated.

Since the isopropylidene group could not be selectively removed from the cyclic derivatives mentioned above, because of the sensitivity of the P–N bond, the reaction of several non-substituted polyols with 2 and 3 mol. of dichloride **1** was examined. In both cases, D-mannitol yielded only amorphous powders, the elemental analyses of which were fairly close to the calculated values. From the corresponding reactions of D-glucitol, two, unidentified, crystalline substances were isolated in poor yield.

The reaction of erythritol with the dichloride (**1**) was then examined in order to decrease the number of possible isomers. In this case, with 2 mol. of dichloride **1**, the formation of two structural isomers could be expected, *viz.*, 1,2:3,4- (**9**) and 1,3:2,4-derivatives (**10**); the first structure seems to be more likely by analogy (*a*) with the corresponding reaction of 1,2-*O*-isopropylidene-α-D-glucose and (*b*) with the *O*-isopropylidene derivatives of erythritol. The formation of a 1,4:2,3-derivative is improbable because of sterical reasons. In both cases, four stereoisomers are possible, *viz.*, an enantiomorphic pair and two *meso* forms. Experimentally, three, sharp-melting, crystalline substances (*A*, *B*, and *C*) were obtained. Substance *A*, however, gave two spots on silica gel, and, consequently, at least four isomers must have been formed. Since there can only be three stereoisomers for each structural isomer, *viz.*, a racemate and two *meso* modifications, the formation of both structures **9** and **10** is highly feasible



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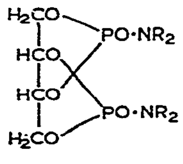

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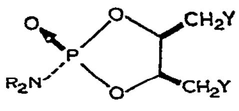
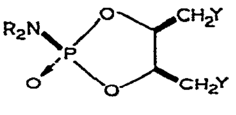


11 $Y = MeSO_2 \cdot O$
12 $Y = Br$



13 $Y = MeSO_2 \cdot O$
14 $Y = Br$

$R = CH_2 \cdot CH_2 \cdot Cl$

The possible formation, in these experiments, of stereoisomers as a function of the presence of a cyclic phosphate group was proved with 1,4-substituted erythritol derivatives. Dichloride (**1**) can react with such substances only at the 2,3-position. 1,4-Di-*O*-methanesulphonyl-[10] and 1,4-dibromo-1,4-dideoxy-erythritol[11] each gave, with the dichloride (**1**), two substances in approximately equal amounts. These substances can only be the optically inactive *cis*- and *trans*-isomers (**11-14**); the phosphorus atom is not asymmetric because of the symmetry of the erythritol moiety. The i.r. spectra of the members of each pair of isomers are very similar.

Thus polyhydroxy compounds usually give stereoisomers on treatment with the dichloride (**1**), and it is therefore rather surprising that no formation of diastereoisomers could be observed in the case of serine which affords[12] five-membered, cyclic amido-ester, similar to the cyclic esters described above.

The compounds described here are poorly soluble, or insoluble, in water, and their biological examination could be carried out only through oral administration. On Yoshida sarcoma, they displayed a weak effect; their therapeutic quotient was much less (5–20%) than that of Endoxan and therefore they were not studied in detail. It is noteworthy that a substantial difference was observed between the toxicity (LD_{50}) and therapeutic effect of the *cis,trans*-1,4-di-*O*-methanesulphonyl and 1,4-dibromo-1,4-dideoxy derivatives of erythritol.

EXPERIMENTAL

Melting points are uncorrected. Thin-layer chromatography (t.l.c.) was carried out on silica gel. Detection reagents used were potassium permanganate or *p*-nitrobenzylpyridine[13].

1,2-O-Isopropylidene-α-D-glucofuranose cyclic 5,6-[bis(2-chloroethyl)phosphoramidate] (**4**)

To 1,2-*O*-isopropylidene-α-D-glucofuranose (22 g, 0.1 mole), dissolved in pyridine (100 ml), dichloride **1** (26 g) was added, with stirring, so that the temperature of the reaction mixture did not exceed 30°. Stirring was continued for 5 h at room temperature, and the reaction mixture was then evaporated under diminished pressure at 30°. The residue was treated with ice-water and extracted with chloroform, and the organic phase was washed with cold N sulphuric acid, water, aqueous sodium hydrogen carbonate, and water. After being dried (Na_2SO_4) and treated with charcoal, the solution was evaporated to dryness under diminished pressure to give a yellow, pulverisable, amorphous material (33 g, 81%). The substance could be partially crystallized from ethyl acetate by gradual addition of light petroleum. After recrystallization from ethyl acetate, compound **4** (8.1 g, 20%) was obtained, m.p. 167–168°, $[\alpha]_D^{20}$ −15.0° (*c* 1, chloroform) (Found: C, 39.0; H, 5.7; Cl, 17.7; N, 3.4; P, 7.3. $C_{13}H_{22}Cl_2NO_7P$ calc.: C, 38.4; H, 5.5; Cl, 17.5; N, 3.45; P, 7.6%).

From the ethyl acetate mother liquor, an amorphous powder, $[\alpha]_D^{20}$ 0° (*c* 1, chloroform), was obtained which showed the same R_F value as compound **4** in water-saturated butanol (Found: Cl, 17.2; N, 3.3; P, 6.9%).

Hydrolysis of phosphoramidate **4**

A solution of ester **4** (1 g) in a mixture of 0.2N hydrochloric acid and dioxane (1:1, v/v; 10 ml) was kept at 50°, and the hydrolysis was followed by t.l.c. The characteristic blue spot of nitrogen mustard appeared after 30 min (development with *p*-nitrobenzylpyridine). After 5 h, half of the starting material could still be recovered, and the residue reduced Fehling's solution.

In another experiment, under the same conditions, hydrolysis for 8 h was required for complete removal of the isopropylidene group.

1,2-O-Isopropylidene-3-O-methanesulphonyl-α-D-glucofuranose cyclic 5,6-[bis(2-chloroethyl)phosphoramidate] (**6**).

(*a*) A solution of 1,2-*O*-isopropylidene-3-*O*-methanesulphonyl-α-D-glucofuranose[6] (obtained from 10 g of 1,2:5,6-di-*O*-isopropylidene-3-*O*-methanesulphonyl-α-D-glucofuranose) in benzene was evaporated to dryness under diminished pressure. The dissolution and evaporation were repeated, and the residual gum was freed from solvent at 50°/0.22 mm. The syrup obtained showed a single spot on t.l.c. (ethyl acetate). To a solution in pyridine (30 ml) of this substance, dichloride **1** (7.7 g) was added with stirring and cooling. The reaction mixture was kept at room temperature overnight and then poured onto ice. The syrupy product was dissolved in chloroform and the solution was washed with N sulphuric acid, water, and aqueous sodium hydrogen carbonate, dried, and evaporated. The residual syrup was crystallised from ethyl acetate–light petroleum, and then from ethyl acetate–ether, to give compound **6** (3.5 g), m.p. 145–147°, $[\alpha]_D^{20}$ −22.15° (*c* 1, chloroform). (Found: C, 34.8; H, 4.9; Cl, 14.5; N, 2.7; S, 6.5; P, 6.1. $C_{14}H_{24}Cl_2NO_9SP$ calc. : C, 34.7; H, 5.0; Cl, 14.6; N, 2.9; S, 6.6; P, 6.1%).

(*b*) To a solution of compound **4** (2 g) in pyridine (10 ml), methanesulphonyl chloride (0.6 ml) was added at −10° with stirring. After 3 h at room temperature, the reaction mixture was poured onto ice and processed as under (*a*) to give a product (2 g) identical (m.p., mixed m.p., and $[\alpha]_D$) with that described in (*a*).

6-Amino-6-deoxy-1,2-O-isopropylidene-α-D-glucofuranose

This substance was prepared by a slight modification of the literature method[8].

A solution of 5,6-anhydro-1,2-*O*-isopropylidene-α-D-glucofuranose[14] (20.2 g) in methanol (200 ml) saturated with ammonia at 0° was kept in a sealed vessel at 20° for 12 h and then refluxed for 2 h. The residue obtained after evaporation to dryness under diminished pressure was dissolved in benzene, and the solution was evaporated. During this procedure, the substance crystallized as thin needles, m.p. 114–122°; Danilov and Lishansky[8] reported m.p. 60–65°. On being dried under diminished pressure over sodium hydroxide and paraffin, the product lost its crystalline structure. The physical constants of the hydrochloride are consistent with the literature data[15]. (Found: C, 48.9; H, 7.9; N, 6.0. $C_9H_{17}NO_5$ calc.: C, 49.3; H, 7.8; N, 6.4%).

6-Amino-6-deoxy-1,2-O-isopropylidene-α-D-glucofuranose cyclic 5,6-[bis(2-chloroethyl phosphoramidate] (5)

To a suspension of 6-amino-6-deoxy-1,2-*O*-isopropylidene-α-D-glucofuranose (11 g) in dioxane (50 ml) triethylamine (14 ml) and dichloride **1** (13 g) weie added with stirring so that the temperature did not rise over 30°. Stirring was continued for 5 h at 20° and the reaction mixture was then poured onto ice and extracted with chloroform. The organic phase was washed with water, dried (Na_2SO_4), and evaporated to a solid, yellow mass (18 g, 80%). A solution of this residue in ethyl acetate–light petroleum was treated with charcoal and evaporated under diminished pressure to give compound **5** as a colourless, amorphous solid, $[\alpha]_D^{20}$ −5.9 (*c* 1, chloroform). Found: C, 38.1; H, 5.9; Cl, 16.5; N, 6.8; P, 7.1. $C_{13}H_{23}N_2O_6Cl_2P$ calc.: C, 38.5; H, 5.7; Cl, 17.5; N, 6.95; P, 7.65%).

3,4-O-Isopropylidene-D-mannitol cyclic 1,2:5,6-di-[bis(2chloroethyl)phosphoramidate] (7)

To a solution of 3,4-*O*-isopropylidene-D-mannitol (11.1 g) in pyridine (60 ml) dichloride **1** (28.6 g) was added slowly with stirring and cooling below 20°. Next day the reaction mixture was poured onto ice and extracted with chloroform, and the organic phase was washed with cold N sulphuric acid, water, and aqueous sodium hydrogen carbonate, dried (Na_2SO_4), and evaporated under diminished pressure. The syrupy residue was crystallised from ethyl acetate (30 ml) and ether (20 ml) to give product *A′* (9 g). Further addition of ether to the mother liquor gave product *B′* (8.5 g), and after evaporation of the remaining solution, crystallisation of the residue from ethyl acetate–ether gave product *C′* (5 g). The total yield of crystalline material was 67%. Recrystallization of *A′* from a ten-fold amount of benzene gave pure isomer *A′*, m.p. 189–190°, $[\alpha]_D^{20}$ −2.5° (*c* 1, chloroform). (Found: C, 33.9; H, 5.4; Cl, 23.2;

N, 4.5; P, 10.2. $C_{17}H_{30}Cl_4N_2O_8P_2$ calc.: C, 34.4; H, 5.1; Cl, 23.9; N, 4.7; P, 10.4%). Recrystallization of *B'* and *C'* from a twenty-fold amount of benzene also furnished isomer *A'*. Concentration of the mother liquor to half-volume gave a mixture of isomers *A'* and *B'*, and further concentration gave isomer *B'*, m.p. 156–157° (from ethyl acetate), $[\alpha]_D^{20}$ +10.8° (*c* 1, chloroform). (Found: C, 33.75; H, 5.3; Cl, 23.3; N, 4.3; P, 10.2%). The mixture of *A'* and *B'* could also be separated by fractional crystallization from benzene. The i.r. spectra of isomers *A'* and *B'* were almost identical. The R_F values (ethyl acetate), were *A'*, 0.60; *B'*, 0.77.

*1,6-Diamino-1,6-dideoxy-3,4-O-isopropylidene-*D*-mannitol cyclic 1,2:5,6-di[bis(2-chloroethyl)phosphoramidate]* (8)

To a solution of 1,6-diamino-1,6-dideoxy-3,4-*O*-isopropylidene-D-mannitol[9] (11 g, 0.05 mole) in dioxane (110 ml) and triethylamine (31 ml) dichloride **1** (28.6 g, 0.11 mole) was added slowly with cooling (below 20°) and stirring. Next day, the triethylamine hydrochloride was filtered off, and the filtrate was concentrated. A solution of the syrupy residue in chloroform was washed with water, dried (Na_2SO_4) and evaporated under diminished pressure. A solution of the residue in ethyl acetate–ether was treated with charcoal and then evaporated under diminished pressure to give a nearly colourless, amorphous solid (72%), R_F 0.80 in ethyl acetate–propan-2-ol–water (3.5:27:1) (Found: Cl, 20.45; N, 7.55; P, 10.55. $C_{17}H_{32}Cl_4N_4O_6P_2$ calc.: Cl, 23.9; N, 9.5; P, 10.5%).

D-*Mannitol cyclic di-[bis(2-chloroethyl)phosphoramidate]*

To a suspension of D-mannitol (9.1 g) in pyridine (40 ml), which had previously been refluxed for 10 min. and then cooled rapidly with vigorous stirring to 10°, dichloride **1** (29.5 g, 2 mole) was added slowly. Next day the reaction mixture was poured onto ice and extracted with chloroform, and the organic phase was washed with water, N sulphuric acid, and aqueous sodium hydrogen carbonate, and evaporated under diminished pressure to give an amorphous solid (13.6 g, 50%). A solution of this material in ethyl acetate–ether was treated with charcoal and evaporated under diminished pressure to give a white, amorphous powder, $[\alpha]_D^{20}$ +2.0° (*c* 2, chloroform) (Found: C, 30.0; H, 4.3; Cl, 25.3; N, 4.9; P, 10.9. $C_{14}H_{26}Cl_4N_2O_8P_2$ calc.: C, 30.3; H, 4.7; Cl, 25.6; N, 5.05; P, 11.2%).

D-*Mannitol cyclic tri-[bis(2-chloroethyl)phosphoramidate]*

This compound was prepared from dichloride **1** (3.1 mole) according to the previous procedure. After purification, as above, a white, amorphous powder (18 g, 60%) was obtained, having $[\alpha]_D^{20}$ +7.2° (*c* 2, chloroform) (Found: C, 28.9, H, 4.5; Cl, 28.1; N, 6.2; P, 11.5. $C_{18}H_{32}Cl_6N_3O_9P_3$ calc.: C, 29.2; H, 4.4; Cl, 28.2; N, 5.7; P, 12.5%).

D-*Glucitol cyclic di-[bis(2-chloroethyl)phosphoramidate]*

This compound (43%), prepared as for the analogous D-mannitol derivative, was a white, amorphous powder, $[\alpha]_D^{20}$ 0° (*c* 1, chloroform) (Found: C, 30.1; H, 4.3; Cl, 24.1; N, 4.9; P, 11.2. $C_{14}H_{26}Cl_4N_2O_8P_2$ calc.: C, 30.3; H, 4.7; Cl, 25.6; N, 5.05; P, 11.2. From ethyl acetate–ether, a portion (2.2 g) of this product gave crystals (0.5 g), m.p. 155–160° (from ethyl acetate), $[\alpha]_D^{20}$ +9.8° (*c* 1, dioxane) (Found: C, 30.8; H, 4.4; Cl, 24.8; N, 5.0; P, 10.9%).

D-*Glucitol cyclic tri-[bis(2-chloroethyl)phosphoramidate]*

Prepared as for the analogous D-mannitol derivative, the product (71%) was a white, amorphous solid, $[\alpha]_D^{20}$ +10.0° (*c* 1, chloroform) (Found: C, 29.0; H, 4.7; Cl, 27.9; N, 5.4; P, 12.1. $C_{18}H_{32}Cl_6N_3O_9P_3$ calc.: C, 29.2; H, 4.4; Cl, 28.7; N, 5.7; P, 12.5%). Crystallisation of a portion (2.8 g) of this material from ethyl acetate–ether gave a product (0.2 g), m.p. 201–202° (from ethyl acetate), $[\alpha]_D^{20}$ +14.5° (*c* 0.5, chloroform) (Found: C, 29.2; H, 4.5; Cl, 28.35; N, 5.6; P, 12.0%).

Erythritol cyclic di-[bis(2-chloroethyl)phosphoramidate] (**9**) or (**10**)

To a stirred solution of erythritol (24.4 g) in pyridine (160 ml) dichloride **1** (103.6 g, 2 mole) was added in small portions below 20°. After being stirred for 6 h, the mixture was poured onto ice, and the precipitated solid (81.2 g, 82%) was collected. The filtrate was extracted with chloroform, and the extract was washed with water, dried (Na_2SO_4), and evaporated to give further product (7.5 g, 7.6%). The reaction product consisted of at least 3 components which could be separated by fractional crystallization. The precipitate was boiled with a 15-fold amount of ethyl acetate, and the insoluble material was filtered off from the hot solution and recrystallized from methanol to give isomer *A*, m.p. 185–186° (Found: C, 29.25; H, 4.7; Cl, 28.2; N, 5.3; P, 12.2. $C_{12}H_{22}Cl_4N_2O_6P_2$ calc.: C, 29.2; H, 4.5; Cl, 28.7; N, 5.6; P, 12.5%). The ethyl acetate solution yielded, on cooling, a mixture of *A*, *B*, and *C*. Evaporation of the mother liquor, and recrystallisation of the residue from benzene gave isomer *C*, m.p. 123–125°. (Found: C, 29.15; H, 4.6; Cl, 28.8; N, 5.4; P, 12.4%). The above chloroform extract also afforded isomer *C*. The mixture of *A*, *B*, and *C* was boiled with a 15-fold amount of ethyl acetate, the solution was filtered to remove undissolved *A* and *B* and evaporated, and the residue (*B* contaminated with *C*) was extracted with hot benzene to remove isomer *C*. Recrystallization from ethyl acetate gave isomer *B*, m.p. 152–154° (Found: C, 29.0; H, 4.55; Cl, 28.15; N, 5.2; P, 12.2%). The ratio of the compounds *A*:*B*:*C* was *ca.* 1:1:2. On chromatography in ethyl acetate, *B* and *C* gave a single spot, whereas *A* afforded two spots of equal intensity; the R_F values were *A*, 0.62, 0.75; *B*, 0.68; *C*, 0.32.

1,4-Di-O-methanesulphonylerythritol cyclic 2,3-[bis(2-chloroethyl)phosphoramidate] (**11, 13**)

To a solution of 1,4-di-*O*-methanesulphonylerythritol[10] (2.8 g) in pyridine (10 ml) dichloride **1** (2.8 g, 1 mole) was added with stirring and cooling. Stirring was continued for 1 h at 0°. After storage at 20° overnight, the reaction mixture was poured onto ice, and the solidified product (3.5 g, 76%) was filtered off and washed with water. Crystallization from ethyl acetate (50 ml) gave isomer *A*, m.p. 135–137° (after repeated recrystallization from ethyl acetate or methanol) (Found: C, 26.0; H, 4.6; Cl, 15.2; N, 2.9; P, 6.5; S, 13.9. $C_{10}H_{20}Cl_2NO_9PS_2$ calc.: C, 25.9; H, 4.3; Cl, 15.3; N, 3.0; P, 6.7; S, 13.8%). The mother liquor was concentrated to half-volume, and addition of an equal volume of ether caused the separation of a mixture of *A* and *B*. Evaporation of the mother liquor gave almost pure isomer *B* which was purified by dissolution in a ten-fold amount of hot methanol. After removal of separated *A*, *B*-mixture, the cooled solution was evaporated to half-volume, and isomer *B* then crystallised; m.p. 102–104° (Found: C, 25.9; H, 4.5; Cl, 15.2; N, 3.0; P, 6.6; S, 13.65%). The *A*, *B*-mixture could be separated by fractional crystallization, first from ethyl acetate and then from methanol. The ratio of *A*:*B* was *ca.* 3:2. The R_F values (ethyl acetate) were *A*, 0.70; *B*, 0.33. The i.r. spectra showed the following bands. Isomer *A* (m.p. 135–137°): 3035, 3020, doublet, m; 2970 m; 2940 m; 1365, 1370 shoulder, s; 1270 s; 1185 s; 1080 s; 1000 s; 970 s; 935 s; 820 s; 655 w; 530, 520 cm^{-1}, doublet, s. Isomer *B* (m.p. 102–104°): 3020, 3030 shoulder, m; 2940 m; 1355, broad, s; 1260 s; 1180 s; 1080 s; 1005 s; 970 s; 920 s; 830, 820 shoulder, s; 660 w; 535, 520 cm^{-1}, doublet, broad, s.

1,4-Dibromo-1,4-dideoxyerythritol cyclic 2,3-[bis(2-chloroethyl)phosphoramidate] (12, 14)

To 1,4-dibromo-1,4-dideoxyerythritol[11] (10 g) in pyridine (40 ml) dichloride **1** (12 g, 1 mole) was added with stirring and cooling in ice. Stirring was continued for 1 h. Next day, the reaction mixture was poured onto ice, and the solidified mass (16.2 g, 93%) was filtered off and washed with water. The crude material was crystallized from a two-fold amount of benzene to give a fraction (6.5 g) rich in isomer *A*. Recrystallisation from aqueous methanol gave the pure substance, m.p. 123–124°. (Found: C, 22.4; H, 3.5; Br, 37.1; Cl, 16.6; N, 3.15; P, 6.9. $C_8H_{14}Br_2Cl_2NO_3P$ calc.: C, 22.1; H, 3.25; Br, 36.8; Cl, 16.3; N, 3.2; P, 7.1%). The residue from the benzene mother liquor was dissolved in ethyl acetate or methanol, and the solvent was allowed to evaporate slowly at room temperature. Isomer *B* crystallized as large, flat crystals which could be readily separated from isomer *A* which crystallized as fine needles. Isomer *B*, purified by crystallisation from ethyl acetate-light petroleum, had m.p. 97–98° (Found: C, 22.3; H, 3.4; Br, 36.6; Cl, 16.6; N, 3.2; P, 6.9%). Chromatography, separately, in ethyl acetate, butanol saturated with water, and propan-2-ol–ethyl acetate–water revealed the two isomers to have identical R_F values (0.91, 0.79, 0.77). The i.r. spectra showed the following bands. Isomer *A* (m.p. 123–124°) : 3030, 3020, doublet, m; 2980, 2965, doublet, m; 1260 s; 1035 s; 995 s; 665 w; 630 m cm^{-1}. Isomer *B* (m.p. 97–98°): 3025 w; 2965 m; 1280, 1270 shoulder s; 1040 s; 990 s; 665 w; 625 w cm^{-1}.

SUMMARY

With polyhydroxy compounds, such as sugars and sugar alcohols, bis(2-

chloroethyl)phosphoramidic dichloride (**1**) gave mixtures of cyclic derivatives in stereoisomeric forms analogous to benzylidene derivatives. In several cases, homogeneous products were isolated and their structures elucidated. The following compounds were obtained: 1,2-*O*-isopropylidene-α-D-glucofuranose cyclic 5,6-[bis(2-chloroethyl)phosphoramidate] (**4**), *cis*- and *trans*-1,4-di-*O*-methanesulphonylerythritol cyclic 2,3-[bis(2-chloroethyl)phosphoramidate] (**11**, **13**), *cis* and *trans*-1,4-dibromo-1,4-dideoxyerythritol cyclic 2,3-[bis(2-chloroethyl)phosphoramidate (**12**, **14**). With amino-polyhydroxy compounds, amorphous products, presumably cyclic amidoesters, were formed, from which no homogeneous compounds could be isolated. The substances examined showed a weak cytostatic effect on Yoshida sarcoma.

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TEIL XI. KONFORMATION VON *N*-ACYL-D-*xylo*-PIPERIDINOSEN UND DEREN STRUKTUR AM ANOMEREN C-ATOM. DARSTELLUNG UND EIGENSCHAFTEN VON D-*xylo*-PIPERIDINOSIDEN[1]

H. PAULSEN UND F. LEUPOLD
Institut fur Organische Chemie der Universität Hamburg (Deutschland)

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Einführung

N-Acetyl-D-*xylo*-piperidinose (5-Acetamido-5-desoxy-D-xylopyranose, **1**)[2] enthält eine cyclische Amidgruppierung, die infolge der Amidmesomerie eine gehinderte Rotation aufweist. Bei Zimmertemperatur liegen in Lösung zwei Isomere, eine *cis*- und eine *trans*-Form, nebeneinander vor. Von beiden Formen lassen sich im NMR-Spektrum die Signale der anomeren Protonen gut beobachten[3]. Beide Signale zeigen eine kleine Dublett-Aufspaltung ($J_{1,2}$ 3.5 Hz), was auf eine äquatorial-axial-Kopplung mit dem Proton am C-2 hinweist. Die Zuordnung einer α-D-Form setzt aber voraus, daß die Piperidinose in einer *C1*-Sesselkonformation vorliegt, bei der in der D-*xylo*-Konfiguration H-2, H-3, und H-4 axial angeordnet sind. Gegen eine α-D-Form spricht die negative Drehung ($[\alpha]_D^{20}$ —19.5°) der *N*-Acetyl-D-*xylo*-piperidinose (**1**), die in Lösung nur in einer der anomeren Formen vorliegt, da keine Mutarotation beobachtet wird. Um die Frage der Konformation von Monosaccnariden mit stickstoffhaltigem Ring grundsätzlich zu klären, wurde eine vollständige Konformationsanalyse mit Hilfe der NMR-Spektroskopie durchgeführt. Bei bekannter Konformation ist die Konfiguration am C-1 aus dem Spektrum zu entnehmen.

Konformation und Konfiguration von N-*Acyl*-D-xylo-*piperidinosen*

Eine vollständige Analyse des NMR-Spektrums der *N*-Acetylpiperidinosen wird durch die gehinderte Rotation um die N–CO Bindung erschwert. Diese führt nicht nur zu unterschiedlichen chemischen Verschiebungen des anomeren Protons in Abhängigkeit von seiner fixierten Stellung zur Carbonylgruppe, sondern auch die Mehrzahl der Signale der anderen Protonen kann verdoppelt sein[4]. Eine Zuordnung der Signale der Ringprotonen bereitet somit große Schwierigkeiten.

Im Gegensatz zu den acetylierten Piperidinosen zeigt die *N*-Carbobenzoxy-D-*xylo*-piperidinose[5-Benzyloxycarbonylamino-5-deoxy-D-xylopyranose, **5**][5] infolge ihrer Urethanstruktur oberhalb —40° eine freie Rotation der Acylgruppe um die N–CO Bindung. Eine gehinderte Rotation scheint bei Urethanen allgemein nicht aufzutreten[6] und wurde bisher erst in einem Falle beobachtet[7]. Das NMR-Spektrum der *N*-Carbobenzoxy-D-*xylo*-piperidinose (**5**) ist deshalb wesentlich vereinfacht und daher einer Deutung zugänglich. Am günstigsten für eine vollständige Analyse erwies sich das Spektrum der 1,2,3,4-Tetra-*O*-acetyl-*N*-carbobenzoxy-D-*xylo*-piperidinose (**8**), welches Abb. 1 zeigt. Die weitgehende Trennung der Signale ermöglicht eine Behandlung nach der ersten Ordnung und lässt unter Anwendung der Doppelresonanztechnik eine genaue Zuordnung der sechs Ringprotonen zu. Die Signalaufspaltungen können angenähert als Kopplungskonstanten betrachtet werden, aus denen unter Anwendung der Karplus-Beziehung[8] für die Kopplung vicinaler Protonen, Dihedralwinkel ermittelt werden, die einen Vergleich mit der angenommenen Konformation erlauben.

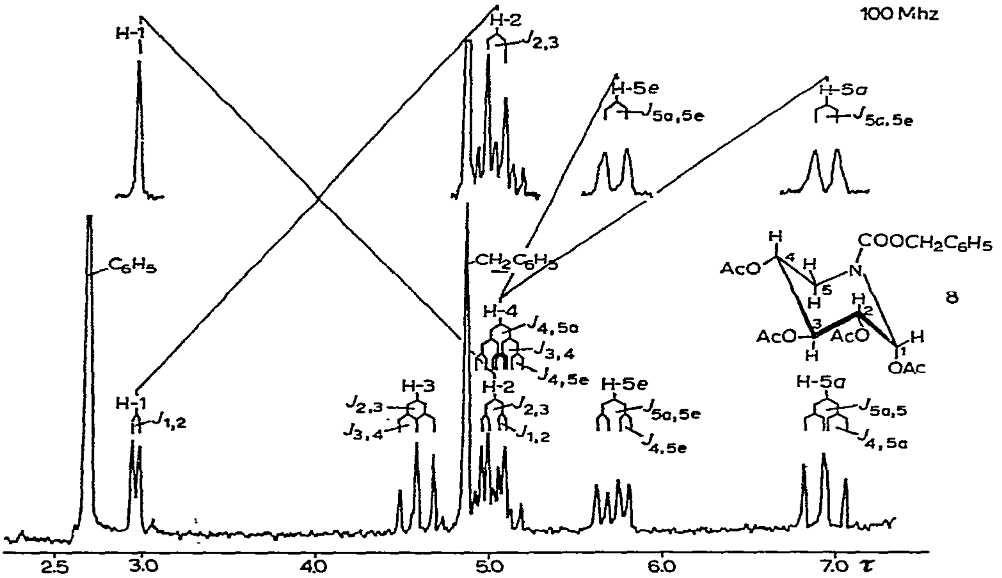


Abb. 1. NMR-Spektrum der 1,2,3,4-Tetra-*O*-acetyl-*N*-carbobenzoxy-α-D-*xylo*-piperidinose (8) bei 100 Mhz in $CDCl_3$ (ohne *O*-Acetyl-Protonen). T 35°. Oberer Teil: Signale bei Spinentkopplung.

Das bei niedriger Feldstärke liegende Signal für das anomere Proton (τ 2.95) zeigt die für eine *syn*-clinale Stellung zum benachbarten Proton am C-2 typische kleine Aufspaltung von $J_{1,2}$ 3.5 Hz[9]. Das H-2-Signal erscheint bei τ 5.03 als Quartett infolge Kopplung mit H-1 und H-3. Durch Einstrahlung beim H-1 geht das Signal über in ein Dublett mit großer Aufspaltung $J_{2,3}$ 10.0 Hz, die nur durch eine Diaxialkopplung mit H-3 zu erklären ist. Die Einstrahlung der Resonanzfrequenz des H-2 führt entsprechend zu einem Singulett für das Signal des anomeren Protons. Die durch Spinentkopplung erhaltenen Signale sind in der oberen Reihe von Abb. 1 wiedergegeben. Für das H-3 findet man ein Triplett bei τ 4.58 mit großer Aufspaltung durch die gleich große vicinale Diaxialkopplung mit H-2 und H-4 von jeweils 10.0 Hz. Das H-4 erscheint als Oktett bei τ 5.06 infolge Kopplung mit den beiden Protonen am C-5 sowie mit dem H-3. Die Signale des H-5*e* und des H-5*a* treten als zwei Quartetts bei τ 5.71 und τ 6.94 mit einer Geminalkopplung von 12.5 Hz und $J_{4,5e}$ 6.0 Hz, bzw. $J_{4,5a}$ 11.0 Hz, auf. Infolge des überraschend großen Unterschiedes in den chemischen Verschiebungen ergibt sich ein einfaches AMX-Spektrum. Den Beweis für die Richtigkeit der Zuordnung liefert die Spinentkopplung. Durch

Einstrahlung beim H-4 gehen beide Signale in Dubletts mit gleicher großer Geminalkopplung über. Beim Vergleich mit dem Spektrum der Tetra-*O*-acetyl-α-D-xylopyranose[10] fällt die erhebliche Verschiebung des H-5*a* zu höherer Feldstärke auf. Diese verstärkte Abschirmung sollte durch die Anisotropiewirkung der Amidgruppierung verursacht sein. Temperaturversuche zeigen, daß das Spektrum zwischen —40° und 116° unverändert bleibt, und damit eine Fixierung der Urethangruppe sowie des Phenylrestes unter diesen Bedingungen nicht eintritt.

TABELLE I

ANALYSE DES NMR-SPEKTRUMS DER 1,2,3,4-TETRA-*O*-ACETYL-*N*-CARBOBENZOXY-α-D-*xylo*-PIPERIDINOSE (8) BEI 100 MHZ (OHNE *O*-ACETYL PROTONEN).
Lösungsmittel : $CDCl_3$. TMS als innerer Standard. T, 35°.

Signal	*τ (p.p.m.)*	*Kopplung (Hz)*	*Anordnung der Protonen*	*Spinentkopplung*[a]
C_6H_5–	2.67 Singulett			
–CH_2–	4.86 Singulett			
H-1	2.95 Dublett	$J_{1,2}$ 3.5	e-a	H-1—{H-2} Singulett
H-2	5.03 Quartett	$J_{2,3}$ 10.0	a-a	H-2—{H-1} Dublett
H-3	4.58 Triplett	$J_{3,4}$ 10.0	a-a	
H-4	5.06 Oktett			
H-5*e*	5.71 Quartett	$J_{4,5e}$ 6.0	a-e	H-5*e*—{H-4} Dublett
H-5*a*	6.94 Quartett	$J_{4,5a}$ 11.0	a-a	H-5*a*—{H-4} Dublett
		$J_{5e,5a}$ 12.5	geminal	

[a] Zur Kennzeichnung der Doppelresonanzversuche wurde die von Baldeschwieler und Randall vorgeschlagene Bezeichnungsweise benutzt. *Chem. Rev.*, 63 (1963) 81.

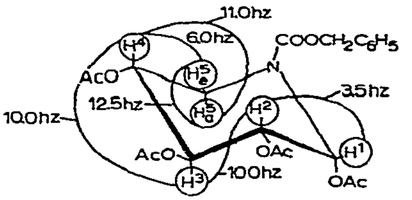


Abb. 2. Konformation der 1,2,3,4-Tetra-*O*-acetyl-*N*-carbobenzoxy-α-D-*xylo*-piperidinose (8) mit zugehörigen Kopplungskonstanten.

In Tab. I und Abb. 2 sind die Ergebnisse für die Tetra-*O*-acetyl-*N*-carbobenzoxy-D-*xylo*-piperidinose (8) zusammengefaßt. Hieraus geht klar hervor, daß die Piperidinose in einer Sesselkonformation vorliegen muß. Insbesondere die großen Kopplungen zwischen H-2 und H-3, H-3 und H-4, und H-4 und H-5*a* sprechen für eine Diaxialanordnung. Es folgt somit für die Konfiguration am C-1 aus der kleinen Kopplung zwischen H-1 und H-2 eine α-Form.

Zu dem gleichen Ergebnis führt auch die Analyse des in D_2O aufgenommenen NMR-Spektrums der *N*-Carbobenzoxy-D-*xylo*-piperidinose (5), welches in Tab. II

TABELLE II

ANALYSE DES NMR-SPEKTRUMS DER *N*-CARBOBENZOXY-α-D-*xylo*-PIPERIDINOSE (5) BEI 100 MHZ. Lösungsmittel : D_2O. DSS[a] als innerer Standard. T, 35°.

Signal	τ *(p.p.m.)*	*Kopplung (Hz)*	*Anordnung der Protonen*
C_6H_5–	2.68 Singulett		
–CH_2–	4.92 Singulett		
H-1	4.19 Dublett	$J_{1,2}$ 3.8	e-a
H-2	6.53 Quartett	$J_{2,3}$ 9.3	a-a
H-3	6.22 Triplett	$J_{3,4}$ 9.3	a-a
H-4	6.46 Multiplett		
H-5*e*	5.90 Quartett	$J_{4,5e}$ 4.9	a-e
H-5*a*	6.91 Quartett	$J_{4,5a}$ 11.0	a-a
		$J_{5e,5a}$ 12.5	geminal

[a] DSS, Natrium-2,2-dimethyl-2-silapentan-5-sulfonat.

wiedergegeben ist. Das Multiplett für H-4 ist nach der ersten Ordnung nicht mehr auflösbar. Während die Protonen am C-5 etwa die gleiche Lage wie in der acetylierten Verbindung haben, werden die Signale der übrigen Ringprotonen infolge größerer Abschirmung durch die Hydroxylgruppen um 1.2–1.6 p.p.m. zu höherer Feldstärke verschoben. Die Verbindung liegt gleichfalls in der Sesselform vor, eine Konformationsänderung des Piperidinolsystems durch Wasserstoffbrücken tritt demnach nicht ein.

Sehr instruktiv ist das NMR-Spektrum des Methyl-*N*-acetyl-2,3,4-tri-*O*-methyl-α-D-*xylo*-piperidinosids (9), welches in Abb. 3, unten bei 35°, oben bei 110° abgebildet ist. Die Verbindung weist eine gehinderte Amidrotation auf, so dass in der Kälte für das anomere Proton zwei Dubletts auftreten. In diesem Spektrum sind auch Quartett-Signale sichtbar, die den Protonen am C-5 zukommen. Diese Signale sind infolge der gehinderten Rotation ebenfalls aufgespalten. Das bei τ 5.49 sichtbare Quartett mit der Intensität für ein halbes Proton ist dem H-5*e* der einen Form zuzuordnen, während das Signal des Protons H-5*e* des Rotationsisomeren in dem Multiplett bei τ *ca.* 6.8 verborgen ist. Beim Erhitzen auf 110° fließen beide Quartetts zu einer Linie bei τ *ca.* 6.2 zusammen, wie aus der oberen Kurve zu erkennen ist.

Von den Protonen H-5*a* ist nur das rechte Quartett bei τ 7.64 sichtbar. Beim Erhitzen wandert dieses Quartett, wie zu erwarten, in das Multiplett bei τ *ca.* 7.0. In der Kälte sind auch die OCH_3-Signale aufgespalten und fließen bei höherer Temperatur infolge freier Rotation zu einem Signal zusammen.

Aus den Quartettaufspaltungen der Protonen H-5*e* und H-5*a* und der Beobachtung von Proton H-2 mittels Doppelresonanz ließen sich die in Tab. III angegebenen Kopplungskonstanten ermitteln. Sie entsprechen den bei der *N*-Carbobenzoxy-α-D-*xylo*-piperidinose (5) gefundenen Werten und lassen sich am besten dahingehend interpretieren, daß die permethylierte *N*-Acetyl-D-*xylo*-piperidinose (9) ebenfalls nur in der Sesselkonformation und nur in der α-Form vorliegt. Ein zusätzliches Argument zum Beweis der α-Form ließ sich bei der *N*-Acetyl-D-*xylo*-piperidinose (1) erbringen.

An **1** konnte erstmalig eine "long-range" -Kopplung zwischen H-5*e* und H-1 über den Ringstickstoff hinweg von 1.0 Hz beobachtet werden[11]. Die Zuordnung erfolgte durch Doppelresonanz. Nach den bisherigen Vorstellungen über die Stereochemie von "long-range" -Kopplungen sollte nur bei 1,3-diäquatorialer Anordnung, also bei einer α-Form, eine Kopplung auftreten.

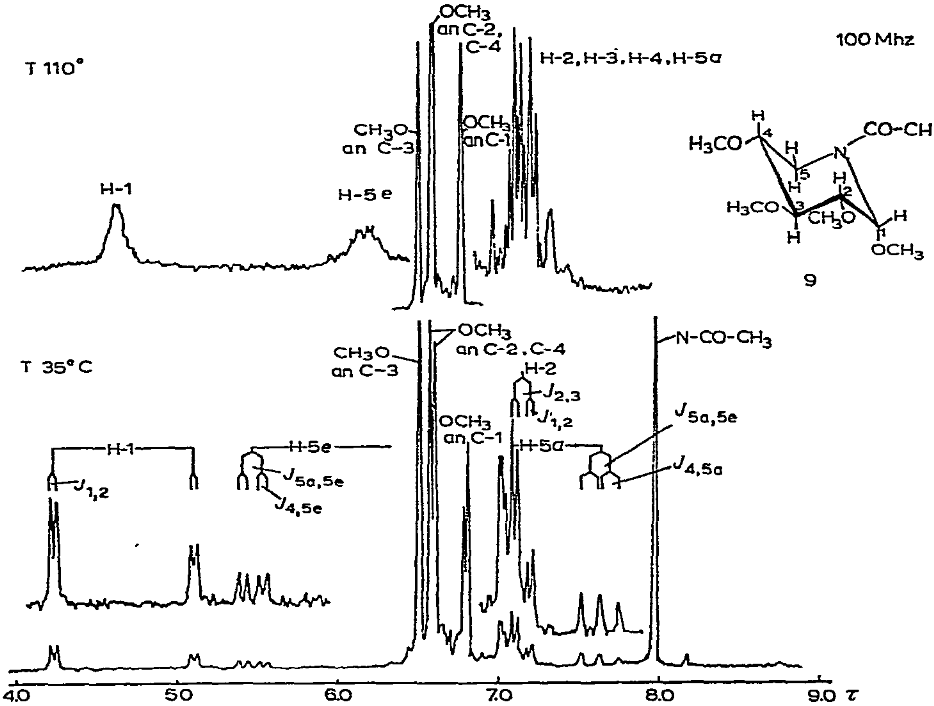


Abb. 3. NMR-Spektrum des Methyl-*N*-acetyl-2,3,4-tri-*O*-methyl-α-D-*xylo*-piperidinosids (**9**) bei 100 Mhz in C_2Cl_4. T 35 (unten) und 110° (oben).

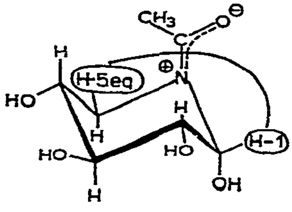
TABELLE III

ANALYSE DES NMR-SPEKTRUMS DES METHYL-*N*-ACETYL-2,3,4-TRI-*O*-METHYL-α-D-*xylo*-PIPERIDINOSIDS (**9**) BEI 100 Mhz.

Lösungsmittel: C_2Cl_4. TMS als innerer Standard. T 35°.

Signal	*τ (p.p.m.)*	*Kopplung (Hz)*	*Anordnung der Protonen*
CH_3–CO–N	7.97 Singulett		
CH_3–O(an C-1)	6.82 Dublett		
CH_3–O(an C-2 und C-4)	6.60 Dublett		
CH_3–O(an C-3)	6.52 Singulett		
H-1	4.25 und 5.12 Dublett	$J_{1,2}$ 3.5	e-a
H-2	7.20 Quartett	$J_{2,3}$ 9.5	a-a
H-5*e* (1/2)	5.49 Quartett	$J_{4,5e}$ 5.5	a-e
H-5*a* (1/2)	7.64 Quartett	$J_{4,5a}$ 11.0	a-a
		$J_{5e,5a}$ 12.5	geminal

Die bisher bekannten *N*-Acylpiperidinosen und *N*-Acylpiperidinoside der D-Xylose-Reihe bevorzugen somit ausschließlich die α-D-Form. Sie zeigen also einen viel stärkeren anomeren Effekt[12] als die entsprechenden sauerstoffhaltigen Pyranoseringe. Eine Erklärung dafür läßt sich geben, wenn man die Stereochemie einer *N*-Acylpiperidinose betrachtet. Bei der *N*-Acetylpiperidinose **1** sollen z.B. infolge der



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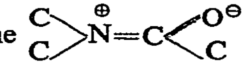
Amidmesomerie die Atome  $\begin{matrix}C\\C\end{matrix}\!>\overset{\oplus}{N}=C\!<\!\begin{matrix}O^{\ominus}\\C\end{matrix}$ in einer Ebene liegen. Dadurch gerät wie man leicht am Atommodell erkennen kann, der äquatoriale Substituent am C-1 in eine direkte ekliptische Stellung zur Amidgruppe. Ein größerer Substituent am C-1 wird daher der ekliptischen β-Form ausweichen und bevorzugt in die sterisch günstigere α-Form übergehen. Ähnliche Verhältnisse findet man bei Sechsring-Keto-Verbindungen, bei denen die benachbarten ekliptischen Substituenten gemäß der Brown–Brewster–Shechter–Regel[13] ein Instabilitätsmoment des Ringes hervorrufen.

Im Widerspruch zur α-Form steht die bei *N*-Acyl-D-*xylo*-piperidinosen beobachtete negative optische Drehung. Es wurden daher die Rotationsdispersionskurven (ORD) der *N*-Acylpiperidinosen und ihrer Derivate ausgemessen und mit denen der Methyl-D-xylopyranoside verglichen (vgl. Abb. 4).

Methyl-α-D-xylopyranosid weist, wie Abb. 4b zeigt, eine positive, die β-D-Verbindung eine negative Plain-Kurve auf. Die Richtung der Kurve wird durch einen bei *ca.* 180 mμ liegenden Cotton-Effekt (CE) bestimmt, welcher vermutlich durch den Ringsauerstoff als asymmetrischen Chromophor verursacht wird[14].

N-Acyl-α-D-*xylo*-piperidinosen (Abb. 4a) und Methyl-*N*-acyl-α-D-*xylo*-piperidinoside (Abb. 4b) zeigen dagegen negative Plain-Kurven und damit ein der Hudson'schen Regel[15] entgegengesetztes Verhalten. Offenbar bestimmt hier eine vorgelagerte langwelligere, optisch aktive Absorption der Amidgruppe die Richtung der Kurve. Bei der Kurve der *N*-Acetyl-α-D-*xylo*-piperidinose (**1**) ist bereits ein beginnender negativer CE bei etwa 200 mμ zu erkennen. Die gefundenen negativen Drehungen der *N*-Acyl-α-D-*xylo*-piperidinosen finden somit eine Erklärung.

*Darstellung und Eigenschaften von Methyl-α-*D-xylo*-piperidinosid*

Die Frage der Konfiguration am C-1 der D-*xylo*-Piperidinose[5] (**6**) mit freier Aminogruppe im Ring liess sich durch kernmagnetische Resonanz nicht lösen. Eine Analyse des Spektrums der mit BaO in D_2O aus D-*xylo*-Piperidinosyl-1-sulfonsäure[5] hergestellten Lösung von **6** war nicht möglich, da die im Vergleich zum Ringsauerstoff verstärkte Abschirmung des anomeren Protons durch die unsubstituierte Aminogruppe sein Signal in den Bereich der übrigen Ringprotonen verschiebt. Hinzu kommen die Bildung von D-*xylo*-3,4,5-Trihydroxy-Δ^1-piperidein (**4**) und die schon bei pH$<$7.8 einsetzende Amadori-Umlagerung zu **7**, die das Spektrum weiter komplizieren[5].

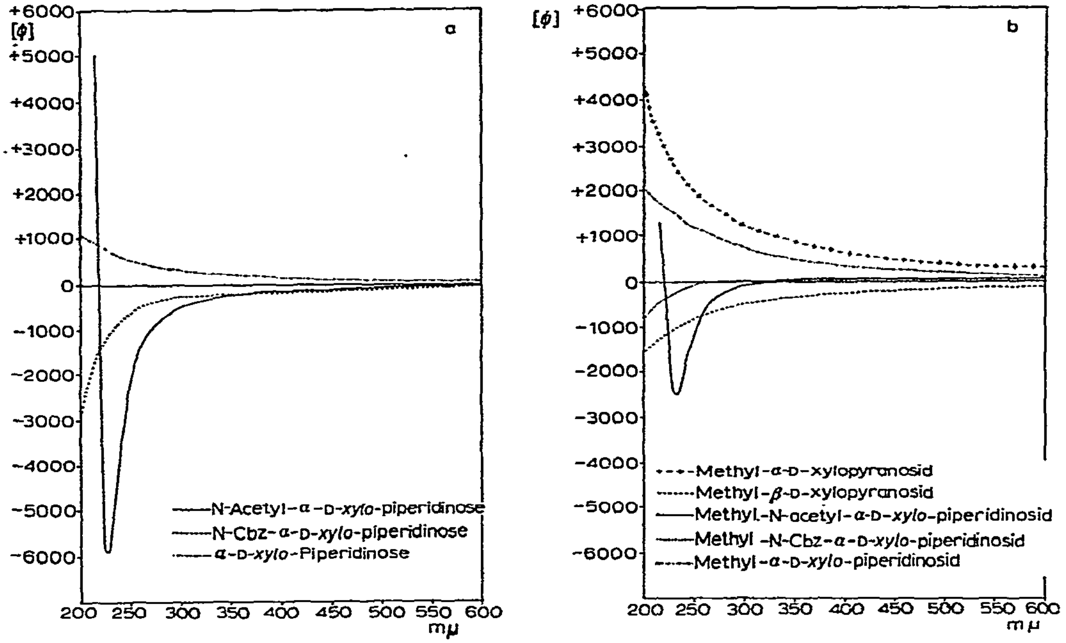
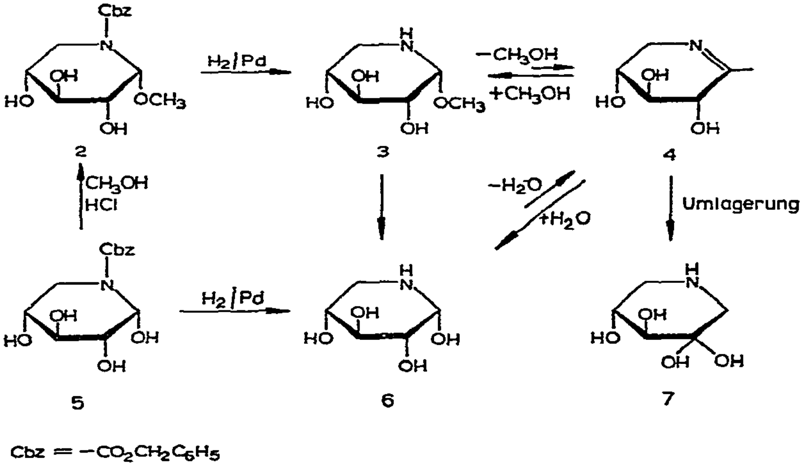


Abb. 4a und 4b. Optische Rotationsdispersion (ORD) der Piperidinosen und ihrer Methylglykoside, *c*, 1 mg/ml in Wasser (Piperidinosen) bzw. Methanol (Piperidinoside).

Versuche, Methyl-α-D-*xylo*-piperidinosid (**3**) durch direkte Umsetzung der Piperidinose **6** mit Methanol unter den verschiedensten Bedingungen darzustellen,



Cbz = $-CO_2CH_2C_6H_5$

waren ohne Erfolg. Stets wurde **6** im Gemisch mit den Sekundärprodukten **4** und **7** zurückerhalten. Es wurde daher versucht, **3** über das Piperidinosid **2** zu gewinnen, welches leicht aus *N*-Carbobenzoxy-α-D-*xylo*-piperidinose **5** mit Methanol–HCl dargestellt werden kann.

Die Hydrierung von **2** in absolutem Methanol mit Pd lieferte eine Lösung von **3**, die als Nebenprodukt wenig 1,5-Didesoxy-1,5-iminoxylit[5] enthielt. Das Piperidinosid **3** mit freier Aminogruppe im Ring erwies sich als recht instabil. Bereits durch Wasser wird **3** schnell unter Bildung von **6** gespalten, welches teilweise zum Piperidein **4** und Amadori-Umlagerungsprodukt **7** weiterreagiert. Eine Isolierung von reinem Glykosid **3** war nicht möglich, da beim Einengen der methanolischen Lösung teilweise Methanolabspaltung zu **4** eintritt. Der erhaltene Sirup enthält stets Piperidein **4** und durch Spaltung mit Spuren von Wasser gebildete Piperidinose **6**. Methylpiperidinoside mit freier Aminogruppe im Ring verhalten sich somit hinsichtlich ihrer Stabilität grundsätzlich anders als normale Methylpyranoside.

Untersucht man eine Lösung von **3** mit Hilfe des Circulardichroismus (CD), so findet man auch bei sorgfältigstem Wasserausschluss bereits nach *ca.* 1 Stunde einen geringen negativen Cotton-Effekt (CE) bei 300 mμ. Dieser CE ist dem Piperidein **4** zuzuordnen, welches einen asymmetrischen C=N Chromophor besitzt[5]. Der Versuch beweist, daß das Piperidinosid **3** in der Lage ist, bereits in methanolischer Lösung spontan Methanol unter Bildung von **4** abzuspalten. Es stellt sich offensichtlich ein Gleichgewicht zwischen Piperidinosid **3** und Piperidein **4** ein, welches weitgehend auf der Seite von **3** liegt, und dessen Anteile an **4** durch CD-Messungen einwandfrei nachweisbar sind.

Während das Methyl-*N*-carbobenzoxy-α-D-*xylo*-piperidinosid **2** eine negative Plain-Kurve bei der ORD-Messung aufweist, zeigt die frisch dargestellte Lösung von Piperidinosid **3** umgekehrt eine positive Plain-Kurve (Abb. 4b). Da nicht anzunehmen ist, daß bei der hydrierenden Abspaltung der Carbobenzoxy-Gruppe eine Umkehr der Konfiguration am C-1 eingetreten ist, sollte **3** ebenfalls in der α-Form vorliegen. Das Piperidinosid **3** gibt dann, da der langwellige Chromophor abgespalten ist, wieder eine Plain-Kurve, die der Kurve des Methyl-α-D-xylopyranosids entspricht.

Untersucht man die ORD-Kurve einer durch Hydrierung von **5** oder Zersetzung von D-*xylo*-Piperidinosyl-1-sulfonsäure frisch dargestellten Lösung der Piperidinose **6**, so findet man ebenfalls eine positive Plain-Kurve (Abb. 4a). Es ist demnach zu vermuten, daß auch die freie D-*xylo*-Piperidinose **6** bevorzugt in einer α-D-Form vorliegt.

EXPERIMENTELLER TEIL

*Methyl-*N*-carbobenzoxy-α-D-*xylo*-piperidinosid* (**2**)

N-Carbobenzoxy-α-D-*xylo*-piperidinose **5** (136 mg) wurde in Methanol (21 ml), das 1.5% HCl enthielt, gelöst und drei Tage bei Raumtemperatur aufbewahrt. Nach dieser Zeit ließen sich dünnschichtchromatographisch (Kieselgel G mit Benzol–Äthanol, 3:1+3.2% Wasser) nur noch Spuren der Ausgangsverbindung nachweisen.

Die Lösung wurde mit Blei(II)-carbonat neutralisiert, die Bleisalze abfiltriert und mit Methanol gewaschen. Die im Vakuum eingeengte Lösung ergab einen farblosen Sirup, der bald kristallisierte. Umkristallisation aus wenig Methanol unter Zusatz von Essigester. Ausbeute 110 mg (77%); Schmp. 85–87°; $[\alpha]_D^{20} + 9.2°$ (*c* 1, Methanol) (Gef.: C, 55.90; H, 6.40; N, 4.56. $C_{14}H_{19}NO_6$. Ber.: C, 56.54; H, 6.44; N, 4.71%).

*Methyl-α-*D-xylo-*piperidinosid* (3)

2 (76 mg) wurde in absolutem Methanol (10 ml) gelöst und mit Pd-Schwarz (10 mg) 10 Minuten bei Normaldruck hydriert. Die farblose Lösung wurde sofort vom Katalysator abfiltriert. Eine Ausbeutebestimmung in der Lösung kann wie bei der D-*xylo*-Piperidinose durch Umsetzung mit *o*-Aminobenzaldehyd durchgeführt werden[5]. Unter Voraussetzung eines quantitativen Umsatzes ergeben sich dabei Ausbeuten von *ca.* 65% an **3**. Das Dünnschichtchromatogramm [Kieselgel G, Dioxan–Wasser (5:1)] ist wegen der leichten Spaltbarkeit des Piperidinosids stets identisch mit dem der D-*xylo*-Piperidinose **6**. Die CD- und ORD-Kurven der frisch hergestellten Lösung zeigen die Abwesenheit des Piperideins **4**. Nach 3 Stunden beobachtet man bei 300 mμ eine circulardichroitische optische Dichte von $\Delta D - 5 \times 10^{-4}$, die nach einem Tag auf $\Delta D - 15 \times 10^{-4}$ und nach zwei Tagen auf $\Delta D - 20 \times 10^{-4}$ angestiegen ist. Dann treten Zersetzungserscheinungen auf, die schnell zu einer Gelbfärbung der Lösung führen und weitere Messungen verhindern.

*Methyl-*N*-acetyl-2,3,4-tri-*O*-methyl-α-*D-xylo-*piperidinosid* (9)[*]

N-Acetyl-α-D-*xylo*-piperidinose[2] **1** (0.5 g) wurde in Dimethylformamid (10 ml) gelöst und Methyljodid (2.5 ml) zugegeben. Nach Abkühlen auf 0° wurde Bariumoxid (2.5 g) und Bariumhydroxid (1 g) zugegeben und zunächst 5 Stunden bei 0°, dann 20 Stunden bei 20° geschüttelt. Dann wurden die Salze abzentrifugiert und mit Chloroform ausgewaschen. Die Lösung wurde mit Wasser (17 ml), dem Natriumthiosulfat (5 mg) zugesetzt worden war, und dann mit Wasser (17 ml) gewaschen. Nach Trocknen über Natriumsulfat ergab das Abdampfen an der Ölpumpe einen Sirup, der durch Vakuumdestillation bei 10^{-6} Torr und 70° gereinigt wurde. Ausb. 0.591 g (91%); $[\alpha]_D^{20} + 5.5°$ (*c* 1, Chloroform) (Gef.: C, 53.10; H, 8.51; N, 5.61. $C_{11}H_{21}NO_5$. Ber.: C, 53.42; H, 8.56; N, 5.66%).

*1,2,3,4-Tetra-*O*-acetyl-*N*-carbobenzoxy-α-*D-xylo-*piperidinose* (8)

N-Carbobenzoxy-α-D-*xylo*-piperidinose **5** (551 mg) wurde in Pyridin (5 ml) unter Zusatz von Acetanhydrid (1.3 ml) 20 Stunden bei Raumtemperatur gehalten. Das Pyridin wurde im Vakuum abdestilliert und der Rückstand in Chloroform (15 ml) aufgenommen. Die Lösung wurde mit Wasser (5 ml), $NaHCO_3$-Lösung (5 ml) und nochmals mit Wasser (10 ml) gewaschen, das Chloroform im Vakuum abdestilliert, der farblose Sirup mehrfach mit Äthanol abgedampft und an der Ölpumpe scharf

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[*] In der kürzlich erschienenen Arbeit von K. Heyns und D. Müller, *Tetrahedron*, 21 (1965) 3151, wurde die Verbindung irrtümlicherweise in der β-D-Form formuliert.

getrocknet. Ausbeute 710 mg (81%); $[\alpha]_D^{20}+20.3°$ (*c* 1, Chloroform) (Gef.: C, 56.00; H, 5.75; N, 3.03. $C_{21}H_{25}NO_{10}$. Ber.: C, 55.87; H, 5.59; N, 3.10%).

Die NMR-Spektren wurden aufgenommen mit einem VARIAN HA-100 Spektrometer unter Verwendung von D_2O (Natrium-2,2-dimethyl-2-silapentan-5-sulfonat als innerer Standard) sowie $CDCl_3$ und C_2Cl_4 (innerer Standard : TMS) als Lösungsmittel. Die optische Rotationsdispersion wurde mit einem Spektropolarimeter Cary 60, der Circulardichroismus mit einem Roussel-Jouan-Dichrographen gemessen.

DANK

Herrn Prof. Dr. K. Heyns sei für sein Interesse und die stete Förderung der Untersuchungen gedankt.

ZUSAMMENFASSUNG

N-Acyl-D-*xylo*-piperidinosen und deren Derivate liegen, wie durch eine vollständige Analyse aller Kopplungskonstanten der NMR-Spektren festgestellt wurde, in der Sesselkonformation vor. Die bisher bekannten Verbindungen der D-Xylose-Reihe treten nur in der α-Form auf. Für den starken anomeren Effekt wird die ungünstige Stereochemie der β-Form mit ekliptischer Stellung des äquatorialen Substituenten zur Amidgruppe verantwortlich gemacht. *N*-Acyl-α-D-*xylo*-piperidinoside zeigen bei ORD-Messungen negative Plain-Kurven und damit ein den normalen α-D-Glykosiden entgegengesetztes Verhalten.

Methyl-α-D-*xylo*-piperidinosid (**3**) liegt in Methanol im Gleichgewicht mit D-*xylo*-3,4,5-Trihydroxy-Δ^1-piperidein (**4**) vor. Piperidinoside mit freier Aminogruppe im Ring wie **3** sind so labil, daß sie bereits durch Wasser gespalten werden.

SUMMARY

5-Acylamido-5-deoxy-D-xylopyranoses (*N*-acyl-D-*xylo*-piperidinoses) and their derivatives are shown, by complete analysis of all the coupling constants in their n.m.r. spectra, to exist in a chair conformation. All known "piperidinoses" in the D-xylose series occur only as the α-D anomer. The unfavourable stereochemistry of the β-D anomer, where the equatorial substituent is in the eclipsed position with respect to the amide group, is proposed as the cause of the pronounced anomeric effect. *N*-Acyl-α-D-*xylo*-piperidinosides show negative, plain o.r.d. curves in contrast to the positive, plain o.r.d. curves shown by α-D-glycopyranosides.

In methanolic solution, methyl 5-amino-5-deoxy-α-D-xylopyranoside (**3**) exists in equilibrium with D-*xylo*-3,4,5-trihydroxy-Δ^1-piperideine (**4**). "Piperidinosides" such as (**3**), having a free amino group in the ring, are so sensitive that the methoxyl group undergoes hydrolysis in water alone.

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STUDIES OF ONUPHIC ACID
PART I. EXTRACTION AND GENERAL CHARACTERISATION

F. G. E. PAUTARD AND H. ZOLA
Astbury Department of Biophysics, The University, Leeds 2 (Great Britain)

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INTRODUCTION

Many marine annelids live within tubes of diverse form and composition. A few members of the *Errantia* manufacture a light, translucent, quill-like tube containing appreciable proportions of phosphorus, together with smaller proportions of calcium and magnesium. The relation of these elements to the rest of the tube substance has been a matter of speculation for nearly a century [1-4].

Recent studies of the tube of *H. tubicola* (see Fig. 1) have suggested that the structure is not homogeneous, but is composed principally of the metal salt of a sugar phosphate polymer[5], of high molecular weight, which has been called onuphic acid[6,7].

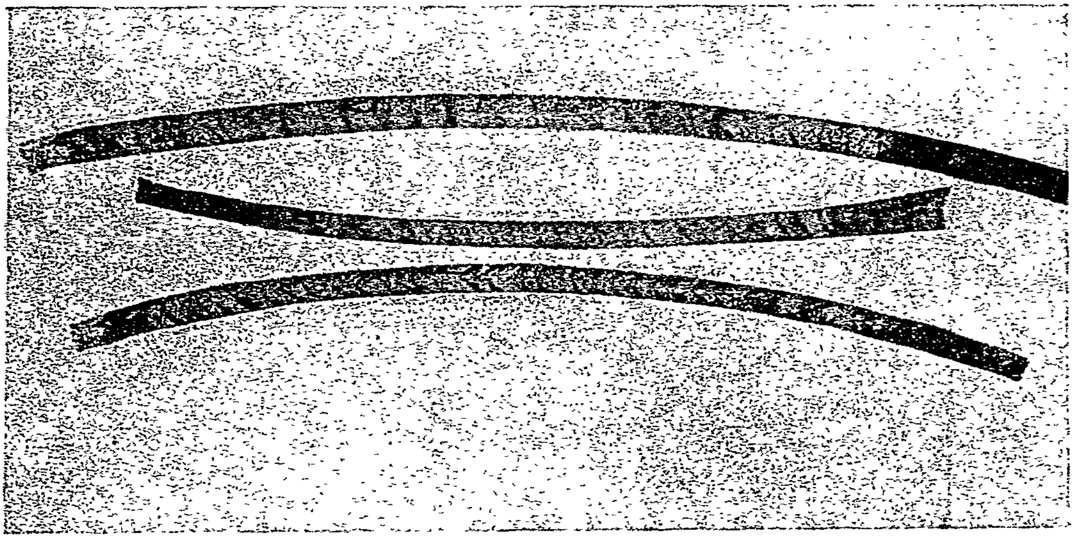


Fig. 1. Empty tubes of *Hyalinoecia tubicola*. × 1.2.

We present here an account of the extraction, purification, and general characteristics of onuphic acid as an introduction to more detailed studies of this new biological macromolecule.

MATERIALS AND METHODS

Whole tubes containing live *Hyalinoecia*, and empty tubes, were collected and identified by staff of the Marine Biological Laboratory, Plymouth; the majority of specimens were harvested by deep trawl at 40 fathoms about 5 miles off the Eddystone Lighthouse. Only clean, dried tubes were used for experiments.

Extraction procedure

The tubes were extracted with various media, generally 50 ml per g at 2°, and usually for 24 h with occasional shaking. Extracts were filtered at the pump through Carlson–Ford filter sheets; subsequent purification by precipitation with quaternary ammonium salts confirmed that the filtrate was not contaminated with cellulose from the filter. Cold ethanol was added, with mixing, to the chilled filtrate, until the supernatant gave no further precipitate with ethanol; the mixtures were kept for 4 h at 2° to aggregate. A more workable product was obtained if solid potassium chloride was added (to a concentration of 1%) before addition of ethanol. Precipitates were collected by centrifugation, dispersed under ethanol, and recentrifuged, and the process was repeated once more in ethanol, twice in acetone, and twice in ether. After the ether had been allowed to evaporate in air, the products were dried *in vacuo* over phosphorus pentoxide. For most studies, each preparation was redissolved in water and dialysed for 24 h against successive changes of deionised water, followed by further dialysis for 24 h against deionised water containing self-indicating ion-exchange resin ("Indicator Biodeminrolit", Permutit, Ltd.). Dialysis was carried out through pre-washed Visking 18/32 tubing at 2° with gentle stirring. All dialysates were filtered through paper and freeze-dried.

Analytical methods

Carbon, hydrogen, and nitrogen microanalyses were kindly carried out by Mr. J. A. Stewart of the Chemistry Department, Leeds University. Oxygen could not be determined because of the formation of phosphorus pentoxide. Other analyses were as follows: amino nitrogen by the micro-Kjeldahl method[8], calcium by the method of Weatherell[9] (magnesium interference [10] was below the limits of detection), magnesium by atomic absorption spectroscopy[11], inorganic phosphate by the Gomori[12] method. Silicate, arsenate, and germanate were shown to be absent[13].

Gel filtration was carried out on Sephadex G-200 gels[14]; water and M potassium chloride were used as eluants. Diethylaminoethyl-Sephadex (DEAE-Sephadex) gels[15] were eluted with potassium chloride, using the following linear gradients: 0 to 3M KCl in water; 0 to saturated KCl in 0.001M HCl; 0 to saturated KCl in 0.01M HCl; 0 to saturated KCl in 0.1M HCl. Eluates were recovered in 1-ml samples, and elution patterns were determined by the cysteine–sulphuric acid reaction[16].

Physical methods

Ultracentrifuge studies were carried out in a Beckman Spinco Model E instrument. A concentration of 1% in 0.2M potassium chloride was normally used.

Moving-boundary electrophoresis was carried out in a Perkin–Elmer Model 238 instrument, kindly made available by Mr. T. J. Bowen, of the Department of Biochemistry, Leeds University. A 15-mm Schlieren cell was used, and buffered solutions were dialysed against excess of buffer before electrophoresis.

Visible and ultraviolet spectra were determined on the Perkin–Elmer 137 u.v. instrument, and infrared spectra were obtained on a Grubb Parsons DB1 spectrophotometer, using the potassium bromide disc technique [17].

Optical rotation was measured in cells fitted with strain-free end windows in a Microptic photoelectric polarimeter (Hilger and Watts) incorporating a mercury discharge lamp (λ 5481Å, didymium filtered).

RESULTS

Extraction characteristics

When treated with acid, the hard, papery *Hyalinoecia* tubes swell to a gelatinous mass, many of the structural features of the tube wall are lost, and quantities of soluble substances appear in the supernatant liquid. The phosphorus in the extract cannot be removed by dialysis, but remains, in association with carbohydrate, as a clear solution. Precipitation with ethanol, followed by drying in acetone and ether, produces a white, hygroscopic powder, designated elsewhere[6,7] as onuphic acid.

Extraction conditions and yields. Hyalinoecia tubes contain approximately 9.5% of phosphorus. Virtually all of this can be recovered as onuphic acid (P, 14.4%), but, for reasons outlined below, a ready supply of tubes has enabled us to use mild conditions to prepare onuphic acid at frequent intervals in low yield, but in a high state of purity, thus avoiding both the severe or prolonged treatment necessary to extract the major bulk of the polymer and the uncertain state of the product after long storage.

Water, at room temperature and at boiling point, extracted no phosphorus or carbohydrate from the tube. At room temperature, dimethyl sulphoxide removed small proportions of a substance having a positive reaction with cysteine–sulphuric acid, but which contained no phosphorus.

A 10% solution of EDTA (ethylenediaminetetra-acetic acid, sodium salt) at pH 7.4 extracted all but a trace of cations, together with a proportion of substances of high molecular weight apparently containing *ca.* 7% of phosphorus. This product consisted of a mixture of EDTA and onuphic acid which could not be separated easily by simple dialysis. Repeated fractional precipitation with ethanol, in solutions containing 1% potassium chloride, removed the EDTA, leaving an onuphic acid salt closely resembling salts prepared in the same way after acid extraction. The rate of dialysis of EDTA was independent of the amount of onuphic acid present (for similar behaviour, see Watson *et al.*[18] and Gilbert and Swallow[19]).

Mineral acids (HCl, H_2SO_4, HNO_3, H_3PO_4) all extracted similar amounts of non-dialysable material which, when purified, contained the same amount (14.4%) of phosphorus. A series of experiments with N hydrochloric acid (Fig. 2) showed that

50% of the tube substance was removed in 24 h at 50°, whereas only 5% was removed at 2° under the same conditions. After 20 days in N hydrochloric acid at 2°, less than 15% of the tube substance was extracted; repeated treatment with fresh acid, however, removed 97% of the onuphic acid in 100 days.

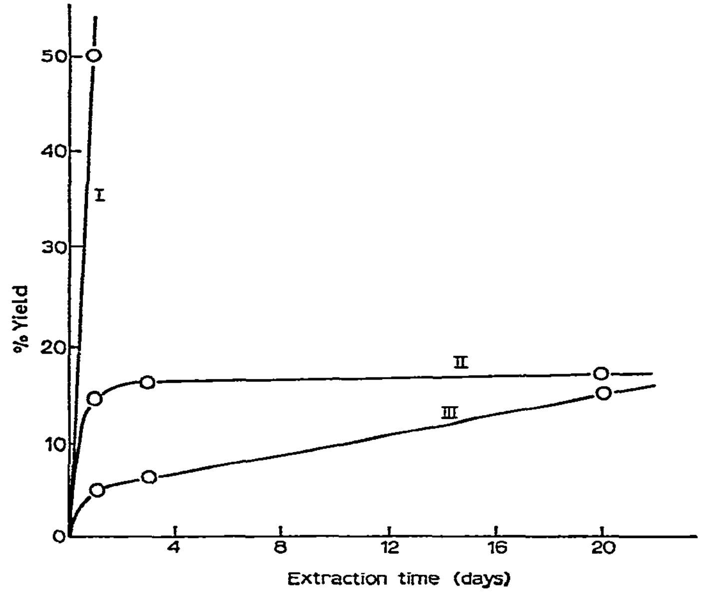


Fig. 2. Extraction of onuphic acid from tubes of *Hyalinoecia tubicola*, using N hydrochloric acid. I; 50°, whole tubes. II; 2°, crushed tubes. III; 2°, whole tubes.

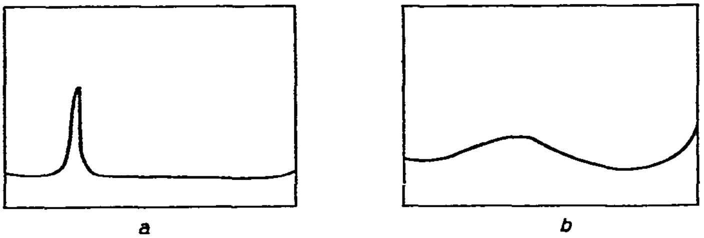


Fig. 3. Sedimentation pattern of onuphic acid (*a*) before and (*b*) after treatment with ballotini beads in a Mickle disintegrator.

Onuphic acid (both as a partial potassium salt and as the free acid) prepared from EDTA extracts, or under mild acid conditions (for example, N HCl at 2° for periods up to 20 days) gave the same sharp sedimentation pattern (Fig. 3*a*) and the same critical ethanol-solubility curve (Fig. 4*a*). With increasing strength of acid and with increasing time and temperature of extraction, the macromolecule became progressively degraded. The increase in polydispersity was reflected in diffuse sedimentation patterns (similar to Fig. 3*b*) and in extended ethanol-solubility curves (similar to Fig. 4*b*). In consequence, the onuphic acid used for further studies was prepared under conditions likely to limit degradation. As standard procedure, extraction with N hydrochloric acid for 24 h at 2°, or with 10% EDTA solution, pH 7.4 for 24 h at 2°, was chosen.

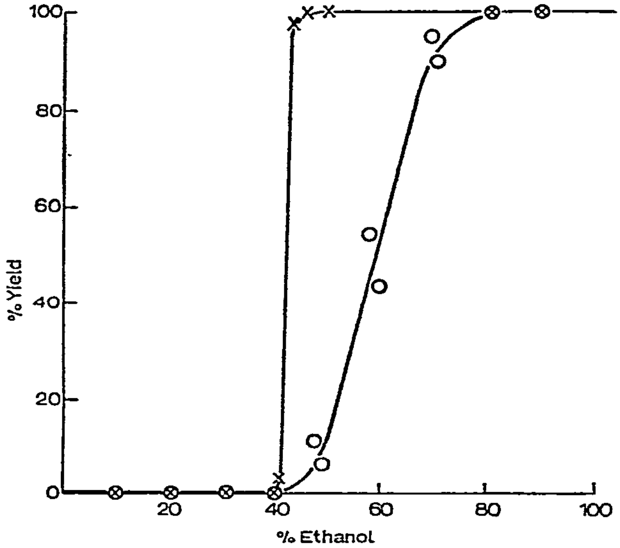


Fig. 4. Ethanol solubility curves of onuphic acid (*a*) extracted from whole tube (x—x) and (*b*) extracted from crushed tubes (o—o). Curves were obtained by adding chilled ethanol, in small aliquots, to chilled aqueous solutions of onuphic acid containing potassium chloride (1%). The solutions were stirred continuously. After precipitates had coagulated, they were collected by centrifugation, dried, and weighed; the supernatant was treated with further ethanol. All precipitations were carried out at 2°.

In a typical preparation, onuphic acid (250 mg, as partial potassium salt) was obtained from 5 g (about 25 tubes) of raw material. Fresh product was usually made for each series of experiments, since, although both the free acid and its potassium salts could be stored in the form of a dry powder for short periods of time at −10°, aqueous solutions underwent degradation, particularly at room temperature.

Mechanical degradation. Early preparations were made from coarsely milled tubes. This practice resulted in greatly increased yields, but the sedimentation patterns (similar to Fig. 3*b*) and the ethanol solubility curves (Fig. 4*b*) differed significantly from the customary, sharp, sedimentation pattern (Fig. 3*a*) and the critical ethanol-solubility curves (Fig. 4*a*) observed in preparations from whole tubes. Since the sedimentation patterns of fractions of "milled" onuphic acid obtained at low concentrations of ethanol were similar to the patterns of the unfractionated onuphic acid extracted from the whole tubes, and since all the forms of onuphic acid (from whole tubes, from crushed tubes, and after fractionation) gave the same figures for phosphorus, the same quantitative results in the cysteine–sulphuric acid reaction, and the same products of hydrolysis[20], it became clear that the increase in polydispersity was not due to the presence of other species of macromolecules, but might be caused by the fragmentation of the onuphic acid during milling.

Dry onuphic acid (partial potassium salt), prepared from whole tubes by the standard method of acid extraction described above and giving a sharp sedimentation peak (Fig. 3*a*), was shaken with ballotini beads in a Mickle disintegrator. The resulting sedimentation pattern (Fig. 3*b*) of the product (dissolved without further treatment) shows a significant increase in polydispersity.

We conclude from these experiments that the differences observed in the polydispersity of onuphic acid from whole and from crushed tubes may be the result of mechanical degradation and may not be caused by the extraction of separate species of macromolecule rendered more accessible by the milling process. Other reports of depolymerization brought about by stirring[21] and by sound waves[22] serve to emphasize the care which must be taken when removing substances of high molecular weight from natural sources.

PURIFICATION

The method of Scott[23] was used in attempts to prepare onuphic acid in a high state of purity. A 3% solution of cetylpyridinium bromide was added to a 0.5% solution of onuphic acid (potassium salt, at pH 7.0) in 0.03M potassium sulphate (to enhance coagulation) until no further flocculation was observed. After storage for 1 h, the suspension was centrifuged and the supernatant decanted. The precipitate was washed with ion-free water and dissolved in warm 2M potassium chloride, and the onuphic acid–detergent salt was reprecipitated by dilution. The washing, dissolution in 2M potassium chloride, and reprecipitation steps were repeated once. Ethanol was added to precipitate onuphic acid as a partial potassium salt, which was then dissolved in water and reprecipitated by the addition of further ethanol. The solution-precipitation procedure was repeated until the absorption at 260 mμ (due to pyridinium ions) was no longer detectable; the detergent-free onuphic acid was then dissolved in ion-free water, dialysed, and lyophilized. The cysteine–sulphuric acid reaction, and the analyses for phosphorus and the products of hydrolysis[20] of this material were closely similar to those obtained from the starting material; yields of 90% or thereabouts were recovered. The substances remaining in the supernatant after detergent precipitation were of similar composition to onuphic acid; no neutral polysaccharides were detected.

When prepared by ethanol precipitation in the presence of potassium chloride, onuphic acid always contained some potassium and detectable quantities of magnesium and calcium, even after dialysis. For all subsequent quantitative and physicochemical experiments, a highly purified product was desirable, and the cation content was therefore reduced by passing a 1 % solution down a column (300 × 18 mm) of Amberlite IR-120 resin (H^+ form). The effluent was examined for inorganic phosphate, and for carbohydrate content by the cysteine–sulphuric acid reaction, and the carbohydrate-containing fractions were combined and freeze-dried; all glassware was prewashed with EDTA and deionized water, and wherever possible, polyethylene apparatus was used. Under these conditions, no inorganic phosphate was detected in the effluent, and potassium, calcium, and magnesium were present in trace amounts only.

Standard, dry product. Onuphic acid contains water, even after extensive desiccation at room temperature, and experiments were carried out to establish a reliable, standard, dry product, from which calculations of elemental composition could be made. Samples of purified, cation-free onuphic acid were allowed to equilibrate for 12 h at various temperatures over phosphorus pentoxide, and the loss of weight was determined. The results (Fig. 5) show that up to 60° the weight loss is reversible; above 60°, the

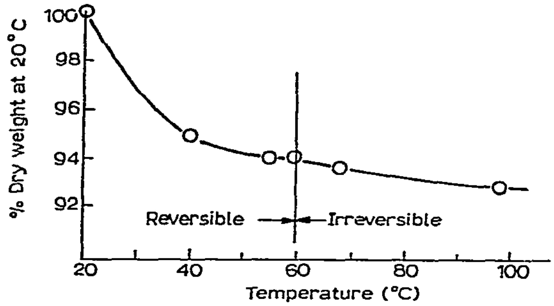
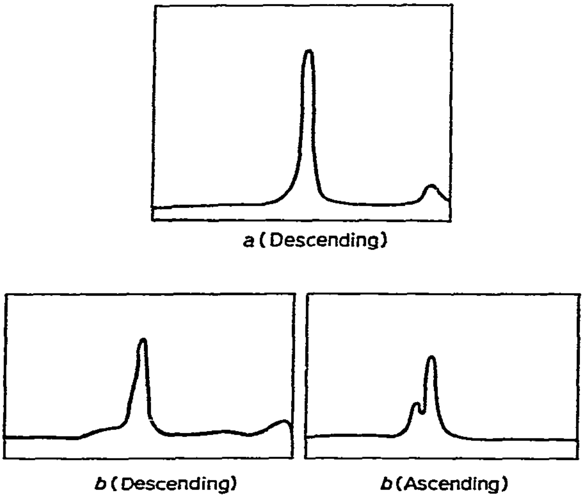


Fig. 5. Weight decrease in onuphic acid dried *in vacuo* over phosphorus pentoxide for 12 h at various temperatures.


a (Descending)

b (Descending) b (Ascending)

Fig. 6. Moving-boundary electrophoresis of onuphic acid; (*a*) veronal buffer, pH 8.6, descending limb; and (*b*) phosphate buffer, pH 6.4, descending and ascending limb.

weight loss is irreversible, suggesting decomposition. At 95°, onuphic acid chars. In general, because of the risk of degradation at elevated temperature, onuphic acid was dried *in vacuo* over phosphorus pentoxide at room temperature, and calculations were based on a water content of 6%.

HOMOGENEITY

The sedimentation patterns (*cf.* Fig. 3*a*), the ethanol solubility curves (*cf.* Fig. 4*a*), the detergent–precipitation characteristics, and the constant composition of ethanol fractions all suggest that onuphic acid extracted under mild conditions is a homogeneous, relatively paucidisperse macromolecule.

No substances of low molecular weight and of similar composition to onuphic acid were detected in any extract of whole tubes removed under mild conditions, or after decationization. Sephadex G200 gels excluded all phosphorus, and no resolution into components was observed for standard preparations as described above. The elution patterns from aqueous solutions and from solutions in M potassium chloride were essentially the same. Similarly, Sephadex G200 excluded all of the material present in the supernatant after treatment of onuphic acid with detergents. On columns of DEAE Sephadex, onuphic acid was bound strongly, resisting elution at normal electrolyte concentrations and remaining stable even in saturated aqueous potassium chloride at pH 1; no neutral polysaccharides were detected in the eluate.

Moving-boundary electrophoresis gave anomalous results. Onuphic acid (both partial potassium salt and decationized material) at concentrations of 0.5–1%, examined for periods up to 6 h, gave one peak only in veronal buffer at pH 8.6 (Fig. 6*a*) with the two arms of the cell showing enantiographic patterns, whereas, in phosphate buffer at pH 6.4, the patterns were non-enantiographic, and two peaks (Fig. 6*b*) of similar mobility appeared.

GENERAL PROPERTIES

The primary cysteine–sulphuric acid reaction[16] indicated that onuphic acid consisted principally of carbohydrate. The only types of monosaccharide found were hexose and 6-deoxyhexose. The carbazole–sulphuric acid reaction[16] gave negative results for hexuronic acid.

Onuphic acid showed no absorption maxima in the visible and near-ultraviolet spectral regions, but the optical density of aqueous solutions increased gradually with decreasing wavelength in the region 270–210 mμ; thereafter the optical density increased rapidly towards 200 mμ.

The principal absorption maxima in the infrared spectrum of decationised onuphic acid are shown in Table I.

Measurement of optical rotation was hampered by the difficulty of obtaining clear solutions of onuphic acid above 1% concentration. Previous figures[6] for impure material suggested a low specific rotation; present studies of purified onuphic acid

TABLE I

PRINCIPAL ABSORPTION PEAKS IN THE INFRARED SPECTRUM OF ONUPHIC ACID

Reference number	1	2	3	4	5	6	7	8	9
Wavelength (μ)	2.9	3.4	4.3	6.10	6.85	7.30	8.20	9.50 (very broad)	11.90
Possible origin	O–H	P–O–H	P–O–H	P(O)–OH	$-CH_2$	$C-CH_3$	P=O	P–O–C	α-D anomeric linkage

give $[\alpha]^{20}_{5461}$ $-4\pm2°$ (water). The specific rotation was not affected by replacing the acid solvent with potassium chloride, or by the addition of urea or dioxane.

ELEMENTAL ANALYSES

Qualitative analysis. Systematic, qualitative, elemental analysis[24] of the free acid detected only C, H, O, and P. In contrast with earlier findings[6,7], no nitrogen was found in any purified preparations of onuphic acid.

Phosphorus. Onuphic acid could not be degraded by calcining, since this method appeared to cause some volatilization of phosphorus pentoxide. In agreement with the results of Ogg[25], we found that in the Kjeldahl digestion procedure, the mixture HNO_3–H_2SO_4 gave higher results than did the mixture H_2SO_4–$HClO_4$. Carius digestion[26a] was considerably more time-consuming than the Kjeldahl digestion[26b] and did not lead to higher values. The finally adopted method involved destruction of the onuphic acid by the procedure of Steyermark[26b], followed by determination of phosphate by the method of Gomori[12]. Estimations by these methods gave a value of 13.5% for phosphorus, in accord with the figure recorded by Graham *et al.*[7]; by applying dry-weight corrections (see above), a figure of 14.4% has been obtained consistently for the phosphorus content of all purified preparations of onuphic acid.

Carbon. There is evidence[1,27] that large proportions of phosphorus can interfere with the analysis of carbon by oxidation. When an equal weight of cupric oxide was added to onuphic acid before oxidation, however, the values obtained for carbon (consistently between 22% and 23%) were not significantly different from those obtained by the oxidation of onuphic acid alone.

From these data, an approximate empirical formula of $C_{12}H_{22}O_{24}P_3$ is proposed for decationised onuphic acid.

DISCUSSION

It is clear from these results that 70% of the tube of *Hyalinoecia tubicola* can be extracted as onuphic acid, a nitrogen-free substance, of high molecular weight, containing carbohydrate and phosphorus. Although the easy removal of small amounts of the macromolecule under mild conditions using EDTA suggests that at least some of the parent substance may be held in place in the tube by salt linkages through divalent cations, native onuphic acid might still be degraded by EDTA by an oxidative-reductive process (see Ogston and Sherman[28]).

With precautions to avoid mechanical damage and with optimal conditions of preparation, the chemical and physical properties of the product suggest that, within the limitations of the methods used, onuphic acid is homogeneous, although it may be polydisperse. There is no evidence for anion-free polysaccharide in association with onuphic acid, and all attempts to divide the purified product into molecular species varying in composition have so far failed. While an increase in polydispersity has been demonstrated as a result of chemical and mechanical degradation, neither the whole products nor fractions from them vary significantly in phosphorus content. The evidence of the electrophoretic patterns might be explained best in terms of interaction, rather than in terms of heterogeneity, since similar observations have produced similar conclusions for such polyanions as heparin[29] and ribonucleate[30], and there are theoretical explanations[31,32] for behaviour of this kind which could be applied to onuphic acid.

Both the u.v. and i.r. spectra of onuphic acid are consistent with a structure containing carbohydrate and phosphorus. The u.v. spectrum resembles that obtained[33] for phosphorus oxyanions; the principal features of the i.r. spectrum compare closely with recent data[34–36] for phosphorus compounds. Peak 9 (Table II) suggests[18] the presence of the α-D anomeric linkage, but the absence of a band at 13 μ makes this conclusion less likely, as does the low optical rotation. There are no absorption features which indicate that aromatic or similar chromophoric groups are present.

The elemental analysis is not entirely compatible with a sugar phosphate polymer, but the data should be treated with reserve. Since phosphorus was determined as orthophosphate after oxidative hydrolysis, there is no clear evidence as to the state of the element in the native macromolecule. Again, the difficulties of a reliable analysis in a phosphorus-rich molecule of unknown configuration make the values for some elements, particularly carbon, uncertain. The absence of nitrogen and sulphur, however, greatly limits the number of structures which might be present.

ACKNOWLEDGMENTS

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SUMMARY

A standard procedure, using ethylenediaminetetra-acetic acid or mineral acid, is described for the extraction of onuphic acid, a phosphorus-rich carbohydrate of high molecular weight, from the tubes of the marine annelid *Hyalinoecia tubicola*. Under the conditions described, the product is nitrogen-free and homogenous, but, with increasing mechanical treatment, there is an increase in polydispersity. The macromolecule contains 14.4% of phosphorus, measured as inorganic phosphate after oxidation, and colour reactions suggest the presence of hexose and 6-deoxyhexose as the major organic constituents.

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SYNTHESIS OF 1-*C*-SUBSTITUTED, UNSATURATED KETOSES

YU. A. ZHDANOV, L. A. UZLOVA, AND G. N. DOROFEENKO
Rostov-on-Don State University (U.S.S.R.)

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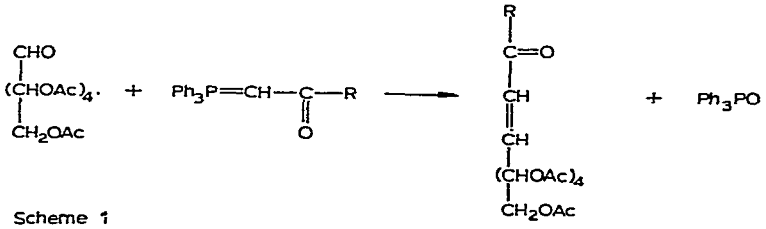
INTRODUCTION

Higher-carbon sugars, particularly ketoses, are often found in vegetable and bacterial raw-materials; for example, a nonulose (D-*erythro*-L-*gluco*-nonulose) was recently isolated[1] from the avocado. Methods for the isolation of higher ketoses[2-5], and for their synthesis by fermentation[6] and by chemical methods[7,8] are difficult and complicated. Preparative carbohydrate chemistry has only few methods for the synthesis of higher ketoses; of these, the Sowden–Fischer nitroalcohol method[9,10] is still the most important.

In seeking simple and convenient methods for lengthening the carbon chain of monosaccharides, we have applied the Wittig reaction to acyclic aldose acetates. The Wittig reaction offers the possibility of synthesising higher, α,β-unsaturated, aldonic acids (from aldoses[11] and acyclic aldoses[12]), *C*-glycosides[13], and unsaturated higher ketoses[14]. The synthesis of higher ketoses from acetylated aldehydo-forms of D-galactose, D-mannose, and D-glucose, using (aroyl- and acetyl-methylene)triphenylphosphoranes, may be represented by Scheme 1. The carbon chain of the hexose may be lengthened by two or three carbon atoms, depending on the structure of the initial phosphorylide. The vinylketonic group, which appears in the final products, may be used for the synthesis of various derivatives of higher ketoses.



Scheme 1

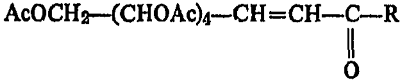
EXPERIMENTAL

General method for the preparation of (aroylmethylene)triphenylphosphoranes

A benzene solution of a bromomethyl ketone (0.01 mole) was mixed with a benzene solution of triphenylphosphine (0.01 mole). After several hours, the phosphonium salt was filtered off and washed with absolute ether. An equimolar quantity

TABLE I

HIGHER-CARBON, 1-*C*-SUBSTITUTED, α,β-UNSATURATED KETOSES



$$AcOCH_2{-}(CHOAc)_4{-}CH{=}CH{-}\underset{\underset{O}{\|}}{C}{-}R$$

No.	Configuration of monose	R	Yield, %	M.p., °	$[\alpha]_D^{18}$, °[d]	Found, % C	Found, % H	Formula	Calculated, % C	Calculated, % H
1	D-*gluco-*	methyl	23	119–120	—	52.66	6.01	$C_{19}H_{26}O_{11}$	53.02	6.04
2	D-*gluco-*	methyl	40	syrup	—	53.47	6.13	$C_{19}H_{26}O_{11}$	53.02	6.04
3	D-*galacto-*	methyl	62.3	142–143	+10.3	52.62	6.07	$C_{19}H_{26}O_{11}$	53.02	6.04
4	D-*gluco-*	phenyl[a]	40	syrup	—	58.77	5.26	$C_{24}H_{28}O_{11}$	58.54	5.69
5	D-*galacto-*	phenyl[a]	79	134–135	+22.0	58.39	5.61	$C_{24}H_{28}O_{11}$	58.54	5.69
6	D-*gluco-*	*p*-nitrophenyl[a]	60	syrup	—	54.24	5.34	$C_{24}H_{27}NO_{13}$	53.63	5.03
7	D-*galacto-*	*p*-nitrophenyl[a]	78.3	149–150	+16.4	53.73	4.92	$C_{24}H_{27}NO_{13}$	53.63	5.03
8	D-*manno-*	*p*-nitrophenyl[a]	35	syrup	—	54.18	5.27	$C_{24}H_{27}NO_{13}$	53.63	5.03
9	D-*gluco-*	*p*-methoxyphenyl[a]	—	syrup	—	—	—	$C_{25}H_{30}O_{12}$	57.47	5.74
10	D-*galacto-*	*p*-ethoxyphenyl	54	135–136	+18.4	58.14	6.00	$C_{26}H_{32}O_{12}$	58.20	5.97
11	D-*galacto-*	*p*-bromophenyl[b]	65	152–153	+17.7	50.83	5.00	$C_{24}H_{27}BrO_{11}$	50.43	4.73
12	D-*galacto-*	*p*-methoxyphenyl	70	140–141	+17.9	57.66	5.69	$C_{25}H_{30}O_{12}$	57.47	5.74
13	D-*galacto-*	1-naphthyl	50	150–151	+20.2	61.82	5.48	$C_{28}H_{30}O_{11}$	62.00	5.53
14	D-*galacto-*	3,4-dimethoxyphenyl	77	134–135	—	56.26	5.61	$C_{26}H_{32}O_{13}$	56.52	5.79
15	D-*galacto-*	2,3,4-trimethoxyphenyl	33	115–117	—	55.27	5.99	$C_{27}H_{34}O_{14}$	55.68	5.84
16	D-*galacto-*	1-furyl	68.8	133–134	+19.1	55.10	5.38	$C_{22}H_{26}O_{12}$	54.77	5.39
17	D-*galacto-*	1-thienyl[c]	70	160–161	+20.3	53.18	5.45	$C_{22}H_{26}O_{11}S$	53.01	5.30

[a] These ketoses were oxidised to the corresponding arylcarboxylic acid.

[b] Found: Br, 13.85. Calc.: Br, 14.00%.

[c] Found: S, 6.40. Calc.: S, 6.43%.

[d] Measured with a Hilger 412M polarimeter; (*c* 1, chloroform).

of a solution of sodium methoxide in methanol was then added to 0.008–0.01 mole of the phosphonium salt. The phosphoranes, thus obtained, were isolated by one of the methods previously described[15,16]. (Acetylmethylene)triphenylphosphorane[17] was synthesised[15] in 60–70% yield.

General method for the synthesis of 1-C-substituted, α,β-unsaturated ketoses

A solution (6–10 ml) of the *aldehydo*-D-hexose pentaacetate (0.001–0.0012 mole) and the (aroylmethylene)triphenylphosphorane (0.0012–0.0014 mole) in chloroform was heated on a boiling-water bath for 2–4 h. Completion of the reaction was indicated by absence of the hexose starting-material [analysis by thin-layer chromatography on alumina, using chloroform–benzene (2:3) or chloroform–toluene (13:10)]. After distillation of the chloroform, the residue was crystallised from methanol or acetone–methanol (1:4). Data on yields and properties of the higher ketoses thus prepared are given in Table I.

The syrupy products **4**, **6**, and **9** (Table I) were purified by Zemplén deacetylation, followed by removal of triphenylphosphine oxide with organic solvents, and subsequent acetylation with acetic anhydride in pyridine. Compound **8** was purified by an alternative procedure in which a solution of the carefully dried residue in absolute ether was repeatedly shaken with lithium bromide (which forms an ether-insoluble complex with triphenylphosphine oxide[18]), and then washed with water, dried, and concentrated.

Oxidation of 1-C-phenyloctuloses with alkaline permanganate

The octulose (0.3 g) was treated with an oxidising mixture consisting of potassium permanganate (1.5 g), water (15 ml), and 40% aqueous potassium hydroxide (0.5 ml). The suspension was heated under reflux on a boiling-water bath for 2 h. After being cooled, the solution was made weakly acid with sulphuric acid, and heated under reflux for 0.5 h. The precipitate was filtered off and washed thoroughly with ether. The ether layer was dried and concentrated, and the resulting, arylsubstituted carboxylic acids were recrystallised from water.

*Deacetylation of 2,3-dideoxy-1-C-phenyl-*D-galacto-*oct-2-en-1-ulose pentaacetate* (**5**)

To a suspension of 1-*C*-phenyloctulose (0.5 g) in absolute methanol (4 ml) was added 0.1N sodium methoxide solution (0.5 ml). After storage for a few minutes, the residue dissolved, and the solution became pink in colour. After 2 h, the solution was filtered and concentrated, and the syrupy residue was shaken with portions (3×25 ml) of ether saturated with water. The ether was decanted off, the residue was dissolved in methanol, and the solution was decolorised with alumina and concentrated to give a slowly crystallising syrup, ν_{max} (liquid film) 1675, 1645, 1632, 1599, and 1586 cm^{-1} (Found.: C, 59.05; H, 6.46. $C_{14}H_{18}O_6$ calc.: C, 59.57; H, 6.38%).

*Periodate oxidation of 2,3-dideoxy-1-C-phenyl-*D-galacto-*oct-2-en-1-ulose*

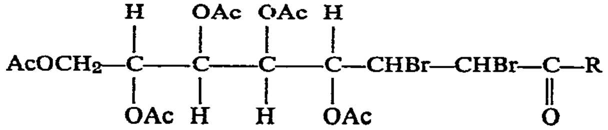
The periodate oxidation and determination of the formic acid liberated were performed according to the method of Fleury and Lange[22]. Oxidations in triplicate, performed with samples (30–50 mg) of this octulose, gave a periodate consumption of 3.96–4.12 mol., with liberation of 3.00–3.05 mol. of formic acid.

*The reaction of 2,3-dideoxy-1-*C*-(*p*-ethoxyphenyl)-*D-galacto*-oct-2-en-1-ulose* (**10**) *with bromine*

To a solution of ketose **10** (0.34 g) in chloroform (3 ml) was added a chloroform solution (1.5 ml) of bromine (0.1 g; 0.03 ml). The solution was then refluxed until it became colourless (10 min). Chloroform and traces of bromine were removed under vacuum to give the dibromide (90%), m.p. 148–149° (from methanol–acetone, 4:1). The dibromides of compounds **5**, **7**, and **12** (Table I) were similarly obtained, and their properties are recorded in Table II.

TABLE II

DIBROMIDES OF DERIVATIVES OF D-*galacto*-OCTULOSES



$$\mathrm{AcOCH_2{-}\overset{H}{\underset{OAc}{C}}{-}\overset{OAc}{\underset{H}{C}}{-}\overset{OAc}{\underset{H}{C}}{-}\overset{H}{\underset{OAc}{C}}{-}CHBr{-}CHBr{-}\underset{\underset{O}{\|}}{C}{-}R}$$

No.	R	Yield, %	M.p., °	Found, %			Formula	Calculated, %			ν_{max}, cm^{-1}
				C	H	Br		C	H	Br	
1	phenyl	90	syrup	44.63	4.35	24.25	$C_{24}H_{28}Br_2O_{11}$	44.17	4.30	24.54	1750, 1697, 1604, 1585
2	*p*-methoxyphenyl	90	144–146	43.67	4.51	23.11	$C_{25}H_{30}Br_2O_{12}$	43.99	4.40	23.46	1743, 1684, 1605, 1580
3	*p*-ethoxyphenyl	92	148–149	44.70	4.85	22.75	$C_{26}H_{32}Br_2O_{12}$	44.82	4.59	23.00	1748, 1683, 1608, 1578
4	*p*-nitrophenyl	85	syrup	40.89	4.19	22.60	$C_{24}H_{27}Br_2NO_{13}$	41.32	3.87	22.95	1748, 1700, 1607, 1590

*Reaction of 1-*C*-phenyl-substituted octuloses with phenylhydrazine*

(*a*) *Preparation of 5-*(D-galacto-*1,2,3,4,5-pentaacetoxypentyl*)*-1,3-diphenyl-2-pyrazoline* (**18**). (*i*) A mixture of the 1-*C*-phenyloctulose pentaacetate **5** (0.3 g) and phenylhydrazine (0.08 g) was dissolved in ethanol (12 ml), and the solution was refluxed for 3.5 h and concentrated to one-third of its original volume. Chloroform was added, and the solution was then washed with water (2×5 ml), dried (Na_2SO_4), and concentrated. On storage of the resulting dark-red syrup in a vacuum desiccator, crystallisation occurred to give compound **18** (0.32 g), m.p. 79–81° (from aqueous ethanol).

(*ii*) A solution of octulose **5** (0.4 g) and phenylhydrazine (0.2 g) in acetic acid (8 ml) was heated on a boiling-water bath for 1 h. The cooled solution was diluted with chloroform, washed with water (3×8 ml), dried, and concentrated to give dark, cherry-coloured crystals of compound **18** (0.28 g), m.p. 81–84° (from aqueous ethanol), ν_{max} (Nujol) 1752 and 1598 cm^{-1}, λ_{max} (methanol) 242 (log *E*, 4.105) and 344 mμ (3.650) (Found: C, 61.49; H, 5.43; N, 4.60 $C_{30}H_{34}N_2O_{10}$ calc.: C, 61.85; H, 5.83; N, 4.81%).

(*b*) *Preparation of phenylhydrazones.* (*i*) Compound **5** (0.15 g) and 2,4-dinitrophenylhydrazine (0.07 g) were dissolved in ethanol (25 ml), and the solution was refluxed for 4 h and cooled. The resulting precipitate was filtered off and washed with cold ethanol to give a quantitative yield of the orange 2,4-dinitrophenylhydrazone, m.p. 156–158° (from aqueous ethanol), ν_{max} (Nujol) 1754, 1624, 1597, and 1510 cm^{-1}, λ_{max} (methanol) 240 (log *E*, 4.264), 369 (4.430), and 378 mμ (4.418) (Found: C, 53.37; H, 4.90; $C_{30}H_{32}N_4O_{14}$ calc.: C, 53.57; H, 4.76%).

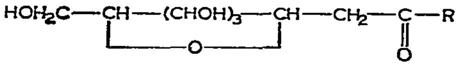
(*ii*) The 1-*C*-(*p*-nitrophenyl)octulose **7** was converted, using the method described in (*i*) above, into its phenylhydrazone, m.p. 144–145°, ν_{max} (Nujol) 1744, 1626, 1601, and 1517 cm^{-1}, λ_{max} (methanol) 242 (log ε, 4.266), 362 (4.427), and 380 mμ (4.420) (Found: C, 57.13; H, 5.50. $C_{30}H_{33}N_3O_{12}$ calc.: C, 57.41; H, 5.26%).

DISCUSSION

The products arising by condensation of Wittig reagents and acyclic aldose acetates are established as 1-*C*-substituted, unsaturated ketoses by infrared spectroscopy, oxidation with alkaline permanganate to give the corresponding substituted aromatic acids, periodate oxidation, and a number of other chemical transformations which confirm the presence of a double bond and a carbonyl group in the products obtained.

The i.r. spectra of the acetylated ketoses have absorption bands in the regions 1743–1750, 1662–1670, and 1624–1628 cm^{-1}. The first band characterises the ester groups of the ketoses, the second band is assigned to the carbonyl group of the unsaturated ketone, and the third band is assigned to the ethylenic bond of the chalcone type.

Deacetylation of certain of the α,β-unsaturated ketoses listed in Table I gave products that had i.r. bands characteristic of C=O and C=C bonds. These products



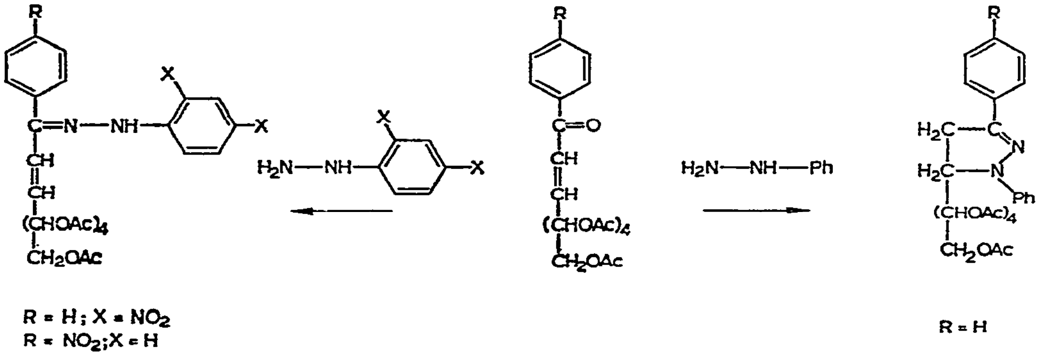
$$\mathrm{HOH_2C-\underbrace{CH-(CHOH)_3-CH}_{O}-CH_2-\underset{\underset{\displaystyle O}{\|}}{C}-R}$$

A

might be expected to form either cyclic hemiacetals or anhydrides (A) resulting from reaction of a hydroxyl group with the double bond. However, the product of deacetylation of **5** consumed 4.12 mol. of sodium periodate, with liberation of 3.05 mol. of formic acid, and this behaviour is consistent with an open-chain structure. The existence of acyclic forms of α,β-unsaturated octuloses may be compared with the *trans* structure assigned[19] to 3,4-dideoxy-D-*glycero*-hex-3-enulose by n.m.r. spectroscopy.

The higher α,β-unsaturated ketoses, synthesised by the Wittig reaction, display certain common reactions (electrophilic addition to the olefinic bond and carbonyl oxygen substitution) in which the 1-*C*-(*p*-nitrophenyl)-substituted octulose derivatives react more slowly than the other ketose derivatives. This influence of the *p*-nitro group in the C-1 substituent is also demonstrated by the instability of the products obtained by addition at the double bond. Thus, the dibromides of ketose derivatives **6**, **7**, and **8** (Table I), and the adduct of ketose derivative **7** with hydrogen bromide, are unstable and decompose when kept in a vacuum desiccator.

The carbonyl group in α,β-unsaturated ketones is somewhat unreactive, and the formation of phenylhydrazones from the 1-*C*-substituted octulose derivatives required vigorous conditions. However, when alkoxy groups are present in the aromatic ring of the ketose derivative, this reaction does not yield a phenylhydrazone. Instead, a diarylsubstituted pyrazoline is formed; a nitro group in the aromatic ring of the ketose derivative stabilises the phenylhydrazone (Scheme 2). The pyrazoline derivative obtained with octulose **5** was assigned the structure 5-(D-*galacto*-1,2,3,4,5-pentaacetoxypentyl)-1,3-diphenyl-2-pyrazoline. This structure was confirmed by the u.v. spectrum which showed maxima at 242 (log *E*, 4.105) and 344 mμ (3.650); 1,3,5-triarylsubstituted pyrazolines are reported[20,21] to show λ_{max} at 242–254 and 342–358 mμ.



Scheme 2

SUMMARY

Using the Wittig reaction, higher α,β-unsaturated ketose derivatives, having substituents at C-1, were synthesised from the acetylated aldehydo forms of D-glucose, D-galactose, and D-mannose, using (acetyl- and aroyl-methylene)triphenylphosphoranes. The structures of the ketose derivatives were examined by i.r. and u.v. spectro-

scopy. In the deacetylated products, the presence of acyclic ketose structures was indicated by periodate oxidation studies. Certain properties of the ketose derivatives are influenced by the nature of the substituent at C-1. The Wittig reaction enables olefinic bonds to be introduced into monosaccharides which may then be converted into 2,3-dideoxy derivatives.

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GRADED, ACID HYDROLYSIS OF SOME *O*-ISOPROPYLIDENE DERIVATIVES (INCLUDING *β-erythro*-KETALS) OF CARBOHYDRATES

J. S. BRIMACOMBE, A. B. FOSTER*, AND L. C. N. TUCKER
Chemistry Department, The University, Birmingham 15 (Great Britain)

(Received May 23rd, 1966)

INTRODUCTION

Although much is known[1] about the relative stabilities towards acid of cyclic acetal derivatives of carbohydrates, corresponding information on cyclic ketals is meagre. In the majority of cases, acid-catalysed condensation of ketones with cyclic, and acyclic, polyhydric alcohols affords 1,3-dioxolan derivatives and, for such compounds containing isolated ketal rings, the stability sequence *α-threo*** $>$ *α* in acid hydrolysis is well established[2]; the sequence *α*$\approx$*α-erythro* has been observed in one case[3]. The formation of 1,3-dioxane derivatives in ketone–carbohydrate reactions is relatively rare, and, until recently, only cyclic polyhydric alcohols had been found to give products of this type; in these cases, the 1,3-dioxan ring was part of a fused-[4] or bridged-ring[5] system. The direct, acid-catalysed condensation of acylic polyhydric alcohols with acetone has now been found to give 1,3-dioxane derivatives in certain cases[3,6,7], and other compounds of this type have been obtained indirectly[8]. A comparison of the relative stability towards acid of *O*-isopropylidene derivatives involving isolated 1,3-dioxane and 1,3-dioxolane rings has thus been made possible.

For example, 1,3-*O*-isopropylideneglycerol is noticeably[3] more sensitive towards acid than is the 1,2 derivative, and the sequence $\alpha > \beta$ has been observed[4] for a compound containing an isolated *α*-ketal and a *β*-ketal which was part of a ring system of the *cis*-decalin type. However, this stability sequence is reversed[8] where the *β*-ketal is part of a ring system of the *cis*-hydrindanone type. We now present corroborative evidence for the latter observation, together with further results which indicate the stability sequence *β-erythro* $>$ *α* for compounds containing isolated ketal rings.

RESULTS AND DISCUSSION

The acid-catalysed acetonation of D-*glycero*-D-*gulo*-heptono-1,4-lactone yields[8], principally, the 3,5:6,7-di-*O*-isopropylidene derivative (**1**). An analogy is provided

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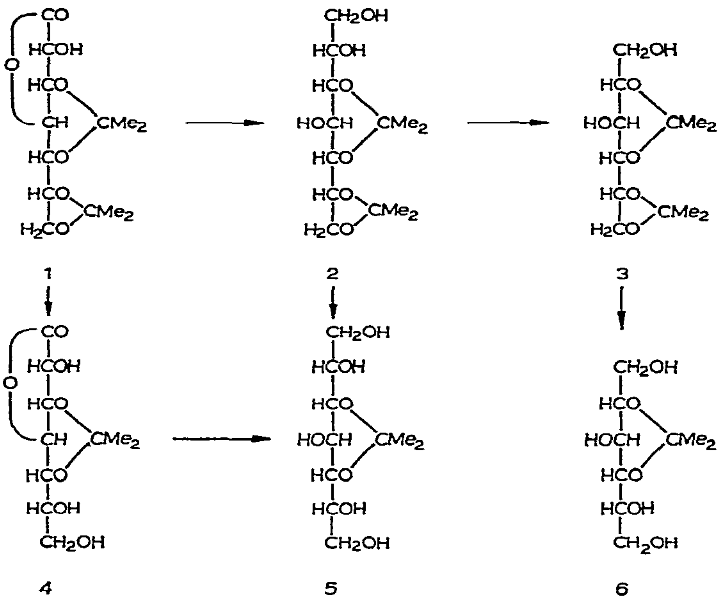
*Present address: Chester Beatty Research Institute, Institute of Cancer Research, Royal Marsden Hospital, Fulham Road, London, S.W.3.
**See *Carbohydrate Research*, 1 (1965) 329, for an explanation of this nomenclature.

by D-glucurono-6,2-lactone diethyl dithioacetal, which yields[6] a 2,4-*O*-isopropylidene derivative, presumably with contraction in size of the lactone ring. Compound **1** provides an indirect route for the synthesis of diketals **2** and **3**. Thus, reduction of diketal **1** with sodium borohydride gave 1,2:3,5-di-*O*-isopropylidene-L-*glycero*-L-*gulo*-heptitol (**2**) (isolated as the monohydrate), which was readily converted into 1,2:3,5-di-*O*-isopropylidene-L-gulitol (**3**) by oxidation with periodate and subsequent reduction with borohydride[8].



The graded, acid hydrolysis of di- and tri-ketal derivatives of polyhydric alcohols may be conveniently followed[3,4] by using n.m.r. spectroscopy to observe the change in pattern of the signals for the protons of the isopropylidene Me groups. The n.m.r. spectrum (methanol–water, 3:1) of diketal **1** showed, *inter alia*, Me proton signals at τ 8.64, 8.74, and 8.79, having integrated areas in the ratio *ca.* 1:1:2. Addition of toluene-*p*-sulphonic acid to the aqueous methanolic solution resulted in simplification of the Me proton resonances to two signals, of similar integrated areas, at τ 8.64 and 8.79. This pattern was also exhibited by the monoketal formed on brief treatment of compound **1** with 0.1N hydrochloric acid at 30–40°. The monoketal was identified as 3,5-*O*-isopropylidene-D-*glycero*-D-*gulo*-heptono-1,4-lactone (**4**), since reduction with sodium borohydride converted it into an optically inactive heptitol derivative **5** which consumed 1.9 mol. of periodate without liberation of acid.

In aqueous methanol containing toluene-*p*-sulphonic acid, both ketal groups were removed from compounds **2** and **3** although, in each case, a slight difference in rate could be discerned from the change in the relative intensities of the signals for the Me protons in the n.m.r. spectrum. Graded, acid hydrolysis was best carried out in 0.1N hydrochloric acid at room temperature. In this manner, compound **2** was converted into 3,5-*O*-isopropylidene-*meso-glycero-gulo*-heptitol (**5**), which was identical with the product obtained by reduction of compound **4**. The monoketal obtained by graded, acid hydrolysis of diketal **3** was identified as 2,4-*O*-isopropylidene-D-glucitol (**6**) by comparison with an authentic sample[6,*], and by its conversion into L-xylose (identified chromatographically) by periodate oxidation and subsequent acid hydrolysis.

Thus, graded, acid hydrolysis of 3,5:6,7-di-*O*-isopropylidene-D-*glycero*-D-*gulo*-heptono-1,4-lactone (**1**) reflects the sequence *β-erythro*>*α* for ketal stability, with a relatively marked stability of the *β-erythro*-ketal. The same, but much less marked, stability sequence is found with the 1,2:3,5-di-*O*-isopropylidene-derivatives [(**2**) and (**3**), respectively] of L-*glycero*-L-*gulo*-heptitol and L-gulitol. The greater stability towards dilute acid of the six-membered cyclic ketal in 1,2:3,5-di-*O*-isopropylidene-L-gulitol (2,4:5,6-di-*O*-isopropylidene-D-glucitol) contrasts with the fact that acid-catalysed acetonation of 3-*O*-methyl-D-glucitol affords[7] mainly the 1,2:5,6 diketal, with only a small proportion of the 2,4:5,6 isomer.

EXPERIMENTAL

Thin-layer chromatography (t.l.c.) was performed on Kieselgel G, using acetone and detection with vanillin–sulphuric acid[9]. N.m.r. spectra were obtained, for *ca.* 20% solutions, with a Varian A-60 spectrometer under normal working conditions and a 6% solution of tetramethylsilane in chloroform as external reference. Infrared spectra were recorded for Nujol mulls, using a Perkin–Elmer Infracord spectrometer. Light petroleum refers to the fraction having b.p. 60–80°, unless otherwise stated.

3,5:6,7-Di-*O*-isopropylidene-D-*glycero*-D-*gulo*-heptono-1,4-lactone, m.p. 153–154°, $[\alpha]_D$ $-76°$ (*c* 2, chloroform); 1,2:3,5-di-*O*-isopropylidene-L-*glycero*-L-*gulo*-heptitol monohydrate, m.p. 67–68°, $[\alpha]_D$ $-6°$ (*c* 2, water); and 1,2:3,5-di-*O*-isopropylidene-L-gulitol, m.p. 117–118°, $[\alpha]_D$ $+2°$ (*c* 1, methanol) were prepared by the procedures previously described[8b].

*Graded, acid hydrolysis of 3,5:6,7-di-O-isopropylidene-*D-glycero-D-gulo-*heptono-1,4-lactone* (1)

(*a*) A solution of compound **1** (1 g) in 0.1N hydrochloric acid (100 ml) was stored at 30–40° for 15 min; t.l.c. then indicated that hydrolysis of the *α*-ketal was essentially complete. The solution was extracted with chloroform (2×50 ml) to remove unchanged starting material, and the aqueous solution was neutralised with Amberlite IRA-400 (CO_3^{2-} form, 25 ml) and concentrated. A solution of the syrupy residue in chloroform (100 ml) was dried ($MgSO_4$) and concentrated, to give a solid which, on recrystallisation from methanol–chloroform–light petroleum, gave 3,5-*O*-isopropylidene-D-*glycero*-D-*gulo*-heptono-1,4-lactone (**4**) (0.8 g), m.p. 158–159°, $[\alpha]_D^{30}$ $-75°$ (*c* 1, ethanol), ν_{max} 1758 and 1778 cm^{-1} (Found: C, 48.8; H, 6.3. $C_{10}H_{16}O_7$

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[*] Kindly provided by Dr. P. A. J. Gorin.

calc.: C, 48.4; H, 6.5%). The monoketal (4) consumed 1.2 mol. of periodate (spectrophotometric determination[10]) within 5 min.

(*b*) A solution of compound **1** (0.2 g) in methanol–water (2 ml, 3:1, v/v) containing toluene-*p*-sulphonic acid (3.3 mg) was stored at room temperature. The change in pattern of the Me proton signals was followed by n.m.r. spectroscopy (see Discussion).

3,5-O-Isopropylidene-meso-glycero-gulo-*heptitol* (5)

Sodium borohydride (0.25 g) was gradually added to a solution of lactone **4** (0.5 g) in ethanol (25 ml), and the solution was stored overnight. The solution was then concentrated, and cations were removed from an aqueous solution of the residue by passage over Amberlite IR-120 (NH_4^+ form, 25 ml). The eluate and washings were concentrated, and methanol was distilled several times from the residue, which slowly crystallised. Two recrystallisations from methanol–chloroform–light petroleum afforded compound **5** (0.36 g), m.p. 172–173°, $[\alpha]_D$ ±0° (*c* 2, methanol) (Found: C, 47.3; H, 8.2. $C_{10}H_{20}O_7$ calc.: C, 47.6; H, 8.0%). Compound **5** consumed 1.9 mol. of periodate (5, 15 min), without liberation of acid.

Graded, acid hydrolysis of 1,2:3,5-di-O-isopropylidene-L-glycero-L-gulo-*heptitol monohydrate* (2)

Conditions which gave an optimal yield of monoketal were ascertained by small-scale experiments, using t.l.c.

A solution of compound **2** (0.31 g) in 0.1N hydrochloric acid (50 ml) was stored at room temperature for 15 min and then neutralised by the addition of a few drops of conc. ammonia. The residue, obtained on removal of the solvent, was extracted with dry acetone, and the extract was concentrated. Recrystallisation of the residue from methanol–chloroform–light petroleum (b.p. 80–100°) gave 3,5-*O*-isopropylidene-*meso-glycero-gulo*-heptitol (**5**) (91 mg), m.p. 169–170°, $[\alpha]_D$ ±0° (*c* 2, methanol). The m.p. was not depressed on admixture with compound **5** prepared in the previous experiment, and the i.r. spectra of the two compounds were indistinguishable.

The n.m.r. spectrum of compound (**2**) in methanol–water (3:1) showed Me proton signals at τ 8.71, 8.75, and 8.79 (integrated areas in the ratio *ca.* 1:2:1); monoketal (**5**) gave corresponding signals at τ 8.80 and 8.75 (strength ratio, *ca.* 1:1).

*Graded, acid hydrolysis of 1,2:3,5-di-O-isopropylidene-*L*-gulitol* (3)

A solution of compound **3** (0.2 g) in 0.1N hydrochloric acid (10 ml) was stored at *ca.* 25° for 5 min. The hydrolysate was neutralised with conc. ammonia and concentrated, and the residue was extracted with acetone. The extract was concentrated, and the residue was chromatographed on Kieselgel G (20 ml). Elution with acetone (15 ml) removed starting material, and subsequent elution with methanol afforded the monoketal. The recovered starting material was re-treated with acid, and the product mixture was fractionated as above. In this way, 2,4-*O*-isopropylidene-D-glucitol (**6**; 45 mg) was obtained; m.p. 167–168° (from acetone), $[\alpha]_D$ +11° (*c* 0.33, methanol).

Gorin[6] recorded m.p. 158–160°, $[\alpha]_D$ +11° (methanol), for this compound prepared by an alternative route. The t.l.c. properties (R_F 0.6) and X-ray diffraction patterns of the two compounds were indistinguishable.

Compound **6** rapidly consumed 0.95 mol. of periodate, and afforded a single product (R_F 0.7) which was hydrolysed in aqueous solution at 50° for 2 h with Amberlite IR-120 (H^+ form, 0.5 ml) to give a product having indistinguishable properties from those of D-xylose on paper chromatograms.

The n.m.r. spectrum of compound (**3**) in methanol–water (3:1) showed Me proton signals at τ 8.69, 8.74, and 8.81 (strength ratio, *ca.* 1:2:1); monoketal (**6**) gave corresponding signals at 8.74 and 8.81 (strength ratio, *ca.* 1:1).

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SUMMARY

Evidence is presented which supports the sequence *β-erythro*>α for ketal stability in the graded, acid hydrolysis of 3,5:6,7-di-*O*-isopropylidene-D-*glycero*-D-*gulo*-heptono-1,4-lactone (**1**), 1,2:3,5-di-*O*-isopropylidene-L-*glycero*-L-*gulo*-heptitol monohydrate (**2**), and 1,2:3,5-di-*O*-isopropylidene-L-gulitol (**3**).

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STUDIES ON STARCHES OF HIGH AMYLOSE-CONTENT

PART VII*. OBSERVATIONS ON THE POTENTIOMETRIC IODINE-TITRATION OF AMYLOMAIZE STARCH

G. K. ADKINS AND C. T. GREENWOOD
Department of Chemistry, The University, Edinburgh 9 (Great Britain)

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INTRODUCTION

One of the most characteristic properties of a starch is its "iodine affinity". This quantity is most readily determined from a potentiometric determination of the iodine-binding properties of starch[1,2], and it enables an estimate of the amylose content to be made. In the case of the amylomaize starches, however, the iodine-binding curves are of an unusual shape. In an earlier part of this Series[3], we showed the graphs of the potentiometric iodine-titration curves for two samples of amylomaize in comparison with that for a sample of regular maize. It was found that the binding of iodine for an amylomaize starch occurred more slowly, and terminated at a higher free-iodine concentration, than is usual for a normal starch. The abnormal shape of the titration curve made the extrapolation to zero free-iodine concentration, to obtain the iodine affinity, extremely haphazard. We think that it is most significant that no potentiometric titration-curves are shown even in the orignal literature for these starches[4]. Estimations of the percentage of amylose material in the amylomaize starch are therefore extremely difficult to make.

In this paper, we have made extensive studies of the iodine-binding capacity for amylomaize starches having a wide variety of amylose contents. From studies on the analogous starch of high amylose-content from the wrinkled-seeded pea[5], it has been shown that an abnormal, potentiometric, iodine-binding curve is a characteristic of amylomaize starch only. Experiments are described which provide an explanation of the iodine-binding behaviour of the amylomaize in terms of the presence of short-chain, amylose-like material.

RESULTS AND DISCUSSION

An essential prerequisite for a satisfactory potentiometric titration with iodine is complete dispersion of the granules. The procedure we have used to date[2,3] has involved dispersion of the starch to 0.1% concentration in 0.2M potassium hydroxide by shaking overnight at 15°. (Some samples of starch may require further heating for 3 min at 98°, or the use of stronger alkali, but these variations in procedure have

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*For Part VI, see *Stärke*, 18 (1966) 240.

been found to have no effect on the resulting titration curves[2].) Solutions were then neutralised prior to titration.

This dispersion procedure was applied to a sample of amylomaize having a reputed amylose-content of 70%, *i.e.*, with an expected iodine affinity of about 14%. The iodine titration curve obtained was comparable to that reported earlier[3]. No adequate extrapolation to obtain the iodine affinity could be made, but it was apparent that the level of iodine sorption of 14% was not attained (Fig. 1, curve 1).

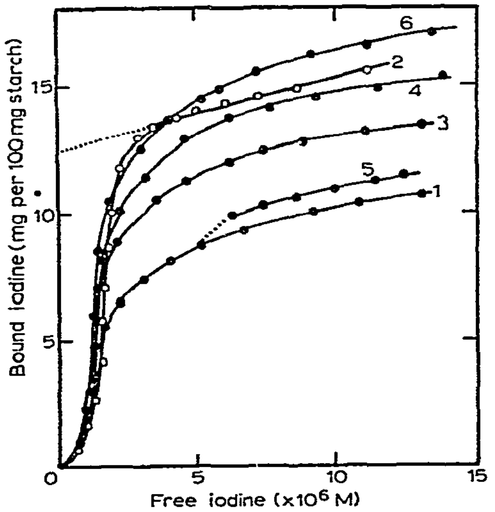


Fig. 1. Potentiometric iodine-titration curves at 20° for (1) Amylomaize *HA70*: dispersion—0.2M KOH and neutralization; (2) Wrinkled-seed pea starch: dispersion—0.2M KOH and neutralization; (3) Amylomaize *HA70*; dispersion—1M KOH and neutralization; (4) Amylomaize *HA70*; dispersion —1M KOH, neutralization and heating at 70°C; (5) Amylomaize *HA70*: dispersion as in (1), dotted line indicates an interruption of 1h in the titration; (6) Amylomaize *HA70*: dispersion in dimethyl sulphoxide (see Discussion).

It was then shown that this abnormal behaviour was not solely a function of a high amylose-content by measuring the adsorption curve for the starch of the wrinkled-seeded pea[5]. After our normal dispersion procedure, the sample gave essentially the same shape of curve as for a normal starch, except that the iodine affinity was correspondingly higher (see curve 2 in Fig. 1); the found value of 12.4% is in excellent agreement with our earlier estimation. It appeared, therefore, that the abnormal curve obtained for the amylomaize sample was a characteristic for that starch.

Various methods were then tried to increase the extent of "dispersion" of the sample.

Dispersion of the starch granules.

The use of M potassium hydroxide for dispersion was found to yield higher iodine-binding curves (see Fig. 1). It thus became apparent that increasing severity of dispersion (by increasing the molarity of alkali, by heat, by shear, or by combinations of these) increased the apparent capacity of the amylomaize starch to bind iodine. It also appeared that this starch required titration to much higher levels of free-iodine concentration before uptake of iodine even tended to become linear. In the differential titration technique used, this meant that the titration points obtained at high concentrations of iodine were less precise. It was observed also that interruptions of 1 h or more during the titration gave rise to an abrupt step-up in the graph (Fig. 1, curve 5). This is in contrast to the behaviour of ordinary, low-affinity starches, where the shape of the curve was found to be completely independent of time. This behaviour suggested that the "solutions" of amylomaize starch still contained aggregates, or micelles, which required time in order to react with iodine.

The use of dimethyl sulphoxide for better dispersion of the amylomaize starch was then examined. Preliminary experiments[6] established that the hydrous amylostarch could be dispersed directly, to 7% concentration (w/v), in dimethyl sulphoxide during 2 h at 15°, with gentle agitation. Aliquot portions of this solution (0.1 ml; *c* 68 mg per ml) were then added directly to the iodine-titration cell (volume, 850 ml). Stability and replication of the adsorption curve were found to be excellent, and the indicated affinity-values were the highest obtained for this starch, as shown by curve 6 in Figure 1.

The presence of dimethyl sulphoxide (*ca.* 0.01%, v/v) was shown to have no effect on the iodine titration. For example, the adsorption curve for potato amylose was identical when the sample was dispersed (*1*) by our usual procedure, and (*2*) in dimethyl sulphoxide solution and added to the titration flask directly. Further, the iodine-titration curve was found to be essentially the same when the dimethyl sulphoxide was removed prior to the titration. This was achieved by heating an aliquot portion of starch dispersion on a boiling-water bath in a gentle current of nitrogen, with additions of water. After final removal of liquid, the starch product, on either direct redispersion in hot water, or on dispersion in 0.5M potassium hydroxide and neutralization, gave replicable graphs, as found on direct titration of the dispersion. Potato and maize starches also gave the same result.

The iodine-binding curve was unaltered, furthermore, when the amylomaize was precipitated from dimethyl sulphoxide solution with acetone, dried with acetone, and then re-dispersed in dimethyl sulphoxide solution prior to titration. (This solubilization procedure will be discussed elsewhere[6]).

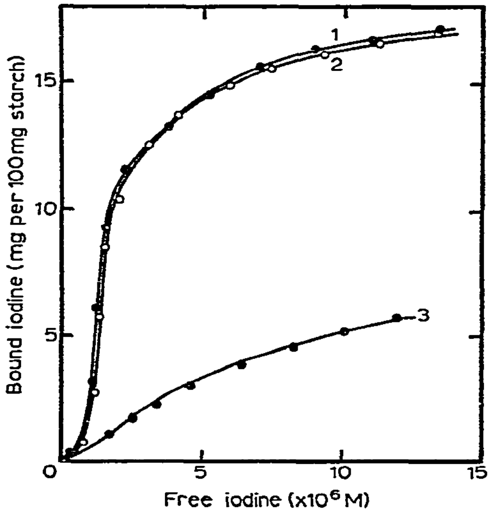
Iodine-titration curves for amylomaize starch

It appeared that the use of dimethyl sulphoxide for dispersion gave the most satisfactory iodine-titration curves. This is in agreement with the results of other techniques for studying the solubilization of amylostarches[6]. The progress of the titrations, and the resulting graphs, however, were much the same as those previously obtained; there was no sharp inflexion-point, and the titrations had to be taken to high levels of free-iodine concentration.

In order to investigate whether the abnormal curves were a result of the presence of contaminating protein or fat, the sample of amylomaize of 70%-amylose content

(*HA* 70) was treated with dilute sodium hydroxide (0.05M) in an attempt to solubilize traces of residual protein, and then the alkali-treated sample was exhaustively defatted with methanol. No difference in the titration curves was found after these treatments, and so it appeared unlikely that the observed effects were caused by protein or fat.

Since it had been observed in other experiments with amylomaize[6], that aqueous dilutions of dimethyl sulphoxide solutions may become turbid on standing, the effect of "ageing" in the electrolyte on the subsequent iodine-titration pattern was examined. Aliquot portions of a solution of *HA*-70 amylomaize were mixed with electrolyte to give concentrations of starch of 0.001, 0.01, and 0.1%. To enhance any possible instability, the solutions were then stored at 6° for 18 h before titration. The curves obtained (Fig. 2) indicated no significant change in the pattern of binding by ageing, at concentrations of starch of 0.001 and 0.01%; ageing at 0.1% concentration, however, gave rise to a greatly depressed binding-pattern as shown in Figure 2. As our titrations are normally carried out within the hour at a starch concentration of 0.001%, at 20°, ageing can have no effect on the result.



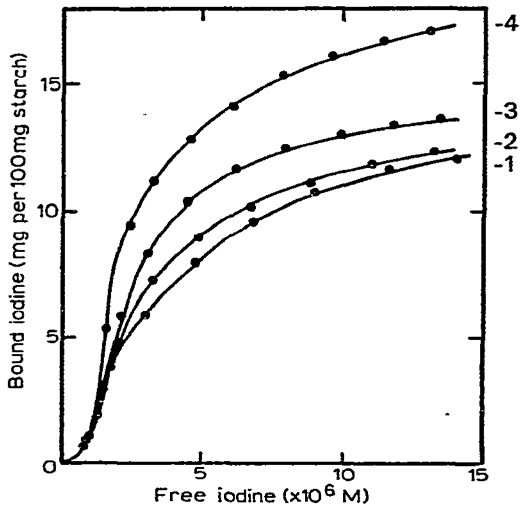


Fig. 2. Effect of pretreatment by ageing of various concentrations of starch *HA*70 for 18h at 6° in 0.01M potassium iodide: (1) 0.001% starch; (2) 0.01% starch; (3) 0.1% starch.

Fig 3. Potentiometric iodine-titration curves at 20° for amylomaize starches after dispersion in dimethyl sulphoxide: (1) *HA*57; (2) *HA*62; (3) *HA*67; (4) *HA*75.

The abnormal iodine-titration curve appeared to be, therefore, a characteristic of this sample of amylomaize. A series of starches having apparent amylomaize-contents of 57, 62, 67, and 75% were then titrated, using the method of prior dispersion in dimethyl sulphoxide. The results are shown in Figure 3. Although replications of the titrations were excellent, there was still no definitely linear portion of the curve from which to make an unambiguous extrapolation to zero free-iodine concentration, in order to obtain the "iodine affinity" by the method of Anderson and Greenwood[2].

It is to be noted that the shapes of the curves also preclude the use of the method suggested by Bates *et al.*[1] for determining iodine affinity. All that can be said is that the relative order of iodine sorption is in agreement with the apparent amylose-content.

Iodine-titration curves for synthetic mixtures of amylose and amylopectin

In earlier work on amylomaize starches[3], we have suggested that the properties of the abnormal, 36-unit amylopectin found in these starches can be attributed to the presence of short-chain, amylose-like material in addition to normal amylopectin. It appeared very likely, therefore, that this material might be causing the characteristic iodine-titration curve for the whole starch.

Short-chain amylose was prepared from the normal amylose from *HA*70-amylomaize (Fig. 4, curve 1) by degradation with *B. subtilis* α-amylase. This procedure yielded degraded amyloses I [average chain-length (C.L.), 27; λ_{max} 565 mμ], II (C.L., 71; λ_{max} 585 mμ), and III (C.L., 125; λ_{max} 600 mμ). The iodine-titration curves for these samples are shown in Figure 4.

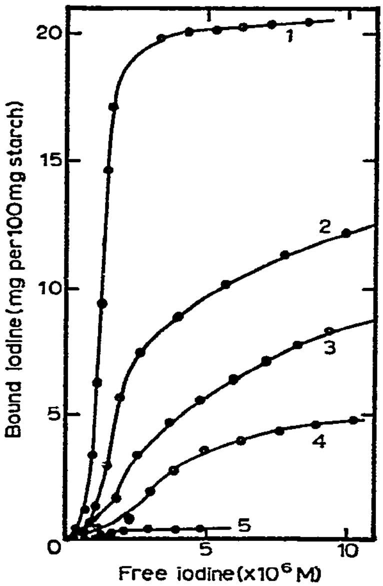


Fig. 4. Potentiometric iodine-titration curves at 20° for (1) amylose from starch *HA*70; (2) degraded amylose III; (3) degraded amylose II; (4) degraded amylose I; (5) normal maize amylopectin.

It was first established that a 30:70 (w/w) mixture of normal amylomaize amylose (Fig. 4, curve 1) and normal maize amylopectin (Fig. 4, curve 5) gave, in contrast to that for starch *HA*70, a curve of regular shape, from which the iodine affinity was readily obtained (Fig. 5, curve 2). This experimental value (13.7%) was, in fact, equivalent to the calculated one of 14%, within experimental error; λ_{max} for this sample was 620 mμ.

The iodine-titration curves for various mixtures of normal amylose, normal amylopectin, and degraded amylose were then determined, as shown in Figure 5.

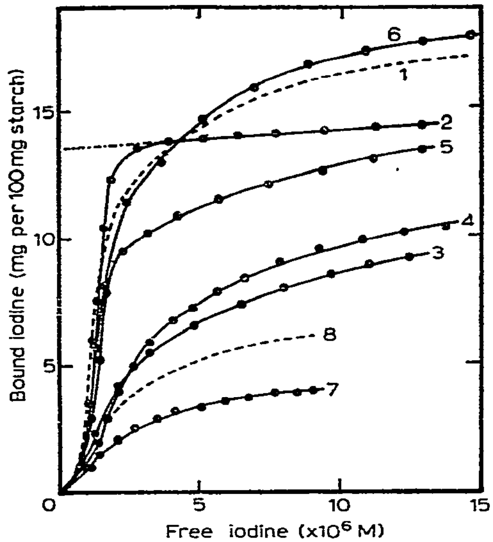


Fig. 5. Potentiometric iodine-titration curves at 20° for various synthetic mixtures: (1) *HA*70-starch, λ_{max} 600 mμ; (2) Amylose:amylopectin (70:30, w/w), λ_{max} 620 mμ; (3) Amylose:amylopectin:degraded amylose I (37:36:27, w/w), λ_{max} 580 mμ; (4) Amylose:amylopectin:degraded amylose II (40:30:30, w/w), λ_{max} 605 mμ; (5) Amylose:amylopectin:degraded amylose I (60:30:10, w/w), λ_{max} 590 mμ; (6) Amylose:amylopectin:degraded amylose III (55:15:30, w/w), λ_{max} 600 mμ; (7) Amylopectin:degraded amylose II (72:28, w/w), λ_{max} 570 mμ; (8) 36-unit amylopectin, λ_{max} 570 mμ.

It can be seen that the adsorption curves for these mixtures gave rounded, inflexionless curves which are similar to those found for amylomaize starch. In particular, the mixture amylose:amylopectin:degraded amylose III (50:15:30 w/w; λ_{max} 600 mμ) gave a graph which approximated very closely to that for *HA*70-starch (λ_{max} 600 mμ).

An analogous result was obtained when the iodine-binding characteristics of a mixture of normal amylopectin and degraded amylose II (72:28 w/w; λ_{max} 570 mμ) were compared to those of the abnormal, 36-unit amylopectin (λ_{max} 570 mμ); the two adsorption curves were comparable, as shown by curves 7 and 8 in Figure 5.

Conclusions

The fact that the addition of short-chain amylose to mixtures of normal amylose and amylopectin, and to amylopectin, permits simulation of the iodine-titration curves for amylomaize starch and 36-unit amylopectin suggests, very strongly, that the anomalous behaviour of these materials is due to the presence of degraded, amylose-like polysaccharide. It is likely that the proportion of this material present will vary with the type of amylomaize starch, and increase with an increase in apparent amylose-content.

The iodine-titration curve for the high amylose-content starch from wrinkled-seeded peas has a normal shape, and this suggests that the proportion of degraded, amylose-like polysaccharide in this starch is less than that in amylomaize.

EXPERIMENTAL

Materials

The isolation and purification of the amylomaize starches has been described elsewhere[7]. Starches *HA*57, *HA*62, *HA*67, *HA*70, and *HA*75 are reputed to contain 57, 62, 67, 70, and 75% of amylose, respectively. The fractionation technique for starch *HA*70 was as described in an earlier part of this Series[3].

Degraded amylose was prepared by the action of crystalline *B. subtilis* α-amylase (Sigma Chemical Corporation) on amylose from starch *HA*70. Portions of the digest were removed at intervals, and the polysaccharide was precipitated with acetone. Properties of the resulting products are shown in Table I.

TABLE I

PROPERTIES OF DEGRADED AMYLOSE

Sample	$[\eta]^a$	*Chain length*[b]	λ_{max}[c]	*Complex formation*[d]
I	17	27	565	None
II	25	71	585	None
III	57	125	600	None

[a]Limiting-viscosity number in dimethyl sulphoxide. [b]Measured by enzymic assay[8]. [c]Wavelength of maximum absorption of the iodine complex in millimicrons. [d]With butan-1-ol.

Titration procedure

The procedure for the potentiometric iodine-titration was that of Anderson and Greenwood[2], except where the dispersion procedure was modified as described in the Discussion.

Solutions at the end of the iodine titration were used directly to study the absorption spectra of the iodine complexes in the range 400–700 mμ.

ACKNOWLEDGMENT

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SUMMARY

The abnormal character of the potentiometric iodine-titration curves obtained at 20° for amylomaize starch are not a direct result of the high amylose-content, since the comparable starch from the wrinkled-seeded pea gives a normally-shaped iodine-titration curve. It has been found that the relative extent of binding of iodine

by an amylomaize depends on the method of dispersion of the starch granules. The use of dimethyl sulphoxide gave the most satisfactory iodine-binding curve. Residual contaminating protein and fat in the starch, and the inherent instability of the dispersion, do not influence the titration.

Potentiometric iodine-titration curves have been obtained for a series of amylomaizes having apparent amylose-contents varying from 57 to 75%. Although the relative order of iodine sorption was in agreement with the apparent amylose-content, the curves possessed no point of inflexion, and estimations of iodine affinity were not practicable.

Short chain-length amylose has been prepared, and added to normal amylopectin and to mixtures of normal amylose and normal amylopectin. It has been found that the iodine-binding characteristics of such mixtures resemble those of the anomalous 36-unit amylopectin and amylomaize starch. The significance of this finding is discussed.

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SEPARATION OF ALDITOLS AND ALDOSES BY PARTITION CHROMATOGRAPHY ON ION-EXCHANGE RESINS

OLOF SAMUELSON AND HANS STRÖMBERG
Department of Engineering Chemistry, Chalmers Tekniska Högskola, Göteborg (Sweden)

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Chromatographic methods are extremely valuable in separations of complicated mixtures containing sugars and sugar alcohols. Several comprehensive reviews have been published in recent years[1]. Great progress in gas-chromatographic work was reported recently by Sawardeker *et al.*[2], who demonstrated excellent separations of alditols after esterification with acetic acid. With complicated mixtures of monosaccharides and alditols, several disadvantages arise in the gas-chromatographic methods.

In earlier papers, it was demonstrated that partition chromatography on ion-exchange resins[3], with automatic determination of the eluate concentration[4], is an efficient method of analysis for complicated mixtures of monosaccharides. The advantages of this method, over the gas-chromatographic methods, are that many other substances, which are present in hydrolysates from materials of biological origin and in various technical liquors, do not interfere, and that the monosaccharides can be separated directly without preparation of any derivatives. This Paper describes the application of automated chromatography to mixtures of alditols and aldoses, using ion-exchange resins.

ANALYSING SYSTEM

No automated methods for the colorimetric determination of sugar alcohols seem to have been published previously, whereas several methods for sugar analysis have been described. Alditols are easily oxidized by periodate and give rise to formaldehyde which can be determined by several colorimetric methods. In this work, the color reaction with pentane-2,4-dione was chosen, and guidance in the choice of working conditions was obtained from the manual method[5]. It was observed that the color developed somewhat more slowly in aqueous ethanol than in water. To decrease the time of reaction, a higher reaction temperature (80°), compared with that used in the manual method, was chosen. A linear relationship between the absorbance and the formaldehyde concentration was observed under these conditions.

Before the colorimetric determination, the unreacted periodate was reduced to iodate or iodide (depending on pH) by addition of sodium arsenite. This precaution is necessary, since periodate destroys the color. All operations were carried out in a Technicon Autoanalyzer. The eluate was pumped by means of Yellow-Tygon tubing, whereas the other tubing was made of Tygon. Two oxidation methods were applied.

In Method *A* (see Fig. 1), the oxidation was carried out at pH 7.5 for about 3.5 min. Under these conditions, formaldehyde was formed in high yield, both from alditols and aldoses. A slight precipitation was observed after the addition of both periodate and arsenite, but, before passing into the heating bath, the solution was clear. A small amount of precipitate in the coils and tubings was removed by washing with dilute hydrochloric acid at intervals of 4 days. With well separated mixtures of sugar alcohols and sugars, this method has the advantage that all solutes can be determined in a single photometric channel. With complicated mixtures containing a great number of compounds of both types, overlapping between elution curves corresponding to sugars and sugar alcohols can jeopardize their quantitative determination.

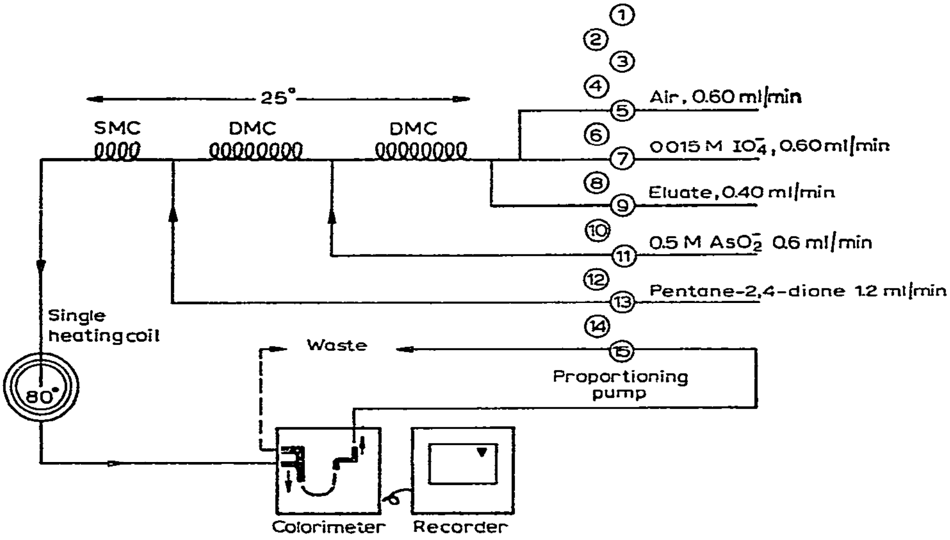


Fig. 1. Outline of analysing system.
Method *A*. Composition of reagents: Periodate: 0.015M HIO_4, neutralized with NH_3 and buffered to pH 7.5 with a phosphate buffer (100 ml/l). Arsenite: 0.5M $NaAsO_2$, neutralized with HCl to pH 7. Pentane-2,4-dione reagent: 2M ammonium acetate, 0.05M acetic acid and 0.02M pentane-2,4-dione Method *B*: Periodate: 0.015M $NaIO_4$ in 0.12M HCl. Other reagents, as in Method *A*.
The absorbance at 420 mμ is measured in a 15-mm flow cell.

In some of the experiments described in this Paper, therefore, the sugars were determined by the orcinol method[6] in one channel, and the sugar alcohols in another channel, using periodate oxidation at pH 1 under otherwise unchanged conditions (Method *B*). Under these conditions[7], the yield of formaldehyde from most alditols is higher, whereas most sugars give rise to negligible, or very slightly interfering, amounts of formaldehyde. No precipitation was obtained after addition of the oxidant, whereas a slight haze was observed after the arsenite addition. Calibration tests showed that there was a linear relation between the absorbance and the amounts of added alditol.

Chromatographic conditions. — The chromatographic separations were made under conditions similar to those reported earlier[8]. The elutions were carried out at various temperatures and ethanol–water concentrations.

Two batches of strongly basic anion-exchange resins, in the sulphate form, were used. One of the resins (T4) had a particle size of 10–35 μ and an exchange capacity of 3.7 mequiv./g of dry resin (Cl^-form). The other resin (T5B; 3–17 μ) had a higher exchange capacity (4.2 mequiv./g). The resins were kindly supplied by Dr. H. W. Holy, Technicon Instruments Co., Chertsey, Great Britain. In the micro-column, a fraction of T5B having a particle size of 10–15 μ was employed.

Chromatographic separations. — A typical chromatogram, obtained by elution of a mixture of several alditols and aldoses with 86% ethanol at 75.5°, is reproduced in Fig. 2. In this experiment, the eluate was analysed by periodate oxidation according to Method *A*, which gives a strong response both with the alcohols and the sugars. It is seen that most of the solutes are well separated from each other. For the sugar

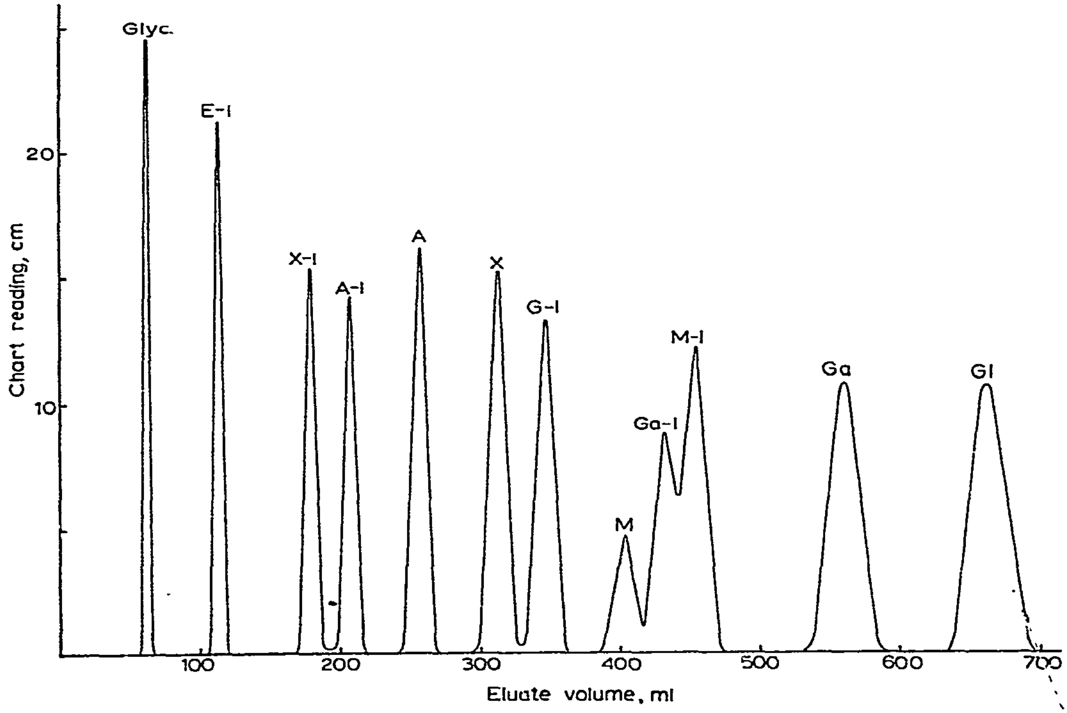


Fig. 2. Separation of various alditols and monosaccharides in 86% ethanol at 75.5°. Resin bed, 6 × 852 mm, T5B. Flow rate, 2.51 ml.cm^{-2}.min^{-1}.
Glyc = glycerol, 0.05 mg; E—l = erythritol, 0.05 mg; X—l = xylitol, 0.1 mg; A—l = arabinitol, 0.1 mg; G—l = glucitol, 0.2 mg; Ga—l = galactitol, 0.2 mg; M—l = mannitol, 0.2 mg; A = arabinose, 0.3 mg; X = xylose, 0.3 mg; M = mannose, 0.1 mg; Ga = galactose, 0.6 mg; Gl = glucose, 0.6 mg.

alcohols, the curves corresponding to galactitol and mannitol overlap to such an extent that accurate determination is prevented. Similarly, mannose and galactitol were incompletely separated from each other.

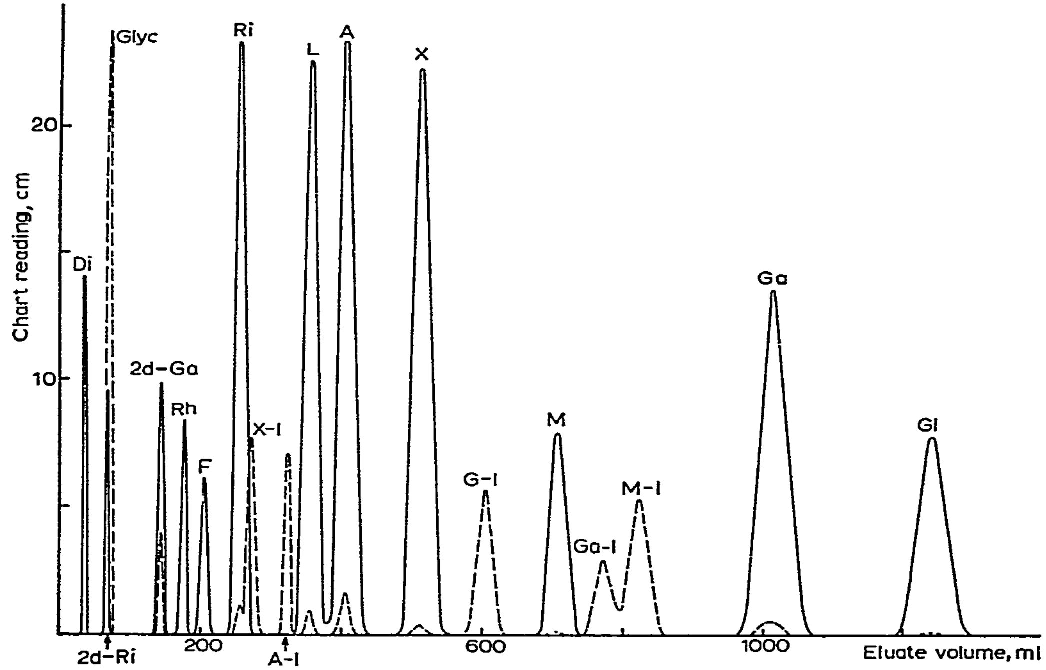
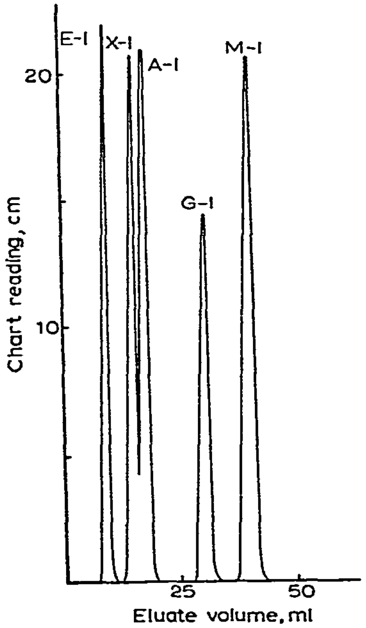


Fig. 3. Separation of various alditols and monosaccharides in 89% ethanol at 78.5°. Determination of monosaccharides with the orcinol method (full line), and alditols with the pentane-2,4-dione method (broken line).
Resin bed, 6 × 915 mm, T5B. Flow rate, 3.16 ml.cm^{-2}.min^{-1}.
Glyc = glycerol, 0.05 mg; X — l = xylitol, 0.1 mg; A — l = arabinitol, 0.1 mg; G — l = glucitol, 0.2 mg; Ga — l = galactitol, 0.2 mg; M — l = mannitol, 0.2 mg; Di = digitoxose, 0.05 mg; 2d — Ri = 2-deoxyribose, 0.2 mg; 2d — Ga = 2-deoxy-galactose, 0.1 mg; Rh = rhamnose, 0.05 mg; F = fucose, 0.05 mg; Ri = ribose, 0.2 mg; L = lyxose, 0.2 mg; A = arabinose, 0.3 mg; X = xylose, 0.3 mg; M = mannose, 0.3 mg; Ga = galactose, 0.6 mg; Gl = glucose, 0.6 mg.

Fig. 3 shows a chromatogram for an analysis in which, in addition to common hexoses and sugar alcohols, various deoxy sugars were present. The eluate was analysed both with the orcinol method, which gives no color reaction with the alcohols, and by periodate oxidation according to Method *B*, which gives only a weak response with the sugars, but a strong response with the alcohols. By using this technique, the sugars can be determined quantitatively in the orcinol channel without interference from overlapping sugar alcohols. Similarly, accurate determinations of the sugar alcohols can be carried out even if the separation from some sugars is far from complete. A higher concentration of ethanol and a somewhat longer column were used for this analysis, compared with that represented in Figure 2. This explains the improved separation of galactitol and mannitol, and the complete separation of mannose from galactitol.

In the analysis reported in Fig. 2, about 11 h were required to elute the last sugar alcohol. In the chromatogram reproduced in Fig. 3, the corresponding time was 16 hours. The time can be shortened by using a resin having a more uniform size of particle; an example of a rapid separation of some sugar alcohols with a uniform resin is given in Fig. 4. In this experiment, the total time of elution was less than 2 h, including the time for loading the column. It is seen that the curves corresponding to xylitol and arabinitol, under these conditions, showed some overlapping which has no serious influence upon the evaluation of the chromatogram for quantitative purposes. By increasing the column length or ethanol concentration, a complete separation was achieved, although a longer time of elution was required. The chromatogram reproduced in Fig. 4 was obtained with a column having an inner diameter of 2 mm. Only a 50-μg sample of each sugar alcohol was added and, although no scale expansion was applied, the height of the peaks were 15–22 cm; this shows that the method is suitable for microchemical work.



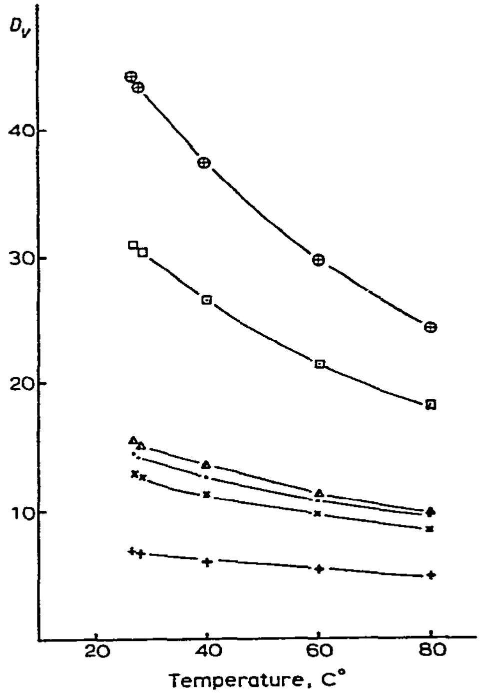


Fig. 4. Separation of various alditols in 86% ethanol at 90°.
Resin bed, 2 × 650 mm, T5B. Flow rate, 16.2 ml. cm^{-2}. min^{-1}.
E—I = erythritol, 0.05 mg; X—I = xylitol; 0.05 mg; A—I = arabinitol, 0.05 mg; G—I = glucitol, 0.05 mg; M—I = mannitol, 0.05 mg.

Fig. 5. Distribution coefficients of alditols in 88% EtOH at various elution temperatures on resin T4. +, erythritol; ×, xylitol; •, ribitol; △, arabinitol; ⊡, glucitol; ⊕, mannitol.

From the peak elution volumes recorded in a great number of analyses at 75°, with water–ethanol mixtures of varying composition, the volume distribution coefficients (D_V) were calculated in the usual manner[9]. The results obtained with the two types of resin are reproduced in Tables I and II. For comparison, results obtained with some sugars are included in Table II. In agreement with the results reported earlier for a few polyhydric alcohols[10], the distribution coefficients increase with an increased ethanol concentration. It is noteworthy that the order of elution of the alcohols, as well as that of the monosaccharides, is independent of the ethanol concentration and is the same with both resins. The distribution coefficients are higher with the resin which has the highest exchange capacity. Similarly, the separation factors (ratio between the distribution coefficients) are somewhat better with the high capacity resin. These results are in agreement with earlier observations for monosaccharides[4,8].

TABLE I

VOLUME DISTRIBUTION COEFFICIENTS OF ALDITOLS, AT 75° AND VARIOUS ETHANOL CONCENTRATIONS, ON RESIN T4

	76%	*80%*	*84%*	*88%*	*90%*
Erythritol	1.87	2.25	3.01	4.38	5.93
Xylitol	2.51	3.32	4.85	7.72	10.55
Ribitol			5.38	8.73	11.08
Arabinitol	2.79	3.80	5.49	9.02	12.87
Glucitol	3.97	5.79	9.25	16.60	24.40
Galactitol			11.83	21.68	
Mannitol			11.94	22.21	33.75

TABLE II

VOLUME DISTRIBUTION COEFFICIENTS OF ALDITOLS AND MONOSACCHARIDES, AT 75° AND VARIOUS ETHANOL CONCENTRATIONS, ON RESIN T5B

	84%	*86%*	*88%*	*90%*		*84%*	*86%*	*88%*	*90%*
Glycerol	1.72	2.07	2.34	2.68	Mannitol	13.83	19.00	27.50	40.20
Erythritol	3.39	4.15	5.19	6.16	Ribose	5.23	6.71	8.56	11.27
Xylitol	5.49	7.09	9.17	12.14	Arabinose	8.04	10.41	13.75	18.50
Ribitol	6.12	7.91	10.38	14.00	Xylose	9.79	12.79	17.32	23.70
Arabinitol	6.31	8.25	10.90	14.70	Mannose	12.43	16.73	23.45	33.40
Glucitol	10.64	14.28	20.15	28.60	Galactose	17.05	23.77	34.00	49.10
Galactitol	13.18	17.20			Glucose	20.29	28.09	41.20	60.70

With the potassium form of a sulfonated styrene–divinylbenzene resin, the distribution coefficients increased in the order: ethylene glycol < glycerol < mannitol < glucitol, indicating that the uptake increases with an increased number of hydroxyl groups in the solutes, and that individual differences appear among isomeric sugar alcohols[10]. The results given in the Tables confirm that the same holds true with the sulfate form of anion-exchange resins. Interestingly, the order of elution of mannitol and glucitol is reversed. This fact demonstrates that the interaction forces between the ions inside the resin and the alditols have a great influence upon the distribution coefficients.

Most of the alditols exhibit lower distribution coefficients than the corresponding aldoses. Ribitol and mannitol are exceptions to this rule and appear after ribose and mannose, respectively. No simple rule seems to be valid for the order of elution of various isomers. Similarly, it has not been possible to find any simple rules valid for the elution of various isomeric monosaccharides. This is to be expected, since the partition of the solutes is determined not only by the differences in solubility at various ethanol concentrations, but also by interaction forces with the resin.

A systematic study was carried out in order to establish the influence of temperature upon the distribution coefficients and upon the shape of the elution curves. The results reproduced in Fig. 5 show that the distribution coefficients decrease with an increased temperature. This means that the solutes are eluted more rapidly at high temperatures. From a practical point of view, it is important that, at constant flow-rate, the pressure drop in the column decreases at high temperature. Similarly, the elution curves are sharper. A disadvantage of working at very high temperatures is that the separation factors are less favorable. As an example, it can be mentioned that the elution bands corresponding to ribitol and arabinitol overlapped seriously in analyses at 93°, whereas an excellent separation was achieved at 40°.

Reproducibility in quantitative analysis. — As already mentioned, a linear relationship exists between the absorbance and the amounts of added alditols. Hence, a quantitative evaluation of the chromatograms can be made using the same methods as in amino-acid analysis[11]. The results obtained in five chromatograms of a complicated mixture, with each of the two analytical methods, are summarized in Table III. All runs were made with the same amount of added alditols and aldoses, and the mean areas corresponding to a 0.2-mg sample are given in the first two columns. It is seen that the response differs with different solutes. Large differences exist even

TABLE III

REPRODUCIBILITY OF THE AREAS OF THE ELUTION CURVES [a] (ABSORBANCE *versus* ELUATE VOLUME)

	Mean areas		*Maximum deviation from mean, %*	
	Method A	*Method* B	*Method* A	*Method* B
Erythritol[b]	8.15	13.2	+1.4 (−1.6)[c]	+0.7 (−0.9)
Xylitol	4.42	10.1	+2.3 (−1.7)	−1.3 (+0.4)
Arabinitol	10.1	10.3	+1.7	−1.0
Glucitol	7.38	6.65	−3.6 (+2.1)	+2.0 (−1.3)
Mannitol	9.60	8.96	+2.1 (−2.6)	+0.4 (+0.6)
Arabinose	4.95	0.73	+2.8 (−1.2)	
Xylose	5.02	< 0.2	−7.4 (−5.9)	
Galactose	2.96	0.37	+3.7 (+1.8)	
Glucose	4.27	0.13	−3.7 (−2.5)	

[a] Column: 4 × 720 mm, T5B. Eluant: 85% ethanol. Temperature: 76°. Flow rate: 8.0 ml.cm^{-2}.min^{-1}.

[b] Added 0.08 mg, instead of 0.20 mg as used with other solutes.

[c] Values in parenthesis refer to calculations with arabinitol as internal standard.

between isomeric solutes, which shows that the yield of formaldehyde is not quantitative under the conditions chosen. It is noteworthy that, with erythritol and xylitol, the oxidation in acid medium (Method *B*) gives a higher response than oxidation at pH 7.5 (Method *A*), whereas, with the hexitols, the opposite holds true.

To demonstrate the reproducibility of the method, the maximum deviation from the mean in the five experiments was calculated. In Method *A*, in which both alditols and sugars can be determined, the maximum deviation from the mean was 3.6% with the alditols. One of the determinations of xylose was less reproducible, with a deviation of 7.4%. The data have also been recalculated using arabinitol as an internal standard. It is seen that, with this procedure, the maximum deviation decreased to 2.6% for the alditols (mannitol) and 5.9% for xylose. The large error obtained with xylose is explained by the overlapping with glucitol.

Method *B*, which gives a much lower response with the sugars, is much more convenient for determination of the alditols in the presence of monosaccharides. It is seen that better reproducibility was achieved; the maximum deviation was 2%, and, by use of an internal standard, the deviation was lowered further. As mentioned previously, the sugars can be determined in a second channel, for example, by the orcinol method[6].

ACKNOWLEDGMENT

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SUMMARY

An automated method for the determination of alditols and aldoses in complicated mixtures is described. The solutes were separated on a column of anion-exchange resin in its sulfate form, using water–ethanol as the eluant. The eluate was analysed automatically by periodate oxidation and subsequent colorimetric determination of released formaldehyde, using pentane-2,4-dione.

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ACCESSIBILITY OF STARCH BY DEUTERATION

M. KHAIRY S. MORSI* AND CLARENCE STERLING

Department of Food Science and Technology, University of California, Davis, California 95616 (U.S.A.)

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INTRODUCTION

The amorphous fraction of a polysaccharide gel may be demonstrated by its physical characteristics or its chemical accessibility. One such measurement of accessibility is the relative amount of exchangeable hydroxyl hydrogen. Lang and Mason[1] used radioactivity determinations in demonstrating that hydrogen could be exchanged with tritium in both cellulose and starch, and indeed they estimated that cellulose had 71–73% accessible protons on the hydroxyl groups and that the accessibility of amylose and of amylopectin was 100%. In essence, the indication was that there were no inaccessible, *i.e.* crystalline, regions in these starch materials despite X-ray evidence of crystallinity in their amylose sample.

Similar results were obtained by Taylor *et al.*[2]. They immersed samples of native starches, amylose, and hydrolyzed amylose in liquid deuterium oxide. On the basis of their calculations, which were based on weight, all samples approached 100% exchangeability with deuterium after 1 h immersion. These investigators concluded that although crystalline regions are present, the hydroxyl hydrogens in these regions are also completely exchangeable, regardless of the crystalline type.

On the other hand, Čeh and Hadži[3] found that the infrared spectrum of deuterated amylose film retains the OH absorption band and that accessibility (measured by the relative areas under the OH and OD peaks) decreases as the film ages. Similarly, Horitsu[4] showed with infrared spectra that regions inaccessible to deuterium (vapor) exchange are present in native starch and to a lesser extent in modified starch. However, Taylor *et al.*[2] suggested that equilibrium with deuterium oxide was not attained by Čeh and Hadži[3] during several half-hour deuterations in the vapor phase and stated "it cannot be concluded that resistant hydroxyls have been demonstrated in their work".

Because inaccessible regions have been shown both in cellulose[5,6] and starch[7] by various techniques, it seemed useful to use infrared methods to re-examine the question of accessibility and deuterium exchange in starches that had been immersed in liquid deuterium oxide, after the suggestion of Taylor *et al.*[2].

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*Present address: Food Technology Department, Cairo University, Giza, Cairo, Egypt, U.A.R.

MATERIALS AND METHODS

The following samples were used: (1) Native potato starch, which was washed from comminuted fresh tubers, mixed with methanol and decanted 3 times, defatted with ethanol for 48 h in a Soxhlet extractor, and dried *in vacuo* (100 μ Hg) at 70°; (2) "Nepol" amylose (Staley Mfg. Co.), which was purified once with 1-pentanol and 3 times with butyl alcohol[8], and then was mixed with butyl alcohol at 3° and decanted 4 times, and finally was stirred for 48 h with ethanol at room temperature, defatted, and dried as in (1); (3) amylomaize (native maize starch of high amylose content), which was obtained from the American Maize Products Co. and defatted as in (1); (4) gelatinized, precipitated ("retrograded") potato starch, which was prepared by heating 2% potato starch in water under nitrogen at 70°, then was held at 3° for 10 days, frozen, and dried *in vacuo* (100 μ Hg), and finally was ground to pass an 80-mesh sieve; (5) cotton cellulose, which was extracted with an ethanol–benzene mixture, hot dilute hydrochloric acid, and hot dilute sodium hydroxide[9].

All starch samples had the *B* X-ray diffraction pattern. X-ray estimates of crystallinity were made by the method of Hermans and Weidinger[10,11] for cellulose and by the same method, modified[7], for starch. The samples were held at a constant relative humidity of 80% in a special chamber[12].

Before deuteration, the samples were sieved at 325 mesh (44 μ). Deuteration was accomplished by suspending a sample in liquid deuterium oxide (5 D_2O:1 sample by gross weight) under nitrogen after the sample had been kept in vacuum under $10^{-1} \mu$ Hg pressure for 24 h at room temperature and then brought in direct contact with an atmosphere of oil-pumped nitrogen. The deuterium oxide used (Matheson, Coleman, and Bell Co.) had a reported purity of 99.5% deuterium, which was confirmed by measuring the density in a specific-gravity balance. The sample remained in deuterium oxide for 24 h and was then dried by evacuation (in the same sample-chamber) under $10^{-1} \mu$ Hg pressure for 48 h at room temperature. Oil-pumped nitrogen gas was admitted to the evacuated sample chamber, and the sample was transferred under positive nitrogen pressure to hexachloro-1,3-butadiene ($CCl_2{=}CClCCl{=}CCl_2$) as a suspension agent (minimal absorption in the range 2000–4500 cm^{-1}).

The suspension (ground sample in hexachloro-1,3-butadiene) was placed between sodium chloride plates in a Beckman IR-5 spectrometer. Infrared spectra were obtained for samples in both the original (protonated) and the deuterated states. Determinations were made for 3 different specimens of each sample, except for single samples of amylose and precipitated potato-starch gel.

RESULTS

In polysaccharides, the CH stretching frequency occupies the region near 2900 cm^{-1} (Ref. 13), and the OH stretching frequency lies between 3300 and 3400 cm^{-1} (ref. 14 and 15). These are the only absorption peaks in the 2000–4500 cm^{-1} region. Upon deuteration, the OH peak decreases to a greater or lesser extent, and a large new peak appears at about 2500–2600 cm^{-1}, the OD stretching frequency[3,4,6,16]. In no case did the absorption peak at 3400 cm^{-1} (the OH stretching frequency) disappear. (Fig. 1 shows the transmission spectra of representative samples.) Measurement of the areas under the OH and OD peaks (after conversion of the vertical coordinate to transmittance and the horizontal coordinate to wave number[17]) permitted an assessment of the relative crystallinity of the samples. The calculation was based on assigning twice the area under the OD peak to the amorphous (accessible)

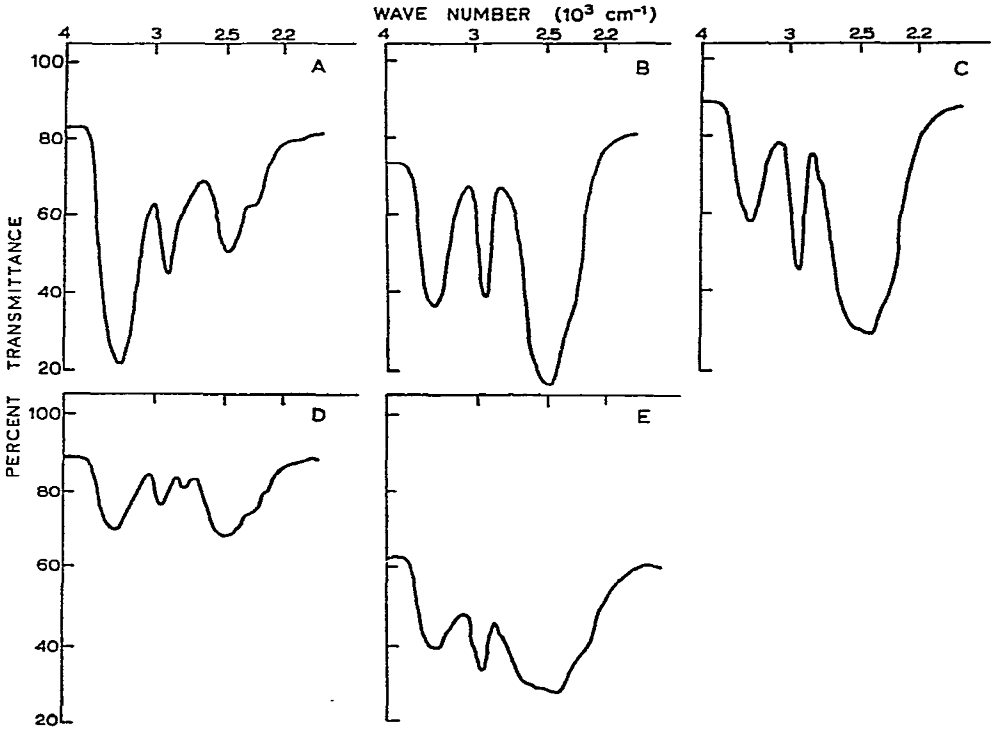

Fig. 1. Infrared transmission spectra of samples after deuteration for 24 h in large excess of deuterium oxide; the OD band, at about 2500 cm^{-1}, does not appear in spectra of undeuterated samples. A, cotton cellulose; B, amylose; C, native amylomaize; D, retrograded potato-starch gel; E, native potato-starch. Although abscissas are given in terms of wave number, they were recorded linearly with wave length. Peak-area calculations were based on wavenumber and transmittance as coordinates[17].

content of the sample and the area under the OH peak to the crystalline (inaccessible) content, the sum of the 2 areas being the basis for computation. [Theoretically, the OH intensity is greater than the OD intensity by a factor of 2 (ref. 18).] The average relative crystalline content of each sample is given in Table 1 (the range of these values is about ±6%). Also listed in this table are the crystallinity values that have been derived from the X-ray diffraction data.

DISCUSSION

Regardless of the exact values for the ratios of integrated intensities (see below), it is evident that even, with longer periods of immersion in liquid deuterium oxide than those used by Taylor *et al.*[2], there remain unsubstituted OH groups in these starch samples. Hence, there are inaccessible regions in starch, which may be conceived as crystalline in large part.

The calculation of the integrated intensities has been based on the theoretical consideration of Wilson *et al.*[18] which relates, with qualifications, peak intensity inversely to the mass of the vibrating atom. Thus, if their qualifications be accepted as valid, K_{OD}/K_{OH} would be 2.0, which is the relationship used in this study. However, Mann and Marrinan[6] estimated this ratio as 1.1 in viscose. Nevertheless, it seemed justifiable to use the higher value of 2.0 not only because of theory but also because of the fact that use of the lower value would yield crystalline contents much higher than are at all reasonable for starch or cellulose.

TABLE I

CRYSTALLINE CONTENT OF POLYSACCHARIDES

Polysaccharide	*Method of determination*	
	Deuterium exchange %	*X-ray diffraction* %
Cellulose	68	70
Potato starch (native)	24	28
Potato starch (retrograded gel)	33	14
Amylomaize	25	24
Amylose	24	25

A comparison of the figures in the two right-hand columns of Table I shows that the computation of crystallinity from infrared spectra gives values that agree essentially with those determined on the basis of X-ray diffraction. A major difference, however, is shown for the retrograded potato-starch gel. This interesting result is based on a single sample and may therefore be doubted. It suggests that, in the retrograded gel, some part which is inaccessible to deuterium exchange is not organized in a crystalline manner. Such a result could have been expected on the basis of what is known of the solubility and other characteristics of retrograded starch. This material is quite resistant to hydrolysis and to dispersion in water even at autoclave temperatures[19,20].

The results of this experiment contradict those of Taylor *et al.*[2] and of Lang and Mason[1]. It is not possible at present to explain this difference. Repeated tests of our starch samples under various conditions of deuteration always showed remaining, a significant OH band whose peak area depended on the crystalline composition of the sample. Moreover, it might be suggested that, if it is impossible for water to disrupt the crystalline structure of starch at room temperature, it is difficult to understand how deuterium oxide could accomplish such a hypothetical disruption as would be understood from the work of Taylor *et al.*[2] and Lang and Mason[1]. Using infrared spectroscopy, Mann and Marrinan[6] (with samples immersed in liquid deuterium oxide) determined a crystalline content of 61% in bacterial cellulose, which was based on peak-heights only. It should be noted that, on the basis of a gravimetric analysis of deuterium oxide uptake, Jeffries[21] found 61% of crystallinity in ramie and 58% in cotton, while Morisson[22] also determined 62% of crystallinity in cotton. These concurring figures are closely in accord with that shown here for cotton cellulose. Hermans[5] has already demonstrated that the crystallinity of native celluloses, as determined by various methods, lies between 60 and 70%. All these values clearly are at variance with those given by Lang and Mason[1] (27–29%).

SUMMARY

Various starch materials and cotton cellulose were deuterated in liquid deuterium oxide. Relative accessibility (amorphous content) was determined by infrared spectroscopy. The values obtained agreed on the whole with those determined by X-ray diffraction methods. In all cases, there was a residuum of inaccessible (usually crystalline) material which could not be deuterated.

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STUDIES ON CARBOHYDRATE-METABOLISING ENZYMES

PART XV.* THE ENZYMIC SYNTHESIS OF SOME *O-D*-GLUCOSYLPENTOSE DISACCHARIDES

D. J. MANNERS** AND J. R. STARK**
Department of Chemistry, University of Edinburgh, Edinburgh 9 (Great Britain)

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INTRODUCTION

Cell-free extracts of the ciliate *Tetrahymena pyriformis* show a wide range of carbohydrase activities, including maltase, but when the extracts are incubated with concentrated solutions of maltose, various oligosaccharides are synthesised[2]. These include isomaltose, maltotriose, panose, 6^3-α-D-glucosylmaltotriose and maltotetraose, and they arise by transfer of an α-D-glucosyl residue from maltose to either D-glucose or a maltosaccharide. Hence, the OH group at C-6 of D-glucose, or at both C-4 and C-6 of the non-reducing residues of maltose and maltotriose, can serve as acceptors of α-D-glucosyl residues. The extracts were unable to polymerise D-glucose. In further studies of the acceptor specificity, digests were prepared containing maltose and pentoses, and, by paper chromatography, evidence for the formation of α-D-glucosylpentose disaccharides, in addition to isomaltose, panose, and maltotriose, was obtained[2]. In an attempt to favour the formation of pentose-containing oligosaccharides at the expense of maltosaccharides, we have used phenyl α-D-glucopyranoside as α-D-glucosyl donor. By this means, several new disaccharides have been prepared. A preliminary account of part of this work has been published[3].

The above experiments represent trans-α-D-glucosylation reactions. We have previously shown[4] that extracts of barley can transfer β-D-glucosyl residues from cellobiose, with the formation of various oligosaccharides. Salicin has now been tested as a β-D-glucosyl donor, and, in the presence of D-xylose and a barley β-glucosidase preparation, β-D-glucosyl-D-xylose disaccharides were formed.

MATERIALS AND METHODS

Materials

We are indebted to Dr. J. F. Ryley for freeze-dried cells of *Tetrahymena pyriformis*. The preparation described previously[2] (Extract *A*) was used for most of the

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*For Part XIV, see Ref. 1.

**Present address, Department of Brewing and Applied Biochemistry, Heriot–Watt University, Edinburgh.

experiments, but a more recent preparation (Extract *B*) showing a similar, but stronger, activity was used for the preparation of the α-D-glucosyl-lyxoses.

Phenyl α-D-glucopyranoside was prepared from penta-*O*-acetyl-α-D-glucopyranose by treatment with phenol and zinc chloride in acetic acid[5], followed by deacetylation[6]. The product had m.p. 173–4° and $[\alpha]_D^{16}$ +186° (*c* 1.80, water). 3-*O*-α-D-Glucosyl-D-arabinose and 5-*O*-α-D-glucosyl-D-arabinose were prepared from maltose and isomaltose, respectively, by the method of Whistler and Yagi[7].

General methods

Solutions of carbohydrates were concentrated under vacuum at temperatures below 40°. Disaccharides (*ca.* 2 mg) were hydrolyzed with 1.5N sulphuric acid (0.2 ml) at 100° for 2 h, and barium carbonate was used for neutralization. Disaccharides (*ca.* 2 mg) were reduced with potassium borohydride (2 mg) in water (1 ml) at 20° for 24 h, and the solution was then deionised with Amberlite IR-120 (H^+ form). Borate ions were removed by successive evaporations with methanol. For the methylation of disaccharides, the method of Kuhn *et al.*[8] was used; the disaccharide (0.5–2.0 mg), methyl iodide (0.2 ml), *N*,*N*-dimethylformamide (0.2 ml), and silver oxide (0.2 g) were shaken in the dark at 20° for 24 h. Two such treatments were generally sufficient. For methanolysis, 3% methanolic hydrogen chloride at 100° for 5–6 h was used, the hydrogen chloride was removed by repeated evaporation with methanol, and the products were examined by gas–liquid chromatography[9] (g.l.c.), using a Pye Argon chromatograph. We are indebted to Dr. G. O. Aspinall for these analyses.

The reduction of periodate was measured spectrophotometrically[10].

Paper chromatography

The following solvent systems were used with Whatman No. 1 and 3 MM papers (v/v): (*A*) ethyl acetate–pyridine–water (10:4:3); (*B*) ethyl acetate–acetic acid–formic acid–water (18:3:1:4); (*C*) butanol–acetic acid–water (4:1:5, upper layer); (*D*) butanone–water–ammonia (200:17:1). In addition to aniline oxalate and silver nitrate, the following reagents were used in spot tests or as chromatographic sprays: (*a*) 2,3,5-triphenyl-2*H*-tetrazolium chloride, as a specific test for (1→2)-linked disaccharides[11]; (*b*) periodate–*p*-rosaniline, as follows: disaccharides were sprayed with a solution containing equal volumes of 2% sodium metaperiodate solution and 0.1M phosphate buffer (pH 8.0). The chromatogram was left at 20° for 5 min, before treatment with sulphur dioxide and spraying with the *p*-rosaniline reagent. The inclusion of phosphate buffer gave clearer yellow colours with 3-*O*-substituted hexoses and pentoses, and, in several cases, spots having blue centres were completely avoided. Reduced disaccharides were treated exactly as described by Hardy and Buchanan[12]. Carbohydrates (*e.g.*, 2-*O*- and 3-*O*-substituted pentoses and 3-*O*-substituted pentitols) which give rise to a malonaldehyde derivative on periodate oxidation at room temperature give a yellow colour with the reagent, whereas those carbohydrates (*e.g.*, 4-*O*-substituted pentoses and 2-*O*- and 4-*O*-substituted pentitols) which do not yield a malonaldehyde derivative give a blue colour. By examining the colour produced before and after borohydride reduction, it is therefore possible to distinguish between (1→2)-, (1→3)- and (1→4)-linked glucosylpentoses.

Paper electrophoresis

Separation of disaccharides was effected using a Shandon small-scale electrophoresis apparatus, with 0.05M borate buffer (pH 10.0) and 10–20 volt/cm for 0.5–2.5 h. Results are expressed as M_G values (glucose = 1.00). Disaccharide alcohols were run in molybdate buffer[13] (12.5 g of sodium molybdate in 1200 ml of water, adjusted to pH 5.0 with sulphuric acid), but using half the recommended concentration of buffer to reduce background colour with silver nitrate reagent. Results are expressed as M_S values (glucitol = 1.00).

From inspection of the formulae, 2-*O*- and 4-*O*-substituted xyloses should have a low mobility (M_G 0.15–0.30) in borate buffer, whilst 3-*O*- and 5-*O*-substituted xyloses should have a high mobility (M_G 0.50–0.90). In molybdate buffer (pH 5), 2-*O*- and 4-*O*-substituted xylitols, 4-*O*-substituted arabinitols, and 2-*O*-substituted lyxitols should have a low mobility, 5-*O*-substituted xylitols, arabinitols, and lyxitols should have high mobility, whilst 3-*O*-substituted xylitols, arabinitols, and lyxitols, and 4-*O*-substituted lyxitols should be immobile.

Isolation of disaccharides

Small-scale digests (total volume, 1 ml) containing phenyl α-D-glucopyranoside, pentose, and extract were prepared, and analysed at intervals by paper chromatography. The time at which the maximal amount of D-glucosylpentose (which gave a pink colour with aniline oxalate) was produced, with minimal formation of isomaltose, was noted. Large-scale digests were then prepared and incubated at 36° for this time. The solution was heated at 100° for 20 min, to inactivate the extract, and cooled, and denatured protein was removed by centrifugation. The solution was concentrated and applied to a charcoal–Celite column, prepared by the method of Andrews *et al.*[14] from either B. D. H. or Ultrasorb Charcoal, which was eluted with water and increasing concentrations of aqueous ethanol. Fractions (*ca.* 200 ml) were collected, concentrated, and examined by paper chromatography (usually with solvent *A*). Mixed fractions were chromatographed on sheets of Whatman 3 MM paper.

RESULTS

Action of protozoal extract on phenyl α-D-glucopyranoside in the absence and presence of other carbohydrates

The following results were obtained by paper-chromatographic analysis of small-scale digests (final volume, 0.5 or 1.0 ml), and tentative identification of products is based on a comparison of R_G values with authentic samples.

When phenyl α-D-glucopyranoside (10%) and extract *A* (1%) were incubated at 36°, glucose was detected within 15 min, maltose and isomaltose within 1 day, and oligosaccharides having R_G 0.30 and 0.22 within 5 days. However, by halving the concentration of phenyl α-D-glucopyranoside, the amount of maltose produced was barely detectable even after 10 days, although small proportions of isomaltose were still formed.

Digests containing phenyl α-D-glucopyranoside (1%), acceptor (1%), and extract (0.2%) were incubated (under toluene to prevent microbial contamination) for 2–3 days, and then fractionated by paper chromatography. New components were isolated, and shown by acid hydrolysis to be composed of glucose and the acceptor. The following served as acceptors: D-galactose, D-mannose, D-xylose, D-ribose, D-lyxose, D-arabinose, L-arabinose, D-mannitol, D-glucitol, xylitol, D-arabinitol, erythritol, glycerol, and ethylene glycol. The following were non-acceptors: D-fructose, L-fucose, L-rhamnose, D-glucuronic acid, sucrose, and methyl α-D-glucopyranoside. Control digests containing extract and either D-glucose, D-xylose, or a mixture of D-glucose and D-xylose, did not produce oligosaccharides. The above results therefore show that the extract can transfer a D-glucosyl residue from phenol to several carbohydrates, including five pentoses, with the formation of new D-glucosyl-acceptor disaccharides.

*Preparation and properties of O-α-*D*-glucosyl-*D*-xylose*

Phenyl α-D-glucopyranoside (4.5 g), D-xylose (5.0 g), extract *A* (1 g), and water (100 ml) were incubated under toluene at 36° for 8 days. After heat treatment and centrifugation, the solution was concentrated and applied to a charcoal–Celite column, which was eluted with water and 5% ethanol. The fractions containing glucosylxylose (R_G 0.68 in solvent *A*) were combined, concentrated, and freed by preparative, paper chromatography from small proportions of isomaltose and a sugar having R_G 0.85, which stained pink with aniline oxalate. The glucosylxylose was isolated as a thin syrup which solidified on addition of ethanol; it was washed with ether and dried under vacuum at 20°. Yield, 231 mg.

The glucosylxylose was homogeneous on paper chromatography in solvents *A*, *B*, and *C*; on total, acid hydrolysis, glucose and xylose were formed in equal amounts (visual estimation), and, on borohydride reduction, followed by acid hydrolysis, glucose and xylitol were produced. The disaccharide had $[\alpha]_D^{16}$ +101 (10 min) → +97° (constant, 100 min) (*c* 0.89, water) and was not hydrolysed by almond *β*-glucosidase. On electrophoresis, the disaccharide had an M_G value of 0.26 suggesting that the linkage was (1→2) or (1→4). Maltose, laminaribiose, isomaltose, and 3-*O*-*β*-D-glucopyranosyl-D-xylose had M_G values of 0.28, 0.56, 0.55, and 0.64, respectively. The borohydride-reduced disaccharide had an M_S value of 0.30 in molybdate buffer (*cf.* M_S 0.33 for cellobi-itol and maltitol, and 0.00 for laminaribi-itol) which also suggested a (1→2) or (1→4) linkage. However, since the disaccharide rapidly produced a rich, pink colour with triphenyltetrazolium chloride, a (1→2)-linkage was unlikely. The disaccharide and the derived alcohol both gave blue colours with the *p*-rosaniline reagent, whereas 3-*O*-*β*-D-glucopyranosyl-D-xylose gave a yellow colour. A (1→2)-linked glucosylxylose would also give a yellow colour.

The disaccharide (10.4 mg) was oxidised with 0.015M sodium metaperiodate

(25 ml) at 20°. After 1.5, 7.5, 20.5, and 27 h, the reduction of periodate was 1.9, 3.2, 3.9, and 3.9 (constant) mol. This value agrees closely with that expected from a (1→4)- or (1→5)-linked glucosylxylose (4.0 mol.).

The disaccharide (60 mg) in *N,N*-dimethylformamide (10 ml) was methylated with methyl iodide (10 ml) and silver oxide (1 g). The methylation was repeated, the methylated disaccharide (recovered in 60% yield) was hydrolysed, and the neutralised solution was applied to a small cellulose-column which was eluted with methyl ethyl ketone–water (10:1, v/v). Fractions (2 ml) were collected and analysed by paper chromatography (solvent *D*). Fractions 5 and 6, which contained tetra-*O*-methyl-D-glucose, were evaporated to a syrup (22 mg) which crystallised after two days. The product was recrystallised three times from light petroleum (b.p. 60–80°) to yield colourless crystals (16 mg) which had mp. 83–84°, undepressed on admixture with 2,3,4,6-tetra-*O*-methyl-D-glucose, and $[\alpha]_D^{16}$ +85° (*c* 0.5, water; lit., +84°). The crystals gave an X-ray powder photograph identical with that from 2,3,4,6-tetra-*O*-methyl-D-glucose.

Fractions 7 to 12 contained a di-*O*-methylxylose and a trace of another sugar, and were purified by paper chromatography to yield a pale-yellow syrup (15 mg) which crystallised on seeding with the *β*-form of 2,3-di-*O*-methyl-D-xylose. The crystals had m.p. and mixed m.p. 78–81°, and gave an X-ray powder photograph identical with that of 2,3-di-*O*-methyl-D-xylose.

Subsequent fractions contained only traces of undermethylated products.

Although the periodate oxidation and methylation studies indicated that the disaccharide was 4-*O*-α-D-glucopyranosyl-D-xylopyranose, the remote, but possible, presence of 5-*O*-α-D-glucopyranosyl-D-xylofuranose (which would also give rise to 2,3-di-*O*-methyl-D-xylose and reduce 4.0 mol. of periodate) had not been eliminated.

The disaccharide (20 mg) in water (20 ml) was treated with potassium borohydride (10 mg) at 20° for 12 h. The excess of borohydride was destroyed, borate was removed by successive evaporation with methanol, and the solution was adjusted to pH 4, before the addition of sodium metaperiodate (40 mg) and dilution to 250 ml. After 24 h at 20°, the solution was deionised with Amberlite IR-120 (H^+ form) and IR-45 (HO^- form) resins and concentrated to 20 ml. The solution was again treated with borohydride, and then hydrolysed with sulphuric acid. By paper chromatography, glucose and glycerol were found to be the major products; ethylene glycol was absent. This result showed the presence of a (1→4)-linked xylopyranose residue, since a (1→5)-linked xylofuranose residue would yield ethylene glycol. The reaction conditions were tested with isomaltose, which gave the expected mixture of glucose and ethylene glycol, and with maltose, which yielded glucose and glycerol. It should be noted that with dilute solutions of periodate, non-reducing glucose residues are not oxidised[15].

Preparation and properties of O-α-D-glucosyl-D-ribose

From a digest containing D-ribose (5 g) in place of the D-xylose used previously (p. *105*), a disaccharide fraction (200 mg) was isolated. The major component had R_G 0.76 in solvent *A* and gave a pink colour with aniline oxalate. Isomaltose and a sugar having R_G 0.85 were trace impurities, and were removed by preparative, paper chromatography. The minor component (R_G 0.85) gave a yellow colour with the periodate–*p*-rosaniline reagent before borohydride reduction, and a blue colour after this treatment. It did not react with the triphenyltetrazolium chloride reagent, and under u.v. light gave an orange fluorescence with aniline oxalate. It was probably a (1→2)-linked α-D-glucosyl-D-ribose.

The major glucosylribose was homogeneous on paper chromatography in solvents *A* and *B*; on total hydrolysis by acid, glucose and ribose were formed in equal amounts (visual estimation) and on borohydride reduction, followed by acid hydrolysis, glucose and ribitol were produced. The disaccharide had $[\alpha]_D^{16}$ $+93°$ (*c* 0.95, water), and M_G 0.28 (*cf.* M_G 0.28 for maltose). The reduced disaccharide had M_S 0.01 which indicated the absence of a (1→5)-linkage. The disaccharide reacted rapidly with triphenyltetrazolium chloride, suggesting the absence of a (1→2)-linkage, and gave a blue colour with periodate–*p*-rosaniline. A (1→2)- or (1→3)-linked disaccharide would give a yellow colour, due to the formation of a malonaldehyde structure[12].

The disaccharide (10.6 mg) was oxidised with 0.015M sodium metaperiodate (25 ml) at 20°. After 0.5, 3, 10, 21, and 31 h, the reduction of periodate was 2.1, 2.9, 3.6, 3.7, and 3.7 (constant) mol. These results confirmed the absence of (1→2)- or (1→3)-linkages, and, together with the previous evidence, characterised the disaccharide as 4-*O*-α-D-glucopyranosyl-D-ribose. This conclusion is strengthened by the fact that the borohydride-reduced disaccharide had the same R_G value (in solvents *A* and *B*) and M_G and M_S values as an authentic sample of 4-*O*-α-D-glucopyranosyl-D-ribitol, kindly provided by Dr. A. R. Archibald.

Preparation and properties of O-α-D-glucosyl-D-arabinoses

A mixture of glucosylarabinose disaccharides (190 mg) was isolated from a digest containing D-arabinose (5 g), phenyl α-D-glucopyranoside, and extract *A*. By paper chromatography in solvent *A*, the presence of four sugars was shown, all of which stained pink with aniline oxalate (see Table I); compound A3 gave a bright-orange fluorescence under u.v. light; this has been observed only with (1→2)-linked disaccharides. The results of a preliminary examination (Table I) suggested that the following linkages might be present: component A1, (1→4); component A2, (1→3); component A3, (1→2); and component A4, (1→5).

The mixture was partially fractionated by thick-paper chromatography into fraction *A* (160 mg., containing components A1, 2, and 3) and fraction *B* (30 mg, containing components A3 and 4). Neither fraction was hydrolysed by almond β-glucosidase. The two fractions were then methylated, methanolysed, and examined by g.l.c. For both methanolysates, the presence of peaks corresponding to the methyl glycosides of 2,3,4,6-tetra-*O*-methyl-D-glucose, 2,3- and 3,4-di-*O*-methyl-D-arabinose was indicated, together with other peaks. Peaks corresponding to the methyl glycosides of 2,4- or 2,5-di-*O*-methyl-D-arabinose would probably overlap with the other products. In fraction *A*, the 2,3-di-*O*-methyl-D-arabinose would arise from a (1→4)-linked, and the 3,4-isomer from a (1→2)-linked disaccharide. In fraction *B*, the 3,4-isomer would

TABLE I

PROPERTIES OF *O-D*-GLUCOSYL-D-ARABINOSES (A1–A4) AND *O-D*-GLUCOSYL-D-LYXOSES (L1–L3).

	Disaccharide								
	A1	*A2*	*A3*	*A4*	*X*[a]	*Y*[a]	*L1*	*L2*	*L3*
R_G value, solvent *A*	0.61	0.70	0.77	0.89	0.70	0.89	0.70	0.96	0.87
Reaction with triphenyltetrazolium chloride	+	+	−	+	+	+	+	−	+
Colour[b] with periodate–*p*-rosaniline									
(*a*) before reduction	b	y	y	b	y	b	b	y	y
(*b*) after reduction	b	y	b	b	y	b	b	b	y
M_G value, borate	–	0.57	0.31	0.45	0.58	0.47	0.45	0.25	0.45
M_S value, molybdate	0.22	0.00	0.01	0.43	0.00	0.41	0.04	0.20	0.06

[a] X, 3-*O*-α-D-glucopyranosyl-D-arabinose; Y, 5-*O*-α-D-glucopyranosyl-D-arabinose.
[b] b, blue; y, yellow.

also arise from component A3, and the presence of 2,3-di-*O*-methyl-D-arabinose is consistent with a (1→5)-linked glucosylarabinofuranose structure for component A4.

By repeated paper chromatography, milligram quantities of the four sugars were isolated in a chromatographically pure state. On acid hydrolysis, each gave glucose and arabinose before, and glucose and arabinitol after, reduction with borohydride. The colours formed with the periodate–*p*-rosaniline reagent, before and after reduction, were consistent with the tentative structures assigned to each component. In addition, (1→4)- and (1→5)-linked disaccharides could be distinguished by molybdate electrophoresis, after borohydride reduction[13]. Component A4 had M_S 0.4, as had an authentic sample of 5-*O*-α-D-glucopyranosyl-D-arabinitol; component A2 was virtually immobile, as was 3-*O*-α-D-glucopyranosyl-D-arabinitol, and component A1 had M_S 0.22, in agreement with the theoretical mobility.

It is therefore concluded that transfer of α-D-glucosyl residues to positions 2, 3, 4, and 5 of D-arabinose had occurred, with the formation of a mixture of four glucosylarabinose disaccharides.

Preparation and properties of O-D-glucosyl-D-lyxoses

From a digest containing phenyl α-D-glucopyranoside (2.25 g), D-lyxose (2.5 g), extract *B* (0.5 g), and water (50 ml), which had been incubated under toluene at 36° for 4 days, a mixture of three disaccharides was isolated by charcoal–Celite chromatography. These were separated by preparative, paper chromatography to give component L {147 mg, R_G 0.44 in solvent *B*, $[\alpha]_D^{16}$ +102° (*c* 1.26, water)}, component L2 {60 mg, R_G 0.68, $[\alpha]_D$ +74° (*c* 1.00, water)}, and component L3 {29 mg, R_G 0.59, $[\alpha]_D$ +88° (*c* 1.00, water)}. The three components were chromatographically and electrophoretically pure, were not hydrolysed by almond β-glucosidase, yielded glucose and lyxose on acid hydrolysis, and glucose and lyxitol after reduction and acid hydrolysis. From a preliminary examination (Table I), and the fact that component L2 gave a fluorescent colour with aniline oxalate, it was probable that L1 was (1→4)-linked, L2, (1→2) linked, and L3 (1→3)-linked.

The disaccharides (5 mg each) were methylated, and the products divided. One portion was hydrolysed and examined by paper chromatography (solvent *D*); each hydrolysate contained tetra-*O*-methylglucose (TG) and an equal proportion (visual estimation) of a second sugar. The R_{TG} values and colours with aniline oxalate were L1, 0.47, brown; L2, 0.50, pink; L3, 0.56, pink. An authentic sample of 2,3-di-*O*-methyl-D-lyxose had R_{TG} 0.47, and gave a brown stain with aniline oxalate. It should be noted that methylated sugars having a free OH at C-4 also give a brown colour, whereas those etherified at position 4 gave a pink colour.

The results suggested that L1 was 4-*O*-α-D-glucopyranosyl-D-lyxose, and this was confirmed by g.l.c. of a methanolysate of the methylated material. Peaks corresponding to the methyl glycosides of 2,3,4,6-tetra-*O*-methyl-D-glucose and 2,3-di-*O*-methyl-D-lyxose were detected. The major peak of the latter had relative retention times of 1.70 and 0.87 on columns of neopentylglycol adipate at 150° and polyphenyl ether [*m*-bis(*m*-phenoxyphenoxy)benzene] at 200 °; *cf* authentic values of 1.69 and 0.88.

The di-*O*-methyl-lyxoses from disaccharides L1, L2, and L3 were isolated by paper chromatography, and reduced with borohydride. The solutions (0.01 ml) were treated with 0.3M sodium metaperiodate (0.01 ml) for 24 h and the products were examined by aniline oxalate spot-tests. The products from L1 and L2 gave a pink colour whereas that from L3 gave no reaction. A (1→3)-linked glucopyranosyl-lyxofuranose would yield a partly methylated lyxose having adjacent OH groups at C-3 and C-4; this would be susceptible to periodate oxidation and thence give a positive reaction with aniline oxalate. The lack of reaction is in accord with the view that L3 is 3-*O*-α-D-glucopyranosyl-D-lyxopyranose.

Preparation and properties of O-β-D-glucosyl-D-xylose

Preliminary experiments by Mr. J. Walker had shown that a barley β-glucosidase preparation[4] could transfer a β-D-glucosyl residue from salicin to various pentoses with the formation of new sugars having the R_G values of β-D-glucosylarabinose, β-D-glucosylxylose, β-D-glucosyl-lyxose and β-D-glucosylribose.

From a large-scale digest of salicin (40%), D-xylose (20%), and barley β-glucosidase (1.2%) in a total volume of 40 ml incubated for three days at 35°, and subsequent charcoal-Celite chromatography, fractions containing small amounts of two glucosylxyloses had been isolated. One of these was chromatographically identical with 3-*O*-β-D-glucopyranosyl-D-xylopyranose[16]. The second sugar was purified by preparative, paper chromatography (yield, 190 mg), and examined by the methods given in Table II. The results show that the new disaccharide is 4-*O*-β-D-glucopyranosyl-D-xylopyranose, and this conclusion was confirmed by small-scale methylation analysis. A methanolysate, on examination by g.l.c., contained the methyl glycosides of 2,3,4,6-tetra-*O*-methyl-D-glucose and 2,3-di-*O*-methyl-D-xylose.

TABLE II

PROPERTIES OF *O-D*-GLUCOSYL-D-XYLOSES

	Enzyme source	
	Tetrahymena pyriformis	*Barley*
$[\alpha]_D$, water	+97°	−7°
R_G value, solvent *A*	0.68	0.68
Colour with aniline oxalate	pink	pink
M_G value, borate	0.28	0.28
M_S value, molybdate	0.30	0.30
Reaction with triphenyltetrazolium chloride	+	+
Colour with periodate–*p*-rosaniline reagent	blue	blue
Products on reduction, followed by acid hydrolysis	glucose, xylitol	glucose, xylitol
Hydrolysis by almond β-glucosidase	—	+
Periodate reduction, mol.	3.9	3.8

DISCUSSION

The present investigation has shown that extracts of *Tetrahymena pyriformis* can transfer α-D-glucosyl residues from phenyl α-D-glucopyranoside to a variety of acceptors including aldohexoses, D-pentoses and certain alcohols, but not to D-fructose, 6-deoxy-L-hexoses, or to D-glucuronic acid. Since trans-α-D-glucosylation to D-glucose results in the formation of maltose and isomaltose, it was of interest to examine the transfer to the stereochemically related D-xylose. From a digest containing D-xylose (5 g), a new disaccharide (*ca.* 230 mg), $[\alpha]_D$ +97° (water), was isolated, which has been characterised as 4-*O*-α-D-glucopyranosyl-D-xylopyranose. Only a trace of a second glucosylxylose was produced. The (1→4)-linked disaccharide had been prepared by Hassid and co-workers[17], using a bacterial enzyme preparation, β-D-glucose 1-phosphate, and D-xylose; this sample had $[\alpha]_D$ +95° (water). In view of the relatively good yield (the above result ignores substantial losses due to overlapping in various fractions from the charcoal–Celite column and from preparative, paper chromatograms), other pentoses were examined as possible acceptors, with a view to preparing hitherto unknown disaccharides.

From D-ribose (5 g), a disaccharide (*ca.* 200 mg), characterised as 4-*O*-α-D-glucopyranosyl-D-ribose, was isolated. Although the conformation of pentoses when bound to the surface of an enzyme-protein is not known, it may be significant that, in solution, D-xylose and D-ribose (as with D-glucose) have an equatorial OH group at C-4, and that this clearly represents a favourable acceptor site.

Enzymic transfer of α-D-glucosyl residues to D-lyxose and D-arabinose yields a mixture of disaccharides. From D-lyxose (2.5 g), a (1→4)-linked disaccharide (147 mg), a (1→2)-linked disaccharide (60 mg), and 3-*O*-α-D-glucopyranosyl-D-lyxose (29 mg) were obtained. These appear to be new compounds [18]. D-Lyxose exists in solution as a mixture of the *C1* and *1C* conformations, in which the OH at C-4 is in an equatorial or an axial position. This appears to influence the transferase specificity of the enzyme system, and although transfer to C-4 predominates, there is appreciable transfer to C-2 and C-3.

From D-arabinose (5 g), a mixture of disaccharides (190 mg) was isolated, including a (1→5)-linked D-arabinofuranose, and (1→2)-, (1→3)-, and (1→4)-linked D-arabinopyranoses. With this pentose, in which the OH at C-4 is in the axial position, transfer to C-4 does not predominate.

The enzyme system therefore shows a fairly high degree of specificity towards α-D-glucosyl acceptors, and favours those sugars which, in solution, have an equatorial OH group at C-4. In general, the system provides a method for the preparation of a limited number of D-glucosylpentose disaccharides, some of which have not been prepared previously by chemical or enzymic means. It is probable that trans-α-D-glucosylation reactions with normal metabolites (*e.g.* degradation products of glycogen) are not physiologically important to *Tetrahymena pyriformis*, and that the present results represent an atypical reaction of an enzyme whose normal function is purely hydrolytic. The question as to whether the maltase and phenyl α-D-glucosidase activities are due to the same enzyme cannot be unequivocally answered. The relative activity of the extract towards the two substrates is very different (maltase = 100, phenyl α-D-glucosidase = 3.2; *cf.* methyl α-D-glucopyranoside = 0.5); there is a small difference in optimum pH (4.7 and 5.0, respectively), and the phenyl α-D-glucosidase activity is slightly more thermostable than the maltase[19]. The difference in these preliminary results is not great enough to distinguish between one enzyme or two different enzymes, and this aspect must await the results of fractionation studies on the protozoal extract.

The experiments with the barley β-glucosidase preparation have led to a preparation of 4-*O*-β-D-glucopyranosyl-D-xylose, in contrast to the (1→3)-linked disaccharide which has been produced by the action of enzyme preparations from *Aspergillus niger*[20] or *Cladophora rupestris*[16] on a mixture of cellobiose and D-xylose.

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SUMMARY

By transfer of an α-D-glucosyl residue from phenyl α-D-glucopyranoside to a pentose, using an enzyme preparation from *Tetrahymena pyriformis*, 4-*O*-α-D-glucopyranosyl-D-xylose and -D-ribose, and mixtures of three *O*-α-D-glucosyl-D-lyxoses and four α-D-glucosyl-D-arabinoses have been prepared. An enzyme preparation from barley transfers a β-D-glucosyl residue from salicin to D-xylose, giving a mixture of (1→3)- and (1→4)-linked *O*-β-D-glucosyl-D-xyloses.

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Notes

The reaction of unsaturated carbohydrates with carbon monoxide and hydrogen.

Part VI. Structure and stereochemistry of the major anhydroheptitols from tetra-*O*-acetyl-1-deoxy-D-*arabino* (and D-*lyxo*)-hex-1-enopyranose*

Parts II–V of this series$^{1-4}$ described the action of carbon monoxide and hydrogen on glycals, and demonstrated the preferential addition of the hydroxymethyl group to C-1 of the glycal. This communication gives the results of an investigation on the reaction of carbon monoxide and hydrogen on the acetylated 2-hydroxyglycals, herein designated as hex-1-enopyranoses.

2,3,4,6-Tetra-*O*-acetyl-1-deoxy-D-*arabino*-hex-1-enopyranose (2-acetoxy-3,4,6-tri-*O*-acetyl-D-glucal[5]) was treated in a high-pressure apparatus with carbon monoxide and hydrogen in the presence of dicobalt octacarbonyl, under conditions similar to

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*Presented in part at the 150th National Meeting of the American Chemical Society, Atlantic City, N.J., September 17-21, 1965.

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Part VI. Structure and stereochemistry of the major anhydroheptitols from tetra-*O*-acetyl-1-deoxy-D-*arabino* (and D-*lyxo*)-hex-1-enopyranose*

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2,3,4,6-Tetra-*O*-acetyl-1-deoxy-D-*arabino*-hex-1-enopyranose (2-acetoxy-3,4,6-tri-*O*-acetyl-D-glucal[5]) was treated in a high-pressure apparatus with carbon monoxide and hydrogen in the presence of dicobalt octacarbonyl, under conditions similar to

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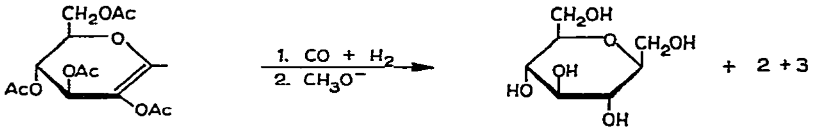
those described previously[1]. The reaction product was separated from the catalyst by chromatography on a Florisil column, and deacetylated with methanolic sodium methoxide; the major product (**1**) was obtained in about 70% yield by recrystallization of the resulting solid from methanol. Two minor products, **2** and **3**, were obtained by preparative paper chromatography.



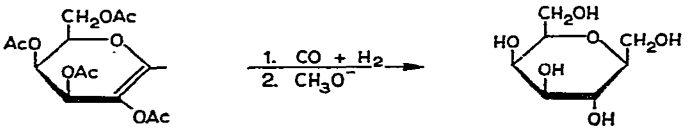
1

The fact that the major product (**1**) is devoid of optical activity suggests that it has a plane of symmetry. Acetylation of compound **1** afforded an optically inactive pentaacetate having a molecular weight of 404. Therefore, compound **1** must be 2,6-anhydro-D-*glycero*-D-*gulo*-heptitol. Using a different sequence of reactions, Fletcher and Coxon[6] have recently prepared a compound to which they assigned structure **1**. The physical constants (m.p., $[\alpha]_D$, and R_F) of their compound and of ours are in agreement.

Because of the fact that the oxo reaction, when applied to glycals, affords epimeric anhydroalditols, it was expected that compound **2** might be an epimer of **1**, differing in configuration only at C-2. Comparison of the molecular weights of the pentaacetates of **1** and **2** and examination of their mass spectra suggested striking similarities between the two compounds. However, as the anhydroalditol **2** consumed only one molar equivalent of periodate, the presence of two vicinal glycols was ruled out, and **2** was therefore not an epimer of **1**. The great difficulty encountered in purifying **2**, as well as the low yield, led us to abandon further structural work.

Recently, Wilkinson and co-workers[7] have found that the olefins undergo hydroformylation with carbon monoxide and hydrogen at much lower temperatures when a rhodium compound is used, instead of a cobalt carbonyl, as the catalyst. Application of their procedure to tetra-*O*-acetyl-1-deoxy-D-*arabino*-hex-1-enopyranose showed, surprisingly, that the unsaturated carbohydrate was deacetylated but did not absorb gases, thus indicating no hydroformylation.

When the oxo reaction (with dicobalt octacarbonyl as catalyst) was applied to 2,3,4,6-tetra-*O*-acetyl-1-deoxy-D-*lyxo*-hex-1-enopyranose[8] (2-acetoxy-3,4,6-tri-*O*-acetyl-D-galactal), the mixture of products was more complex than that obtained from the C-4 epimer. Deacetylation of the oxo product gave a complex mixture of alditol derivatives which was only partially separated by paper chromatography. Direct chromatographic comparison of one component with an authentic sample[9] indicated that it was probably 2,6-anhydro-D-*glycero*-L-*manno*-heptitol (**4**).



4

The conversion of 2,3,4,6-tetra-*O*-acetyl-1-deoxy-D-*arabino*(and D-*lyxo*)-hex-1-enopyranose into 2,6-anhydro-D-*glycero*-D-*gulo*-heptitol and 2,6-anhydro-D-*glycero* L-*manno*-heptitol, respectively, provides chemical proof that the oxo reaction proceeds *via* a *cis* addition of hydroxymethyl and hydrogen to the carbon–carbon double bond. This finding is in agreement with previous results[1,4] in which the stereochemistry of the oxo reaction was adduced on the basis of an analysis of the p.m.r. spectra of partially deuterated, epimeric anhydro-heptitols and -hexitols.

EXPERIMENTAL

General considerations

Conditions for chromatographic separations and for measurement of p.m.r. spectra have been described previously[1] All molecular weights were determined by mass spectrometry with an Atlas CH4 spectrometer.

*Treatment of tetra-O-acetyl-1-deoxy-*D-arabino*-hex-1-enopyranose with carbon monoxide and hydrogen to yield 2,6-anhydro-*D-glycero-D-gulo*-heptitol* (**1**)

A. *With dicobalt octacarbonyl as catalyst*

To a mixture of 2,3,4,6-tetra-*O*-acetyl-1-deoxy-D-*arabino*-hex-1-enopyranose (11.2 g) with purified benzene (40 ml) contained in a 300-ml shaker bomb, was added pre-formed dicobalt octacarbonyl (1.5 g). After the bomb had been flushed with carbon monoxide, carbon monoxide (750 lb. in^{-2}) and hydrogen (2120 lb. in^{-2}) were added, and the reactants were heated, with rocking, for 1.2 h at 115–120°.

The product was freed from catalyst as described previously[1], and then deacetylated with methanolic sodium methoxide. Recrystallization of 0.92 g of the deacetylated product from ethanol gave chromatographically pure platelets of **1**, yield 0.43 g. Evaporation of the filtrate yielded a syrupy mixture (A), which consisted mainly of **1** and **2**. This syrup is further described under compound **2**.

Characterization of compound **1**.

Compound **1** (2,6-anhydro-D-*glycero*-D-*gulo*-heptitol) had m.p. 203–205°, $[\alpha]_D^{20}$ 0° (*c* 2, water), and it consumed 1.6 molar equivalents of periodate in 20 h, as measured by the spectrophotometric method of Dixon and Lipkin[10]. The reported values[6] for 2,6-anhydro-D-*glycero*-D-*gulo*-heptitol are: m.p. 204–205°, $[\alpha]_D^{20}$ 0° (water).

Anal. Calc. for $C_7H_{14}O_6$: C, 43.29; H, 7.27. Found: C, 43.15; H, 7.20.

Compound **1** was oxidized with periodate, and the resulting "dialdehyde" was reduced with sodium borohydride according to a previously described procedure[1] to yield a syrup; p.m.r. singlet at $\delta = 5.2$ from H on OH, and singlet at $\delta = 6.3$ (with visible shoulders).

*Preparation of 1,3,4,5,7-penta-O-acetyl-2,6-anhydro-*D-glycero-D-gulo-*heptitol*

Compound **1**, acetylated according to the procedure described by Fletcher and Coxon[6], gave a product, m.p. 91–92°, $[\alpha]_D^{21}$ 0° (*c* 1.5, chloroform).

Anal. Calc. for $C_{17}H_{24}O_{11}$ (404.36): C, 50.49; H, 5.98. Found: C, 50.35; H, 6.1; mol. wt. 404.

Fletcher and Coxon[6] reported m.p. 89° and $[\alpha]_D^{20}$ ±0.2° for 1,3,4,5,7-penta-*O*-acetyl-2,6-anhydro-D-*glycero*-D-*gulo*-heptitol.

Attempted characterization of compound **2**

The syrup A (0.50 g) was fractionated by preparative paper chromatography to yield compound **1** (about 0.25 g), compound **2** (0.128 g), and compound **3** (0.034 g).

Compound **2** was crystallized from methanol–isopropyl ether; m.p. 85–90°, $[\alpha]_D^{20}$ +23° (*c* 0.5, water); p.m.r. unresolved multiplet of peaks at $\delta = 5.8$–6.6.

Periodate oxidation of **2**, followed by sodium borohydride reduction of the "dialdehyde", according to a previously described procedure[1], gave an oil; n.m.r., two singlets (with shoulders) at $\delta = 6.4$ and 6.7 in the ratio of about 9:1.

Compound **2** consumed 0.91 molar equivalent of periodate in 20 h, as measured by the spectrophotometric method of Dixon and Lipkin[10]. No further consumption of periodate was noted after an additional 24 h.

Acetylation of substance **2** with acetic anhydride and sodium acetate afforded a syrupy product which could not be crystallized.

Purification of the pentaacetate of **2** (0.105 g) was performed by preparative, thin-layer chromatography on silica gel, with isopropyl ether containing a trace of methanol as the developing solvent. The main zone was located by means of the acetate spray of Bishop and Tate[11]. An infrared lamp was used for heating strips of the plate. The main zone (0.048 g) could not be crystallized; $[\alpha]_D^{22}$ +7.1° (*c*, 2.4, chloroform). The molecular weight of the pentaacetate (by mass-spectral analysis) was 404. The molecular weight of a penta-*O*-acetylanhydroheptitol is 404.36. The p.m.r. spectrum of the pentaacetate of **2** in deuteriochloroform resembled that of the pentaacetate of compound **1**.

B. With $RhPy_4Cl_2$ as catalyst

2,3,4,6-Tetra-*O*-acetyl-1-deoxy-D-*arabino*-hex-1-enopyranose (7 g) in 40 ml of (3:1 v/v) ethanol–benzene was treated with a mixture of carbon monoxide (1000 lb. in^{-2}) and hydrogen (1800 lb. in^{-2}) for 3 h at 130° in the presence of $RhPy_4Cl_2$ (0.5 g) as catalyst[7]. There was no significant absorption of gases. Processing of the reaction mixture showed that complete transesterification of the carbohydrate acetate had taken place, yielding about 7.5 g of ethyl acetate (theoretical yield, 7.5 g).

*Reaction of 2,3,4,6-tetra-O-acetyl-1-deoxy-*D-lyxo*-hex-1-enopyranose with carbon monoxide and hydrogen to yield 2,6-anhydro-*D-glycero-L-manno*-heptitol*

2,3,4,6-Tetra-*O*-acetyl-1-deoxy-D-*lyxo*-hex-1-enopyranose[8] was treated with carbon monoxide and hydrogen in the presence of dicobalt octacarbonyl as described in the first part of the experimental section. Deacetylation of the oxo product gave a mixture of three alditol derivatives, one of which (present in about 20% yield) was shown, by direct chromatographic comparison with an authentic sample[9], to be 2,6-anhydro-D-*glycero*-L-*manno*-heptitol.

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Department of Chemistry,
University of British Columbia,
Vancouver 8,
British Columbia (Canada)

ALEX ROSENTHAL
DEREK ABSON

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Preparation of glycosyl phosphates: β-D-glucopyranosyl phosphate

The formation of glycosyl phosphates by reaction of a fully acetylated sugar with anhydrous phosphoric acid at moderate temperatures is a convenient procedure for the preparation of these esters, and a number of such phosphates of biochemical interest have been prepared in this way. For example, the potassium salts of α-D-glucopyranosyl phosphate and α-D-galactopyranosyl phosphate were prepared from the corresponding pentaacetates of β-D-glucopyranose and β-D-galactopyranose, respectively, in yields[1,2] approaching 60%. Subsequently, it was shown[3] that salts of 2-acetamido-2-deoxy-α-D-glucopyranosyl phosphate and 2-acetamido-2-deoxy-α-D-galactopyranosyl phosphate could be prepared from the β-D-tetraacetates of the appropriate 2-acetamido sugars.

In a later paper, O'Brien[4] reported on a fusion reaction using 2-acetamido-2-deoxy-α-D-glucopyranose tetraacetate. This compound, having substituents on C-1 and C-2 in the 1,2-*cis* orientation, reacted much more slowly than its anomer having the 1,2-*trans* (diequatorial) conformation, and reasonable yields of product (33%) were obtained only by raising the reaction temperature to 83° (as compared to 50°, originally used) and increasing the proportion of phosphoric acid. Recently, the pentaacetates of α-D-glucopyranose and α-D-galactopyranose have been shown to react readily only under the more strenuous conditions reported by O'Brien[4], and the product isolated was, in each instance, the α-D anomer[2,5]. Several workers have reported the preparation of salts of α-D-mannopyranosyl phosphate by using this general phosphorylating procedure[6].

In the above experiments, the α-D (axial) anomer was generally the one isolated, although it is probable that, in most instances, both anomers are formed in the reaction. The isolation of only one anomer may be the result of loss of the other anomer (a) during the crystallization process, or (b) through the preferential hydrolysis of the (more labile) β-D (equatorial) anomer during the fractionation procedure[4]. The actual isolation of both anomers using an ion-exchange fractionation procedure was reported by O'Brien[4] for the anomeric 2-acetamido-2-deoxy-D-glucopyranosyl phosphates.

The replacement of an acetoxy group on C-1 of an acetylated sugar bearing acetoxy groups on C-1 and C-2 in a *trans* configuration is greatly facilitated by neighboring-group participation of the acetoxy group on C-2. As a consequence, β-D-glucopyranose pentaacetate, for example, reacts much more rapidly than the corresponding α-D anomer in various replacement reactions[7], as has been amply shown in the preparation of glycosyl halides, as well as of glycosyl phosphates, as outlined above. In the preparation of a tetra-*O*-acetyl-α-D-glucopyranosyl halide from β-D-glucopyranose pentaacetate, it is to be expected that the tetra-*O*-acetyl-β-D-glucopyranosyl halide will be the first product of the reaction. Under the conditions of the reaction, this compound would then be anomerized to the thermodynamically more stable α-D anomer. The actual preparation of the β-D anomer requires, in general, shorter reaction times, and conditions for such preparations have been published[8].

In an analogous way, it would be expected that, in the preparation of a D-glucosyl phosphate from β-D-glucopyranose pentaacetate using the fusion procedure, the first product would be a derivative of β-D-glucopyranosyl phosphate, and that this material would then be anomerized to the more stable α-D anomer. In most of the previously published preparations of glycosyl phosphates by this method, the reaction has been allowed to proceed for up to two hours. Generally, only that anomer having a stable conformation (with an axial phosphate group) has been isolated, except in the case of the 2-acetamido-2-deoxy-D-glucose derivative[4].

We have now found that, as anticipated, a derivative of β-D-glucopyranosyl phosphate is an early and major product in the reaction of penta-*O*-acetyl-β-D-glucopyranose with anhydrous phosphoric acid at 50°. After removal of the protecting acetyl groups, and purification of the barium salt, an examination of the optical rotation showed that the yield of the more levorotatory anomer decreases as the duration of the fusion reaction is increased. Some results indicating this transformation are indicated in the Table.

TABLE I

EFFECT OF REACTION TIME ON FORMATION OF THE ANOMERS OF D-GLUCOPYRANOSYL PHOSPHATE

D-*Glucopyranosyl phosphate*	*Time (min)*		
	5	*10*	*20*
Barium salt, yield %	52	56	55
$[\alpha]_D$[a]	+17	+28	+43
% of β anomer[b], calc.	88	71	48
Dipotassium α-, yield %	1	8	31
$[\alpha]_D$	+73	+63	+67
Di(cyclohexylammonium) β-, yield %	45	36	19
$[\alpha]_D$	+8.8	+6.2	+5.8

[a] Calc. on basis of anhydrous salt.

[b] On the basis of reported molecular rotations[9], the rotations of the anhydrous barium salts would be +74° and +9.6° for the α and β anomer, respectively.

The barium salts were converted into the potassium salts, and the readily crystallizing dipotassium salt of α-D-glucopyranosyl phosphate dihydrate was obtained. Although no effort was made to purify this compound, the yield varied directly with the elapsed time for the fusion reaction. The salt of β-D-glucopyranosyl phosphate which remained in solution was then isolated by conversion into the di(cyclohexylammonium) salt, followed by crystallization. The air-dried salt was found to be anhydrous and to have a specific rotation of +5.9°.

Putman and Hassid[9] reported that their air-dried product, one molecule of which crystallized with one molecule of ethanol and one of water, had a specific rotation of +7.3° (corresponding to +8.3° for solvent-free material).

EXPERIMENTAL

Di(cyclohexylammonium) β-D-glucopyranosyl phosphate

Ten g of anhydrous phosphoric acid (which had been dried overnight *in vacuo* over magnesium perchlorate) was melted at 50°, and to the melt was added 5.00 g (12.8 mmoles) of powdered penta-*O*-acetyl-β-D-glucopyranose. The mixture was stirred magnetically *in vacuo* at 50°; after 5 min, the mixture was rapidly cooled, and 205 ml of ice-cold 2 N lithium hydroxide was added. The contents of the flask were thoroughly mixed to disperse the sirup, and then allowed to stand overnight. The precipitated lithium phosphate was removed by filtration through Celite, and washed with cold, lithium hydroxide solution (*ca.* 0.01 N). The resulting solution contained 6.7 mmoles (52%) of acid-labile phosphate[10].

The pH of the solution was lowered to *ca.* 8.5 with Dowex 50W-H^+ and, after the resin had been removed by filtration, barium acetate (3.0 g) was added and the solution concentrated to about 50 ml. The barium salt was precipitated by the addition of four volumes of ethanol; after several hours at 5°, the solids were collected by centrifugation, washed successively with acetone and ether, and dried *in vacuo* over calcium chloride. The solid was dissolved in water (40 ml), faint traces of insoluble matter were removed by centrifugation, and the barium salt was reprecipitated by the addition of ethanol (120 ml). After a second such reprecipitation, there was obtained 3.92 g of barium salt containing 6.7 mmoles of phosphate and showing $[\alpha]_D^{24}$ + 17° (*c* 2.6, water; calculated on the basis of the anhydrous barium salt). The salts were dissolved in water at *ca.* 5°, and the solution was passed through a precooled column of Dowex 50W-H^+ (1 × 25 cm) into water containing 1.2 g of potassium hydroxide. The column was washed with 50 ml of cold water, and the pH of the resulting combined effluent was adjusted to 9 with Dowex 50W-H^+. The resin was removed by filtration, and the solution was concentrated to about 40 ml. The potassium salt crystallized at 5° on addition of 1.5 volumes of ethanol over a period of two days; it weighed 61.8 mg (1.3%) and showed $[\alpha]_D^{24}$ + 73.3° (*c* 0.5, water). Pure α-D-glucopyranosyl dipotassium phosphate dihydrate shows[11] $[\alpha]_D^{20}$ + 78°.

The mother liquors remaining from crystallization of the potassium salt were concentrated under diminished pressure, and the residue was dissolved in 50 ml of cold water. This solution was passed through a precooled column of Dowex 50W-H^+ (1 × 25 cm) and the column was washed with 100 ml of water, the effluent being collected in water containing 2 ml of cyclohexylamine. The combined percolate was concentrated *in vacuo* to *ca.* 2 ml, and 100 ml of absolute ethyl alcohol was added.

After two days at 5°, the crystalline salt was collected by filtration, washed with absolute ethanol, and air dried; yield 2.65 g (5.8 mmoles, 45%) $[\alpha]_D^{24}$ + 8.8° (*c* 2, water). A further 0.23 g (0.5 mmole, 4%), showing $[\alpha]_D^{24}$ + 28.6°, was obtained from the mother liquors. Recrystallization of the first crop gave 2.48 g (42%) of di(cyclohexylammonium) β-D-glucopyranosyl phosphate, showing $[\alpha]_D$ + 5.9°.

Putman and Hassid[9] reported that their air-dried product showed +7.3° and that one molecule contained 1 molecule each of ethanol and water.

Anal. Calc. for $C_{18}H_{39}N_2O_9P$ (458.5): C, 47.15; H, 8.57; N, 6.11; P, 6.76. Found*: C, 47.10; H, 8.81; N, 5.99; P, 6.87.

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Department of Chemistry and
Science Research Institute,
Oregon State University
Corvallis, Oregon 97331 (U.S.A.)

D. L. MACDONALD**

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*Analyses by Elek Microanalytical Laboratories, Torrance, California.
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Facile route to sugar thionocarbonates

In our studies with bis(*O*-thiocarbonyl) disulfide derivatives of carbohydrates, we reported[1] that bis(1,2-*O*-isopropylidene-3-*O*-thiocarbonyl-α-D-glucofuranose) disulfide rearranges to give one molar equivalent of 1,2-*O*-isopropylidene-α-D-glucofuranose 5,6-thionocarbonate simply on standing at room temperature in pyridine for a few hours. It was thought that this type of reaction might provide a facile route for synthesis of cyclic thionocarbonates, which have utility as intermediates for the preparation of olefins[2]. To explore this reaction, suitably protected carbohydrate derivatives possessing structurally different vicinal diol groups were prepared: 1,2:5,6-di-*O*-isopropylidene-D-mannitol (**1**); 3,4-*O*-isopropylidene-D-mannitol (**2**); 1,2-*O*-isopropylidene-α-D-glucofuranose (**3**); methyl 4,6-*O*-benzylidene-α-D-mannopyranoside (**4**); and methyl 5-*O*-benzyl-D-ribofuranoside (**5**).

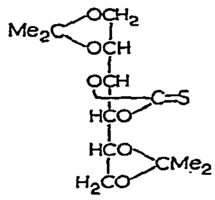
Each of these derivatives was converted readily into the bis(*O*-thiocarbonyl) disulfide derivative by oxidation of the corresponding xanthate with iodine. When the products were treated with pyridine, rearrangement occurred rapidly, as evidenced by the crystallization of elemental sulfur from the pyridine solutions. The rearrangement was monitored by observing the disappearance of the characteristic absorption maxima near 240 and 280 mμ for the bis(*O*-thiocarbonyl) disulfide group and the appearance of the maximum near 235 mμ for the thionocarbonate group. In each case, the reaction was complete after 0.5 h at room temperature.

Removal of the elemental sulfur and evaporation of the pyridine afforded the corresponding thionocarbonate in admixture with an equimolar amount of the starting sugar diol. These results are consistent with our earlier finding[1] that a bis(*O*-thiocarbonyl) disulfide derivative rearranged and fragmented to give equimolar amounts of the cyclic thionocarbonate, elemental sulfur, carbon disulfide, and the starting diol. Separation of the thionocarbonates from the diols was readily accomplished with the products from **1**,**3**,**4**, and **5** by extraction with water. Since the thionocarbonate from **2** was water-soluble, it was separated by column chromatography. The free diols were readily recovered, and could be recycled without further purification.

By these two procedures, substances **1** through **5** gave, respectively, 1,2:5,6-di-*O*-isopropylidene-D-mannitol 3,4-thionocarbonate (**6**); 3,4-*O*-isopropylidene-D-mannitol 1,2-thionocarbonate (**7**); 1,2-*O*-isopropylidene-α-D-glucofuranose 5,6-thionocarbonate (**8**); methyl 4,6-*O*-benzylidene-α-D-mannopyranoside 2,3-thionocarbonate (**9**); and methyl 5-*O*-benzyl-D-ribofuranoside 2,3-thionocarbonate (**10**). The thionocarbonates were identified by elemental analyses, spectroscopic data, and for **8**, comparison with an authentic sample.

EXPERIMENTAL

Melting points were taken in a Thomas–Kofler* melting-point apparatus. Ultraviolet spectra were recorded with a Perkin–Elmer 202 spectrophotometer.



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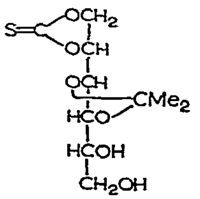

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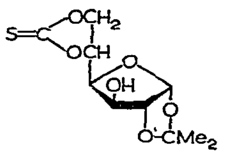

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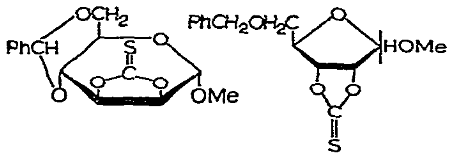

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*1,2:5,6-Di-O-isopropylidene-*D*-mannitol 3,4-thionocarbonate* (6)

1,2:5,6-Di-*O*-isopropylidene-D-mannitol[3] (1, 2.00 g) was dissolved in *p*-dioxane (10 ml), and sodium hydroxide (2.0 ml of 5N) and carbon disulfide (2 ml) were added. After having been stirred for 20 min, the solution was neutralized with acetic acid (5N), and the xanthate formed was oxidatively coupled by addition of iodine solution (0.3M). Partial evaporation of the reaction mixture produced a syrup. The syrup was washed with water, and then dissolved in 2 ml of pyridine, and the solution was kept at room temperature. After 0.5 h, most of the elemental sulfur formed was removed by filtration, and the filtrate was evaporated to a crystalline mass. Extraction of the mass with water removed **1**, leaving the thionocarbonate **6** (0.99 g, 85% based on the rearrangement). Recrystallization from ethanol–water gave m.p. 165–166°, $[\alpha]_D^{23}$ $-15°$ (c 2, $CHCl_3$), $\lambda_{max}^{ethanol}$ 235 mμ (ε 16,700). Haines[2c] reported m.p. 166–168°, $[\alpha]_D^{26}$ $-15°$ for this thionocarbonate.

*3,4-O-Isopropylidene-*D*-mannitol 1,2-thionocarbonate* (7)

3,4-*O*-Isopropylidene-D-mannitol[4] (2, 2.22 g) was dissolved in 2.3 ml of sodium hydroxide (5N). *p*-Dioxane (10 ml) and carbon disulfide (3 ml) were added, and the mixture was stirred for 20 min, neutralized, and treated with iodine as for **1**. The solution was evaporated to about 5 ml, and extracted with chloroform. The extract was evaporated to a syrup, which was dissolved in pyridine. After 0.5 h, most of the elemental sulfur was removed by filtration and the pyridine was evaporated off. The syrup was placed on a 2×15 cm column of Adsorbosil (Applied Science Laboratories, Inc., State College, Pennsylvania) with 1:1 v/v benzene–ethyl acetate as the eluent. The thionocarbonate **7** was eluted first, to give 1.16 g (88%). Recrystallization from ether–hexane gave m.p. 95–96°, $\lambda_{max}^{ethanol}$ 234 mμ (ε 14,900).

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*Mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not named.

Anal. Calc. for $C_{10}H_{16}O_6S$: C, 45.5; H, 6.1; S, 12.1. Found: C, 45.7; H, 6.1; S, 12.2.

1,2-O-Isopropylidene-α-D-glucofuranose 5,6-thionocarbonate (**8**)

By a procedure similar to that used for **1**, compound **3** (2.0 g) gave the 5,6-thionocarbonate (**8**, 1.1 g, 92%). Recrystallization from *p*-dioxane–water yielded a product identical with an authentic sample.

Methyl 4,6-O-benzylidene-α-D-mannopyranoside 2,3-thionocarbonate (**9**)

Methyl 4,6-*O*-benzylidene-α-D-mannopyranoside[5] (**4**, 2.00 g) was treated by a procedure similar to that used for **1**. The thionocarbonate **9**, recrystallized from carbon disulfide, gave 1.07 g (93%), m.p. 151–152°, $\lambda_{max}^{ethanol}$ 235 mμ (ε 15,700).

Anal. Calc. for $C_{15}H_{16}O_6S$: C, 55.6; H, 4.9; S, 9.9. Found: C, 55.3; H, 5.0; S, 10.0.

Methyl 5-O-benzyl-D-ribofuranoside 2,3-thionocarbonate (**10**)

Treatment of methyl 5-*O*-benzyl-D-ribofuranoside[6] (**5**, 1.0 g) by the foregoing general procedure gave the 2,3-thionocarbonate **10** (0.5 g, 86%) as a syrupy, anomeric mixture, $\lambda_{max}^{ethanol}$ 236 mμ (ε 15,900).

Anal. Calc. for $C_{14}H_{16}O_5S$: C, 56.8; H, 5.4; S, 10.8. Found: C, 57.4; H, 5.5; S, 10.8.

Northern Utilization Research and
Development Division,
Agricultural Research Service,
U.S. Department of Agriculture,
Peoria, Illinois 61604 (U.S.A.)

B. S. SHASHA
W. M. DOANE
C. R. RUSSELL
C. E. RIST

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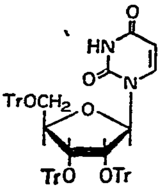
Nucleosides. XXXIV. 1-(2,3,5-Tri-*O*-trityl-β-D-ribosyl)uracil (2′,3′,5′-tri-*O*-trityluridine)

To the best of our knowledge, the introduction of such bulky groups as triphenylmethyloxy (trityloxy) groups on adjacent carbon atoms has not been reported. It appears noteworthy, therefore, that we have now isolated crystalline 1-(2,3,5-tri-*O*-trityl-β-D-ribosyl)uracil (2′,3′,5′-tri-*O*-trityluridine) monoethanolate (**1**), albeit in small yield (1.5%), from the reaction of 3.0 equivalents of trityl chloride with uridine in pyridine at elevated temperatures. Compound **1**, which bears the 2- and 3-*O*-trityl groups in a *cis*-vicinal relationship, was formed in a reaction which also produced the known 2,5-(**2**)[1,2,3] and 3,5-di-*O*-trityluridine (**3**)[2,4] in approximately 45 and 25% yields, respectively.

A tri-*O*-trityluridine structure for **1** was established both from analytical data (C, H, and N) and from integration of the proton magnetic resonance (n.m.r.) curve ($CDCl_3$). N.m.r. data suggested the presence of about 45 aromatic (trityl) protons, in agreement with the theoretical number. That the three trityl groups are attached to the sugar moiety, and not to the pyrimidine residue, is based on several lines of evidence. A signal at τ 1.20 probably represents a proton on N-3 of the uracil moiety[5], which would rule out the presence of a trityl group at O-2, O-4, or N-3. Indeed, a decrease in optical density at 260 mμ produced by the addition of alkali to an 80% ethanolic solution of **1** is consistent with the presence of a dissociable proton in the aglycon. A similar type of shift, attributed to dissociation of the N-3 proton, has been described for uridine[6]. Furthermore, methylation of **1** with excess methyl iodide and silver oxide in *N,N*-dimethylformamide for three days gave a new product having an ultraviolet absorption spectrum almost identical with that of **1** in neutral solution. Unlike compound **1**, however, the u.v. spectrum of the methylated derivative is unaltered in alkaline solution, thus proving the presence of a replaceable proton on N-3 of **1**.

Proof that compound **1** had maintained the *ribo* configuration was shown by the detritylation of **1** to uridine, as the sole product, in anhydrous ethereal hydrogen chloride at 0° within 60 min.

The n.m.r. spectrum of **1** reflects an extremely crowded situation in this molecule, probably involving *gauche* interactions between the 2- and 3-trityl groups, interaction between the 5-trityl group and the pyrimidine residue, and a marked distortion of normal bond angles in the furanoid ring. In the light of the recent generalization of Fromageot and co-workers[2] that the chemical shift of H-5 in ribofuranosyl-uracils (and -cytosines) occurs at higher field for 2- than for 3-substituted derivatives (an observation corroborated by us, using deuterated methyl sulfoxide, H-5 in **2** τ 0.31 upfield from H-5 in **3**), it is interesting to note that, in the solvent employed by us in this study, namely $CDCl_3$, the chemical shift for H-5 in **2** at τ 4.78 is almost identical with that for H-5 in **3**, τ 4.73. In **1**, however, H-5 is shifted much further upfield, to τ 5.12, reflecting a strong shielding effect by the trityl groups. This effect is also observed in the upfield position of what is most probably the H-1 signal, τ 5.37, which is far removed from the H-1 signal in **2**, τ 3.28, and in **3**, τ 3.95. Unfortunately, positive identification of signals for H-2, H-3, and H-4 in **1** was not possible. H-5 appeared as a widely spaced quadruplet, however, with a splitting of 11–12 c.p.s., which is centered at τ 7.51. This multiplet probably reflects a severe restriction of free rotation about the C-4–C-5 bond, probably due to interaction between the 5-trityl group and the pyrimidine residue.



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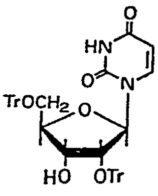

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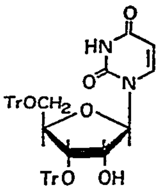

3

EXPERIMENTAL

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. Ultraviolet absorption spectra were recorded on a Cary Model 15 spectrophotometer. N.m.r. spectra were measured with a Varian A-60 apparatus operating at 60 Mc/sec. An internal indicator, tetramethylsilane (τ 10.00), was employed.

Tritylation of uridine

To a solution of uridine (12.0 g, 0.05 mole) in 125 ml of pyridine at 94° (internal temp.) was added trityl chloride (42.0 g, 0.15 mole) with stirring. The solution was maintained at 120° for 60 min, then at 100° for 8 h. Excess trityl chloride was converted into the alcohol by the addition of ice (4–5 g). The solution was poured, with stirring, into 2 l of ice-water, and the gummy solid collected and washed on the filter with water.

1-(2,5-Di-O-trityl-β-D-ribofuranosyl)uracil (2′,5′-di-O-trityluridine) (**2**)

The above gum was dissolved in chloroform, and the solution dried with sodium sulfate. After removal of the solvent *in vacuo*, the residue was dissolved in benzene, and **2** in crude form (11.72 g) was obtained on addition of ether to the warm solution, followed by cooling, as previously described[1]. Recrystallization of **2** from benzene–ether gave colorless needles, double m.p., 221–223° and 244–247°. Crystallization of **2** from ethanol gave needles, double m.p. 158–162° and 248–250°, $[\alpha]^{25}_{D}$ +94° (*c* 0.5, acetone); $[\alpha]^{24}_{D}$ +69° (*c* 0.5, chloroform). $\lambda^{\text{ethanol}}_{\text{max}}$ 260 $m\mu$ (ε 9200); $\lambda^{\text{ethanol}}_{\text{max}}$ 240 $m\mu$ (ε 8370). Reported melting points are[1] 224–225° and[3] 223–224°. Reported optical rotations are +91° (acetone)[1] and +91.4° (acetone)[3].

1-(2,3,5-Tri-O-trityl-β-D-ribosyl)uracil (2′,3′,5′-tri-O-trityluridine) (1)

The mother liquor from the crystallization of **2** was evaporated to dryness and dissolved in chloroform (70 ml). Preliminary fractionation was performed on a column (12×10 cm) of 450 g of neutral aluminum oxide (Bio-Rad, AG 7, 100–200 mesh). Elution was made successively with chloroform (1000 ml, Fraction *A*), 2:1 chloroform–ethanol (1000 ml, Fraction *B*), and 1:1 chloroform–ethanol (2000 ml, Fraction *C*). Monotritylated nucleoside and colored material remained adsorbed to the alumina.

The chloroform effluent (*A*) contained both triphenylmethanol and **1**. After removal of solvent, the residue was triturated with boiling heptane (550 ml), and the supernatant was decanted. Crystallization of the residue from ethanol gave crude **1** (1.0 g, 2.3%) as colorless needles, m.p. 238–244°, Purification of **1** was best achieved by chromatography on neutral alumina, as described below.

A sample (0.25 g) of the above material in chloroform was placed on an alumina column (1.7×24.0 cm). The column was first eluted with chloroform (50 ml), and the effluent discarded; it was then eluted with 4:1 chloroform–ethanol. From the 29–65 ml fraction, colorless needles (0.16 g), m.p. 284–287°, were obtained. Total yield, 0.64 g (1.5%). Chromatographic separation of material from a second experiment gave needles, m.p. 285–288°, $[\alpha]_D^{25}$ —29° (c 0.4, chloroform); $\lambda_{max}^{ethanol}$ 260 mμ (ε 9160); $\lambda_{max}^{ethanol}$ 240 mμ (ε 8980). N.m.r. ($CDCl_3$): doublet, τ 5.11 (J 8 c.p.s.); multiplet, τ 5.37; doublet, τ 5.91 (J 1 c.p.s.); doublet, τ 6.50 (J 5 c.p.s.); multiplet, τ 6.79; quadruplet, τ 7.51 (J 11–12 c.p.s.).

Anal. Calc. for $C_{66}H_{54}N_2O_6 \cdot C_2H_6O$: C, 80.29; H, 5.95; N, 2.75. Found: C, 79.96; 79.82; H, 5.80, 5.81; N, 2.69, 2.83.

1-(3,5-Di-O-trityl-β-D-ribofuranosyl)uracil (3′,5′-di-O-trityluridine) (3)

Fraction *B* was evaporated to dryness, to give a pale yellow solid (16.7 g). Addition of ether to a solution of this material in benzene gave an additional 2.25 g of crude **2**. Based on integrated n.m.r. (deuterated methyl sulfoxide) values of H-5 for compounds **2** and **3** (separated by about 20 c.p.s), the yellow residue obtained after removal of solvent from the mother liquor (14.18 g) was calculated to contain **2** (5.43 g, 37.5%) and **3** (9.05 g, 62.5%). A practical separation of **2** and **3** from this material was possible by column chromatography using alumina, as described below for the residue obtained from fraction *C*.

Removal of solvent from fraction *C* gave a pale-yellow solid (2.5 g). A solution of this material in chloroform (15 ml) was placed on a column (5×32 cm) of neutral alumina (530 g). Elution with chloroform (3300 ml) was followed by elution with 3:1 chloroform–ethanol. The 3600–3800 ml fraction from this solvent contained a yellow, fluorescent material which was discarded. Elution with 1:1 chloroform–ethanol, gave a small amount of **2** in the 300–500 ml fraction. Compound **3** was slowly eluted from the column and was collected in the 1000–4000 ml fraction. The residue was triturated with water, collected and dried to give a colorless amorphous solid (**3**, 1.3 g). Crystallization was achieved by dissolving **3** in warm methyl sulfoxide, adding water (up to 10%), and allowing the solution to cool slowly. Long, colorless needles, m.p. 113–116°, separated. This material gave a qualitative test for sulfur; $[\alpha]_D^{25}$ $-1°$ (c 0.4, chloroform), $[\alpha]_D^{24}$ $+28°$ (c 0.3, acetone). The presence of one molecule of methyl sulfoxide per molecule of the crystalline product was indicated by n.m.r. ($CDCl_3$), which gave a single peak at τ7.41 integrating for 6.6 protons. $\lambda_{max}^{ethanol}$ 260 mμ (ε 9900); $\lambda_{max}^{ethanol}$ 240 mμ (ε 8260). Compound 3 had previously been obtained[4] in an amorphous form, but was recently isolated as a crystalline monoethanolate[7].

Anal. Calc. for $C_{47}H_{40}N_2O_6 \cdot C_2H_6OS$: C, 72.93; H, 5.75; N, 3.47. Found: C, 72.68, 72.69; H, 5.81, 5.82; N, 3.49.

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Division of Biological Chemistry,
Sloan-Kettering Institute for Cancer Research,
New York, New York 10021 (U.S.A.)

JOHN F. CODINGTON
JACK J. FOX

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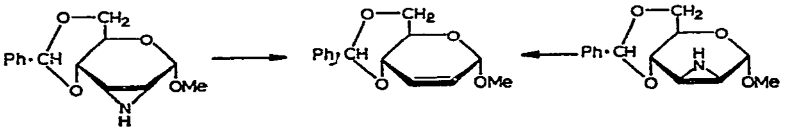
Nitrogen-containing carbohydrate derivatives
Part XII*. Reaction of epimino sugars with nitrous acid

From previous work on the nitrous acid deamination of simple alkyl aziridines[1], and of steroid epimines[2], to the corresponding alkenes, it would be expected that epimino sugars would give unsaturated sugars. Deaminations of methyl 4,6-*O*-benzylidene-2,3-dideoxy-2,3-epimino-α-D-alloside (**1**) and the corresponding mannoside (**3**) have both given good yields of methyl 4,6-*O*-benzylidene-2,3-dideoxy-α-D-*erythro*-hex-2-enopyranoside (**2**).



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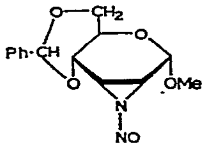

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In the case of the epimino alloside (**1**), it was possible to isolate the intermediate yellow *N*-nitrosoepimine (**4**), having an ultraviolet spectrum which corresponded well with that recorded[1] for *N*-nitroso-*trans*-2,3-dimethylaziridine. The spectrum also showed a band due to contaminating unsaturated glycoside (**2**). The *N*-nitrosoepimino mannoside was too unstable for isolation.

This facile reaction provides a ready method of characterising epimino sugars and may, in some cases, provide a method of preparing unsaturated sugars inaccessible by other routes.

EXPERIMENTAL

Deaminations

(*a*) *Methyl 4,6-O-benzylidene-2,3-dideoxy-2,3-epimino-α-D-alloside* (**1**). The epimino alloside (**1**)[3] (263 mg; 0.001 mole) was dissolved in aqueous acetic acid (50%; 10 ml). Sodium nitrite (103 mg; 0.0015 mole), dissolved in water (4 ml), was added. A yellow paste formed; this was diluted with water (20 ml), and the mixture was processed in two ways.

(*i*) The mixture was rapidly filtered, and the yellow precipitate was washed with a small volume of water and dissolved in methanol (100 ml), with shaking at room temperature. The methanol solution was filtered and cooled to —20°, and the yellow methyl 4,6-*O*-benzylidene-2,3-dideoxy-2,3-*N*-nitrosoepimino-α-D-alloside (**4**) (28%) (Found: C, 57.8; H, 5.5; N, 9.2. $C_{14}H_{16}N_2O_5$ calc. C, 57.9; H, 5.5; N, 9.6%) was collected in a pre-cooled filter and dried in a pre-cooled desiccator (P_2O_5). The product, which could be kept for a few days at —20°, had ν_{max} 1510 cm^{-1} (N=O), λ_{max}^{MeOH} 217, 257, and 451 mμ (ε_{max} not recorded, because of the compound's instability);

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*Part XI. G. J. F. Chittenden and R. D. Guthrie, *J. Chem. Soc.*, 1966 (c) 1508.

Clark and Helmkamp[1] reported $\lambda_{\max}^{\mathrm{MeOH}}$ 254 and 457 mμ for *N*-nitroso-*trans*-2,3-dimethylaziridine. Methyl 4,6-*O*-benzylidene-2,3-dideoxy-α-D-*erythro*-hex-2-enopyranoside has $\lambda_{\max}^{\mathrm{MeOH}}$ 217 mμ.

The *N*-nitrosoepimine was thermally unstable and became colourless after storage for about 4 h at room temperature. In an attempted m.p. determination, the compound turned colourless at 60–70°; continued heating gave m.p. 115–116° (the unsaturated glycoside **2** has m.p. 119–120°).

(*ii*) The mixture was made alkaline by addition of 2N sodium hydroxide, and then extracted with chloroform. The extracts were dried (Na_2SO_4) and evaporated. The crystalline residue (249 mg) was recrystallised from ethanol to give methyl 4,6-*O*-benzylidene-2,3-dideoxy-α-D-*erythro*-hex-2-enopyranoside (**2**) (81%), m.p. 118–119°, $[\alpha]_D^{26}$ +129° (*c* 0.2, chloroform) (Lit.[4], m.p. 119-120°, $[\alpha]_D^{20}$ +129°).

(*b*) *Methyl 4,6-O-benzylidene-2,3-dideoxy-2,3-epimino-α-D-mannoside.* This compound* was treated as in (*a*) above. Procedure (*i*) gave a very unstable, yellow product, presumed to be the *N*-nitrosoepimine. Procedure (*ii*) gave the unsaturated glycoside (**2**), in 78% yield, m.p. 119–120°.

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The Chemical Laboratory,
University of Sussex,
Brighton (Great Britain)

R. D. GUTHRIE
D. KING

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*This epimine, usually made from the diaxial methyl 2-azido-4,6-*O*-benzylidene-2-deoxy-α-D-altroside 3-methanesulphonate[3], has also been synthesised from the corresponding diequatorial 3-azido-D-glucoside 2-methanesulphonate by using the same conditions. The epimine was isolated, in 71% yield, as its *N*-acetyl derivative. (This experiment was carried out by D. Murphy.)

Crystalline trimethylsilyl ethers of cyclitols: hexakis(trimethylsilyl) ethers of *myo*-inositol and *scyllo*-inositol and pentakis(trimethylsilyl) ether of *myo*-inosose-2

Trimethylsilylation is a useful means of converting carbohydrates into compounds sufficiently volatile for gas chromatography. A comprehensive account presented by Sweeley, Bentley, Makita, and Wells[1] in 1963 clearly demonstrated the research potential of trimethylsilyl ethers of sugars. This procedure is now widely accepted among those concerned with separation and identification of carbohydrates. As one reviews the growing literature, it becomes apparent that an easily prepared, stable reference compound is needed.

Fully trimethylsilylated derivatives of *myo*-inositol, *scyllo*-inositol, and *myo*-inosose-2, as well as all other isomers of inositol, have been prepared and their retention times have been reported[1,2]; but to this author's knowledge, no report has yet appeared in which the physical properties of these compounds are detailed. Most fully silylated sugars are liquids at room temperature[3]. In the course of a program devoted to the study of carbohydrates related to plant cell-wall biosynthesis, a number of the fully trimethylsilylated derivatives were prepared in bulk. Of those investigated, *myo*-inositol, *scyllo*-inositol, *myo*-inosose-2, and galactitol formed crystalline compounds. All were easy to prepare, readily purified by sublimation, and completely stable under normal conditions of storage. In short, these crystalline trimethylsilyl derivatives have precisely the qualities desired for a reference substance.

EXPERIMENTAL

myo-Inositol (Nutritional Biochemical Corp., Cleveland, Ohio) was recrystallized from water. *myo*-Inosose-2 was prepared from *myo*-inositol by the action[4] of *Acetobacter suboxydans* ATCC-621. Inosose was purified by preparation of the phenylhydrazone which was recrystallized and treated with benzaldehyde to form free inosose. *scyllo*-Inositol was prepared from *myo*-inosose-2 by reduction with sodium borohydride[5]. The insoluble *scyllo*-inositol diborate was converted directly into *scyllo*-inositol hexaacetate and recrystallized from hot acetic anhydride. Hydrolysis of the hexaacetate[6] gave crystalline *scyllo*-inositol, which was recrystallized from water and alcohol.

Galactitol was prepared from D-galactose by reduction with sodium borohydride[7].

To prepare fully trimethylsilylated ethers, the carbohydrate (0.01 mole) was dissolved in dry, redistilled pyridine (75 ml). To this solution, at 0°, was added hexamethyldisilazane (0.1 mole, 16.1 g) (Aldrich Chem. Co., Milwaukee, Wis.) and chlorotrimethylsilane (0.05 mole, 5.4 g) (Applied Science Labs, Inc., State College, Pa.) The mixture was stirred for one 1 h, during which the contents of the flask reached room temperature. Stirring was continued for 3 h at 40°. Solvent and excess reagents were removed at reduced pressure in a rotary evaporator. The residue was slurried in heptane (20–30 ml) to extract the product and then the residue was filtered off. After washing the residues with more heptane, washings and filtrate were combined, and the heptane was removed in a rotary evaporator. As the solvent was removed, the product crystallized; the yields were nearly quantitative. Sublimation at 0.1 mm and 100° gave sharp-melting crystals which remained unchanged after repeated sublimation.

M.p.'s are corrected and infrared spectra were recorded on potassium bromide discs.

*1,2,3,4,5,6-Hexakis-O-(trimethylsilyl)-*myo*-inositol*

A white crystalline solid, m.p. 118–119°. Infrared spectrum: a strong absorption characteristic of trimethylsilyl ethers at 1245 cm^{-1}. Five strong absorptions in the 950–1200 cm^{-1} region, at 995, 1045, 1075, 1115, and 1180 cm^{-1}.

Anal. Calc. for $C_{24}H_{60}O_6Si_6$: C, 47.00; H, 9.86. Found: C, 47.26; H, 9.92.

*1,2,3,4,5,6-Hexakis-O-(trimethylsilyl)-*scyllo*-inositol*

A white crystalline solid, m.p. 179–180°. Infrared spectrum: strong characteristic absorption at 1245 cm^{-1} and two strong absorptions at 1030 and 1145 cm^{-1}.

Anal. Calc. for $C_{24}H_{60}O_6Si_6$: C, 47.00; H, 9.86. Found: C, 46.90; H, 9.90.

*1,3,4,5,6-Pentakis-O-(trimethylsilyl)-*myo*-inosose-2*

A white crystalline solid, m.p. 98°. Infrared spectrum: carbonyl stretching absorption at 1750 cm^{-1}, strong characteristic absorption at 1240 cm^{-1}, and three strong absorptions at 1005, 1045, and 1130 cm^{-1}.

Anal. Calc. for $C_{21}H_{50}O_6Si_5$: C, 46,78; H, 9.35. Found: C, 46.91; H, 9.77.

Hexakis-O-(trimethylsilyl)galactitol

A white crystalline solid, m.p. 78°. The elemental analysis and the infrared spectrum were not determined.

Gas–liquid chromatography

Each of the compounds just described gave a single symmetrical peak having a retention time identical to that of the trimethylsilyl derivatives of *myo*-inositol, *scyllo*-inositol, *myo*-inosose-2, or galactitol prepared in the usual way[1]. The retention times of trimethylsilyl derivatives of a number of carbohydrates relative to the retention time of each of the three cyclitol derivatives are listed in Table I. These values were determined after injection, into the gas chromatograph, of a mixture of the carbohydrate derivative (0.1–0.4 μg) and each of the three cyclitol derivatives (0.2 μg) in heptane (total volume of 0.4–0.6 μl). The instrument was a dual column chromatograph (Packard Inst. Co., Downers Grove, Ill., Model 7821) with flame ionization detection. Columns were glass coils, 4 mm by 1.8 m, packed with 3%

silicone polymer, JXR, on Gas Chrom Q, 100–120 mesh (Applied Science Labs, Inc., State College, Pa.). They were flushed with nitrogen at 12 lb. in.$^{-2}$, 60 ml/min. The inlet temperature was 240°, the detector temperature 260°, and the outlet temperature 265°. The samples were introduced into the column at 140° and the elution was programmed linearly, after a 2-min delay, to 210° at 3°/min. The sensitivity of the electrometers was 1×10^{-9} amperes. Under these conditions, the trimethylsilylated *myo*-inositol peak appeared about 19 min after injection.

TABLE I

RETENTION TIMES OF SELECTED FULLY TRIMETHYLSILYLATED CARBOHYDRATES

Compounds	*Retention time relative to:*		
	myo-*inosose-2*	scyllo-*inositol*	myo-*inositol*
β-L-Arabinopyranose[a]	0.34	0.26	0.24
2-*O*-Methyl-D-xylose	0.36	0.28	0.26
α-D-Xylopyranose[a]	0.55	0.44	0.41
Xylitol[a]	0.60	0.48	0.46
L-Arabinitol[a]	0.63	0.51	0.48
Methyl α-D-mannopyranoside[a]	0.72	0.58	0.54
L-Rhamnitol[a]	0.73	0.58	0.55
Quebrachitol	0.77	0.62	0.58
Pinitol	0.86	0.69	0.64
L-Galactono-1,4-lactone[a]	0.91	0.72	0.66
Methyl α-D-glucopyranoside[a]	0.91	0.73	0.67
L-Gulono-1,4-lactone[a]	0.96	0.76	0.70
α-D-Glucopyranose[a]	0.96	0.77	0.71
myo-Inosose-2[b]	1.00	0.81	0.75
D-Mannitol[a]	1.07	0.85	0.79
Ononitol	1.08	0.87	0.80
D-Glucitol[a]	1.09	0.86	0.79
Galactitol[b]	1.09	0.87	0.80
L-Inositol	1.11	0.88	0.82
D-Bornesitol	1.15	0.93	0.86
β-D-Glucopyranose[a]	1.17	0.94	0.87
scyllo-Inositol[b]	1.24	1.00	0.93
myo-Inositol[b]	1.36	1.08	1.00

[a] The fully trimethylsilylated derivative is a liquid at room temperature.

[b] The fully trimethylsilylated derivative is a crystalline solid at room temperature.

DISCUSSION

Three trimethylsilylated cyclitols described in this paper show considerable promise as internal references for the determination of the retention times of other silylated carbohydrates. All three are stable crystalline compounds, easily purified by sublimation. When normal precautions are observed to prevent solvent losses, heptane solutions of these ethers can be stored for indefinite periods.

Generally it is advantageous to use a reference consisting of more than one standard compound, since derivatives having retention times close to one or other of the references employed are often encountered. In the values reported in Table I, only cyclitols were used as reference substances. Galactitol would also have served as well. It is interesting that the fully trimethylsilylated derivatives of mannitol and sorbitol are liquids.

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Department of Biology,
State University of New York at Buffalo,
Buffalo, New York 14214 (U.S.A.)

F. LOEWUS

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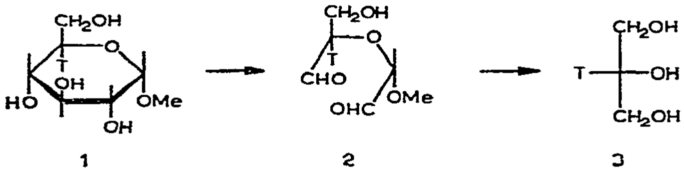
A synthesis of D-glucose-5-*t* and D-glucose-5-*t* 6-phosphate

During studies of the mechanism of enzymic transformations in the sugar series, D-glucose-5-*t* 6-phosphate was required. At that time, syntheses of D-glucose-5-*t* and D-glucose-5-*d* had not been reported, and synthesis by the following route was undertaken.

Calcium D-*xylo*-5-hexulosonate (5-oxo-gluconate), which is readily prepared by oxidation of D-glucose[1] with *Acetobacter suboxydans*, was reduced by sodium borotritiide, after conversion into the sodium salt. The resulting mixture of D-gluconic-5-*t* and L-idonic-5-*t* acids was subjected to mild lactonization, which permitted the selective formation of D-glucono-1,5-lactone-5-*t*. Sodium borohydride reduction, followed by deionization, gave chromatographically pure D-glucose-5-*t* which was characterized as methyl α-D-glucopyranoside. After more vigorous lactonization in preliminary experiments, some 1,6-anhydro-L-idose was detected after the second reduction. D-Glucose-5-*t* 6-phosphate was prepared using hexokinase[2].

A partial degradation of the D-glucose molecule was carried out to ensure that, under the conditions of the initial reduction, no isomerization of the 5-oxo group occurred which might lead to introduction of labelling at C-4 or C-6. The hydrogen atoms at C-6 were isolated as the formaldehyde–dimedone (5,5-dimethylcyclohexane-1,3-dione) complex after periodate oxidation of D-glucose.

Carbon atoms 4, 5, and 6 were isolated as glycerol (**3**) by periodate oxidation of methyl α-D-glucopyranoside (**1**), followed by reduction of the dialdehyde (**2**) formed, and acid hydrolysis. The glycerol thus produced had only 76% of the activity per μmole of the methyl glycoside, probably because of acid-catalysed exchange of tritium with the solvent in the intermediate dialdehyde (**2**), in which the tritium is located α to the aldehyde group. Periodate oxidation of the glycerol showed that no activity was located at C-4 or C-6 of the D-glucose molecule. Periodate oxidation of D-glucose-5-*t* 6-phosphate gave glycolaldehyde 2-phosphate; this was converted into ethanediol 1-phosphate dicyclohexylamine salt which retained 92% of the activity. Thus, the tritium was localized at C-5 of the D-glucose and D-glucose 6-phosphate.



While this work was in progress, the preparation of 1,2-*O*-isopropylidene-α-D-glucofuranose-5-*d* was reported from 1,2-*O*-isopropylidene-α-D-glucofuranurono-6,3-lactone[3] and also by a similar procedure to that outlined above[4]. In the latter case, the product was contaminated with L-idose and D-glucitol derivatives.

EXPERIMENTAL

Radioactivity was measured on an I.D.L. Liquid Measuring Head 2022. Generally, dioxane-based liquid scintillator NE 220 [Nuclear Enterprises (G.B.) Ltd.], containing 0.05 ml of water in 4 ml, was used; this gave 20% efficiency. Since D-glucose 6-phosphate and ethanediol 1-phosphate dicyclohexylamine salt are not sufficiently soluble in dioxane, a solution of 2,5-bis(5-*tert*-butylbenzoxazolyl-2)thiophen(B.B. O.T., Ciba Ltd.) (4 g/l) in 50% (v/v) toluene–methanol, containing 0.05 ml of water in 5 ml, was used; this gave only 3.2% efficiency.

D-*Glucose*-5-t

Calcium D-*xylo*-5-hexulosonate (269 mg, 1 mmole) was suspended in water (5 ml) and stirred with Amberlite IR-120 (H^+ form) until it was all converted into the free acid (about 1 h). The resin was filtered off, and the acid was neutralized to Methyl Red with sodium hydroxide. Sodium borotritiide (10 mg, 0.25 mmole, 5 mc) in water (2 ml) was added over 10 min at room temperature, with stirring, and the mixture was left for a further 10 min. Amberlite IR-120 (H^+ form) was added to stop the reaction and remove sodium ions. The solution was evaporated to dryness at 30–35° *in vacuo*, and dioxan (6×5 ml) was distilled from the residue at 30–35° *in vacuo*. The mixture of acids and lactones was redissolved in ice-cold water (10 ml), and sodium borohydride (15 mg, 0.37 mmole) in water (2 ml) was added over 45 min with stirring at 0° and pH 3–4. After a further 15 min, Amberlite IR-120 (H^+ form) was added, followed by Amberlite IRA-400 (CO_3^{2-} form). The solution was stirred for 30 min to decompose any remaining lactone and to deionize the solution. The resin was filtered off and washed well, and the filtrate was evaporated to dryness *in vacuo* at 35–40° to give chromatographically pure D-glucose-5*t* which was further purified by chromatography on Whatman No. 3 MM paper, using ethyl acetate–pyridine–water (120:50:40) as solvent. Yield: 30 mg, 17%, 1.8 mc/mmole.

*Methyl α-*D*-glucopyranoside*-5-t

Carrier D-glucose (360 mg) was added to D-glucose-5-*t* (6 mg) and refluxed with 3% methanolic hydrogen chloride for 3 h. The solution was deionized with Amberlite IRA-400 (CO_3^{2-} form) in methanol and evaporated to a syrup which was recrystallized from ethanol and ethyl acetate to give methyl α-D-glucopyranoside-5-*t* (205 mg), m.p. 161°, 30 μc/mmole.

D-*Glucose*-5-t *6-phosphate*

D-Glucose-5-*t* (30 mg, 120μc) was dissolved in 0.05M 2-amino-2-hydroxymethylpropane-1,3-diol–hydrochloric acid buffer (pH 8.2). Adenosine 5-triphosphate disodium dihydrogen salt (74 mg) was added, followed by hexokinase (100 units in 0.2 ml). The solution was incubated for 2 h at 35°, with shaking, and boiled to denature protein, and, after centrifugation, the supernatant solution was chromatographed on Whatman

No. 3 MM paper, using butanol–acetic acid–water (4:1:5). The area corresponding to D-glucose-5-*t* 6-phosphate was eluted. Yield: 30 mg, 80 μc, 66%.

Degradation[5] *of* D-*glucose*-5-t

To a 1-ml sample of D-glucose-5-*t*, containing 8.5×10^5 c/min, was added carrier D-glucose (18 mg); final activity 8.5×10^3 c/min/μmole. Periodic acid (0.2 ml, 50%, w/w) was added to the solution which was left overnight at room temperature and then poured into a solution (20 ml) containing dimedone (100 mg), Na_2HPO_4 (93 mg), and $NaH_2PO_4 \cdot 2H_2O$ (70 mg). After 1 h, the formaldehyde–dimedone complex (22 mg) was recrystallized from methanol three times; m.p. 182–3°, 6.1 c/min/μmole. Activity (*cf.* D-glucose), 0.07%.

Degradation of methyl α-D-*glucopyranoside*-5-t

Methyl α-D-glucopyranoside-5-*t* (70 mg, 5.5×10^3 c/min/μmole) was dissolved in water (2 ml), and periodic acid (240 mg) in water (2 ml) was added. After 2.5 h at 35°, the solution was deionized. To the resulting solution (20 ml) was added sodium borohydride (30 mg), and, after 1.5 h at room temperature, 5 ml of 2N hydrochloric acid. The solution was maintained at 60° for 30 min, the acid was neutralized to Methyl Red with N sodium hydroxide, and sodium borohydride (20 mg) was added. After 1 h, the solution was evaporated to dryness *in vacuo*, and methanol was added. The sodium chloride was filtered off and well washed with methanol, and the methanol was again removed. The remaining solution was deionized, and chromatographed on Whatman No. 3 MM paper by using butanol–ethanol–water (4:1:5); the glycerol (R_F 0.47) was eluted. The glycerol content was estimated by titration of the formic acid released during periodate oxidation, compared with a weighed standard; it had 4.2×10^3 c/min/μmole. The glycerol was oxidized with periodic acid, and the formaldehyde–dimedone complex was isolated as described for the degradation of D-glucose-5-*t*. After three recrystallizations from methanol, the formaldehyde–dimedone complex had m.p. 181–2°, 2.4 c/min/μmole. Activity (*cf.* glycerol), 0.05%.

Degradation of D-*glucose*-5-t *6-phosphate*

To a 1-ml sample of D-glucose-5-*t* 6-phosphate disodium salt, containing 4.96×10^3 c/min, was added D-glucose 6-phosphate disodium salt (60 mg); final activity, 2.48×10^4 c/min/mmole. Sodium periodate (160 mg) in water (1 ml) was added and, after 20 min, the solution was titrated (Methyl Red) with N sodium hydroxide (0.6 ml). The sodium iodate was precipitated with alcohol (5 ml) and removed by centrifugation. Sodium borohydride (20 mg) was added, and after 1 h at room temperature, Amberlite IR-120 (H^+ form) was added, and the deionized solution was evaporated to dryness *in vacuo* at 35–40°. Methanol was added and evaporated five times, and the yellow residue was dissolved in water (1 ml). Cyclohexylamine (0.1 ml) was added, and, after 1 h, the water was removed *in vacuo* at 35–40°. The syrup was dissolved in hot alcohol, and ether was added to produce turbidity. After two recrystallizations, the needles of ethanediol 1-phosphate dicyclohexylamine salt had m.p.

150–155° (lit.[6], 160–165°); activity, 2.28×10^4 c/min/mmole. Activity (*cf.* D-glucose 6-phosphate), 92%.

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Department of Physiology and Biochemistry,
University of Southampton (Great Britain)

J. E. G. BARNETT
D. L. CORINA

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Preliminary communications

A new route to 3-deoxy-3-fluoro-D-glucose

Whereas the replacement of primary hydroxyl groups of hexose and pentose derivatives by fluorine atoms is readily accomplished[1], the replacement of secondary hydroxyl groups has only recently been reported. Derivatives of 2-deoxy-2-fluoro-D-ribose[2], 3-deoxy-3-fluoro-D-xylose[3], 2-deoxy-2-fluoro-D-allose and -D-altrose[4], and 3-deoxy-3-fluoro-D-glucose[4] are now known. Preparation of the last compound involved treatment of a 2,3-anhydro-D-allose derivative with hydrogen fluoride–boron trifluoride at −70° which afforded the deoxyfluoro-D-*gluco*-derivative as the minor reaction-product.

We have found that conversion of 1,2:5,6-di-*O*-isopropylidene-α-D-allofuranose[5] into the 3-toluene-*p*-sulphonate, m.p. 120–121°, $[\alpha]_D^{30}$ +87° (*c* 1.0, chloroform) (Found: C, 54.75; H, 6.3; S, 8.0. $C_{19}H_{26}SO_8$ calc.: C, 55.1; H, 6.3; S, 7.7%), followed by treatment[6] with tetra-*n*-butylammonium fluoride in boiling acetonitrile, gave 3-deoxy-3-fluoro-1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose (**1**, 40%), b.p. 80–85° (bath)/0.1 mm, $[\alpha]_D^{30}$ −22° (*c* 1.0, chloroform) (Found: C ,55.5;H , 7.3; F, 7.6. $C_{12}H_{19}FO_5$ calc.: C, 55.0; H, 7.3; F, 7.3%). Similar treatment of 1,2:5,6-di-*O*-

150–155° (lit.[6], 160–165°); activity, 2.28×10^4 c/min/mmole. Activity (*cf.* D-glucose 6-phosphate), 92%.

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Department of Physiology and Biochemistry,
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J. E. G. BARNETT
D. L. CORINA

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isopropylidene-3-*O*-toluene-*p*-sulphonyl-α-D-glucofuranose afforded 3-deoxy-1,2:5,6-di-*O*-isopropylidene-α-D-*erythro*-hex-3-enofuranose[7].

The n.m.r. spectrum (^{19}F, $CDCl_3$) of compound (1) contained 8 signals (centre of gravity, ϕ + 207.8 p.p.m. with respect to CCl_3F) having the following coupling constants J_{H_3F} 49.8, J_{H_4F} 29.8, and J_{H_2F} 10.8 c.p.s. The ^1H-spectrum showed, *inter alia*, a doublet at τ 4.06 (J 3.8 c.p.s.) for the anomeric proton. The signal for H-3 was a doublet of multiplets with the low-field portion at τ 4.60.

Mild, acid hydrolysis of compound (1) gave a non-crystalline product having a mobility and detection characteristics on paper chromatograms identical with those of 3-deoxy-3-fluoro-D-glucose prepared by Johansson and Lindberg[4].

The chemistry of 3-deoxy-3-fluoro-1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose is being further investigated since, by graded, acid hydrolysis, it could provide access to hexose and pentose derivatives containing more than one fluorine atom.

The authors thank Professor M. Stacey, F.R.S., for his interest, Professor B. Lindberg for a sample of 3-deoxy-3-fluoro-D-glucose, Dr. E. F. Mooney for measurement of the ^{19}F resonance spectrum, and the Sugar Research Foundation for financial support.

Chemistry Department,
The University,
Birmingham 15 (Great Britain)

K. W. BUCK
A. B. FOSTER
R. HEMS
J. M. WEBBER

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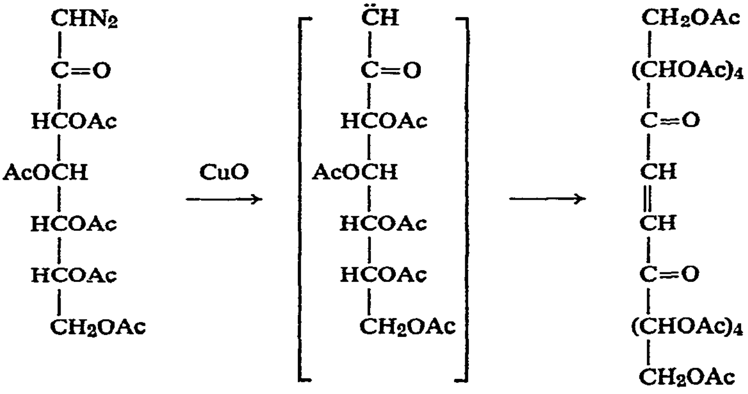
α-Oxo carbenes of the carbohydrate series

In studying the formation and reactions of carbene derivatives in the carbohydrate field, we have examined the decomposition of 1-deoxy-1-diazoketose acetates. In the presence of copper oxide or copper powder, these compounds give rise to α-oxo carbenes.

The decomposition of penta-*O*-acetyl-1-deoxy-1-diazo-*keto*-D-*gluco*-heptulose (**1**) in boiling benzene, in the presence of copper oxide, results in the formation of 1,2-bis(penta-*O*-acetyl-D-gluconyl)ethylene (**3**), m.p. 115–117°, $[\alpha]_D^{18}$ +26° (*c* 5, chloroform) (Found: C, 50.53; H, 5.40. $C_{34}H_{44}O_{22}$ calc.: C, 50.74; H, 5.47%). Presumably, the α-oxo carbene (**2**) is formed initially and subsequently dimerises[1,2].

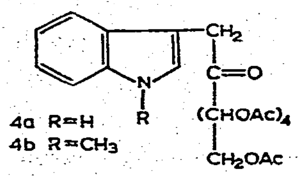


1 **2** **3**

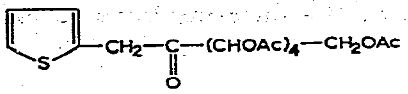
In a similar reaction, a carbohydrate residue was linked to the β-position of indole[3]. In toluene at 95–100°, with copper powder as catalyst, penta-*O*-acetyl-1-deoxy-1-diazo-*keto*-D-*galacto*-heptulose and indole reacted to give penta-*O*-acetyl-1-deoxy-1-(indol-3-yl)-*keto*-D-*galacto*-heptulose (**4a**) (27–30%) as a yellow powder, λ_{max} 222 (ε 26,800) and 280 mμ (ε 7500) Found: C, 58.20; H, 5.49; N, 2.50. $C_{25}H_{29}NO_{11}$ calc.: 57.80; H, 5.58; N, 2.69). Oxidation of compound (**4a**) with oxygen in an alkaline medium afforded indole-3-carboxylic acid.

In a similar manner, penta-*O*-acetyl-1-deoxy-1-(*N*-methylindol-3-yl)-*keto*-D-*galacto*-heptulose (**4b**) was obtained as a yellow, amorphous powder, λ_{max} 222 (ε 26,500) and 282 mμ (ε 6200) Found: C, 58.20; H, 5.88; N, 2.80. $C_{26}H_{31}NO_{11}$ calc.: C, 58.53; H 5.81; N, 2.62%).

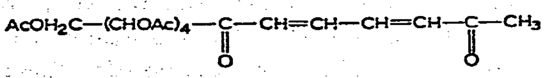
Likewise, a carbohydrate residue could be introduced into the α-position of thiophene. Thus, decomposition of penta-*O*-acetyl-1-deoxy-1-diazo-*keto*-D-*galacto*-heptulose in boiling thiophene, in the presence of copper powder, gave penta-*O*-acetyl-1-deoxy-1-(2-thienyl)-*keto*-D-*galacto*-heptulose (**5**) as a yellow powder, m.p.



4a R=H
4b R=CH$_3$



5



$AcOH_2C{-}(CHOAc)_4{-}\underset{\underset{O}{\|}}{C}{-}CH{=}CH{-}CH{=}CH{-}\underset{\underset{O}{\|}}{C}{-}CH_3$

6

130–132°, λ_{max} 248 (ε 3500) and 275 mμ (ε 4600) (Found: C, 51.54; H, 5.67; S, 6.39. $C_{21}H_{26}O_{11}S$ calc.: C, 51.86; H, 5.35; S, 6.58%).

According to the literature[4], furan and its derivatives give open-chain products with diazoketones. Hence, the syrupy product from the reaction of penta-*O*-acetyl-1-deoxy-1-diazo-*keto*-D-*galacto*-heptulose with 2-methylfuran is assigned the structure 1-(penta-*O*-acetyl-D-galactonyl)-hexa-1,3-dien-5-one (6), λ_{max} 223 (ε 5000) and 286 mμ (ε 9300), ν_{max} 1756 (strong), 1690 (medium), 1621 (weak), 1380 (strong) and 1430 cm^{-1} (medium). Found: C, 54.30 H, 5.93. $C_{12}H_{28}O_{12}$ calc.: C, 54.54; H, 5.78%).

A full report of this work will be published elsewhere.

Department of Chemistry,
Rostov-on-Don State University,
(U.S.S.R.)

YU. A. ZHDANOV
V. I. KORNILOV
G. V. BOGDANOVA

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Announcement

Carbohydrate Discussion Group (a Chemical Society Subject Group). A meeting will be held at Bristol University on April 7th and 8th, 1967, following the Chemical Society Anniversary Meeting at Exeter (April 4th–6th). For details of this meeting, please write to Dr. R. D. Guthrie, The Chemical Laboratory, The University of Sussex, Falmer, Brighton, Sussex.

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ACTION DE L'α-GALACTOSIDASE DU CAFÉ SUR QUELQUES GALACTOMANNANES

J E Courtois et P Le Dizet
Laboratoire de Chimie Biologique, Faculté de Pharmacie, Paris (France)

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INTRODUCTION

Les galactomannanes des graines de Légumineuses présentent une assez grande homogénéité de structure · unités α-D-galactopyranosyl branchées en 6 sur une chaîne de mannane constituée d'unités β-D-mannopyranosyl reliées en ($1 \rightarrow 4$). Les principales différences résident dans le rapport moléculaire mannose/galactose ou M/G. Dans les galactomannanes purifiées les valeurs de M/G s'étendent du voisinage de 1,0–1,2 (*Trifolium, Medicago*) à 3,5–4,0 (*Ceratonia, Gleditschia*)[1-3].

Dans les galactomannanes à rapport M/G > 2 la répartition des molécules de galactose le long de la chaîne n'est peut-être pas aussi régulière qu'il avait été envisagé primitivement[4-5]

L'α-galactosidase (3 2 1 22) du Café libère du galactose à partir des galactomannanes[6-8] L'hydrolyse aisée au début, se ralentit ensuite et jusqu'ici n'a jamais permis de détacher la totalité du galactose.

Ces essais avaient été réalisés avec des préparations enzymatiques partiellement purifiées Petek et To Dong[9] ont réussi à purifier les α-galactosidases du Café. Nous avons utilisé ces enzymes pour étudier systématiquement l'hydrolyse de galactomannanes à valeurs distinctes du rapport M/G Parmi les galactomannanes utilisées seule celle de *Gleditschia ferox* a fourni un précipité relativement abondant; nous avons étudié cette fraction insolubilisée par l'enzyme

MATÉRIEL ET TECHNIQUES

Galactomannanes

Elles ont été obtenues par extraction aqueuse des graines, précipitation par 3 vol. d'éthanol à 95%, reprise par l'eau, dialyse et nouvelle précipitation par l'éthanol Ces opérations sont répétées trois fois successivement. Les préparations obtenues sont pratiquement dépourvues de protéines d'après la réaction au biuret

La plupart de ces galactomannanes ont été décrites dans notre publication antérieure[2]

Trifolium repens Extraction aqueuse à la température du laboratoire (20°), M/G 1,07 (1)

Genista scoparia. Extrait à 20°, M/G 1,59 (**2a**).
Le résidu de l'extraction à 20° est traité par l'eau a 50°, M/G 1,66 (**2b**)

Gleditschia ferox Les graines à maturité sont extraites à 20°, M/G 3,9 (**3a**), puis ensuite à 50°, M/G 3,86 (**3b**). Les graines vertes sont extraites avant maturité selon Courtois et Le Dizet[2]. L'extraction est faite par l'hydroxyde de sodium dilué, suivie de précipitation par le réactif cupro-alcalin Le précipité est repris par une solution d'acide chlorhydrique et la solution est dialysée et précipitée par l'éthanol; M/G 3,82 (**3c**)

Gleditschia triacanthos Extrait à 20°, M/G 3,82 (**4a**), Extrait à 50°, M/G 3,76 (**4b**)

Ceratonia siliqua Extrait à 20°, M/G 3,88 (**5**)

Enzymes

(1) Préparation partiellement purifiée des graines de Cafe[10] (2) Fractions purifiées obtenues par élution à partir d'une colonne d'alumine[10]. Par suite du faible rendement, nous employons le mélange des fractions éluées à pH 5,1, puis à pH 6,0 dialysées et lyophilisées Ce mélange renferme une faible proportion de β-galactosidase, il est dépourvu d'activité sur les β-D-mannanes et β-D-mannosides étudiés

Techniques

Les oses réducteurs sont déterminés par la méthode au réactif cupro-alcalin de Nelson et Somogyi[11] Les monosaccharides et oligosaccharides sont caractérisés par chromatographie descendante avec le mélange de solvant 1-butanol–pyridine–eau (9 5 4, v/v) Pour l'établissement du rapport M/G, les bandes correspondant au galactose et au mannose sont découpées, extraites par l'eau à 20°, puis 40° Les extraits sont concentrés et dosés par la méthode Nelson-Somogyi

RÉSULTATS

pH Optimum

Le Café contient deux α-galactosidases, dont les pH optimums vis-à-vis du phényl α-D-galactoside sont respectivement de 5,3 et 6,1[10]. Chacune des deux fractions d'α-galactosidase libère du galactose à partir de la galactomannane de Luzerne[9]

Avec les galactomannanes de *Gleditschia ferox* **3a** et de *Ceratonia* **5**, le mélange des deux α-galactosidases et les tampons acide citrique–phosphate disodique fournissent des courbes avec un seul optimum tres aplati au voisinage de pH 5,0 Les courbes ont le même aspect avec des doses differentes de préparation enzymatique

Hydrolyse en fonction du temps

La quantité de substrat est choisie en fonction du rapport M/G, de façon à opposer l'enzyme à une quantité constante de galactose combiné La solution de galactomannane (1 ml) contenant le galactose combiné (1,89 mg) est additionnée de tampon acide acétique–hydroxyde de sodium (0,25 ml) de pH 4,8, de solution d'α-galactosidases purifiées (1 ou 2 ml) et de l'eau pour obtenir un volume total de 5 ml. Après hydrolyse à 37° nous obtenons les résultats groupés dans le tableau I. À titre comparatif, nous y avons fait figurer en bas les résultats obtenus avec deux oligosaccharides · raffinose et stachyose.

Avec tous les substrats la chromatographie n'a permis de déceler que la libération de galactose

Les pourcentages de galactose libéré ne permettent pas de différencier les galactomannanes à rapport M/G voisin de 1,0 (*Trifolium et Genista*) de celles où il s'élève vers 4,0 (*Gleditschia*) Hue[7] n'avait pas observé de différences significatives entre les vitesses initiales de libération du galactose des galactomannanes de *Medicago sativa* (M/G 1), de *Trigonella foenum graecum* (M/G 1) et de *Coesalpinia spinosa* (M/G 3)

Dans une même graine où les deux fractions de galactomannanes ont des M/G et pouvoirs rotatoires très voisins, la fraction extraite à 20° est hydrolysée plus rapidement que celle extraite ensuite à 50°.

TABLEAU I

ACTION DE L'α-GALACTOSIDASE DU CAFE OPPOSEE, À pH 4,8, À DES QUANTITÉS ÉQUIMOLÉCULAIRES DE GALACTOSE COMBINÉ[a]

Galactomannanes		*1 ml de préparation enzymatique*			*2 ml de préparation enzymatique*		
		Durée d'hydrolyse					
		6 h	*24 h*	*48 h*	*6 h*	*24 h*	*48 h*
Trifolium repens	1	11,7	16,4	23,3	18,0	24,4	45,5
Genista scoparia	2a	12,7	17,5	23,3	16,4	24,8	44,5
	2b	6,9	11,1	18,0	10,0	17,5	35,0
Gleditschia ferox	3a	13,2	20 0	29,1	18,0	31,7	45,0
	3b	8,0	10,0	18,0	9,5	21,7	35,4
	3c	18,5	25,0	35,0	24,8	37,6	56,5
Gleditschia triacanthos	4a	14,3	20,0	29,1	17,5	31,7	45,0
	4b	9,5	18,0	18,5	13,7	27,5	37,6
Raffinose		64,0	87,0	100,0	78,5	100,0	100,0
Stachyose		12,0	22,6	31,0	21,5	37,0	68,0

[a] Les chiffres du tableau correspondent au pourcentage du galactose libéré Les conditions expérimentales et les produits sont décrits dans le texte

Il est à signaler que dans le genre *Gleditschia* les fractions extraites à 20° de *G. ferox* et *G triacanthos* (3a et 4a) sont hydrolysées à la même vitesse par l'α-galactosidase.

Nous avons observé par ailleurs que selon l'origine de la galactomannane il n'existe pas de relation directe entre la diminution de viscosité au cours de l'hydrolyse et la libération du galactose C'est ainsi qu'après 48 h, avec des pourcentages de galactose libéré voisins, les diminutions de viscosité relative sont de 10% pour *Trifolium* et de 45% pour *Gl ferox* 3a.

L'hydrolyse α-galactosidasique ne provoque pas la chute brutale de viscosité observée avec la dépolymérase de *Leucaena glauca* agissant sur la galactomannane de cette graine[12] Dans des recherches en cours, nous avons noté la même chute brutale en faisant réagir l'hémicellulase Novo de *Bacillus subtilis* sur diverses galactomannanes.

Le poids moléculaire du substrat paraît influer nettement sur la vitesse galactosidasique. Dans le bas du Tableau I nous pouvons observer que le raffinose est hydrolysé beaucoup plus rapidement que le stachyose, dont le galactose n'est pas totalement détaché avec 2 ml d'enzyme en 48 h

À pH 4,6 et 37°, la valeur de la constante de Michaelis déterminée par la méthode graphique de Lineweaver et Burk est $Km = 5 \cdot 10^{-3}$ M pour le raffinose. Par la même méthode, nous avons obtenu à pH 4,6 et 37° un Km de $2,5 \cdot 10^{-3}$ M pour le stachyose Il ne s'agit que d'un Km approximatif puisque le stachyose renferme deux liaisons α-galactosidiques hydrolysées successivement L'α-galactosidase du Café hydrolyse en effet le stachyose en deux étapes[13]. stachyose→raffinose→saccharose L'affinité $K_M = 1/Km$ est donc de 400 vis-à-vis du stachyose, elle est en réalité supérieure pour la rupture de la liaison α-galactosidique terminale puisque le K_M du raffinose hydrolysé dans la seconde étape est de 200 Avec une préparation brute de Café et à pH 4,0, nous avions obtenu antérieurement [14] K_M 150 pour le mélibiose et des K_M 325 identiques pour le raffinose et le plantéose L'affinité K_M augmente donc avec le poids moléculaire de l'oligosaccharide, alors que la vitesse maximum *V* diminue

Il serait actuellement prématuré de tenter de généraliser aux galactomannanes ces quelques observations Ceci d'autant plus que la cinétique d'action des α-galactosidases de graines se révèle déjà comme fort complexe avec un substrat de faible poids moleculaire comme le phényl α-D-galactoside[15] Le galactose libéré inhibe l'α-galactosidase, ce qui nous a conduit à faire réagir l'enzyme en plusieurs étapes

Hydrolyses par additions successives d'enzyme

Trifolium repens. La galactomannane (0 125 g) est mise en contact à 37° avec le mélange des deux α-galactosidases purifiées (50 mg), le tampon acide acétique–hydroxide de sodium de pH 4,8 (1 ml), du toluène (0,5 ml) et de l'eau (50 ml) Après 6 jours, il n'est pas apparu de précipité et 38% du galactose sont libérés, le mélange est alors traité par 3 vol d'éthanol à 96% qui précipite une galactomannane, dont le rapport M/G s'est élevé à 1,54 Ce polysaccharide est remis en contact avec de l'α-galactosidase (50 mg), puis tous les quatre jours nous ajoutons à nouveau de l'enzyme (50 mg) Après cinq affusions d'enzyme il n'y a pas eu de précipitation de mannane L'addition de trois vol d'éthanol insolubilise une galactomannane de M/G 1,72

Ces résultats sont à rapprocher de ceux obtenus avec la galactomannane de *Medicago*[6] possédant elle aussi un rapport M/G voisin de 1,0

Gleditschia ferox Nous opérons de façon identique avec la galactomannane 3a (0 375 g), mais il apparaît après 2 jours un précipité qui est collecté par centrifugation, lavé avec de l'éthanol qui extrait régulièrement du galactose fixé par le précipité. Ce précipité est remis en suspension dans le tampon de pH 4,8 et additionné d'enzyme (50 mg) Apres 48 h nous déterminons le galactose libéré et le rapport M/G du précipité Ce dernier est remis en contact avec de l'α-galactosidase comme précédemment

La fraction demeurée en solution est insolubilisée par 3 vol. d'éthanol à 95%. Le précipité est repris par l'eau, et la solution est dialysée Après une nouvelle précipitation éthanolique, le précipité est remis en contact avec l'enzyme

Nos résultats sont réunis dans le Tableau II L'examen chromatographique des fractions demeurées en solution, après traitement par l'éthanol, n'a pas permis de déceler d'autre saccharide que le galactose.

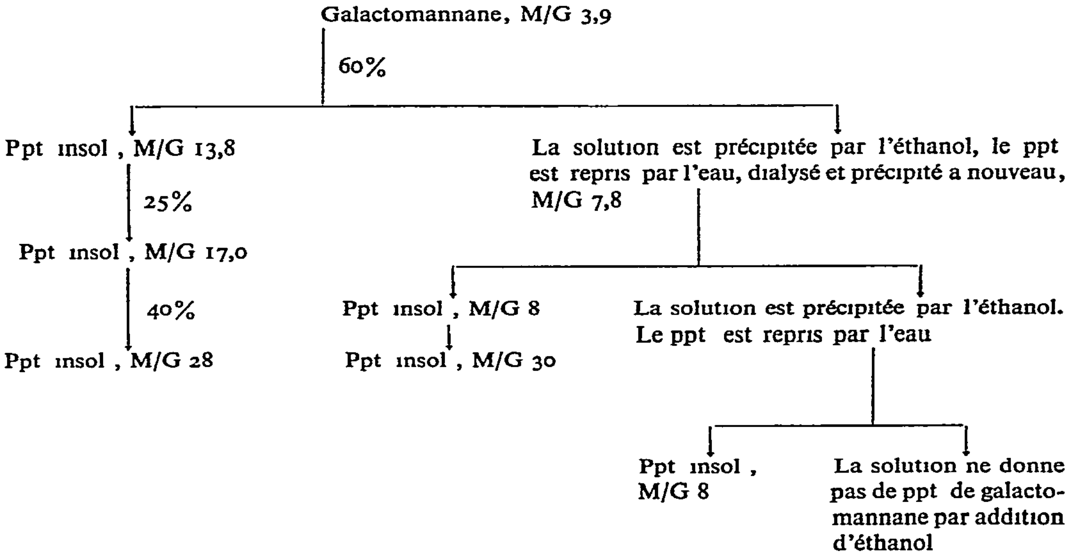
L'α-galactosidase a donc libéré du galactose, aussi bien à partir des galactomannanes solubles, que des fractions qui se sont insolubilisées

Le rapport moléculaire M/G augmente régulierement avec le nombre de traitements successifs par l'enzyme. Il n'a cependant pas été possible de détacher la totalité du galactose des fractions insolubles et de dépasser un rapport M/G de 30

Les résultats du Tableau II tendraient à indiquer que la galactomannane est un mélange de fractions à degrés de polymérisation distincts Nous avons en effet séparé une fraction soluble à M/G 7,8 conduisant ensuite à deux fractions insolubles de M/G 8,0.

TABLEAU II

HYDROLYSE DE LA GALACTOMANNANE DE *Gleditschia* PAR AFFUSIONS SUCCESSIVES D'α-GALACTOSIDASE[a]



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[a] Les pourcentages indiquent la proportion de galactose libérée par action de l'enzyme Les flèches verticales indiquent l'action de l'enzyme

Nous avons en outre constaté qu'au cours de l'hydrolyse acide des diverses fractions la totalité du galactose est libérée par chauffage de 4 h à 100° dans H_2SO_4 5 N Si nous séparons la fraction insoluble, son hydrolyse acide ultérieure ne libère plus que le seul mannose

Ceratonia siliqua Nous opérons comme pour *Trifolium repens*. Après 5 jours à 37°, il apparaît un précipité peu abondant (M/G 4,57); il est remis en contact avec de l'enzyme et après 5 jours l'insoluble a un M/G qui s'est élevé légèrement à 5,27 Les galactomannanes de *Gleditschia* et *Ceratonia* ont des rapports M/G initiaux de 3,9 Elles paraissent avoir une répartition différente des unités galactose sur l'ensemble des molécules Au cours de divers essais, l'α-galactosidase du Café n'a jamais insolubilisé, à partir de la galactomannane de *Ceratonia*, une fraction où le rapport M/G soit notablement accru

Étude de la fraction insolubilisée à partir de la galactomannane du Gleditschia ferox

Préparation La galactomannane **3a** (20 g) est dissoute dans l'eau distillée (1 l), et l'on ajoute la préparation d'α-galactosidase éluée à pH 6,0 (200 ml) Le mélange est introduit dans un boyau de cellophane baignant dans une cuve contenant un tampon acide citrique–phosphate disodique 0.01 M de pH 6,0 Nous maintenons à 37° en renouvelant chaque jour le liquide extérieur au boyau. Dès le premier jour du galactose diffuse à l'extérieur du boyau Notre protocole opératoire a pour but d'éliminer la majeure partie du galactose inhibiteur de l'enzyme. À partir du deuxième jour il apparaît un précipité, dont la proportion s'amplifie régulièrement

Au bout de 20 jours, nous collectons le précipité, le suspendons dans de l'eau, dialysons et récoltons par centrifugation l'insoluble Nous obtenons une poudre blanc grisâtre (1 5 g), soluble dans NaOH 2 N; cette solution a un $[\alpha]_D^{20}$ −40°, proche de ceux des β-D-mannanes de Palmiers, *Phytelephas macrocarpa*[16] (−46°) et *Phoenix canariensis*[17,18] (−50°) Le corps est totalement hydrolysé en 4 h à 100° dans H_2SO_4 5 N, M/G 24

Méthylation Elle est réalisée dans une première étape par le sulfate de méthyle et la soude, puis dans une seconde étape par l'iodure de méthyle et oxyde d'argent ou par l'oxyde de baryum dans la *N,N*-diméthylformamide[19]

Les deux oxydes ont permis d'obtenir les mêmes résultats L'examen spectroscopique à l'infra-rouge n'indique pas la présence d'hydroxyles non combinés Le produit méthylé est lévogyre, $[\alpha]_D^{20}$ −25° (*c* 1 0, chloroforme) Aspinall *et al.*[16] avaient obtenu −20° et −22° pour les β-D-mannanes de *Phytelephas* méthylées

Le produit méthylé (100 mg) est dissous dans le méthanol contenant HCl à la concentration 0,66 N (5 ml) Après chauffage sous réfrigérant à reflux pendant 8 h, la solution est concentrée sous pression réduite et le résidu additionné de HCl N est chauffé 10 h au bain-marie bouillant Après désionisation par l'Amberlite IR-4-B, l'hydrolysat est concentré sous pression réduite et examiné par chromatographie sur papier Une hydrolyse du produit méthylé pendant 8 h à 100° dans H_2SO_4 2 N a fourni les mêmes résultats

Les dérivés méthylés sont identifiés par chromatographie sur papier, selon le protocole de Petek et To Dong[20,21] avec le solvant octane–alcool isopropylique–ammoniaque à 10% (50 25·2) Les bandes correspondant aux divers dérivés méthylés sont découpées et extraites par le chloroforme, avec un épuisement de 16 h à la température du laboratoire, puis deux autres à 30° Nous évaporons à sec et reprenons par l'eau

Les oses méthylés sont déterminés par l'hypoïodite 0 1 N en présence de CO_3Na_2 à 15%, pour 4h à 0°[22] et par colorimétrie avec le phosphate d'aniline[23] Nos résultats sont rassemblés dans le Tableau III

TABLEAU III

DÉTERMINATION DES DÉRIVÉS *O*-METHYLES DANS L'HYDROLYSAT DE LA GALACTOMANNANE DE *Gleditschia ferox*, MÉTHYLÉE APRÈS DÉGRADATION PAR L'α-GALACTOSIDASE[a]

Dérives O-méthyles	*Méthode de dosage*	
	Iodométrie	*Colorimétrie*
2,3-Di-*O*-méthyl-D-mannose	6,0	8,0
2,3,6-Tri-*O*-méthyl-D-mannose	87,0	85,0
2,3,4,6-Tétra-*O*-méthyl-D-galactose	3,0	3,5
2,3,4,6-Tétra-*O*-méthyl-D-mannose	4,0	3,75

[a] Les résultats sont exprimés en molécules pour cent

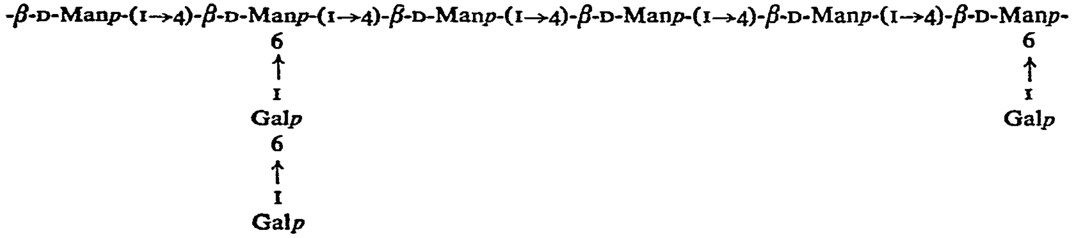
Pour une molécule de 2,3,4,6-tétra-*O*-méthylgalactose, il y a donc environ 1,2 molécule de 2,3,4,6-tétra-*O*-méthylmannose, 2,15 molécules de 2,3-di-*O*-méthylmannose et 26,4 molécules de 2,3,6-tri-*O*-méthylmannose

Pour les dérivés du mannose ceci, correspond aux résultats obtenus avec la β-D-mannane a liaisons (1→4) du *Phoenix canariensis*[17,18] La faible proportion de galactose est retrouvée sous forme de tétra-*O*-méthylgalactose, indiquant qu'il est présent sous forme d'unités branchées sur la chaine d'une (1→4)-β-D-mannane.

La galactomannane de *Gleditschia ferox* soumise à la méthylation nous avait permis antérieurement[2] de déceler de faibles quantités de 2,3,4-tri-*O*-méthylgalactose Cerezo[24] a de même signalé la présence de traces de ce tri-*O*-méthylgalactose dans la galactomannane de *Gleditsia amorphoides* soumise aux opérations successives de méthylation puis d'hydrolyse L'application de la méthode de méthylation, puis d'hydrolyse à la galactomannane **3a** de *Gl. ferox* nous a permis de déceler régulièrement environ 1 à 1,5% de molécules de 2,3,4-tri-*O*-méthylgalactose Ce dérivé ne paraît pas être un artefact résultant d'une déméthylation partielle du 2,3,4,6-tétra-*O*-méthylgalactose Le raffinose soumis aux mêmes opérations de méthylation, puis d'hydrolyse, n'a jamais permis de déceler un tri-*O*-méthylgalactose La galactomannane de *Trifolium repens* a permis de déceler également environ 1,2% des molécules d'oses méthylés sous forme de ce 2,3,4-tri-*O*-méthylgalactose

Il apparaît donc vraisemblable que sur les chaînes de (1→4)-β-D-mannanes se relient en 6 quelques rares courtes chaînes de galactose associés en (1→6), ce qui donnerait ce type de structure **1** possible.

Le galactose substitué qui conduit à ce dérivé triméthylé ne paraît pas être intercalé dans les chaînes de mannanes entre deux unités mannose S'il en était ainsi, nous aurions retrouvé ce dérivé triméthylé en plus forte proportion dans la galactomannane à rapport M/G élevé, insolubilisée par l'α-galactosidase En effet, l'enzyme du Café hydrolyse les liaisons α-galactosidiques terminales et ne semble pas rompre les liaisons α-galactosidiques d'unités de galactose substituées par un autre monosaccharide[10 14].



$$\begin{array}{cccccc}
\text{-}\beta\text{-D-Man}p\text{-}(1\rightarrow4)\text{-} & \beta\text{-D-Man}p\text{-}(1\rightarrow4)\text{-} & \beta\text{-D-Man}p\text{-}(1\rightarrow4)\text{-} & \beta\text{-D-Man}p\text{-}(1\rightarrow4)\text{-} & \beta\text{-D-Man}p\text{-}(1\rightarrow4)\text{-} & \beta\text{-D-Man}p\text{-} \\
 & 6 & & & & 6 \\
 & \uparrow & & & & \uparrow \\
 & 1 & & & & 1 \\
 & \text{Gal}p & & & & \text{Gal}p \\
 & 6 & & & & \\
 & \uparrow & & & & \\
 & 1 & & & & \\
 & \text{Gal}p & & & &
\end{array}$$

1

Le Tableau III indique que dans le précipité de galactomannane dégradée par l'enzyme une molécule de tétra-*O*-méthylmannose correspond à 24 molécules des di- et tri-*O*-méthylmannoses

Le galactomannane 3a de *Gl ferox* a permis d'obtenir une molécule de tétra-*O*-méthylmannose pour 13 molécules des autres méthyl-*O*-mannoses Ce rapport est de 1 pour 50 avec la galactomannane de *Trifolium repens*

Il en résulte que la formation d'une galactomannane insoluble par action de l'enzyme du Café ne paraît pas s'accompagner d'une rupture des chaînes de mannanes, la formation de ces fragments aurait accru la proportion de tétra-*O*-méthylmannose Il convient de noter que le rendement en galactomannane insoluble est faible, 7 5% Il est donc possible que ce soient les chaînes de mannanes les plus longues qui s'insolubilisent après détachement de la plus grande partie des galactoses substituants

L'existence de branchements dans les chaînes de mannanes ne doit pas être exclue Le Tableau III montre que la proportion moléculaire du 2,3-di-*O*-méthylmannose est environ double de celle du 2,3,4,6-tétra-*O*-méthylgalactose Ceci tendrait à indiquer que, dans les chaînes de mannanes, 2 à 3% des unités mannosyles sont substituées autrement que par une unité de galactose

Nous rapprocherons ce fait d'une observation antérieure Au cours de recherches non publiées réalisées en collaboration avec A Wickstrom, nous avons étudié la méthylation de la galactomannane de *Medicago*, dont une partie du galactose avait été éliminé par l'α-galactosidase du Café Ce polysaccharide de M/G 5 avait fourni pour une molécule de 2,3,4,6-tétra-*O*-méthylmannose, 3,4 molécules de 2,3,4,6-tétra-*O*-méthylgalactose, 7,7 molécules de 2,3-di-*O*-méthylmannose et 12,6 molécules de 2,3,6-tri-*O*-méthylmannose

Les deux galactomannanes à M/G accru par action de l'α-galactosidase renferment donc une proportion moléculaire plus élevée de di-*O*-méthylmannose que de tétra-*O*-méthylgalactose

Il est à remarquer que la plupart des auteurs ayant soumis les galactomannanes intactes à la méthylation ont en général retrouvé une proportion moléculaire légèrement supérieure du tétra-*O*-méthylgalactose par rapport au di-*O*-méthylmannose[25,26].

Nous avons observé le même fait avec les galactomannanes de *Trifolium repens* et le produit 3a de *Gl ferox*

Oxydation par l'acide periodique L'oxydation (Fig 1) s'effectue comme pour un polysaccharide constitué d'hexoses pyraniques liés en $(1\rightarrow4)^{17\ 18,27}$

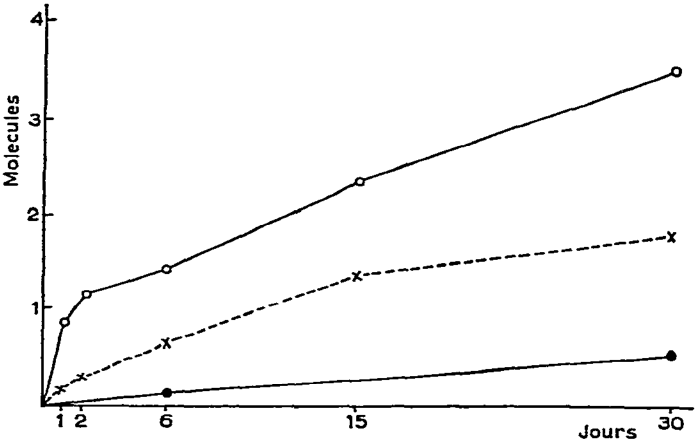


Fig 1 Oxydation periodique de la fraction insoluble de galactomannane obtenue par action de l'α-galactosidase La réaction est accomplie a la glacière et dans l'obscurité, le milieu de réaction contient une suspension de 0,162 g de galactomannane dégradée dans 10 ml, la concentration d'acide periodique dans le milieu est 1N (ou 0 5M) Les résultats sont exprimés en molécules par molécules d'anhydrohexose, la courbe o-o- indique l'acide periodique réduit, la courbe x-x- l'acide monovalent libéré et la courbe •–•– le formaldéhyde libéré

La superoxydation évolue sensiblement comme avec la β-D-mannane $(1\rightarrow4)$ de *Phoenix canariensis*[17] Cette superoxydation est plus accentuée qu'avec la galactomannane 3a de *Gl ferox*[2], la substitution en 6 des unités de mannose limitant la superoxydation Tout comme pour la cellulose ou la mannane de *Phoenix*[17,18], le polyaldéhyde formé par action de l'acide periodique est soluble, on assiste donc à une dissolution du précipité initial lorsque l'oxydation progresse

DISCUSSION

Les galactomannanes de Légumineuses étudiées ont un type général de structure commun Il semble cependant exister des différences individuelles selon la répartition des unités de galactose, la longueur et l'imbrication des chaînes de mannanes C'est, ainsi que pour deux galactomannanes à rapport M/G identiques, celle de *Gleditschia* donne un abondant précipité à M/G nettement accru lorsque l'α-galactosidase a détaché une partie des molécules de galactose Par contre, celle de *Ceratonia* ne conduit pas à un précipité abondant et à un M/G accru

L'on doit aussi envisager la présence dans le *Gleditschia* de quelques courtes chaînes renfermant plus d'une unité de galactose.

Enfin, la proportion de di-*O*-méthylmannose est en faveur de l'existence de ramifications dans la partie mannane La structure complète des galactomannanes laisse donc planer encore quelques incertitudes

RÉSUMÉ

L'α-galactosidase des graines de Café hydrolyse initialement à des vitesses voisines les liaisons α-galactosidiques de diverses galactomannanes Les galactomannanes de Trèfle et Caroubier ne donnent pas de composé insoluble du type mannane au cours de l'hydrolyse α-galactosidasique Avec *Gleditschia ferox* nous avons obtenu une fraction insoluble dans l'eau contenant 24 molécules de mannose pour chaque molécule de galactose Ce composé est constitué de chaînes d'unités β-D-mannopyranosyl liées en (1→4), il semble probable que ces chaînes sont faiblement ramifiées Quelques unités de galactose sont reliées en (1→6) à ces chaînes

SUMMARY

Coffee-bean α-galactosidase (3 2 1 22) splits, at similar initial rates, α-D-galactosyl units from various galactomannans isolated from *Trifolium repens, Genista scoparia, Gleditschia ferox, Gleditschia triacanthos*, and *Ceratonia siliqua* Except for white-clover, genista, and carob-tree galactomannans, α-galactosidase treatment resulted in insoluble mannan-like polysaccharides A water-insoluble fraction containing 24 residues of mannose for each residue of galactose was obtained from the galactomannan of *Gleditschia ferox* It contains chains having a β-D-(1→4)-mannopyranosyl repeating unit. These chains have probably a few side-branches. The few remaining D-galactose residues are linked (1→6) as side-branches to the D-mannose chains

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STUDIES ON STARCHES OF HIGH AMYLOSE-CONTENT

PART VIII * THE EFFECT OF LOW TEMPERATURE ON THE INTERACTION OF AMYLOMAIZE STARCH WITH IODINE A UNIQUE CHARACTERIZATION

G K Adkins and C T Greenwood
Department of Chemistry, The University, Edinburgh, 9 (Great Britain)

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INTRODUCTION

In the previous part of this Series[1], we showed that the potentiometric iodine-binding curves, obtained at 20°, for amylomaizes of differing amylose-content were abnormal This means that the unambiguous estimation of iodine affinity is not possible for such starches We also suggested that the presence of low molecular-weight, amylose-like material was responsible for this behaviour Experiments which confirm this proposal are described here

The effect of low temperatures on the iodine-binding capacity of starch has now been studied It has been found that, under these conditions, amylomaize gives the more usual shape of iodine-binding curve, from which an estimate of iodine affinity can be made This change in iodine-binding power with the lowering of temperature has been shown to be a characteristic of low molecular-weight amylose, the apparent iodine affinity of a high molecular-weight amylose is not affected to any appreciable extent by the lowering of temperature

EXPERIMENTAL

The preparation and sources of the amylomaizes have been described earlier[2]. Starch samples were labelled *HA*57, *HA*62, *HA*67, *HA*70, and *HA*75, where the number represents the reputed content of amylose Samples of degraded amylose (I–III) were the polysaccharides prepared previously[1]

The starches were dispersed into dimethyl sulphoxide and used directly (0 1 ml, *c*, approx 60 mg/ml) in the titration cell, as described in the previous part of this Series[1]

Estimations of the "iodine-binding capacity", or "iodine affinity", of a starch were obtained by extrapolation of the linear portion of the adsorption curve to zero concentration of free iodine[3]

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*For Part VII, see Ref 1.

RESULTS AND DISCUSSION

When amylomaize starch *HA*70 was potentiometrically titrated with iodine at various temperatures, the results shown in Figure 1 were obtained. It can be seen that, when titrations were carried out below the usual temperature[1,3] of 20°, the starch commenced to complex with iodine at much lower levels of free-iodine concentration. Furthermore, the low-temperature adsorption curves were markedly more normal in shape than the unusual, inflexionless curves obtained at 20° or higher. Indeed, although it has not proved possible[1] to obtain iodine-affinity values for amylomaize starch from titration curves at 20°, extrapolations of the adsorption curves at low temperatures could be made without difficulty.

A temperature of 2° was therefore chosen as being the most convenient, routine condition for the titration of an amylomaize starch.

Titrations of amylomaize starches at 2°

The normal shaped, titration curves obtained for the other samples of amylomaize starch at 2° are shown in Figure 2.

From Figures 1 and 2, it can be seen that the iodine-binding capacity for the amylomaize starches *HA*57, *HA*62, *HA*67, *HA*70, and *HA*75 was 14.9, 15.6, 16.6, 17.3, and 17.5%, respectively. As the iodine affinity of amylomaize amylose at this temperature is 20% (see below), the apparent contents of amylose are 75, 78, 83, 86, and 88%,

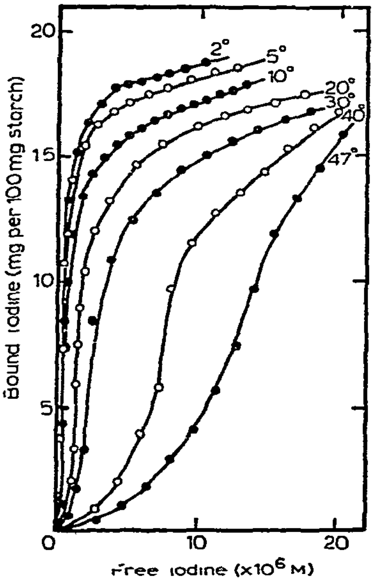


Fig. 1. Potentiometric iodine-titration curves for amylomaize starch *HA*70 at various temperatures.

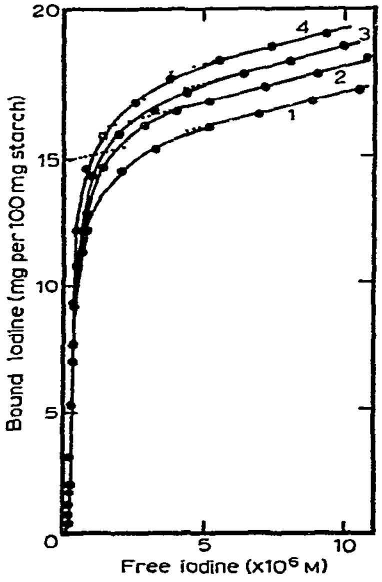


Fig. 2. Potentiometric iodine-titration curves for various amylomaize starches at 2°. (1) *HA*57, (2) *HA*62; (3) *HA*67, (4) *HA*75.

for the various starches. It is significant that these amylose contents are considerably higher (some 15–18%) than the alleged amylose-contents of these starches.

The implications of this finding will be discussed elsewhere.

Titrations of maize starch and its components at 2°

The behaviour of regular maize starch and its component amylose and amylopectin on potentiometric titration with iodine at 2° was then examined. As shown in Figure 3, although there was an increase in the apparent extent of adsorption of iodine at low temperatures, the iodine affinity of the starch did not alter. A comparable effect was found for the amylopectin, this effect accounted for the change in adsorption observed for the whole starch. The iodine-affinity characteristics of the amylose component were little altered by the change in titration temperature.

Potato starch and its component amylose and amylopectin behaved similarly when titrated at low temperature, the limiting iodine-binding levels were attained at considerably lower concentrations of free iodine, but these levels were almost dentical to those obtained in the titration at 20°.

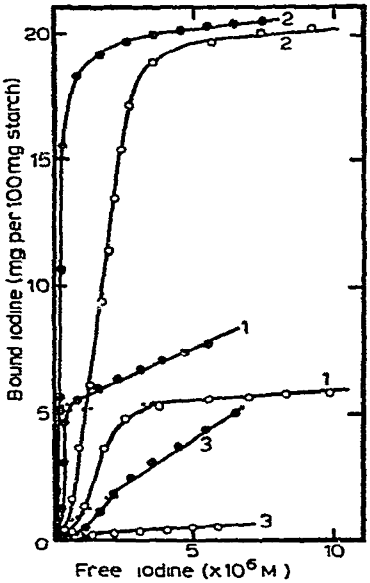
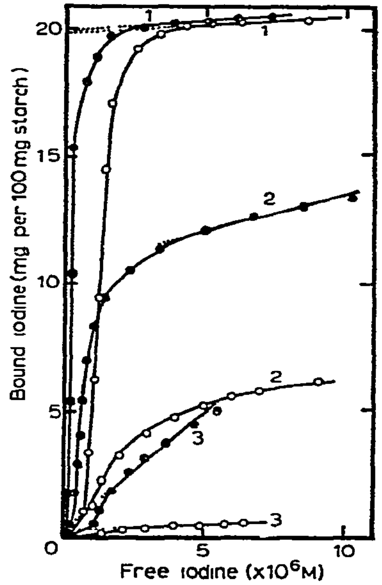


Fig 3 Potentiometric iodine-titration curves for regular maize starch and its components at 20° (–o–) and 2° (–•–) (1) maize starch, (2) maize amylose, (3) maize amylopectin

Titrations of the amylomaize components at 2°

Figure 4 shows that the titration curves for the amylomaize amylose were also essentially independent of temperature, but this was not so for the 36-unit amylopectin[4]. At 20°, this material gave an abnormal, inflexionless curve, but, at 2°, a curve of regular shape was obtained, the corresponding iodine-binding capacity indicates the presence of some 50% of amylose. In contrast, the purified, normal amylopectin, obtained by ultracentrifugation from the 36-unit material[4], behaved as did the amylopectin from regular maize In the previous part of this Series[1], we showed that the abnormal titration curves for amylomaize starch could be simulated by the addition of degraded amylose to mixtures of normal amylose and amylopectin These degraded amyloses were therefore titrated at 2°, as shown in Fig 5 The same dramatic change in character was observed as found for the amylomaize starches; at low temperatures, the curves tended to show a point of inflexion



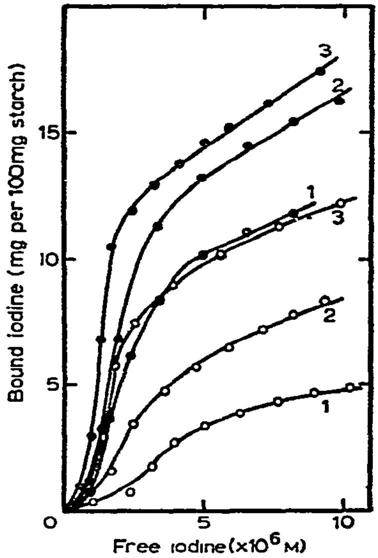


Fig 4 Potentiometric iodine-titration curves for the amylomaize components at 20° (-o-) and 2° (-•-) (1) amylo-amylose, (2) 36-unit amylopectin, (3) amylopectin obtained by ultracentrifugation

Fig 5 Potentiometric iodine-titration curves for degraded amyloses[1] at 20° (-o-) and 2° (-•-) (1) degraded amylose I, (2) degraded amylose II, (3) degraded amylose III

CONCLUSIONS

Since the iodine affinity of normal amylose and amylopectin is unchanged with decrease in temperature, whilst that for degraded amylose is radically altered, it appears very probable that the abnormal behaviour of amylomaize starch on titration is due to the presence of short-chain, amylose-like material (*cf* Refs 1 and 4) In any case, the iodine-binding capacity of a maize starch of high amylose-content can only be satisfactorily determined by carrying out the potentiometric iodine-titration at 2°.

ACKNOWLEDGMENT

This work was supported in part by a grant made by the United States Department of Agriculture, under P L 480

SUMMARY

The iodine-binding characteristics of amylomaize starch are affected dramatically by a lowering of temperature At 2°, a normal-shaped, potentiometric, iodine-titration curve is obtained, from which, in contrast to results at 20°, estimation of the iodine affinity can be readily made These conditions provide a unique method of characterization of this type of starch

Amylomaize starches having a variety of reported amylose-contents have been examined by this technique The iodine-affinity values indicated that the starches contain some 15–18% more linear-material than expected

The iodine affinity at 2° of normal amylose and amylopectin is not appreciably different from that at 20°, but very large changes occur in the iodine-binding characteristics of samples of 36-unit amylopectin and degraded amylose at this low temperature It is suggested that these changes account for the abnormal behaviour of the whole amylomaize starch

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ÉTUDES SUR LA STRUCTURE DE LA LACTOTRANSFERRINE HUMAINE

I PRÉPARATION DE GLYCOPEPTIDES ET MISE EN ÉVIDENCE D'UNE LIAISON GLYCOSIDIQUE ENTRE LES GLUCIDES ET LA THRÉONINE

R GOT, Y GOUSSAULT ET J FONT
Laboratoire de Biochimie, Faculté de Médecine, Paris VI^e (France)

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INTRODUCTION

La lactotransferrine est une des principales protéines du lactosérum humain[1]. Son existence fut pressentie par Schafer[2] et son isolement fut l'objet de plusieurs travaux[3-7] Son association avec le fer, sa spécificité lactique et son taux élevé dans le lactosérum lui confèrent un intérêt particulier, intérêt accru par le rôle qu'elle doit jouer en nutrition infantile Des études antérieures[4 7 8] ont montré que la lactotransferrine est une glycoprotéine contenant hexoses, hexosamine, 6-desoxyhexose et acide sialique Le but de ce travail est de déterminer le nombre et la nature des chaînes polysaccharidiques et leur liaison avec la partie protéique de la lactotransferrine. Cet article décrit la préparation et la purification d'une fraction glycopeptidique, la détermination des séquences peptidiques au voisinage de la liaison glucides–protéine et l'identification de l'acide aminé engagé dans la liaison

MATERIEL ET MÉTHODES

Lactotransferrine

La lactotransferrine utilisee dans cette étude est préparée selon une méthode précédemment décrite[1] La caséine est éliminée par dialyse du colostrum humain contre un tampon acétate de pH 4,5, puis le lactosérum est fractionné par relargage par le sulfate d'ammonium . la fraction qui précipite entre les concentrations 2M et 2,7M en sulfate d'ammonium à pH 4 est constituée essentiellement par la lactotransferrine, accompagnée d'α-lactalbumine et de sérumalbumine que l'on élimine par une filtration sur colonne de Sephadex G 200

L'homogénéité des préparations ainsi obtenues est vérifiée par immunoélectrophorèse[9] contre un immunsérum anti-colostrum total, par ultracentrifugation et par électrophorèse en veine liquide (Fig 1) La constante de sédimentation, 4,85 S, est légèrement supérieure à celle donnée dans la littérature, 4,5 S (4,7) Au contraire, la mobilité électrophoretique à pH 8 6, $-2\ 8 \times 10^{-5}$cm^2 volts^{-1} sec^{-1}, est inférieure à celle donnée par Montreuil *et al*[4], $-3\ 5 \times 10^{-5}$, mais voisine de celle donnée par Johansson[3] $-2\ 95 \times 10^{-5}$ à pH 9,8

Analyses des glucides

Les hexoses, les osamines, les 6-désoxyhexoses et l'acide sialique sont dosés par les méthodes colorimetriques precedemment décrites[10–13], respectivement contre des témoins D-galactose–D-mannose en proportion (1 1), 2-amino-2-désoxy-D-glucose, L-fucose et acide *N*-acétylneuraminique (Sigma).

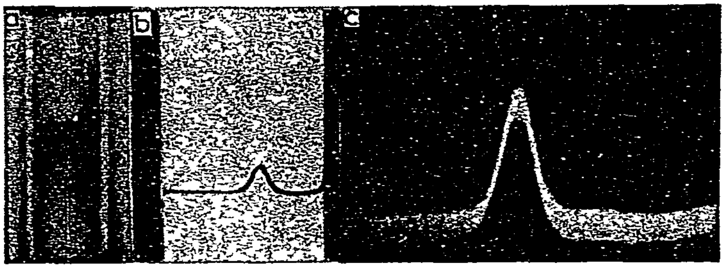

Fig 1 Homogénéité des préparations de lactotransferrine
(a) Immunoélectrophorèse contre un immunsérum anti-colostrum
(b) Ultracentrifugation 5 mg/ml de tampon phosphate de sodium 0 02M–NaCl 0 08 M pH 7, 20° Ultracentrifugeuse Spinco, Modèle E Photo prise après 38 minutes a 59,780 r p m
(c) Électrophorèse en veine liquide, appareil Perkin–Elmer, Modèle 38 Tampon de Michaelis, pH 8 6 10 volts/cm, 5,400 sec

Les osamines sont libérées par une hydrolyse de 4 h à 100°, par HCl 3N et l'acide sialique à 80° par HCl 0 05N pendant 40 min

La chromatographie des glucides est effectuee en couche mince, sur cellulose MN 300[14] avec révélation par le nitrate d'argent[15]

Analyse des acides amines

La composition en acides aminés des glycopeptides est determinée dans l'appareil Beckman–Spinco modèle 120, après hydrolyse de 20 h à 110° en tube scellé, par HCl 6N, selon la méthode de Moore *et al*[16].

Les chromatographies sont effectuées, soit sur papier Schleicher et Schull 2043b dans les solvants butanol–pyridine–HCl 0,1N (5 3 2) et butanol–acide acétique–eau (4.1 5), soit en couche mince sur cellulose MN 300[17]

La dégradation séquentielle des glycopeptides est effectuée en tube selon une variante de la méthode d'Edman[18–20] : le glycopeptide (3 mg) est repris dans l'eau (0,07 ml) à laquelle on ajoute de la triméthylamine à 25% (0.03 ml) et du phénylisothiocyanate à 1% dans la pyridine (0 1 ml), au bout d'une h à 40°, on ajoute une solution de phénylisothiocyanate (0 02 ml) Après une autre h à 40°, on ajoute de l'eau (0 2 ml) et on lave trois fois par le cyclohexane et quatre fois par le benzène Après évaporation au dessiccateur, la cyclisation et l'hydrolyse sont effectuées en laissant 2 h à 40° dans un mélange d'acide acétique et HCl conc (5 1, 0 4 ml) Après une nouvelle évaporation à sec, on reprend par HCl 0 01N (0 4 ml) et les phénylthiohydantoïnes formees sont extraites 4 fois par l'éther (0 4 ml) et 4 fois par l'acétate d'éthyle (0 4ml), chromatographiées dans les solvants *o*-xylène et acide formique 75%–1,2-dichloroéthane-heptane (4 4 2)[21] et révélees par le réactif iodure–azide de sodium[22].

Pour la recherche des acides amines C-terminaux, le glycopeptide (3 mg) est soumis à l'action de la carboxypeptidase A (Worthington) pendant 18 h à pH 8,0, en tampon 2-amino-2-hydroxy-1,3-propanediol 0,05M à 40° Le rapport enzyme sur substrat est de 1 à 10. Un témoin contenant l'enzyme seul est mis en incubation dans les mêmes conditions L'hydrolyse est arrêtée par addition d'acide acétique Les hydrolysats sont alors évaporés à sec et les acides aminés éventuellement libérés sont extraits par un melange acétone–HCl concentré (100:1) et analysés par chromatographie sur papier.

Préparation des glycopeptides

La lactotransferrine humaine (4 g) est dissoute dans un tampon 2-amino-2-hydroxy-1,3-propanediol 0 05M, pH 8,0, 0,01M en chlorure de calcium 25 ml, la Pronase 40 mg (Kaken Kagaku Co. Ltd. Tokyo) est ajoutée et la solution est mise en incubation à 40° sous couche de toluène Après trois jours d'incubation, 10 mg de Pronase sont ajoutés Après six jours d'hydrolyse, le précipité produit est éliminé par centrifugation et le surnageant évapore à sec est repris par l'acide acétique 0,1N 4 ml (contenant 2% de butanol) et filtré sur colonne de Sephadex G-25 (2 × 90 cm, fin) La colonne est éluée par le même solvant avec une vitesse d'écoulement de 40 ml par h. Des fractions de 5 ml sont collectées et leur contenu en sucres et en peptides est déterminé par les méthodes appropriées[10,23]

Après un écoulement de 80 ml, les substances glycopeptidiques émergent de la colonne en un pic unique et symétrique les fractions correspondantes sont réunies (fractions 16 à 24, Fig 2), évaporées à sec et reprises par l'eau distillée. Une chromatographie sur colonne d'Amberlite IR 120 (25 × 1,5 cm, ajustee à pH 2,3 sous la forme H$^+$ par l'acide formique) permet d'eliminer quelques contaminations pepti-

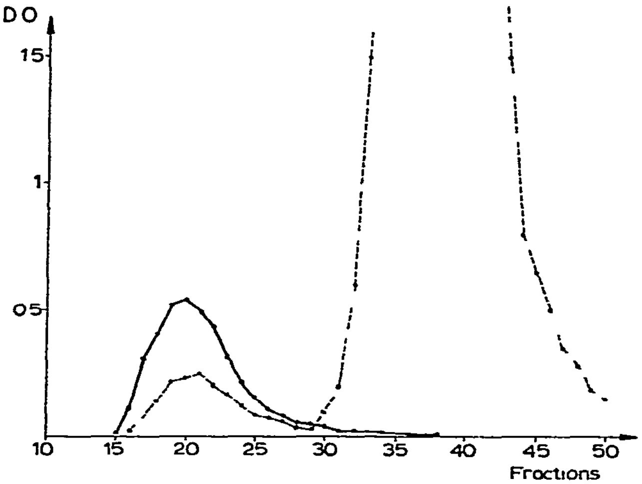

Fig 2 Chromatographie sur Sephadex de l'hydrolysat de la lactotransferrine par la Pronase Colonne 2 × 90 cm, acide acétique 0,1N, fractions 5ml Trait plein dosage des oses neutres Trait discontinu dosage des groupements aminés libres par la ninhydrine

diques qui restent fixées sur la résine alors que les glycopeptides filtrent directement à travers la colonne

La fraction glycopeptidique ainsi obtenue, nommée GPT, est lyophilisée et soumise à diverses analyses . ultracentrifugation en frontière préformée, électrophorèse à haut voltage dans l'appareil Pherographe*, en tampons acide acétique–acide formique (pH 1,9 et 2,4) et acide acétique–pyridine (pH 3,5 et 6,4), sur papier MN 214 Les révélations sont effectuées par la ninhydrine et par le réactif de Schiff après oxydation performique

Le fractionnement des glycopeptides GPT est poursuivi par electrophorèse à haut voltage sur bloc de Pevikon C 870, selon la technique de Cornillot[24] le Pevikon, après avoir été longuement lavé afin d'en éliminer les impuretés, est mis en suspension dans un tampon acide acétique–acide formique de pH 2,4 et coulé sur la plaque de verre refroidie de l'appareil; l'excès de liquide est éliminé avec du papier filtre On obtient ainsi un bloc compact, épais de 1 cm, long de 38 cm, la largeur étant fonction de la quantité de substances à séparer (1 cm pour 10 mg) La température du liquide réfrigérant est fixée à −5°, ce qui maintient une température de +4° à l'intérieur du bloc pendant le passage du courant La solution de glycopeptides à fractionner (110 mg/par ml) est déposée dans une fente pratiquée dans le bloc, à 10 cm du bord anodique, 1200 volts sont appliqués durant 2 h et 40 min Puis des sections de 1 cm sont découpées et éluées par l'eau les éluats ainsi obtenus sont lyophilisés après avoir été réunis en fonction de leur composition en acides aminés et en glucides

Les glycopeptides purifiés par électrophorèse sont appelés GPE1, GPE2 et GPEM.

Hydrolyses des glycopeptides

Afin de simplifier leur composition en aminoacides, les glycopeptides sont soumis à des incubations de 20 h à 40°, en présence de divers enzymes protéolytiques pepsine, papaïne, leucine-aminopeptidase. Les hydrolyses sont suivies par le dosage des groupements aminés libres[23]

Le glycopeptide GPE1 est également soumis à une hydrolyse alcaline ménagée par KOH 0,45N, durant 20 h à température ambiante[25] Puis les acides aminés sont libérés par hydrolyse acide et dosés

Enfin, la présence de liaisons peptidiques avec l'acide aspartique nous a amenés à hydrolyser le glycopeptide GPE2 par HCl 0,03N durant 8 h à 100°[26] Les glucides libérés sont dosés et l'hydrolysat est fractionné par électrophorèse sur bloc de Pevikon suivant la technique décrite ci-dessus La teneur en oses neutres et en groupements aminés libres de chaque fraction est déterminée et la composition en acides aminés et en glucides est étudiée qualitativement par chromatographie sur papier ou en couche mince, avant et après hydrolyse par HCl N durant 8 h à 100°

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*L Hormuth, Heidelberg, Allemagne

RÉSULTATS

Fraction glycopeptidique GPT

Après hydrolyse de 4 g de lactotransferrine par la Pronase et purification de l'hydrolysat par filtration sur Sephadex G 25 suivie de chromatographie sur échangeur de cations, 330 mg de glycopeptides ont été obtenus Le Tableau I rapporte les pourcentages en glucides de la lactotransferrine et de cette fraction GPT (les valeurs trouvées dans la littérature pour la lactotransferrine sont également données à titre de comparaison)

TABLEAU I

COMPOSITION EN GLUCIDES DE LA LACTOTRANSFERRINE ET DES FRACTIONS GLYCOPEPTIDIQUES[a]

Glucides	*Lactotransferrine*		*Glycopeptides*		
	Valeurs publiées[8]	*Cette étude*	*GPT*	*GPE1*	*GPE2*
	%	%	%	%	%
Hexoses	3 9	3 7	40	33	35
Hexosamines	2 4	2 0	22	22 4	23
Acide sialique	0 87	0 9	11	9 6	10
Fucose			6,6	8	9

[a] Pourcentages du poids sec

La fraction glycopeptidique GPT est homogène à l'ultracentrifugation (Fig 3) mais hétérogène en électrophorèse sur papier (Fig 4) Un échantillon de 300 mg de GPT est alors soumis à l'électrophorèse préparative sur Pivekon à pH 2,4, pH qui s'est avéré le plus résolutif

Glycopeptides GPE

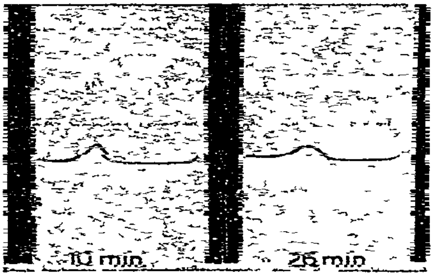
Le fractionnement obtenu par cette technique est sensiblement identique à celui que donne l'électrophorèse sur papier dans les mêmes conditions Les fractions suivantes sont ainsi préparées, dans l'ordre de vitesse de migration croissante vers la cathode · GPE1 = 110 mg, GPE2 = 28 mg, GPM = 95 mg

En fait GPM représente la somme de plusieurs fractions qui s'avèrent hétérogènes en électrophorèse la révélation par le réactif de Schiff après oxydation périodique donne pour GPM une tache largement étalée, alors que GPE1 et GPE2 (Fig 4) ne présentent qu'une bande étroite.

Nous ne rapporterons ici que les résultats obtenus sur GPE1 et GPE2, bien que la composition des autres fractions ne soit pas essentiellement différente.

La composition en glucides et en acides aminés de GPE1 et GPE2 est donnée dans les Tableaux I et II.

Par dégradation séquentielle d'Edman, les phénylhydantoïdes des acides aminés suivants sont identifiés de façon similaire dans ces deux glycopeptides . à la première étape, alanine et glycine, à la deuxième étape, glycine et acide aspartique, à la troisième étape, acide aspartique et proline



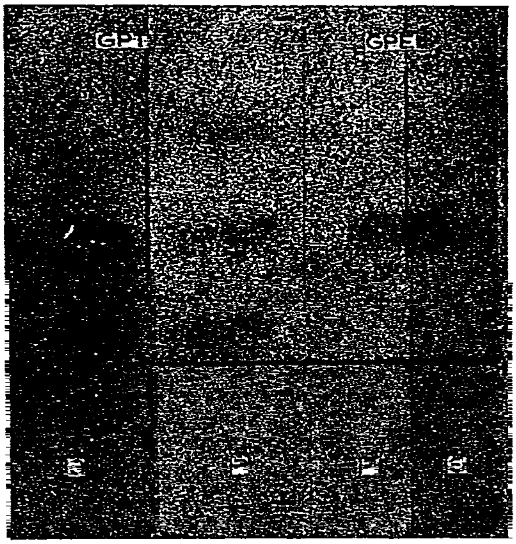


Fig 3 Ultracentrifugation de la fraction glycopeptidique totale GPT 3 mg/ml de tampon phosphate de sodium 0,02M–NaCl 0,08M pH 7 Frontière préformée Photos prises après 10 et 26 min a 59,780 r p m

Fig 4 Électrophorèse sur papier des glycopeptides de la lactotransferrine pH 2,4, tampon acide acétique–acide formique, 60 Volts/cm, 2h Révélation par le réactif de Schiff après oxydation périodique (G) et par la ninhydrine (P)

TABLEAU II

COMPOSITION EN ACIDES AMINES DES GLYCOPEPTIDES GPE1, GPE2[a]

Acides aminés	*GPE1*	*GPE2*	*GPE1 (après hydrolyse alcaline ménagée)*
Acide aspartique	0,85	0,75	0,82
Thréonine	0,96	1	0,54
Sérine	0,36	0,2	0,36
Acide glutamique	0,6	0,5	0,63
Proline	0,85	1,05	0,86
Glycine	0,35	0,35	0,38
Alanine	0,35	0,35	0,38
Valine	0,72	0,55	0,69
Leucine et isoleucine	traces	traces	traces
Phénylalanine	1	0,1	1,05

[a] En μmol/3 mg de glycopeptide.

Après action de carboxypeptidase, la phénylalanine est libérée à partir de GPE1 ; aucun acide aminé ne semble être libéré à partir de GPE2 La pepsine, la papaïne et la leucine-aminopeptidase sont pratiquement sans action sur les glycopeptides.

Après hydrolyse alcaline ménagée, GPE1 perd environ 40% de son contenu en thréonine (Tableau II).

Après 8 h d'hydrolyse de GPE2 par HCl 0,03N à 100°, plus de 80% des oses neutres sont libérés et seulement 30% des oses aminés (Tableau III) Le fractionnement de cet hydrolysat par électrophorèse sur bloc de Pevikon, à pH 2,4 est représenté sur la Fig 5 La première fraction est constituée essentiellement par les oses neutres cette fraction n'a pas migré La deuxième fraction contient principalement l'acide aspartique libre Aucun glucide ou acide aminé libre ne peut être mis en évidence par chromatographie dans les autres fractions Après hydrolyse par HCl N, on identifie un seul glucide, la glucosamine, dans les fractions III et IV, la thréonine et la proline dans la fraction III, et la thréonine dans la fraction IV La fraction V semble constituée par un mélange de peptides et ne contient aucun sucre

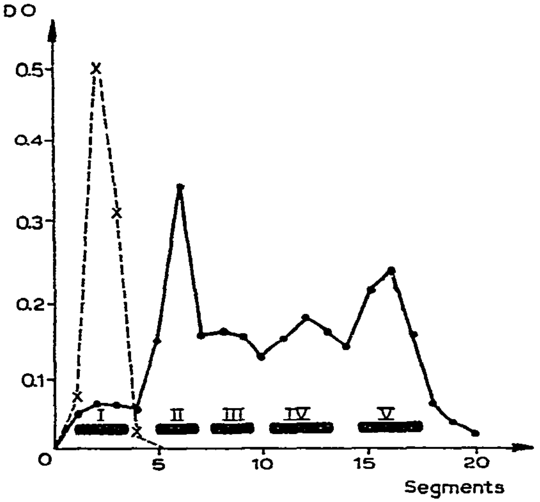


Fig 5 Électrophorèse sur bloc de Pevikon de l'hydrolysat du glycopeptide GPE2 obtenu après action de HCl 0 03N, 8 h a 100° Tampon acide acétique–acide formique, pH 2 4, 40 volts/cm, 9.600 sec Trait plein · dosage des oses neutres Trait discontinu dosage des groupements aminés libres par la ninhydrine Les barres horizontales indiquent la réunion des éluats constituant chaque fraction

TABLEAU III

LIBÉRATION DES GLUCIDES DU GLYCOPEPTIDE GPE2 APRES 8 H D'HYDROLYSE PAR HCl 0 03 N À 100°[a]

Acide sialique	100
Oses neutres	80
Osamines	30

[a] Pourcentage de chaque composé

DISCUSSION

Les préparations de lactotransferrine utilisées dans cette étude répondent à divers critères de pureté qui en garantissent l'homogénéité Les propriétés physicochimiques, en particulier leur teneur en glucides, sont très voisines de celles que l'on trouve dans la littérature[8].

Dans une étude préliminaire, nous avions montré que la trypsine, la chymotrypsine, la papaïne, la pepsine n'ont qu'une action limitée sur cette glycoprotéine[27]. Au contraire, l'hydrolyse par la Pronase est très efficace et se poursuit bien au-delà de 48 h; toutefois, il faut rajouter périodiquement cet enzyme, car il s'autolyse assez rapidement[28]

La filtration sur Sephadex G 25 d'un hydrolysat par la Pronase est un processus désormais classique dans les études sur la structure des glycoprotéines Le passage sur Amberlite IR 120 n'intervient que pour éliminer quelques contaminations peptidiques Le rendement de ces opérations est de l'ordre de 90% pour l'ensemble des glucides qui se retrouvent dans la fraction glycopeptidique GPT dans les mêmes proportions que dans la lactotransferrine native

Si elle est hétérogène en électrophorèse, cette fraction GPT est exclue de la colonne de Sephadex G 25 en un pic unique et symétrique et semble homogène à l'ultracentrifugation on peut donc supposer que les divers glycopeptides qui la constituent ont sensiblement le même poids moléculaire Nous n'avons pas determiné ce poids moléculaire, cependant, la teneur en acide sialique, 11%, nous conduit à un poids moléculaire minimum de l'ordre de 3000, chaque glycopeptide contenant au moins une molécule d'acide sialique Or, les glucides totaux de la lactotransferrine représentent environ 7,5% de la molécule, – poids moleculaire 95,000[4]–, soit un poids moléculaire de 7,200 D'autre part, la lactotransferrine possède trois résidus d'acide sialique Si l'on suppose que chaque acide sialique est à l'extrémité d'une chaîne polysaccharidique, on aurait ainsi trois chaînes ayant un poids moléculaire de 2,400 Les glycopeptides de la fraction GPT possèdent environ 80% de glucides, représentant alors 2,400 sur un poids moléculaire de 3,000 Ces chiffres sont en accord avec la constante de sédimentation de GPT, 0,83 S, et surtout avec les résultats des dosages rapportés dans le Tableau II, puisque plusieurs acides aminés sont en proportion stoechiométrique avec 3 mg de glycopeptide (1 μmole)

Deux autres possibilités peuvent être envisagées soit une chaîne unique contenant les trois molécules d'acide sialique et ayant un poids moléculaire voisin de 9,000, soit deux chaînes ayant respectivement un poids moléculaire de 6,000 et 3,000 Mais ces deux hypothèses ne semblent pas compatibles avec un seul pic symétrique de constante de sédimentation 0,83 S, et moins encore avec les dosages d'acides aminés

On pourrait donc admettre que les glucides de la lactotransferrine sont répartis en trois chaînes terminées par un acide sialique et comportant 1 ou 2 résidus de fucose, 6 résidus d'hexose et 4 résidus d'*N*-acétylglucosamine.

Des résultats obtenus par ultracentrifugation de la lactotransferrine dans différents solvants corroborent cette hypothèse Les ultracentrifugations sont effectuées en tampon phosphate de sodium 0,1M pH 7. En présence dodécylsulfate de sodium 0,01M, on obtient 6% de lactotransferrine native (*s* moins de 4,8 S) et 94% d'un produit ayant pour constante 3,6 S Dans l'urée 8 M, on retrouve 18% de ce constituant et 82% d'un constituant plus léger (*s* 1,7 S) Enfin, dans la guanidine 6 M, on n'obtient plus que le constituant le plus léger. Il semble donc bien que la lactotransferrine est constituée de trois sous-unités et que, par suite, elle possède trois chaînes polysaccharidiques Notons que Jamieson[29] suggère que la transferrine humaine possède deux chaînes polysaccharidiques, de constitution très voisine de celles-ci

Le rendement de l'électrophorèse sur Pevikon est d'environ 75%. Les deux glycopeptides homogènes, GPE1 et GPE2, représentent 60% des produits récupérés, soit 45% de l'échantillon initial

Dans le principal glycopeptide, GPE1, quatre acides aminés seulement sont en proportion stoechiométrique avec un glycopeptide ayant un poids moléculaire de 3,000 l'acide aspartique, la thréonine, la proline et la phénylalanine Les autres acides aminés, présents en proportion plus faible, proviennent soit de contaminations peptidiques que l'on n'a pu éliminer totalement, soit de glycopeptides dont la partie peptidique est moins dégradée Cette hetérogéneité de la chaîne peptidique est confirmée par la degradation d'Edman · à chaque étape, deux phénylthiohydantoïnes-aminoacides sont identifiés Cependant, l'apparition des phénylthiohydantoines respectivement de l'acide aspartique à la deuxième étape et de la proline à la troisième suggère une séquence –Asp–Pro–.

Le glycopeptide GPE2 diffère de GPE1 par l'absence de phénylalanine, ce qui permet d'envisager une position terminale pour cet acide aminé Malgré un très mauvais rendement, l'action de la carboxypeptidase confirme sa présence du côté C-terminal

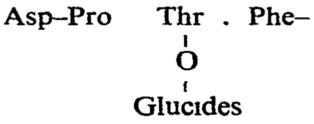
Ainsi, seule la thréonine n'apparaît ni du côté C-, ni du côté N-terminal C'est, de plus, l'acide aminé présent en proportion la plus elevée dans les deux glycopeptides on peut donc supposer qu'il existe une liaison glucide–peptide de type glycosidique avec l'hydroxyle de la thréonine La présence d'une telle liaison dans une des chaînes est également suggérée par l'hydrolyse alcaline ménagée ce traitement entraîne, par β-élimination, la formation d'un acide aminé non saturé (2-amino-2,3-buténoïque) à partir de la thréonine L'hydrolyse acide subséquente provoque la décomposition de cet acide[30] et l'analyse des acides aminés met alors en évidence une perte de la thréonine Cette méthode a permis de montrer que la mucine de la glande sous-maxillaire ovine contenait des liaisons de ce type[31] Dans le cas présent, la perte de 40% de thréonine est en accord avec cette hypothèse Ajoutons que, récemment, Montreuil et Monsigny[32] ont pu montrer la formation d'acide α-aminobutyrique après hydrolyse alcaline et réduction par le borohydrure de sodium[33] d'un hydrolysat pronasique de la lactotransferrine.

L'hydrolyse de peptides ou de protéines par HCl 0,03N a la propriété de rompre spécifiquement les liaisons amides avec l'acide aspartique[26]. Ce traitement provoque une dégradation, non seulement de la chaîne peptidique de GPE2, mais encore de sa chaîne polysaccharidique Il est d'ailleurs intéressant de noter que les glucides sont libérés beaucoup plus facilement à partir des glycopeptides qu'à partir de la glycoprotéine native. Le fractionnement de cet hydrolysat par électrophorèse sur Pevikon permet d'obtenir, d'une part l'acide aspartique libre, d'autre part deux produits constitués, l'un de thréonine et de glucosamine, l'autre de proline, de thréonine et de glucosamine.

Nous n'avons pu étudier en détail ces deux produits par manque de substance. Dans un travail en cours, nous cherchons à confirmer la structure de ces deux composés Toutefois, ces résultats préliminaires sont en accord avec les résultats précédents

La transferrine du sérum présente des propriétés physico-chimiques voisines de celles de la lactotransferrine. Toutefois, la composition en oses et en acides aminés de ces deux glycoprotéines montre des différences significatives[34], en rapport peut-être avec la spécificité immunologique de la lactotransferrine. D'autre part Jamieson[29] a montré que la transferrine possédait deux chaînes polysaccharidiques liées à la protéine par l'acide aspartique, sans exclure cependant la possibilité de liaisons glycosidiques avec la sérine

À partir des résultats obtenus, nous pouvons émettre deux hypothèses d'une part, les glucides de la lactotransferrine seraient répartis en trois chaînes, d'autre part, il existerait dans la lactotransferrine des liaisons de type glycosidique entre les résidus de thréonine et les glucides (vraisemblablement la glucosamine) et la séquence des acides aminés au voisinage de la liaison serait la suivante



$$\text{Asp–Pro}\quad \underset{\substack{|\\ \text{O}\\ |\\ \text{Glucides}}}{\text{Thr}}\ .\ \text{Phe–}$$

Il peut être intéressant de rappeler qu'une liaison semblable se retrouve dans les mucines des glandes sous-maxillaires Or, la lactotransferrine est elle-même un produit de sécrétion d'une glande exocrine et sa présence a été montrée dans les sécrétions bronchiques[35] et dans la salive, la bile ou le sperme[36] . ce type de liaison est peut-être caractéristique des glycoprotéines d'origine glandulaire

RÉSUMÉ

Des glycopeptides de la lactotransferrine humaine sont prépares par hydrolyse pronasique, suivie de chromatographie sur Sephadex G25, Amberlite IR120 et électrophorèse à haut voltage sur Pevikon. La composition de ces glycopeptides suggère que les glucides sont répartis en trois chaînes Une liaison de type glycosidique par un groupement hydroxyle de la thréonine est mise en évidence

SUMMARY

The structure of human lactotransferrin has been investigated by Pronase digestion, followed by chromatography on Sephadex G25 and on Amberlite IR-120 and by high-voltage electrophoresis on Pevikon The composition of the resulting glycopeptides suggests that the carbohydrate moiety is composed of three similar chains Some evidence for the presence in lactotransferrin of glycosidic linkages, involving the hydroxyl groups of threonine residues, is presented

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SORPTION OF AMMONIA BY "V" AMYLOSE

P. C NICOLSON*, G U YUEN, AND B ZASLOW
Department of Chemistry, Arizona State University, Tempe, Arizona 85281 (U S A)

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INTRODUCTION

Adsorption studies conducted over a broad range of temperature provide a means for surveying for chemical reactivity between a solid surface and a gaseous molecule It may be anticipated that adsorption due to weak physical interactions will drop sharply as the temperature is raised However, processes involving strong physical or chemical interactions may not be activated at low temperatures, should such activation occur at higher temperatures, an increase in the amount of adsorption would result as the temperature is raised

In the present study, direct heterogeneous interaction of gaseous ammonia with the helical amylose species, namely "V" amylose dehydrate, was undertaken "V" Amylose dehydrate, also called anhydrous "V" amylose, has a helix packing-diameter[1] of 13 0 Å, water reacts at both the helix exterior and the interhelical cavities[2] Since there is a general similarity of the ammonia and water molecules, adsorption of ammonia on the helix surfaces is to be expected, particularly at low temperatures

EXPERIMENTAL

Although "V" amylose dehydrate was the principal carbohydrate matrix under investigation, auxiliary data were obtained from palmitic acid–amylose[3], and from amorphous amylose Vacuum techniques[4] were used for handling and treating the amylose samples These techniques permit study over a wide range of temperature, but they restrict to less than one atmosphere the pressure of ammonia gas that may be applied Gas–solid studies were conducted under a fixed pressure of ammonia gas at various temperatures Reactions were performed in the absence of air, with the sample temperature and the ammonia pressure under careful control A treatment of "V" amylose by liquid ammonia was also conducted under vacuum to supplement the gas–solid study Reaction products of ammonia with "V" amylose and with the palmitic acid–amylose complex were characterized by X-ray diffraction powder-patterns, and quantitative estimates of the uptake of ammonia by the starch

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*Present Address Geigy Research Laboratories, Yonkers, New York, N Y samples were made gravimetrically. Kjeldahl analysis for nitrogen was used for testing for chemical incorporation of ammonia.

The "V" amylose dehydrate was prepared from amylose (10 g, supplied by the A E Staley Co) which had first been defatted and dried, sufficient water was then added to give a suspension (1% by wt) The mixture was autoclaved (15 lb/in ^{2}, 1 h), the supernatant liquor was decanted from residual insoluble matter, and the hot solution was saturated with hot 1-butanol and placed in a Dewar flask (to allow extremely slow cooling). After four washings with 1-butanol followed by filtration, the amylose was dried for 4 h in an Abderhalden dryer at 64° C under vacuum X-ray powder patterns identified the precipitate as having a hexagonal-cell constant and, hence, a helix packing-diameter of 13 0 Å

A sample of amorphous amylose was obtained by proceeding as above, including the autoclaving Then the dispersion was rapidly cooled to 10° C in an ice bath The resulting precipitate was separated by centrifugation, and freeze-dried (24 h, pressure $<$ 1 mm), this product did not have a discrete, diffraction pattern To prepare the amylose–palmitic acid complex, molten palmitic acid (1% by wt) was added to the autoclaved dispersion The mixture was stirred at reflux temperature for three days, and subsequently allowed to cool slowly to room temperature The resulting precipitate was separated by centrifugation, washed with two portions of 1-butanol, filtered through a fritted-glass funnel, and freeze-dried (24 h, pressure $<$ 1 mm) X-ray powder patterns indicated the presence of a fatty acid–amylose complex similar to that reported by Mikus, Hixon, and Rundle[3], but with orthorhombic-cell constants $a = 13\ 3$, $b = 23\ 3$, $c = 8\ 0$ Å.

The function of the palmitic acid is to serve as a protecting agent by closing off the interior of the "V" amylose helix to attack by ammonia, the amylose–palmitic acid complex is, however, not truly isomorphous with the crystalline "V" amylose dehydrate, as is demonstrated by a comparison of cell dimensions[3] The cell dimensions of the two are closely related, but those of the palmitic acid complex are not pseudohexagonal On a comparative basis, the external surfaces of the helix and the interhelical sorption volumes of the two helix types can be taken to be very similar, although not identical. The exterior of the helix of the amylose–iodine complex behaves identically to that of the "V" amylose dehydrate[2], but iodine was not used as a protecting agent because it is highly reactive toward ammonia As is found with many polymeric materials, lack of crystal perfection limits the observable diffraction maxima obtainable from powder patterns of "V" amylose, and regions of differing disorder and imperfection are likely to be present in the solid However, crystal defects in the palmitic acid–amylose complex may be considered to match those in the "V" amylose dehydrate In addition, the data from amorphous amylose should provide a rough basis for assessing the adsorptive properties of disordered and imperfect regions in the "V" amylose dehydrate

The vacuum line was constructed with standard features[4], including a volume ballast-bulb, a manometer, a McLeod gauge, and a sub-manifold into which anhydrous ammonia was introduced for storage at −80° C Amylose was placed in the reaction chamber, which was attached to the main manifold by a ball-and-socket joint; entry to the sample chamber occurred at a tapered, ground-glass joint which was directly adjacent to a stopcock For the gas–solid studies, ammonia gas was admitted into the main manifold and into the reaction chamber containing an amylose sample at a fixed pressure of 700 mm Hg The temperature of the amylose sample was controlled by placing the sample chamber in a low-temperature bath or in an oil bath (thermostatic control), in this way, a temperature range of −30 to +80°C was readily obtained The sample was allowed to remain in contact with ammonia vapor for 24 h The amount of vapor adsorbed was determined gravimetrically, weighings being made in the reaction chamber on an Ainsworth, semi-micro, single-pan balance at room temperature. The amount of ammonia taken up by the amylose sample was determined after correcting for the weight of gas above the sample in the sample bulb

When a gravimetric determination was made at a temperature below 25°C (at 700 mm Hg), it was necessary to allow the chamber to warm to room temperature before it could be accurately weighed, in order to avoid condensation of moisture on the outside walls of the chamber This warming created the problem of build-up of an internal pressure above 760 mm during equilibration to room temperature, this was solved as follows The volume of the chamber and manifold were first accurately determined At the conclusion of an adsorption experiment, the chamber was closed off from the manifold and was maintained at the low temperature while the manifold was evacuated to a pressure below 0 1 mm Hg The chamber was then carefully opened to the manifold, and was allowed to warm to room temperature as the desorbed ammonia diffused into the manifold When the temperature of the entire system had reached equilibrium, the pressure of the system was measured, and the chamber was closed and weighed The weight of ammonia that had been desorbed from the sample during equilibration is calculable from the P–V–T relationship (ideal-gas equation of state), after the proper corrections for the expansion of ammonia vapor over the sample have been made

Amylose samples to be treated with liquid ammonia were placed in the reaction chamber, which was then evacuated and cooled in a Dry Ice–2-propanol bath When the ammonia was distilled into the chamber, it liquefied; after 1 h, the ammonia was removed by pumping In addition, a special reaction-chamber was constructed which could be so tilted as to decant the liquid ammonia from the amylose, very short, experimental reaction-times could be obtained with this vessel

The reaction products of ammonia with "V" amylose and the "V" amylose–palmitic acid complex were examined with filtered Cu or Cr radiation at room temperature, the radius of the X-ray camera was 57 3 mm. Samples were removed from the reaction chamber and placed in thin-walled, glass capillaries; these were sealed with Apiezon W wax The process of transfer of sample involved a two-minute exposure to the atmosphere prior to sealing

Kjeldahl analyses for nitrogen were conducted by EFCO Laboratories, Tucson, Arizona

RESULTS

The amounts of ammonia adsorbed by "V" amylose dehydrate, the "V" amylose–palmitic acid complex, and amorphous amylose are presented in Fig. 1. Observed increases of weight due to adsorbed ammonia have been converted into molecules of ammonia per D-glucose residue for this tabulation, in order to indicate directly the stoichiometry involved in interaction, estimates of the error of the ordinate in terms of reproducibility of data are ±0.05 molecule of ammonia per D-glucose residue. The pressure of ammonia was fixed at 700 mm Hg in each experiment, and results are plotted as a function of the temperature of the sample for the range −30 to +80° C. Gravimetric data for the fatty acid–amylose complex were corrected for an assumed 7% content of fatty acid (by wt)[3], which corresponds to one palmitic acid molecule per 20 D-glucose residues, with this ratio, a correction term based on the possibility of a 1:1 reaction between ammonia and palmitic acid would not be significant.

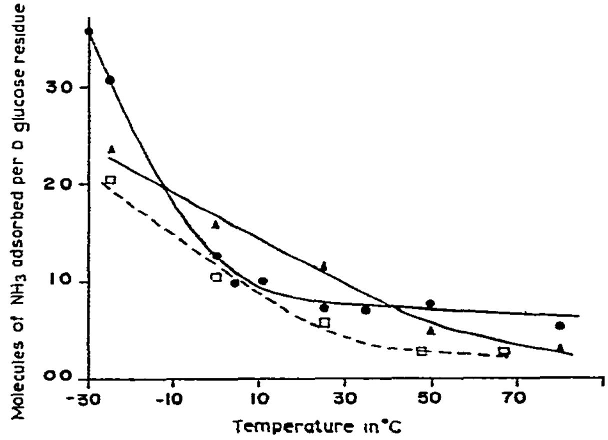

Fig. 1. Adsorption data as a function of temperature of exposure to ammonia at a pressure of 700 mm Hg. Ammonia uptake by "V" amylose dehydrate (●, ————), by the amylose–palmitic acid complex (□, — — — —), and by amorphous amylose (▲, ————)

At the conclusion of each gravimetric determination, a small portion of the sample was taken for X-ray analysis. X-ray results are shown in Fig. 2 and Tables I and II. The *d*-spacings of the hexagonal (110) and (210) lines on powder films were used to calculate the helix packing-diameters found in the reaction products after "V" amylose dehydrate samples had been exposed to 700 mm pressure of ammonia; helix packing-diameters are plotted *versus* temperature in Figure 2. Maximum expected limits of error in helix size due to measurement are estimated at ±0.07 Å, except for the sample treated at −25° C, this sample yielded a diffraction pattern of three halos having $\sin^2\theta$ values of 0.0040, 0.0117, and 0.0273. The X-ray powder pattern of the "V" amylose–ammonia adduct having the greatest number of, and sharpest, diffraction lines (and, therefore, the most crystallinity) was obtained at 80°C Table I shows the sin$^2\Theta$ values for CuKα radiation, and the relative intensity of the pattern demonstrated by this product The calculated helix packing-diameter is 13 12 Å. Agreement between observed and calculated sin$^2\Theta$ values indicates that hexagonal dimensions have been retained

TABLE I

SIN$^2\Theta$ VALUES (CuKα RADIATION) FOR THE PRODUCT OF THE REACTION OF "V" AMYLOSE DEHYDRATE AT 80 °C WITH AMMONIA (700 MM Hg)

Hexagonal Indices	*Intensity*	*Sin$^2\Theta$*	
		Obs	*Calc*
(100)	m	0 00459	0 00459
(110)	ms	0 0137	0 0138
(200)	vw	0 0182	0 0183
(210)	vs	0 0321	0 0321
(300)	w	0 0412	0 0413
(410)	vw	0 0960	0 0964
(510)	vvw	0 143	0 142

TABLE II

SIN$^2\Theta$ VALUES (CuKα RADIATION) FOR AMYLOSE–PALMITIC ACID COMPLEXES

Orthorhombic indices	*Literature values*[3]	*Starting material*	*Temperature of sample exposed to NH_3*				
			vapor				*liquid*
			67°C	*25°C*	*0°C*	*−25°C*	*−80°C*
(110)	0 0045	0 0045	0 0046	0 0047	0 0046	0 0041	0 0040[a]
(011)	0 0109	0 0109					
(200)	0 0137	0 0135	0 0138	0 0138	0 0135	0 0118	0 0116[a]
(131)	0 0221	0 0224	0 0219	0 0222	0 0219	0 0217	
						0 0273	0 0262
(310)	0 0323	0 0320	0 0325	0 0327	0 0325	0 0324	0 0320
(320)		0 0349					
		0 0439			0 0400	0 0399	0 0404
		0 0692					

[a] Measured at the center of the halo

The observed sin$^2\Theta$ values, derived from CuKα radiation, of the palmitic acid–amylose complex which was used for starting material, as well as the sin$^2\Theta$ values for each of the reaction products from fatty acid complex plus ammonia interaction, are shown in Table II The sin$^2\Theta$ values reported by Mikus, Hixon, and Rundle[3] for the palmitic acid–amylose complex are also recorded, along with their orthorhombic indices Since the X-ray powder patterns for the reaction products of fatty acid complex and ammonia were difficult to measure, and could not be indexed reliably, the indices in Table II, column 1, do not necessarily apply to the sin$^2\Theta$

values listed for the reaction products at the various temperatures. However, it may be noted that the $\sin^2\theta$ values listed for the lines showing the (110) and (200) orthorhombic indices have *d*-spacings that are approximately in the ratio $3^{1/2}$ to 1, and roughly correspond to *d*-spacings of the hexagonal indices (100) and (110) An approximate helix packing-diameter was calculated for these lines The concept of approximate helix packing-diameter as applied to the palmitic acid–amylose complex is probably valid within ±0 15 Å The two innermost halos, observed when the fatty acid complex is treated with liquid ammonia, are very similar to those observed for the "V" amylose treated with liquid ammonia, which is described below.

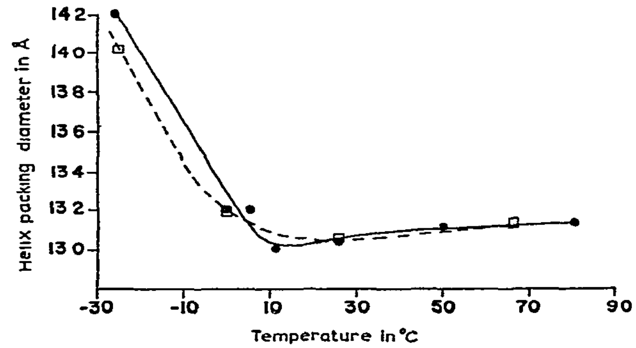

Fig 2 Packing diameter spacings as a function of temperature of exposure to ammonia at a pressure of 700 mm Hg Variation in helix packing diameter of "V" amylose dehydrate (• , ———) Approximate helix packing-diameter of the amylose–palmitic acid complex (□, — — — —)

Treatment of "V" amylose dehydrate with liquid ammonia yields a hard, gritty product that is readily soluble in water The X-ray diffraction pattern of this product consisted of three diffuse halos appearing at the approximate positions of the (100), (110), and (210) diffraction lines for the "V" amylose pattern, but at slightly lower angles The observed $\sin^2\theta$ are 0 0040, 0 0117, and 0 0273, with CuKα radiation The helix packing-diameter in the hexagonal cell could be roughly 14 2 Å Treatment of "V" amylose dehydrate with liquid ammonia for one second, in a special chamber, gave a product whose X-ray powder pattern showed a mixture of the halo species and "V" amylose dehydrate The reaction of liquid ammonia, which led to the halo species, could not be reversed by pumping from the sample, even though all the ammonia was shown, by subsequent weighing, to have been removed, "V ' Amylose dehydrate showed no apparent solubility in a large excess of liquid ammonia

X-ray studies showed no change of size of packing diameter in an ammoniated species having a packing diameter of 13 2 Å when it was allowed to stand exposed to air for four hours. However, the gain in weight due to adsorbed ammonia was not retained to any detectable extent by samples of "V" amylose that had been subjected to vacuum at room temperature, the X-ray pattern of samples de-ammoniated by this means showed only the characteristic *d*-spacings of "V" amylose dehydrate

To check for chemical incorporation of nitrogen into amylose samples, Kjeldahl analyses for nitrogen were performed. Prior to analysis, samples were subjected to vacuum for 24 h at room temperature The results were as follows "V" amylose dehydrate plus liquid NH_3 for 1 h at —78°C, 0 042% N, "V" amylose dehydrate plus NH_3 vapor at 80°C and 700 mm Hg for 24 h, 0 085% N, control (*i e*, untreated "V" amylose dehydrate), 0 050% N.

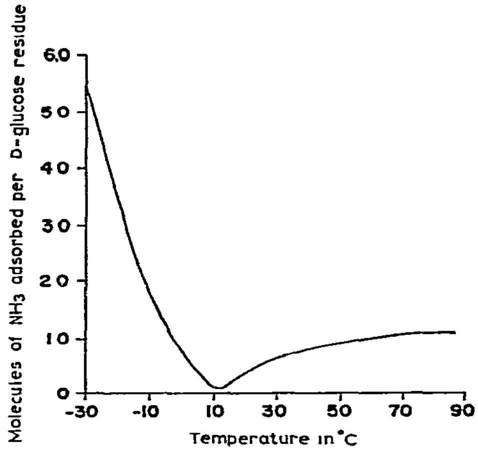

Fig 3 Adsorption results for crystalline "V" amylose Ammonia uptake by crystalline "V" amylose dehydrate at a pressure of 700 mm Hg, assuming the total "V" amylose dehydrate to be 40% crystalline

DISCUSSION

The gravimetric curve shown in Figure 1 for the "V" amylose dehydrate indicates extensive adsorption of ammonia vapor at —25°C. A comparison with the palmitic acid–amylose, where the interior of the helix is blocked, shows that, in the total sample, approximately one molecule of ammonia per D-glucose residue is adsorbed on the interior of the helices of "V" amylose dehydrate at this low temperature The adsorption capacity of the interior of the helix toward ammonia drops off rapidly as the temperature is changed from —25 to +5°C At about 5°C, both species show an adsorption of about one molecule of ammonia per D-glucose residue, the two curves do not cross, but almost touch at this temperature

Since iodine bestows stability on the helical structure of the amylose–iodine complex in the presence of water vapor[2], it is reasonable to expect that the palmitic acid stabilizes the carbohydrate helix in the palmitic acid–amylose complex. The helix packing-diameter of about 14 Å observed for the adduct of palmitic acid–amylose and ammonia at —25°C can then best be explained by extensive adsorption on the helix exterior, rather than by an expansion due to enlargement of the carbohydrate skeleton by a fractional increase in the number of D-glucose residues per helix turn At —25°C, the helix packing-diameter of the product of reaction between "V" amylose dehydrate and ammonia is 14 2 Å, which, because of its closeness to that for palmitic acid–amylose, suggests that the "V" amylose dehydrate has undergone a similar process, that is, the increase in packing diameter from 13 0 Å to 14 2 Å is also due to extensive adsorption of ammonia on the helix exterior. If this is so, the structure of the "V" amylose helix is stable, even in the absence of the complexing agent, at a pressure of ammonia of 700 mm Hg. Then, as the temperature is changed from —25 to +10° C, interaction on the helix exterior drops off markedly, as is indicated by the negative slopes of the gravimetric and helix-diameter curves for the "V" amylose and the fatty acid complex, respectively, in Fig 2, the helix-diameter curve for the "V" amylose dehydrate reaches a minimum at about 11 °C.

A small increase in the capacity of crystalline "V" amylose dehydrate to adsorb ammonia is found at temperatures in the range of 15 to 80 °C; this phenomenon is reflected in both the crystallographic and the gravimetric data. Above 30 °C, the lattice expansion for the crystalline "V" amylose dehydrate is greater than the error in measurement, therefore, the slow rise in reaction with ammonia on the helix exterior at higher temperatures is real, and is complemented by the improved crystallinity observed at 80 °C The curve for amorphous amylose, plotted in Fig 1, is a limiting curve, and may be taken to represent a description of the adsorption in the more disordered and imperfect regions in the "V" amylose dehydrate It is noteworthy that the proportion of adsorbed ammonia diminishes more sharply for the amorphous amylose than for the "V" amylose dehydrate in the range of 25 to 80 °C This means that the regions in the total "V" amylose dehydrate that are least disordered, or most crystalline, show an increase in ability to adsorb ammonia at the higher temperatures The effect may be put on a semiquantitative basis, on the assumption that the "V" amylose dehydrate may be treated as though it were composed of two fairly homogeneous portions an amorphous fraction and an essentially crystalline fraction; with this assumption, the adsorption data at 11 °C imply that the "V" amylose dehydrate is *ca* 40% crystalline At 11 °C, the helix packing-diameter indicates that no adsorption has occurred on the exterior surface of the helix in "V" amylose dehydrate, and the gravimetric data indicate that the correction for adsorption on the helix interior is very small, all of the ammonia adsorbed on "V" amylose dehydrate can then be attributed to the presence of amorphous amylose, which represents about 60% of the total sample A plot of the molecules of ammonia adsorbed per D-glucose residue of crystalline "V" amylose, made by applying a 0 40 fraction for crystallinity, is shown in Fig 3 The rise in sorption above 15°C means that a new type of interaction between the more crystalline fraction of "V" amylose dehydrate and ammonia has been activated

It is interesting to note that, at —80 °C, pure liquid ammonia can irreversibly disrupt the interhelical "V" amylose structure. The presence of the halos in the X-ray powder pattern may be interpreted as being the result of fragmentation into smaller particles, the helices being forced apart from one another. This would account for the observed dispersability in water of the product obtained on treatment with liquid ammonia Despite the disruption of the regular structure, the lack of retention of nitrogen by the carbohydrate structure shows that selective incorporation of ammonia into the "V" amylose matrix does not occur at low temperature However, pretreatment of "V" amylose with liquid ammonia may prove to be a means of enhancing the reactivity toward other reagents by exposing the outer helix surface to chemical attack.

Changes in lattice constants in the "V" amylose–ammonia system are continuous or nondiscrete, therefore, ammonia adsorption is nonspecific under the conditions studied, and except in general terms, it cannot be related to a detailed molecular picture of the "V" amylose helix By contrast, water vapor attacks crystalline "V" amylose dehydrate at specific sites, so that a lattice discontinuity occurs and a stoichiometric hydrate is formed[2,5] According to the Hybl model of "V" amylose, which is based on a single-crystal analysis of potassium acetate–cyclohexaamylose, a number of oxygen atoms are exposed at the exterior surface of the helix[6], and are available for hydrogen-bonding interactions. Although both water and ammonia are readily capable of forming hydrogen bonds, water undoubtedly forms fewer but stronger hydrogen bonds with the carbohydrate polymer.

ACKNOWLEDGMENTS

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SUMMARY

The reactivity of "V" amylose toward ammonia has been investigated by an adsorption study with gaseous ammonia over the temperature range of −30° to +80°C, and with liquid ammonia at −80°C The study with gaseous ammonia was conducted at a fixed pressure of 700 mm Hg, where the gas pressure and the temperature control of the samples were maintained by using high-vacuum techniques Both gravimetric and X-ray diffraction data are reported Much physical adsorption occurred on both the interior and exterior of the helix at low temperatures Adsorption dropped markedly as the temperature of the sample was raised to 10°C, at higher temperatures, there was evidence that a new sorption process was activated Variable packing-diameters for the helix were observed, indicating that adsorption on the helix exterior is not restricted to specific sites

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METHYLATION OF CARBOHYDRATES BEARING BASE-LABILE SUBSTITUENTS, WITH DIAZOMETHANE–BORON TRIFLUORIDE ETHERATE I

IRMA O MASTRONARDI, SUSANA M FLEMATTI, JORGE O DEFERRARI, AND EDUARDO G GROS*
Departamento de Química Orgánica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Perú 222, Buenos Aires, Argentina

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Methylation of carbohydrates containing base-labile substituents (*e g* , acetyl groups) with the conventional reagents, such as methyl iodide or methyl sulfate in the presence of such bases as silver oxide or barium hydroxide, respectively, is not a suitable reaction owing to the ready mobility of the substituents in the alkaline media

Previously, we have reported[1] that this difficulty could be avoided by the use of diazomethane and boron trifluoride etherate, since, under the acidic conditions of the reaction, the sensitive groups seem to be unaffected, whereas the free hydroxyl groups are readily methylated[2] We now report an extension of the previous work to show the versatility of the method, which may be used to methylate primary and secondary alcoholic groups in compounds known to suffer acetyl migration when methylated with Purdie's reagents

1,2,3,4-Tetra-*O*-acetyl-β-D-glucopyranose (**1**), when methylated with diazomethane–boron trifluoride etherate produced 1,2,3,4-tetra-*O*-acetyl-6-*O*-methyl-β-D-glucopyranose (**8**)[1], but when compound **1** was treated with methyl iodide and silver oxide a C-1 to C-6 acetyl migration occurred and methyl 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside (**6**) was obtained[3]

Methylation of 1,3,4,6-tetra-*O*-acetyl-β-D-glucopyranose (**2**) and of its anomer (**3**) with the new reagent afforded, respectively, 1,3,4,6-tetra-*O*-acetyl-2-*O*-methyl-β-D-glucopyranose (**9**) and the α-D anomer (**10**) in high yields, it was already known that compounds **2** and **3** are both converted into **6** upon treatment with Purdie's reagents[4] We found a similar acetyl migration with 1,3,4,6-tetra-*O*-acetyl-α-D-galactopyranose (**5**), which yielded methyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside (**7**) when submitted to the action of Purdie's reagents On the other hand, when compound **5** was methylated with diazomethane–boron trifluoride etherate, 1,3,4,6-tetra-*O*-acetyl-2-*O*-methyl-α-D-galactopyranose (**12**) was readily obtained The new methylation method permitted us to transform 1,2,3,6-tetra-*O*-acetyl-β-D-glucopyranose (**4**) into the hitherto-unknown 1,2,3,6-tetra-*O*-acetyl-4-*O*-methyl-β-D-glucopyranose (**11**), it had earlier been reported[5] that an acetyl migration occurs upon methylation, with Purdie's reagents, of compound **4** (which was also converted into compound **6**)

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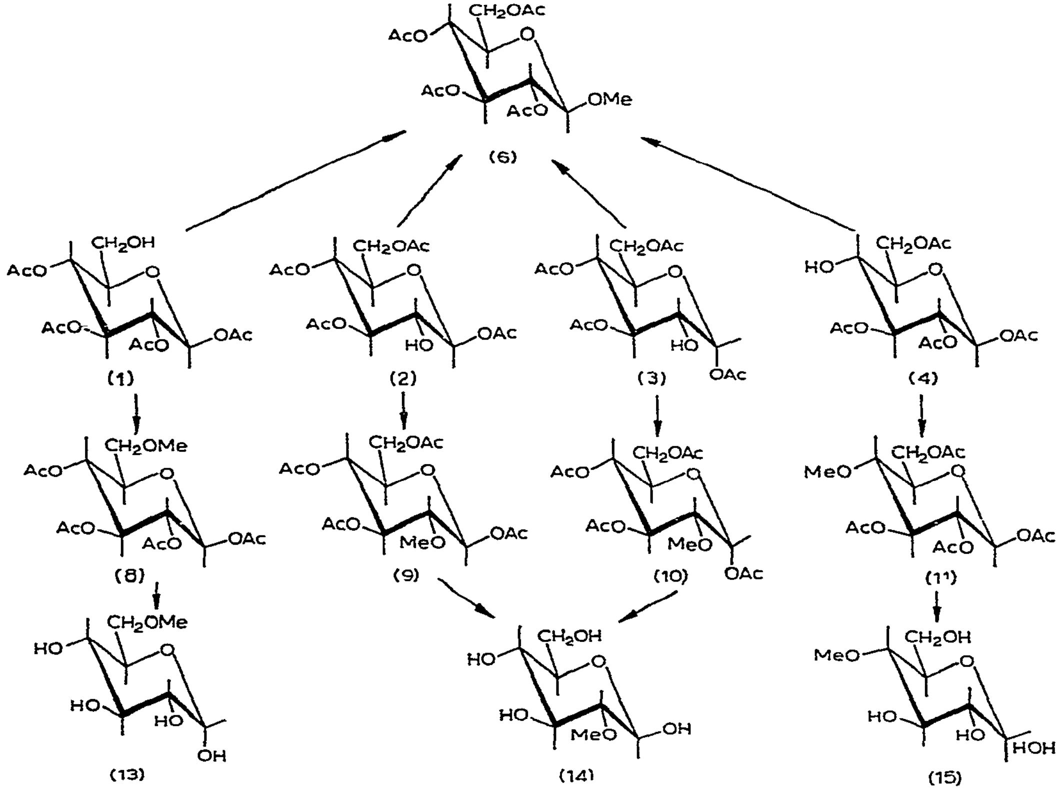
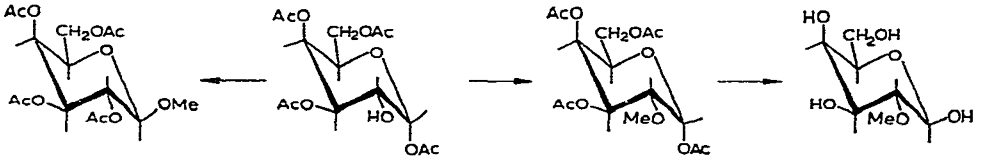
*Research member of the Consejo Nacional de Investigaciones Científicas y Técnicas

To confirm the structures of compounds **8**, **9**, **10**, **11**, and **12**, they were deacetylated with sodium methoxide in methanol in yields of about 80%, thus, compounds **9** and **10** yielded 2-*O*-methyl-β-D-glucose (**14**); compound **8** afforded 6-*O*-methyl-α-D-glucose (**13**), compound **11** produced 4-*O*-methyl-D-glucose (**15**) (mixture of the anomers), and compound **12** gave 2-*O*-methyl-β-D-galactose (**16**) The physical properties of the monomethyl ethers were identical with those of authentic specimens



The n m r. spectra of the mono-*O*-methylhexoses show that each of the crystalline products (**13**, **14**, and **16**) is a pure anomer Compounds **14** and **16** are in the β-D form, since, immediately after dissolution, the hemiacetal proton seems to be axial, being coupled to H-2 with an axial–axial coupling of about 8 c p s Measurement of the solution in deuterium oxide after 6–7 h reveals (a) a decrease in the relative intensity of the previous signal, and (b) the appearance of another doublet at lower field due to the equatorial position of H-1 (α-D form) which is coupled to H-2 with an equatorial–axial coupling of about 3 c p s , this accompanies the mutarotation that, in both cases, produces an increase in the value of the optical rotation For compound **13**, the crystalline product is in the α-D form, because the spectrum reveals that H-1 is in the equatorial position; 6 h after dissolution, the spectrum exhibits another doublet at higher field (β-D-form) having an axial–axial coupling of about 8 c p s , in this case, the mutarotation consists of a shift of the optical rotation to lower values. The syrup **15** proved to be a mixture of the anomers, since its n m r spectrum shows two doublets, corresponding to equatorial and axial H-1, the former is coupled to H-2 with a coupling constant of 3 c p s, whilst the latter exhibits a coupling constant of 8 c p s, this result is in agreement with the lack of mutarotation of compound **15.**



(7) (5) (12) (16)

In accordance with our previous finding[1], the method is suitable for methylation of the alcoholic hydroxyl groups of carbohydrates bearing base-sensitive substituents Other advantages of the method are the short time needed to complete the reaction and the high yields (70–90%) in which the methylated products are obtained

EXPERIMENTAL

Melting points are not corrected Infrared spectra were recorded with a Perkin–Elmer Infracord spectrophotometer. Nuclear magnetic resonance spectra were determined in deuteriochloroform or deuterium oxide, with tetramethylsilane as the internal reference, by using a Varian A-60 spectrometer. Solvents were removed under diminished pressure below 50°

*1,2,3,4-Tetra-*O*-acetyl-β-*D*-glucopyranose* (**1**) — Compound **1** was obtained by a method described[6] in the literature Our product had m p 129–130°, $[\alpha]_D^{20}$ +11 8° (*c* 1 1, chloroform), in agreement with the reported values[6]

*1,3,4,6-Tetra-*O*-acetyl-β-*D*-glucopyranose* (**2**) — This was prepared according to Lemieux and Huber[7] Our compound showed m.p. 135–136°, $[\alpha]_D^{20}$ +24.2° (*c* 1 1, chloroform), lit [7] m p 136–137°, $[\alpha]_D$ +26°.

*1,3,4,6-Tetra-*O*-acetyl-α-*D*-glucopyranose* (**3**) — This product was prepared after the method of Helferich and Zirner[8] Our compound had m p 97–98°, $[\alpha]_D^{20}$ +139 4° (*c* 1 0, chloroform) in agreement with the properties of a sample kindly provided by Professor Helferich

*1,2,3,6-Tetra-*O*-acetyl-β-*D*-glucopyranose* (**4**) — This compound was obtained following the method described by Bonner[9] Our product had m p. 129–130°, $[\alpha]_D^{20}$ —32 6° (*c* 1 1, chloroform) in accordance with reported values

*1,3,4,6-Tetra-*O*-acetyl-α-*D*-galactopyranose* (**5**) — This was obtained as described by Helferich and Zirner[8] Our compound had m p 145–146°, $[\alpha]_D^{20}$ +140 2° (*c* 1 0, chloroform) in agreement with the properties of a sample provided by Professor Helferich

Methylation of 1,3,4,6-tetra-O-acetyl-α-D-galactopyranose with Purdie's reagents. Preparation of methyl 2,3,4,6-tetra-O-acetyl-β-D-galactopyranoside (**7**)

Compound **5** (5 g) was treated with methyl iodide (17.5 g) and freshly prepared, wet silver oxide (3.7 g), which was added in portions during 30 min. The mixture was shaken during 1 h, the temperature being kept at about 40°. The solid was filtered off, and the filtrate was evaporated. The syrupy residue was dissolved in methanol (5 ml) and crystallized by addition of water (20 ml); it was then recrystallized from 1:4 methanol–water to yield pure compound **7** (4.2 g, 80%), m.p. 96–97°, $[\alpha]_D^{20}$ −14.2° (*c* 1.0, chloroform), in accordance with the reported values[10]. When deacetylated with sodium methoxide in methanol, compound **7** (0.5 g) gave methyl β-D-galactopyranoside (220 mg, 84%), m.p. 178–179°, $[\alpha]_D^{20}$ +0.1° (*c* 1.1, water); lit.[11] m.p. 178–179°, $[\alpha]_D^{20}$ +1.2°.

Methylation with diazomethane–boron trifluoride etherate. General procedure

The substrate (compounds **1**, **2**, **3**, **4**, and **5**) (1 g) was dissolved in dichloromethane (10 ml), and the solution was kept at −5° while boron trifluoride etherate (0.04 ml) was added. A solution of diazomethane in dichloromethane was then slowly added at the same temperature. A vigorous reaction occurred with formation of a white solid (polymethylene), and the addition was stopped when a faint yellow color persisted in the solution for a short time. After 30 min at −5°, the solid was filtered off, and the filtrate was washed with 10% sodium hydrogen carbonate solution and with water, and dried with magnesium sulfate. The residue obtained by evaporation of the filtrate crystallized spontaneously. The method was applied to the preparation of the following compounds.

(*a*) *1,2,3,4-Tetra-O-acetyl-6-O-methyl-β-D-glucopyranose* (**8**). — When recrystallized from absolute ethanol, the product (925 mg, 88%) had m.p. 92–94°, $[\alpha]_D^{20}$ +20.5° (*c* 1.1, chloroform) in agreement with the values reported[12]. The i.r. and n.m.r. spectra were in accordance with the structure; in the latter spectrum, the *O*-methyl signal appeared at τ 6.65 and the H-1 signal as a doublet at τ 4.24 ($J_{1,2}$ 8 c.p.s.).

(*b*) *1,3,4,6-Tetra-O-acetyl-2-O-methyl-β-D-glucopyranose* (**9**). — This was recrystallized from 95% ethanol; the pure product (820 mg, 78%) had m.p. 106–107°, $[\alpha]_D^{20}$ +29.1° (*c* 1.1, chloroform). The i.r. spectrum showed no absorption in the hydroxyl region, and the n.m.r. spectrum showed the *O*-methyl group at τ 6.49, the H-1 signal appearing as a doublet at τ 4.36 ($J_{1,2}$ 8 c.p.s.).

Anal. Calc. for $C_{15}H_{22}O_{10}$: C, 49.72; H, 6.12. Found: C, 49.77; H, 6.09.

(*c*) *1,3,4,6-Tetra-O-acetyl-2-O-methyl-α-D-glucopyranose* (**10**). — When recrystallized from 95% ethanol, the product (800 mg, 75%) had m.p. 107–108°, $[\alpha]_D^{20}$ + 109.2° (*c* 0.9, chloroform), with no hydroxyl bands in its i.r. spectrum. In the n.m.r. spectrum, the *O*-methyl signal appeared at τ 6.57, and the H-1 signal showed as a doublet at τ 3.53 ($J_{1,2}$ 3.5 c.p.s.).

Anal. Calc. for $C_{15}H_{22}O_{10}$: C, 49.72; H, 6.12. Found: C, 49.52; H, 5.85.

(*d*) *1,2,3,6-Tetra-O-acetyl-4-O-methyl-β-D-glucopyranose* (**11**). — After recrystallization from 95% ethanol, the compound (862 mg, 82%) had m.p. 103–104°,

$[\alpha]_D^{20}$ −10 2° (*c* 1 1, chloroform) The i r. spectrum showed no absorption in the hydroxyl region, the n m r spectrum showed a band at τ 6 55 (*O*-methyl), H-1 signal appearing as a doublet at τ 4 29 ($J_{1,2}$ 8 c p s).

Anal Calc. for $C_{15}H_{22}O_{10}$· C, 49.72, H, 6 12. Found. C, 49.59, H, 5 80

(*e*) *1,3,4,6-Tetra-*O*-acetyl-2-*O*-methyl-α-D-galactopyranose* (**12**) — This was recrystallized from 95% ethanol, the compound (780 mg, 74%) had m p 101–102°, $[\alpha]_D^{20}$ +98 0° (*c* 1 0, chloroform) The i r spectrum showed no hydroxyl absorption, and the n m r spectrum showed a band at τ 6 55 (*O*-methyl) and a doublet at τ 3 50 ($J_{1,2}$ 4 c p s) (hemiacetal proton)

Anal Calc for $C_{15}H_{22}O_{10}$· C, 49 72, H, 6 12 Found C, 49 72, H, 6.38

*Deacetylation of the tetra-*O*-acetyl-*O*-methylhexopyranoses General procedure*

The methylated sugar (compound **8**, **9**, **10**, **11**, and **12**) (500 mg) was dissolved in dry methanol (10 ml), and the solution was treated with 0 9 M sodium methoxide (0 5 ml) and kept at room temperature with occasional shaking After 6 h, Dowex-50 W resin was added to neutralize the sodium methoxide, the mixture was filtered, and the filtrate was evaporated The residue was dissolved in water, the solution filtered, and the filtrate lyophilized. Where possible, the product was crystallized from suitable solvents The following compounds were obtained in this way

(*a*) *6-*O*-Methyl-α-D-glucose* (**13**) — This was obtained from compound **8** The product was recrystallized from absolute ethanol, the pure substance (216 mg, 82%) had m p 142–144°, $[\alpha]_D^{20}$ +95 2→+61.0° (*c* 1.2, water) in agreement with the reported values[13] The n m r spectrum showed that the compound was the α-D anomer, since 10 min after dissolution the H-1, signal appeared as a doublet at τ 4 67 ($J_{1,2}$ 3 5 c p s), after 4 h, the intensity of the previous signal had decreased and another doublet (β-D form) at τ 5 32 ($J_{1,2}$ 7 5 c p s) had appeared The *O*-methyl signal showed at τ 6 63

(*b*) *2-*O*-Methyl-β-D-glucose* (**14**) — This was prepared from compounds **9** and **10** The product was recrystallized from absolute ethanol, and the pure compound (210 mg, 80%) had m p 158–159°, $[\alpha]_D^{20}$ +21 0→+66 0° (*c* 1 0, water) in agreement with the values reported in the literature[14] The n m r spectrum showed, immediately after dissolution, the H-1, signal as a doublet at τ 5 30 ($J_{1,2}$ 8 c p s) (β-D-form), but after 7 h, this signal had diminished, and another doublet at τ 4 51 ($J_{1,2}$ 3 5 c p s) (α-D form) had appeared, the *O*-methyl signal showed at τ 6 38

(*c*) *4-*O*-Methyl-D-glucose* (**15**) — This was prepared from compound **11** The syrupy product (260 mg, 99%) had $[\alpha]_D^{20}$ +58 4° (*c* 1 2, water) without mutarotation, in accordance with the reported value[15] The n m r spectrum showed that there was an equilibrium between the anomers, the H-1, signal of the α-D-form appeared as a doublet at τ 4 77 ($J_{1\ 2}$ 3 c p s), and for the β-D form, it appeared as a doublet at τ 5.35 ($J_{1,2}$ 8 c p s), for both doublets, the intensity did not change 7 h after dissolution, the *O*-methyl signal showed at τ 6 43 The phenylosazone had[15] m p 157–158°

(*d*) *2-*O*-Methyl-β-D-galactose* (**16**) — This was produced from compound **12** After recrystallization from absolute ethanol, the product (210 mg, 80%) had m p.

148–150°, $[\alpha]_D^{20}$ $+54\ 5 \rightarrow +82\ 9°$ (c 1 2, water), in agreement with the values reported in the literature[16] The n m r spectrum showed that the product was in the *β*-D form, since the H-1 signal appeared as a doublet at τ 5 35 ($J_{1,2}$ 8 c p s); after 7 h, this signal had decreased, and the spectrum showed another doublet (α-D form) at τ 4 48 ($J_{1\ 2}$ 3 5 c p s), the *O*-methyl signal appeared at τ 6 38

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SUMMARY

Various tetra-*O*-acetyl-D-hexopyranoses were known to undergo acetyl migration upon methylation with Purdie's reagents, thus, 1,2,3,4-tetra-*O*-acetyl-*β*-D-glucopyranose, 1,2,3,6-tetra-*O*-acetyl-*β*-D-glucopyranose, and 1,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranose and its α anomer are converted into methyl 2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranoside when methylated with methyl iodide and silver oxide

It is now found that, when each of the above compounds is treated with diazomethane–boron trifluoride etherate, the methylated product is produced in high yield, with the methyl ether group in the expected position Under the same conditions, 1,3,4,6-tetra-*O*-acetyl-α-D-galactopyranose is converted into the 2-methyl ether without the acetyl migration which had been observed when the tetra-*O*-acetyl-D-galactopyranose was methylated with Purdie's reagents

The tetra-*O*-acetyl-*O*-methyl-D-hexopyranoses were then transformed by deacetylation into the following mono-*O*-methylhexoses· 2-*O*-methyl-*β*-D-glucose, 4-*O*-methyl-D-glucose, 6-*O*-methyl-α-D-glucose, and 2-*O*-methyl-*β*-D-galactose

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ACYLATED SACCHARIDE HYDRAZONES AND OSAZONES

Z. M. El-Shafei and S. H. El Ashry
Faculty of Science, Alexandria University, Alexandria (U.A.R.)

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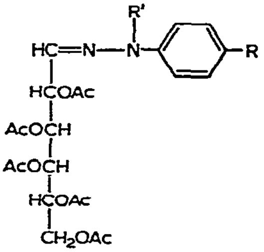
When acetylated with acetic anhydride in pyridine, saccharide hydrazones yield *O*-acetylated derivatives[1] On the other hand, treatment of D-galactose phenylhydrazone with boiling acetic anhydride affords a hexaacetate having acetyl groups attached to a nitrogen atom in addition to the oxygen atoms[2]. We have studied the acetylation of saccharide hydrazones with boiling acetic anhydride, and with acetyl chloride in pyridine, and have found that D-galactose phenyl- and (*p*-chlorophenyl)-hydrazones yield the hexaacetates (**1a**) and (**1b**), and that the corresponding *p*-nitro derivative affords the pentaacetate (**1c**) It seems that the nitro group deactivates the imino hydrogen atom and prevents *N*-acetylation The hexaacetates obtained showed *N*-acetyl bands at 1700 and 1690 cm^{-1} and *O*-acetyl bands at 1750 and 1740 cm^{-1}, respectively, whereas D-galactose (*p*-nitrophenyl)hydrazone pentaacetate showed only the *O*-acetyl group at 1745 cm^{-1} In all three cases, acetylation with acetyl chloride in pyridine gave better yields than acetylation with boiling acetic anhydride

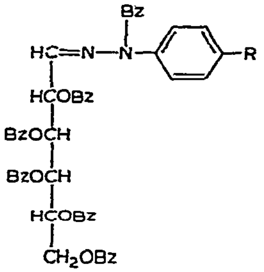


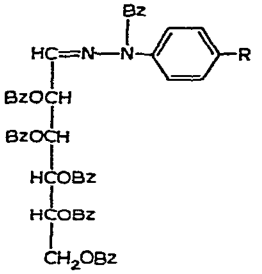
1a R = H R' = Ac
1b, R = Cl R' = Ac
1c, R = NO_2, R' = H

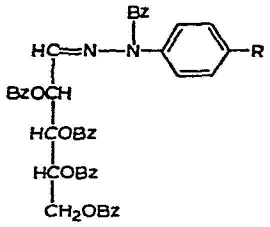
Benzoylation of saccharide hydrazones with benzoyl chloride in pyridine was found to give *N*-benzoyl derivatives and benzoates[3] We have extended this reaction to the *p*-tolyl-, (*p*-chlorophenyl)-, and (*p*-nitrophenyl)-hydrazones of D-galactose, as well as to D-mannose (*p*-chlorophenyl)hydrazone and D-arabinose *p*-tolylhydrazone, and have obtained the benzoates (**2a–c**), (**3**), and (**4**) which showed three carbonyl bands, the *O*-benzoyl band at 1720 cm^{-1} and two *N*-benzoyl bands, one between 1620 and 1630 cm^{-1} and the other between 1660 and 1675 cm^{-1}

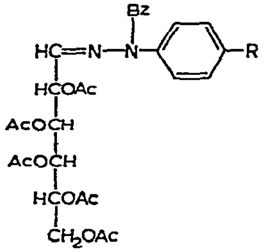
Benzoyl chloride, being a stronger acylating agent than acetyl chloride or boiling acetic anhydride, caused *N*-benzoylation of D-galactose (*p*-nitrophenyl)hydrazone.

When it became apparent that benzoylation always causes introduction of an *N*-benzoyl group in the hydrazone residue, we decided to introduce such an *N*-benzoyl group in the hydrazone residue of saccharide hydrazone acetates, with the object of preparing *N*-benzoylated *O*-acetylated arylhydrazones We therefore treated penta-*O*-acetyl-*aldehydo*-D-galactose phenyl- and (*p*-bromophenyl)-hydrazone with benzoyl chloride in pyridine, and obtained penta-*O*-acetyl-*N*-benzoyl-*aldehydo*-D-galactose phenyl- (**5a**) and (*p*-bromophenyl)-hydrazone (**5b**), respectively. These compounds showed the *O*-acetyl band at 1740 and 1735 cm^{-1}, and the *N*-benzoyl band at 1675 and 1670 cm^{-1}


2a R = CH_3
2b, R = Cl
2c, R = NO_2


3, R = Cl


4 R = CH_3

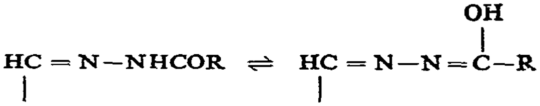

5a R = H
5b, R = Br

We have also studied the benzoylation (of saccharide hydrazones and osazones) with insufficient proportions of benzoyl chloride, in order to obtain the *O*-benzoylated derivatives only This was found possible in the case of osazones, but not with hydrazones Thus, we could obtain, from D-*lyxo*-hexulose phenylosazone, a crystalline tetrabenzoate which showed only two *O*-benzoyl bands (at 1700 and 1725 cm^{-1}) denoting the absence of the *N*-benzoyl group—which usually appears[4] between 1670 and 1680 cm^{-1}. Similarly, L-*erythro*-pentulose phenylosazone afforded a tribenzoate which showed one *O*-benzoyl band, at 1700 cm^{-1} On the other hand, all the hexose hydrazones treated with insufficient proportions of benzoyl chloride gave the corresponding *N*-benzoyl-pentabenzoates, but in smaller yields

To compare the behavior of acylhydrazones with that of arylhydrazones in regard to (a) formazan formation, (b) formation of azoethylene derivatives, and

(c) benzoylation, we have prepared a number of new acylhydrazones, including the *m*-toluoylhydrazones of D-glucose, D-galactose, D-mannose, and D-arabinose, as well as the (*m*-nitrobenzoyl)hydrazones of D-galactose and D-xylose and the nicotinoylhydrazone of D-glucose

Unlike arylhydrazones, which readily give intensely colored formazans, the acylhydrazones fail to give such a reaction, this is probably due to the enolization of acylhydrazones in the alkaline medium used in the coupling reaction The formazan reaction is known[5] to require the presence of an imino hydrogen atom (which is absent in the enol form)



$$\underset{|}{HC}=N-NHCOR \rightleftharpoons \underset{|}{HC}=N-N=\overset{\overset{\displaystyle OH}{|}}{C}-R$$

Enolization would also explain the failure of penta-*O*-acetyl-*aldehydo*-D-galactose benzoylhydrazone and of penta-*O*-acetyl-*aldehydo*-D-glucose nicotinoylhydrazone to give azoethylene derivatives on boiling with ethanolic pyridine D-Galactose benzoylhydrazone yielded, on benzoylation, a derivative formulated as *N*-benzoylpenta-*O*-benzoyl-*aldehydo*-D-galactose benzoylhydrazone

EXPERIMENTAL

Infrared spectra were measured with a Unicam SP 200 spectrophotometer Microanalytical determinations were made by A Bernhardt, Mulheim, W Germany.

N-*Acetylpenta*-O-*acetyl*-aldehydo-D-*galactose* (p-*chlorophenyl*)*hydrazone* (**1b**)

(a) D-Galactose (*p*-chlorophenyl)hydrazone (3 g) was refluxed with acetic anhydride (20 ml) for 30 min, and the solution was poured onto crushed ice The viscous residue that separated was washed repeatedly with water until it solidified Compound **1b** crystallized from methanol in needles (0 5 g), m p 125°; ν_{max}^{KBr} 1740 (OAc), 1690 (NAc), 1640 (C=N) cm^{-1}

Anal Calc for $C_{24}H_{29}ClN_2O_{11}$ C, 51 76, H, 5 25, Cl, 6 37, N, 5 03 Found C, 51 40, H, 5 16, Cl, 6 09, N, 4 88

(b) A solution of D-galactose (*p*-chlorophenyl)hydrazone (1 g) in dry pyridine (10 ml) was added, with stirring, to cold acetyl chloride (30 ml) during one h It was then poured onto crushed ice, the aqueous solution was decanted, and the residue was crystallized from methanol, 0 5 g, m p 125°, alone or mixed with the product from (a)

N-*Acetylpenta*-O-*acetyl*-aldehydo-D-*galactose phenylhydrazone*[2] (**1a**)

A solution of D-galactose phenylhydrazone (2 g) in dry pyridine (20 ml) was treated with acetyl chloride as above, and the product was crystallized from ethanol–water in needles, m p 135°, alone or mixed with an authentic specimen[2].

Penta-O-*acetyl*-aldehydo-D-*galactose* (p-*nitrophenyl*)*hydrazone*[1] (**1c**)

D-Galactose (*p*-nitrophenyl)hydrazone (2 g) was refluxed with acetic anhydride

(20 ml), or treated with acetyl chloride as for the (*p*-chlorophenyl) derivative The acetate crystallized from ethanol in yellow plates, m p 194°, alone or mixed with an authentic specimen[1] Both products had the same infrared spectrum

Arylhydrazone benzoates (*Table I*)

A suspension of the saccharide arylhydrazone (0 5 g) in dry pyridine (10 ml) was treated with benzoyl chloride (3 ml) and kept overnight at room temperature The reaction mixture was poured onto crushed ice, and the viscous residue that separated was washed repeatedly with water until it solidified The benzoates were recrystallized from benzene–methanol, unless otherwise stated

*Penta-O-acetyl-N-benzoyl-*aldehydo-D-*galactose phenylhydrazone* (**5a**)

A solution of penta-*O*-acetyl-*aldehydo*-D-galactose phenylhydrazone (2 g) in dry pyridine (15 ml) was treated with benzoyl chloride (4 ml), and the mixture was kept overnight at room temperature It was poured onto crushed ice and the viscous residue that separated washed repeatedly with water until the product solidified It was recrystallized from methanol, needles (2 g), m p 138–140°, ν_{max}^{KBr}1740 (OAc), 1675 (NBz), 1625 (C=N) cm^{-1}.

Anal Calc for $C_{29}H_{32}N_2O_{11}$ C, 59 59, H, 5 52, N, 4 79 Found C, 59 22, H, 5 74; N, 4 91

*Penta-O-acetyl-N-benzoyl-*aldehydo-D-*galactose* (p-*bromophenyl*)*hydrazone* (**5b**)

A solution of penta-*O*-acetyl-*aldehydo*-D-galactose (*p*-bromophenyl)hydrazone (2 g) was benzoylated as for the phenylhydrazone, and the product was recrystallized from methanol, needles (2 g), m p 169–171°, ν_{max}^{KBr} 1735 (OAc), 1670 (NBz), 1630 (C=N) cm^{-1}.

Anal Calc for $C_{29}H_{31}BrN_2O_{11}$ C, 52 50, H, 4 71, N, 4 22 Found C, 52 53, H, 4 91, N, 4 10

*Tetra-O-benzoyl-*D-lyxo-*hexulose phenylosazone*

D-*lyxo*-Hexulose phenylosazone (2 g) in dry pyridine (20 ml) was treated with benzoyl chloride (4 ml) After being kept overnight at room temperature, the reaction mixture was poured onto crushed ice and stirred, with frequent decantation of water After two days, the product solidified, and was recrystallized from methanol, yellow needles, m p 170–172°, ν_{max}^{KBr} 1700, 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, 71 85; H, 5 11, N, 7 66

*Tri-O-benzoyl-*D-erythro-*pentulose phenylosazone*

D-*erythro*-Pentulose phenylosazone (1 g) in dry pyridine (10 ml) was benzoylated as above with benzoyl chloride (2 ml) overnight at room temperature The tribenzoate crystallized from methanol in yellow needles, m p 148–150°, ν_{max}^{KBr} 1700 (OBz), 1600 (C=N) cm^{-1}

TABLE I

ARYLHYDRAZONE BENZOATES

Sugar	*Substituent on phenyl residue*	*m p, degrees*	*Yield, %*	*Formula*	*Calc*			*Found*			ν_{max}^{KBr}, *cm*$^{-1}$		
					C	*H*	*N*	*C*	*H*	*N*	*OBz*	*NBz*	*C=N*
D-Arabinose	*p* methyl-	179–180	90	$C_{47}H_{38}N_2O_9$	72.86	4.94	3.62	72.64	5.10	3.81	1720	1620, 1660	1600
D Galactose	*p*-methyl-	150–152	85	$C_{55}H_{44}N_2O_{11}$	72.67	4.88	3.08	73.27	5.14	3.34	1720	1625, 1670	1600
D-Galactose	*p*-chloro-	142–143	80	$C_{54}H_{41}ClN_2O_{11}$	69.79	4.45	3.01	69.94	4.89	3.41	1720	1625, 1675	1600
D-Galactose	*p*-nitro	173–176	80	$C_{54}H_{41}N_3O_{13}$	69.00	4.40	4.47	69.00	4.48	4.67	1720	1630, 1670	1600
D-Mannose	*p*-chloro-	115–117[a]	70	$C_{54}H_{41}ClN_2O_{11}$	69.79	4.45	3.01	69.48	4.65	3.18	1720	1620, 1670	1600

[a] Crystallized from methanol

TABLE II

ACYLHYDRAZONES

Hydrazone	*m p, degrees*	*Yield, %*	*Formula*	*Calc*			*Found*			ν_{max}^{KBr} *cm*$^{-1}$	
				C	*H*	*N*	*C*	*H*	*N*	*CONH*	*C=N*
D Arabinose *m* toluoyl-	187–189	90	$C_{13}H_{18}N_2O_5$	55.31	6.43	9.93	55.55	6.69	10.22	1640	1600
D Glucose *m*-toluoyl-[a]	215–218	95	$C_{14}H_{20}N_2O_6$	53.84	6.45	8.97	53.91	6.61	8.99	1645	1590
D-Galactose *m* toluoyl-	182–185	55	$C_{14}H_{20}N_2O_6$	53.84	6.45	8.97	53.45	6.91	8.80	1660	1590
D-Mannose *m*-toluoyl-	170	30	$C_{14}H_{20}N_2O_6$	53.84	6.45	8.97	53.91	6.84	9.11	1630	1600
D-Xylose *m*-nitrobenzoyl-	159–161	60	$C_{12}H_{15}N_3O_7$	46.01	4.83	13.41	46.49	5.09	13.48	1660	1615
D-Galactose *m* nitrobenzoyl-	182–185	90	$C_{13}H_{17}N_3O_8$	45.48	4.99	12.24	45.33	5.00	12.41	1655	1620
D Glucose nicotinoyl-[a]	115–117	85	$C_{12}H_{17}N_3O_6 \cdot H_2O$	45.42	6.04		45.33	6.31		1660	1600

[a] Crystallized from water–methanol

Anal. Calc. for $C_{38}H_{32}N_4O_6$: C, 71.24; H, 5.04; N, 8.75. Found: C, 71.01; H, 5.22; N, 8.81.

Acylhydrazones (*Table II*)

A solution of the saccharide (2 g) in water (4 ml) was refluxed with a solution of the acylhydrazine (1–2 g) in methanol (40 ml) for 30 min. The hydrazone that separated on cooling was collected, and crystallized from methanol unless otherwise stated.

*Penta-*O*-acetyl-*aldehydo-D-*glucose nicotinoylhydrazone*

D-Glucose nicotinoylhydrazone (2 g) in dry pyridine (20 ml) was treated with acetic anhydride (15 ml), and the mixture was kept for two days at room temperature. It was poured onto crushed ice, whereupon the product crystallized after four days. It was filtered off, washed with water, and recrystallized from methanol–water in needles, m.p. 110–113°, $\nu_{\max}^{\mathrm{KBr}}$ 1755 (OAc), 1600 (C=N) cm^{-1}.

Anal. Calc. for $C_{22}H_{27}N_3O_{11} \cdot H_2O$: C, 50.09; H, 5.54. Found: C, 49.90; H, 5.94.

N-*Benzoylpenta-*O*-benzoyl-*aldehydo-D-*galactose benzoylhydrazone*

D-Galactose benzoylhydrazone (0.5 g) in dry pyridine (10 ml) was treated with benzoyl chloride (4 ml), and the mixture was kept overnight at room temperature. It was poured onto crushed ice, and the product that separated was washed repeatedly with water until it solidified. It was recrystallized from methanol in needles (0.5 g); m.p. 205–207°; $\nu_{\max}^{\mathrm{KBr}}$ 1720 (OBz), 1645 (NBz), 1600 (C=N) cm^{-1}.

Anal. Calc. for $C_{55}H_{42}N_2O_{12}$: C, 71.58; H, 4.59; N, 3.04. Found: C, 71.91; H, 4.83; N, 3.00.

ACKNOWLEDGMENT

The authors thank Professor H. El Khadem for his interest and valuable suggestions.

SUMMARY

Acetylation of D-galactose phenyl- and (*p*-chlorophenyl)-hydrazone with boiling acetic anhydride, or with acetyl chloride in pyridine, afforded *N*-acetyl-*O*-acetyl derivatives, whereas with the (*p*-nitrophenyl)hydrazone, only the *O*-acetyl derivative was obtained. Benzoyl chloride, being a stronger acylating agent, caused the *N*-benzoylation of D-galactose (*p*-nitrophenyl)hydrazone, and, with hydrazone penta-acetates, gave the *N*-benzoylated *O*-acetylated derivatives. Some new acylhydrazones were prepared, but, because of their enolization, they failed to undergo the formazan reaction, and their acetates could not be converted into azoethylene derivatives.

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A SYNTHESIS OF 4-ACETAMIDO-4-DEOXY-L-XYLOPYRANOSE*

A. E. El-Ashmawy and D. Horton
Department of Chemistry, The Ohio State University, Columbus, Ohio 43210 (U.S.A.)

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A program of synthesis in this laboratory is concerned with 4-amino-4-deoxy sugars[1-3]. The 4-amino-4-deoxyhexoses having the D-*manno* and D-*talo* configurations have been prepared[3,4]. In an earlier report[5], the synthesis of 4-acetamido-4,5-dideoxy-L-xylose was described, and it was shown that the sugar exists in aqueous solution mainly as the anomeric furanose forms, with nitrogen as the ring hetero-atom. Concordant observations have been reported[6] for the D enantiomorph. In the case of 4,5-diacetamido-4,5-dideoxy-L-xylose, which has been reported as a syrup[6] and also in crystalline form[7], the more favorable, pyranose ring-form is possible, and this appears to be the ring form adopted in aqueous solution, nitrogen is again the hetero-atom in the ring. An acetamido sugar has been prepared by Dick and Jones[8] by treatment of methyl 2,3,4-tri-*O*-(methylsulfonyl)-α-D-xylopyranoside with sodium azide, followed by a series of transformations on the product, and it was deduced on mechanistic grounds and from data of periodate oxidation that the product was 4-acetamido-4-deoxy-L-xylose. Infrared spectral data indicated that the favored ring form of the acetamido sugar is pyranoid, with oxygen as the hetero atom in the ring. A degradative route to derivatives of this amino sugar, by way of uronic acid intermediates, has been noted[9].

The present report describes the synthesis of 4-acetamido-4-deoxy-L-xylose by a stereochemically definitive route involving C-5 to C-6 cleavage of a 2-amino-2-deoxy-D-glucitol derivative. It is shown that the sugar exists in aqueous solution as a mixture of the anomeric pyranose forms.

The starting point in the synthesis was 2-deoxy-2-(2,4-dinitroanilino)-D-glucitol (**1**), prepared[10] from 2-amino-2-deoxy-D-glucitol hydrochloride. The latter could be obtained by reduction[11] of 2-amino-2-deoxy-D-glucose hydrochloride with hydrogen over Raney nickel. Yields were only moderate when this reduction was performed on a 100-g scale. *N*-Acetylation[12] of the amino sugar, followed by reduction[11] with hydrogen over Raney nickel, gave 2-acetamido-2-deoxy-D-glucitol, which was hydrolyzed to give 2-amino-2-deoxy-D-glucitol hydrochloride, all products were obtained in crystalline form and in a high yield.

Acetonation of **1** gave the crystalline 3,4:5,6-diisopropylidene acetal (**2**), which was further characterized as its 1-*O*-(*p*-tolylsulfonyl) derivative. The positions

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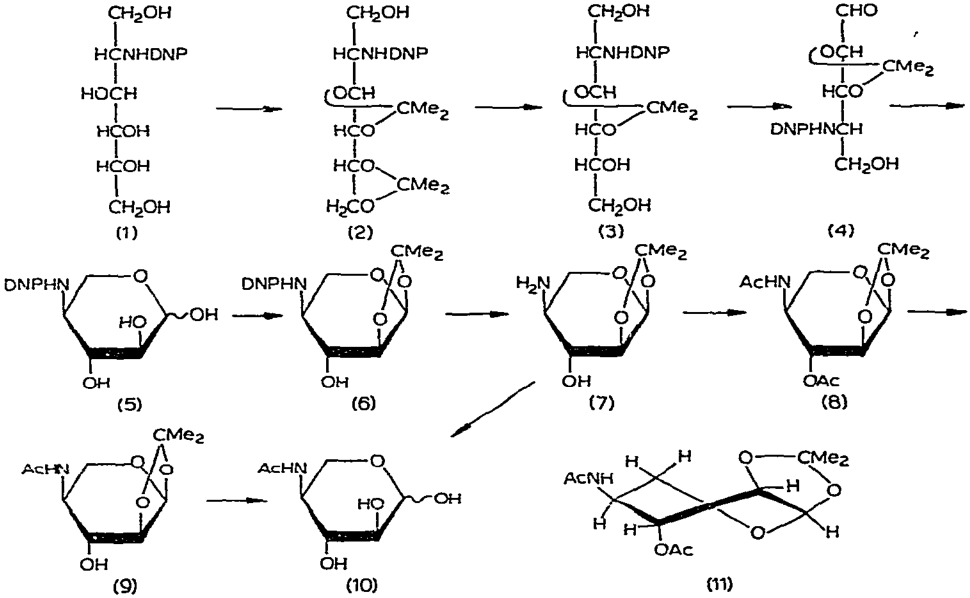
*For a preliminary communication, see ref. 1.

of the *O*-isopropylidene groups in **2** were assigned by analogy with the known 2-acetamido analog^{13} of **2**, and also with 2-acetamido-2-deoxy-3,4 5,6-di-*O*-isopropylidene-D-glucose diethyl dithioacetal$^{5\ 14\ 15}$ Subsequent conversions from **2** verified this assignment

Partial hydrolysis by acid of the diisopropylidene acetal (**2**) under mild conditions removed one *O*-isopropylidene group, and the product was shown to be 2-deoxy-2-(2,4-dinitroanilino)-3,4-*O*-isopropylidene-D-glucitol (**3**) by the fact that on periodate oxidation it consumed one mole of oxidant with the formation of a pentose derivative. Substance **3** was encountered as the orange, anhydrous form, m p 128–129°, and also as a stable, yellow, solvated form, m p 95–96° The latter was converted into the former by vigorous drying or by heating to 110°, but it could be recrystallized unchanged from chloroform Acetylation of either form gave the 1,5,6-triacetate of **3** Removal of the *N*-substituent from **3** by treatment with Dowex-1 (OH$^-$) ion-exchange resin, with subsequent acetylation, gave crystalline 2-acetamido-1,5,6-tri-*O*-acetyl-2-deoxy-3,4-*O*-isopropylidene-D-glucitol.



DNP = 2,4-dinitrophenyl

Preparative periodate oxidation of **3** (either form) gave 4-deoxy-4-(2,4-dinitroanilino)-2,3-*O*-isopropylidene-*aldehydo*-L-xylose (**4**) as a glass, which in the infrared spectrum showed absorption for the aldehyde group Cyclization of this derivative is presumably prevented by the fact that a *trans*-fused 2,3-*O*-isopropylidene group would result Hydrolysis of the *O*-isopropylidene group with aqueous acetic acid gave crystalline 4-deoxy-4-(2,4-dinitroanilino)-L-xylopyranose (**5**) The ring size of this product was indicated by the absence of carbonyl absorption in the infrared spectrum, and by the fact that acetylation with acetic anhydride–pyridine gave a syrupy triacetate which showed absorptions for the NH group in the infrared and n m r. spectra A furanose form would have given a tetraacetate having no NH group, and an acyclic form of the free sugar would have exhibited carbonyl absorption.

Acetonation of **5** gave a crystalline mono-*O*-isopropylidene derivative in high yield, which was shown to be 4-deoxy-4-(2,4-dinitroanilino)-1,2-*O*-isopropylidene-α-L-xylopyranose (**6**), acetylation gave the crystalline 3-acetate of **6** The structure assigned to **6** was based on the following facts; the substance was nonreducing; it was different from **4**; and removal of the *N*-substituent from **6** gave a nonreducing 4-amino-4-deoxypentose derivative (**7**) which consumed one mole of periodate Further concordant data were provided by the n m r spectra of some of the transformation products No further acetonation of **6** was observed when treatment with acetone was prolonged, even though a 1,2 3,5-di-*O*-isopropylidene derivative of the furanose form might be considered possible

Removal of the *N*-(2,4-dinitrophenyl) group from **6** gave crystalline 4-amino-4-deoxy-1,2-*O*-isopropylidene-α-L-xylopyranose (**7**) in good yield, the product consumed one mole of periodate rapidly (5 min) The oxidant consumption remained constant for several h, and slow overoxidation was subsequently observed

Acetylation of **7** gave crystalline 4-acetamido-3-*O*-acetyl-4-deoxy-1,2-*O*-isopropylidene-α-L-xylopyranose (**8**), and *O*-deacetylation of **8** gave the corresponding syrupy 3-hydroxy derivative (**9**) Removal of the *O*-isopropylidene group from **9** by mild, acid hydrolysis gave 4-acetamido-4-deoxy-L-xylose (**10**) as a chromatographically homogeneous, crystalline product, m p 155–157°, $[\alpha]_D$ $-53\rightarrow-49°$ (water) The acetamido sugar **10** could also be prepared directly, in one step, from 4-amino-4-deoxy-1,2-*O*-isopropylidene-α-L-xylopyranose (**7**), by treatment of **7** in aqueous solution with acetic anhydride, selective *N*-acetylation took place and the acetic acid formed by hydrolysis of the excess acetic anhydride caused cleavage of the *O*-isopropylidene group

The yields for all steps in the synthesis were at least 50%, and most were considerably higher The overall yield in the conversion of 2-amino-2-deoxy-D-glucose hydrochloride into 4-acetamido-4-deoxy-L-xylose (**10**) was about 10% when purification at some of the intermediate stages was omitted The melting point observed for the final product (**10**) is in good agreement with the value (157–158°) reported by Dick and Jones[8], but the specific rotation ($-22\rightarrow-16°$ in water) reported[8] for **10** by these authors differs from that found in the present work*

The infrared spectrum of the crystalline acetamido sugar **10** showed absorptions typical of the amide carbonyl and amide NH groups A solution of the sugar in deuterium oxide, at room temperature and at mutarotational equilibrium, showed a narrow doublet in the n m r spectrum, at τ 4 78, $J_{1,2}$ 3 c p s , assigned to the equatorial H-1

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*In a personal communication (June 20, 1965) Professor J K N Jones has stated that the i r spectra of our substance **10**, and the product of Dick and Jones[8], are identical. In a repeat preparation, Dick and Jones have observed an equilibrium specific rotation of −43 5° (*c* 0 93, water) for their product A sample provided by Professor Jones had an X-ray powder diffraction pattern identical to that of substance **10** of the α-L-pyranose anomer of **10**. The signal of the axial H-1 of the β-L anomer was not observable at room temperature because of interference by the HOD signal. The latter signal was shifted upfield[16,17] in the spectrum measured at 80° (Fig. 1), and the (axial) H-1 signal of the β-L pyranose anomer was clearly observable at τ 5.44 as a wide doublet, $J_{1,2}$ 7.6 c.p.s.; the total integral of both H-1 signals corresponded to one proton. The magnitudes of the observed $J_{1,2}$ couplings leave no doubt that the anomeric pyranoses are involved, and the fact that the chemical shifts of the H-1 signals of the anomers correspond closely to those reported[16] for the anomeric D-xylopyranoses provides further confirmation. Integration of the spectrum indicated that the α-L and β-L anomers of **10** are present in a 2:3 ratio at equilibrium.

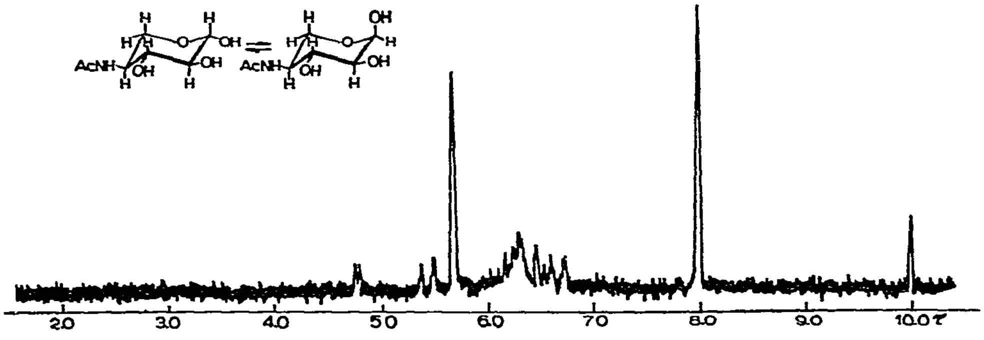

Fig. 1. The 60-Mc.p.s. n.m.r. spectrum of 4-acetamido-4-deoxy-L-xylose (**10**) at equilibrium in deuterium oxide, at 80°.

The small amount of mutarotation observed with **10**, in relation to the anomeric composition at equilibrium in aqueous solution, suggests that the crystalline product is a co-crystallized mixture of anomers (compare ref. 17). A solution of **10** in methyl sulfoxide-d_6 showed signals for H-1 of both pyranose anomers.

The n.m.r. spectrum of 4-acetamido-3-*O*-acetyl-4-deoxy-1,2-*O*-isopropylidene-α-L-xylopyranose (**8**), when measured in chloroform-*d*, showed (Fig. 2) a broad, one-proton signal at low field (τ 3.60), which was assigned to the NH proton, since it disappeared on deuteration. This observation provided independent evidence that compounds **6**, **7**, **8**, and **9** do not have the nitrogen atom in the ring. A two-proton multiplet at τ 4.91 in the spectrum of **8** (in chloroform-*d*) was assigned to H-1 and H-3, the two protons most strongly deshielded of those attached to carbon. The overlap of these two signals made analysis difficult, but the signals were well separated when the spectrum was measured in benzene (Fig. 2). A sharp doublet at τ 4.96 was assigned to H-1 ($J_{1,2}$ 2.5 c.p.s.), and the narrow signal at τ 4.72 (total width 10 c.p.s.) was assigned to H-3. The fact that the H-3 signal was not observed as a wide (18–20 c.p.s.), symmetrical triplet indicated that H-2, H-3, and H-4 are not *trans*-diaxial [**8** in the *1C* (L) conformation], and that the observed data accord with the formulation of **8** in a skew conformation (**11**). It has been proposed[18,19] that 1,2-*O*-alkylidene acetals of arabinopyranose, glucopyranose, and related derivatives adopt a skew form as the favored conformation.

The 2,4-dinitroanilino derivatives studied in this work all showed signals at low field for the three aryl protons and the NH proton. None of the signals disappeared when the samples were deuterated in the usual way, even during several days, a fact which indicates that the NH proton could not be exchanged by deuterium oxide alone. However, the signal of the NH proton could be assigned definitively by adding a small amount of tributylamine to the prepared sample in chloroform-*d* containing deuterium

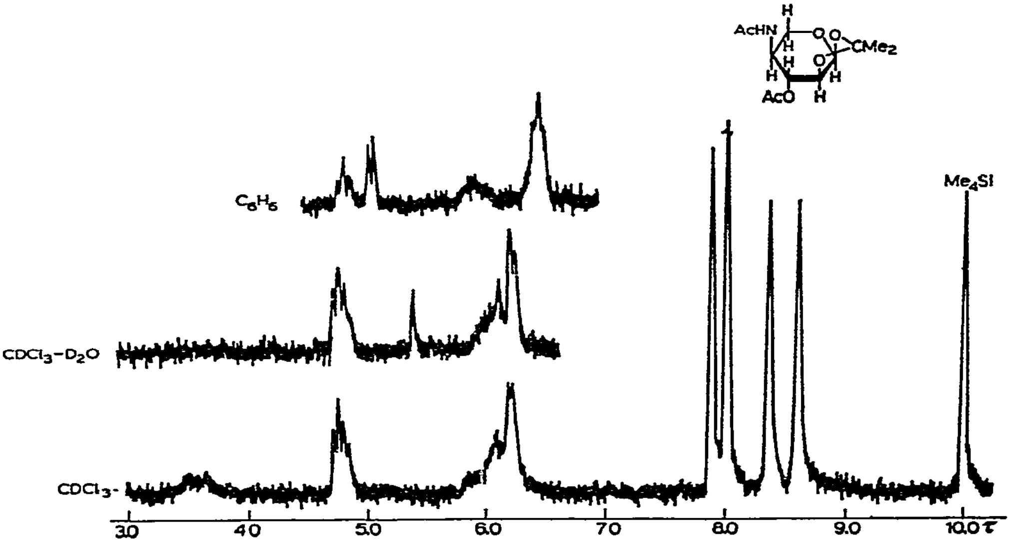

Fig. 2. The 60-Mc.p.s. n.m.r. spectrum of 4-acetamido-3-*O*-acetyl-4-deoxy-1,2-*O*-isopropylidene-α-L-xylopyranose (8).

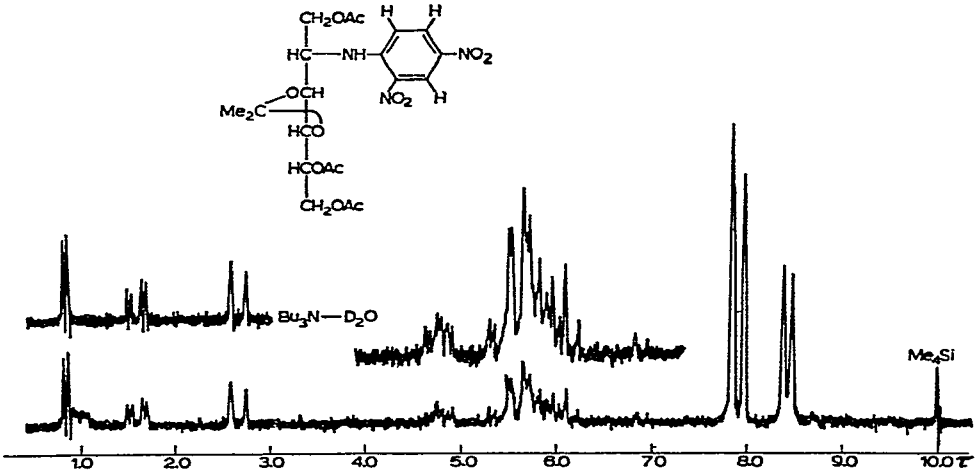

Fig. 3. The 60-Mc.p.s. n.m.r. spectrum of 1,5,6-tri-*O*-acetyl-2-deoxy-2-(2,4-dinitroanilino)-D-glucitol in chloroform-*d*, and in chloroform-*d* containing tributylamine in deuterium oxide.

oxide; this caused immediate exchange of the NH proton A typical example is provided by the spectrum of 1,5,6-tri-*O*-acetyl-2-deoxy-2-(2,4-dinitroanilino)-3,4-*O*-isopropylidene-D-glucitol, which is shown (Fig. 3) before and after addition of tributylamine in deuterium oxide The broadened doublet at τ 1 01, which disappears on treatment with base, may clearly be assigned to the NH proton The narrow doublet at lowest field (τ 0 85), the doublet of doublets (τ 1.60), and the wide doublet (τ 2 68) can thus be assigned unambiguously to H-3, H-5, and H-6, respectively, of the 2,4-dinitrophenyl group. In a number of 2,4-dinitroanilino derivatives of sugars[20,21], the signal of the NH group appears as a wide doublet, and unambiguous differentiation of this signal from the signal of H-6 of the aryl residue cannot be made without exchange of the NH proton Exchange experiments have now shown that the tentative assignments made for the aryl H-6 and the NH proton, of the anomeric 2-deoxy-2-(2,4-dinitroanilino)-D-glucopyranose tetraacetates[20] and the derived 1-bromide[21], must be reversed. N m r data on *N*-methyl-2,4-dinitroaniline[22] and *N*-(2,4-dinitrophenyl)serine derivatives[23] are in agreement with the present work

Further details of n.m r measurements and assignments, for a number of compounds in this work, are given in the experimental section

EXPERIMENTAL

General

Melting points were determined with a Thomas–Hoover "Unimelt" apparatus (Arthur H Thomas Co , Philadelphia, Pennsylvania) Specific rotations were determined in a 2-dm polarimeter tube Infrared spectra were measured with a Perkin–Elmer "Infracord" infrared spectrometer N m r spectra were measured with a Varian A-60 spectrometer equipped with a Varian V-6040 variable-temperature probe Tetramethylsilane (τ 10 00) was used as the internal standard, except for spectra measured in deuterium oxide, for which the internal standard was sodium 4,4-dimethyl-4-silapentane-1-sulfonate (τ 10 00) The first-order coupling constants recorded are the measured peak spacings and are considered accurate to $\pm$ 0 5 c p s Unless otherwise stated, the spectra were measured at about 40° Deuteration was performed by adding one drop of deuterium oxide to the prepared sample Microanalytical determinations were made by W. N. Rond X-ray powder diffraction data give interplanar spacings, Å, for CuKα radiation The camera diameter was 114 59 mm Relative intensities were estimated visually s, strong, m, moderate; w, weak, v, very. The strongest lines are numbered (1, strongest), double numbers indicate approximately equal intensities Thin-layer chromatography was performed on Silica Gel G (E Merck, Darmstadt, Germany) activated at 110°, with sulfuric acid as the indicator Unless otherwise stated, ethyl acetate was used as the developer

Preparation of 2-acetamido-2-deoxy-D-glucitol[11]

2-Amino-2-deoxy-D-glucose hydrochloride (100 g) was *N*-acetylated[12] and the resultant, crystalline 2-acetamido-2-deoxy-α-D-glucose (95–100 g), dissolved in 20%

aqueous ethanol (1 l), was shaken in a 2-l autoclave with Raney nickel* (40 g) for 36 h at 100° under an atmosphere of hydrogen at 1000 lb in^{-2} pressure The catalyst was filtered from the cooled suspension, the filtrate was concentrated, and the product, which crystallized on standing, was filtered off and washed with a little ethanol, yield 92 g (91%), m p 150–151°, $[\alpha]_D^{21}$ $-9\pm1°$ (*c* 1.3, water) [lit [11] m p 153°, $[\alpha]_D^{21}$ $-11°$ (water), λ_{max}^{KBr} 3 00 (OH, NH), 6 05, 6 30 μ (NHAc), no absorption in the range 11 7–12 1 μ, X-ray powder diffraction data 16 06 m, 10 16 m, 8 93 vw, 6 70 vs (1,1), 4 92 vs (1,1), 4 69 m, 4 41 m, 4 27 m, 4 06 s (2,2), 3 91 s (2,2), 3 46 s (3).

Preparation of 2-deoxy-2-(2,4-dinitroanilino)-D-glucitol[10] (1)

2-Acetamido-2-deoxy-D-glucitol (30 g) was heated with 6N hydrochloric acid (400 ml) for 1 h at 100°, the solution was evaporated (codistillation with propyl alcohol), and the crystalline residue was recrystallized from methanol–ether to give 2-amino-2-deoxy-D-glucitol hydrochloride, yield 25 g (85%), m p 160–162° (lit [11] 160–161°), $[\alpha]_D^{23}$ $-2\ 7\pm0\ 6°$ (*c* 1 8, water), ninhydrin-positive, λ_{max}^{KBr} 3 0 (OH), 3 30, 4 90, 6 18 μ (NH_3^+); X-ray powder diffraction data 7 47 m, 5 64 m, 5 29 m, 4 53 w, 4.33 vs (1,1), 4 15 vs (1,1), 3 74 vs (1,1), 3 63 vs (1,1), 3 44 w, 3 35 m, 3 26 s (2), 3 13 m, 3 00 m

To a solution of the foregoing product (20 g) in 50% aqueous ethanol (240 ml) was added sodium hydrogen carbonate (15 4 g) and 1-fluoro-2,4-dinitrobenzene (17 0 g) and the mixture was stirred for 18 h at room temperature. The yellow solid which separated was filtered off, washed with small amounts of water and ethanol, and then washed thoroughly with ether, yield 22 g (69%) Recrystallization from methanol gave **1** as fine yellow needles, m p 162–163° (lit [10] 163–164°), $[\alpha]_D^{24}$ $+95°$ (*c* 1, methanol), R_F 0 12, λ_{max}^{KBr} 3 0 (NH, OH), 6 14, 6 30, 6 70 (aryl C=C), 7 44 (NO_2), 13 45 μ (substituted benzene), X-ray powder diffraction data 9 11 w, 8 19 m, 7 19 m, 6 23 w, 5 40 vw, 4 74 s (2,2), 4 62 m, 4 21 s (2,2), 3 98 m, 3 83 s (2,2), 3 61 vw, 3 38 vs (1)

2-Deoxy-2-(2,4-dinitroanilino)-3,4 5,6-di-O-isopropylidene-D-glucitol (2)

A solution of **1** (11 g) in dry acetone (200 ml) was shaken with concentrated sulfuric acid (2 ml) and anhydrous cupric sulfate (15 g) for 21 h at room temperature The mixture was filtered, the filtrate was poured into an excess of aqueous sodium hydrogen carbonate, the acetone was evaporated, and the resultant solution was extracted with 3 100-ml portions of chloroform The combined extracts were washed with water, dried (sodium sulfate), and evaporated, and the crystalline residue was recrystallized from ether; yield 8 5 g (63%), m p 164–165°, $[\alpha]_D^{20}$ $+103\pm1°$ (*c* 0 7, chloroform); R_F 0.9, λ_{max}^{KBr} 2 91 (OH), 3 08 (NH), 6 20, 6 32, 6 70 (aryl C=C), 7 30 (CMe_2), 7 55 (NO_2), 13 48, 13.80 μ (substituted benzene), n m r data (chloroform-*d*), τ 0 85 (1-proton doublet, $J_{3',5'}$ 2 8 c p s , H-3′), τ 0 90 (1-proton broadened doublet, unchanged on deuteration, disappears after addition of 0 01 ml of tributylamine, NH),

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* Raney nickel catalyst, no 28, Raney Catalyst Division of the W R Grace Co , Chattanooga, Tennessee

τ 1.72 (1-proton quartet, $J_{5',6'}$ 9.5 c.p.s., H-5'), τ 2.79 (1-proton doublet, H-6'), τ 5.48–6.52 (8-proton multiplet, H-1,2,3,4,5,6), τ 7.39 (1-proton triplet, J 6 c.p.s., disappears on deuteration, OH), τ 8.48, 8.57, 8.65, 8.68 (3-proton singlets, CMe_2), X-ray powder diffraction data: 8.84 m, 7.86 w, 6.88 vs (1), 6.60 vw, 6.02 m, 5.60 s (2), 5.14 w, 4.72 m, 4.15 m, 3.97 m, 3.86 m, 3.76 m, 3.59 w.

Anal. Calc. for $C_{18}H_{25}N_3O_9$: C, 50.58; H, 5.85; N, 9.83. Found: C, 50.88; H, 5.73; N, 9.89.

2-Deoxy-2-(2,4-dinitroanilino)-3,4:5,6-di-O-isopropylidene-1-O-(p-tolylsulfonyl)-D-glucitol

A solution of **2** (0.6 g) in pyridine (5 ml) was treated at 0° with *p*-toluenesulfonyl chloride (0.38 g), and, after 24 h at 25°, the mixture was poured into water to yield a solid. Recrystallization from ethanol gave very fine, yellow crystals, yield 0.6 g (75%), m.p. 161–162°, $[\alpha]_D^{25}$ $+60\pm1°$ (*c* 1.2, chloroform), R_F 0.88 (9:1 benzene–methanol); λ_{max}^{KBr} 3.03 (NH), 6.18, 6.28, 6.67 (aryl C=C), 8.50 (sulfonate), 13.43, 13.70 μ (substituted benzene).

Anal. Calc. for $C_{25}H_{31}N_3O_{11}S$: C, 51.63; H, 5.33; N, 7.23; S, 5.50. Found: C, 51.56; H, 5.45; N, 7.55; S, 5.45.

2-Deoxy-2-(2,4-dinitroanilino)-3,4-O-isopropylidene-D-glucitol (3)

To a solution of **2** (2.0 g) in methanol (95 ml) was added N aqueous hydrochloric acid (25 ml), and the mixture was kept for 5 h at 25°. An excess of lead carbonate was added and the mixture was filtered. The filtrate was concentrated until most of the methanol had been removed, water was added to a total volume of 75 ml, and the small amount of solid starting-material which separated was removed by filtration. The filtrate was extracted with 3 75-ml portions of chloroform, and the aqueous phase, which contained a small amount of **1**, was discarded. The dried (magnesium sulfate) chloroform extract was concentrated to 30 ml, and the yellow solid (solvate of **3**) which separated on standing was filtered off, yield 0.1 g. The remaining chloroform solution was evaporated and the residual syrup was crystallized from ether to give solvent-free **3** as orange granules, yield 0.85 g (47%, total yield of both forms 53%), m.p. 128–129°, $[\alpha]_D^{25}$ $+78\pm2°$ (*c* 1, acetone), R_F 0.22, λ_{max}^{KBr} 3.00 (OH, NH), 6.18, 6.30, 6.60 (aryl C=C), 7.20 (CMe_2), 7.50 (NO_2), 13.48 μ (substituted benzene); n.m.r. data (acetone-d_6) τ 0.98 (1-proton doublet, $J_{3',5'}$ 2.7 c.p.s., H-3'), τ 1.00 (1-proton broad doublet, NH), τ 1.82 (1-proton quartet, H-5'), τ 2.71 (1-proton doublet, $J_{5',6'}$ 9.5 c.p.s., H-6'), τ 8.51, 8.60 (3-proton singlets, CMe_2), X-ray powder diffraction data: 11.26 m, 9.99 w, 8.42 vw, 6.86 vs (2), 6.15 vw, 5.86 vw, 5.54 vs (1), 4.79 m, 4.48 m, 4.07 m, 3.78 m, 3.53 vw, 3.37 s (3), 3.27 s.

Anal. Calc. for $C_{15}H_{21}N_3O_9$: C, 46.51; H, 5.42; N, 10.85. Found: C, 46.30; H, 5.49; N, 11.06.

Periodate oxidation of **3**, at 25° with a 5-molar excess of oxidant, showed a consumption of oxidant (Fleury–Lange method[24]) of 1.00 mole/mole after 5 min, 1.05 moles/mole after 24 h.

The preparation was repeated 20 times, and although the total yield of both forms was approximately the same each time, the proportion of the solvated form was frequently much higher than that described, and the ratio of the two forms appeared to depend on minor variations in experimental procedure. Recrystallization of the solvate from chloroform gave fine, yellow needles, m.p. 95–96° (with effervescence, solidifying at higher temperature and remelting at 126–127°); R_F 0.22; X-ray powder diffraction data: 12.27 m, 9.40 w, 8.11 w, 6.81 m, 6.32 w, 5.90 w, 5.57 s (2), 5.12 vs (1).

Anal. Calc. for $(C_{15}H_{21}N_3O_9)_3 \cdot CHCl_3$: C, 43.12, H, 5.03, N, 9.84. Found: C, 42.72; H, 4.92; N, 10.34.

A sample of the solvate, kept for 2 h at 110° over phosphoric oxide, lost 10% of its weight (calc. 9.4%) and gave the solvent-free form, m.p. and mixed m.p. 126–127°.

Anal. Calc. for $C_{15}H_{21}N_3O_9$: C, 46.51, H, 5.42; N, 10.85. Found: C, 46.33, H, 5.27, N, 11.11.

The solvate showed the same molar uptake of periodate as the non-solvated form, and the n.m.r. spectra of the two forms were identical except for the fact that the solvate showed an additional signal at τ 2.80 (singlet, 1/3 proton, $CHCl_3$). The i.r. spectra (KBr disc) of the two forms were very similar, but were not completely superposable.

2-Acetamido-1,5,6-tri-O-acetyl-2-deoxy-3,4-O-isopropylidene-D-glucitol

A solution of **3** (8 g) in acetone (280 ml) and water (120 ml) was passed slowly through a column (35×3.5 cm) of Dowex-1 (OH^-) ion-exchange resin which had been pre-washed with 7:3 (v/v) acetone–water. The column was washed with 7:3 (v/v) acetone–water until the effluent gave a negative ninhydrin reaction, and the combined effluent was evaporated. The dried residual syrup (4.2 g) was dissolved in pyridine (25 ml), acetic anhydride (45 ml) was added, and the solution was kept for 18 h at room temperature. The solution was poured into ice and water (200 ml), and the mixture was extracted with 3 75-ml portions of chloroform. The extract was washed successively at 0° with dilute sulfuric acid, aqueous sodium hydrogen carbonate, and water, the dried (sodium sulfate) solution was evaporated, and the residue was crystallized from ether, yield 2.21 g, m.p. 93–94°, $[\alpha]_D^{25}$ +31±1° (*c* 1.7, chloroform), R_F 0.42, $\lambda_{\max}^{KBr}$ 3.08 (NH), 5.73 (OAc), 6.08, 6.53 (NHAc), 7.28 μ (CMe_2); n.m.r. data (chloroform-*d*) τ 3.89 (1-proton doublet, *J* 8.5 c.p.s., NH), τ 4.80 (1-proton octet, width 16 c.p.s., H-5), τ 5.30–6.30 (7-proton multiplet, H-1,2,3,4,6), τ 7.89, 7.95 (singlets, 3 and 9 protons, acetyls), τ 8.59, 8.61 (3-proton singlets, CMe_2), X-ray powder diffraction data: 7.69 s (1), 6.97 w, 5.71 m, 5.43 m, 4.64 m, 4.25 s (2), 3.81 m.

Anal. Calc. for $C_{17}H_{27}NO_9$: C, 52.44, H, 6.94, N, 3.60. Found: C, 52.52; H, 6.89; N, 3.84.

1,5,6-Tri-O-acetyl-2-deoxy-2-(2,4-dinitroanilino)-3,4-O-isopropylidene-D-glucitol

A solution of **3** (430 mg) in pyridine (4 ml) was treated with acetic anhydride (2.5 ml) at room temperature, and after 24 h, the solution was poured into water.

The product was extracted with dichloromethane and processed in the usual way, to give the triacetate of **3** as a yellow glass, yield 435 mg (76%); R_F 0 54 (1 3 ethyl acetate–benzene), λ_{max}^{film} 3 02 (NH), 5 77 (OAc), 6 20, 6 30, 6 60 (aryl C=C), 7.34 (CMe_2), 13.25 μ (substituted benzene); n m r data in chloroform-*d* (See Fig 3): τ 0 85 (1-proton doublet, $J_{3',5'}$ 2 7 c p s , H-3′), τ 1 01 (1 proton, broad, NH), τ 1 60 (1-proton quartet, H-5′), τ 2 68 (1-proton doublet, $J_{5',6'}$ 9 5 c p s , H-6′), τ 4 78 (1-proton octet, width 16.5 c p s., H-5), τ 5 26-6 30 (7-proton multiplet, H-1,2,3,4,6), τ 7.85, 7 87, 7 98 (3-proton singlets, acetyls), τ 8 40, 8 50 (3-proton singlets, CMe_2)

Anal Calc for $C_{21}H_{27}N_3O_{12}$ C, 49 12, H, 5 62, N, 8 19 Found C, 48 66; H, 5 89, N, 8.16.

The signal at τ 1 01 was not affected by deuteration, even after 57 h at room temperature, but the signal disappeared (Fig 3) rapidly when 0 01 ml of tributylamine was added to the prepared sample, and a 1-proton singlet appeared at τ 5 31 (HOD)

Acetylation of the yellow solvate of **3** gave a product whose n m r spectrum was identical to that recorded above

*4-Deoxy-4-(2,4-dinitroanilino)-2,3-O-isopropylidene-*aldehydo-L-*xylose* (**4**)

A solution of sodium metaperiodate (1.81 g, 1 1 molar equiv) in water (30 ml) was added to a solution of **3** (3 0 g) in 1 4 ethanol–water (75 ml) The mixture was kept for 25 min in the dark at room temperature, and was then evaporated at 30° Anhydrous sodium sulfate (~3 g) was added to the residue, and the solid mixture was extracted repeatedly with ether Evaporation of the extract gave **4** as a yellow glass, yield 2 4 g (87%), R_F 0 40, λ_{max}^{film} 2 95 (OH), 3 05 (NH), 3 50, 5 80 (CHO), 6 20, 6 30, 6 65 (aryl C=C), 7 33 (CMe_2), 7 55 (NO_2), 12 25, 13 20 μ (substituted benzene)

Anal Calc for $C_{14}H_{17}N_3O_8$ C, 47 32, H, 4 79, N, 11 83 Found C, 47 18; H, 5 46, N, 11 77.

*4-Deoxy-4-(2,4-dinitroanilino)-*L-*xylopyranose* (**5**)

A solution of **4** (3 29 g) in 50% aqueous acetic acid (16 ml) was heated for 2 5 h at 95°, and then evaporated Addition of ethanol to the residual syrup gave the crystalline product, yield 2 54 g (84%), m p 194–195° (dec), $[\alpha]_D^{23}$ $+37\pm1°$ (*c* 1, acetone), R_F 0 15, R_F 0 69 (papergram, 40 11 19 butyl alcohol–ethanol–water); λ_{max}^{KBr} 2 9 (OH), 3 05 (NH), 6 20, 6 30, 6 60 (aryl C=C), 7 52 (NO_2), 13 50, 14 0 μ (substituted benzene); X-ray powder diffraction data 10 46 m, 6 02 m, 5 08 vs (1), 4 44 m, 4 06 m, 3 44 s (3), 3 20 s (2), 3 06 vw, 2 91 vw

Anal Calc for $C_{11}H_{13}N_3O_8$ C, 41 90, H, 4 21, N, 13 33 Found C, 41 42, H, 4 38, N, 13 59

*1,2,3-Tri-O-acetyl-4-deoxy-(2,4-dinitroanilino)-*L-*xylopyranose*

To a solution of **5** (0 5 g) in pyridine (6 ml) was added acetic anhydride (3 ml) The mixture was kept for 24 h at room temperature, and then processed in the usual way to give the product as a yellow, distillable glass, yield 0 35 g (50%), R_F 0 60 and 0 70 (1 1 ethyl acetate–benzene), λ_{max}^{film} 3 05 (NH), 5 70 (OAc), 6 15, 6 30, 6 60 (aryl

C=C), 13.40 μ (substituted benzene), n.m.r. data (chloroform-*d*) $\tau \sim 0.88$, 1.0-1.2, 1.68, 2.78 (multiplets, 4 protons, H-3′,5′,6′, and NH, of anomers), τ 7.77, 7.81, 7.91, 7.95 (singlets, 9 protons, acetyls).

Anal. Calc. for $C_{17}H_{19}N_3O_{11}$ N, 9.52. Found N, 9.74.

4-Deoxy-4-(2,4-dinitroanilino)-1,2-O-isopropylidene-α-L-xylopyranose (**6**)

A solution of **5** (1.84 g) in acetone (400 ml) was shaken with anhydrous cupric sulfate (5 g) and conc. sulfuric acid (0.5 ml) for 1 day at room temperature. The mixture was filtered, the filtrate was neutralized with aqueous sodium hydrogen carbonate, the acetone was evaporated, and the product was extracted with 3 75-ml portions of ethyl acetate. The extract was washed with water, dried (sodium sulfate), and evaporated, and the crystalline residue was recrystallized from ethyl acetate, yield 1.64 g (79%), m.p. 220–222° (dec.), $[\alpha]_D^{25}$ $+175 \pm 2°$ (*c* 1.1, acetone), R_F 0.70, λ_{max}^{KBr} 3.00 (OH, NH), 6.20, 6.32, 6.62 (aryl (C=C), 7.22, 7.30 (CMe$_2$), 7.56 (NO$_2$), 13.42, 14.10 μ (substituted benzene), n.m.r. data (pyridine) τ 4.39 (1-proton doublet, $J_{1,2}$ 2 c.p.s., H-1), τ 5.24-6.13 (5-proton multiplet, H-2,3,4,5), τ 8.23, 8.60 (3-proton singlets, CMe$_2$), X-ray powder diffraction data 13.29 w, 8.97 w, 7.86 w, 6.70 m, 5.57 vs (1), 5.15 s (2), 4.79 s (3,3), 4.53 s (3,3), 4.27 s (3,3), 4.09 s (3,3), 3.77 s (3,3), 3.63 s (3,3).

Anal. Calc. for $C_{14}H_{17}N_3O_8$ C, 47.32, H, 4.82, N, 11.83. Found C, 47.55, H, 5.09, N, 12.23.

Only one product could be detected by t.l.c. in the above preparation, and no difference in the yield of **6** was observed when the time of reaction was extended to 4 days.

3-O-Acetyl-4-deoxy-4-(2,4-dinitroanilino)-1,2-O-isopropylidene-α-L-xylopyranose

A solution of **6** (175 mg) in pyridine (2 ml) and acetic anhydride (3 ml) was kept for 1 day at room temperature, and then poured into ice and water. The precipitated solid was filtered off, washed with water, dried, and recrystallized from methanol, yield 130 mg (66%), m.p. 171–172°, $[\alpha]_D^{21}$ $+190 \pm 2°$ (*c* 1, chloroform), R_F 0.84; λ_{max}^{KBr} 3.02 (NH), 5.77 (OAc), 6.18, 6.30, 6.60 (aryl C=C), 7.30 (CMe$_2$), 13.45, 14.10 μ (substituted benzene), n.m.r. data (chloroform-*d*) τ 0.84 (1-proton doublet, $J_{3',5'}$ 2.7 c.p.s., H-3′), τ 0.88 (1-proton, broad, disappears on addition of tributylamine in deuterium oxide, NH), τ 1.66 (1-proton quartet, H-5′), τ 2.68 (1-proton doublet, $J_{5',6'}$ 9.5 c.p.s., H-6′), τ 4.69 (2-proton multiplet, width 10 c.p.s., H-1, H-3), τ 5.92-6.38 (4-proton multiplet, H-2,4,5), τ 7.78 (3-proton singlet, OAc), τ 8.33, 8.62 (3-proton singlets, CMe$_2$).

Anal. Calc. for $C_{16}H_{19}N_3O_9$ N, 10.58. Found N, 10.72.

4-Amino-4-deoxy-1,2-O-isopropylidene-α-L-xylopyranose (**7**)

A solution of **6** (0.72 g) in acetone (60 ml) and water (20 ml) was stirred with Dowex-1 (OH$^-$) ion-exchange resin, added in small portions at 45–50° until the solution became colorless. The mixture was filtered, and the resin was washed with hot methanol (500 ml). The filtrate and washings were evaporated to a colorless syrup which crystallized from ether, yield 0 247 g (65%), m p 131–132°, $[\alpha]_D^{24}$ +32 5±1° (*c* 1, methanol); λ_{max}^{KBr} 3 4, 6 3 (NH), 7 25 μ (CMe_2), X-ray powder diffraction data· 7.97 vs (2), 6 23 w, 5.79 vw, 5 27 vs (1), 5 01 m, 4.77 m, 4 39 s (3), 4 00 m, 3 64 m, 3.14 vw, 3 01 vw, 2 85 m.

Anal. Calc for $C_8H_{15}NO_4$· C, 50 80, H, 7 93; N, 7 40 Found· C, 50 62, H, 7 73, N, 7 67

Periodate oxidation of **7** (40 mg) at 25° in water (20 ml) containing a 5-molar excess of oxidant showed consumption of oxidant (Fleury–Lange method[24]) as follows (time and moles per mole of oxidant consumed given) 5 min, 1 03, 1 h, 1 05, 2 h, 1 07, 4 h, 1 14, 8 h, 1.28, 22 h, 1 51; 46 h, 1 75; 70 h, 1 82

4-Acetamido-3-O-acetyl-4-deoxy-1,2-O-isopropylidene-α-L-xylopyranose (8)

A solution of **7** (590 mg) in pyridine (5 ml) and acetic anhydride (5 ml) was kept for 1 day at room temperature, and then poured into ice and water. The product was extracted with 3 60-ml portions of dichloromethane, the extract was washed with aqueous sodium hydrogen carbonate, dried (sodium sulfate), and evaporated (codistillation with toluene) The resulting syrup crystallized on storage, and recrystallization from chloroform gave **8** as fine needles, yield 596 mg (70%), m p 109–110°, $[\alpha]_D^{22}$ +68±1° (*c* 1, chloroform), R_F 0 40, λ_{max}^{KBr} 3 04 (NH), 5 73 (OAc), 6 08, 6 52 (NHAc), 7 22 μ (CMe_2), n m r data in chloroform-*d* (see Fig 2) τ 3 60 (1-proton broad doublet, *J* 8 c p s , disappears on deuteration, NH), τ 4 91 (2-proton multiplet, H-1,3), τ 5 85–6.50 (4-proton multiplet, H-2,4,5), τ 7 90, 8 03 (3-proton singlets, acetyls), τ 8 38, 8.63 (3-proton singlets, CMe_2), in benzene (see Fig 2) τ 4 72 (1-proton multiplet, width 10 c p s , H-3), τ 4 96 (1-proton doublet, $J_{1,2}$ 2 5 c p s , H-1), τ 5 70–5 95 (1-proton multiplet) and τ 6 20–6.50 (3-proton multiplet) (H-2,4,5), τ 8 33, 8.37 (3-proton singlets, acetyls), τ 8 51, 8 82 (3-proton singlets, CMe_2); X-ray powder diffraction data 8 88 m, 7 47 vs (3), 6.23 m, 5 54 vs (2), 5.15 s, 4.72 m, 4 37 vs (1), 4 08 m, 3 92 m, 3 58 m, 3 43 w, 3 18 m, 2 98 m.

Anal. Calc for $C_{12}H_{19}NO_6$ C, 52 74, H, 6 96, N, 5.12 Found. C, 52 79, H, 6 86, N, 5 05

4-Acetamido-4-deoxy-1,2-O-isopropylidene-α-L-xylopyranose (9)

A solution of **8** (0 5 g) in anhydrous methanol (10 ml) was treated with a very small piece of metallic sodium, and after 2 h at room temperature, the solution was neutralized with Amberlite IR-120 (H^+) ion-exchange resin Evaporation of the solution gave **9** as a colorless syrup, yield 0 36 g (85%), R_F 0 15; λ_{max}^{film} 2 85–3 10 (OH, NH), 6 10, 6 50 (NHAc), 7 30 (CMe_2)

4-Acetamido-4-deoxy-L-xylopyranose (10)

(*a*) *From 4-acetamido-4-deoxy-1,2-O-isopropylidene-α-L-xylopyranose* (**9**). A solution of **9** (350 mg) in water (10 ml) was stirred with Amberlite IR-120 (H^+) ion-exchange resin (1 5 g) for 2 5 h at 60°. The resin was filtered, and washed with methanol, and the filtrate was evaporated to a chromatographically homogeneous syrup which crystallized after trituration with ethanol and ethyl acetate, yield 152 mg (53%) After recrystallization from methanol–ether, the product had m p 155–157°, $[\alpha]_D^{24}$ −53→ −49° (*c* 3 3, water), $R_{Rhamnose}$ 0 85 (papergram, 3.1·1 butyl alcohol–ethanol–water); λ_{max}^{KBr} 3 00 broad (NH, OH), 6 18, 6 44 μ (NHAc), X-ray powder diffraction data 9 30 m, 7 86 vw, 6 02 s (3,3), 5 71 vs (2), 5 09 w, 4 67 vs (1), 4 33 s (3,3), 4 03 s (3,3), 3 80 m, 3 64 m, 3 37 vw, 3 18 m, 3 05 m, 2 93 s

Anal Calc. for $C_7H_{13}NO_5$. C, 43 97, H, 6 85, N, 7 32 Found C, 43 67, H, 6 91; N, 7.69

For this compound, prepared by a different route, Dick and Jones reported[8] m p. 157–158°, $[\alpha]_D$ −22→−16° (*c* 1, water) and $R_{Rhamnose}$ 0.81 (papergram, 3 1:1 butyl alcohol–ethanol–water); a revised value of $[\alpha]_D$ − 43 5° (equil , *c* 0 93, water) was subsequently reported (see footnote p 193)

(*b*) *From 4-amino-4-deoxy-1,2-O-isopropylidene-α-L-xylopyranose* (7) To a solution of 7 (200 mg) in methanol (1 ml) and water (4 ml) was added acetic anhydride (1 ml), and the mixture was kept for 2 h at room temperature Examination by t l c (1 2 isopropyl alcohol–benzene) revealed that the starting material **7** (R_F 0 17) was completely converted into **9** (R_F 0 72) Water (2 ml) was added and the solution was heated for 3 h at 90°, by which time conversion of **9** into **10** (R_F 0 07) was complete Evaporation of the solution (codistillation with toluene), and crystallization of the residue from ethanol–ether gave **10**, yield 120 mg (60%), m p 155–157°, identical by mixed m p and i r spectrum with the product prepared by procedure (*a*)

N m r spectrum of 4-acetamido-4-deoxy-L-xylose

The n m r. spectrum of **10** (Fig 1), measured at 80° in deuterium oxide with an equilibrated solution gave the following data τ 4 78 (doublet, $J_{1\,2}$ 3 0 c p s , H-1 of α-L anomer) and τ 5 44 (doublet, $J_{1,2}$ 7 6 c p s., H-1 of β-L anomer) (total integral, 1 proton, relative intensities 2 3), τ 5 89-6 28 (5-proton multiplet, H-2,3,4,5), τ 7 99 (3-proton singlet, NAc) The signal at τ 5 44 was partially obscured by the HOD signal when the spectrum was measured at 40°.

A spectrum of **10**, measured 2 min after dissolution in deuterium oxide. showed the signal at τ 4 78 The relative intensity of the latter was not appreciably different from its intensity in the spectrum of the equilibrated solution The spectrum of **10**, measured 30 min after dissolution in methyl sulfoxide-d_6, showed signals at τ 4 79 (H-1 of α-L pyranose anomer) and τ 5 46 (H-1 of β-L pyranose anomer), a singlet at τ 8 00 (NAc), and a broad signal, $\tau \sim 4$ 7 which disappeared on deuteration (NH) The spectrum of the deuterated sample, measured at 80°, showed the H-1 signals as sharp doublets at τ 4 79 ($J_{1,2}$ 2 8 c p s) and τ 5 46 ($J_{1,2}$ 7 2 c p s), total integral 1 proton, in approximately 2 3 ratio. The H-2,3,4,5 signals were observed as a 5-proton multiplet, τ 5 95-6 86

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SUMMARY

Acetonation of 2-deoxy-2-(2,4-dinitroanilino)-D-glucitol (**1**) gave the 3,4 5,6-diisopropylidene acetal (**2**), which was converted by mild, acid hydrolysis into the 3,4-monoisopropylidene acetal (**3**) Periodate oxidation of **3**, followed by mild hydrolysis of the oxidation product (**4**) gave 4-deoxy-4-(2,4-dinitroanilino)-L-xylopyranose (**5**), which underwent condensation with acetone to give the 1,2-*O*-isopropylidene derivative (**6**) Removal of the *N*-substituent from **6** gave 4-amino-4-deoxy-1,2-*O*-isopropylidene-α-L-xylopyranose (**7**), which was acetylated to give 4-acetamido-3-*O*-acetyl-4-deoxy-1,2-*O*-isopropylidene-α-L-xylopyranose (**8**) *O*-Deacetylation of **8**, followed by mild hydrolysis of the product (**9**), gave 4-acetamido-4-deoxy-L-xylose (**10**), substance **10** was also prepared from **7** in one step In aqueous solution, the acetamido sugar (**10**) underwent equilibration to give a mixture of the α-L and β-L pyranose anomers in 2 3 ratio Structural assignments were supported by n m r. and other physical data

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APIOSE I

1,2:3,5-DI-*O*-ISOPROPYLIDENE-α-D-APIO-L-FURANOSE

F. A. Carey, D. H. Ball, and L. Long, Jr.
Pioneering Research Division, U. S. Army Natick Laboratories, Natick, Mass. 01760 (U.S.A.)

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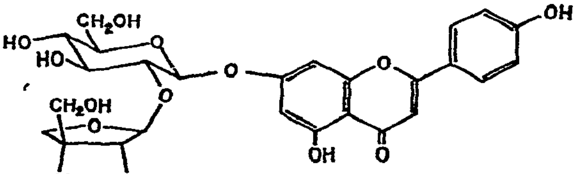
INTRODUCTION

Known since 1901 as a constituent of the flavone glycoside apiin (**1**), apiose is the most abundant of the naturally occurring, branched-chain carbohydrates[1,2].

Various groups have contributed to the clarification of the structure of **1**, with the last facet, the stereochemistry at C-3 in the apiose fragment of the molecule, being recently determined by Jones and his associates by using a combination of synthetic and degradative methods[3].

A more plentiful source of apiose is a cell-wall polysaccharide[4] present in several marine and fresh-water plants, *e.g.*, *Zostera marina* L.[5], *Posidonia australis* L.[6], and *Lemna minor*[2], from which apiose is freed by acid hydrolysis and commonly isolated as a crystalline diisopropylidene derivative. The free sugar is capable of existing in four diastereoisomeric furanose forms. Two diisopropylidene derivatives (**2** and **3**), differing only in the stereochemistry at C-3, seem equally possible. In practice, only one diisopropylidene derivative is isolated. In conjunction with work in progress involving the synthesis of branched-chain carbohydrate derivatives of biological interest, it was necessary to determine the stereochemistry of this and related compounds as a pre-



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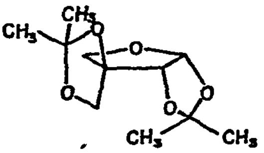

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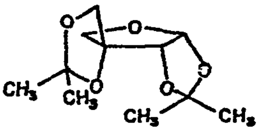

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requisite to structure-activity correlations This paper reports our finding that the known diisopropylidene derivative of apiose is 1,2 3,5-di-*O*-isopropylidene-α-D-apio-L-furanose (**2**)*, namely, 1,2·3,3^1-di-*O*-isopropylidene-[3-*C*-(hydroxymethyl)-β-L-threo-furanose].

DISCUSSION

The procedure described for the extraction of apiose from *Posidonia australis* L [7] was found amenable to large-scale adaptation, from 14 kg of *Zostera marina* L was obtained the known crystalline di-*O*-isopropylideneapiose (in 1% yield) with m p 80–82° and $[\alpha]_D^{25}$ +58° (ethanol) in accord with the reported physical constants[7]. Both thin-layer and gas chromatographic examination showed the compound to be a single isomer

No *a priori* choice between structures **2** and **3** was possible on steric grounds, since examination of models of each revealed no severe non-bonded interactions which would destabilize one relative to the other Similarly, the n m r spectrum (see Fig. 1) showed no characteristics which offered a clue to the stereochemistry at C-3. The coupling constant between the anomeric proton and the C-2 proton was 3 7 c p s , which corresponds to a dihedral angle of 40–50° and indicates that the furanose ring had adopted a C_2 or "twist" conformation[8].

Selective removal of one of the isopropylidene groups was effected by hydrolysis with acetic acid–water (2 1 by volume) at 25° for 48 h The resulting crystalline mono-*O*-isopropylideneapiose [actually 1,2-*O*-isopropylidene-α-D-apio-L-furanose (**4**)] was formed in 70% yield, was homogeneous on thin-layer chromatogram, and was readily purified by recrystallization from dichloromethane–hexane

A partial hydrolysis (in 3 1 dioxane–water, containing *p*-toluenesulfonic acid) was monitored by n m r , which indicated that the 3,5-*O*-isopropylidene group was removed Of the four partially resolved signals for the isopropylidene methyl groups (τ 8 70, 8 65, 8 60, and 8 57), the high and low field resonances remained substantially unchanged during the course of the reaction, while the peaks at τ 8 65 and 8 60 progressively disappeared (*cf* Fig 1) In accord with the observations of Foster and co-workers[9], the high and low field resonances may be assigned to the methyl groups of the 1,2-*O*-isopropylidene ring (α- and γ-type) and the internal peaks to the methyl groups of the 3,5-*O*-isopropylidene ring (β-type)

In agreement with expectation, the mono-*O*-isopropylideneapiose consumed 1 equivalent of aqueous sodium metaperiodate with the liberation of one equivalent of formaldehyde and no formic acid A crystalline ketone, m p. 60 5–61.5° and $[\alpha]_D$ +140° (chloroform), was isolated from the oxidation mixture in 75% yield and found to be identical with 1,2-*O*-isopropylidene-D-*glycero*-tetros-3-ulose (**5**), prepared by ruthenium tetroxide oxidation of 1,2-*O*-isopropylidene-L-threose (**6**)[10]

Removal of the elements of asymmetry at C-3 was accomplished by conversion

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*The nomenclature is essentially that suggested by Cahn (see ref 6). See also, ref. 1b of diol 4 into olefin 8 by Corey's method[11]. Successive treatment of 4 with butyllithium, carbon disulfide, and methyl iodide in tetrahydrofuran, followed by chromatography afforded the cyclic thionocarbonate 7 in 51% yield (73% based on unrecovered 4). Alternatively, 7 may be prepared from 4 and bis(imidazol-1-yl)thione in 93% yield. Refluxing a solution of 7 in trimethyl phosphite under nitrogen for 16 h effected desulfurization–decarboxylation and yielded 3-deoxy-1,2-*O*-isopropylidene-3-*C*-methylene-D-*glycero*-tetrose (8) in 62% yield. Structure 8 follows from the mode of synthesis, known to be position-specific[12], from its infrared spectrum, which contained weak bands at 1660 cm^{-1} (C=C) and 920 cm^{-1} (C=CH$_2$ out-of-plane deformation)[13], and from its n.m.r. spectrum. It showed peaks at τ 8.77 and 8.64 (isopropylidene methyls)

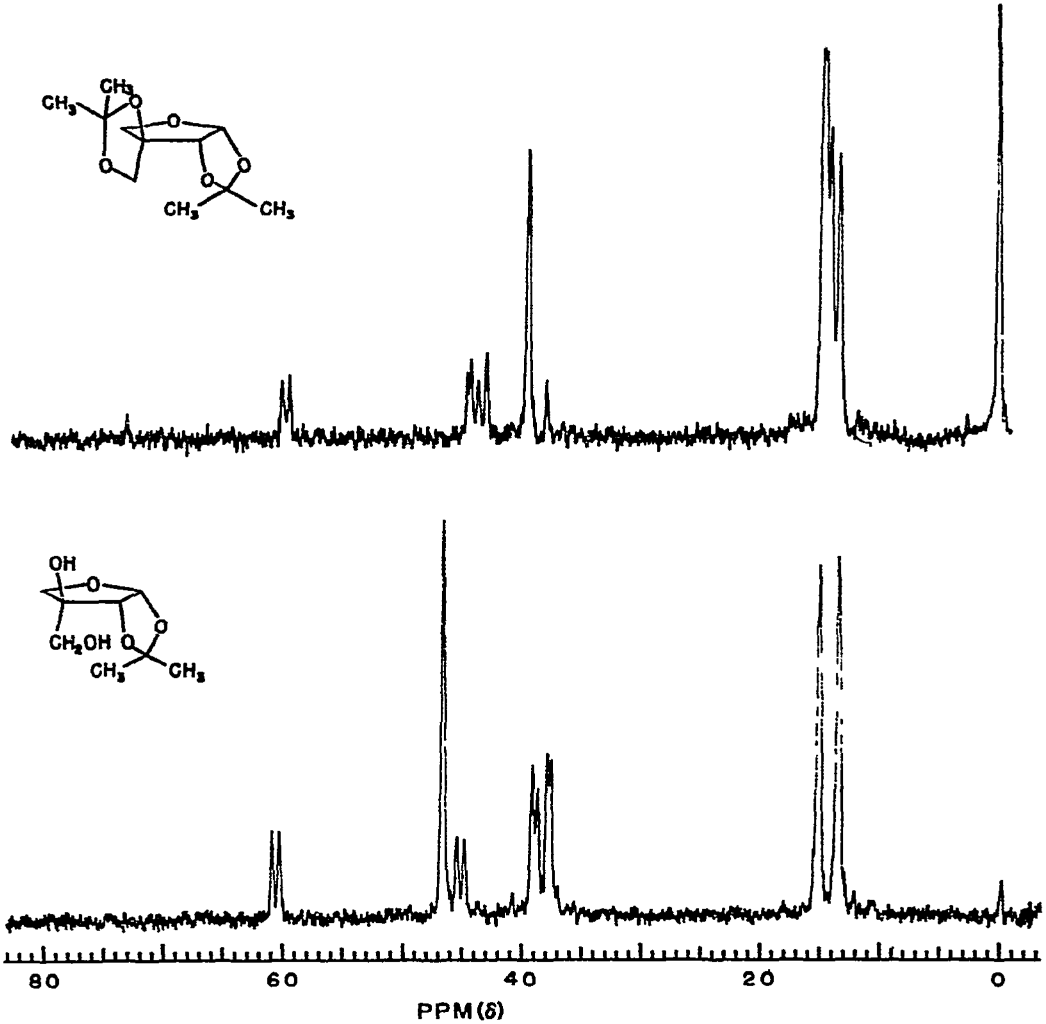
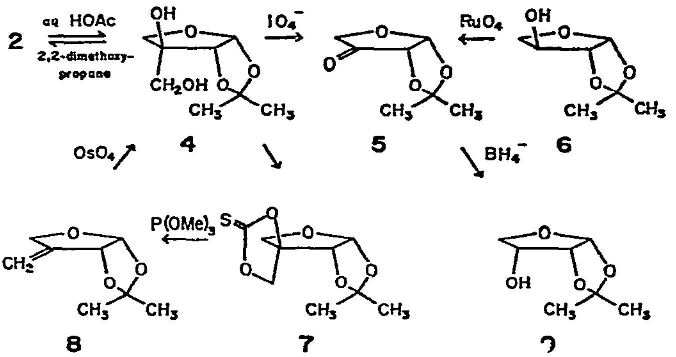


Fig. 1. The 60 Mc/sec p.m.r. spectra of 1,2:3,5-di-*O*-isopropylidene-α-D-apio-L-furanose (in CDCl$_3$) and of 1,2-*O*-isopropylidene-α-D-apio-L-furanose (in D$_2$O)

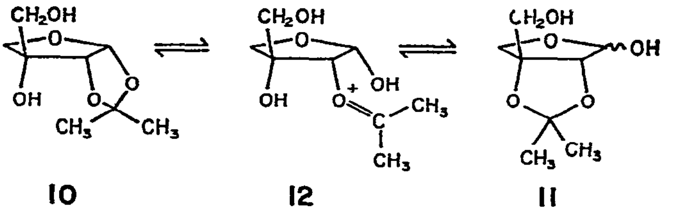
and an AB quartet (C-4 protons) centered at τ 5 69 (J 12 c p s) which was broadened by allylic coupling with the olefinic protons These appeared as two broad peaks at τ 4.95 and 4 77. The C-2 proton gave a broad doublet at τ 5 32, coupled to the anomeric proton at τ 4 32 (J 3.8 c p s)

It was anticipated that restoration of asymmetry at C-3 by addition to the double bond schould be stereoselective and, in particular, it was thought that hydroxylation with osmium tetroxide, a bulky reagent, should occur *trans* to the 1,2-*O*-isopropylidene ring 1,2-*O*-Isopropylidene-α-D-apio-L-furanose (**4**) should therefore be formed in preference to 1,2-*O*-isopropylidene-α-D-apio-D-furanose (**10**)*.



As hoped, hydroxylation proceeded cleanly to give a single, crystalline diol in 53% yield This material was identical in melting point, mixture melting point, i r , n m r , t l c , and optical rotation with **4** (prepared by partial hydrolysis of **2**). Since **4** may be reconverted to the original diisopropylidene derivative by condensation with 2,2-dimethoxypropane, it follows that the known di-*O*-isopropylideneapiose is 1,2 3,5-di-*O*-isopropylidene-α-D-apio-L-furanose (**2**)

It is evident from examination of models that the side of the double bond which is *trans* to the isopropylidene ring is the less hindered; this was verified experimentally by borohydride reduction of ketone **5** The previously unknown 1,2-*O*-isopropylidene-D-erythrose (**9**) was formed exclusively No trace of the threose isomer, which would be formed by attack of borohydride *cis* to the isopropylidene ring, could be detected by gas chromatography



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*Precedent exists for deducing the stereochemistry of a natural product possessing a > C(OH)–CH_2OH grouping by osmium tetroxide hydroxylation of the derived olefin as in the case of the diterpene cafestol [14]

As mentioned previously, no significant difference in energy between **2** and **3** is predictable from examination of models On this basis, it would be expected that the reaction of apiose with acetone would give a mixture of the two. The isomer **2** is the only one which has so far been conclusively identified*, and a possible explanation for the preponderance of **2** is that an intermediate such as **10** may rearrange as shown. If this rearrangement were favored over condensation with a second molecule of acetone, very little of isomer **3** would be formed While such an acetal migration has not as yet been observed[9], such intermediates as **12** have recently been found to be involved in acetal hydrolysis[15].

Although this scheme is necessarily speculative at the present time, further experiments are in progress to clarify the course of this novel acetalation

EXPERIMENTAL

Melting points were determined in glass capillaries with a Thomas–Hoover apparatus and are corrected Infrared spectra were recorded with a Perkin-Elmer Infracord spectrophotometer and calibrated against the 1600-cm^{-1} band of polystyrene Nuclear magnetic resonance spectra were recorded on a Varian A-60 spectrometer, and peak positions are given in τ units relative to internal tetramethylsilane (for solutions in organic solvents) or to sodium 4,4-dimethyl-4-silapentanesulfonate (for solutions in D_2O)

Gas–liquid chromatography was performed on a Pye Argon chromatograph equipped with a 4′×3/16″ column of neopentyl glycol sebacate (10% on 80–100 mesh Chromosorb W, DMCS-A/W) The column temperature was 150°, the flow rate was 90 ml of argon/min

Extraction of apiose from eel grass

Air-dried eel grass (*Zostera marina* L) (14 kg) was ground in a rotary cutter and digested for 1 h at 95–99° in water (160 l) containing sulfuric acid (3 2 l) The solution was allowed to cool and was filtered through cloth, the residue was washed with hot water The filtrate was neutralized (pH 7) with saturated barium hydroxide solution, and most of the supernatant solution was collected by decantation The residual solids were removed by centrifugation, and the total clear solution (*ca* 170 l) was acidified with acetic acid (35 ml) Concentration under reduced pressure afforded a granular solid which was mixed with acetone (88 l) containing *p*-toluenesulfonic acid (880 g), the suspension was boiled under reflux for 78 h Potassium carbonate (2 kg) was added, the mixture was stirred overnight and filtered, and the residue was washed with acetone (10 l) After the addition of 20% sodium hydroxide solution (250 ml), the solution was concentrated to 14 l This solution (in 20 portions) was then concentrated under reduced pressure at 25° to a syrup Addition of cold water caused

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*A brief report (ref 5) that L-apiose forms a diisopropylidene derivative different from that of D-apiose warrants further investigation di-*O*-isopropylideneapiose to separate as an oil, which soon crystallized The yield of tan crystals was 112 g

The mother liquors were extracted continuously with ether for 40 h, and concentration of the extracts afforded a syrup which was fractionated by chromatography on silica gel with dichloromethane as eluent An additional 35 g of di-*O*-isopropylideneapiose was obtained, giving a total yield of 147 g (1 05%)

Recrystallization of the crude material from water containing a little ammonia gave pure di-*O*-isopropylidene-D-apiose as needles, m p 80–82°, $[\alpha]_D^{29}$ +59° (*c* 1 0, ethanol) in good agreement with reported values[7]. (2)

*Partial hydrolysis of di-O-isopropylidene-*D*-apiose* (2)

A solution of di-*O*-isopropylidene-D-apiose (2) (1 0 g) in a mixture of acetic acid (10 ml) and water (5 ml) was kept for 48 h at 25° Concentration afforded a crystalline residue, which was recrystallized from dichloromethane–hexane to give 1,2-*O*-isopropylidene-D-apiose (4) (0 59 g, 70%) A further recrystallization of **4** from the same solvents gave needles, m p 124–125°, $[\alpha]_D^{29}$ +46° (*c* 1 0, ethanol)

Anal. Calc for $C_8H_{14}O_5$ C, 50 52, H, 7 42 Found C, 50 27, H, 7 37

Treatment of **4** with pyridine–acetic anhydride (4 1) for 2 h under reflux gave the 3,5-diacetate which crystallized from cyclohexane as cubes, m p 88–89 5°, $[\alpha]_D^{29}$ +75° (*c* 1 4, chloroform)

Anal Calc. for $C_{12}H_{18}O_7$· C, 52 55, H, 6 62 Found C, 52 54; H, 6 45

Acetonation of compound 4

To a solution of **4** (190 mg, 1 mmole) in 2,2-dimethoxypropane (7 ml) was added 1 drop of conc sulfuric acid. After 2 h at 25°, the solution was poured into saturated sodium bicarbonate solution (15 ml) and the layers were separated. The aqueous phase was extracted twice with ether (5-ml portions), and the combined organic phases were dried with magnesium sulfate Concentration gave a pale-yellow crystalline solid (229 mg, 100%) which was purified by sublimation at 70° and 15 mm to give colorless crystals (143 mg, 62%), m.p. 79 5–80 5° (not depressed by admixture with **2**) $[\alpha]_D^{27}$ +51° (*c* 1.0, ethanol)

Periodate oxidation of compound 4

Oxidation of 1 mole of **4** with sodium metaperiodate was complete after 3 h with an uptake of 1 mole of periodate and the liberation of 0 9 mole of formaldehyde. No formic acid was produced

On a preparative scale, an aqueous solution of sodium metaperiodate (2 5 g, 12 mmole) was added to a solution of **4** (1.73 g, 9 1 mmole) in water (total volume, 10 ml) After 3 h at 25°, dichloromethane (100 ml) was added, and to the stirred mixture was added sufficient anhydrous magnesium sulfate to eliminate the water present The suspension was stirred overnight and then filtered; the residue was washed with dichloromethane, and the combined filtrate and washings were concentrated to a crystalline solid (1 07 g, 75%) Recrystallization from hexane afforded long needles, m p. 60 5–61 5°, $[\alpha]_D^{28}$ +140° (*c* 1 6, chloroform) and $\nu_{C=O}$ 1780 cm^{-1} (chloroform) This compound was identical by m p, mixture m p, and infrared spectroscopy with an authentic sample of 1,2-*O*-isopropylidene-D-*glycero*-tetros-3-ulose (**5**)

Anal Calc for $C_7H_{10}O_4$. C, 53 16, H, 6.37 Found C, 53.30; H, 6 26

Borohydride reduction of compound **5**

An aqueous solution of **5** was prepared by treatment of **4** (0.19 g, 1 mmole) with sodium metaperiodate (0 25 g, 1 2 mmole) in water (1 ml) for 1 h Water (1 ml) and potassium borohydride (0 22 g, 4 mmole) were then added and the solution was left overnight at room temperature Excess borohydride was decomposed with 1 N hydrochloric acid, and the reaction mixture was extracted with three 5-ml portions of ethyl acetate The combined extracts were washed with saturated sodium hydrogen carbonate solution and dried over anhydrous magnesium sulfate Gas–liquid chromatography of the solution revealed a single peak with a retention time of 2.9 min under conditions in which 1,2-*O*-isopropylidenethreose had a retention time of 6 8 min

Concentration of the solution gave a syrup which crystallized readily (0 13 g, 82%), and recrystallization from hexane afforded pure 1,2-*O*-isopropylidene-D-erythrose (**9**), m p 74 5–75 5°, $[\alpha]_D^{23}$ +25 5° (*c* 1 8, ethanol)

Anal Calc for $C_7H_{12}O_4$· C, 52 49, H, 7 55 Found C, 52 50, H, 7 45

Treatment of **9** with 0.1 N hydrochloric acid for 1 h at 100° removed the isopropylidene group, paper chromatograms developed in 1-butanol–ethanol–water (3 1 1) showed a single spot indistinguishable from that of authentic erythrose

*1,2-*O*-Isopropylidene-α-D-apio-L-furanose 3,5-thionocarbonate* (**7**)

A To a solution of **4** (0 57 g, 3 mmole) in dry tetrahydrofuran (15 ml) was added butyllithium in hexane (2 3 ml, 3 mmole) and the solution was stirred at room temperature. After 5 min, carbon disulfide (0 25 ml, 3 8 mmole) was added, the solution was stirred for 30 min at room temperature and then boiled under reflux for 30 min Tetrahydrofuran was removed by distillation through a 10-cm Vigreux column, and the residue was fractionated by chromatography on a column of Woelm silica gel (20 g) with dichloromethane as eluent. After elution of the thionocarbonate (0 35 g), the column was washed with ethyl acetate to remove unchanged **4** (0 17 g). The yield of **7**, based on unrecovered **4**, was 73% Recrystallization from cyclohexane gave needles, m p 100–100 5°, $[\alpha]_D^{25}$ +108° (*c* 0 4, chloroform). The infrared spectrum exhibited characteristic peaks at 1308 cm^{-1} and 1330 cm^{-1} (Ref[11] [16])

Anal. Calc for $C_9H_{12}O_5S$ C, 46 55, H, 5 21; S, 13 78 Found C, 46 43, H, 5 25, S, 13 78

B A solution of **4** (1 90 g, 10 mmole) and bis(imidazol-1-yl)thione[17] (2 00 g, 11 mmole) in dry tetrahydrofuran (40 ml) was boiled under reflux in a stream of nitrogen for 4 h Tetrahydrofuran was removed as in method A, and the residue was chromatographed on Woelm silica gel (60 g). Elution with dichloromethane (400 ml) gave **7** (2 17 g, 93%), m p 97–99°

*3-Deoxy-1,2-O-isopropylidene-3-C-methylene-*D-glycero-*tetrose* (**8**)

A solution of **7** (1.60 g, 6.9 mmole) in redistilled trimethyl phosphite (50 ml) was boiled under reflux in a stream of nitrogen for 16 h. Sodium hydroxide solution (15%, 400 ml) was added, and the solution was heated on a steam bath for 45 min to decompose phosphorus esters. The solution was cooled, and extracted with methylene chloride (3 × 50 ml), the extracts were washed with saturated sodium chloride solution and dried with magnesium sulfate. Dichloromethane was removed by distillation through a 10-cm Vigreux column, and the olefin **8** was obtained as a clear colorless liquid (0.68 g, 62%). This material was homogeneous by gas–liquid chromatography and, after purification by "Kugelrohr" distillation at 105° and 20 mm, had $[\alpha]_D^{28}$ $+140°$ (*c* 1.6, chloroform).

Anal. Calc. for $C_8H_{12}O_3$: C, 61.52, H, 7.74. Found: C, 61.90, H, 7.38.

Hydroxylation of compound **8**

Addition of osmium tetroxide (0.5 g, 2.0 mmole) to a solution of **8** (0.20 g, 1.3 mmole) in pyridine (7.5 ml) resulted in an immediate exothermic reaction and the formation of a brown precipitate. After 16 h at 25°, the osmate ester was decomposed by the addition of a solution of sodium bisulfite (1 g) in a mixture of water (15 ml) and pyridine (10 ml)[18]. The solution was extracted with dichloromethane (5 × 20 ml), and the extracts were dried with magnesium sulfate and concentrated to a syrup. Trituration with hexane afforded crystals (0.13 g, 53%), m.p. 122–125° not depressed by admixture with **4**, $[\alpha]_D^{26}$ $+49°$ (*c* 2.0, ethanol). The i.r. and n.m.r. spectra and t.l.c. mobilities of the product and of **4** were identical.

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SUMMARY

A procedure for the extraction of apiose from eel grass (*Zostera marina* L.) on a large scale is described. The crystalline diisopropylidene derivative was obtained in a yield of 1% and was shown to be 1,2:3,5-di-*O*-isopropylidene-α-D-apio-L-furanose (2), namely, 1,2:3,3^1-di-*O*-isopropylidene-[3-*C*-(hydroxymethyl)-β-L-threofuranose]

Graded acid hydrolysis of **2** afforded 1,2-*O*-isopropylidene-α-D-apio-L-furanose (**4**), which was oxidized with periodate to give 1,2-*O*-isopropylidene-D-*glycero*-tetros-3-ulose (**5**) Borohydride reduction of **5** gave 1,2-*O*-isopropylidene-D-erythrose (**9**). Treatment of **4** with bis(imidazol-1-yl)thione gave the cyclic thionocarbonate **7**, which was converted into 3-deoxy-1,2-*O*-isopropylidene-3-*C*-methylene-D-*glycero*-tetrose (**8**) by treatment with trimethyl phosphite Stereoselective hydroxylation of **8** gave **4**

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STUDIES ON 2-AMINO-2-DEOXY-D-GLUCOSE DERIVATIVES

XIV*. COMPARISON OF STABILITIES OF *O*-AMINOACYL DERIVATIVES OF 2-ACETAMIDO-2-DEOXY-D-GLUCOSE TOWARD HYDROLYSIS

JUJI YOSHIMURA, MASUO FUNABASHI, SADAO ISHIGE, AND TETSUO SATO
Department of Chemistry, Tokyo Institute of Technology, Ookayama, Meguroku, Tokyo (Japan)

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INTRODUCTION

Amino acid esters of sugars, supposedly of wide occurrence in nature, have seldom been found[1] up to the present, probably because of their high lability It is therefore significant to examine in detail the stability of the esters formed from amino acids and sugars

In the field of aminoacyl derivatives of nucleosides and nucleotides, studies have been conducted on the hydrolysis properties[2] of these compounds, as model systems for aminoacyl derivatives of soluble ribonucleic acids It has been concluded that the stability of these derivatives is enhanced by the bulkiness of the aminoacyl moiety, by the absence of a neighboring *cis*-hydroxyl group, and by the presence of a phosphate group

On the other hand, recent work[3] on the stabilities of 6-*O*-aminoacyl-D-glucose derivatives having neutral amino acids has shown that these compounds show minimum hydrolytic rate at a pH at which the electrostatic repulsion of the protonated amino group stabilizes the ester linkage, in proportion to the relative distance of the amino group from the ester bond

The preparation of various *O*-(2-aminoacyl) derivatives of 2-acetamido-2-deoxy-D-glucose, as model compounds for hydrolytic studies, has been reported[4–6].

We report herein the hydrolytic stability of these derivatives We have attempted to evaluate the data in terms of the influence of the nature of the amino acid (neutral, basic, or acidic), the acetamido group, the position of the ester bond in the sugar moiety, and the anomeric group

EXPERIMENTAL

Materials

The following nine model compounds were used 2-acetamido-2-deoxy-6-*O*-glycyl-D-glucose[4] (**1**), 2-acetamido-2-deoxy-6-*O*-(L-lysyl)-D-glucose[4](**2**), 2-acetamido-6-*O*-(L-α-aspartyl)-2-deoxy-D-glucose[4] (**3**), 2-acetamido-2-deoxy-4-*O*-glycyl-D-glucose[5] (**4**), 2-acetamido-2-deoxy-3-*O*-glycyl-D-glucose[5] (**5**), 2-acetamido-2-deoxy-1-*O*-glycyl-

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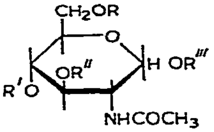
*Part XII; see ref 5. Part XIII, see ref 6 β-D-glucose[6] (6), ethyl 2-acetamido-6-O-(L-α-aspartyl)-2-deoxy-α,β-D-glucoside[4] (7), 2-acetamido-6-O-(N-benzyloxycarbonylglycyl)-2-deoxy-D-glucose[4] (8) and 2-acetamido-1-O-(N-benzyloxycarbonylglycyl)-2-deoxy-β-D-glucose[6] (9).



	R	R'	R''	R'''
1	$COCH_2NH_2$	H	H	H
2	$\underset{NH_2}{COCH}(CH_2)_4NH_2$	H	H	H
3	$\underset{NH_2}{COCH}CH_2COOH$	H	H	H
4	H	$COCH_2NH_2$	H	H
5	H	H	$COCH_2NH_2$	H
6	H	H	H	$COCH_2NH_2$
7	$\underset{NH_2}{COCH}CH_2COOH$	H	H	C_2H_5
8	$COCH_2NHCOOCH_2C_6H_5$	H	H	H
9	H	H	H	$COCH_2NHCOOCH_2C_6H_5$

Procedure

The following method, which is essentially the same as that reported by Kochetkov *et al* [3] and by Suzuki[7], was used

Each sample (6 8–9 5 mg, 0 02 mmole) was dissolved in 1 ml of a buffer solution (acetate buffers pH 0 76, 1 20, 1.51, 1 95, 3 38, 4 25, and 5 48; phosphate buffers. pH 6 80 and 7.94), and was kept in a stoppered test tube at 40±0 5°. Aliquots (0 05 ml) of the solution were withdrawn with a micropipet at 4–5 suitable intervals, and were spotted onto a line ruled on the center of a sheet (35×12 cm) of Toyo Roshi No 51A filter paper

At the same time, another sample of the solution was spotted as a standard for reference, about 3 cm from the main spot Paper electrophoresis was then performed, with pyridine–acetic acid buffer[3] (pH 4 3) at 32 V/cm and 0 2 mA/cm After detection of the positions of 2-acetamido-2-deoxy-D-glucose, of the amino acid, and of the starting material, by the method of Rydon and Smith[8] or with 0 1% ninhydrin solution in butyl alcohol saturated with water, the section of the hydrolyzed product was cut off at 4-cm width for determination

The migration distances of the starting materials and the hydrolyzates are shown in Table I

In the case of glycine or lysine esters, the liberated 2-acetamido-2-deoxy-D-glucose, which remains close to the starting line, was eluted with 10 ml of distilled water, and the quantities were determined by a modified method[9] of Morgan and

Elson[10], in which the presence of an equimolar amount of an amino acid does not disturb the assay of 2-acetamido-2-deoxy-D-glucose.

In the case of aspartic acid esters, the quantity of the liberated L-aspartic acid was determined by a bioassay[11] using *Leuconostoc mesenteroides* P-60 (ATCC 8042), because 2-acetamido-2-deoxy-D-glucose and the starting materials were inadequately resolved on the chromatograms

TABLE I

MIGRATION DISTANCE OF 2-ACETAMIDO-*O*-AMINOACYL-2-DEOXY-D-GLUCOSES AND HYDROLYZATES IN PAPER ELECTROPHORETOGRAMS[a]

Compound	1	2	3	4	5	6	7
Migration distance (cm)	−5.7	−8.9	−1.4	−5.9	−5.9	−6.0	−1.5

Compound	Glycine	L-Lysine	L-Aspartic acid	2-Acetamido-2-deoxy-D-glucose
Migration distance (cm)	−0.90	−7.7	+4.6	−1.0

[a] Conditions 32V/cm, 0.2 A/cm, 60 min, at pH 3.4

In the case of **8** and **9**, the separation of the hydrolyzates was performed by paper chromatography with 2-butanol saturated with water as developer Detection of the substances was performed by the method of Rydon and Smith[8], and the quantity of the hydrolyzed 2-acetamido-2-deoxy-D-glucose was determined as before.

The first-order rate constants for the hydrolysis were calculated, and the times of half-reaction ($t_{0.5}$) at each pH are summarized in Table II The relation between $t_{0.5}$ and pH is shown in Fig. 1 and Fig 2

The total relative errors in this procedure are less than 8%

TABLE II

TIME (IN HOURS) OF HALF-REACTION ($t_{0.5}$) OF 2-ACETAMIDO-*O*-AMINOACYL-2-DEOXY-D-GLUCOSES AT VARIOUS PH'S

	pH								
Compound	*0.76*	*1.20*	*1.51*	*1.95*	*3.38*	*4.25*	*5.48*	*6.80*	*7.95*
1	220	430	720	890	180	48	20	4.4	0.58
2	190	1100	1500	740	160	50	11		
3				190	100	82		7.5	1.7
4	180		190	600	230	140	1.7		
5	220		740	380	500	22			
6	290	410	600	600	220	22		0.50	
7	210	210		240	140	89		11	2.0
8	120		150	240	620	480	310		
9	50	180	400	480	360	140			

RESULTS AND DISCUSSION

It is evident from the data shown in Fig 1 that **2** is hydrolyzed most slowly at pH 1 0–1 8, indicating that the electrostatic effect of the protonated α- and ε-amino groups in the lysyl residue prevents the attack of protons more so than that in **1** In contrast, the nucleophilic character of the two amino groups predominates over the former effect in the higher pH range, so that **2** is more labile than **1** at pH 1.8

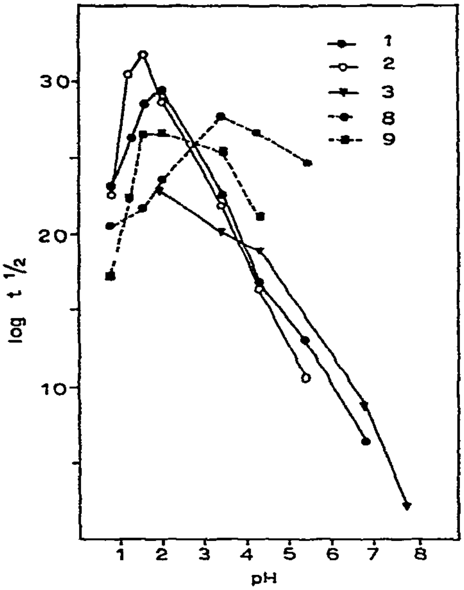


Fig 1 Time of half-reaction at various pH's of 2-acetamido-2-deoxy-6-*O*-glycyl-D-glucose (**1**) 2-acetamido-2-deoxy-6-*O*-)L-lysyl)-D-glucose (**2**), 2-acetamido-6-*O*-(L-α-aspartyl)-2-deoxy-D-glucose (**3**), 2-acetamido-6-*O*-(*N*-benzyloxycarbonylglycyl)-2-deoxy-D-glucose (**8**), and 2-acetamido-1-*O*-(*N*-benzyloxycarbonylglycyl)-2-deoxy-β-D-glucose (**9**)

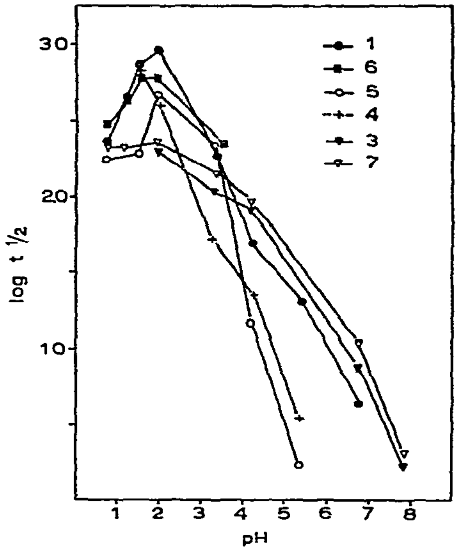


Fig 2 Time of half-reaction of various pH's of 2-acetamido-2-deoxy-6-*O*-glycyl-D-glucose (**1**), 2-acetamido-2-deoxy-1-*O*-glycyl-β-D-glucose (**6**), 2-acetamido-2-deoxy-3-*O*-glycyl-D-glucose (**5**), 2-acetamido-2-deoxy-4-*O*-glycyl-D-glucose (**4**), 2-acetamido-6-*O*-(L-α-aspartyl)-2-deoxy-D-glucose (**3**), and ethyl 2-acetamido-6-*O*-(L-α-aspartyl)-2-deoxy-α,β-D-glucoside (**7**)

In the case of **3**, intramolecular proton exchange between the amino and β-carboxyl groups in the aspartyl residue allows the protonated amino group to have an effect, even in the higher pH range, whereas in the lower pH region the proton attractive effect of the carboxylate anion diminishes the electrostatic repulsion of the protonated amino group Therefore, **3** is rather more stable than **1** or **2** at pH > *ca* 4 and much more labile at pH < *ca* 4

The effect of the protonated amino group is more directly apparent when **1** is compared with **8**, or **6** with **9** (Fig 1) In both cases, the benzyloxycarbonyl group decreases the nucleophilicity of the amino group, and consequently, $t_{0\ 5}$ values of **8** and **9** are larger than those of **1** and **6** in the higher pH range and much smaller in the lower pH range. Moreover, the pH value at the maximum point in **8** is diminished from *ca.* 3.4 to 1.95 by removal of the benzyloxycarbonyl group. However, the fact that the above change cannot be observed in the case of **9** and that **9** is hydrolyzed more slowly than **8** at pH 1.2–2.4 seems to suggest that the acetamido group at C-2 in 2-acetamido-2-deoxy-D-glucose exerts a protonation effect. This effect is also supported by the observation that 6-*O*-glycyl-D-glucose[3] ($t_{0.5}$ 500 h at pH 2.3) is hydrolyzed more rapidly than 2-acetamido-2-deoxy-6-*O*-glycyl-D-glucose ($t_{0.5}$ 890 h at pH 1.95).

On the other hand, comparison of the influence of the position of the ester linkage (Fig. 2) indicates that **1** is the most stable of all because of the primary hydroxyl ester, but **6** is not as labile as expected. It is also noteworthy that **4** is most labile at pH 0.8–1.8, attributable to the absence of the effect of the neighboring acetamido group mentioned above. The fact that **7** is hydrolyzed a little more slowly than **3** (Fig. 2) suggests that the glycosidic hydroxyl group exerts an effect.

Although the compounds containing amino and carbonyl groups generally show high lability because of an intramolecular amino–carbonyl reaction[12], the results indicate that the contribution of the free aldehyde form of a sugar is rather small.

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SUMMARY

The times of half-reaction of 2-acetamido-1-, -3-, -4-, and -6-*O*-aminoacyl-2-deoxy-D-glucoses on hydrolysis under acidic conditions were measured. The maximum points were generally observed at pH 1.5–2.0 and resulted from the electrostatic repulsion of the protonated amino group. In a higher pH range, the effect of the nucleophilic character of the amino group predominates over the above effect. Consequently, the order of the stability is 6-*O*-(L-lysyl) (**2**)$>$6-*O*-glycyl (**1**)$>$6-*O*-(L-α-aspartyl) (**3**) at pH$<$1.8, **3**$>$**1**$>$**2** at pH$>$*ca.* 4, and **1**$>$6-*O*-(*N*-benzyloxycarbonylglycyl) (**8**) at the maximum point. The 2-acetamido group of these compounds showed a similar effect to the neighboring ester linkage: 1-*O*-(*N*-benzyloxycarbonylglycyl) (**9**)$>$**8**, at pH 1.3–2.5.

In relation to the position of the ester linkage, the order of the stability at pH 1.5–2.0 is **1**$>$1-*O*-glycyl (**6**), 3-*O*-glycyl (**5**)$>$4-*O*-glycyl (**4**). A weak effect of the anomeric hydroxyl group was estimated from the comparison of **3** with the ethyl 6-*O*-(L-α-aspartyl)derivative (**7**).

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A SPECTROPHOTOMETRIC METHOD FOR THE DETERMINATION OF FORMIC ACID IN THE PERIODATE OXIDATION OF CARBOHYDRATES

S. A. Barker and P. J. Somers
Department of Chemistry, University of Birmingham, Birmingham 15 (Great Britain)

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INTRODUCTION

In the cleavage of α,β,γ-triols by periodate, one molecular proportion of formic acid is produced. Thus, in the periodate oxidation of polysaccharides, non-reducing end groups or intercatenary (1→6)-linked units give rise to formic acid, when three contiguous hydroxyl-groups are present. Measurement of the formic acid released in such reactions can thus yield structural data concerning the polysaccharide. Potentiometric and other titrimetric methods have been described[1-3] for the determination of formic acid. Sensitive manometric[4] and enzymic[5] procedures have also been reported.

EXPERIMENTAL

Standardised analytical procedure. — Reagents: (1) Ethylene glycol (10% v/v) in distilled water, (2) sodium borohydride (5% w/v) in sodium tetraborate (0.05M)–hydrochloric acid buffer (pH 8.0), (3) sulphuric acid (8.0N), prepared from Microanalytical Reagent, concentrated acid, (4) a suspension of 2-thiobarbituric acid (2.5 g) in distilled water (80 ml) was adjusted to pH 5.4 with sodium hydroxide (2.0N) to obtain complete dissolution at room temperature, and the solution was diluted to 100 ml with distilled water, (5) redistilled butyl alcohol containing 5% (v/v) hydrochloric acid (11.6N).

Method. — Portions (0.4 ml) of sample solutions from periodate oxidation reactions, containing 0 to 200 μg of formic acid, were pipetted into clean, stoppered test-tubes (125×16 mm), and the periodate oxidation was terminated by the addition of ethylene glycol solution (0.05 ml). Standard solutions of formic acid, weighed as sodium formate, were similarly treated. After 5 min, sodium borohydride solution (0.1 ml) was added, excess of borohydride being destroyed after a further 5 min by the addition of dilute sulphuric acid (8.0N, 0.05 ml). The addition of 2-thiobarbituric acid solution (1.0 ml), followed by heating for 20 min at 100°, resulted in the development of a characteristic chromophore (λ_{max} 450 mμ). After the solution had been cooled to room temperature, the chromophore was quantitatively extracted into acidic butyl alcohol (reagent 5, 1.5 ml). The optical density was determined at 450 mμ after clarification by centrifugation (1000 r.p.m., 3 min).

Variation of colour development with pH and extracting solvent — Solutions containing 200 μg of formic acid were analysed as in the standard procedure, but using a different pH for chromophore development. After the addition of sodium borohydride, sulphuric acid of various strengths (1–10N, 0 05 ml) was added After colour development, extraction was effected into acidic butyl alcohol (1.5 ml) or cyclohexanone (1.5 ml). The results obtained are shown in Table I

TABLE I

VARIATION OF COLOUR DEVELOPMENT WITH pH AND EXTRACTING SOLVENT IN THE REACTION OF FORMIC ACID AND 2-THIOBARBITURIC ACID

Sulphuric acid added (mM)	*Reaction pH*	*Optical density at 450 mμ (10-mm cuvettes)*	
		Cyclohexanone	*Acidic butyl alcohol (reagent 5)*
0 025	9 36	0 002	0 002
0 050	8 60	0 026	0 056
0 075	7 00	0 107	0 194
0 100	3 60	0.151	0 302
0 125	3 12	0 255	0 424
0 150	2 88	0 372	0 475
0 175	2 61	0 396	0 540
0 200	2 45	0 395	0 545
0 225	2 32	0 348	0 465
0 250	2 22	0 281	0 251

Variation in colour development with concentration of 2-thiobarbituric acid — A solution of 2-thiobarbituric acid (5% w/v) (adjusted to pH 5 4 with 2N sodium hydroxide to obtain complete dissolution at room temperature) was diluted to obtain concentrations of 0–5%, at intervals of 0 5% By the standard procedure, solutions containing 200 μg of formic acid were analysed with solutions (1 0 ml) of 2-thiobarbituric acid having different concentrations The results are recorded in Table II

TABLE II

COLOUR DEVELOPMENT WITH RESPECT TO 2-THIOBARBITURIC ACID CONCENTRATION IN THE REACTION WITH FORMIC ACID

Concentration of 2-thiobarbituric acid (%)	0	0 5	1 0	1 5	2 0	2.5
Optical density at 450 mμ (10-mm cuvettes)	0 001	0 092	0 210	0 379	0 524	0 563
Concentration of 2-thiobarbituric acid (%)	3 0	3 5	4 0	4 5	5 0	5 0(blank)
Optical density at 450 mμ (10-mm cuvettes)	0 565	0 531	0 524	0 471	0 468	0 005

Variation of colour development with heating time — Solutions containing 200 μg of formic acid were analysed by the standard procedure, using heating times of 0–60 min at 100°. The rate of chromophore development is shown in Table III

TABLE III

VARIATION OF COLOUR DEVELOPMENT WITH TIME OF HEATING IN THE REACTION OF FORMIC ACID WITH 2-THIOBARBITURIC ACID

Heating time (min)	2.0	4.0	7.0	10	12	14	16	18	20	30	60
Optical density at 450 mμ (10-mm cuvettes)	0.065	0.140	0.220	0.290	0.330	0.375	0.405	0.445	0.475	0.490	0.475

Calibration of the method and determination of reproducibility — Portions of standard formic acid solutions, prepared from sodium formate, containing 0–200 μg of formic acid, were analysed by the standard procedure. The optical density of the chromophore developed was determined at 450 mμ by using 10-mm cuvettes (capacity, 0.45 ml) for solutions containing 50–200 μg of formic acid, and 40-mm cuvettes capacity, 1.4 ml) for solutions of 5–50 μg of formic acid. The resulting calibration, together with a typical absorption spectrum, is shown in Fig. 1.

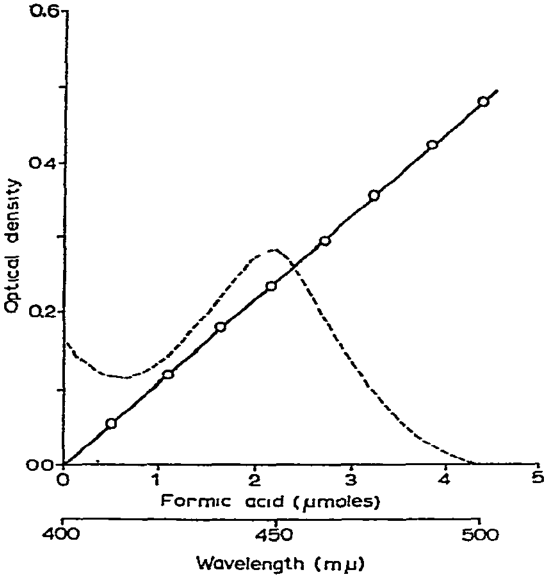

Fig. 1. Calibration and chromophore absorption spectrum for the formic acid—2-thiobarbituric acid reaction

Determination of the formic acid released on periodate oxidation of methyl α-L-rhamnopyranoside

Cooled (2°) portions (in duplicate) of a solution of methyl α-L-rhamnopyranoside (2.68 mg/ml) were treated with aqueous sodium metaperiodate (40 mg/ml, 0.2 ml) at 2° The oxidations were terminated by the addition of ethylene glycol (10% v/v, 0 05 ml) at various time intervals The formic acid contents were determined by the standard procedure (see Table IV)

TABLE IV

RELEASE OF FORMIC ACID ON PERIODATE OXIDATION OF METHYL α-L-RHAMNOPYRANOSIDE

Time (min)	*0*	*12*	*30*	*60*	*94*	*120*
Optical density at 450 mμ (10-mm cuvettes)	0 022	0 127	0 200	0 300	0 340	0 347
Formic acid (μmoles)	0 198	1 15	1 80	2 70	3 07	3 12
Formic acid (μmoles per μmole of substrate)	0 062	0 356	0 561	0 842	0 956	0 975

Determination of the effect of possible interfering compounds on the analytical procedure — Possible interfering compounds derived from the oxidation of carbohydrates were tested for their action in the analytical method Acetaldehyde, formaldehyde, acetic acid, oxalic acid, glyoxylic acid, malonaldehyde, and formylpyruvic acid all gave no reaction when present in ten-fold excess, and did not inhibit chromophore development from formic acid itself

DISCUSSION

In the Warren assay[6] for *N*-acetylneuraminic acid, the sugar is oxidised by periodate under acidic conditions, the excess of periodate is converted into iodate with sodium arsenite, and the formylpyruvic acid produced from the sugar is condensed (by heating) with 2-thiobarbituric acid to give a characteristic red chromophore (λ_{max} 549 mμ) which is extractable with cyclohexanone Under such conditions, it was noticed that formic acid eluates of *N*-acetylneuraminic acid from ion-exchange columns gave an additional chromophore (λ_{max} 450 mμ), which was later shown to be derived from formic acid

Trial of the Warren[6] method with formic acid standards showed only a low sensitivity (500 μg/ml gave an optical density of 0 201 at 450 mμ in a 40-mm cuvette) In addition, assay of the formic acid liberated in the oxidation of methyl α-L-rhamnopyranoside gave approximately 10 times the theoretical colour yield. This was attributed to the interfering chromophores developed from the dialdehydes formed on periodate oxidation Attempted reduction of the aldehyde and dialdehyde groups with sodium borohydride, in conjunction with the Warren procedure, was not satisfactory, since the sodium arsenite used to terminate the oxidation produced various coloured precipitates on the addition of the sodium borohydride

A trial calibration of the assay was made using sodium formate, with the use of ethylene glycol as a terminator, followed by reduction with sodium borohydride Although the sensitivity of the method was still low, this procedure was satisfactory. The problem of sensitivity was overcome by establishing the optimal thiobarbituric acid concentration and heating time, and by the demonstration that chromophore development was pH sensitive. The pH (2 45) selected for the assay was considered unlikely to give rise to formic acid other than that already present after reduction of the aldehyde groups. Further improvement in sensitivity was obtained by extraction into acidified butyl alcohol, rather than cyclohexanone. Such extraction is necessary, since precipitates and turbidities were occasionally encountered on cooling the assay solutions after colour development.

Various methods have been reported for the determination of formic acid, but all suffer from several disadvantages Potentiometric or other titrimetric methods[1-3] use large amounts of material and need great care in interpretation due to the titration of other acids[7] The more-sensitive manometric method[4], using liberation of carbon dioxide from carbonate buffer at pH 5 7, is experimentally difficult to use and is restricted to a narrow pH range for the periodate oxidation Its chief advantage lies in the continous monitoring of the release of formic acid without sampling

The present assay is a sensitive, reliable, and interpretable method It is free from interference from other acids and aldehydes, even when these are present in a 10-fold excess This is particularly important for those studies where overoxidation by periodate can occur and high selectivity (*e g*, formic acid instead of oxalic acid) is required. The method also avoids alkaline conditions which might cause liberation of formic acid. Besides the oxidation of methyl α-L-rhamnopyranosides and other reference compounds, the assay has proved satisfactory in periodate-oxidation studies of polysaccharides

ACKNOWLEDGMENTS

We thank Professor M Stacey, F.R S , for his interest in this work, Dr J P. R Herrmann for valuable discussions, and the Medical Research Council for a fellowship (P J S)

SUMMARY

A spectrophotometric method of assaying formic acid, in the range 5–200 μg, has been devised by using the chromophore (λ_{max} 450 mμ) formed on heating formic acid with 2-thiobarbituric acid under acidic conditions The method may be used to monitor formic acid production from carbohydrates during periodate oxidation, since any interfering aldehydes are reduced with sodium borohydride following termination of the reaction with ethylene glycol

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STUDIES ON THE MECHANISM OF METHANOLYSIS OF SOME METHYL D-GLYCOPYRANOSIDES BY THE METHOD OF ISOTOPE EXCHANGE

J Świderski and A Temeriusz
Department of Organic Chemistry, The University of Warsaw (Poland)

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INTRODUCTION

Methyl 2,3,4,6-tetra-*O*-acetyl-α- and -β-D-glucopyranosides, and the D-galactose and D-mannose analogues, are deacetylated[1] under Fischer's methanolysis conditions to give the methyl D-glycopyranosides In the case of methyl 2,3,4,6-tetra-*O*-acetyl-β-D-mannopyranoside, considerable anomerisation was observed, affording 30% of methyl α-D-mannopyranoside We were thus prompted to undertake a more-detailed investigation of the deacetylation of methyl 2,3,4,6-tetra-*O*-acetyl-D-glycopyranosides under conditions of methanolysis

Previously, we demonstrated[2] by means of paper chromatography that, in the first stage, all completely acetylated methyl glycopyranosides undergo deacetylation, but that further transformations of the products are not dependent on the deacetylation process, even if the reaction is conducted in a continous manner. The individual methyl glycopyranosides obtained by deacetylation, and free methyl glycopyranosides prepared by a different route, behaved in a similar manner. The compounds investigated were divided into two groups The first group included methyl α-D-glucopyranoside and methyl α-D-mannopyranoside, both of which failed to undergo any further transformations under the described conditions The second group included methyl β-D-glucopyranoside, methyl α- and β-D-galactopyranoside, and methyl β-D-mannopyranoside, formation of the corresponding furanoside was observed[2] in each case

The present investigation was aimed at determining whether, under conditions of methanolysis, the methoxyl group of methyl hexopyranosides was completely split off, apart from the previously reported reactions[2] (formation of methyl glycofuranosides, anomerisation of methyl glycofuranosides, and marked anomerisation of methyl β-D-mannopyranoside) The problem is more interesting, since the compounds investigated do not exhibit identical patterns of behaviour Demonstration of methoxyl group mobility in the unreactive methyl D-hexopyranoside could largely elucidate the mechanism of methanolysis in the investigated glycosides; methoxyl group mobility may be conveniently studied by conducting methanolysis in ^{14}C-labelled methanol.

EXPERIMENTAL

Exchanges — Methanol labelled with ^{14}C (specific activity, 0 018 mcuries) was employed, it contained 1% anhydrous hydrogen chloride.

Methyl D-*hexopyranosides* The methyl D-hexopyranosides (0 4854 g, 0 025M) were separately dissolved in 5 06 ml (0 125M) of labelled alcohol The solution was boiled for 3 h, and hydrogen chloride was then removed by Dowex-1 (HO$^-$) Alcohol was distilled off under reduced pressure, and the oily residue was crystallized from methanol Physical properties of the products are presented in Table I

*Methyl 2,3,4,6-tetra-O-acetyl-*D-*hexopyranosides* The methyl 2,3,4,6-tetra-*O*-acetyl-D-hexopyranosides (0 9058 g, 0 025M) were separately dissolved in 5 06 ml (0 125M) of labelled alcohol The treatment was continued according to the procedure given above and the methyl D-hexopyranosides were obtained by crystallization from methanol. Physical properties are given in Table I.

TABLE I

PHYSICAL PROPERTIES OF GLYCOSIDES OBTAINED AFTER ISOTOPE EXCHANGE

Substrates before exchange			*Products obtained after exchange*					
Compound	*M p*,°	[α]D, °[a]	*Compound*	*M p*,°	[α]D, °[a]	*Activity of product*[b] *1*	*2*	*3*
Methyl D-hexopyranoside			Methyl D-hexopyranoside					
α-gluco	167–168	+ 158 0	*α-gluco*	168–169	+ 158 0	66	5,300	1
β-gluco	110–112	— 32 0	*β-gluco*	110–112	— 32 0	84	6,700	1 3
α-galacto	115–116	+ 174 4	*α-galacto*	117	+ 174 4	269	21,600	4 1
β-galacto	177–179	+ 0 6	*β-galacto*	176–178	+ 1 0	528	42,300	8 0
α-manno	193–194	+ 82 0	*α-manno*	192–194	+ 82 0	141	11,300	2 1
Methyl 2,3,4,6-tetra-*O*-acetyl-D-hexopyranoside			Methyl D-hexopyranoside					
α-gluco	101–102	+ 175 0(benzene)	*α-gluco*	168–169	+ 158 0	27	2,100	1
β-gluco	104–105	— 22 8(methanol)	*β-gluco*	110–112	— 32 0	45	3,600	1 7
α-galacto	85–86	+ 133 3	*α-galacto*	117	+ 174 4	187	15,000	6 9
β-galacto	94–95	— 14 5	*β-galacto*	176–178	+ 1 0	256	21,400	9 5
α-manno	62–64	+ 48 7	*α-manno*	192–194	+ 82 0	78	6,200	2 9
β-manno	161–162	— 47 0	*α-manno*	190–192	+ 82 0	1,860	150,000	69 0

[a] Rotations measured in water for glycosides, and in chloroform for tetra-acetates [b] 1, the c p m values per 10 mm of radioactive CO_2 introduced into the counter, 2, c p m per mmole, 3, the ratio of exchange of the methyl glycoside to the exchange of methyl α-D-glucopyranoside

Combustion of glycosides — Combustion of the radioactive compound was conducted by the method of Van Slyke and Folch[3] The resulting carbon dioxide was passed through conc. sulphuric acid Anhydrous carbon dioxide was purified by double sublimation (with liquid air) and introduced into the counter for radioactivity measurements

Activity measurement. — An internal-filling counter was employed A layer of stannic dioxide served as cathode The tungsten anode had a diameter of 40 μ The capacity of the counter was 160 ml, active length, 130 mm

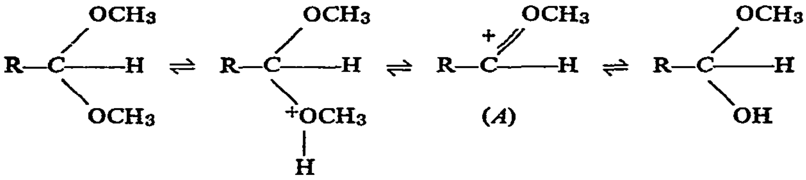
Before being filled with the gas under investigation, the counter was pumped out to the pressure of 4×10^{-4} mm. It was then filled with a mixture of 20 mm of *n*-hexane and 60 mm of carbon dioxide (total pressure, 80 mm) Measurements were made within the Geiger region, at a tension of 2,500 volts The length of the plateau was 600 volts, its slope did not exceed 2% per 100 volts The background was about 45 c p m

An extinction system was applied for eliminating multiple counts, it served to produce an electric impulse extinguishing discharges in Geiger–Muller counters A modified model of the Herman–Renker extinction system was used

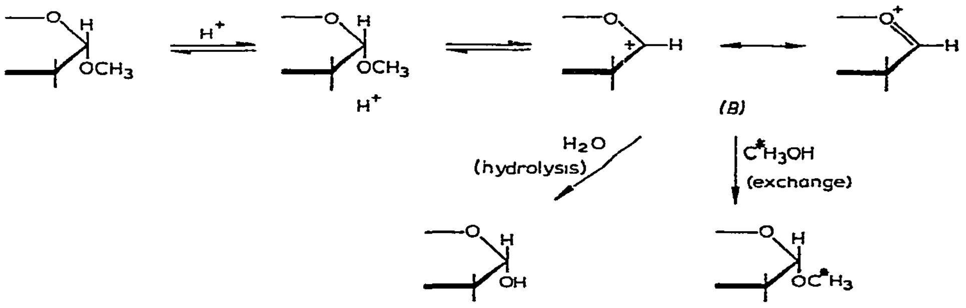
DISCUSSION

In the process of isotope exchange, as applied in the present investigation, the methoxyl group is replaced by a ^{14}C-containing methoxyl group When the exchange process is conducted in acidic medium, the similarity to hydrolysis is emphasised

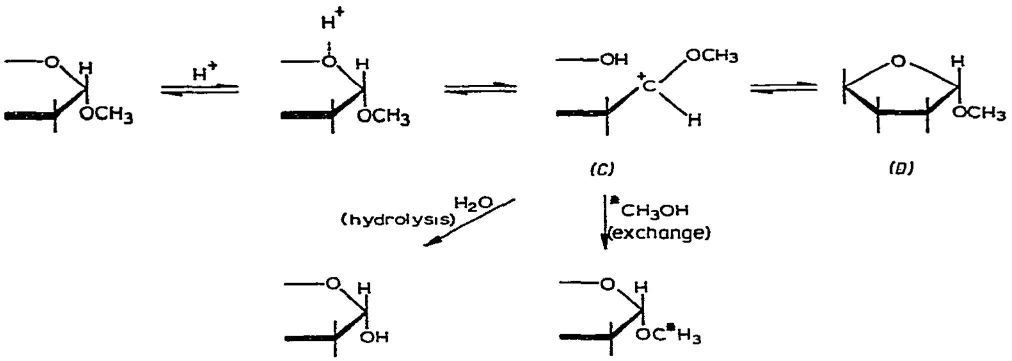
The formation of ion *A* is characteristic of the splitting of an acetal with acid The further course of the reaction is dependent on the medium It may proceed by the addition of a molecule of water to ion *A* and ultimately lead to hydrolysis of the acetal On the other hand, by the addition of an alcohol molecule, the acetal is re-formed, in the presence of labelled alcohol, an exchange takes place



In the hydrolysis of glycosides, two reaction pathways, namely through the cyclic ion *B* or the acyclic ion *C*, have been discussed[4]. In view of the low stability of the hydrolysis products in acidic medium. it is difficult to prove experimentally which reaction mechanism is involved



On methanolysis in ^{14}C-labelled methanol containing 1% of hydrogen chloride, methoxyl group exchange occurred in all of the methyl D-hexopyranosides studied (see Table I). Hence, loss of the initial methoxyl group and formation of the cyclic or acyclic ion had occurred. Since anhydrous, ^{14}C-labelled methanol was used, hydrolysis was precluded, and the ions (*B* or *C*) could only recombine with labelled methoxyl ion, thus affording the radioactive methyl D-hexopyranoside.



As previously mentioned, the compounds investigated could be divided into two groups. Compounds (methyl α-D-glucopyranoside and methyl α-D-mannopyranoside) included[4], in the first group were apparently inactive during methanolysis. Compounds (methyl β-D-glucopyranoside, methyl α- and β-D-galactopyranosides, and methyl β-D-mannopyranoside) of the second group were partly transformed into the corresponding methyl hexofuranosides on methanolysis. Under these conditions, isotope exchange occurred in both groups of compounds.

When considering the results of the previous chromatographic analysis and the isotope exchange experiments, it appears that, in the first group of compounds, the exchange involves the cyclic ion *B*, whereas, for the second group of compounds, the formation of furanose forms suggests that the acyclic ion *C* must also be taken into account.

Unlike hydrolysis, isotope exchange involves relatively stable products, and study of the reaction mechanism is simplified. If the results of our isotope-exchange investigations are applied, as is reasonable, to hydrolysis, it appears that there is not a common reaction mechanism for hydrolysis of all methyl D-hexopyranosides.

The differences between individual glycosides as to the extent of exchange of α- and β-D anomers may be explained in a similar manner as in the hydrolytic process. The exchange is more extensive in β- than in α-D anomers because, in the predominating chair conformation of the β-D anomer, the C-1 methoxyl group adopts the more-exposed equatorial orientation.

On methanolysis, methyl β-D-mannopyranoside undergoes substantial anomerisation, and the methyl α-D-mannopyranoside so obtained in the presence of labelled methanol was *ca* 70-times more radioactive than the other compounds. This may be explained if anomerisation proceeds by a complete, or almost complete, loss of the methoxyl group and attachment of a radioactive methoxyl group to a compound of different configuration Since only a fraction of the glycoside molecules participates in exchange without anomerisation, the activity is smaller than in the case of a product obtained as a result of anomerisation

SUMMARY

Methyl α- and β-D-hexopyranosides, as well as methyl 2,3,4,6-tetra-*O*-acetyl-α- and -β-D-glucopyranosides and the D-galactose and D-mannose analogues, were heated in ^{14}C-labelled methanol containing 1% of hydrogen chloride Exchange of the methoxyl group occurred under these conditions, the acetates were also deacetylated According to chromatographic analysis and the extent of isotope exchange, the glycosides investigated were divided into two groups The first group included methyl α-D-glucopyranoside and methyl α-D-mannopyranoside, for which exchange proceeds probably by way of a cyclic carbonium ion. The second group includes methyl β-D-glucopyranoside, methyl α- and β-D-galactopyranosides, and methyl β-D-mannopyranoside, for which the exchange mechanism probably involves an acyclic ion

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CARBOHYDRATE SPECIFICITY OF INDUCIBLE *KLEBSIELLA AEROGENES* GLUCOSIDASES

S A Barker, J W Hopton, P J Somers, and A Repaš
Departments of Chemistry and Microbiology, The University, Birmingham 15 (Great Britain)

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INTRODUCTION

During studies[1-4] of sequential enzyme induction with *Klebsiella aerogenes* (NCIB 9479), a wide range of inducible glycosidases has been reported Thus, methyl α-L-rhamnopyranoside induced an α-L-rhamnosidase and methyl β-L-rhamnopyranoside induced a β-L-rhamnosidase With both enzymes, the inducers were also substrates, whereas the glycoside of opposite anomeric configuration was not attacked Both rhamnosidases were sequentially induced by the α- and then the β-linked L-rhamnose residues in Type II *Pneumococcus* polysaccharide, which also served as a substrate for both enzymes The ability to attack both monomer and polymer substrates appears to be a general feature of the induced glycosidases from *Klebsiella aerogenes* For structural studies, it was important to ascertain the point of linkage specificity of such induced α- and β-glycosidases, and so a model study has been undertaken to demonstrate (*a*) the range of α- and β-linked D-glucose carbohydrates capable of inducing an α- or β-D-glucosidase and (*b*) the spectrum of specificity of the glucosidases so induced.

EXPERIMENTAL

All incubations were carried out at 37°

Standard method of induction — Resuspended cells of *K aerogenes*, prepared as described previously[4] were used to inoculate induction units of media (50 ml) consisting of 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 A solution of inducer (12 5 mg/ml, 1 ml) was added to separate 50-ml cultures (×2) The cell suspensions were added in such volume as to give a cell concentration of 0 3–0 5 mg/ml at 650 mμ (10-mm cells)

After incubation for 24 h at 37°, a further addition of inducer was made at the same level of concentration, and growth was allowed to continue for a further 24 h at 37° The cells were removed by centrifugation at 2°, and the supernatant solution was dialysed against phosphate buffer (0 02M, pH 6.5, 3×100 volumes) The non-dialysable fraction was concentrated (10-fold) by ultrafiltration, and sterilised by membrane filtration

Glucosidases induced by maltose and cellobiose. — The specificity of glucosidases induced by maltose and cellobiose (the standard method of induction for 48 h) was determined by incubating solutions (0.5 ml) of the enzyme preparations and 0.02M phosphate buffer (pH 6.8) with maltose (200 µg, 1 ml), cellobiose (200 µg, 1 ml), and methyl β-maltoside (500 µg, 1 ml). For quantitative analyses (Table I), the D-glucose content of a portion (0.2 ml) was determined by employing D-glucose oxidase[6]. The reducing power, as compared with a heat-denatured enzyme control, was determined[7] on portions (0.2 ml) which were diluted to 1 ml. With second preparations of both enzymes assayed using more concentrated solutions of substrates (2 mg/2 ml of digest), the percentage conversions into D-glucose after 6 h with various substrates were maltose, 19.3%; methyl β-maltoside, 23.3%, isomaltose, 7.14%, αα-trehalose, 40.4%, and methyl α-D-glucopyranoside, 15.3% (with the α-D-glucosidase induced by maltose), and cellobiose, 21.8%, methyl β-D-glucopyranoside, 17.5%, and laminaribiose, 8.3% (with the β-D-glucosidase induced with cellobiose). Maltose alone was obtained (17.1% conversion) from methyl β-maltoside and the β-D-glucosidase. Methyl α-D-mannopyranoside was not a substrate for α-D-glucosidase, and methyl β-D-galactopyranoside was not a substrate for β-D-glucosidase.

TABLE I

SPECIFICITY OF GLUCOSIDASES INDUCED BY MALTOSE AND CELLOBIOSE

Substrate	*Inducer*	*Conversion (%) into* D-*glucose at*		
		2 h	*6 h*	*24 h*
Maltose	Maltose	22.3	37.2	38.1
Maltose	Cellobiose	0	0	0
Cellobiose	Cellobiose	27.2	56.2	56.2
Cellobiose	Maltose	0	0	0
Methyl β-maltoside	Maltose	18.6	70.0	72.0
Methyl β-maltoside	Cellobiose	20.8[a]	28.6[a]	49.1[a]

[a] Conversion (%) into maltose, assayed by the Nelson[7] method, no reaction with D-glucose oxidase.

*β-*D*-Glucosidase induced by methyl β-maltoside.* — Aliquot portions of a cell suspension were removed at 1, 6, 24 and 48 h from a standard induction using methyl β-maltoside as an inducer. The glucosidase activity in the centrifuged, dialysed supernatant solutions at these times was assessed by incubation in a digest (2 ml) containing 200 µg of substrate, 0.02M phosphate buffer (pH 6.8, 0.5 ml), and enzyme solution (0.5 ml). The results are given in Table II. With a second preparation of the glucosidase obtained after induction for 24 h with methyl β-maltoside, a range of carbohydrates was examined (Table II) as substrates at a concentration of 1 mg/2 ml of digest. Neither methyl α-D-mannopyranoside nor methyl β-D-galactopyranoside (200 µg/2 ml of digest) were substrates for the β-D-glucosidase.

*α-*D*-Glucosidase induced by αα-trehalose.* — The enzyme induced by αα-trehalose after 24 h was examined with a range of substrates at 2 mg/2 ml of digest. αα-Trehalose itself was converted into D-glucose (47 6%), but maltose, methyl α-D-glucopyranoside, isomaltose, and methyl β-maltoside were unattacked.

TABLE II

SPECIFICITY OF GLUCOSIDASE INDUCED BY METHYL β-MALTOSIDE

Substrate	*Induction time (h)*	*Conversion (%) into maltose at*		
		2 h	*6 h*	*24 h*
Methyl β-maltoside (200 μg/2 ml)	1	1 5	1 9	1 95
—	6	8 3	15 1	16 4
—	24	8 8[a]	16 7[a]	16 7
—	48	0	0	0
Methyl β-maltoside (1 mg/2 ml)	24	6 2[a]	21 1[a]	22 9[a]
		Conversion (%) into D-glucose at		
		2 h	*6 h*	*24 h*
Cellobiose	24	4 8	14 3	14 3
Maltose	24	0	0	0
Methyl β-D-glucopyranoside	24	—	24 7	—

[a] No reaction with D-glucose oxidase

α- and β-D-Glucosidase induced by isopropyl α-cellobioside —The enzymes induced by isopropyl α-cellobioside after induction for 24 h by the standard method were examined with a range of substrates at 2 mg/2 ml of digest The percentage conversion into D-glucose after a 6-h incubation with the enzyme solution was 10 7% with isopropyl α-cellobioside itself, 5 2% with maltose, 28 6% with αα-trehalose, 4% with cellobiose, and 6% with methyl β-maltoside These conversions were assayed with D-glucose oxidase[6] With the Nelson reagent[7], the total percentage "apparent" conversions expressed as D-glucose were 11 2% with isopropyl α-cellobioside and 9 2% with methyl β-maltoside.

Other attempted inductions — Neither methyl α-D-glucopyranoside nor phenyl α-cellobioside acted as inducers The possible substrates assessed were methyl α-D-glucopyranoside and isomaltose with the former, phenyl α-cellobioside and cellobiose with the latter, and αα-trehalose, methyl β-maltoside, and maltose with both glycosides

Control incubations.—Cells obtained by going through the procedure of induction without addition of any carbohydrate inducer were fragmented in a Mickle Tissue Disintegrator The resulting, centrifuged enzyme extract had no action (assessed by D-glucose oxidase) on maltose, cellobiose, methyl β-maltoside, methyl α-D-glucopyranoside, or αα-trehalose Neither the active α-L-rhamnosidase prepared by induction with methyl α-L-rhamnopyranoside, nor the active β-L-rhamnosidase prepared by induction with methyl β-L-rhamnopyranoside, had any action on maltose or cellobiose

DISCUSSION

The pattern of response by induced enzymes in *Klebsiella aerogenes*, to challenge by various carbohydrates, appears to be governed by the following general rules The specificity of the induced carbohydrase reflects the structural characteristics of that sugar involved in the C-1–O linkage which constitutes the inducing site With polysaccharides, the initial inducing site is at or near the non-reducing ends of chains From both previous and present studies, it is difficult to predict the specificity of the first induced enzyme where one sugar type, with variation in configuration of the anomeric linkage, constitutes the non-reducing ends of the chains. Thus, with Type II *Pneumococcus* polysaccharide, the β-L-Rhap-(1→3)-β-L-Rhap-(1→3)-α-L-Rhap-(1→4)-X sequence first induced an α-L-rhamnosidase and then a β-L-rhamnosidase Again, methyl β-maltoside induces a β-D-glucosidase not an α-D-glucosidase, whereas isopropyl α-cellobioside induces both α- and β-D-glucosidases What emerges from the present studies is the narrow specificity of the induced carbohydrases as regards their enzyme action on a range of carbohydrates This specificity is generally confined to the sugar (*e g*, D-glucose) and the anomeric character (α or β) of the linkage However, the β- and α-D-glucosidases induced by cellobiose or maltose, respectively, exhibit action on a range of carbohydrates containing β- and α-D-glucosidic linkages even where these occur together in the same substrate αα-Trehalose appears somewhat unique in producing an α-trehalase, rather than a more widely acting α-D-glucosidase. In structural studies, these enzymes should therefore prove very useful, with the proviso that the technique of sequential enzyme induction, while providing evidence in many cases as to the sequence of sugars in polymers and the anomeric character of the linkages joining them, does not give any evidence as to point of attachment Thus, as illustrated here, the α-D-glucosidase induced by maltose breaks α-(1→4)- and α-(1→6)-links, and the β-D-glucosidase induced by cellobiose breaks β-(1→4)- and β-(1→3)-links.

Even where genetically, as with *K aerogenes*, the cell can make both α- and β-D-glucosidases, it does not respond in every case to potential inducers containing such D-glucosidic linkages Thus, neither methyl α-D-glucopyranoside nor phenyl α-cellobioside acted as inducers of extracellular enzymes

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SUMMARY

The α-D-glucosidase induced in *K aerogenes* by maltose had no β-D-glucosidase or α-D-mannosidase activity, but did attack α-D-glucosidic links in maltose, methyl β-maltoside, isomaltose, αα-trehalose, and methyl α-D-glucopyranoside The β-D-

glucosidase induced by cellobiose had no α-D-glucosidase or β-D-galactosidase activity, but hydrolysed the β-D-glucosidic links in methyl β-maltoside, cellobiose, methyl β-D-glucopyranoside, and laminaribiose A specific α-trehalase was induced by αα-trehalose, this did not attack other α-D-glucosidic links Whereas methyl β-maltoside induced only a β-D-glucosidase, isopropyl α-cellobioside induced both an α- and β-D-glucosidase Methyl α-D-glucopyranoside and phenyl α-cellobioside were not inducers

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Notes

A comparative study of some animal brains with regard to content of acidic mucopolysaccharide

Acidic mucopolysaccharides are found in animal body fluids and in a great variety of animal organs[1], connective tissue contains relatively large proportions[2] of these substances Their precise biological function is still not certain, and it was thought that information on this role might be obtained from a study of mucopolysaccharide distribution in organs, such as the brain, which have a low content of polysaccharide but which have biochemically important functions The present work describes a comparative study of acidic mucopolysaccharides isolated from bovine, Japanese monkey (*Macaca fuscata yabui*), pig, and rabbit brains

EXPERIMENTAL AND RESULTS

Fresh, whole brains were kept frozen and ground in a meat grinder. The mixture was suspended in water and digested for 48 h with Pronase-P (45,000 p.u k /g) at pH 7 0 in the presence of toluene at 50° The isolation and purification of acidic mucopolysaccharides were carried out according to Meyer's procedure[2]; the yields were 0 07 to 0 22% of fresh tissue, in good agreement with reported data[3-5]. The mixed glucosidase induced by cellobiose had no α-D-glucosidase or β-D-galactosidase activity, but hydrolysed the β-D-glucosidic links in methyl β-maltoside, cellobiose, methyl β-D-glucopyranoside, and laminaribiose A specific α-trehalase was induced by αα-trehalose, this did not attack other α-D-glucosidic links Whereas methyl β-maltoside induced only a β-D-glucosidase, isopropyl α-cellobioside induced both an α- and β-D-glucosidase Methyl α-D-glucopyranoside and phenyl α-cellobioside were not inducers

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EXPERIMENTAL AND RESULTS

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Characterization was carried out on the basis of analytical results and infrared spectra. Samples 1 and 5 were digestible with testicular hyaluronidase, and paper-chromatographic examination of the product revealed oligosaccharides which corresponded to those produced from hyaluronic acid of umbilical cord[7]. In addition to the evidence mentioned above, the i.r. spectra of samples 1 and 5 showed no absorption for S=O vibration, and the amino-sugar component was 2-amino-2-deoxy-D-glucose,

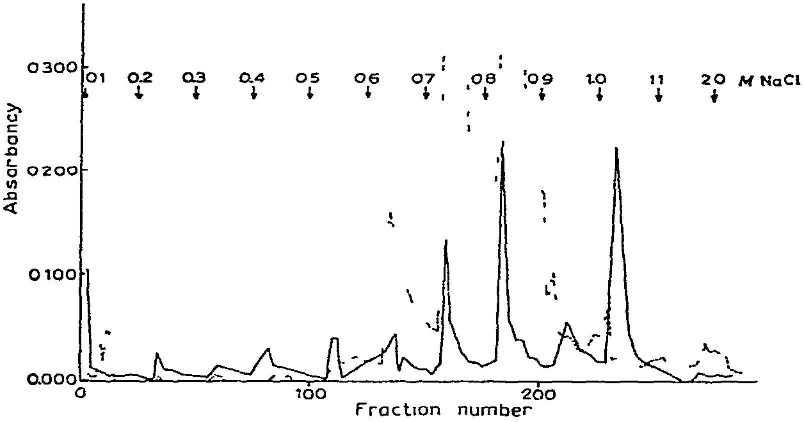

Fig. 1. Fractionation (DEAE-Sephadex A-25 column) of the mixed, acidic mucopolysaccharides isolated from pig brain. The sample (20 mg) dissolved in water (2 ml) was added to the top of a DEAE-Sephadex A-25 (Cl$^-$ form) column (2 × 18 cm) and eluted with sodium chloride (stepwise gradient from 0 1 M). Fractions (5 ml) were taken at the rate of about 1 ml/min. Each fraction was analyzed for uronic acid at 660 mμ with the carbazole method[11] (————), for hexosamine at 530 mμ with the Elson–Morgan method[13], and for nucleic acid at 260 mμ (— — — —). The analytical pattern for hexosamine was similar to that for uronic acid.

TABLE I

ANALYTICAL DATA FOR ACIDIC MUCOPOLYSACCHARIDES ISOLATED FROM SOME ANIMAL BRAINS

	Brain	*Method of fractionation*[a]	*Uronic acid (%)*[b] *Carbazole*	*Orcinol*	*Hexosamine (%)*[c]	*Hexose*[d] *(%)*	*N (%)*	*S (%)*
1	bovine	30% ethanol	40 6	29 0	36 5 (GN + trace of GalN)		3 84	0 94
2	bovine	35% ethanol	35 5	22 1	26 0 (GalN)		2 89	5 36
3	bovine	40% ethanol	37 5	22 0	28 6 (GalN)		2 80	5 65
4	monkey	30% ethanol	20 5		17 3 (GalN + trace of GN)		4 20	+[e]
5	monkey	40% ethanol	26 4		21 6 (GN)		3 40	—
6	pig	0 8M NaCl	23 4	16 9	20 3 (GalN)	6 9	4 13	+
7	pig	1 0M NaCl	25 2	18 0	17 7 (GalN)	6 9	3 67	+
8	rabbit	acetone	26 4	13 8	29 3 (GalN)	6 7	2 93	+

[a] For origins of these fractions, see text (all samples were analyzed as the Ca salt) [b] Uronic acid was determined by the carbazole[15] and orcinol[16] methods, and calculated as D-glucuronic acid [c] Hexosamine was determined by the Elson–Morgan method[17], and calculated as 2-amino-2-deoxy-D-glucose The nature of hexosamine was determined by conversion into the corresponding pentose

together with a trace of 2-amino-2-deoxy-D-galactose Samples 1 and 5 were thus characterized as hyaluronic acid

Samples 2, 6, and 7 showed i r absorption for $S=O$ (strong) and $C–O–S$ in axial orientation (weak)[8]. The only amino-sugar component was 2-amino-2-deoxy-D-galactose Thus, samples 2, 6, and 7 are identified as chondroitinsulfuric acid A (chondroitin 4-sulfuric acid) The existence of 6 9% of hexose in samples 6 and 7 indicates the presence of other substances in the fraction Samples 3, 4, and 8 showed i r absorption for $S=O$ (strong) and $C–O–S$ in equatorial orientation (weak) The amino-sugar component of samples 3, 4, and 8 was 2-amino-2-deoxy-D-galactose, but a trace of 2-amino-2-deoxy-D-glucose was present in sample 4 Samples 3, 4, and 8 are believed to be chondroitinsulfuric acid C (chondroitin 6-sulfuric acid) It is considered that sample 4 may be contaminated with heparin or heparitinsulfate, or both, from the evidence given by the positive specific rotation and by the presence of trace amounts of 2-amino-2-deoxy-D-glucose Table II shows the distribution of acidic mucopolysaccharides in some animal brains

Hyaluronic acid was found in the bovine and monkey brains, but not in the pig and rabbit brains. Chondroitinsulfuric acid A was found in the bovine and pig brains, but not in the monkey and rabbit brains Chondroitinsulfuric acid B was not found in any of the brains examined This was confirmed by uronic acid analyses, with the carbazole and orcinol methods, of the samples which showed i r. absorption for axial $C–O–S$ Chondroitinsulfuric acid C was found in the bovine, monkey, and rabbit brains, but not in the pig brain. The acidic mucopolysaccharide isolated from rabbit brain was characterized as chondroitinsulfuric acid C. In addition to these acidic mucopolysaccharides, other substances were present in the fractions isolated from

$[\alpha]^{19°}_{D}$ (c 1.0, water)	ν_{max} (cm^{-1})^f				
	OH	*COO⁻*	*S=O*	*C–O–S*	*Phase*
−62	3250–3350(s)	1610–1640(s)	—	—	KBr
−33	3300–3400(s)	1610–1670(s)	1220–1280(s)	855(w)	KBr
−25	3200–3350(s)	1590–1670(s)	1220–1280(s)	825(w)	KBr
+10	3300–3400(s)	1640–1680(s)	1220–1240(s)	830(w)	Nujol
−20	3100–3300(s)	1620–1660(s)	—	—	Nujol
−17	3300–3400(s)	1600–1660(s)	1220–1260(s)	860(w)	Nujol
− 7	3000–3500(s)	1560–1680(s)	1220–1280(s)	850(w)	Nujol
−13	3200–3400(s)	1600–1660(s)	1220–1260(s)	830(w)	Nujol

derivative[18]. GN, 2-amino-2-deoxy-D-glucose, GalN, 2-amino-2-deoxy-D-galactose. [d]Hexose was determined by the anthrone method[19], and calculated as D-galactose. [e]Some fractions were not determined quantitatively, and the presence of SO_4^- group was confirmed from the i.r. spectrum. [f]s, strong, w, weak.

bovine, monkey, and pig brains[3,9–11]. These substances contained carbohydrates (9–22%) and N (7–12%), the i.r. spectra showed weak or moderately strong S=O absorption. It thus seems that these may be mixtures containing other substances, such as sulpholipids. Therefore the substances were not considered as acidic mucopolysaccharides.

TABLE II

THE DISTRIBUTION OF ACIDIC MUCOPOLYSACCHARIDES IN SOME ANIMAL BRAINS (%)[a]

Brain	*Experiment No.*	*Hyaluronic acid*	*Chondroitinsulfuric acids*	
			A	*C*
Bovine	*4*	19	61	20
Monkey[b]	*1*	78	—	23
Pig	*2*	—	100	—
Rabbit[c]	*2*	—	—	100

[a]The ratio of each of the amounts of hyaluronic acid, and chondroitinsulfuric acids A and C to the total amount of acidic mucopolysaccharides isolated from the corresponding animal brain.
[b]Obtained from a male, adult monkey.
[c]Obtained from male rabbits, aged 8–12 months.

No significant differences with regard to sex were observed with the content and nature of acidic mucopolysaccharides for bovine brain. Our analytical results for bovine brain are in good agreement with reported data[4], although the presence of chondroitinsulfuric acid C was not previously recognised. The acidic mucopolysaccharides isolated from sheep and rat brains consisted of hyaluronic acid, chondroitinsulfuric acid A, and heparin or chondroitinsulfuric acid C, together with a trace of chondroitinsulfuric acid B or heparitinsulfuric acid[12] It is of interest to note that human brain contains mainly hyaluronic acid (15–60%) These data indicate that there is a pattern for the distribution of acidic mucopolysaccharides in animal brains, which is characteristic for the species as well as for some diseases Further studies on other animal brains, and establishment of the analytical method, will be necessary to confirm these observations Also, the age of animals may be of significance in relation to the differing distribution of acidic mucopolysaccharides

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Laboratory of Biochemistry,
Department of Agricultural Chemistry,
Kyoto University,
Kyoto (Japan)

Konoshin Onodera
Shigehiro Hirona
Fukashi Horiuchi
Naoki Kashimura

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The synthesis of D-ribose-*3-t*

Blakley and Barker[1,2] demonstrated that (5-deoxyadenosyl)cobalamin (coenzyme B_{12}) is required as a cofactor for the enzymic reduction of ribonucleoside triphosphates to 2-deoxyribonucleoside triphosphates by cell-free extracts of *Lactobacillus leichmannii*. This reaction may be visualized[3] as a dioldehydrase type of reaction of the ribonucleotide to the corresponding 2-deoxy-3-pentulonucleotide, followed by a reduction to the 2-deoxyribonucleotide During the conversion, by the dioldehydrase reaction, of 1,2-propanediol into propionaldehyde, the hydrogen atom that replaces the secondary hydroxyl group of the diol does not come from the solvent, but is one of the hydrogen atoms linked to C-1 of 1,2-propanediol. If a similar transfer of hydrogen occurs during the ribonucleotide reductase reaction, it would be expected that the hydrogen atom linked to C-3 displaces the hydroxyl group on C-2. Thus, one of the hydrogen atoms linked to C-2 of the 2-deoxyribonucleotide would have been derived from the hydrogen atom linked to C-3 of the ribonucleotide. The experimental verification of such a mechanism requires the synthesis of ribonucleotides labeled with tritium or deuterium at C-3

This communication describes the synthesis of D-ribose-*3-t* from 1,2 5,6-di-*O*-isopropylidene-α-D-glucofuranose (**1**) Compound **1** was oxidized to 1,2 5,6-di-*O*-isopropylidene-α-D-*ribo*-hexofuranos-3-ulose (**2**) with methyl sulfoxide and acetic anhydride as described by Sowa and Thomas[4] Reduction of **2** with sodium borohydride-*t* afforded 1,2 5,6-di-*O*-isopropylidene-α-D-allofuranose-*3-t* (**3**) Selective removal of the 5,6-*O*-isopropylidene group with mineral acid yielded 1,2-*O*-isopropylidene-α-D-allofuranose-*3-t* (**4**) Compound **4** was oxidized with sodium periodate to 1,2-*O*-isopropylidene-α-D-*ribo*-pentodialdo-1,4-furanose-*3-t* (**5**) Reduction of **5** with sodium borohydride to 1,2-*O*-isopropylidene-α-D-ribofuranose-*3-t* (**6**), followed by mild hydrolysis with acid, yielded D-ribose-*3-t* (**7**).

In the present study, it was found that removal of the 5,6-*O*-isopropylidene group from **3** with 0 15N sulfuric acid is virtually complete after 2 h at room temperature That both of the isopropylidene groups are not hydrolyzed under these conditions is indicated by the fact that only a trace of D-allose is liberated Hydrolysis with 0 15N sulfuric acid for 22 h, as described by Schmidt[5] for the preparation of 1,2-*O*-isopropylidene-α-D-glucofuranose, yields a mixture of D-allose (53%) and 1,2-*O*-isopropylidene-α-D-allofuranose (47%)

EXPERIMENTAL

Sodium borohydride-*t* (100 mc, 26 mg) was purchased from New England Nuclear Corporation Hexamethyldisilazane was obtained from Applied Science Laboratories, Inc, and chlorotrimethylsilane from Aldrich Chemical Company, Inc Compound (**1**) was prepared by the method of Schmidt[5]

Infrared absorption spectra were determined with a Perkin–Elmer Model 52 spectrometer. Radioactivity was measured in a Packard Model 3003 Tri-Carb liquid scintillation spectrometer. Aqueous samples were counted in the scintillation solvent of Bray[6], the counting efficiency was 36%. For gas chromatography of the trimethylsilyl derivatives[7], an Aerograph Hy Fi Model 600-D with a hydrogen flame ionization detector, manufactured by Wilkens Instrument and Research, Inc., was used. The 5-ft column was packed with 10% poly(ethylene glycol sebacate) on Gas-Chrom Q supplied by Applied Science Laboratories, Inc. Silica gel (0.05–0.20 mm), Merck, was used for column chromatography. Melting points were taken on a hot stage equipped with a microscope, and are not corrected.

1,2:5,6-Di-O-isopropylidene-α-D-allofuranose-3-t (**3**). — A solution of 5.0 g (19.7 mmoles) of **2** in 200 ml of 70% (v/v) ethanol was adjusted to pH 8 with N sodium hydroxide. The solution was cooled, and treated with 26 mg (100 mc) of sodium borohydride-*t*. After 1 h, 5.8 g of sodium borohydride was added, and the reaction was allowed to proceed for an additional 30 min. The reaction mixture was then poured into 300 ml of water and extracted with ethyl acetate (8 × 200 ml). The combined extracts were dried with sodium sulfate, and concentrated to a syrup (5.4 g). The desired product was crystallized from benzene–petroleum ether (2.5 g, 49% yield), m.p. 72–75° (lit.[4], 77–78°), specific activity, 3.64 μc/μmole.

1,2-O-Isopropylidene-α-D-allofuranose-3-t (**4**). — A solution of 2.0 g (7.4 mmoles) of **3** in 10.4 ml of methanol was treated with 10.4 ml of 0.3N sulfuric acid, and allowed to stand for 2 h at room temperature. Gas chromatography of the trimethylsilyl derivatives of the products of hydrolysis showed **4**, and traces of D-allose and **3**. The solution was neutralized with barium carbonate, and the suspension was boiled, and filtered through Celite. The filtrate was evaporated under diminished pressure to a syrup, which was crystallized from methanol–ether, yield 1.4 g (87%), m.p. 130–132° (lit.[8], 133–134°), specific activity, 3.65 μc/μmole.

*1,2-O-Isopropylidene-α-D-*ribo*-pentodialdo-1,4-furanose*-3-t (**5**). — A solution of 1.2 g (5.55 mmoles) of **4** in 12 ml of water was treated with 12 ml of 0.5 M sodium periodate, and allowed to stand for 8 h at room temperature. Approximately 100 ml of methanol was added, and the solution was kept overnight at −10°. Sodium iodate, which had crystallized out of solution, was filtered off, and the filtrate was evaporated to dryness under diminished pressure. The residue was dissolved in a small volume of methanol, the solution was filtered to remove a small amount of sodium iodate, and the filtrate was evaporated to a syrup (1.3 g). Gas chromatography of the trimethylsilyl derivative showed that the product contained only a trace of impurity.

1,2-O-Isopropylidene-α-D-ribofuranose-3-t (**6**). — To 1.3 g of **5** dissolved in 25 ml of 70% (v/v) ethanol, was added 0.5 g of sodium borohydride. After being kept for 1 h at room temperature, the solution was neutralized by adding Rexyn RG-57 (H$^+$). The mixture was filtered, and the filtrate was evaporated to dryness. The residue was dissolved in 25 ml of methanol, and the solution was evaporated to dryness to remove methyl borate; this procedure was repeated twice. The residue was crystallized from benzene–petroleum ether; yield 0.733 g (69%), m.p. 86–88° (lit.[9], 86–87°), specific activity 3.67 μc/μmole.

In a subsequent preparation, the mother liquor was evaporated to dryness, the residue was dissolved in benzene–methanol (9·1, v/v), and the solution was applied to a column of dry silica gel (30 g) Elution with benzene–methanol (9 1, v/v) and crystallization from benzene–petroleum ether gave an additional crop of **6**, to raise the yield to 92%

D-*Ribose*-3-t (7) – To a solution of 0 67 g (3 5 mmoles) of **6** in 20 ml of methanol was added 20 ml of 0 3N sulfuric acid, and the reaction mixture was heated for 2 h under reflux. The solution was then neutralized with barium carbonate, and the suspension was filtered through Celite The filtrate was evaporated under diminished pressure to a syrup (0 54 g); on gas chromatography of the trimethylsilyl derivatives, the retention times were identical with those of the trimethylsilyl derivatives of authentic D-ribose

Treatment of the product with methanol and sulfuric acid, as described by R. Barker and Fletcher[10], afforded crystalline methyl β-D-ribofuranoside-*3-t* in high yield

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Department of Biochemistry,
College of Medicine,
University of Iowa,
Iowa City, Iowa 52240 (U S A)

H P C HOGENKAMP

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The action of β-amylase on some unusual oligosaccharides

β-Amylase catalyzes the stepwise hydrolysis of maltose from the nonreducing end of a malto-oligosaccharide The nature of its action on native-starch fractions and on oligosaccharides has been summarized by French[1]. More recently, Thoma and Koshland[2] have reported on extensive studies of its actions on methyl α-malto-oligosaccharides

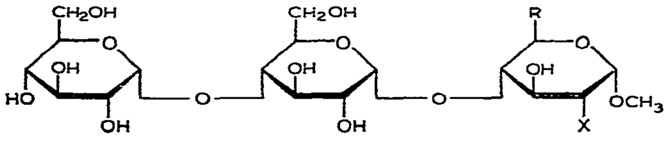
Chemically modified amyloses have also been used as substrates for β-amylase[3 4] The action of the enzyme on such polymers, in which one or more of the D-glucose residues has been modified, could yield information on the nature of those substrate groups that are necessary in the binding or hydrolysis steps The present investigation is concerned with the action of crystalline, sweet-potato β-amylase on some low molecular-weight oligosaccharides containing one pyranoside moiety which differs slightly from a D-glucopyranoside residue These molecules are shown in Figure 1 The trisaccharide **1b** lacks the hydroxymethyl group on C-5 of the methyl α-D-pyranoside residue Tetrasaccharide **2b** lacks the same group The tetrasaccharide **2c** has a methyl 2-deoxy-α-D-*arabino*-hexopyranoside residue A pentasaccharide, methyl *O*-α-maltotetraosyl-(1→4)-2-deoxy-α-D-*arabino*-hexopyranoside, was also used as a substrate for the crystalline enzyme

RESULTS AND DISCUSSION

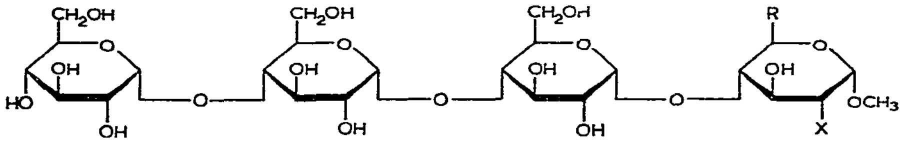
The action of crystalline, sweet-potato β-amylase on small samples of **2b** and **2c** was observed by examining thin-layer chromatograms of the reaction mixtures Both of these products were completely hydrolyzed to maltose and the respective methyl *O*-α-D-glucopyranosyl-(1→4)-α-D-aldopyranoside The pentasaccharide methyl *O*-α-D-maltotetraosyl-(1→4)-2-deoxy-α-D-*arabino*-hexopyranoside liberated maltose and the trisaccharide **1c** When the concentration of enzyme was increased by a factor of one hundred, the **1c** was further cleaved, to maltose and methyl 2-deoxy-α-D-*arabino*-hexopyranoside That this great increase in concentration of enzyme was necessary in order to accomplish this latter hydrolysis is not considered to be particularly important, Peat, Whelan, and Jones[5] have previously demonstrated that low concentrations of β-amylase do not liberate maltose from either maltotriose or unmodified **1a**

The trisaccharide (**1b**) containing the D-xylopyranoside residue was completely resistant to the action of the enzyme, even when 1 9 mg of enzyme was used per 1 0 mg of substrate

Saccharides **2b** and **2c** were compared with unmodified **2a** as substrates for β-amylase by measuring the liberation of maltose Figure 2 shows that the unmodified **2a** is the most readily hydrolyzed substrate, but it also indicates that the enzyme is reasonably active toward **2c** It would seem that the absence of the hydroxyl group at C-2 of the methyl α-aldopyranoside residue does not prevent this modified methyl maltotetraoside from serving as a substrate These data, and the previously demonstrated hydrolysis of **1c**, show that the presence of a hydroxyl group at C-2 is not essential for enzyme action as long as there is a terminal maltose residue that can be liberated.



(1)



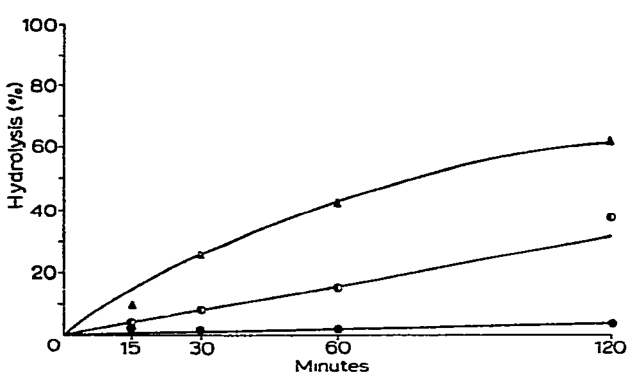
(2)

Fig 1 **1a**, Methyl α-maltotrioside, $R = CH_2OH$, $X = OH$, **1b**, methyl α-maltosyl-(1→4)-α-D-xylopyranoside, $R = H$, $X = OH$, **1c**, methyl α-maltosyl-(1→4)-2-deoxy-α-D-"glucopyranoside", $R = CH_2OH$, $X = H$, **2a**, methyl α-maltotetraoside, $R = CH_2OH$, $X = OH$, **2b**, methyl α-maltotriosyl-(1→4)-α-D-xylopyranoside, $R = H$, $X = OH$, and **2c**, methyl α-maltotriosyl-(1→4)-2-deoxy-α-D-"glucopyranoside", $R = CH_2OH$, $X = H$

Figure 2 also shows that the β-amylase has very little activity on **2b** at the specified concentration of enzyme. When the amylase concentration was increased by a factor of twenty, there was 60% hydrolysis in two hours These data, and the previously demonstrated stability of **1b** in the presence of very high concentrations of β-amylase, suggest that the presence of the hydroxymethyl group at C-5 of the pyranoside residue is much more important to the action of the enzyme than is the hydroxyl group at C-2

The resistance of **1b** to hydrolysis might have been caused by its failure to be bound by the β-amylase However, Figure 3 shows that **1b** serves as a competitive inhibitor of the action of the crystalline, sweet-potato amylase on corn amylose Inhibitions of this sort are usually interpreted as demonstrating reversible binding

The difference in behavior between the two different structural modifications permits the speculation that this may be an illustration of Koshland's[6] "induced fit". It seems possible that the absence of the hydroxymethyl group at C-5 causes a more serious misalignment of the active center of the enzyme than does the absence of the hydroxyl group at C-2 The importance of the hydroxymethyl group has been further shown by the failure of β-amylase to catalyze the hydrolysis of an amylose molecule that we have prepared in which some of the primary hydroxyl groups are methylated Whether this misalignment is a result of simple steric factors or of hydrogen bonding may be at least partially clarified by results obtained with some other modifications now in preparation



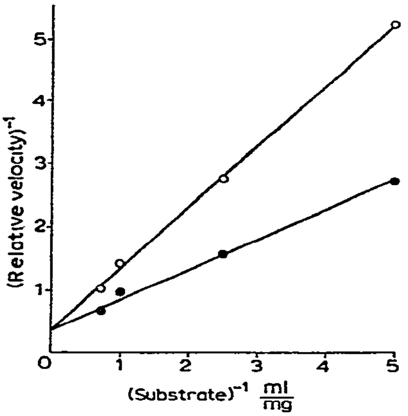


Fig 2 Action of crystalline, sweet-potato amylase on **2a**, **2b**, and **2c** ▲ **2a**, ● **2b**, ◐ **2c** Percent of hydrolysis is calculated as the percent of the theoretical yield of maltose Enzyme concentration is 1 5 × 10^{-4} mg per mg of substrate

Fig 3 Compound **1b** as an inhibitor of the action of crystalline, sweet-potato amylase on amylose, a Lineweaver–Burke plot[10] ● β-amylase on amylose ○ β-amylase on amylose, with a concentration of **1b** of 1 0 mg/ml

EXPERIMENTAL

The corn amylose was a gift of the A E Staley Co (Decatur, Illinois) Crystalline, sweet-potato β-amylase was obtained from Worthington Biochemical Corp (Freehold, New Jersey)

Methyl α-maltotrioside and α-tetraoside were prepared and fractionated by the technique of Pazur, Marsh, and Ando[7]. The modified methyl α-oligosaccharides were prepared by the technique of Wheeler, Hanke, and Weill[8], and were fractionated by the technique of Pazur *et al*[7]. In each case, the isolated fraction was shown to be homogeneous (t l c) by comparison with the series prepared by the method of Wheeler *et al*[8]

Methyl *O*-α-maltotriosyl-(1→4)-α-D-xylopyranoside, **2b**, was obtained in a crystalline and an amorphous form Samples of these and of the methyl *O*-α-maltosyl-(1→4)-α-D-xylopyranoside, **1b**, were sent to Weiler and Strauss (Oxford, England) for analysis

Anal. (**2b**, crystalline) Calc for $C_{24}H_{42}O_{20}$ $2H_2O$ C, 42 0; H, 6.7. Found C, 42 1; H, 6 9 (**2b**, amorphous) Calc for $C_{24}H_{42}O_{20}$ $3H_2O$ C, 41 0; H, 6 8 Found· C, 41 0; H, 6 8 (**1b**) Calc for $C_{18}H_{32}O_{15}\cdot 2H_2O$. C, 41 3, H, 6 9 Found C, 41 4, H, 7 3

T l c experiments were conducted on silica gel G (Warner Chilcott Laboratory, Instrument Division, Morris Plains, New Jersey) The solvent irrigation system was ethanol–ether–toluene (40 50 10, v/v) The colors were developed by spraying with a 5% solution of concentrated sulfuric acid in ethanol, followed by heating in an oven at 105° for 30 min

The t l.c experiments with **1b**, **2b**, **2c**, and the pentasaccharide were conducted at pH 4 8 at 26°, with a substrate concentration of 1 0 mg per ml The enzyme concentration was $3\ 0\times 10^{-4}$ mg per mg of substrate for the experiments with **2b**, **2c**, and the pentasaccharide containing the 2-deoxy-α-D-*arabino*-hexopyranoside residue This concentration was increased to $3\ 0\times 10^{-2}$ mg per mg for the experiments with **1c**, and to 1.9 mg per mg for the experiments with **1b**. At the end of 48 h, the samples were heated in a boiling-water bath, and de-ionized by passage through a column (11×0 9 cm) of Amberlite MB-3 resin (mixed anion- and cation-exchangers; Rohm and Haas Co , Philadelphia, Pennsylvania) The respective effluents were concentrated to a small volume before application to the thin-layer plates

The data for Figure 2 were obtained with a solution containing 0 20 mg per ml of substrate in a 0 016 M acetate buffer of pH 4 8. The β-amylase concentration was $1\ 5\times 10^{-4}$ mg per mg of substrate Reducing values were determined by the method of Hagedorn and Jensen[9], using appropriate conversion factors for maltose

Vacuum-dried amylose was dissolved in a measured, small amount of 2M KOH, and the solution was diluted, and neutralized with acetic acid to pH 4 8. Enough acetate buffer and water were added so that the buffer concentration was 0.016M at pH 4 8, the amylose concentration was 2 mg per ml Enzyme activity was measured on four concentrations of this amylose in a solution which was 0 016 M with respect to acetate buffer of pH 4 8. The enzyme concentration was $1\ 2\times 10^{-4}$ mg per ml Inhibition studies with **1b** were conducted on solutions containing 1 0 mg of **1b** per ml, in addition to the same amylose, buffer, and enzyme as were used in the absence of the inhibitor

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Department of Chemistry,
Rutgers–The State University,
Newark, New Jersey 07102 (U S A)

C EDWIN WEILL
ROBERT REBHAHN

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Preparation and characterization of 2-*O*-(4-*O*-methyl-α-D-glucopyranosyl)-D-xylopyranose

In connection with a study of the acid-catalyzed hydrolysis of (4-*O*-methylglucurono)xylans, it became desirable to ascertain the kinetic parameters for 2-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-D-xylopyranose and its neutral analog, 2-*O*-(4-*O*-methyl-α-D-glucopyranosyl)-D-xylopyranose The latter compound has so far not been available, 2-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-D-xylitol having been used instead for comparison[1].

It has now been found that the fully acetylated aldobiouronic acid can be reduced with diborane in tetrahydrofuran[2-4], giving, after deacetylation, 2-*O*-(4-*O*-methyl-α-D-glucopyranosyl)-D-xylopyranose in an overall yield of 57% This new disaccharide crystallizes readily from water as the trihydrate Unfortunately, this has no sharp melting point, but gradually loses its water of hydration at 95–105° It has $[\alpha]_D^{26}$ +114° (in water). The compound was further characterized through its infrared spectrum and its X-ray diffraction pattern

2-*O*-(4-*O*-Methyl-α-D-glucopyranosyluronic acid)-D-xylopyranose has been obtained from some 40 different plant products Its only known, crystalline derivative is the tetraacetate of its methyl ester, methyl glycoside[5] The ease with which the aldobiouronic acid can be transformed into 2-*O*-(4-*O*-methyl-α-D-glucopyranosyl)-D-xylopyranose now makes possible its identification *via* this crystalline disaccharide also

EXPERIMENTAL

All evaporations were conducted *in vacuo* at 40° Thin-layer chromatography (t l c) was performed with benzene–methanol (9 1) and silica gel H (E Merck) Separations by paper chromatography were effected with (A) ethyl acetate–pyridine–water (8 2 1) and (B) ethyl acetate–acetic acid–water (9 2 2)

2-O-(2,3-*Di*-O-*acetyl*-4-O-*methyl*-α-D-*glucopyranosyluronic acid*)-1,3,4-*tri*-O-*acetyl*-β-D-*xylose*

2-*O*-(4-*O*-Methyl-α-D-glucopyranosyluronic acid)-D-xylopyranose ($[\alpha]_D$ +110° in water) was isolated by partial hydrolysis by acid of a (4-*O*-methylglucurono)xylan from aspen wood[6] Paper chromatography, instead of carbon[7] chromatography, was used in the final purification step The aldobiouronic acid (0 85 g) was boiled under reflux with acetic anhydride (20 ml) in the presence of anhydrous sodium acetate for 4 h After being cooled, the reaction mixture was poured into ice-water (150 ml), and the acetate was extracted with four 100-ml portions of chloroform, to yield 1 1 g (80%) of a syrup, which was almost pure as shown by t l c

2-O-(4-O-*Methyl*-α-D-*glucopyranosyl*)-D-*xylopyranose*

The acetylated aldobiouronic acid (1 1 g) was dissolved in dry tetrahydrofuran

(25 ml) Diborane, generated by adding boron trifluoride etherate to sodium borohydride in bis(2-methoxyethyl) ether (diglyme), was introduced into the solution at room temperature for[8] one h Excess diborane was decomposed by dropwise addition of a 1 1 mixture (v/v) of methanol and tetrahydrofuran, after which the solution was concentrated to dryness, and boric acid was removed as the volatile methyl ester. The reaction product (1 0 g) was deacetylated with methanolic sodium methoxide in the usual way, and was subsequently purified by preparative paper-chromatography (solvent A) The final product crystallized readily from water, giving 0 46 g (57% overall yield), which was recrystallized twice The compound lost water of crystallization at 95–105° (corr.) and had $[\alpha]_D^{26}$ +114° (*c* 2 0, water) Its rates of movement on the paper chromatogram (solvent B), relative to xylose and to the aldobiouronic acid, were 0 76 and 0 94, respectively On acid hydrolysis, the compound gave equimolar amounts of 4-*O*-methyl-D-glucose and D-xylose

Anal Calc. for $C_{12}H_{22}O_{10}$ $3H_2O$. C, 37 96, H, 7 38 Found C, 38 13, H, 7 56 Calc for $C_{12}H_{22}O_{10}$ OMe, 9 56 Found OMe, 9 52

The infrared spectrum of the compound (potassium bromide pellet) exhibited absorption maxima at *ν* 3405, 3215, 2935, 2890, 1665, 1460, 1440, 1380, 1360, 1350, 1290, 1265, 1155, 970, 955, 917, 880, 862, and 775 cm^{-1} X-ray diffraction data*: 13 18 s, 8 36 m, 737 s, 6 65 w, 6 47 w, 5 70 s, 5 30 m, 4 82 s, 4 36 m, 3 89 s, 3 68 m, 3 49 m, 3 19 w, 3 18 w, 2 87 w, 2 67 vw, 2 53 vw, and 2 29 vw.

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Department of Forest Chemistry,
State University College of Forestry,
Syracuse, N Y 13210 (U S A)

NIRMOLENDU ROY
T E TIMELL

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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, v, very

Preliminary communications

Studies on the optical rotatory dispersion of carbohydrate *C*-nitro alcohols. II*

We have previously reported[1] the optical rotatory dispersion curves of some epimeric pairs of carbohydrate *C*-nitro alcohols and discussed the relation between the sign of the Cotton effect and the absolute configuration Our conclusions may be summarized in the following rules

1) Compounds that have the carbon adjacent to the nitromethyl group belonging to the *R*-series of the Cahn's convention[2] exhibit negative Cotton effects, while those having the adjacent carbon of the *S*-series show positive Cotton effects

2) The just-mentioned rule appears to be independent of the nature of the substituent at C-2, because replacement of the 2-hydroxyl group by a 2-acetamido group does not affect the sign of the Cotton effect

3) The absolute configurations of other asymmetric centers do not contribute to the sign of the Cotton effect

These rules are illustrated by the o r d curves of the following compounds 1-deoxy-1-nitro-D-mannitol (**1**)[3], 1-deoxy-1-nitro-D-*glycero*-D-*talo*-heptitol (**2**)[4], 1-deoxy-1-nitro-D-*glycero*-L-*gluco*-heptitol (**3**)[5], 2-acetamido-1,2-dideoxy-1-nitro-D-mannitol (**4**)[6–8], 2-acetamido-1,2-dideoxy-1-nitro-D-*glycero*-D-*talo*-heptitol (**5**)[9], 1-deoxy-1-nitro-D-glucitol (**6**)[3], 1-deoxy-1-nitro-D-*glycero*-D-*galacto*-heptitol (**7**)[4], 1-deoxy-1-nitro-D-*glycero*-L-*manno*-heptitol (**8**)[5], 2-acetamido-1,2-dideoxy-1-nitro-D-glucitol(**9**)[6–8], and 2-acetamido-1,2-dideoxy-1-nitro-D-*glycero*-D-*galacto*-heptitol(**10**)[9]

The reduced intensity of the Cotton effect of compound **9** relative to **4** may be due to the subtle conformational change of the molecular structure surrounding C-2 The curves of all these compounds showed a shoulder (due to the nitro group chromophore) in the 270–290 mμ region of their ultraviolet absorption spectra However, this absorption is not responsible for the Cotton effect of these compounds, which has the first extremum in the 340-mμ region. Besides the absorption band in the 270–290 mμ region, a second, longer-wavelength absorption band of the nitro group chromophore around 330 mμ has been identified by means of the circular dichroism curves of nitro steroids[10]. With compounds **9** and **10**, the absorption band near 320 mμ was also indicated by the c d curves as shown in Figure 2. The Cotton effect of carbohydrate *C*-nitro alcohols illustrated in Figure 1 is associated with this band The absolute configuration of compounds **1**, **2**, **3**, and **4** at C-2 is *R*, and that of **6**, **7**, **8**, and **9** at C-2 is *S*

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* This work was presented at the 85th Annual Meeting of the Pharmaceutical Society of Japan in Tokushima on October 29th, 1965

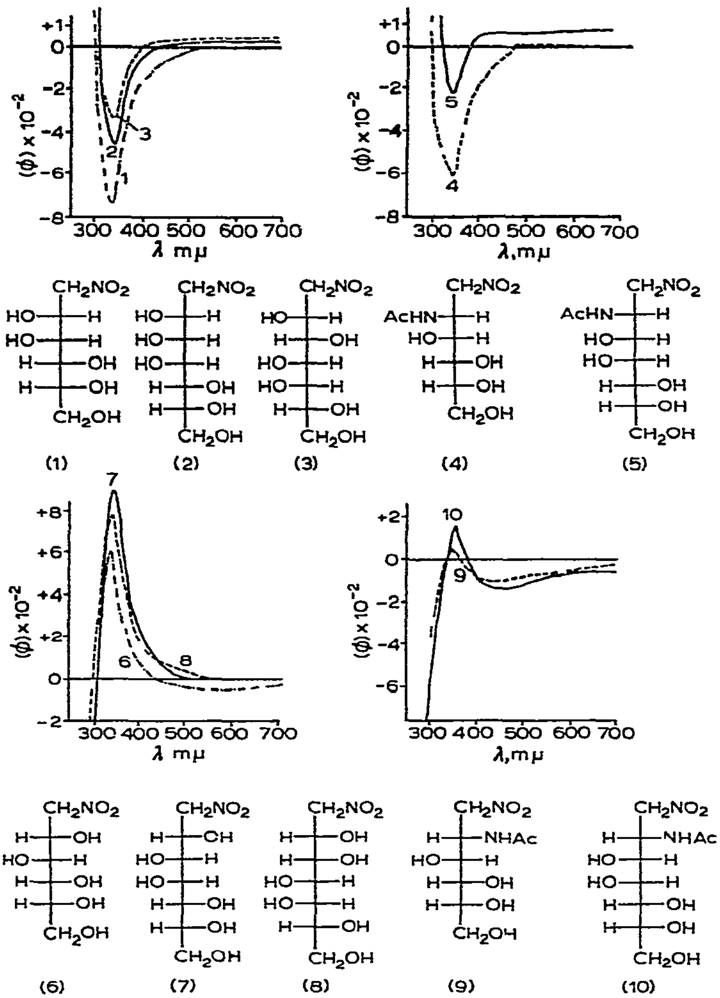


Fig 1 Optical rotatory dispersion curves of carbohydrate *C*-nitro alcohols The curves were determined on water solutions with an automatically recording spectropolarimeter (JASCO Model-ORD/UV-5) made by Japan Spectroscopic Manufacturing Co (Nippon Bunko)
$[\phi] = [\alpha] \times M/100$, molecular rotation

In the figure, the absolute configuration at C-2 of the epimeric pair of 2-acetamido-1,2-dideoxy-1-nitro-D-heptitols (**5** and **10**) is shown only tentatively, based on the analogy to the corresponding D-hexitols (**4** and **9**) The present report presents unequivocal evidence for the predicted configuration of 2-acetamido-1,2-dideoxy-1-nitro-D-*glycero*-D-*talo*-heptitol and 2-acetamido-1,2-dideoxy-1-nitro-D-*glycero*-D-*galacto*-heptitol, and adds another example to support the rules just established.

The 2-acetamido-1,2-dideoxy-1-nitro-D-heptitols (**5** and **10**), in which only the absolute configuration at C-2 had to be determined, were synthesized[9], and their o r d curves were examined Based on the above rules, the structure of 2-acetamido-

1,2-dideoxy-1-nitro-D-*glycero*-D-*talo*-heptitol (**5**) was predicted for one of the two epimeric heptitols that exhibited a negative Cotton effect, whereas the structure of 2-acetamido-1,2-dideoxy-1-nitro-D-*glycero*-D-*galacto*-heptitol (**10**) was predicted for the other epimer, which showed a positive Cotton-effect curve

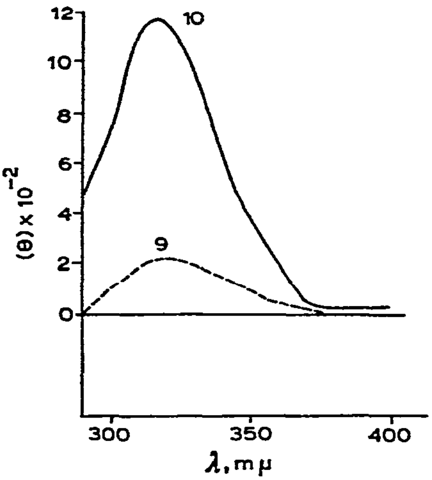
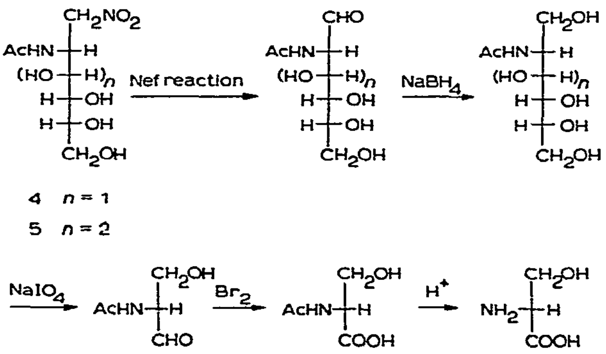


Fig 2 Circular dichroism curves of 2-acetamido-1,2-dideoxy-1-nitro-D-glucitol (**9**) and 2-acetamido-1,2-dideoxy-1-nitro-D-*glycero*-D-*galacto*-heptitol (**10**)
The curves were determined on water solutions with an automatically recording spectropolarimeter (JASCO Model ORD/UV-5) made by Japan Spectroscopic Manufacturing Co (Nippon Bunko) [θ], molecular ellipticity

In order to confirm these structures, the chemical determination of the absolute configuration at C-2 of compound (**5**) was performed according to the sequence of reactions illustrated in the following scheme D-Serine is the final product of degradation of **5** As a model experiment, **4** was subjected to the reactions illustrated in the scheme The absolute configuration of **4** at C-2 had already been established by the isolation of 2-acetamido-2-deoxy-D-mannose from **4** after treatment according



to Nef[6] The final product resulting from the degradation of **4** was shown to be D-serine, thus indicating that no inversion occurred throughout the whole process.

In the degradation of **5**, the yield from the first reaction was about 60%, whereas the yields from the other steps were quantitative The product of the last reaction was placed on a column of Dowex-50 (H^+) and eluted with 5% ammonium hydroxide The effluent was evaporated to a crystalline residue Recrystallization from ethanol–water gave colorless needles, dec. 225°, $[\alpha]_D^{23}$ −15 6° (*c* 5 237, N HCl).

Anal. Calc for $C_3H_7NO_3$ C, 34 28, H, 6 71, N, 13 33 Found C, 34 57; H, 6 29; N, 13 23

The infrared spectrum and paper-chromatographic properties [R_F 0 28, 1-butanol–acetic acid–water (4 1.2)] of this product were indistinguishable from those of D-serine (Sigma Chemical Co , St Louis, U S A) This isolation of D-serine indicates that the starting heptitol is 2-acetamido-1,2-dideoxy-1-nitro-D-*glycero*-D-*talo*-heptitol, as was predicted from the rules Thus, the rules describing the relation between the absolute configuration at C-2 and the sign of the Cotton effect are also valid for this epimeric pair of deoxynitroheptitols

We thank Dr T. Okuda for valuable discussions

Biological Reasearch Laboratory,
Tanabe Seiyaku Co , Ltd ,
Toda, Saitama (Japan)

CHIYOKO SATOH
AKIO KIYOMOTO

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Carbonyl forms of sugars

In 1926–7, Levene and Meyer prepared the aldehyde sugars, penta-*O*-methyl-D-glucose[1], -galactose[2], and -mannose[2], by methylation of the aldose diethyl dithioto Nef[6] The final product resulting from the degradation of **4** was shown to be D-serine, thus indicating that no inversion occurred throughout the whole process.

In the degradation of **5**, the yield from the first reaction was about 60%, whereas the yields from the other steps were quantitative The product of the last reaction was placed on a column of Dowex-50 (H^+) and eluted with 5% ammonium hydroxide The effluent was evaporated to a crystalline residue Recrystallization from ethanol–water gave colorless needles, dec. 225°, $[\alpha]_D^{23}$ $-15.6°$ (*c* 5.237, N HCl).

Anal. Calc for $C_3H_7NO_3$ C, 34.28, H, 6.71, N, 13.33 Found C, 34.57; H, 6.29; N, 13.23

The infrared spectrum and paper-chromatographic properties [R_F 0.28, 1-butanol–acetic acid–water (4:1:2)] of this product were indistinguishable from those of D-serine (Sigma Chemical Co, St Louis, U S A) This isolation of D-serine indicates that the starting heptitol is 2-acetamido-1,2-dideoxy-1-nitro-D-*glycero*-D-*talo*-heptitol, as was predicted from the rules Thus, the rules describing the relation between the absolute configuration at C-2 and the sign of the Cotton effect are also valid for this epimeric pair of deoxynitroheptitols

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Biological Reasearch Laboratory,
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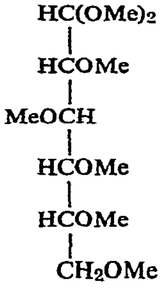
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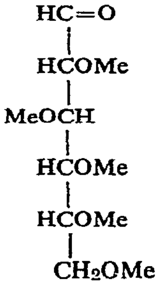
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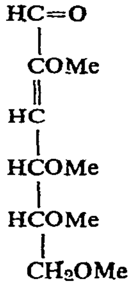
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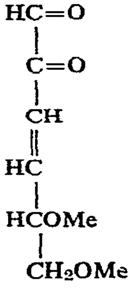
(4)

Penta-*O*-acetyl-D-glucose dimethyl acetal[6] was methylated to yield penta-*O*-methyl-D-glucose dimethyl acetal (**1**), which, on hydrolysis with 0 02N hydrochloric acid for 35 min at 50°, gave penta-*O*-methyl-*aldehydo*-D-glucose (**2**) The physical properties of both products, obtained by this new method, were identical with those reported by Levene and Meyer[1] (see Table I) The acetal **1** was re-formed instantly when **2** was dissolved in methanol containing 1% of hydrogen chloride[1] Owing to the presence of the free aldehyde group, **2** polymerizes rapidly on standing, and is extremely labile towards alkali, in 0 02N lime-water at 20°, it is completely converted in 20 min into 3-deoxy-2,4,5,6-tetra-*O*-methyl-D-*erythro*-hex-*trans*-2-enose (**3**) With 0 1N hydrochloric acid for 9 min at 100°, **3** underwent an allylic rearrangement to 3,4-dideoxy-5,6-di-*O*-methyl-D-*glycero*-hex-*trans*-3-enosulose (**4**), a yellow oil giving a colorless solution in water or alcohols (hydrate, or hemiacetal)

The n m r. spectra of compounds of **2** to **4** in deuteriochloroform and in deuterium oxide were consistent with the assigned structures, and clearly showed that **4** was the *trans* isomer ($J_{3,4}$ 16 c p s), and that, in aqueous solution, the aldehyde groups of **2**, **3**, and **4** were half hydrated, not hydrated, and all hydrated, respectively Other physical properties of these compounds are shown in Table I*

Bayer and Widder[3 4] claimed that the direct methylation of sugars yields not only methyl per-*O*-methylglycosides but also "per-*O*-methylcarbonyl sugars", which showed, on gas–liquid chromatography (g l c), retention times lower than those of the isomeric glycosides, a rather unexpected result The g l c behavior of their "aldehydo glucose" corresponds, however, to that of the enal **3** (see Table I). Their "ketofructose" derivative is clearly not a simple carbonyl compound (ν_{max} 1670 cm^{-1})[5]

When Bayer and Widder[5] attempted to repeat Levene and Meyer's[1,2] preparations, they obtained mixtures of widely varying compositions The infrared spectrum for one of their preparations (from D-glucose) was reproduced in their paper[5], and it

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*Compounds **1** to **4** gave satisfactory elementary analyses

TABLE I

SOME PROPERTIES OF COMPOUNDS **1** TO **4**

Compound	$[\alpha]_D^{25}$(*c* 1), *degrees*	$\lambda_{max}^{H_2O}$ (185–400 mμ)	ν_{max}(1500–1800 cm^{-1})	*Retention times*[a]	
				A	*B*
1	$+15$ (MeOH)[b]	none	none	180	101
2	-35 5($CHCl_3$)[c]	< 190	1728 ($C=O$)	100	114
3	$+38$ 8 (H_2O)	242	1699 and 1642 ($C=C-C=O$)	48	63
4	$+35$ 3 (H_2O)	228	1722 ($C=O$) 1685 and 1629 ($C=C-C=O$)	15	40

[a] Retention times × 100 on g l c, relative to that of methyl tetra-*O*-methyl-α-D-glucofuranoside, *A*, with methylsilicone, and *B*, with poly(ethylene glycol) as the stationary phase[9] The methyl α- and β-D-pyranosides and β-D-furanoside of tetra-*O*-methylglucose gave *A*, 83, 66, and 90, and *B*, 82, 55, and 85, respectively

[b] $[\alpha]_D^{20}$ + 15 09° (*c* 4 44, methanol)[1]

[c] $[\alpha]_D^{20}$ − 35 1° (*c* 4 7, tetrachloroethane)[1]

indicates that the sample contained both **2** and **3** (*cf* Table 1) The g l c peak assigned by them to **2** could be due to **3**, whereas **2** is probably responsible for their "furanoside peak" In the same paper, there is also reproduced an infrared spectrum of **2** obtained by Stephens and Whiffen[8], who presumably prepared **2** by Levene and Meyer's method The latter spectrum indicates that their sample[8] of **2** was substantially pure, if somewhat polymerized, and was free from **3**

I have repeated the preparation of penta-*O*-methyl-*aldehydo*-D-galactose by the method of Levene and Meyer[2], and have confirmed their results The g l c behavior of the product corresponded to that of the "α-D-septanoside" peak[5] of Bayer and Widder The "pure aldehydo galactose" compound of Bayer and Widder is probably an unsaturated carbonyl compound

E F. L J. Anet

Commonwealth Scientific and Industrial Research Organization,
Division of Food Preservation,
Ryde, N S W (Australia)

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The enzymic hydrolysis of 2-acetamido-1-*N*-(4′-L-aspartyl)-2-deoxy-β-D-glucopyranosylamine*

The linkage between the aspartyl and 2-acetamido-2-deoxy-D-glucopyranosyl residues of the title compound is hydrolysed by an enzyme preparation from sheep epididymis[1]. Since the enzyme differed from 2-acetamido-2-deoxy-D-glucopyranosidase, Murakami and Eylar[1] concluded that it is a specific *N*-glycosidase. In a study of a glycopeptide prepared from ovalbumin, we showed that the *O*-glycosidase present in a boar epididymal preparation released 2-acetamido-2-deoxy-D-glucose units from the terminal, non-reducing end of the glycopeptide[2]. We have now observed that this enzyme preparation also hydrolyses 2-acetamido-1-*N*-(4′-L-aspartyl)-2-deoxy-β-D-glucopyranosylamine* giving 2-acetamido-2-deoxy-D-glucose (1 mol., determined colorimetrically[3]), but did not hydrolyse the isomeric 1′-L-aspartyl derivative[4] or 2-acetamido-1-*N*-acetyl-2-deoxy-β-D-glucopyranosylamine[4], thus suggesting that the enzyme is specific for the 4′-aspartyl derivative. The enzyme appears to be equally active at pH 5.0 and 7.7, in contrast to the findings of Murakami and Eylar[1] who reported that their enzyme was more active at pH 7.7. The *N*-glycosidase activity was inhibited by 2-acetamido-2-deoxy-D-glucono-1→5-lactone and diminished in the presence of 2-acetamido-1-*N*-acetyl-2-deoxy-β-D-glucopyranosylamine[4]. The enzyme preparation also hydrolysed the linkage between oligosaccharide and aspartic acid in an ovalbumin glycopeptide. Lee *et al.*[5] reported that sweet almond gradually cleaves the β-D-glucosylamine bond of the asparagin-carbohydrate fragment from ovalbumin. However, our sweet-almond emulsin (California Corporation for Biochemical Research) did not hydrolyse 2-acetamido-1-*N*-(4′-L-aspartyl)-2-deoxy-β-D-glucopyranosylamine over a prolonged period, but, as expected[6], methyl 2-acetamido-2-deoxy-β-D-glucopyranoside was rapidly hydrolysed. Hence, we conclude that pig epididymis contains an *N*-glycosidase that hydrolyses the β-D-glucosylamine linkage in the title compound and in ovalbumin glycopeptide.

We are indebted to Dr. G. A. Levvy, Rowett Research Institute, Aberdeen, for generous supplies of epididymal enzyme preparations.

School of Chemistry and Department of Medicine,
The University,
Bristol (Great Britain)

J. R. CLAMP
G. DAWSON
L. HOUGH
M. Y. KHAN

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*We have used 1′ and 4′ to indicate the α and β carboxyl groups of aspartic acid, because the latter system conflicts with the carbohydrate nomenclature for the anomeric linkage.

Carbohydrate Res., 3 (1966) 254–255

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4 C. H BOLTON, L HOUGH AND M Y KHAN, *Biochem J*, 101 (1966) 184
5 R MONTGOMERY, Y C LEE, AND Y C WU, *Biochemistry*, 4 (1965) 566
6 H BAUMANN AND W PIGMAN, in W PIGMAN (Ed), *The Carbohydrates*, Academic Press, New York, 1957, p 562

(Received July 6th, 1966)

Specific labeling of sugars through enolization of aldosulose derivatives. Synthesis of 1,6-anhydro-β-D-talopyranose*

The advent of convenient, high-yielding routes to aldosulose derivatives, by oxidation of suitably protected aldoses[1–3], offers a potentially useful route, through base-catalyzed enolization, to sugar derivatives that are specifically deuterated or tritiated at positions adjacent to the carbonyl group. Such derivatives are of potential interest for biochemical work, for n m r and mass-spectroscopic studies, and for studies of reaction mechanisms

Exchange of hydrogen atoms, accompanied by epimerization, at both positions adjacent to a carbonyl group, would lead to a mixture of several diastereoisomers Exchange at one of these positions could be prevented if the hydrogen atom were to be at the bridgehead of a strained system of fused rings (Bredt rule) Exchange at the other position could be made essentially stereospecific if one of the resultant two epimers were considerably more stable than the other At equilibrium, a single epimer would preponderate

In the present communication, it is shown that 1,6-anhydro-2,3-*O*-isopropylidene-*β*-D-*lyxo*-hexopyranos-4-ulose[2] (**1**), on treatment with NaOD in deuterium oxide, is deuterated rapidly at C-3 without change of configuration, whereas the hydrogen atom at C-5 does not undergo exchange. Ring strain in the [3 2 1] bicyclic system makes it impossible for C-5 to achieve sp^2 hybridization, so that the intermediate 4,5-enol required for exchange of H-5 cannot be formed Planarity at C-3 can be achieved readily, so that replacement of H-3 is facile The stereospecificity of the replacement at C-3 is predictable since, in a reversible process at equilibrium, the thermodynamically more stable, *cis*-fused 2,3-*O*-isopropylidene acetal (D-*lyxo* 3-epimer) may be expected to preponderate greatly over the less stable, *trans*-fused acetal (D-*arabino* 3-epimer)

The 60-Mc.p s n m r spectrum of the ketone **1** in deuterium oxide was similar to that observed[2] in chloroform-*d* The signals for H-2,3,5, and the *endo* hydrogen at C-6, were incompletely resolved The H-1 signal was observed at τ 4 61 as a narrow triplet, through coupling[2] with H-2 and H-3 The signal of the *exo* hydrogen atom at

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*Presented, in part, at the 4th Gordon Research Conference on Carbohydrates, Tilton, New Hampshire July 1966, see also ref 1

3 W T J MORGAN AND L A ELSON, *Biochem J.*, 28 (1934) 988
4 C. H BOLTON, L HOUGH AND M Y KHAN, *Biochem J*, 101 (1966) 184
5 R MONTGOMERY, Y C LEE, AND Y C WU, *Biochemistry*, 4 (1965) 566
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Exchange of hydrogen atoms, accompanied by epimerization, at both positions adjacent to a carbonyl group, would lead to a mixture of several diastereoisomers Exchange at one of these positions could be prevented if the hydrogen atom were to be at the bridgehead of a strained system of fused rings (Bredt rule) Exchange at the other position could be made essentially stereospecific if one of the resultant two epimers were considerably more stable than the other At equilibrium, a single epimer would preponderate

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*Presented, in part, at the 4th Gordon Research Conference on Carbohydrates, Tilton, New Hampshire July 1966, see also ref 1

C-6 was observed at τ 6.25 as a quartet; it represents the A portion of an ABX system (H-6, 6′, 5). Addition of a small proportion of NaOD to the solution caused rapid (5 min) collapse of the H-1 signal to a sharp doublet, $J_{1,2}$ 3 c.p.s., with concomitant appearance of a one-proton HOD signal. The H-6 (*exo*) signal was unchanged. No further change in the spectrum was observed during 24h. After 24h, the product was recovered, crystalline and chromatographically homogeneous, in good yield. The product had the same X-ray powder diffraction pattern as **1**, and contained one fewer hydrogen atoms than **1** (by n.m.r.). The data indicate that H-3 had been replaced by deuterium with retention of configuration, and that no replacement of H-5 had occurred.

Preliminary experiments on the alkylation of **1** with methyl sulfate and base indicate that, in this kinetically controlled process, both 3-epimeric 3-*C*-methyl derivatives of **1** are obtained.

Reduction of the ketone **1**, with sodium borohydride in water, or with lithium aluminum hydride in ether, gave 1,6-anhydro-2,3-*O*-isopropylidene-β-D-talopyranose* (**2**), yield 83-87%, m.p. 109°. Reduction of **1** with hydrogen over palladium-on-carbon gave **2** together with a small proportion of 1,6-anhydro-2,3-*O*-isopropylidene-β-D-mannopyranose[4], m.p. 160–161°. Treatment of **2** with Dowex-50 (H^+) for 30 min at 30° gave chromatographically homogeneous 1,6-anhydro-β-D-talopyranose (**3**). Hydrolysis of **3** with N hydrochloric acid for 30 min at 80° gave a mixture from which crystalline D-talose was isolated, identical with an authentic sample** by t.l.c., mixed m.p., and X-ray powder diffraction pattern. Substance **3** is the last 1,6-anhydro-D-aldohexopyranose to be described; it had m.p. 185–186°.

Reduction of the 3-deuterated analog of **1** with sodium borohydride gave the 3-deuterated analog of **2**. Direct acetylation of the latter gave the 4-acetate; acetylation after cleavage of the *O*-isopropylidene group gave a 2,3,4-triacetate. The n.m.r. spectra of these products, in comparison with those of the non-deuterated analogs, showed the differences anticipated for the absence of H-3 in the deuterated derivatives. Reduction of **1** with lithium aluminum deuteride gave **2** deuterated at C-4.

Reduction of the oxime[2] of **1** with hydrogen over platinum in the presence of 0.5N hydrochloric acid gave a 4-amino-1,6-anhydro-4-deoxy-β-D-hexopyranose hydrochloride (**4**), yield 70%, m.p. 175–180° (dec.), $[\alpha]_D^{20}$ −68 ±2° (*c* 1.5, water), which, on acetylation with acetic anhydride—sodium acetate, gave the triacetate, m.p. 146°. Substance **4** presumably has the D-*talo* configuration, since it differs from the known[5] D-*manno* analog.

The reactions described provide a route to derivatives of D-talose and D-mannose that are deuterated (or tritiated) at C-3, at C-4, or both positions, and also to branched-chain sugar derivatives having *C*-substituents at C-3. The principle of stereochemical control of base-catalyzed enolization as applied in this work, through appropriate ring fusion and selection of substituent groups, should be amenable to general extension, as, for example, with 1,6-anhydro-3,4-*O*-isopropylidene-β-D-galactopyranose.

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*All of the new, crystalline compounds gave satisfactory elemental analyses.

**The authors thank Dr. R. S. Tipson for a reference sample of D-talose.

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This work was supported, in part, by funds from the National Institutes of Health, U. S. Public Health Service, Department of Health, Education, and Welfare, Bethesda, Maryland 20014; Grant No. GM-11976-02 (The Ohio State University Research Foundation Project 1820A). Funds for the n.m.r. spectrometer were provided by the National Science Foundation, Washington, D. C.

Department of Chemistry,
The Ohio State University,
Columbus, Ohio 43210 (U. S. A.)

D. HORTON
J. S. JEWELL

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(Received August 30th, 1966)

Acid-catalyzed degradation of a 2,3-unsaturated sugar*

The conversion of hexoses into 5-(hydroxymethyl)-2-furaldehyde and levulinic acid by the action of strong mineral acid is well known[1]. This communication describes the sequence of conversions observed when a 2,3-unsaturated sugar of the alkene type[2] is treated with acidic reagents.

Treatment of methyl 4,6-O-benzylidene-2,3-dideoxy-α-D-erythro-hex-2-enopyranoside[4] (1) with aqueous acetic acid for 10 min at 70° has been shown[3] to give quantitative conversion into 2-(D-glycero-1,2-dihydroxyethyl)furan (2); the structure of 2 has been established[1] by degradation, by spectroscopy, and by preparation of derivatives. It is now shown that treatment of 1 with 0.05N ethanolic H_2SO_4 for 10 min at 70° also gives 2; the u.v. spectrum** of the product in ethanol shows a positive

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*Part V in the series Synthesis and Reactions of Unsaturated Sugars. For Part IV, see ref. 2.

**The u.v. spectrum was measured with a Cary Model 15 spectrophotometer, by courtesy of N. Merkel of Applied Physics Corporation, Monrovia, Calif. Thin-layer chromatographic data refer to t.l.c. on Silica Gel G (E. Merck, Darmstadt, Germany) activated at 110°, with 3:1 benzene–ether as the developer and sulfuric acid as the indicator. Elemental analyses were performed by W. N. Rond and by Galbraith Laboratories, Inc. Nuclear magnetic resonance data refer to solutions in chloroform-d with tetramethylsilane (τ 10.00) as internal standard. Spectra were measured by J. S. Hughes with a Varian A-60 spectrometer.

ACKNOWLEDGMENTS

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Department of Chemistry,
The Ohio State University,
Columbus, Ohio 43210 (U. S. A.)

D. HORTON
J. S. JEWELL

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5 S. P. JAMES, F. SMITH, M. STACEY, AND L. F. WIGGINS, *J. Chem. Soc.*, (1946) 625.

(Received August 10th, 1966)

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The conversion of hexoses into 5-(hydroxymethyl)-2-furaldehyde and levulinic acid by the action of strong mineral acid is well known[1]. This communication describes the sequence of conversions observed when a 2,3-unsaturated sugar of the alkene type[2] is treated with acidic reagents.

Treatment of methyl 4,6-O-benzylidene-2,3-dideoxy-α-D-*erythro*-hex-2-enopyranoside[4] (**1**) with aqueous acetic acid for 10 min at 70° has been shown[3] to give quantitative conversion into 2-(D-*glycero*-1,2-dihydroxyethyl)furan (**2**); the structure of **2** has been established[3] by degradation, by spectroscopy, and by preparation of derivatives. It is now shown that treatment of **1** with 0.05% ethanolic H_2SO_4 for 10 min at 70° also gives **2**; the u.v. spectrum** of the product in ethanol shows a positive

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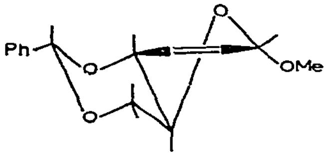
*Part X of a series on Syntheses and Reactions of Unsaturated Sugars. For Part IX, see ref. 2.

**The u.v. spectrum was measured with a Cary Model 14 spectrophotometer, by courtesy of V. Marcus of Applied Physics Corporation, Monrovia, Calif. Thin-layer chromatographic data refer to t.l.c. on Silica Gel G (E. Merck, Darmstadt, Germany) activated at 110°, with 2:1 benzene–ether as the developer and sulfuric acid as the indicator. Elemental analyses were performed by W. N. Rond and by Galbraith Laboratories. The Nuclear magnetic resonance data refer to solutions in chloroform-d with tetramethylsilane (TMS) as internal standard. Spectra were measured by J. R. Hughes with a Varian A-60 spectrometer.

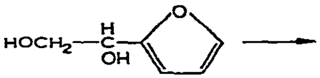
Cotton effect, with an amplitude ([α]×mol wt) of 85,000 and [α] = 0 at 243 and 222 mμ Substance **2** undergoes further conversion by the reagent to give the racemic 1′-ethyl ether **3**, followed by a slower conversion of **3** into the 1′,2′-diethyl ether (**4**) The latter undergoes opening of the furan ring, to give 2,6-diethoxy-4-oxohexanal diethyl acetal (**5**) The reagent converts **5** into a mixture containing a component chromatographically identical with **4**

The stepwise conversions were verified by studies on the individual, isolated products The R_F values** of the products were **1**, 0 75, **2**, 0 10, **3**, 0 40, **4**, 0 81; and **5**, 0 50 Methyl analogs of **3**, **4**, and **5** were prepared from **1** (or **2**) by acid-catalyzed methanolysis.

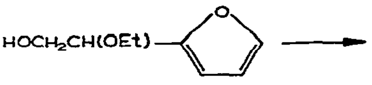
The products were separated by preparative t l c or g l c , and were all obtained as distilled liquids which gave correct elemental analyses**. Substances **3** and **4** showed u v spectra, and n m r ** spectra (aryl protons), very similar to those of **2** and 2-(hydroxymethyl)furan The n m r spectrum of **4** showed the signals anticipated for two ethyl groups, together with the pattern of signals for H-1′ and H-2′ observed with **2** (after deuteration) and with di-*O*-acyl derivatives of **2** Alternative formulations, as 2-ethoxy-1-[5-ethoxy-2(5*H*)-furylidene]ethane or 2-[2,2-bis(ethoxy)ethyl]furan, were not considered probable The n m r spectrum of **3** showed the presence of one ethyl group, and of a hydroxyl group attached to a methylene group Acetylation of **3** caused a downfield shift of the H-2′ signal to the position observed with the diacetate of **2** Substance **5** showed carbonyl absorption in the infrared spectrum, and gave a bis[(2,4-dinitrophenyl)hydrazone], m p 146° The n m r spectrum of **5** showed the presence of four ethyl groups, two of which were exchanged for methyl groups when **5** was treated with methanolic H_2SO_4 A one-proton doublet at τ 5 50, $J_{1,2}$ 5 c p s ,



(1)



(2)



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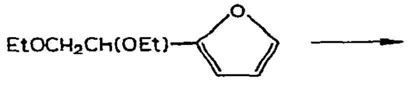

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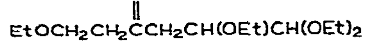

$EtOCH_2CH_2\overset{\overset{O}{\|}}{C}CH_2CH(OEt)CH(OEt)_2$

(5)

in the n m r. spectrum of **5** was assigned to H-1, and a 4-proton multiplet at $\tau \sim 7$ 27 was assigned to the C-3 and C-5 methylene protons

The extreme ease of hydrolysis of the glycoside **1** is attributable to allylic stabilization of an intermediate, C-1 carboxonium ion The facile formation of **3** from **2** presumably proceeds through a stabilized, benzylic type of cation, and complete racemization at C-1′ therefore takes place The conversion of **4** into the ring-opened product **5**, and the apparent reverse process, presumably follows a conventional mechanism observed with furan derivatives[4]

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Department of Chemistry,
The Ohio State University,
Columbus, Ohio 43210 (U.S A)

D HORTON
TSUTOMU TSUCHIYA

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Book review

Conformational Analysis, by E L. ELIEL, N. L ALLINGER, S. J. ANGYAL AND G A. MORRISON, Wiley, New York, 1965, xiii + 524 pages, 113s.

The development of conformational analysis has been one of the major steps in the advance of chemical theory. Particularly for polyfunctional compounds like the carbohydrates, a knowledge of the detailed molecular geometry is vital to the understanding of the interactions which affect reactivity; indeed, carbohydrate chemists were among the first to appreciate the significance of conformation, and it is appropriate that this volume should contain a chapter on carbohydrates The book is the joint work of four well-known experts and, as one would expect from their previous writings, it provides a lucid, authoritative, and stimulating view of the field

After a brief historical introduction, the basic principles of the conformational analysis of acyclic compounds and cyclohexane derivatives are presented in detail The various physical methods for the investigation of conformation are discussed critically in a separate chapter Ring systems other than cyclohexane (both alicyclic and heterocyclic) are then reviewed. There are two chapters on applications to the chemistry of natural products, one on steroids, triterpenoids and alkaloids, and one on carbohydrates In the final chapter, conformational energies in cyclohexane derivatives are tabulated and their *a priori* calculation is discussed.

In all the chapters there is incisive thinking about fundamentals and therefore, despite the rapid development of the subject, this book will remain valuable It is also a unique storehouse of facts, these include unpalatable, inexplicable observations which emphasize the lesson that caution and humility are required in the application of conformational analysis The volume can be warmly recommended to carbohydrate chemists, not only for the excellent chapter on carbohydrates by Professor Angyal, but also for the broad view which it gives of the whole subject

J C P SCHWARZ (Edinburgh)

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SEQUENCE STUDIES ON *Diplococcus pneumoniae* TYPE II POLYSACCHARIDE

S. A. BARKER, P. J. SOMERS, AND M. STACEY
Department of Chemistry The University, Birmingham 15 (Great Britain)

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INTRODUCTION

The recent application[1] of the technique of sequential enzyme induction to Type II *Pneumococcus* polysaccharide revealed that all of the L-rhamnose residues were arranged as [β-L-Rha*p*-(1→3)-β-L-Rha*p*-(1→3)-α-L-Rha*p*-]-sequences at the non-reducing ends of the polysaccharide chains. Although methylation data[2] showed that (*a*) the D-glucose residues were involved in 1,4,6-branch points and (*b*) some of the D-glucuronic acid residues were linked at positions 1 and 4 and the remainder occurred as non-reducing terminal units, the precise arrangement of these residues had not been ascertained. Most of these remaining structural sequences have now been determined by using specific, induced α- and β-D-glucosidases, together with the α-L-rhamnosidase obtained previously.

MATERIALS AND METHODS

Quantitative analysis of carbohydrates. — 6-Deoxyhexoses and hexoses were determined by the cysteine-sulphuric acid reaction[3,4], and hexuronic acids by the modified carbazole–sulphuric acid reaction[5]. For assay of these sugars in periodate-oxidation mixtures, reaction was terminated by addition of ethylene glycol, and a two-fold excess of lead dithionate solution (20%, w/v) was added to enable the standard spectrophotometric determinations to be performed directly on the oxidation products after centrifugation.

Borohydride reduction was sometimes performed prior to assay of a sugar mixture, since any resulting glycitol, whether free or bound, did not react with any of the above assay reagents. The standard reduction procedure was to add to the mixture (0.2 ml) a freshly prepared 1% solution of sodium borohydride in borate buffer (0.05M, pH 8.0, 0.2 ml). After 30 min, excess of borohydride was destroyed by the addition of acetic acid (5%, 0.1 ml). After 5 min with periodic shaking, the standard assays were performed. Formic acid was assayed by the thiobarbituric acid method[6].

Periodate oxidation of Type II polysaccharide (SII). — A solution of SII (prepared as described previously[7], 10.3 mg) and sodium metaperiodate (80.6 mg) in distilled water (25 ml) was kept at 2° in the dark. At intervals, samples (1.2 ml) were assayed by the following procedure. Aliquot portions (3 × 0.05 ml) were diluted to 5.0 ml with distilled water, the optical density at 225 mμ was determined, and the periodate uptake was calculated with reference to standard periodate and iodate controls.

Excess of periodate was destroyed in the remainder of the samples by addition of ethylene glycol (111 mg/ml, 0.05 ml), and assays for rhamnose and glucose (0.025-ml aliquots in triplicate), and glucuronic acid (0.075-ml aliquots in triplicate) were performed. The results are shown in Table I.

TABLE I

PERIODATE OXIDATION OF SII

Time (h)	*Periodate uptake (μmoles/mg of original SII)*	*Composition of product (μg per mg of original SII)*		
		Rhamnose	*Glucose*	*Sodium glucuronate*
0	0.16	478	347	162
1	1.57	374	339	105
2	2.79	327	308	87
3	—	319	293	84
4	3.53	318	264	77
16	4.34	—	—	—
76	4.94	319	102	34
144	5.15	317	057	17
360	5.30	318	010	05

After 360 h, the remaining solution of oxidised SII (*ca.* 14 ml) was treated with ethylene glycol (111 mg/ml, 10 ml) and dialysed against saline (0.9%, 3 × 100 volumes). The non-dialysable portion (20 ml) was reduced by the addition of sodium borohydride (10 × 20 mg at 30-min intervals). The pH of the solution was monitored continuously and kept between 6.0 and 7.0 by the passage of carbon dioxide. After a total of 6 h, the pH was adjusted to 7.0 with acetic acid (5% w/v), and the resulting solution was dialysed against three changes of phosphate buffer (0.05M, pH 7.0) containing saline (0.9%), and lyophilised. The resulting solid was dissolved in water (10 ml), and a portion (5.0 ml) was adjusted to pH 1.0 with hydrochloric acid (6.0N) and hydrolysed at 20°. Samples (1.0 ml) were removed at 4, 8, and 12 h, adjusted to pH 7 with sodium hydroxide (4N), and fractionated on a G-25 Sephadex column (66 ml). Fractions (1.0 ml) were collected and analysed by the cysteine–sulphuric acid reaction. The remaining portion of the oxidised and reduced SII solution (5.0 ml) was hydrolysed at 20° in 0.1N hydrochloric acid for 4 h and fractionated on G-25 Sephadex to remove small molecular species.

The oxidised, reduced, and hydrolysed SII obtained was submitted to a repetition of this sequence. Degraded SII (containing *ca.* 1.5 mg of L-rhamnose) was dissolved in a solution of sodium metaperiodate (3.2 mg/ml, 5 ml) and oxidised for 4 h at 2°. The excess of periodate was destroyed with aqueous ethylene glycol, and the product was reduced with sodium borohydride (10 × 5 mg) as previously described. After hydrolysis in 0.1N hydrochloric acid for 4 h at 20°, the mixture was desalted by fractionation on G-25 Sephadex. A third application of the degradative sequence was performed using the same conditions. At all stages, samples containing 20 to 50 μg of rhamnose were removed and assayed by the cysteine–sulphuric acid method (Table II).

TABLE II

SEQUENTIAL REMOVAL OF RHAMNOSE FROM SII

	Rhamnose content[a] (*μg/mg of original* SII)
SII, original	478
SII, oxidised	318
SII, oxidised, and borohydride reduced	320
SII, oxidised, borohydride reduced, and hydrolysed	317

[a]After 2nd and 3rd applications of degradation sequence, 157 and <2, respectively.

Liberation of formic acid on periodate oxidation of intact and α-L-rhamnosidase-degraded polysaccharide. — SII (16.0 mg) was dissolved in water (10.0 ml) and a preparation[1] of induced α-L-rhamnosidase (25 ml), in phosphate buffer (pH 6.5, 0.02M), was added. The solution was sterilised by membrane filtration and incubated at 37°. Aliquot portions (8.1 ml) were removed at 0, 15, 42, and 65 h. An enzyme blank was similarly treated, and a control solution of SII (2.0 mg) in phosphate buffer (pH 6.5, 0.02M, 5 ml) was included. Portions of each sample (2.0 ml × 4) were cooled to 2°, aqueous sodium metaperiodate (16.0 mg/ml, 0.5 ml) was added, and oxidation was allowed to proceed at 2° in the dark.

For each enzyme-digestion sample, the oxidation was terminated by addition of aqueous ethylene glycol (111 mg/ml, 0.05 ml) to one of the oxidation tubes after 0, 1, 4, and 24 h oxidation. The resulting samples were stored at 2°, and all assays for formic acid were performed simultaneously. Samples of the enzyme and SII controls were obtained after incubation for 0 and 65 h, and oxidised for 0 and 24 h with sodium periodate under similar conditions. Each oxidised sample was concentrated to dryness (by rotary evaporation directly in the oxidation tube), and redissolved in water (0.4 ml). The normal procedure for determination of formic acid was carried out, and the results are presented in Table III.

A portion (2.0 ml) of the remaining solution of digested SII was fractionated on a G-25 Sephadex column (100 ml), and the small molecular-weight fraction was analysed for carbohydrate by the standard procedures. The assays were performed in conjunction with borohydride reduction and periodate oxidation. Rhamnose trisaccharide (302 μg) was detected as the sole product; the rhamnose content after reduction was 210 μg, and after oxidation, 99 μg.

Preparation of carboxyl-reduced Type II polysaccharide.—The sodium salt of SII (150 mg) in H_2O (100 ml) was converted into the free acid by passage through Amberlite IR-120 (H^+, 200 ml) and recovered by lyophilisation. Gaseous ethylene oxide was bubbled into an aqueous solution (50 ml) of the free acid (initial pH, 3.60) for 4 days at 8-10°. The final solution (pH 6.91) was reduced by addition of sodium borohydride (1.0 g) in 20-mg portions over a period of 5 h using carbon dioxide to maintain pH 6.0–6.6. The reduced product was dialysed against distilled water (3 × 100 volumes) and recovered by lyophilisation. The product was redissolved in water (100 ml), and a sample (1.0 ml) was removed for analysis. The remainder was passed through a column of IR 120 (H^+, 200 ml) and concentrated by lyophilisation to 50 ml, and the pH was adjusted from 4.9 to 3.5 with (0.1N) hydrochloric acid. Ethylene oxide gas was bubbled through the solution for 3 days at 10°, and the product was reduced with sodium borohydride (1.0 g) as previously described. After dialysis against distilled water (3 × 100 volumes), a discoloured white powder (132 mg) was obtained by lyophilisation. Spectrophotometric analyses (Table IV) were performed for rhamnose and glucose by the cysteine–sulphuric acid method[3,4], but application of the carbazole method failed to reveal a characteristic spectrum for hexuronic acids.

TABLE III

LIBERATION OF HCOOH ON OXIDATION OF INTACT AND α-L-RHAMNOSIDASE-DEGRADED SII

	Incubation time (h)	*HCOOH (μg) released*				*HCOOH (μg/mg of SII) released after oxidation for 24 h*
		Oxidation time (h)				
		0	1	4	24	
SII	0	0	37	57	58	63.4
Enzyme	15	0	39	63	79	86.4
	42	0	39	66	89	97.4
	65	0	41	68	91	99.6
Enzyme	0	0	—	—	0	—
Control	65	0	—	—	0	—
SII	0	0	—	—	50	62.5
Control	65	0	—	—	51	63.7

TABLE IV

REDUCTION OF D-GLUCURONIC ACID RESIDUES IN SII

	Composition (%)		
	L-*Rhamnose*	D-*Glucose*	*Sodium* D-*glucuronate*
SII, original	47.8	37.7	16.2
SII, 1st reduction	48.4	43.4	6.7
SII, 2nd reduction	49.8	49.7	<0.5

A sample solution of carboxyl-reduced SII was fractionated (Fig 1) on a DEAE A-50 Sephadex column (Cl^-, 2.5 ml). The carboxyl-reduced SII had $[\alpha]^{21}_{\mathrm{D}} + 41.6^\circ$ (*c* 0.1, 0.04N sodium hydroxide).

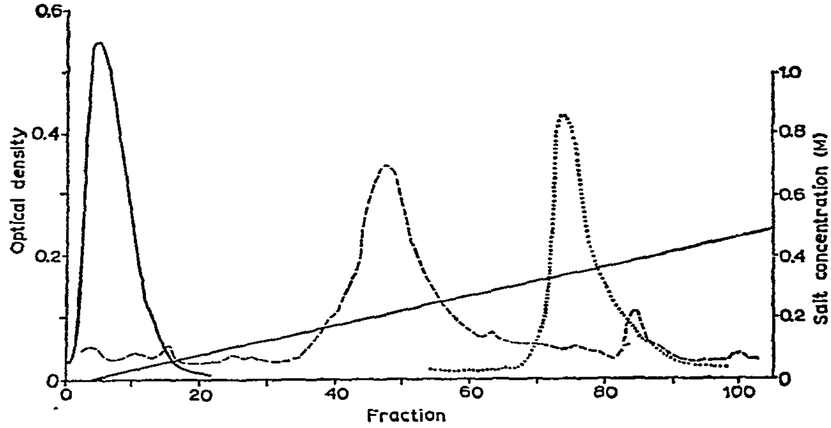


Fig. 1. Comparative fractionation of carboxyl-reduced Type II polysaccharide on DEAE Sephadex A-50 : ————————, carboxyl-reduced SII; — — — — — — —, partially carboxyl-reduced SII; ......, original SII, as analysed by the cysteine-sulphuric acid reaction.

Preparation of induced α- *and* β-D-*glucosidases*. — Resuspended cells of *K. aerogenes* were used to inoculate induction units of media (50 ml) consisting of 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. Solutions of maltose (12.5 mg/ml, 1.0 ml) and cellobiose (12.5 mg/ml, 1.0 ml) were added to separate 50-ml cultures (×4). After incubation for 16 h at 37°, a further addition of each inducer was made at the same level of concentration, and growth was continued for a further 24 h at 37°. The cells were removed by centrifugation (3,000 r.p.m., 30 min) at 2°, and the supernatant solutions were dialysed against phosphate buffer (0.02M, pH 6.5, 3 × 100 volumes).

The non-dialysable fractions were concentrated 10-fold by ultrafiltration and sterilised by membrane filtration. The specificity of the induced enzymes was determined by using aliquot portions (0.5 ml) of the preparation with solutions (0.5 ml) of maltose (100 μg/ml) and cellobiose (100 μg/ml). Analyses were performed by the borohydride-reduction technique, and the results are presented in Table V.

The action of α- *and* β-D-*glucosidase preparations on intact and the carboxyl-reduced polysaccharide*. — Sterile, aqueous solutions of SII (1.0 mg/ml, 1.0 ml) and carboxyl-reduced SII (1.0 mg/ml, 1.0 ml) were mixed with portions of sterile, enzyme preparations (1.0 ml) and incubated for 24 h at 37°. Controls of enzyme solutions without substrate, and of substrate with denatured enzymes, were included. Each digest was fractionated by gel filtration by the standard procedure, and the fractions containing small molecular-weight products were isolated and lyophilised. After being reconstituted with water (1.0 ml), portions (0.2 ml) were analysed by the cysteine-sulphuric acid[3,4] and carbazole reactions[5], before and after reduction with ~~sodium borohydride.~~

TABLE V

SPECIFICITY OF α-AND β-D-GLUCOSIDASE PREPARATION

Substrate	*Inducer*	*Composition of digest (μg)*		*Hydrolysis (%)*
		Total glucose	*Glucose after reduction*	
Maltose	Maltose	38.5	8.82	52.8
Maltose	Maltose[a]	38.3	19.0	—
Maltose	Cellobiose	37.8	19.3	—
Maltose	Cellobiose[a]	38.2	19.1	—
Cellobiose	Cellobiose	38.9	7.81	59.8
Cellobiose	Cellobiose[a]	38.6	19.2	—
Cellobiose	Maltose	45.7	22.5	—
Cellobiose	Maltose[a]	45.2	22.6	—

[a]Heat-denatured enzyme control.

The results of the analyses are presented in Table VI, the compositions being corrected to 1.0 mg of original SII or carboxyl-reduced SII. No carbohydrates were detected in the low molecular-weight fractions from the denatured enzyme or enzyme blank controls. No rhamnose was detectable in any low molecular-weight fraction.

TABLE VI

ACTION OF α-AND β-D-GLUCOSIDASE PREPARATIONS ON SII AND CARBOXYL-REDUCED SII

Substrate	*Enzyme*	*Composition of product (μg)*			
		Direct analysis		*After borohydride reduction*	
		Glucose	*Glucuronic acid*	*Glucose*	*Glucuronic acid*
SII	α	59	57	0	55
	β	0	0	0	0
	$\alpha+\beta$	29	27	0	28
SII (carboxyl-reduced)	α	42.5	0	22.5	0
	β	60.5	0	0	0
	$\alpha+\beta$	40.5	0	0	0

DISCUSSION

The ability to account[1] categorically for the arrangement of *all* of the L-rhamnose units in *Diplococcus pneumomiae* Type II polysaccharide (SII) enables some conclusions to be drawn from analysis of the residual sugars at various stages of periodate oxidation. The oxidation of the terminal non-reducing end group of L-rhamnose is complete in 3 h (Table I), at least as far as the breakage of its vicinal glycol group is concerned. Negligible amounts of glucose and glucuronic acid remain after prolonged periodate oxidation. One interpretation of the 5.3 µmoles of periodate per mg of polysaccharide SII consumed after 360 h is that oxidation of the terminal L-rhamnose units would account for 2.06 µmoles of periodate. The oxidised D-glucose residues, whether linked 1,4,6 or 1,4, would consume 1 mol., of oxidant and would require a further 2.08 µmoles of periodate, leaving 1.16 µmoles of periodate to be consumed by 0.8 µmoles of sodium D-glucuronate residues. Since (1→4)-linked uronic acid residues would consume 1 mol., the remaining periodate would be consumed by non-reducing end groups of D-glucuronic acid (at least 2 moles/mole). After 24 h, 1.42 µmoles of formic acid are liberated per mg of SII, as assayed with thiobarbituric acid[6], which does not react with any oxalic acid that might arise from overoxidation of terminal D-glucuronic acid residues (Table II). Of this, 1.03 µmoles would come from the terminal L-rhamnose residues, leaving 0.39 µmoles from terminal D-glucuronic acid residues (at least 1 µmole of formic acid/µmole of sugar). The postulated 1:1 distribution of terminal and non-terminal D-glucuronic acid residues (Fig. 2) is in fair agreement with these results. The figures for L-rhamnose residues remaining after one, two, and three sequences of periodate oxidation, borohydride reduction, and acid-catalysed removal of the attacked L-rhamnose residues agree with previous work[1] and with the postulated arrangement of these residues.

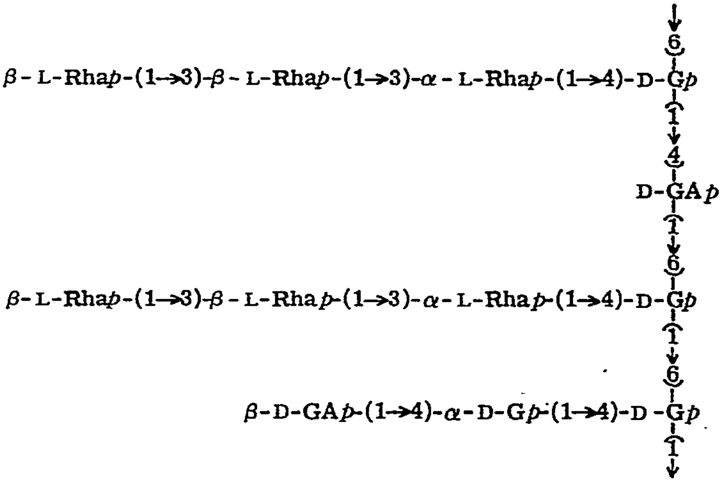
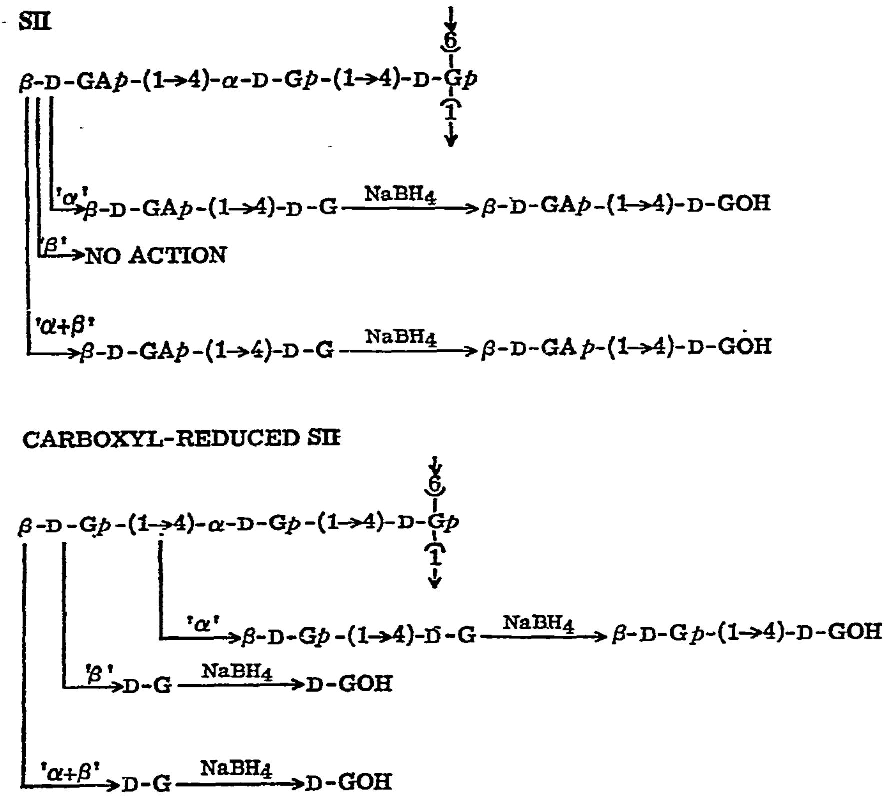


Fig. 2. A possible repeating unit for *Pneumococcus* Type II polysaccharide.

The sequence of three L-rhamnose residues must, on the basis of methylation studies[2] and the results of periodate oxidation, be either (1→4)-linked to a D-glucose or D-glucuronic acid residue, or (1→6)-linked to a D-glucose residue. If this trisaccharide is broken off from SII by treatment with α-L-rhamnosidase, it will expose a new terminal unit, which (*a*), if previously linked through position 4, will give formic acid on periodate oxidation, or (*b*), if previously linked through position 6, would provide no further contribution to formic acid released after periodate oxidation. An increase in the total formic acid obtained on oxidation with increasing time of enzyme degradation would indicate attachment at position 4; no increase indicates attachment at position 6. The results listed in Table III show that the trisaccharide is linked at position 4 of either a D-glucose or a D-glucuronic acid residue, and Fig. 2 pictures the former.



GOH = D-Glucitol, 'α' = α-D-glucosidase, 'β' = β-D-glucosidase

Fig. 3. Action of α- and β-D-glucosidases on SII and carboxyl-reduced SII.

In order to reduce the D-glucuronic acid residues in SII, formation of the 2- hydroxyethyl ester with ethylene oxide, followed by borohydride reduction, was effected. Complete reduction was effected after two such sequences; dilute acid was added to the second-stage esterification to obtain a sufficiently low pH (3·5) for esterification. No glucuronic acid (<0.5%) was detectable (carbazole reaction[5]) in the completely reduced product, and the neutral polysaccharide was not absorbed on DEAE Sephadex (Fig. 1) in contrast to the original and the partially reduced SII.

The resulting polysaccharide {$[\alpha]_D^{21}$ +41.6° (water)} contained 49.8% of rhamnose and 49.7% of glucose (cysteine-sulphuric acid reaction). Fractionation of the polysaccharide, in particular the partially reduced preparation, on DEAE Sephadex revealed quite homogenous preparations suggesting, perhaps, that reduction proceeded at certain carboxyl groups in preference to others (*e.g.*, terminal *vs.* intracatenary); a completely random reduction would be expected to give a very disperse fractionation.

Reference, induced α- and β-D-glucosidases were prepared, and their anomeric specificities were confirmed by using maltose and cellobiose as inducers and substrates. The action of these enzymes on SII and carboxyl-reduced SII was determined, the products being characterised after separation by gel filtration. With SII as substrate, α-D-glucosidase liberated a product containing equal proportions of glucose and glucuronic acid, which, on reduction with sodium borohydride, contained only glucuronic acid. β-D-Glucosidase gave no detectable products with SII. Carboxyl-reduced SII was attacked by both the glucosidase preparations. A disaccharide containing glucose was obtained with α-D-glucosidase, as evidenced by the 50% decrease in glucose content on borohydride reduction. Both β-D-glucosidase and a mixture of α- and β-D-glucosidases gave solely glucose with carboxyl-reduced SII. A summary of these postulated reaction schemes is presented in Fig. 3. These studies provide good evidence for the presence in SII of terminal residues of cellobiouronic acid which could be arranged as postulated in Fig. 2.

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We thank Professor M. Heidelberger for the provision of the original sample of SII, and Dr. J. W. Hopton for valuable discussions.

SUMMARY

Assay of formic acid released by periodate oxidation of *Diplococcus pneumoniae* Type II polysaccharide (SII), during liberation of an L-rhamnose-containing trisaccharide from SII by α-L-rhamnosidase, has demonstrated an α-(1→4) linkage between this trisaccharide and the remainder of the SII molecule. Carboxyl-reduced SII, $[\alpha]_D^{21}$ +42°, containing D-glucose (49.7%) and L-rhamnose (49.8%) has been prepared by reduction of the 2-hydroxyethyl ester in two stages. A comparison of the cleaved products of the action of specific, induced α- and β-D-glucosidases on SII and carboxyl-reduced SII demonstrated the presence of *O*-β-(D-glucopyranosyluronic acid)-(1→4)-*O*-α-D-glucopyranosyl-(1→4) residues in SII and *O*-β-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→4) residues in the reduced polysaccharide. A possible repeating unit for SII is discussed.

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STUDIES OF ONUPHIC ACID

PART II*. THE PHOSPHORUS AND THE MONOSACCHARIDE COMPONENTS

F. G. E. PAUTARD AND H. ZOLA

Astbury Department of Biophysics, The University, Leeds 2 (Great Britain)

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INTRODUCTION

An increasing number of polysaccharides containing phosphorus is being described in the literature[1-7], but, with the exception of the material obtained from a squid liver[1], they are all of plant origin. In the previous communication[8], we have shown that onuphic acid contains 14.4% of phosphorus, together with hexose and methylpentose. The principal component has been preliminarily identified[9,10] as glucose.

The present communication examines in detail the nature of the phosphorus and the monosaccharide components. As there is evidence that unusual states of phosphorus exist in biological systems (for instance, the phosphinic acids in marine organisms[11]), some caution has been exercised in appraising the nature of the phosphorus in this novel molecule isolated from an unusual animal site.

MATERIALS AND METHODS

Onuphic acid was prepared by extraction with N hydrochloric acid for 24 h at 2°, as previously described[8]. The undecationised preparation, which is a partial K^+ salt with traces of Ca^{++} and Mg^{++}, was used for the qualitative experiments; decationised onuphic acid was used in the quantitative experiments.

Inorganic phosphate and total phosphorus were determined directly or after sulphuric acid–nitric acid oxidation[8].

Potentiometric titrations were carried out by using a Radiometer TTT1 direct-reading pH meter, fitted with a magnetic valve to control the flow of titrant from the burette. A sodium-insensitive glass electrode and a calomel standard electrode were used throughout. Carbon dioxide was excluded at all stages of the experiments. To ensure adequate mixing, and to flush out carbon dioxide and oxygen, nitrogen was bubbled continuously through the titration solution. Alkaline solutions were made up with freshly distilled water and stored under carbon dioxide-free conditions, and a

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[*] Part I, ref. 8.

carbon dioxide trap was fitted to the burette during titration. Under these conditions, controls showed no absorption of carbon dioxide by the solutions.

The titrants used (*ca.* 0.1N potassium hydroxide and 0.1N hydrochloric acid, both M with respect to potassium chloride) were standardised by potentiometric titration using anhydrous sodium carbonate as primary standard. Free onuphic acid was titrated at a concentration of *ca.* 5mg/ml in M potassium chloride.

Relative viscosities were measured in B.S.S. Nos. 0 and 1 Ostwald viscometers at 20°.

Calf-intestinal alkaline phosphomonoesterase (B.D.H., Poole) was used in glycine–sodium hydroxide buffer at pH 9.5 in the presence of magnesium sulphate (0.1mM). Monoesterase activity was estimated by the procedure of Morton[12], and diesterase activity was shown to be absent, using bis(*p*-nitrophenyl) hydrogen phosphate[13].

Wheat-germ acid phosphomonoesterase (Sigma, London) was used in acetate buffer at pH 5.0. Monoesterase activity was demonstrated by the use of (±)-glycerol 1-phosphate and D-glucose 6-phosphate as substrates, and the enzyme was shown to be without action on bis(*p*-nitrophenyl) hydrogen phosphate.

Bovine spleen phosphodiesterase (Sigma, London; prepared by the method of Hilmoe[14]) was used[14] in maleate buffer (pH 6.6). No monoesterase activity could be detected when D-glucose 6-phosphate or (±)-glycerol 1-phosphate were used as substrates.

Acid hydrolysis was carried out in sealed glass ampoules at various temperatures. Hydrochloric acid was removed by repeated lyophilisation and sulphuric acid by neutralisation with barium carbonate before chromatography. Alkaline hydrolysis was carried out in a sealed glass vessel in the absence of air.

Paper chromatography was carried out on Whatman No. 1 paper by using the following solvent systems: (*a*) ethyl acetate–pyridine–water. (20:50:40; ascending, Smith[15]) for rapid work; (*b*) butyl alcohol–ethanol–water (50:10:40 top layer; descending, Hirst and Jones[16]) for identification of sugars. Thin-layer chromatography (t.l.c.) was carried out on silica gel[17]. Paper electrophoresis was carried out on Whatman 3MM paper in borate buffer[18].

Sugars were detected with alkaline silver nitrate[19] and aniline hydrogen phthalate[20]. Both procedures were modified slightly to improve sensitivity and were suitable for use in conjunction with t.l.c. In the aniline hydrogen phthalate reaction, a heating period of 10 min at 120° was used for thin-layer chromatograms.

Inorganic phosphate and sugar phosphates were detected by the method of Hanes and Isherwood[21] as modified by Bandurski and Axelrod[22]. The method was found to be particularly suited to t.l.c. on silica gel, since stronger heating conditions could be used, and no difficulty was experienced in detecting acid-stable phosphate esters, which are difficult to locate on paper.

Infrared spectra of samples from chromatograms.—Sugars isolated by preparative t.l.c. gave poor i.r. spectra after lyophilisation. Specimens of better quality were obtained by dissolving the samples in the minimal quantity of hot ethanol, allowing them to cool slowly, and evaporating the solvent under reduced pressure after 24 h. As control markers of the same degree of crystallinity, authentic samples were chromatographed and isolated by the same procedures. I.r. spectra were obtained as described previously[8].

Quantitative determination of monosaccharide components. — Monosaccharide components were determined directly on the unhydrolysed onuphic acid by variations of the cysteine–sulphuric acid reaction. Hexose was determined by the procedure of Dische *et al.*[23], modified as described below, and 6-deoxyhexose was determined by the procedures of Dische and Shettles[24]. In each case, quantitative determinations were made by comparison with standard D-glucose and L-fucose. The spectra of the solutions were plotted by using a Unicam SP 600 spectrophotometer. Interference from unwanted components was eliminated by dichromatic readings at wavelengths selected from the spectra of the standards.

Glucose oxidase test for D-*glucose*

Neutralised hydrolysates were tested by using glucose oxidase–peroxidase–*o*-tolidine test strips (Clinistix, Ames Co., Slough). Chromatograms were sprayed with the same reagents as follows: solution *1*, Na_2HPO_4 (1.65 g) + $NaH_2PO_4.2H_2O$ (1.09 g) + peroxidase (Sigma, London) (6 mg) + glucose oxidase (Sigma, London) (38 mg) made up to 150 ml with water, (pH = 7.0); solution *2*, *o*-tolidine (3 ml) in butyl alcohol (50 ml).

The best results were obtained by spraying the chromatogram with solution *2*, drying in a current of warm air, spraying with solution *1*, leaving for 5 min, and spraying again with solution *2*.

In this test, D-glucose (10 μg) gave a blue colour, whilst the following sugars (50 μg) gave no colour: D-mannose, D-ribose, D-xylose, D-fructose, L-sorbose, D-glucitol, L-fucose, L-arabinose, L-rhamnose, D-galactose.

Hydrolysates were separated into high- and low-molecular weight fractions by using Sephadex G25 columns[25]. Water was used to elute the fractions, which were detected by changes in refractive index.

RESULTS AND DISCUSSION

Linkage of the phosphorus

Since the only elements found in purified onuphic acid are P, H, C, and O, the P must be linked either through C or through O. No entirely satisfactory method appears to be available at present for the determination of oxygen-bound and carbon-bound phosphorus when both are present although ^{31}P n.m.r. spectroscopy or mass spectrometry may provide an answer in due course. The procedure adopted at present depends on the determination of total P by oxidation and of P–O phosphorus by hydrolysis, but, since the stability of the bond may be dependent partly on its environment, unusually stable P–O or acid-labile P–C bonds can exist and will escape detection.

Determination of P_i after oxidative hydrolysis (sulphuric acid–nitric acid) and non-oxidative hydrolysis (6N hydrochloric acid for 48 h at 100°[11]) gave identical results, indicating that onuphic acid contains no detectable P–C bonds.

Pentavalent low-oxygen states of P, such as derivatives of phosphorous acid, are possible. The presence or absence of the P–H bond cannot be decided from the infrared spectrum of onuphic acid[8], since the P–H stretch mode gives rise[26] to an absorption in the region 4.1–4.4 μ, which overlaps with the frequencies due to P–(O–H) modes[27,28]. Again the K_d for the strong-acid dissociation of phosphites and phosphates are too close to allow differentiation. Since a phosphite bound to the macromolecule through one of its –OH groups has only one remaining dissociable proton, phosphite ester can be present only to the extent estimated for diesterphosphate (see below). Phosphites, however, are generally stable to non-oxidising mineral acids[29], and, at present, their identification must rely largely on differences between P_i determined after oxidation and P as P_i determined by hydrolysis. We have detected no differences in P_i after acid hydrolysis in 6N hydrochloric acid at 100° in the absence of oxygen, and P determined by the best available method of oxidation (see ref. 8), and we conclude that phosphite is absent in onuphic acid or present only in traces.

Orthophosphates or chain phosphate?

Pyrophosphates and polyphosphates are normally characterised by their lability on treatment with mild acid (for example[30], N hydrochloric acid for 7 min at 100°). These conditions do not release any detectable P_i from onuphic acid, thus eliminating the possibility that polyphosphate or monoesterified pyrophosphate are present.

However, mild, acid treatment (for instance, autohydrolysis at pH 2.5 at 80°, see Fig. 1) causes depolymerisation of onuphic acid without immediate release of P_i, and this could be due to hydrolysis of diesterified pyrophosphate to acid-stable monoesters, as follows:

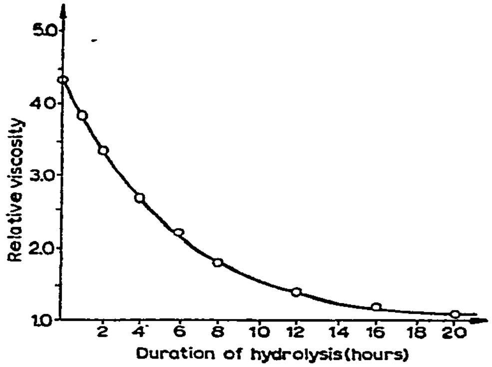
$$\mathrm{R{-}O{-}P{-}(O_2H){-}O{-}P(O_2H){-}O{-}R'} \rightarrow \mathrm{R{-}O{-}P(O_3H_2)} + \mathrm{R'{-}O{-}P(O_3H_2)}$$

where R and R′ are carbohydrate moieties.

Thus, the failure of N hydrochloric acid to release P_i from onuphic acid during 7 min at 100° does not eliminate the possibility of the presence of diesterified pyrophosphate groups.

Degree of esterification

Potentiometric acid–base titration. — The results of potentiometric acid–base titration of onuphic acid are summarised in Table I; the titration curve is shown in Fig. 2. The titration curve was faithfully reproduced on back-titration and on further forward-titration of the back-titrated material. This suggests that no irreversible changes in structure are occurring at either pH extreme during the period of the titration, and also that the results are independent of the polymer concentration over the range used. The decationised onuphic acid preparation used for these studies remained soluble throughout the titrations, although undecationised preparations showed a tendency to precipitate at high pH values. Since each phosphorus atom appears to have at least one ionisable acid group, triesterified phosphate is absent. These data indicate that approximately 10% of the phosphorus is present in the diester form, with 90% in the monoester form.



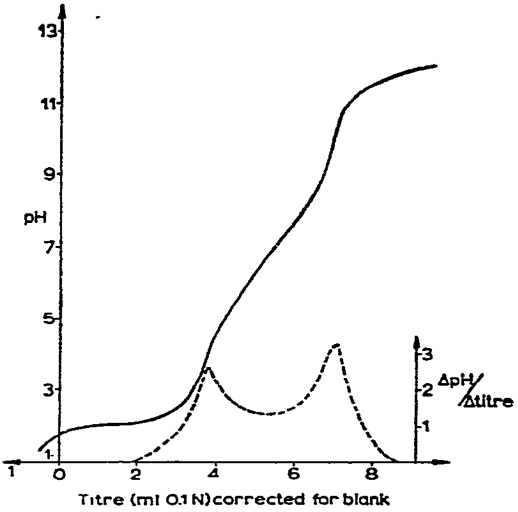


Fig. 1. Fall in viscosity of onuphic acid (1% solution in deionized water) with time at 80°.

Fig. 2. Potentiometric titration curve (————) and differential titration curve (— — — —) of onuphic acid.

TABLE I

DATA FROM THE POTENTIOMETRIC ACID–BASE TITRATION OF ONUPHIC ACID[a]

(1) Number of protons dissociated at the first inflexion per atom of P:	1.1	±0.1[b]
(2) Number of protons dissociated at the second inflexion per proton dissociated at the first inflexion:	1.90	±0.02[b]
(3) *Hence*, % of P atoms having only one ionisable hydrogen atom:	10	±2[b]

[a] The standard deviation for (1) includes errors due to variable moisture content, weighing, and preparation of solutions. These errors do not feature in (2) and (3), since these result from a ratio of two quantities measured on the same solution. Thus the comparatively large standard deviation of (1) does not invalidate the significance of (2).

[b] The results and standard deviations were obtained from four different experiments on different batches of material.

Depolymerisation by phosphodiesterase. — The presence of diester phosphate in onuphic acid is confirmed by the fall in viscosity of onuphic acid on incubation with bovine spleen diesterase (Fig. 3). Neither solutions of onuphic acid in buffer alone, nor solutions of the enzyme in the absence of substrate, show any significant changes in viscosity over the period of the experiment.

Dephosphorylation with phosphomonoesterase. — The results of incubation of onuphic acid with an alkaline and an acid phosphatase under a variety of conditions are set out in Table II. In no case was it found possible to remove 90% of the total P, as has been expected from the titration results. The alkaline phosphatase was inhibited by onuphic acid at concentrations above about 50 μg/ml (Pautard and Zola, unpublished results) which thus resembles other phosphate polymers[31-33]. The macromolecule did not, however, inhibit the action of the acid phosphatase on D-glucose 6-phosphate under the conditions used. The non-dialysable contents remaining after the last run shown in Table II released P_i from D-glucose 6-phosphate, suggesting that the cessation of activity with respect to onuphic acid was not a result of inactivation of the enzyme.

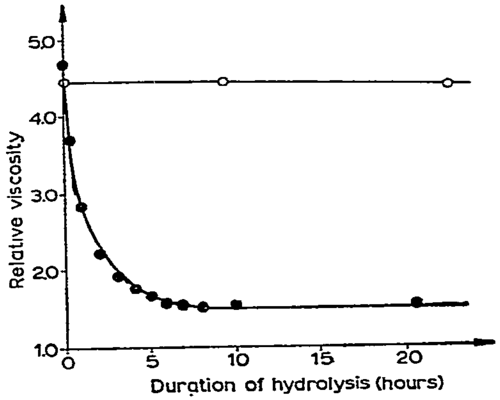


Fig. 3 Effect of spleen phosphodiesterase on the viscosity of onuphic acid. Enzyme absent (o-o) enzyme present (●-●).

TABLE II

RELEASE OF P_i FROM ONUPHIC ACID BY MONOESTERASES

Conditions of experiment	*P_i released (as % of total phosphate)*
Alkaline phosphatase (calf-intestinal), pH 9.5, 24 h at 37°	4
Acid phosphatase (wheat germ) at pH 5.0:	
37°, 24 h	10.5
37°, 48 h	14.6
37°, 48 h, fresh enzyme added, incubation continued for 24 h	34.0
As previous treatment, further 48 h	38.4
37°, in dialysis bag, dialysed against 90 ml of buffer, 72 h	50.3[a]
As previous treatment, fresh enzyme added, incubation continued for 24 h	50.6

[a] No increase with prolonged dialysis.

Products of hydrolysis

The components detected in chromatograms of hydrolysates of onuphic acid are set out in Table III. In addition, formic acid hydrolysates contained a series of fast-moving spots, probably formyl esters, which were not detected after brief alkaline treatment of the hydrolysates. Some properties of the components are shown in Table IV.

TABLE III

HYDROLYSIS RESULTS

Component	*Conditions*									
	2N *HCl, 80–90°*					*90% Formic acid*	2N H_2SO_4, *80–90°*			0.2N *NaOH*[a]
	2 h	*4 h*	*6 h*	*12 h*	*24 h*	*80°, 6 h*	*2 h*	*4 h*	*24 h*	*24 h*
A	+	+	+	+	+	+	−	−	−	+
B	+	+	+	+	+	+	−	−	−	+
C	+	+	−	−	−	+	+	+	−	−
D	−	+	+	+	+	trace	−	+	+	+
E	−	+	+	+	+	trace	−	−	−	+

[a] Room temperature.

Identity of the major monosaccharide component

Specimens of the major component (*D*) were isolated by preparative t.l.c. and gave the reaction characteristic of hexoses in the cysteine–sulphuric acid reaction. On paper chromatograms, the component failed to react with the phloroglucinol reagent, suggesting that it is not a ketose. The infrared spectra of Component *D* and authentic D-glucose were closely similar.

Using D-glucose, D-mannose, D-galactose, L-sorbose, and D-fructose, together with the solvent systems and the data of Smith[15], Hirst and Jones[16], and Frahn and Mills[35], it was shown that Component *D* can be separated, chromatographically or electrophoretically, from all of the unbranched aldohexoses and ketohexoses, with the exception of glucose.

Deionised hydrolysates of onuphic acid gave positive reactions with the glucose oxidase–peroxidase–*o*-tolidine test strip; while still on the paper after chromatography, Component *D* gave a positive reaction to the test applied in the spray form.

Identity of the minor monosaccharide component

Specimens of the minor component (*C*) were isolated by preparative thin-layer chromatography and gave the reaction characteristic of 6-deoxyhexoses in the cysteine–sulphuric acid reaction. The infrared spectra of Component *C* and authentic L-fucose were closely similar.

Using L-fucose and L-rhamnose, together with the solvent systems and the data of Hirst and Jones[16], Moore *et al.*[36], and Elbein *et al.*[37], it was shown that Component *C* can be separated chromatographically from all of the unbranched 6-deoxyaldohexoses, with the exception of fucose.

TABLE IV

CHROMATOGRAPHIC AND ELECTROPHORETIC CHARACTERISATION OF COMPONENTS OF ACID HYDROLYSATES

A Mobilities in various systems

Component[a]	*Solvent, paper*		*Electrophoresis (10 volts/cm, 3 h) 0.05 M borax*	*T.l.c. (silica gel)*[c]
	a[b]	*b*[b]		
A	Origin	Origin	Not detected	Origin
B	R_G 0.22[d]	R_G 0.26[d]	Not detected	Slow moving
C	R_G 1.5[e]	R_G 2.0[f]	Elongated spot M_G 0.83[g]	Did not separate from fucose or ribose[h]
D	R_G 1[i]	R_G 1[i]	M_G 1[i]	[i]
E	R_{P_i}	R_{P_i}	Not detected	Did not separate from P_i

B Reactions on chromatograms

Component[j]	*Reaction*		
	Alkaline AgNO₃	*Aniline hydrogen phthalate*	*Molybdate*
A	+ (weak)	+ (weak)	+
B	+	+ (weak)	+
C	+	+ (brown, i.e., not pentose)	—
D	+	+ (brown)	—
E	white spot	—	+ (gave reaction for P_i)

[a] To determine whether components separated from standards, the standards were superimposed on the dried hydrolysate spots before development; [b] see experimental; [c] mobilities are not given for thin-film chromatograms, as they were not found to be reproducible; [d] D-glucose 6-phosphate had same R_G; [e] did not separate from fucose or xylose, but separated from ribose; [f] separated from xylose, but not from fucose or ribose; [g] separated from ribose and xylose, but not from fucose; [h] when isolated from the chromatogram, gave cysteine–H_2SO_4 reaction of pentose or 6-deoxyhexose; [i] did not separate from glucose; [j] none of the components gave positive ninhydrin tests.

Determination of hexose by the cysteine–sulphuric acid reaction applied to the unhydrolysed macromolecule

The absorption maximum at 410–414 mμ, due to hexose, was found to be more intense if concentrated sulphuric acid was used in place of the diluted acid recommended by Dische *et al.*[23]. Using the concentrated acid and various heating times, it was found that the highest extinction coefficient was obtained after 4 min; application of these conditions gave a value of 59 ± 1% for the hexose content of onuphic acid, expressed on a weight basis with glucose as the standard.

Determination of 6-deoxyhexose

Comparison of the Cy R3 and Cy R10 reactions, allowing different time intervals for the development of the absorption maximum, indicated that the maximum extinction coefficient was obtained using the Cy R10 procedure and 20-h colour development. Under the conditions given above, and using dichromatic readings to eliminate hexose interference, the 6-deoxyhexose content of onuphic acid was found to be 2.4 ±0.4%, expressed as fucose. The reproducibility of the analysis, when carried out on several different batches of onuphic acid, was not smaller than that obtained in repeat analyses of the same batch.

Quantitative aspects of the release of monosaccharides

The results of kinetic studies of the hydrolysis of onuphic acid by acid under various conditions are set out in Tables V and VI. Multistage, acid hydrolysis was done according to the following scheme in order to improve the yields of the monosaccharides. Onuphic acid (1% in 2N hydrochloric acid) was heated for 4 h at 80° and passed down a Sephadex G25 column; the excluded fraction was freeze dried and dissolved in 2N hydrochloric acid to a concentration of 1%. The process was repeated several times.

TABLE V

RELEASE OF P_t AND DESTRUCTION OF CARBOHYDRATE DURING HYDROLYSIS IN 2N HCl AT 100°

Time (h)	$[\alpha]^{20}_{D}$, °	*mmoles of carbohydrate*[a] (*x*)	*mmoles of P_t* (*y*)	*x/y*
0	−4.1	0.186	0.00	—
1	+0.73	0.233	0.010	23.0
2	+1.39	0.391	0.015	26.0
5	+1.68	0.305	0.022	13.9
8	+2.45	0.260	0.031	8.4
12	+2.48	0.230	0.046	5.0

[a] Determined by the cysteine–sulphuric acid reaction[23].

TABLE VI

RELEASE OF P_t AND REDUCING SUGAR, AND CHANGE OF OPTICAL ROTATION DURING TREATMENT OF ONUPHIC ACID IN 6N HCl AT 100°

Time (h)	*% P_t*[a]	*Reducing sugar*[b]	$[\alpha]^{20}_{D}$, °
0	0	0.02	−4.63
0.5	1.3	0.10	+5.51
1.0	3.9	0.40	+21.5
1.5	5.4	0.41	+22.3
2.0	6.7	0.15	+17.3
12.0	14.4	0.00	0.00

[a] mg of P_t/100 mg of onuphic acid; [b] mg of reducing sugar/100 mg of onuphic acid, determined by the Somogyi–Nelson procedure[34].

After a total of 6 hydrolysis stages, the excluded fraction was hydrolysed further at 95° in 2N hydrochloric acid for 6 h, and the hydrolysate was combined with the retarded, low-molecular weight fractions from the previous 6 separations. Column chromatography of the combined hydrolysate on anion-exchange resin[38] showed that only a fraction of the carbohydrate present was in the form of free monosaccharide or monosaccharide phosphate. The rest (approximately 75% by weight of the total hydrolysate), which was probably in the form of acid-stable phosphorylated oligosaccharides, was held strongly by the ion-exchange resin.

These results show clearly that onuphic acid contains acid-stable groups, as well as the acid-labile linkages involving fucose, and, in consequence, acid hydrolysis alone cannot be used to obtain mixtures of sugars in proportions which represent the monosaccharide composition of the parent macromolecule.

The results of alkaline hydrolysis (summarised in Table VII) suggest that the reaction is accompanied by considerable degradation of reducing sugar, and the method is therefore impracticable as a stage in the quantitative hydrolysis of onuphic acid to monosaccharides.

TABLE VII

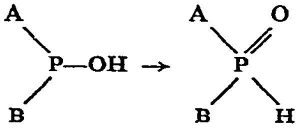
COMPONENTS DETECTED ON CHROMATOGRAMS OF ALKALI HYDROLYSATES OF ONUPHIC ACID

Conditions[a]	*2.5 h*	*5 h*	*24 h*
0.2N KOH, 20°	—	*B+D+E*	*B+D+E*
0.2N KOH, 100°	*E*	*E*	*E*
2N KOH, 20°	—	*B+D+E*	*D+E*
2N KOH, 100°	*E*	*E*	*E*

[a] All under nitrogen.

CONCLUSIONS

Phosphorus in the trivalent state tends to be unstable and rearranges, where possible, to give a coordination number of four (see, for instance, van Wazer[39]). In particular, trivalent phosphorus compounds with an unsubstituted –OH group rearrange to the pentavalent structure as follows:



Such a compound would have a K_d differing from those observed, and it is unlikely therefore that trivalent phosphorus is present.

The evidence presented above indicates that the phosphorus in onuphic acid is present as phosphate ester. Although the titrimetric data suggest that 10% of the phosphorus is in the form of diester, and 90% is in the form of monoester, these values may not represent the true proportions in onuphic acid in its native state in the tube. The diesterphosphate may be present as orthophosphate, or as di-esterified pyrophosphate similar to the pyrophosphate dinucleotides. Some, at least, of the diesterphosphate may form part of the molecular chain, since treatment of the macromolecule with a purified phosphodiesterase leads to a marked decrease is viscosity. The extent to which depolymerization might be due to glycosidase contained in the spleen preparation cannot be determined from the present experiments.

By all of the criteria used, the identities of the monosaccharide components of onuphic acid are established as glucose and fucose. The lack of sufficient material precludes studies of optical rotation to establish the enantiomeric nature of the sugars; the other tests are inconclusive, since it is not known whether L-glucose responds to the glucose oxidase test.

The quantitative analyses of the monosaccharide components do not exclude the possibility of traces of other sugars, although glucose and fucose are the only monosaccharides detected in hydrolysates produced under a wide variety of conditions. The analyses for hexose and deoxyhexose (expressed as glucose and fucose, respectively) are reproducible for various batches of onuphic acid.

ACKNOWLEDGMENTS

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SUMMARY

The phosphorus and the monosaccharide components of onuphic acid have been examined. Current methods have been used to establish that the phosphorus appears to be present in the form of phosphate esters (90% of monoester and 10% of diester). The monosaccharides have been identified as glucose and fucose (93.9 and 3.8%, respectively, of the organic material).

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NEUARTIGE ISOMERISIERUNGEN VON ZUCKERDERIVATEN
TEIL IV[1]. ÜBER DIE KATIONISCHE ISOMERISIERUNG VON HEPTOSE-DERIVATEN

F. MICHEEL, H. PFETZING, UND G. PIRKE
Organisch-Chemisches Institut der Universität Münster, Westf. (Deutschland)

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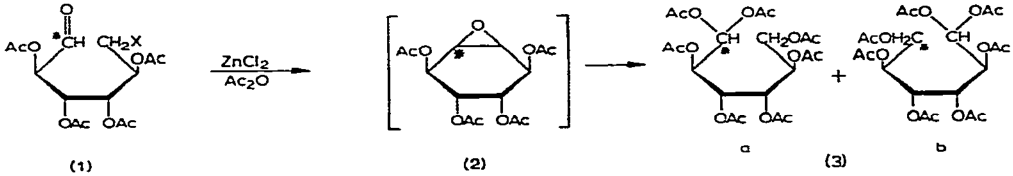
EINFÜHRUNG

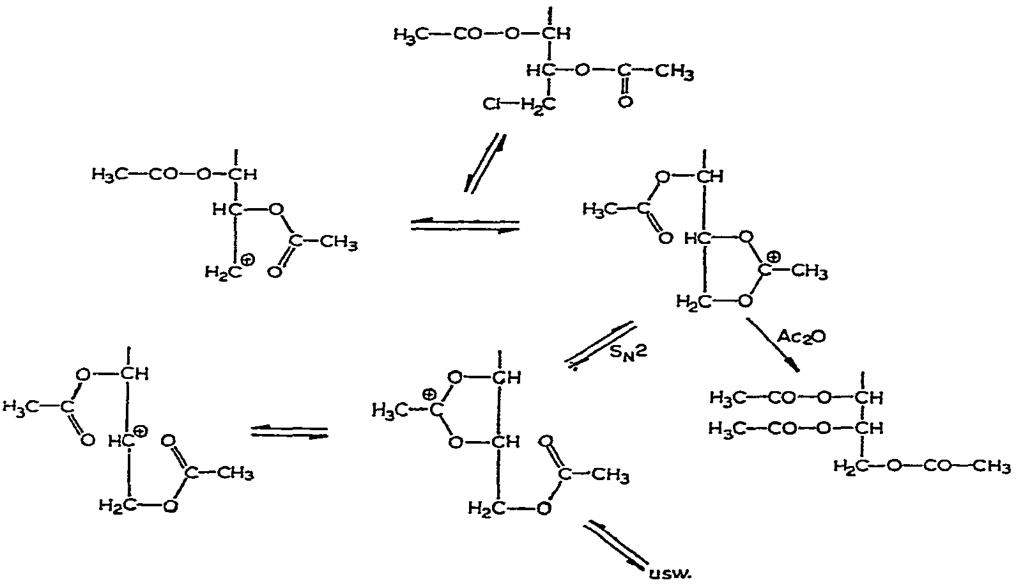
In einer früheren Mitteilung[2] wurde mit Hilfe von [1–^{14}C]-D-Galaktose gezeigt, daß bei der Bildung van Hepta-*O*-acetyl-al-D,L-galaktose (**3**) aus Derivaten der β D-Galaktose (**1a,b**)[3-5] die Reihenfolge der Kohlenstoffatome in der Zuckerkette nicht unter Bildung eines cyclischen Zwischenproduktes (**2**) vertauscht wird.



a: $X = SO_2 \cdot C_6H_4 \cdot CH_3(p)$ b: $X = J$

Um den Reaktionsmechanismus dieser Isomerisierung aufzuklären, wurde 1,1,2,3,4,5-Hexa-*O*-acetyl-6-*O*-toluolsulfonyl-al-D-galaktose mit Zinkchlorid in die 6-Chlor-Verbindung übergeführt. Letztere bildet unter Substitution des Cl-Atoms ein optisch fast inaktives Gemisch aller Hepta-*O*-acetyl-al-D,L-hexosen[1]. Setzt man Penta-*O*-acetyl-al-D-galaktose oder das 2,3,4,5-Tetra-*O*-acetyl-6-*O*-triphenylmethyl-al-D-galaktose-äthyl-halbacetal mit Acetanhydrid/$ZnCl_2$ um, dann bildet sich nur Hepta-*O*-acetyl-al-D-galaktose. Hiermit ist bewiesen, daß nur dann Isomerisierung erfolgt, wenn sich bei der Reaktion ein Carbonium-ion am C-6-Atom bilden kann. Durch eine Folge von Acylwanderungen unter Bildung von Orthoessigsäureester-Derivaten[6] als Zwischenprodukten kann intermediär die Asymmetrie aller Kohlenstoffatome aufgehoben werden. Hierbei kann jedes Carbonium-ion mit einem Acetoxyrest als nucleophilem Substituenten in einer inneren S_N1-Reaktion unter Erhaltung der Konfiguration oder in einer inneren S_N2-Reaktion unter Platzwechsel reagieren, bis sich die D- und L-Formen aller möglichen isomeren Hexosen als Hepta-*O*-acetyl-Derivate gebildet haben: (Siehe Formel-schema S. 284).
Ganz analog verhalten sich die entsprechenden Derivate der D-Glucose und D-Mannose[1].

Um zu zeigen, daß diese neuartige totale Racemisierung nicht nur auf Derivate der Hexosen beschränkt ist, haben wir die Reaktion an entsprechenden Derivaten der Heptose-Reihe untersucht.

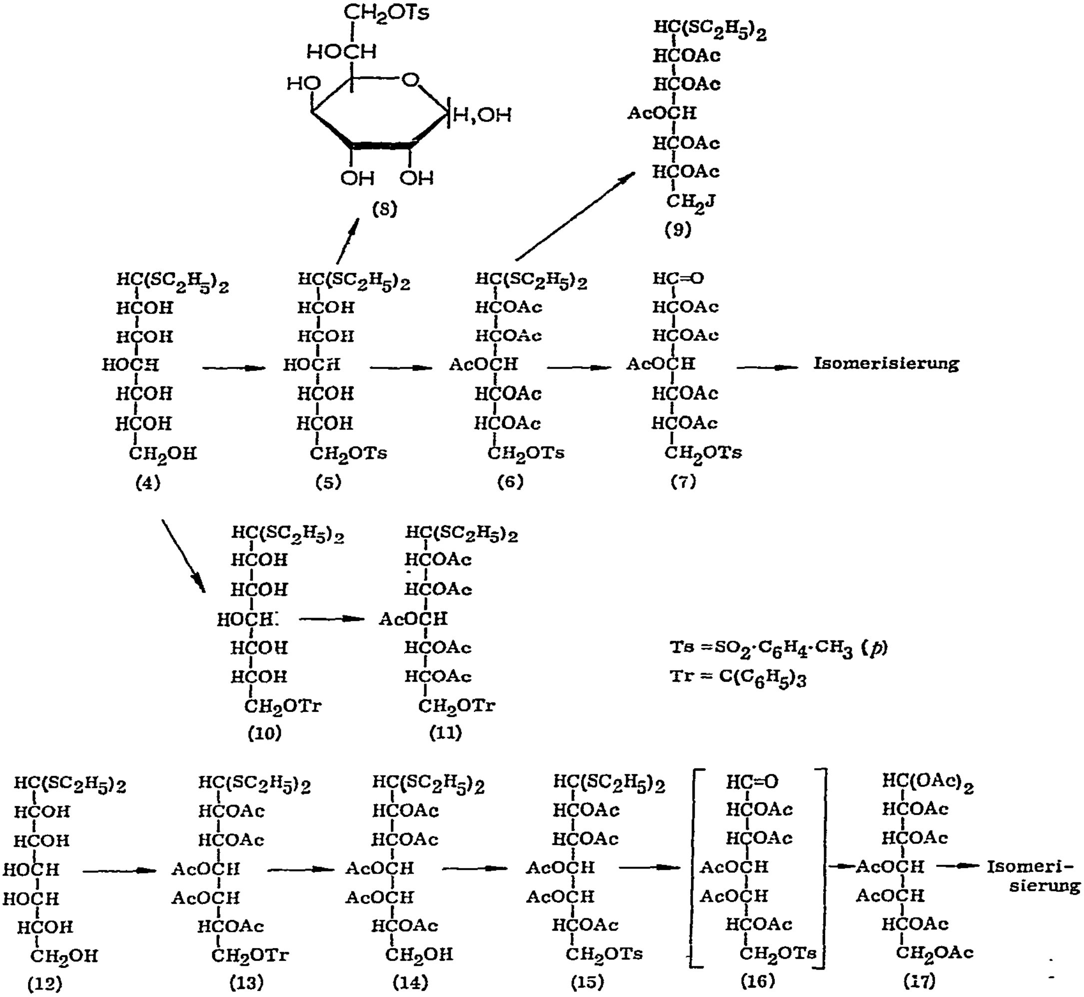


ERGEBNISSE UND DISKUSSION

D-*glycero*-D-*gulo*-Heptose-diäthylmercaptal (**4**)[7] ergibt mit *p*-Toluolsulfonsäurechlorid in Pyridin die 7-*O*-Toluolsulfonyl-Verbindung (**5**). Nach Acetylierung und Demercaptalisierung mit $HgCl_2$–$CdCO_3$[8] entsteht hieraus 2,3,4,5,6-Penta-*O*-acetyl-7-*O*-toluolsulfonyl-al-D-*glycero*-D-*gulo*-heptose (**7**). **4** läßt sich zum 7-*O*-Triphenylmethyl-D-*glycero*-D-*gulo*-heptose-diäthylmercaptal (**10**) umsetzen, das mit Acetanhydrid–Pyridin die 2,3,4,5,6-Penta-*O*-acetyl-Verbindung (**11**) bildet. **5** Wurde zur 7-*O*-Toluolsulfonyl-D-*glycero*-D-*gulo*-heptose (**8**) demercaptalisiert und **6** ergibt mit NaJ in Aceton die 7-Jod-Verbindung (**9**).

Als weiteres, zur Isomerisierung geeignetes Heptose-Derivat wurde die 1,1,2,3,4,5,6-Hepta-*O*-acetyl-7-*O*-toluolsulfonyl-al-D-*glycero*-L-*manno*-heptose (**17**) dargestellt. D-*glycero*-L-*manno*-Heptose-diäthylmercaptal (**12**)[9] wurde auf bekanntem Wege in 2,3,4,5,6-Penta-*O*-acetyl-7-*O*-triphenylmethyl-D-*Glycero*-L-*manno*-heptose-diäthylmercaptal (**13**) übergeführt. Nach Abspaltung des Triphenylmethylrestes läßt sich das enstandene 2,3,4,5,6-Penta-*O*-acetyl-D-*glycero*-L-*manno*-heptose-diäthylmercaptal (**14**) glatt zum 7-*O*-Toluolsulfonyl-Derivat (**15**) umsetzen und dieses mit H_2SO_4–Ac_2O demercaptalisieren[10] und zu **17** acetylieren.

Eine direkte Tosylierung, wie im Falle des D-*glycero*-D-*gulo*-Heptose-diäthylmercaptals (**4**), ist bei der analogen D-*glycero*-L-*manno*-Verbindung (**12**) nicht möglich. Bei der Reaktion bildet sich unter verschiedenen Bedingungen stets ein Gemisch verschieden stark tosylierter Produkte, aus dem nur 4,7-Anhydro-D-*glycero*-L-*manno*-heptose-diäthylmercaptal (**18**) chromatographisch abgetrennt werden konnte.



Ts = $SO_2 \cdot C_6H_4 \cdot CH_3$ (*p*)
Tr = $C(C_6H_5)_3$

2,3,4,5,6-Penta-*O*-acetyl-7-*O*-toluolsulfonyl-al-D-*glycero*-D-*gulo*-heptose (**7**) und 1,1,2,3,4,5,6-Hepta-*O*-acetyl-7-*O*-toluolsulfonyl-al-D-*glycero*-L-*manno*-heptose (**17**) wurden zur Isomerisierung mit Acetanhydrid–$ZnCl_2$ 3 Stunden auf 100° erhitzt und wie beschrieben aufgearbeitet[1]. Im Falle des D-*glycero*-D-*gulo*-Heptose-Derivates (**7**) ließen sich nach der Verseifung mit Natriummethylat chromatographisch neben 7-*O*-Toluolsulfonyl-D-*glycero*-D-*gulo*-heptose (**8**) und einer unbekannten Verbindung folgende isomere Heptosen* nachweisen: D-*glycero*-D-*talo*-Heptose, D-*glycero*-L-

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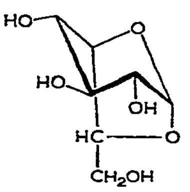
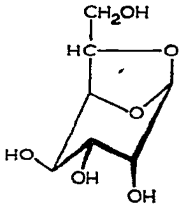
*Wir danken Herrn Dr. H. S. Isbell, National Bureau of Standards, Washington, D.C., USA, sehr, daß er uns mehrere dieser Heptosen für chromatographische Vergleiche zur Verfügung gestellt hat.

manno-Heptose, D-*glycero*-D-*gulo*-Heptose, D-*glycero*-D-*galacto*-Heptose, bzw. deren Enantiostereomere. Bei dem Versuch, die Isomeren an Cellulose präparativ säulenchromatographisch zu trennen, wurde mit einer Ausbeute von mehr als 40% ein kristallines Heptose-anhydrid (**19** bzw. **20**) isoliert, das weder Fehlingsche Lösung noch ammoniakalisches Silbernitrat reduziert. Mit Acetanhydrid–Pyridin entsteht ein Tetra-*O*-acetyl-Derivat (**21**). Erwärmt man das Anhydrid mit 5-proz. wässriger Schwefelsäure, dann erhält man ein Produkt, das Fehlingsche Lösung schwach reduziert. Die hierbei im Gleichgewicht gebildete Heptose ist chromatographisch nicht identisch mit einer der vorstehend angeführten Heptosen. Es muß sich also um eine der schwer zugänglichen oder noch unbekannten Heptosen handeln. Bei dem Versuch, das Anhydrid mit Acetanhydrid–Eisessig–Schwefelsäure acetolytisch aufzuspalten[11] wurde selbst nach 80-stündiger Reaktionszeit das Ausgangsprodukt praktisch quantitativ in Form seines Tetra-acetates (**21**) zurückgewonnen. Mit überschüssigem *p*-Toluolsulfonsäurechlorid in Pyridin bildet das Anhydrid ein Tetra-*O*-toluolsulfonyl-Derivat (**22**), das mit NaJ in Aceton in ein Mono-jod-tri-*O*-toluolsulfonyl-heptose-anhydrid (**18**) übergeht. Es muß daher eine primäre Hydroxylgruppe im Anhydrid vorliegen, das Jod also am C-7-Atom gebunden sein. Bei der Oxydation mit Perjodat werden 2 Mol. davon verbraucht. Aus diesen Ergebnissen folgt, daß im Anhydrid 3 vicinale sekundäre Hydroxylgruppen vorliegen. Das Anhydrid besitzt also die Struktur eine 1,5:1,6- oder 1,2:1,6-Anhydro-heptosen. Ein 1,2:1,6-Anhydrid scheidet wegen des reaktionsfähigen Äthylenoxyd-ringes aus (vgl. **Brigl**'s Anhydrid[12].) In diesem Falle müßte der Epoxy-ring leicht aufspaltbar sein und auch Fehlingsche Lösung reduziert werden. Das Anhydrid reduziert selbst nach längerem Erhitzen nicht Fehlingsche Lösung. Auch auf Grund der Resistenz bei der Hydrolyse und der Lage des Gleichgewichtes scheidet ein 1,2:1,6-Anhydrid aus. Die offenbar sehr leicht erfolgende Anhydridbildung legt nahe, daß es sich um eine 1,6-Anhydro-D-*glycero*-*β*-L-*ido*-heptopyranose (**19**) oder eine 1,6-Anhydro-L-*glycero*-*β*-D-*gulo*-heptopyranose (**20**) handelt, weil die entsprechenden Hexosen sehr leicht in wässriger Lösung die Anhydride bilden (hierzu s. weiter unten).



(19)



(20)

Nach der Isomerisierung ausgehend von 1,1,2,3,4,5,6-Hepta-*O*-acetyl-7-*O*-toluolsulfonyl-al-D-*glycero*-L-*manno*-heptose (**27**) wurde das Reaktionsgemisch ohne vorhergehende Verseifung mit Cyclohexan–Benzol–Pyridin (5,4:5:0,5) an Kieselgel getrennt. Hierbei wurde kristalline 1,1,2,3,4,5,6-Hepta-*O*-acetyl-7-chlor-7-desoxy-al-D-*glycero*-L-*manno*-heptose erhalten, die nach der Theorie[1] als Zwischenprodukt bei der Isomerisierung auftreten kann. Vier weitere Komponenten konnten nach der Säulentrennung durch wiederholte fraktionierte Kristallisation in einer Gesamtausbeute von 37% (berechnet als Octa-*O*-acetyl-al-heptose) erhalten werden: (1) zwei Substanzen, die sich als Gemische von 1,1,2,3,4,5,6,7-Octa-*O*-acetyl-al-D-*glycero*-L-*manno*-heptose und dem gleichen Derivat der enantiomeren Heptose erwiesen; (2) die racemische Verbindung der beiden spiegelbildlichen Octa-*O*-acetyl-al-heptose; (3) reine 1,1,2,3,4,5,6,7-Octa-*O*-acetyl-al-L-*glycero*-D-*manno*-heptose als Hauptprodukt.

Alle 4 Substanzen lieferten nach der alkalischen Verseifung D-*glycero*-L-*manno*-Heptose bzw. die zugehörige L-Form. Der Beweis für das Vorliegen einer racemischen Verbindung aus der D- und L-Form des Octa-acetates ließ sich durch Synthese führen. Aus gleichen Teilen der D-Form und der aus dem Isomerisierungsgemisch isolierten L-Form des Octa-acetates in Benzol ließ sich optisch inaktives Material gewinnen, das IR-spektroskopisch identisch ist mit dem Racemat, das sich bei der Isomerisierungsreaktion bildet.

Das Gemisch amorpher Octa-*O*-acetyl-al-heptosen aus den übrigen Fraktionen der Säulentrennung wurde mit Natriummethylat verseift und papierchromatographisch untersucht (absteigende Durchlaufchromatographie). Von den 16 möglichen isomeren Heptosen der D-Reihe sind 12 bekannt und von diesen standen uns 8 zum Vergleich zur Verfügung. Es ließen sich folgende Heptosen im Isomerisierungsgemisch nachweisen: D-*glycero*-L-*manno*-Heptose, D-*glycero*-D-*talo*-Heptose, D-*glycero*-D-*galacto*-Heptose, D-*glycero*-L-*talo*-Heptose (Spur), bzw. deren Enantiostereomere. Mit Hilfe einer R_G-Wert-Tabelle von Davies[13] (Bezugssubstanz: D-*glycero*-D-*gulo*-Heptose) ließen sich außerdem drei weitere Heptosen zuordnen: D-*glycero*-D-*gluco*-Heptose, D-*glycero*-D-*allo*-Heptose, D-*glycero*-D-*altro*-Heptose (Spur), bzw. deren Enantiostereomere. Mit Sicherheit liegen im Heptosengemisch der Ausgangsprodukte **7** und **17** weder die D-*glycero*-L-*gluco*- noch die D-*glycero*-D-*ido*-Heptose (bzw. deren Enantiomere) vor. Auch das bei der Isomerisierung von 2,3,4,5,6-Penta-*O*-acetyl-7-*O*-toluolsulfonyl-al-D-*glycero*-D-*gulo*-heptose (**7**) in hoher Ausbeute entstehende 1,5;1,6 Heptose-anhydrid konnte bei der Isomerisierung von 1,1,2,3,4,5,6-Hepta-*O*-acetyl 7-*O*-toluolsulfonyl-al-D-*glycero*-L-*manno*-heptose (**17**) nicht nachgewiesen werden. In beiden Fällen tritt dagegen in erheblicher Menge D-*glycero*-D-*talo*- und D-*glycero*-D-*galacto*-Heptose auf.

Die teilweise Übereinstimmung in den Ergebnissen bei der Isomerisierung verschiedener al-Heptose-Derivate und die Unterschiede bezüglich der Hauptprodukte bei der Isomerisierungsreaktion lassen vermuten, daß bei dieser Reaktion konfigurationsspezifische Faktoren von Einfluß sind.

Abb. 1 zeigt entsprechend dem von Micheel und Böhm[1] vorgeschlagenen Reaktionsmechanismus ein Schema der Isomerisierungsreaktion: die Strichformeln sollen die vollacetylierten al-Heptosen darstellen, die Ringe geben die orthoesterartigen Zwischenstrukturen wieder. Die von diesen Zwischenstrukturen abgeleiteten Reaktionsprodukte sind durch einen Isomerisierungsschritt entstanden. Die umrahmten Konfigurationen werden gebildet, wenn ein Zwischenprodukt durch den intermolekularen Angriff eines Acetanhydrid-Moleküls abgefangen wird. Die Isomerisierung ist damit abgebrochen. Wie das Schema zeigt, tritt bereits nach 3 Isomerisierungsschritten eine so große Zahl möglicher Zwischenstufen auf, daß der Verlauf der Isomerisierung nur mit Hilfe eines elektronischen Rechners weiter verfolgt werden kann (theoretisch sind 352 unterschiedliche Strukturen möglich, von denen jede aber auch mehrfach in einem Isomerisierungsschritt auftreten kann).

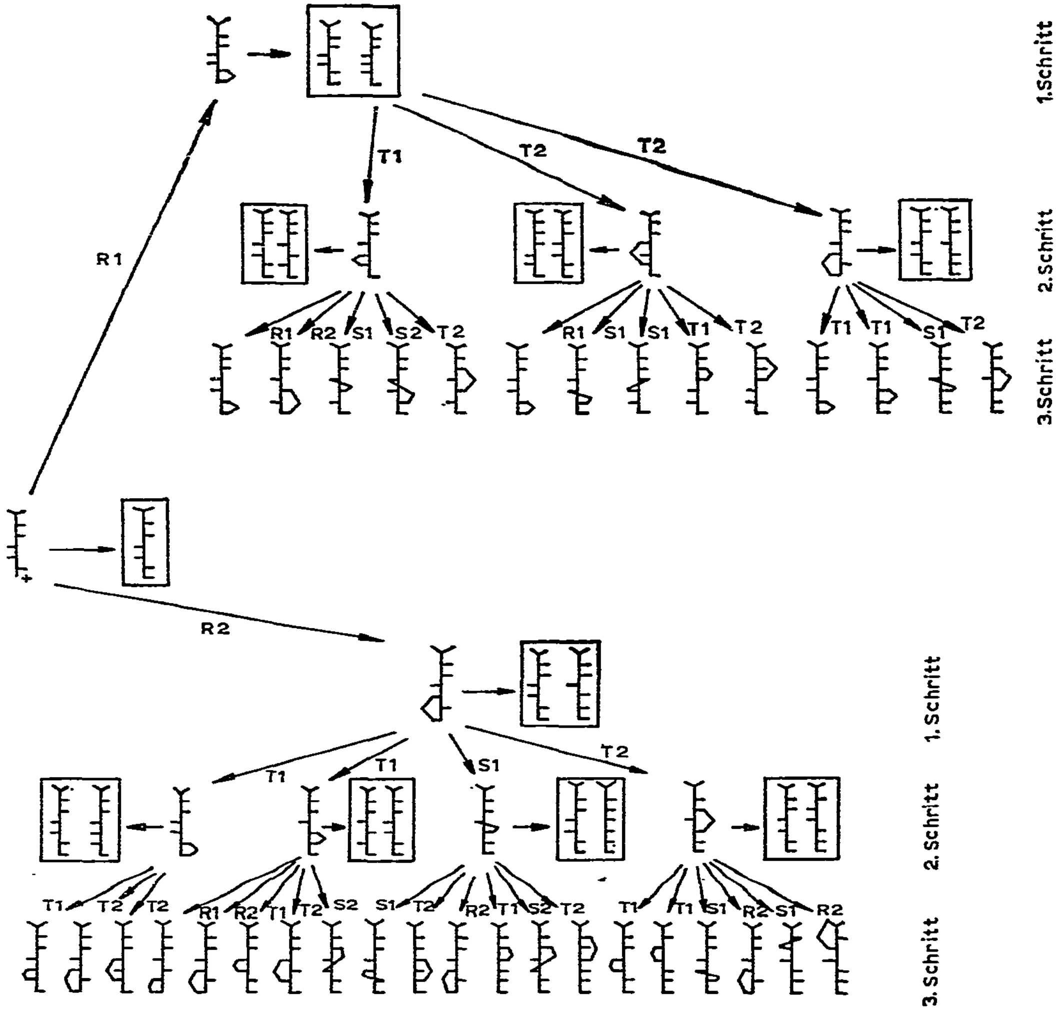


Abb. 1. Isomerisierung von Heptose-Derivaten.

Für die rechnerische Reaktionssimulierung wurden u.a. folgende vereinfachende Annahmen zugrunde gelegt: (1) die Kohlenstoffkette der Octa-*O*-acetyl-al-heptosen liegt in der "Zick-zack"-Konformation vor[14–16]. (2) Unter dieser Voraussetzung läuft die Isomerisierung nur in 1,2- und 1,3-Schritten durch die Kohlenstoffkette. Größere als 6-gliedrige Ringe sind sterisch unwahrscheinlich. (3) Sitzt der angreifende Substituent, bezogen auf die Fischer-Projektion in *cis*-Stellung zu der austretenden Gruppe, so erfolgt ein 1,2 C- oder 1,3 C-Schritt. Hat die reagierende Acetylgruppe *trans*-Konfiguration, so erfolgt ein 1,2 T- oder 1,3 T-Schritt. Die frei drehbaren Acetylgruppen der C-Atome am Kettenende reagieren in einfachen 1,2- bzw. 1,3-Schritten. (4) Das Verhältnis der Wahrscheinlichkeiten möglicher Isomerisierungsschritte wurde an Schwerpunktmodellen der Octa-*O*-acetyl-al-heptosen abgeschätzt. Ein Schritt, der ohne Verdrehung der "Zick-zack"-Konformation möglich ist, wurde für wahrscheinlicher angesetzt als ein solcher, der eine Verdrehung der Kohlenstoff-Kette erfordert.

Unter diesen Voraussetzungen liefert die Rechnung weitgehende Übereinstimmung mit den experimentellen Befunden. Die Zahl der auftretenden Konfigurationen vermehrt sich mit fortschreitender Isomerisierung so rasch, daß bereits nach 6 Schritten alle 32 Heptosen als Abbruchprodukte auftreten. Setzt man dagegen die Schritte geringer Wahrscheinlichkeit, die nur unter starker Verdrehung der Heptose-Kette aus der Zick-zack-Form möglich sind, gleich O, so tritt die D-*glycero*-L-*ido*-Heptose selbst nach 8 Schritten noch nicht auf. Außerdem bestätigt die Rechnung das eindeutige Fehlen der D-*glycero*-L-*gluco*- und der D-*glycero*-D-*ido*-Heptose und ihrer Enantiomeren. Das Auftreten der L-*glycero*-D-*manno*-Heptose als Hauptprodukt der Isomerisierung zeigt, daß ein großer Teil der Ausgangssubstanz mehr als 18 Isomerisierungsschritte durchlaufen muß.

Prüft man die Ergebnisse bei der Racemisierung von 2,3,4,5,6-Penta-*O*-acetyl-7-*O*-toluolsulfonyl-al-D-*glycero*-D-*gulo*-heptose (7) rechnerisch nach, dann ist das Auftreten der D-*glycero*-L-*ido*-Konfiguration bzw. der enantiomeren Form äußerst unwahrscheinlich. Dagegen tritt die L-*glycero*-D-*gulo*-Heptose bereits in den ersten Schritten mit großer Wahrscheinlichkeit auf, da lediglich Isomerisierung am C-Atom 6 erfolgt ist. Das bei der Isomerisierung von 7 als Hauptprodukt entstehende Heptoseanhydrid besitzt daher wahrscheinlich die Konfiguration eine 1,6-Anhydro-L-*glycero*-*β*-D-*gulo*-heptopyranose (**20**).

EXPERIMENTELLER TEIL*

*7-O-Toluolsulfonyl-*D-glycero-D-gulo*-heptose-diäthylmercaptal* (**5**) [*Pf.*]. — D-*glycero*-D-*gulo*-Heptose-diäthylmercaptal (**4**)[7] wird in 90 ccm wasserfreiem Pyridin gelöst und unter Kühlung und mechanischem Rühren innerhalb von 30 Min. tropfenweise mit 9,6 g *p*-Toluolsulfochlorid in 45 ccm Pyridin versetzt. Die Lösung bleibt über Nacht bei —15° stehen. Anschließend wird in 1 Liter Eiswasser eingegossen und der ausgefallene Sirup in Chloroform gelöst. Die Chloroformphase wird in üblicher Weise von Pyridinresten befreit, mit Na_2SO_4 getrocknet und im Vak. eingedampft. Der Rückstand wird in warmem Essigester gelöst, mit Aktivkohle gereinigt und der

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*[*Pf.*: bearbeitet von H. Pfetzing; *Pi.*: bearbeitet von G. Pirke.

Kristallisation überlassen. Ausb. 16,5 g (60%); Schmp. 102°; $[\alpha]_D^{20}$ + 1.6° (Chloroform) (Gef.: C, 45.89; H, 6.26; S, 20.43. Ber. für $C_{18}H_{30}O_8S_3$: C, 45.93; H, 6.42; S, 20.44%).

*2,3,4,5,6-Penta-O-acetyl-7-O-toluolsulfonyl-*D-glycero-D-gulo-*heptose-diäthylmercaptal* (**6**) [*Pf.*]. — **5** (16.5 g) wird in 80 ccm absol. Pyridin gelöst und unter Eiskühlung tropfenweise mit 110 ccm Acetanhydrid versetzt. Die Lösung bleibt über Nacht bei Raumtemperatur stehen und wird dann in 1 Liter Eiswasser eingegossen. Nach kurzer Zeit tritt Kristallisation ein. Das Produkt wird aus absol. Äthanol umkristallisiert. Ausb. 18.5 g (79%); Schmp. 89°; $[\alpha]_D^{20}$ −8.0° (Chloroform) (Gef.: C, 49.61; H, 5.88; S, 14.0. Ber. für $C_{28}H_{40}O_{13}S_3$: C, 49.39; H, 5.92; S, 14.12%).

*2,3,4,5,6-Penta-O-acetyl-7-O-toluolsulfonyl-al-*D-glycero-D-gulo-*heptose* (**7**) [*Pf.*] — **6** (10 g) wird in 100 ccm Aceton gelöst und nacheinander mit 20 g gepulvertem $CdCO_3$, 30 ccm Wasser und tropfenweise unter Rühren mit 20 g $HgCl_2$ in 40 ccm Aceton versetzt. Die Mischung wird bei Raumtemperatur 24 Stdn. gerührt. Anschließend wird von den Salzen abgesaugt und der Rückstand mit Aceton gewaschen. Das Filtrat wird bei 30° im Vak. eingedampft und der Rückstand dreimal mit je 75 ccm Chloroform extrahiert. Zur Entfernung von gelösten Quecksilbersalzen wird die Chloroformphase zweimal mit gesättigter Kochsalzlösung und anschließend dreimal mit Wasser ausgeschüttelt. Nach dem Trocknen mit Na_2SO_4 wird das Lösungsmittel im Vak. entfernt. Beim Trocknen im Hochvak. bläht sich der Sirup stark auf und fällt in voluminöser amorpher Form an. Ausb. 8.0 g (94%); $[\alpha]_D^{20}$ +12.5° (Chloroform) (Gef.: C, 50.23; H, 5.52; S, 5.94. Ber. für $C_{24}H_{30}O_{14}S$: C, 50.17; H, 5.28; S, 5.58%).

*7-Toluolsulfonyl-*D-glycero-D-gulo-*heptose* (**8**) [*Pf.*]. — **5** (10 g) wird in 30 ccm Aceton gelöst und nacheinander mit 20 g gepulvertem $CdCO_3$, 10 ccm Wasser und tropfenweise unter Rühren mit 20 g $HgCl_2$ in 24 ccm Aceton versetzt. Das Gemisch wird 24 Stdn. bei Raumtemperatur gerührt und anschließend 1 Std. auf 50° erwärmt. Die auf 0° abgekühlte Lösung wird filtriert und der Rückstand mit Aceton und Methanol ausgewaschen. Nach dem Abdampfen der Hauptmenge Lösungsmittel wird mit Wasser auf etwa 800 ccm verdünnt und die Quecksilbersalze werden durch Einleiten von H_2S entfernt. Die hierbei freiwerdende Salzsäure wird mit einem Anionenaustauscher (IR-45) entfernt. Die Lösung wird eingedampft und der Rückstand aus Äthanol umkristallisiert. Ausb. 2,3 g (30%); Schmp. 132–134° (Zers.); $[\alpha]_D^{20}$ −13.0° (Wasser) (Gef.: C, 46.15; H, 5.66; S, 8.45; Ber. für $C_{14}H_{20}O_9S$: C, 46.12; H, 5.53; S, 8.79%).

*2,3,4,5,6-Penta-O-acetyl-7-desoxy-7-jod-*D-glycero-D-gulo-*heptose-diäthylmercaptal* (**9**) [*Pf.*].—In einer Druckflasche werden 3 g **6** in einem Gemisch von 1.5 g NaJ in 15 ccm wasserfreiem Aceton gelöst und 3 Stdn. auf 100° erwärmt. Nach dem Abkühlen auf 0° wird von ausgefallenem Na-Tosylat (0.9 g; 96%) abfiltriert und das Aceton im Vak. verdampft. Der Rückstand wird in absol. Äthanol gelöst und nach mehrfachem Reinigen mit Aktivkohle der Kristallisation überlassen. Ausb. 2,5 g (89%); Schmp. 80°; $[\alpha]_D^{20}$ −19.0° (Chloroform) (Gef.: C, 39.49; H, 5.04; S, 9.67; J, 19.77. Ber. für $C_{21}H_{33}JO_{10}S_2$: C, 39.63; H, 5.22; S, 10.06; J, 19.94%).

*2,3,4,5,6-Penta-O-acetyl-7-O-triphenylmethyl-*D-glycero-D-gulo-*heptose-diäthylmercaptal* (**11**) [*Pf.*].—**4** (6 g) wird in 35 ccm absol. Pyridin gelöst und mit 5.3 g Triphenylchlormethan versetzt. Die Lösung wird 30 Stdn. bei Raumtemperatur aufbewahrt. Anschließend werden unter Eiskühlung tropfenweise 70 ccm Acetanhydrid zugegeben. Die Lösung wird erneut 30 Stdn. bei Raumtemperatur aufbewahrt und anschließend in 500 ccm Eiswasser eingegossen. Dabei tritt nach einiger Zeit Kristallisation ein. Das Produkt wird aus absol. Äthanol umkristallisiert. Ausb. 8 g (71%); Schmp. 111°; $[\alpha]_D^{20}$ −4.1° (Chloroform) (Gef.: C, 62.54; H, 6.11; S, 8.07. Ber. für $C_{40}H_{48}O_{11}S_2$: C, 62.48; H, 6.29; S, 8.33%).

*2,3,4,5,6-Penta-O-acetyl-7-O-triphenylmethyl-*D-glycero-D-manno*-heptose-diäthylmercaptal* (**13**) [*Pi.*].—D-*glycero*-L-*manno*-Heptose-diäthylmercaptal (**12**)[9] (3 g) wird in 90 ccm absol. Pyridin mit 3 g Triphenylchlormethan umgesetzt und wie vorstehend beschrieben aufgearbeitet. Ausb. 6,4 g (87.7 %); Schmp. 176°; $[\alpha]_D^{20}$ −26.8° (*c* 1.46, Chloroform) (Gef.: C, 62,15; H, 6.56; S, 8.36. Ber. für $C_{40}H_{48}O_{11}S_2$: C, 62.48; H, 6.29; S. 8.34%).

*2,3,4,5,6-Penta-O-acetyl-*D-glycero-L-manno*-heptose-diäthylmercaptal* (**14**) [*Pi.*].—**13** (4.9 g) wird in 7.5 ccm eiskaltem Eisessig mit 1.25 ccm gekühltem Bromwasserstoff–Eisessig versetzt. Das ausgeschiedene Triphenylbrommethan (58%) wird abfiltriert, und das Filtrat in 500 ccm Eiswasser gegossen, das mit Chloroform unterschichtet ist. Die wässrige Phase wird zweimal mit je 100 ccm Chloroform ausgeschüttelt. Die vereinigten Chloroformphasen werden mit gesättigter $NaHCO_3$-Lösung behandelt, mit Na_2SO_4 getrocknet und eingedampft. Der sirupöse Rückstand wird in Äthanol gelöst und bis zur beginnenden Trübung mit Wasser versetzt. Das Kristallisat wird zur Entfernung von Triphenylcarbinol mehrmals aus Benzol–Petroläther umkristallisiert. Ausb. 3.0 g (89%); Schmp. 110°; $[\alpha]_D^{20}$ −12.0° (*c* 7, Chloroform) (Gef.: C, 47.75; H, 6.15; S, 10.20. Ber. für $C_{21}H_{34}O_{11}S_2$: C, 47.90; H, 6.51; S, 12.18%).

*2,3,4,5,6-Penta-O-acetyl-7-O-toluolsulfonyl-*D-glycero-L-manno*-heptose-diäthylmercaptal* (**15**) [*Pi.*].—**14** (10.5 g) in 60 ccm absol. Pyridin werden bei 0° tropfenweise mit 7 g *p*-Toluolsulfochlorid in 10 ccm Pyridin versetzt. Die Lösung wird 48 Stdn. bei 0° und 6 Stdn. bei Raumtemperatur aufbewahrt. Beim Eingießen in Eiswasser kristallisiert **15** bald aus. Es wird aus Äthanol–Wasser umkristallisiert. Ausb. 11.1 g (85.8%); Schmp. 58°; $[\alpha]_D^{20}$ −5.6° (*c* 1.8, Chloroform). Gef.: C, 49.61; H, 5.97; S, 13.35. Ber. für $C_{28}H_{40}O_{13}S_3$: C, 49.32; H, 5.92; S, 14.13%).

*1,1,2,3,4,5,6-Hepta-O-acetyl-7-O-toluolsulfonyl-al-*D-glycero-L-manno*-heptose* (**17**) [*Pi.*].—**15** (1 g) wird bei 0° im Gemisch von 100 ccm Acetanhydrid und 5 ccm konz. Schwefelsäure gelöst und 20 Stdn. bei Raumtemperatur gelassen. Beim Eingießen in Eiswasser scheidet sich ein dunkles Produkt ab, das in Chloroform aufgenommen wird. Die wässrige Phase wird noch zweimal mit je 100 ccm Chloroform extrahiert. Die vereinigten Auszüge werden zweimal mit Wasser, dreimal mit gesättigter $NaHCO_3$-Lösung und noch zweimal mit Wasser ausgeschüttelt, mit Na_2SO_4 getrocknet und eingedampft. Der Rückstand wird in Äthanol aufgenommen, über Nacht mit Aktivkohle behandelt und bis zur beginnenden Kristallisation mit Wasser versetzt. Es wird aus Benzol–Petroläther umkristallisiert. Ausb. 8.5 g (77.8%); Schmp. 100°, $[\alpha]_D^{20}$ +17.8° (*c* 1.21, Chloroform) (Gef.: C, 49.70; H, 5.30; S, 4.45. Ber. für $C_{28}H_{36}O_{17}S$: C, 49.70; H, 5.30; S, 4.74%).

*Isomerisierung von 2,3,4,5,6-Penta-O-acetyl-7-O-toluolsulfonyl-al-*D-glycero-D-gulo-*heptose* (**7**) [*Pf.*]. — **7** (2 g) wird in 30 ccm Acetanhydrid mit 2 g wasserfreiem Zinkchlorid unter Feuchtigkeitsausschluß 3 Stdn. auf 100° erhitzt. Die dunkelgefärbte Lösung wird auf 0° abgekühlt, filtriert und in Eiswasser eingegossen. Der Filterrückstand wird mit kaltem Aceton gewaschen und aus Wasser umkristallisiert: 1.3 g (75%) *p*-toluolsulfonsäures Zink-hexahydrat. Die wässrige Lösung wird nach Zersetzung des Acetanhydrids mit Chloroform extrahiert, die Chloroformphase von Säurespuren befreit und mit Na_2SO_4 getrocknet. 8 g des dunkelgefärbten Produktes werden an einer Magnesol–Celite-Säule (5:1) mit 1.5 l thiophenfreiem Benzol-*tert*-Butanol (125:1) gereinigt: 5.1 g gelber Sirup, $[\alpha]_D^{20}$ +4.8° (Chloroform). Dieser wird in 50 ccm Methanol gelöst und mit 30 ccm 0.5N Natriummethylat verseift. Die Lösung bleibt über Nacht bei Raumtemperatur stehen. Sie wird anschließend mit einigen Tropfen Eisessig neutralisiert und im Vak. eingedampft. Der bräunlich gefärbte Rückstand wird in 100 ccm Wasser aufgenommen und mit Aktivkohle gereinigt. Die Na-Ionen werden mit einem Kationenaustauscher (IR-120) entfernt. Das Eluat wird mit einem Anionenaustauscher (IR-45) neutralisiert und die Lösung im Vak. eingedampft. Ausb. 0.96 g eines amorphen Produktes, $[\alpha]_D^{20}$ +12.9° (Wasser). 600 mg hiervon werden in üblicher Weise an einer Cellulose-Säule getrennt (1-Butanol–Pyridin–Wasser (4:3:3). Die Fraktionen, die das Heptose-anhydrid (**19** bzw. **20**) [R_F 0.35; Kieselgel G; 2-propanol–Diisopropyläther–65-proz. Ameisensäure (4:3:3)] enthalten, werden im Vak. eingedampft. Der Rückstand wird in wenig heißem Äthanol gelöst, mit Aktivkohle gereinigt und der Kristallisation überlassen. Ausb. 246 mg. (41%); Schmp. 151°; $[\alpha]_D^{20}$ +35.5° (Wasser) (Gef.: C, 43.94; H, 6.34. Ber. für $C_7H_{12}O_6$: C, 43.75; H, 6.29%).

Zur Identifizierung der isomeren Heptosen wird das Gemisch nach der Verseifung mit Natriummethylat chromatographiert: absteigende Durchlaufchromatographie an Whatman Nr. 1 Papier; 1-Butanol–Pyridin–Wasser (3:1:1); Laufzeit 3,5 Tage; Entwickler: $AgNO_3$.

2,3,4,7-Tetra-O-acetyl-1,6-anhydroheptopyranose (**21**) [*Pf.*]. — **19** bzw. **20** (55.5 mg) wird in 6 ccm absol. Pyridin unter Eiskühlung mit 6 ccm Acetanhydrid versetzt und über Nacht bei Raumtemperatur aufbewahrt. Es wird in 50 ccm Eiswasser eingegossen und im Vak. zum Trocknen gedampft. Der Rückstand wird in 5 ccm absol. Äthanol gelöst, mit Aktivkohle gereinigt und im Vak. über $CaCl_2$ der Kristallisation überlassen. Ausb. 79.8 mg (76%); Schmp. 102°; $[\alpha]_D^{20}$ +24.0° (Chloroform) Gef. : C, 49.76; H, 5.38; Acetyl, 50.3. Ber. für $C_{15}H_{20}O_{10}$: C, 49,99; H, 5.59; Acetyl, 47.8%).

2,3,4,7-Tetra-O-toluolsulfonyl-1,6-anhydroheptopyranose (**22**) [*Pf.*]. — **19** bzw. **20** (120 mg) wird in 1 ccm absol. Pyridin bei 0° mit 720 mg Toluolsulfochlorid versetzt. Nach 50 Stdn. bei Raumtemperatur wird in 50 ccm Eiswasser eingegossen. Nach Zersetzung des überschüssigen Toluolsulfochlorids wird mit Chloroform extrahiert, die Chloroformphase säurefrei gewaschen, mit Na_2SO_4 getrocknet und im Vak. verdampft. Der amorphe Rückstand wird in 5 ccm heißem Äthanol gelöst, mit Aktivkohle gereinigt und der Kristallisation überlassen. Ausb. 220 mg (44%); Schmp. 124°; $[\alpha]_D^{20}$ + 24.7° (Chloroform) (Gef.: C, 51.88; H, 4.84; S, 15.62. Ber. für $C_{35}H_{36}O_{14}S_4$: C, 51.98; H, 4.49; S, 15.83%).

1,6-Anhydro-7-desoxy-7-jod-2,3,4-tri-O-toluolsulfonylheptopyranose (**23**) [*Pf.*]. — **22** (202 mg) wird in einer Druckflasche in 5 ccm wasserfreiem Aceton mit 500 mg wasserfreiem NaJ versetzt. Die Lösung wird 2 Stdn. auf 100° erwärmt, von abgeschiedenem Na-Tosylat abgetrennt (41 mg; 84.5%) und eingedampft. Der Rückstand wird in Chloroform gelöst, mit Wasser gewaschen, getrocknet und das Chloroform im Vak. entfernt. Der Rückstand wird in Aceton gelöst. Beim Verdampfen im Vak. bläht sich der Sirup stark auf und erstarrt zu einem glasig-amorphen Produkt. Ausb. 140 mg (73%); $[\alpha]_D^{20}$ +47.7° (Chloroform) (Gef.: C, 43.35; H, 4.13; J, 15.24; S, 12.49. Ber. für $C_{28}H_{29}JO_{11}S_3$: C, 43.98; H, 3.82; J, 16.60; S, 12.58%).

*Isomerisierung von 1,1,2,3,4,5,6-Hepta-O-acetyl-7-O-toluolsulfonyl-al-*D-glycero-L-manno-*heptose* (**27**) [*Pi.*]. — **27** (4 g) wird mit 4 g $ZnCl_2$ in 60 ccm Acetanhydrid umgesetzt und aufgearbeitet wie vorstehend beschrieben. 5 g des Heptose-Gemisches werden an Kieselgel getrennt (Cyclohexan–Benzol–Pyridin (5:4.5:0.5). Die zueinander gehörenden Fraktionen werden vereinigt, im Vak. eingedampft und zur Entfernung von Pyridin-Spuren im Vak. über konz. Schwefelsäure aufbewahrt. Die Rückstände werden in Benzol–Petroläther zur Kristallisation gebracht. Hierdurch werden vier Fraktionen erhalten: (1) 1,1,2,3,4,5,6-Hepta-*O*-acetyl-7-chlor-7-desoxy-al-D-*glycero*-L-*manno*-heptose: Ausb. 35 mg; $[\alpha]_D^{20}$ + 17.0° (*c* 0.57, Chloroform) (Gef.: C, 46.82; H, 5.50; Cl, 6.09. Ber. für $C_{21}H_{29}O_{14}Cl$: C, 46.64; H, 5.40; Cl, 6.56%). (2) 220 mg (3) 320 mg. (4) 650 mg (Sirup). Aus den Fraktionen (2) and (3) kristallisieren mit Benzol–Petroläther vier Komponenten: (A) und (B): Gemische aus 1,1,2,3,4,5,6,7-Octa-*O*-acetyl-al-D-*glycero*-L-*manno*-heptose und der L-Form; Schmp. 113 bzw. 161°: $[\alpha]_D^{20}$ − 8.7 bzw. − 2.5° (Chloroform); (C) das Racemat aus D- und L-Form; Schmp. 167°; $[\alpha]_D^{20}$ 0° (Chloroform); und (D) die reine L-Form; Schmp. 126°; $[\alpha]_D^{20}$ − 29.2° (Chloroform).

Die Mutterlaugen und die Fraktion (4) werden zur Trockne gebracht und mit Na N-methylat verseift. Nach 12 Stdn. wird mit verd. Essigsäure neutralisiert, im Vak. zum Sirup eingedampft und in Wasser gelöst. Die Ionen werden mit Amberlite IR-120 und IR-45 entfernt. Zur Identifizierung der Heptosen wird an Whatman No. 1 Papier chromatographiert. (absteigende Durchlaufchromatographie; 1-Butanol–Pyridin–Wasser (3:1:1); Laufzeit: 4 Tage; Entwickler: Anilinphthalat).

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Die Rechnungen wurden mit einem Elektronenrechner Zuse Z 23 durchgeführt.

ZUSAMMENFASSUNG

2,3,4,5,6-Penta-*O*-acetyl-7-*O*-toluolsulfonyl-al-D-*glycero*-D-*gulo*-heptose (**7**) und 1,1,2,3,4,5,6-Hepta-*O*-acetyl-7-*O*-toluolsulfonyl-al-D-*glycero*-L-*manno*-heptose (**17**) wurden der totalen Racemisierung mit $ZnCl_2$ in Acetanhydrid unterworfen. Aus

dem entstandenen Gemisch isomerer Heptosen ließen sich zahlreiche Isomere durch chromatographischen Vergleich zuordnen. Berechnungen mit Hilfe eines Elektronenrechners bestätigen den von Micheel und Böhm[1] vorgeschlagenen Reaktionsmechanismus und zeigen weitgehende Übereinstimmung mit den experimentellen Ergebnissen. Für das bei der Isomerisierung von 7 auftretende Heptose-anhydrid wurde die Struktur eine 1,6-Anhydro-L-*glycero*-β-D-*gulo*-heptopyranose wahrscheinlich gemacht.

SUMMARY

Penta-*O*-acetyl-7-*O*-toluene-*p*-sulphonyl-*aldehydo*-D-*glycero*-D-*gulo*-heptose (7) and hepta-*O*-acetyl-7-*O*-toluene-*p*-sulphonyl-*aldehydo*-D-*glycero*-L-*manno*-heptose (17) were subjected to total racemisation with zinc chloride in acetic anhydride. From the resulting mixture of isomeric heptoses, numerous isomers were identified by chromatographic comparison. Calculations with the aid of a computer confirm the previously proposed reaction mechanism and show extensive agreement with the experimental results. The anhydride obtained by isomerisation of compound 7 was tentatively identified as 1,6-anhydro-L-*glycero*-β-D-*gulo*-heptopyranose.

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THE OPTICAL ROTATION OF SOME CARBOHYDRATE DERIVATIVES IN *p*-DIOXAN–WATER MIXTURES

A. J. HANNAFORD*
Department of Chemistry, Birkbeck College, Malet Street, London, W.C.1. (Great Britain)

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INTRODUCTION

The specific optical rotation of a compound with respect to light of a particular wavelength sometimes varies considerably with change of solvent[1]. However, rotations in mixed solvents have not been investigated extensively. We have measured the variation in optical rotation of some carbohydrate derivatives in *p*-dioxan (dioxan)–water mixtures, since this information was needed for hydrolysis studies of carbohydrate phenylboronates[2].

RESULTS AND DISCUSSION

The specific rotations, $[\alpha]_D$, of thirteen carbohydrate derivatives in dioxan and in water are recorded in Table I and values of $[\alpha]_D$ for dioxan–water mixtures are plotted in the Figs. against weight percentage of water. The densities of dioxan and water are 1.028 and 0.997 at 25°; weight percentages and volume percentages are therefore similar. With the exception of Fig. 1, all values of $[\alpha]_D$ are plotted on ascending scales of increasing magnitude, irrespective of the sign of rotation. The signs of the $[\alpha]_D$ values are shown on each curve.

The specific rotation of a compound can vary with its concentration in solution[1], but this effect is generally not large[1]. No concentration effects were observed for the thirteen carbohydrate derivatives in dioxan–water mixtures, since the concentrations were varied randomly from less than 1% to about 4% by weight and yet $[\alpha]_D$ was found to vary smoothly with solvent composition. Nor was any concentration dependence of $[\alpha]_D$ found for any compound in pure dioxan or in pure water. Many solutions of different concentrations were prepared for each substance, but $[\alpha]_D$ remained constant for each solvent, *e.g.*, $[\alpha]_D$ for methyl α-D-glucopyranoside in pure water was found to be constant for solutions varying in concentration from 2.5 to 410 g/l. All solutions of a particular compound were prepared at a constant temperature, and the rotation measurements for this compound were carried out at one temperature. Thus, variations in $[\alpha]_D$ due to temperature changes were eliminated.

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[*] Present address, Department of Chemistry, Woolwich Polytechnic, London, S.E.18.

Variations of specific rotation in different solvents have been attributed in part to the local electric field around the optically active solute, caused by the polarisation of neighbouring solvent molecules[3]. This effect is eliminated by considering the rotivity, Ω, of the solution, defined as $\Omega = 3\,[\alpha]_D/(n^2 + 2)$, where n is the refractive index of the solvent[3]. A further aspect of the solvent effect on optical rotation is provided by more specific solvent–solute interactions. Such interactions may consist of weak dipole–dipole linkages or stronger chemical bonds, and they influence optical rotation either by providing new active centres associated with groups attached to solute

TABLE I

OPTICAL ROTATION DATA FOR SOME CARBOHYDRATE DERIVATIVES

Compound	*Temp.* (°)	$[\alpha]_D$ (*Dioxan*)(°)	$[\alpha]_D$ (*Water*)(°)
A Methyl α-D-glucopyranoside	25	$+174.8^{a}$	$+159.3^{b}$
B Methyl β-D-glucopyranoside	25	-40.2^{c}	-33.5^{a}
C Methyl 2,3-di-*O*-methyl-α-D-glucopyranoside	25	+153.4	+149.9
D Methyl 2,3-di-*O*-methyl-β-D-glucopyranoside	25	−34.7	−32.8
E D-Glucal	25	+1.02	−7.63
F Methyl α-D-galactopyranoside	23	+171.9	+192.6
G Methyl β-D-galactopyranoside	24	-34.2^{d}	-1.0^{a}
H Methyl α-D-mannopyranoside	23	+99.4	+79.5
I Methyl β-L-arabinopyranoside	23	$—^{e}$	+245.1
J Methyl 4,6-*O*-benzylidene-α-D-glucopyranoside	22	+99.9	$—^{e}$
K Methyl 4,6-*O*-benzylidene-β-D-glucopyranoside	20	−73.2	$—^{e}$
L Methyl 4,6-*O*-benzylidene-2,3-di-*O*-methyl-α-D-glucopyranoside	22	+91.3	$—^{e}$
M Methyl 4,6-*O*-benzylidene-2,3-di-*O*-methyl-β-D-glucopyranoside	22	−70.3	$—^{e}$

[a]Mean of readings for 5 solutions; [b]for 4 solutions; [c]for 6 solutions; [d]for 9 solutions; [e]unobtainable because of insolubility of compound.

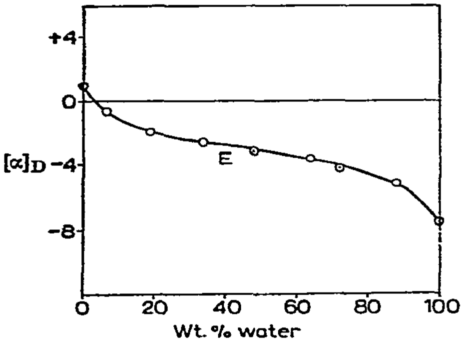

Fig. 1

Figs. 1–5. Variation in $[\alpha]_D$ for various carbohydrate derivatives in dioxan–water mixtures. See Table I, for identification of the code letters.

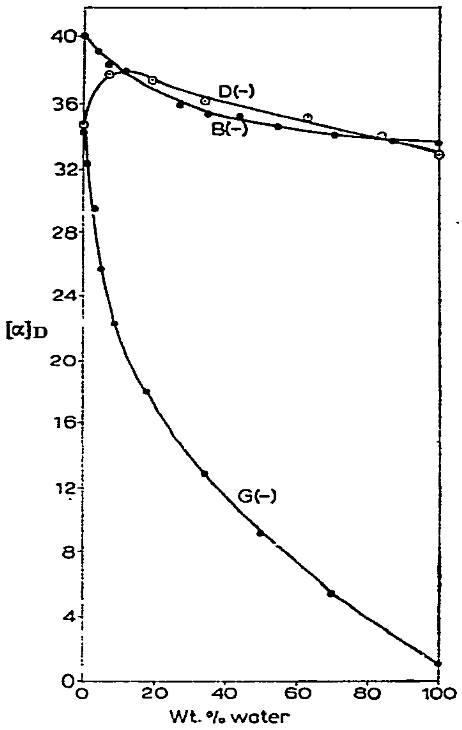

Fig. 2

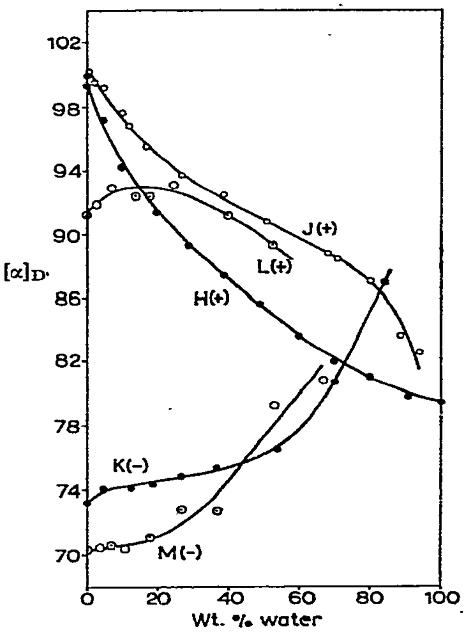

Fig. 3

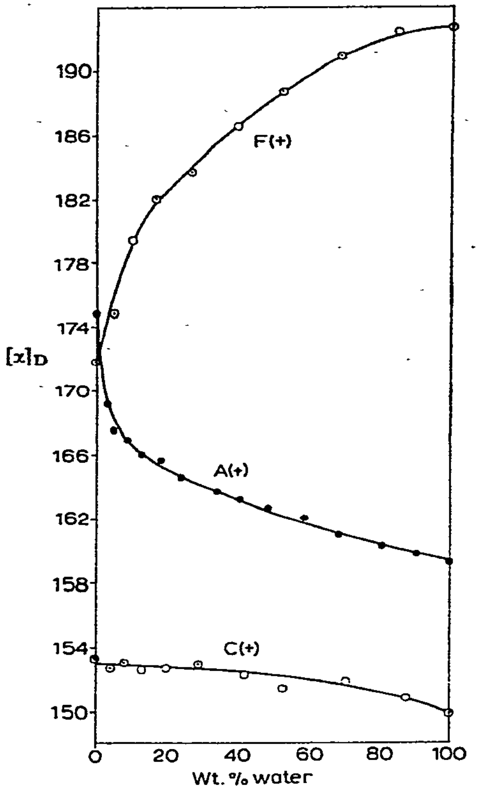

Fig. 4

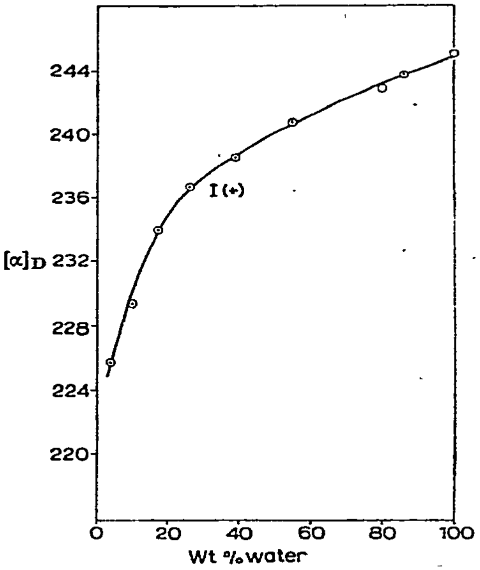

Fig. 5

molecules, or by actual distortion of the geometry of the active solute molecules[3]. All these effects are reflected in the variation of Ω with the electrostatic field acting on the active solute molecules and therefore with the dielectric constant (ε) of the medium. Thus, it has been found[3,4] that most of the solvent effect on specific optical rotation can be eliminated by considering the rotivity calculated from the equation $\Omega = \Omega_0 + B\,(\varepsilon - 1)/(\varepsilon + 2)$ where B is a function of the polarity of the solute and Ω_0 is the rotivity in a medium of unit dielectric constant and is therefore equivalent to the specific rotation in the vapour phase. An approximately linear relationship between Ω and $(\varepsilon - 1)/(\varepsilon + 2)$ is found in some cases[3,4], and a more general dependence of specific rotation on dielectric constant has been known for many years[4].

The rotivity Ω of each carbohydrate derivative, calculated from specific rotation data and refractive index data, was plotted against solvent composition and also against $(\varepsilon - 1)/(\varepsilon + 2)$ for the dioxan–water mixtures; the dielectric constants of dioxan and water are 2 and 78, respectively, at 25°, and the function $(\varepsilon - 1)/(\varepsilon + 2)$ increases from 0.25 to 0.95. In both cases, the resulting curves have generally similar shapes to the curves shown in the Figs. Straight lines are not obtained, and the curves do not show any particular pattern of behaviour. It is thus clear that the observed range of behaviour for these carbohydrate derivatives cannot be explained simply in terms of the refractive index and dielectric constant of the solvent mixtures.

However, one consistent feature does emerge from the curves. The change in $[\alpha]_D$ (and therefore Ω) with solvent composition is most rapid at that end of the composition scale where the optically active solute is least soluble. The fully substituted glycosides, which are insoluble in pure water, show their most rapid change in $[\alpha]_D$ towards the water end of the scale; whereas the unsubstituted glycosides (very soluble in water, but only soluble to about 0.2% in dioxan) have $[\alpha]_D$ changing most rapidly at the dioxan end. The 2,3-di-*O*-methylglycosides and D-glucal, which are soluble in dioxan and water, show only small changes in $[\alpha]_D$ along their composition scales. These observations hold whether a curve of $[\alpha]_D$ increases or decreases in magnitude (irrespective of sign) from the dioxan end. If, however, $[\alpha]_D$ is plotted against molar % composition of solvent, this one consistent feature vanishes, and the resulting curves show no particular trends.

EXPERIMENTAL

For the measurement of $[\alpha]_D$ values, a quantity of each compound was weighed into a volumetric flask, an approximately known volume of distilled water was added, and the flask was weighed again. After complete dissolution had been effected, dioxan was added, slowly and with swirling to ensure complete mixing, until the level of the solution was just below the mark. The flask was then left overnight at a constant temperature, the volume was made up to the mark with dioxan, and the flask was weighed again. Thus, volume errors due to heat of mixing were eliminated. For each compound, single stocks of dioxan and water were used, and all rotation measurements were made on the same day. The dioxan used was purified in the usual manner by repeated distillation from molten sodium and contained <0.025% of water as shown by titration with Karl Fischer reagent. Optical rotations were measured in a 4- or 1-dm, centre-filling, polarimeter tube and errors in $[\alpha]_D$ were calculated as ±0.4°. Where a compound was not very soluble in one of the pure solvents, many separate solutions were prepared, and the mean value of $[\alpha]_D$ is quoted. The carbohydrate derivatives were all prepared by standard methods and carefully purified by recrystallisation.

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SUMMARY

The specific optical rotations of thirteen carbohydrate derivatives in *p*-dioxan–water mixtures have been measured and found to vary in a complex manner with solvent composition. Elimination of possible refractive-index effects of the solvent and of effects due to solvent–solute interactions, by established methods, did not account for this complex behaviour.

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EQUILIBRIUM BETWEEN PYRANOID AND FURANOID FORMS
PART I. 2,3-*O*-ISOPROPYLIDENE-L-RHAMNOSE*

S. J. Angyal, V. A. Pickles, and Rajendra Ahluwalia
School of Chemistry, The University of New South Wales, Kensington, N.S.W. (Australia)

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INTRODUCTION

One fundamental problem in carbohydrate chemistry has not yet been satisfactorily solved[1]. Aqueous solutions of reducing sugars consist of equilibrium mixtures of at least five compounds: the two pyranoses, the two furanoses, and the *aldehydo*- (or *keto*-) form. The physical and chemical properties of sugars in aqueous solution will depend on the composition of this equilibrium mixture, yet, until quite recently, this composition had not been determined for any sugar. In particular, the proportion of furanoid forms in equilibrium is not known, except in the case of D-ribose[2].

Proton magnetic resonance (p.m.r.) spectroscopy offers now a promise towards the solution of this problem[2], since it can be applied to aqueous (D_2O) solutions. Unfortunately, the method is not particularly suitable for detecting minor components in a mixture. Hence, initially, we have been studying sugars which have a higher than usual proportion of furanoid forms. In this paper we report an instance in which the amounts of the furanoid and pyranoid forms have been determined with the aid of p.m.r.

DISCUSSION

Both the pyranoid (**1**) and the furanoid (**4**) forms are possible for 2,3-*O*-isopropylidene-L-rhamnose[3], but it is usually regarded as being predominantly in the furanoid forms. As evidence, the formation of a 5-methyl ether[3], a 5-benzoate[4], and a 5-toluene-*p*-sulphonate[5] are cited. Such evidence, however, is not conclusive, because the various forms present in equilibrium may not react at the same rate. In this case, the exocyclic hydroxyl group of the furanoid form may be more reactive than the endocyclic one in the pyranoid form.

The p.m.r. spectrum of 2,3-*O*-isopropylidene-L-rhamnose in aqueous solution, as already described by Perlin[6], shows it to be a mixture. Although the compound is crystalline, its mutarotation in aqueous solution is too fast to allow the spectrum of the crystalline modification to be determined. However, when methyl sulphoxide is used as solvent, mutarotation is slow, and, initially, the spectrum of one compound can be observed. This spectrum (Table I) shows H-1 as a singlet, H-2 as a doublet,

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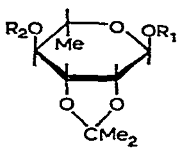
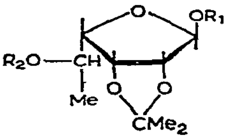
*Presented at the IUPAC Symposium on the Chemistry of Natural Products, Kyoto, Japan, April 12–18 (1964), *Abstract Papers*, p. 238.

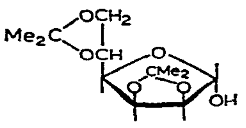
and H-3 as a pair of doublets, H-4 and H-5 being unresolved. The spectrum is similar to that of D-mannose 2,3-carbonate described by Perlin[6] who concluded, on indirect evidence, that it represents that of a furanoid form. It is also very similar to the p.m.r. spectrum of 2,3:5,6-di-*O*-isopropylidene-α-D-mannose (7) (Table I) which, of course, must be in the furanoid form.



(1) $R_1 = R_2 = H$
(2) $R_1 = Me, R_2 = H$
(3) $R_1 = H, R_2 = Me$



(4) $R_1 = R_2 = H$
(5) $R_1 = R_2 = Ac$
(6) $R_1 = H, R_2 = Ts$



(7)

Definite evidence that crystalline 2,3-*O*-isopropylidene-L-rhamnose is a furanose was obtained by preparing its diacetate (5) and studying its p.m.r. spectrum. In this spectrum, every proton is clearly resolved (Table I). One proton appears, in the form of a quartet of doublets, at lower field than any other proton except the anomeric one. This proton must be H-5, which is coupled to H-4 and to the three protons of the methyl group. Its position at low field indicates that the carbon atom to which it is attached (C-5) also carries one of the acetoxyl groups. The diacetate is therefore 1,5-di-*O*-acetyl-2,3-*O*-isopropylidene-L-rhamnofuranose (5); the very small coupling between H-1 and H-2 shows that it is the α-anomer (in the β-anomer, H-1 and H-2 are *cis* and $J_{1,2}$ must be larger[7-9] than 3). The coupling constants are listed in Table I.

The spectra of solutions (methyl sulphoxide-d_6) of the crystalline 2,3-*O*-isopropylidene-L-rhamnose and of its diacetate are very similar in the H-1, H-2, H-3 region. The comparison indicates that the crystalline modification is 2,3-*O*-isopropylidene-α-L-rhamnofuranose (4).

In order to obtain information on the p.m.r. spectrum of the 2,3-*O*-isopropylidene-L-rhamnopyranose structure, the spectrum of the *O*-isopropylidene derivative (2) of methyl α-L-rhamnopyranoside[10] was determined. This differs considerably from that of the furanoid derivatives: H-3 is a triplet and it is at higher field than the H-2 doublet. The other chemical shifts also are different (see Table I). For our purpose, the most significant difference is in the separation between the two methyl peaks of the isopropylidene group which is 0.09–0.11 p.p.m. in the furanoid compounds, but 0.14 in the methyl pyranoside. This distinction appears to be general for derivatives of 2,3-*O*-isopropylidenerhamnose. Arzoumanian *et al.*[11] described the p.m.r. spectra of methyl 2,3-*O*-isopropylidene-α-L-rhamnofuranoside and of its 5-toluene-*p*-sulphonyl (5-tosyl) derivative in which the separation between the methyl peaks is 0.07 p.p.m., and we found that this value is 0.18 for 2,3-*O*-isopropylidene-4-*O*-methyl-L-rhamnopyranose (3).

As 2,3-*O*-isopropylidene-α-L-rhamnofuranose mutarotates, the methyl peaks

TABLE I

P.m.r. DATA OF L-RHAMNOSE DERIVATIVES: CHEMICAL SHIFTS (δ) AND COUPLING CONSTANTS (c.p.s.)[a]

Compound	Solvent	H-1	H-2	H-3	H-4	H-5	H-6	C-Me	OH	OR[b]	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6}$	$J_{H,OH}$
2	D$_2$O	5.00	4.29	4.14	3.42	3.73	1.29	1.42,1.55		3.47	<0.5	5.5	6.5	9.8	5.8	
	(CD$_3$)$_2$SO	4.80	4.08	3.89	3.12	3.43	1.18	1.29,1.43	3.43	3.32	<0.5	5.8	6.2	9.8	6.0	5.5
3	D$_2$O	5.29 β5.10	4.21	4.27	3.17	3.88	1.26	1.40,1.57		3.52 β3.51	<0.5 β1.2	4.8^c	5.0	9.0	6.0	
	CDCl$_3$	5.30 β4.90	4.11	4.20	3.03	3.86	1.27 β1.32	1.36,1.54	3.93	3.54 β3.51	<0.5	5.3^c	5.0	8.8	6.0 β5.6	
4	D$_2$O	5.32	4.68	4.94			1.30 1.25^d	1.36,1.47 1.39^d,1.54^d			<0.5	5.9	3.0		6.2 6.2^d	
	(CD$_3$)$_2$SO	5.28	4.44	4.75			1.16	1.26,1.35	4.58(C-5) 6.26(C-1)		<0.5	5.9	3.0		5.6 6.2^d	
5	CDCl$_3$	6.19	4.70	4.82	4.06	5.18	1.36	1.31,1.46		2.06(C-1) 2.08(C-5)	<0.5	5.8	3.2	7.3	6.3	
	(CD$_3$)$_2$SO	6.02	4.75	4.89	4.06	5.04	1.26	1.28,1.39		2.02(C-1) 2.06(C-5)	<0.5	5.8	3.2	7.1	6.4	
6	CDCl$_3$	5.03				4.87	1.45 β1.44	1.10,1.26 β1.14,1.32	3.28		<0.5				6.0 β6.0	
	(CD$_3$)$_2$SO	5.05	4.37	4.47	3.96	4.70	1.39	1.06,1.12	6.45		<0.5	5.6	2.9	6.8	6.0	4.3
7	CDCl$_3$	5.38	4.61	4.81				1.33,1.49 1.39,1.49	3.54		<0.5	5.9	3.2			
	(CD$_3$)$_2$SO	5.13	4.44	4.70				1.24,1.34 1.27,1.34	6.34		<0.5	5.8	3.2			4.4

[a] All data refer to α anomers, unless otherwise indicated. [b] R = Me for **2**,**3**,**4**. R = Ac for **5**. [c] $J_{2,4}$(?) = 1.6. [d] Pyranoid form (**1**).

split (Fig. 1), with the appearance of smaller side-peaks. The most pronounced side-peak (at 1.54 p.p.m. in D_2O) is 0.15 p.p.m. downfield from the other methyl peak and is therefore assigned to the pyranoid forms. From its area, it can be estimated that the equilibrium mixture contains 35 ±5% of pyranoid forms at 40°. Another indication of the presence of the pyranoid forms is the appearance of a doublet at δ 5.12 (H-1 of the β-L-pyranose) and a multiplet at *ca.* 3.4 (H-4 of the pyranoses), but these signals are not sufficiently distinct for quantitative estimation.

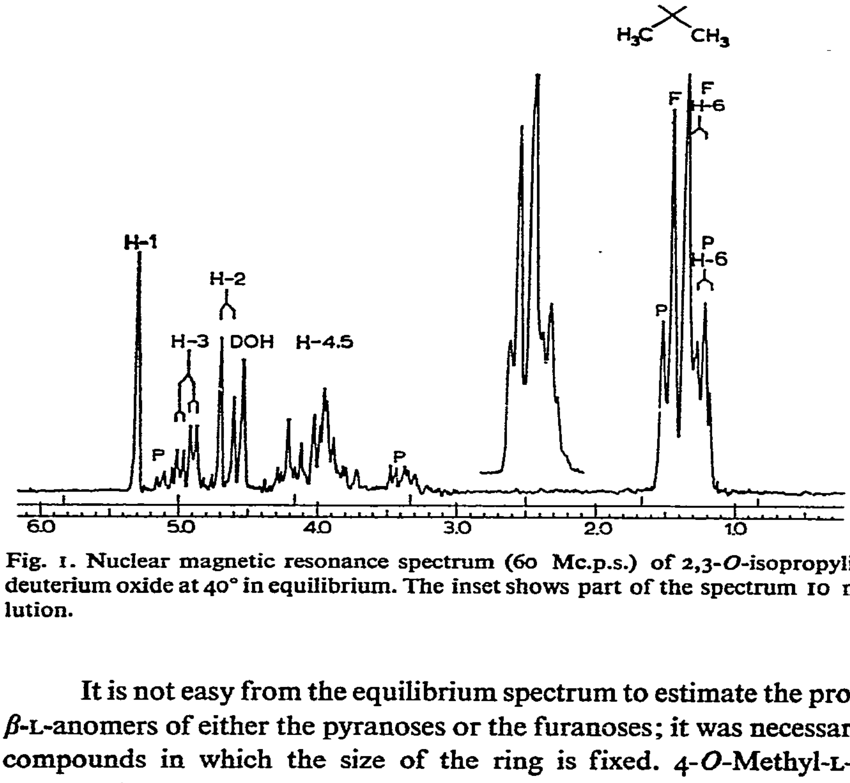


Fig. 1. Nuclear magnetic resonance spectrum (60 Mc.p.s.) of 2,3-*O*-isopropylidene-L-rhamnose in deuterium oxide at 40° in equilibrium. The inset shows part of the spectrum 10 minutes after dissolution.

It is not easy from the equilibrium spectrum to estimate the proportion of α- and β-L-anomers of either the pyranoses or the furanoses; it was necessary to study model compounds in which the size of the ring is fixed. 4-*O*-Methyl-L-rhamnose[10] was treated with acetone in the presence of sulphuric acid and gave a compound to which the 2,3- (rather than 1,2-) *O*-isopropylidene structure was assigned because it showed mutarotation. The aqueous equilibrium solution showed two signals corresponding to anomeric protons: the one at lower field, a singlet, was assigned to the α-L pyranose (3), the other, a doublet, to the β-L form. The α,β ratio was found to be 72:28 at 40°; in chloroform solution, the ratio was the same. The crystalline form is the α-L anomer; its rotation increases with time and initially it shows only an anomeric singlet.

L-Rhamnose itself showed the same two anomeric peaks in a ratio of 60:40 at 40°. It is interesting to note that there is little difference in respect of this ratio between L-rhamnose and its *O*-isopropylidene derivative. Formation of the cyclic ketal involves a distortion which brings the two *cis* oxygen atoms of the pyranoid ring closer together. The presence of another *cis* oxygen atom, as in β-L-rhamnose, would be expected to increase the energy required for the ring formation[12]. The above figures show that, in this case, the increase is only 0.3 kcal/mole.

In the furanoid form, the *β*-L isomer would be much less stable than the *α*-L form, since the former has all of the substituents *cis* on the five-membered ring; the presence of a cyclic isopropylidene ring would make the *β*-L form even less stable. 2,3-*O*-Isopropylidene-5-*O*-tosyl-*α*-L-rhamnose[5] (6) was studied as a model compound; its p.m.r. spectrum, either in pure or in aqueous methyl sulphoxide, gave no indication of the presence of another anomer*. Similarly, the spectrum of 2,3:5,6-di-*O*-isopropylidene-*α*-D-mannose (7) did not alter with time (Table I). Levene and Compton[13] stated that 5-*O*-methyl-L-rhamnose does not mutarotate. It appears therefore that only small proportions of *β*-L-rhamnofuranose are present in equilibrium with the *α*-form. Assuming that the ratios of the anomers of 2,3-*O*-isopropylidene-L-rhamnose are the same as those of the model compounds, it was calculated that the aqueous equilibrium solution at 40° contains approximately 25% of *α*-pyranose, 10% of *β*-pyranose, 65% of *α*-furanose, and very little of the *β*-furanose form.

Clearly, fusion with a dioxolane ring stabilizes a five-membered, compared with a six-membered, ring. Rhamnose itself contains very little furanose in its equilibrium solution; the exact amount is not known, but, if it is taken as less than 1%, the stabilization of the furanoid form of 2,3-*O*-isopropylidenerhamnose by the attached five-membered ketal ring is calculated to be more than 3.1 kcal/mole. It is to be noted that the *manno* configuration present in rhamnose is the least favourable for furanoid forms (three *cis* substituents[14]), and the most favourable for pyranoid forms. The pyranoid forms of allose, gulose, and talose all have higher free energies than mannopyranose[1]. Other 2,3-*O*-isopropylidenehexoses will therefore have even less pyranoid form in equilibrium than has the rhamnose derivative.

2,3-*O*-Isopropylidene-L-rhamnose does not crystallize readily. Baxter and Perlin[15] found that only part of their substance crystallized, the rest remained in a syrupy state for a long period; the syrup, however, gave the same reactions as the crystals. Obviously, mutarotation is very slow in the solvent mixture (ether–light petroleum) used for crystallization. Mutarotation in non-hydroxylic solvents is catalysed by 2-hydroxypyridine which acts both as an acid and a base[16]. Following a discussion with Dr. J. A. Mills, we added a very small amount of 2-hydroxyquinoline (2-hydroxypyridine being not available) to a solution of 2,3-*O*-isopropylidene-L-rhamnose in ether–light petroleum, and found that crystallization occurred within a few minutes. This method may be generally applicable whenever a sugar derivative having a free anomeric hydroxyl group has to be crystallized from aprotic solvents.

Freudenberg and Wolf[3] described two forms of 2,3-*O*-isopropylidene-L-rhamnose, m.p. 87–89° and 79–80°; the lower melting form was not encountered in our work. Levene and Compton[13] suggested that the two forms represented an *α*,*β* pair;

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*In chloroform solution, however, all of the methyl signals gradually develop side-peaks at lower field, indicating the presence of *ca.* 14% of *β*-L furanose in equilibrium. It is probable that hydrogen bonding from OH-1 to O-2 increases the stability of the *β*-L anomer in non-hydroxylic solvents.

however, since they both mutarotate in the *same* direction, this suggestion is untenable. It is likely that the lower-melting form (which was isolated from mother-liquors) was impure.

We refrain from attempting to determine the shape of the furanoid ring in the rhamnose derivative, as has been done in other cases[17,18]. It is clear now that the dihedral angles calculated from the coupling constants by the use of the Karplus equation[19] are not sufficiently accurate for this purpose[20]. Perlin[6] found substantial disagreement between proton–proton coupling observed for D-mannofuranose 2,3-carbonate and data derived from the Karplus curve. Similar difficulties arise with 2,3-*O*-isopropylidene-L-rhamnose. The $J_{3,4}$ value of 3.0 c.p.s. corresponds to an angle of 50°, which appears too high to be incorporated into a furanoid ring. It is worth commenting, however, that $J_{2,3}$ has a value close to 5.5 in the furanoid and in the pyranoid derivatives; it appears that the 1,3-dioxolane ring has an approximately constant shape, irrespective of whether it is attached to a five- or a six-membered ring. Lemieux *et al.*[21] found 6.3 for the corresponding coupling constant in 2,2-dimethyl-1,3-dioxolane, and values close to 5.5 were obtained for many ketals of inositols[22]. This value represents an angle of about 40° by the Karplus equation. A slightly higher value (6.5) was found for $J_{2,3}$ in D-mannose 2,3-carbonate[6], and, apparently, the ring in the carbonate is flatter than that in the ketal, owing to resonance. Hence, the carbonate ring stabilizes the furanoid form to a greater extent. The p.m.r. spectrum of D-mannose 2,3-carbonate gives no indication of the presence of pyranoid forms.

EXPERIMENTAL

2,3-O-Isopropylidene-α-L-rhamnose (4), prepared according to Freudenberg and Wolf[3], and crystallized from ether–light petroleum (b.p. 60–80°) containing a minute amount of 2-hydroxyquinoline, had m.p. 92–93°.

1,5-Di-O-acetyl-2,3-O-isopropylidene-α-L-rhamnofuranose (5).—2,3-*O*-Isopropylidene-α-L-rhamnose (0.25 g) was left standing overnight in a mixture of acetic anhydride (0.47 ml) and pyridine (0.58 ml). Water (5 ml) was added, and the mixture was kept at 0° until it crystallized. After two crystallizations from light petroleum the compound (0.23 g) had m.p. 54–55°, $[\alpha]_D^{29}$ −43° (*c* 3.2, chloroform) (Found: C, 54.2; H, 6.85. $C_{13}H_{20}O_7$ calc.: C, 54.15; H, 7.0%).

Deacetylation of 1,5-di-O-acetyl-2,3-O-isopropylidene-α-L-rhamnofuranose.— 1,5-Di-*O*-acetyl-2,3-*O*-isopropylidene-α-L-rhamnofuranose (0.65 g) was dissolved in dry methanol (3 ml) and, at 12°, barium hydroxide solution (8%) was added dropwise until the solution was alkaline to phenolphthalein. After 60 h, the solution was neutralized with solid carbon dioxide, and concentrated under reduced pressure to a syrup. From light petroleum (b.p. 60–80°), crystals (0.3 g), m.p. 92–93°, were obtained.

Methyl 2,3-O-isopropylidene-α-L-rhamnopyranoside[10] (2), b.p. 90°/0.3 mm, probably contained some of the *β*-anomer, but insufficient to be noticeable in the p.m.r. spectrum.

2,3-O-Isopropylidene-4-O-methyl-α-L-rhamnose (3).—Syrupy 4-*O*-methyl-L-rhamnose[10] (5.1 g) was shaken for 24 h with dry acetone (100 ml) containing 0.2% sulphuric acid and anhydrous copper sulphate (10 g). The mixture was filtered, and the filtrate was neutralized with calcium oxide. After evaporation of the solvent, the compound was distilled at 106–108°/0.5 mm as a syrup which crystallized from light petroleum in long needles, m.p. 73–74°. For analysis, it was sublimed *in vacuo* (Found: C, 55.1; H, 8.4. $C_{10}H_{18}O_5$ calc.: C, 55.0; H, 8.3%), $[\alpha]_D^{19}$ −9.4° (1.5 min), −4.0° (34 h) (c 1.9, chloroform).

P.m.r. spectra. — The spectra were obtained with a Varian A-60 n.m.r. spectrometer. The sweep width was calibrated from time to time by using a chloroform solution of tetramethylsilane and the recommended side-band technique. Solutions (15–20% w/v) of the samples in $(CD_3)_2SO$ or deuteriochloroform were used with tetramethylsilane (δ 0.0) internal standard; for solutions in deuterium oxide, sodium 3-(trimethylsilyl)-1-propanesulphonate (δ 0.00), *tert*-butyl alcohol (δ 1.23) or acetone (δ 2.17) were used; internal standards were added after equilibrium spectra had been obtained, in order to avoid possible interference. The spectra were determined at 40°. Initial spectra were obtained immediately after dissolution of the compound; in aqueous solution, mutarotation was rapid, but several days were allowed to elapse before equilibrium spectra were measured. The concentration of anomers was measured quantitatively by repeated integration of the anomeric and methyl proton signals. Where the HDO peak interfered with the quantitative estimation of the anomeric proton, repeated evaporation with deuterium oxide was used. In the case of 2,3-*O*-isopropylidene-α-L-rhamnose, where the methyl and H-6 peaks overlap, the spectral curve was decomposed into individual peaks by using an IBM 1620 computer with a programme written by Associate Professor G. R. Roper and Mr. T. Newburn, Faculty of Applied Science, The University of New South Wales.

Chemical shifts are expressed as δ values in p.p.m.; J values are given in c.p.s.; accuracy is ±0.01 p.p.m. for chemical shifts and ±0.1 c.p.s. for coupling constants. First-order analysis was used to evaluate the spectra. By treating groups of three adjacent protons as isolated ABX systems, it was possible to analyse the multiplets in most cases, except where multiplet overlap was excessive. The δ values of the AB portion of the ABX systems were calculated as the centres of gravity.

SUMMARY

From the p.m.r. spectrum of 2,3-*O*-isopropylidene-L-rhamnose, by comparison with that of some model compounds, it was deduced that the aqueous solution in equilibrium contains approximately 25% of α-pyranose, 10% of β-pyranose, 65% of α-furanose, and very little of the β-furanose.

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STUDIES, ON URONIC ACID MATERIALS

PART XVIII. LIGHT-SCATTERING STUDIES ON SOME MOLECULAR-WEIGHT FRACTIONS FROM *Acacia senegal* GUM

D. M. W. ANDERSON, SIR EDMUND HIRST, AND S. RAHMAN,
Department of Chemistry, The University, Edinburgh 9 (Great Britain)
AND G. STAINSBY
Procter Department, The University, Leeds 2 (Great Britain)

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INTRODUCTION

Despite the industrial importance of *Acacia senegal* gum (gum arabic), there have been few studies to date of its molecular size and shape.

From osmotic pressure measurements, Thomas and Murray[2] found gum arabic solutions to be isotonic over the range pH 6–9, and Oakley[3] obtained values of 191,000 and 217,000 for the number-average molecular weight in 0.1N and 0.5N sodium chloride, respectively. Ultracentrifugal studies by Säverborn[4] indicated values in the range of 256,000–326,000. More recently, two light-scattering results have been reported; Veis and Eggenberber[5] found a weight-average molecular weight ($\bar{M}_w$) of $(1.00 \pm 0.05) \times 10^6$ in 0.02N hydrochloric acid, whereas Deb and Mukherjee[6] found $\bar{M}_w = 0.58 \times 10^6$ in a solution 0.02 N with respect to a mixture of potassium, calcium, and magnesium chlorides. Contrary to the opinion expressed by Deb and Mukherjee[6], these values cannot be regarded as being in satisfactory agreement; the reason for this requires investigation.

Electrolyte concentrations of only 0.02N were used in the light-scattering studies[5,6] but, in our experience (*cf.* Oakley[7]), considerably higher concentrations are required to suppress ionisation of the carboxyl groups in the gum. Furthermore, Deb and Mukherjee[6], without explanation, used a mixed solvent containing divalent cations. The reason for this is difficult to understand in view of the cross-linking tendencies of calcium ions in acidic polysaccharide systems. A more fundamental factor may also be involved. The investigators to date appear to have used commercial samples, without giving adequate attention to their characterisation. It is now recognised[8,9] that early samples of gum arabic were mixtures of the exudates from various *Acacia* species. The composition[10] and physical properties[11] of *Acacia* gums vary from species to species and, furthermore, within a particular species[12,13]. The commercial gum arabic produced at the present time originates almost exclusively from *A. senegal*, but admixture with other species, particularly *A. seyal* and *A. laeta*, or with other genera, *e.g.*, *Albizia sericocephala*[14], can never be discounted. Commercial samples should not be used in fundamental studies.

Recently, an authenticated specimen of *A. senegal* gum was characterised carefully, and it was reported[15] that an arbitrary number of molecular-weight fractions could be obtained by fractional precipitation with sodium sulphate. Preliminary experiments with molecular-sieve chromatography[16] indicated that the gum had a broad molecular-weight distribution, with considerable contributions from high molecular-weight material[17], in agreement with Veis and Eggenberger[5]. In view of these indications, it appeared that light scattering was preferable to osmometry as a means of obtaining a self-consistent series of reliable measurements of the whole gum and of its fractions.

EXPERIMENTAL

Characterisation and purification of the whole gum.—Details of the characterisation of the specimen of *A. senegal* gum have been given[15]. After dissolution in cold distilled water, the gum was filtered, dialysed against tap water, exhaustively electrodialysed against distilled water[12], and then freeze-dried.

Fractionation of the gum. — Fractionation was achieved by the procedure described by Anderson and Stoddart[15]. The purified gum (40 g, in the free-acid form) was dissolved in water (400 ml) and maintained at 28–30°. Anhydrous sodium sulphate was added in small amounts to the solution with continuous stirring. When precipitation began, the rate of stirring was decreased to enable the precipitate to rise to the surface and coagulate; it was then transferred quickly to a warm Buchner funnel (15 cm), and the excess solution was removed. The remaining mother-liquor was filtered through a similar funnel; the residue on both funnels gave Fraction 1. By repetition of the above procedure, Fractions 2 and 3 were obtained (the number of fractions isolated at this stage is arbitrary). Eventually, the solution contained gum which could not be precipitated with sodium sulphate, and this gave Fraction 4. The fractions were dialysed, exhaustively electrodialysed, and freeze-dried. On a dry-weight basis, the yields of Fractions 1–4 were 16, 8, 8, and 0.5 g, respectively; the total recovery of gum was 81%.

Preparation of the sodium salt of the whole gum and each of its fractions.—The sodium salt of the whole gum, and of its fractions, was prepared by neutralisation, followed potentiometrically, with sodium hydroxide solution. The neutralised products were dialysed to remove any excess of alkali, and the sodium salts were finally recovered by freeze-drying.

Solutions for light-scattering experiments were made by direct weighing, allowance being made for the pre-determined moisture content of each of the products. The weighed amounts were then dissolved in molar sodium chloride.

Light-scattering photometer.—Light-scattering experiments were carried out at 25.00 ±0.02° by using an instrument based on the design of McIntyre and Doderer[18], and constructed in the Procter Department, University of Leeds. Details of this instrument and its calibration have been described[19]. In use, one galvanometer (G_1) monitors the main beam, whilst a second galvanometer (G_2) receives the light scattered at any angle in the range 20–160° and also the main beam (at 0°) after suitable attenuation. By using the ratio of the galvanometer readings, compensations for the inevitable fluctuations (of the order of ±5%) in the intensity of the main beam are possible: the "reduced intensity-of-scatter", G'_θ, for a solution at $\theta°$ is given by

$$G'_\theta = \frac{(G_2/G_1)_\theta}{(G_2/G_1)_0}.$$

Unpolarised blue light (4358 Å) was used, and was selected from the mercury spectrum by a combined 47B and 2E Wratten filter.

Clarification of solutions. — Each solution was clarified by successive passage through 5- and 1.2-μ "Millipore" filters, using a stainless-steel filter holder attached to a 50-ml syringe. The filtrate ran directly into the cylindrical light-scattering cell, which had been cleaned by steaming followed by rinsing with condensing acetone vapour. The solvent was treated in a similar manner and its reduced intensity-of-scatter, G''_θ, was also determined from the ratios of galvanometer readings.

The scatter due to the gum at $\theta°$, G_θ, was then taken as equal to $(G'_\theta - G''_\theta)$, and the usual corrections for volume viewed and partial polarisation, which both vary with θ, were applied[20]. The use of the difference $G'_\theta - G''_\theta$ implies that the solution and the solvent each contain identical amounts of scattering impurities. Ideally, each should be free from such scatter, but this is rarely achieved in practice, particularly for aqueous solutions [21].

Ultracentrifugation is the alternative technique to ultrafiltration for clarifying macromolecular solutions, and the two methods were compared for effectiveness by centrifuging a sample at 17,000 r.p.m. (30,000 g) in a Spinco Model L ultracentrifuge for 3 h. The upper half of the solution from each centrifuge tube was transferred to the light-scattering cell, and the scattering envelope was determined. A portion of the solvent was similarly treated; in neither case was there any significant difference between the envelopes resulting from clarification by centrifugation or by filtration. Since the "Millipore" filters are much more rapid and convenient to use than the centrifuge, all subsequent clarifications were achieved by ultrafiltration.

In the centrifugal clarification, sedimentation did not occur under the conditions used. The level-of-scatter did not change on subsequent passage through the "Millipore" filters, which therefore do not retain any of the gum molecules. Colorimetric experiments did not detect differences from the initial concentration after clarification. The use of the initial concentration of gum therefore appears to be justified, although, ideally, the concentration after clarification should be used in calculating results. Any concentration difference is unlikely to exceed 1μg/ml; this does not introduce any serious error into the molecular-weight calculation.

RESULTS AND DISCUSSION

Fig. 1 shows how the reciprocal, reduced intensity-of-scatter, G_θ^{-1}, varies with the angle-of-scatter θ (plotted as $\sin^2(\theta/2)$ as suggested by Zimm[22]) for the whole gum and Fractions 1, 2, and 3. The curves bend downwards steeply at the lower angles.

If the observed scatter arose solely from the gum, these curves would indicate that the gum molecules behave as stiff rods in solution. It is well known, however, that gum arabic solutions have a comparatively low viscosity, even when fairly concentrated, whereas rod-like molecules give solutions of high viscosity, even at low concentrations, and the viscosity rises sharply with increasing concentration. It is suggested, therefore, that the curvature seen in Fig. 1 is not a reflection of the shape of the gum molecules, but an indication of incomplete clarification of the solutions from dust and suspended impurities despite the care taken with clarification processes. Unwanted scatter from such sources would be manifest most particularly at the lower angles and be of less importance at the higher angles. Repeated passage of the solutions through the filters failed to reduce the scatter, which was independent of time over several hours. More reliable figures for the angular variation of scatter could not, therefore, be obtained for the present fractions. This illustrates the need for caution and for consideration of all relevant information when interpreting the data obtained from such experiments.

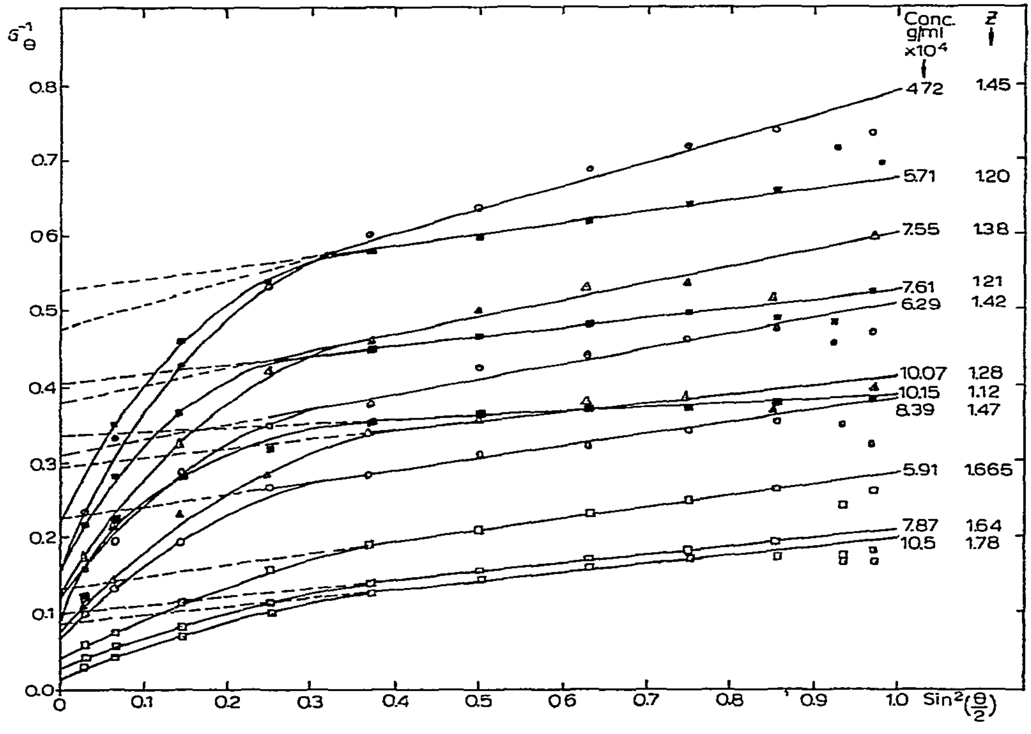

Fig. 1. Plot of the reciprocal, reduced intensity-of-scatter, G_θ^{-1}, *versus* $\sin^2(\theta/2)$ for *A. senegal* gum (○) and Fractions 1 (□), 2 (△), and 3 (■) obtained therefrom.

In order to obtain estimates of molecular weight from the data in Fig. 1, it is assumed that the linear parts of the curves are due to scatter from the gum molecules. In this region, contributions from unwanted impurities will be minimal, and almost independent of angle. Thus, the whole of the linear region most nearly represents scatter from the gum molecules, although the level of scatter will be in error by an amount equal to the contribution from residual impurities. (At the present time, the magnitude of this contribution is indeterminate, but it is possibly 10% of the observed total scatter). Since the linear portion is not parallel to the $\sin^2(\theta/2)$ axis, the effective size of the scattering units in solution exceeds $\lambda/20$ (*i.e.* exceeds 160 Å).

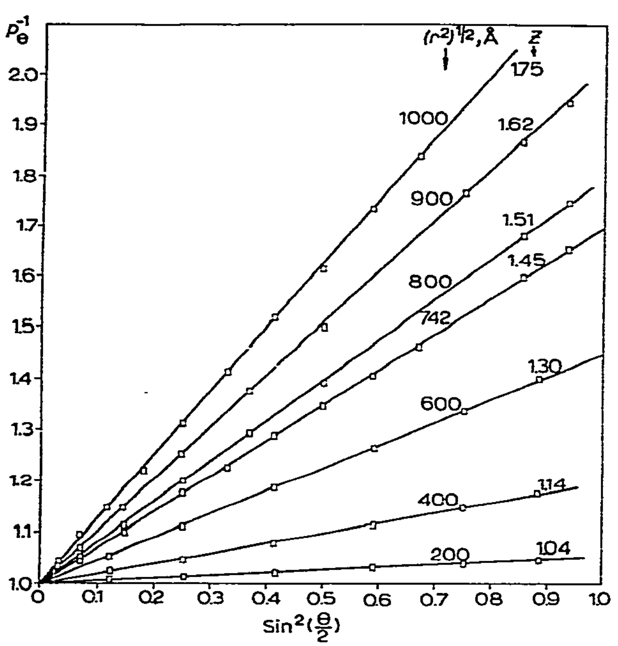

Fig. 2. Plot of P_θ^{-1} *versus* $\sin^2(\theta/2)$ for polydisperse coils of various root-mean-square end-to-end distances $(r^2)^{\frac{1}{2}}$. The dissymmetry (Z) is shown on each curve.

It is therefore necessary to extrapolate to $\sin^2(\theta/2) = 0$, using the intercept to compute the molecular weight from expression (1),

$$\frac{1}{M_w} = \frac{KC}{R_o} \tag{1}$$

where $R_o = G_o \times$ the instrument constant (which depends only on geometrical factors, and on the galvanometer and photo-tube sensitivities).

In this way, molecular weights are obtained without reference to any model for the scattering units in solution. The assumption made above means that the calculated values are likely to be somewhat higher than the true values. The experimental data from which Fig. 1 was plotted, together with the intercepts and the corresponding values of $\overline{M}_w$, are given in Table I.

The use of equation (1) to obtain $\overline{M}_w$ from R_o also presumes that C/R_o is independent of C, *i.e.*, that virial coefficients are zero. This presumption appeared to be reasonable in the present experiments, since the use of molar sodium chloride as solvent minimises the interionic forces between the charged molecules[7]. Moreover, any small variations of pH with dilution will not affect the total charge carried by each gum molecule[2], since totally neutralised gums have been used. In such circumstances, the gum may be treated as a neutral polymer. The work of Veis and Eggenberger[5], using concentrations 10 to 100 times greater than our own, shows that the value of the virial coefficient, B, decreases sharply with increasing ionic strength, μ, and is approximately 4×10^{-5} for $\mu = 2 \times 10^{-2}$ for un-ionised arabic acid in 0.02M hydrochloric acid (the most appropriate parallel available[5] to that studied here). At the highest concentration of whole gum used in the present study, neglect of the term $2BC$ in the full expression[22] (2), where P_θ is the particle scattering factor, is equivalent to an

$$\frac{KC}{R_o} = \frac{P_\theta^{-1}}{M_w} + 2BC \qquad (2)$$

error of about 4% in $\overline{M}_w$, in the opposite direction to the error in $\overline{M}_w$ arising from the inclusion of scatter by impurities. Whilst it would be of interest to determine the correct value of B, this cannot be done from the results given in Table I because of the unsystematic variation of $\overline{M}_w$ with C. Indeed, it is difficult to estimate the probable correctness of the values of B and $\overline{M}_w$ reported by Veis and Eggenberger[5], as they recorded scatter at only three angles. Their use of more concentrated solutions implies, however, that the contribution from unwanted scatter was quite low.

TABLE I

RECIPROCAL SCATTERED INTENSITIES OF GUM FRACTIONS AT VARIOUS CONCENTRATIONS AND ANGLES

	$G_\theta^{-1} \times 10$											
Angle	*Fraction 1*			*Fraction 2*		*Fraction 3*			*Whole gum*			
$\theta°$	*Conc. (g/ml × 10⁴)*			*Conc. (g/ml × 10⁴)*		*Conc. (g/ml × 10⁴)*			*Conc. (g/ml × 10⁴)*			
	10.5	*7.87*	*5.91*	*10.07*	*7.55*	*10.15*	*7.61*	*5.71*	*8.39*	*6.29*	*4.72*	
20	0.3	0.4	0.6	1.0	1.7	1.2	2.2	2.2	1.0	1.6	2.3	
30	0.4	0.6	0.8	1.4	2.1	2.2	2.8	3.5	1.4	2.0	3.3	
45	0.7	0.8	1.1	2.3	3.2	2.8	3.6	4.6	2.0	2.9	4.3	
60	1.0	1.2	1.6	2.8	4.2	3.1	4.2	5.3	2.7	3.5	5.4	
75	1.3	1.4	1.9	3.4	4.5	3.5	4.5	5.8	2.8	3.7	6.0	
90	1.5	1.6	2.1	3.5	5.0	3.6	4.6	5.9	3.2	4.3	6.4	
105	1.6	1.7	2.3	3.8	5.2	3.7	4.7	6.1	3.2	4.4	6.9	
120	1.7	1.8	2.5	3.8	5.3	3.7	4.9	6.4	3.4	4.6	7.2	
135	1.7	1.9	2.6	3.6	5.1	3.8	4.8	6.6	3.5	4.7	7.4	
150	1.6	1.8	2.4	3.5	4.8	—	4.8	7.2	3.4	4.5	7.1	
160	1.6	1.8	2.6	3.9	6.0	3.8	5.2	9.0	3.2	4.7	7.4	
G_θ^{-1}	0.09	0.10	0.14	0.29	0.37	0.33	0.40	0.52	0.23	0.31	0.48	
Z	1.78	1.65	1.67	1.28	1.38	1.12	1.21	1.20	1.47	1.42	1.43	
$M_w \times 10^5$	10.74	12.35	12.45	3.50	3.63	2.99	3.26	3.36	5.42	6.22	5.77	

It is possible to use the molecular weights found for the fractions to derive a "reconstituted weight-average molecular weight", which should be given if all the fractions were recombined. This test provides a guide to the reliability of the molecular weights relative to one another, and indicates whether significant degradation or aggregation, or loss of material of high or low molecular weight, occurred during fractionation. A value is required, however, for the molecular weight of Fraction 4; this could not be obtained by light scattering on account of the high degree of unwanted scatter. A value of 100,000 was, however, estimated for $\bar{M}_w$ by molecular-sieve chromatography[16,17], the column being calibrated with dextrans of known $\bar{M}_w$. Calculation then gives the reconstituted molecular weight as 7.2×10^5, compared with the value of 5.8×10^5 found for the original whole gum. This indicates that there may have been some loss, during fractionation, of material of low molecular weight; this seems reasonable in view of the extensive dialysis procedures necessary after the sodium sulphate precipitations. Table I shows, however, that the largest experimental uncertainty is associated with Fraction 1; the relatively high proportion of this fraction of high molecular weight means that any error in its $\bar{M}_w$ will contribute significantly to the error in the "reconstituted" $\bar{M}_w$.

Although there is some uncertainty regarding the extent to which the scatter at higher angles represents scatter from the gum alone, it is, nevertheless, worthwhile to obtain an estimate of the dimensions of the gum in solution using the observed scatter-envelope. This necessitates the choice of a model for the dissolved gum (no choice would be required if the observed envelopes arose solely from the gum). We have chosen the "polydisperse* random coil" system, which best fits Veis and Eggenberger's results[5], although it may not be in keeping with recent chemical evidence[1] that the gum is very highly branched.

The particle scattering factor, P_θ, for a system of polydisperse random coils varies linearly with $\sin^2(\theta/2)$. The plots shown in Fig. 2 have been calculated from equation[23] (3) where $x = k^2 s^2 \bar{r}^2/6$; $k = 2\pi/\lambda'$, (λ' being the wavelength of light in

$$P_\theta = \frac{2}{x^2}\left[e^{-x} - (1-x)\right] \tag{3}$$

solution); $s = 2\sin(\theta/2)$; $(\bar{r}^2)^{\frac{1}{2}}$ = root-mean-square of the separation of the ends of the coil. The slope of the line relating P_θ, or P_θ^{-1}, to $\sin^2(\theta/2)$ increases with increase in $(\bar{r}^2)^{\frac{1}{2}}$. Since G_θ^{-1}, plotted in Fig. 1, equals $(G_0^{-1})(P_\theta^{-1})$, it follows that polydisperse coils would give a linear plot of G_θ^{-1} *versus* $\sin^2(\theta/2)$, G_0^{-1} being a constant for each solution examined. The straight lines in Fig. 1, each extrapolated from scatter at the larger angles, can thus be used to determine $(\bar{r}^2)^{\frac{1}{2}}$. The most convenient way of doing this is to calculate the dissymmetry ${}_{45}Z_{135}$ (*i.e.*, the ratio of G_{45}/G_{135}) and to read off the corresponding value of $(\bar{r}^2)^{\frac{1}{2}}$ from the theoretical graph of ${}_{45}Z_{135}$ *versus* $(\bar{r}^2)^{\frac{1}{2}}$ (Fig. 3). The values of ${}_{45}Z_{135}$ and the corresponding values of $(\bar{r}^2)^{\frac{1}{2}}$ are shown in Table II. Unlike the values of $\bar{M}_w$, these values of $(\bar{r}^2)^{\frac{1}{2}}$ are unlikely to be in error even if there is a small amount of scatter from impurities.

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*The significance of the term "polydisperse" in this context follows the usage of Veis and Eggenberger[5], and differs from the convention used in an earlier part of this series[15].

TABLE II

SUMMARY OF RESULTS OBTAINED BY LIGHT SCATTERING

Fraction Number	$\overline{M}_w \times 10^5$	Z	$(\bar{r}^2)^{\frac{1}{2}}$, Å	$(\bar{r}_g^2)^{\frac{1}{2}}$, Å
1	11.85	1.70	948	387
2	3.56	1.33	633	258
3	3.20	1.18	455	186
4	1.00[a]			
Whole gum	5.8	1.44	739	339

[a]Estimated by molecular-sieve chromatography[17].

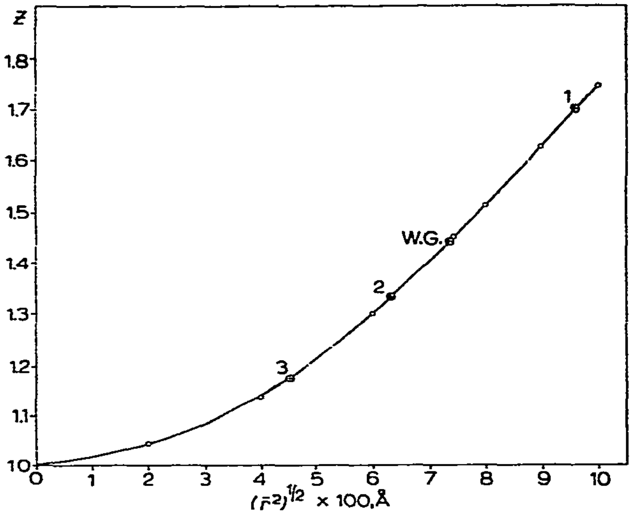

Fig. 3. Plot (○) of Z *versus* $(\bar{r}^2)^{\frac{1}{2}}$ from Fig. 2. Experimental points shown as ⊗ for whole gum (W.G.) and Fractions 1, 2, and 3.

The radius-of-gyration $(\bar{r}_g^2)^{\frac{1}{2}}$, calculated from $(\bar{r}^2)^{\frac{1}{2}}$ by the expression $\bar{r}^2 = 6\bar{r}_g^2$, is included in Table II. These data may be compared with that of Veis and Eggenberger[5], who concluded that for the whole gum in its un-ionised form $\overline{M}_w = 1 \times 10^6$ and that Z (for blue light) was 1.65. These values agree closely with our values for Fraction 1, but there is a discrepancy in the corresponding calculated values of $(\bar{r}^2)^{\frac{1}{2}}$. Veis and Eggenberger[5] apparently used the relation of Z to $(\bar{r}^2)^{\frac{1}{2}}$ for monodisperse coils in this instance [$(\bar{r}^2)^{\frac{1}{2}}$ for monodisperse coils and polydisperse coils = 1035 and 920, respectively, for $Z = 1.65$], although later in their paper the polydisperse coil model was used for ionised arabic acid. Veis and Eggenberger also give much larger values of $(\bar{r}^2)^{\frac{1}{2}}$ for fully ionised arabic acid [$(\bar{r}^2)^{\frac{1}{2}} = 2400$ Å for $Z = 2.7$], but their solutions were of low ionic strength, and it is reasonable to expect the polyelectrolyte to stretch out in these conditions. We deliberately maintained the ionic strength at a high level to avoid a sharp dependence of Z on C at the low concentrations used.

Thus, our results are to be compared with the results for un-ionised arabic acid in dilute hydrochloric acid rather than with the results for the ionised gum[5]. The close agreement of our value of $\bar{M}_w$ for the whole gum with that of Deb and Mukherjee[6] is interesting in view of the solvent system they used.

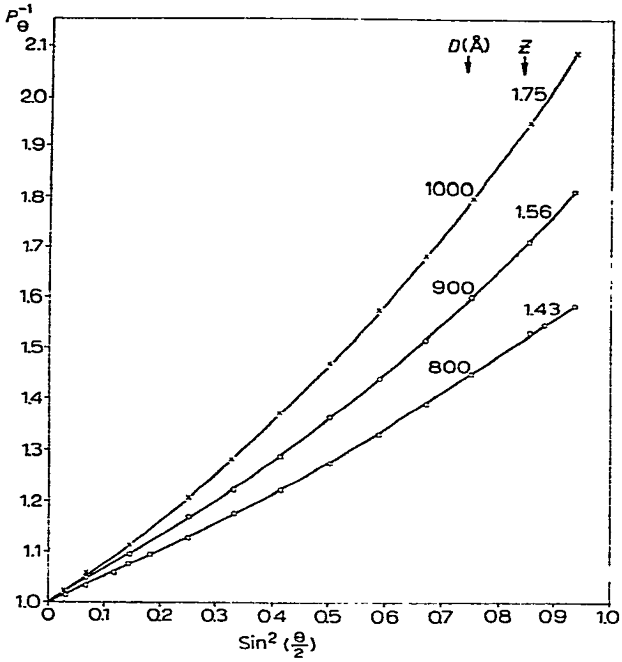

Fig. 4. Plot of P_θ^{-1} *versus* $\sin^2(\theta/2)$ for spherical molecules having diameters D of 800, 900, 1000 Å, and dissymmetries of 1.43, 1.56, and 1.75, respectively.

In view of the recent suggestions that gum arabic molecules are more globular in shape than previously recognised[1], and that they behave as a suspension of non-entangling spheroids in solution[24], it is of interest, finally, to consider the form of the scatter envelope for the highly branched gum if it were to behave as equivalent to a sphere, since this molecular form, like the coil, would impart low viscosity to a solution. The scatter envelope for spheres has a slight upward curvature (see Fig. 4) which has been calculated according to equation[25] (4), where $x = ksD/2$, D being the

$$P_\theta = \frac{3}{x^3}[(\sin x - x\cos x)]^2 \tag{4}$$

diameter of the sphere. The present results, do not, however, enable a significant distinction to be drawn between the spherical and the polydisperse coil models in view of the very slight curvature for the envelope due to spheres and the uncertainties arising from the difficulty of obtaining ideally clear solutions.

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SUMMARY

Light-scattering studies on the sodium salt of *A. senegal* gum, in molar sodium chloride solutions, give $\overline{M}_w = 580{,}000$. The $\overline{M}_w$ values obtained for three molecular-weight fractions, obtained from the whole gum by sodium sulphate precipitation, confirm previous observations that the gum has a very broad molecular-weight distribution. The results obtained are compared with those of earlier investigators, and reasons for some of the differences are discussed. Studies of the shape of the gum molecules in solution did not distinguish between the spheroidal and the polydisperse coil models.

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THE OXIDATION OF SOME CARBOHYDRATE DERIVATIVES, USING ACID ANHYDRIDE–METHYL SULPHOXIDE MIXTURES AND THE PFITZNER–MOFFATT REAGENT. FACILE SYNTHESES OF 3-ACETAMIDO-3-DEOXY-D-GLUCOSE AND 3-AMINO-3-DEOXY-D-XYLOSE

J. S. Brimacombe, J. G. H. Bryan, A. Husain, M. Stacey, and M. S. Tolley
Chemistry Department, The University, Birmingham 15 (Great Britain)

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INTRODUCTION

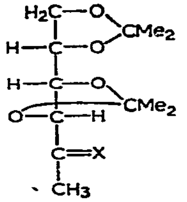
There has been considerable interest recently in applying newly developed oxidation procedures to the synthesis of substituted keto-sugars[1,2]. The chromium trioxide–pyridine complex[3], the oxidant generally employed[4] in the oxidation of carbohydrate secondary hydroxyl groups, often gives only moderate yields of the carbonyl compound and, in one instance, an unusual isomerisation has been observed[5], using this reagent.

Following the demonstration by Pfitzner and Moffatt[6] that dicyclohexylcarbodi-imide and methyl sulphoxide, in the presence of certain acids, led to efficient oxidation of alcohols to the corresponding aldehydes or ketones, this reagent has been used to prepare[1,2] several substituted keto-sugars in high yield. In certain cases, Baker and Buss[2a] were unable to achieve any oxidation of partially protected carbohydrate derivatives (for example, 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose), although the formation of dicyclohexylurea was clearly indicated. More recently, Albright and Goldman[7] have reported a general method for the oxidation of sterically hindered alcohols which uses a mixture of methyl sulphoxide and certain acid anhydrides. This oxidation procedure has been applied [8–10] to carbohydrate derivatives and is mechanistically related to the Pfitzner–Moffatt method, since both oxidations proceed through the intermediate formation of alkoxysulphonium salts[7,11].

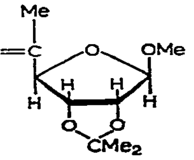
The long-standing need for a mild oxidant, which operates under neutral condiditions, has culminated in the discovery of ruthenium tetroxide[12], and a combination of ruthenium dioxide and sodium periodate[13] has proved to be a potent oxidant of partially protected sugars. A route[14] to glycopyranosid-3-uloses by rearrangement of certain methyl 4,6-*O*-benzylidene-3-deoxy-3-phenylazoaldopyranosides with alkali is clearly of potential, but more limited, value.

RESULTS AND DISCUSSIONS

In connexion with studies on certain 4- and 5-acetamido-deoxy sugars, we sought to prepare these compounds *via* oximation[4,15] of the keto-sugars derived from 1,2:3,4-di-*O*-isopropylidene-L-rhamnitol and methyl 6-deoxy-2,3-*O*-isopropylidene-β-D-allofuranoside. Repeated oxidation with the chromium trioxide–pyridine complex[3] gave only low yields of keto sugars (**1**)* and (**2**), and necessitated chromatographic separation of the products. Subsequently, it was found that 1-deoxy-3,4:5,6-di-*O*-isopropylidene-L-fructose (**1**)* and methyl 6-deoxy-2,3-*O*-isopropylidene-β-D-*ribo*-hexofuranosid-5-ulose (**2**) were prepared in substantially better yield by using either the Pfitzner–Moffatt reagent[6] or a combination of an acid anhydride (phosphorus pentaoxide or acetic anhydride) and methyl sulphoxide[7]. Compounds (**1**) and (**2**) were obtained as distillable oils and were identified by borohydride reduction and acid hydrolysis to L-rhamnitol and 1-deoxy-L-glucitol, and to 6-deoxy-L-talose and 6-deoxy-D-allose, respectively. The keto-sugars gave crystalline toluene-*p*-sulphonylhydrazones (**3**) and (**4**), respectively.



(1) $X=O$
(3) $X = N{\cdot}NH{\cdot}SO_2C_6H_4Me$



(2) $X= O$
(4) $X = N{\cdot}NH{\cdot}SO_2C_6H_4Me$

Acid anhydride–methyl sulphoxide mixtures were also effective in the oxidation of 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose to 1,2:5,6-di-*O*-isopropylidene-α-D-*ribo*-hexofuranos-3-ulose (**5**). Similar results were communicated by other workers[8,9] during the course of these investigations and, in addition, this oxidation is achieved, in high yield, by using ruthenium tetroxide[12] and ruthenium dioxide–sodium periodate[13]. Methyl 4,6-*O*-benzylidene-2-*O*-toluene-*p*-sulphonyl-α-D-*ribo*-hexopyranosid-3-ulose was also obtained on oxidation of the corresponding glucopyranoside with a mixture of an acid anhydride and methyl sulphoxide but, in this instance, the yields (*ca.* 50%) were less than those reported[2a] for the Pfitzner–Moffatt reagent.

It is known[16] that keto-sugar (**5**) is reduced stereospecifically and in high yield with sodium borohydride to 1,2:5,6-di-*O*-isopropylidene-α-D-allofuranose (**6**). In contrast to the *exo*-sulphonate group of the epimeric D-glucose derivative[17], the *endo*-sulphonate group of 1,2:5,6-di-*O*-isopropylidene-3-*O*-toluene-*p*-sulphonyl-α-D-allofuranose (**7**) was readily displaced with azide ion in boiling *N*,*N*-dimethylformamide. The resulting azide (**8**) was converted by reduction with lithium aluminium hydride and *N*-acetylation into crystalline 3-acetamido-3-deoxy-1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose (**9**). Mild treatment of diketal **9** with acid afforded crystalline 3-acetamido-3-deoxy-β-D-glucopyranose and thus provides a facile, stereospecific synthesis of this compound (*c.f.* Ref. 18). 3-Amino-3-deoxy-D-glucose (kanosamine) occurs naturally as a constituent of the antibiotic kanamycin[19].

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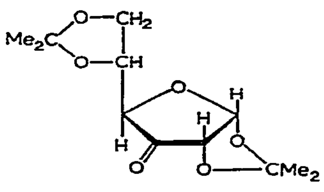
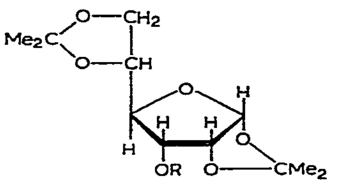
*Oxidation of 1,2:3,4-di-*O*-isopropylidene-L-rhamnitol with chromium trioxide–pyridine readily gave 1-deoxy-3,4:5,6-di-*O*-isopropylidene-L-fructose (**1**), $[\alpha]_D$ *ca.* 0° (methanol), isolated in 42% yield after conversion of unchanged alcohol into its *p*-nitrobenzoate (T. D. Inch, Ph. D. Thesis, Birmingham, 1963).

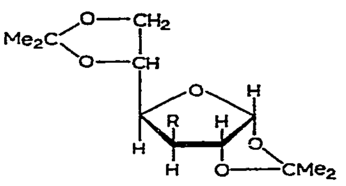
Selective removal of the 5,6-*O*-isopropylidene group from diketal **9** was accomplished with 50% acetic acid at room temperature to yield 3-acetamido-3-deoxy-1,2-*O*-isopropylidene-α-D-glucofuranose. On successive treatments with sodium periodate and sodium borohydride, the latter diol was transformed into 3-acetamido-3-deoxy-1,2-*O*-isopropylidene-α-D-xylofuranose and thence, by hydrolysis with dilute hydrochloric acid, into 3-amino-3-deoxy-D-xylose (isolated as the crystalline hydrochloride). This amino sugar has been synthesized previously[20] by epoxide scission of methyl 2,3-anhydro-β-D-ribo-furanoside and -pyranoside with ammonia.



(5)



(6) R = H
(7) R = Ts



(8) R = N_3
(9) R = NHAc

Displacement reactions involving allofuranose disulphonates are currently being investigated in our laboratories as a route to diaminodideoxy sugars possessing the D-*gluco*- and D-*xylo*- configurations.

EXPERIMENTAL

Thin-layer chromatography (t.l.c.) was performed on Kieselgel, with detection by vanillin–sulphuric acid[21]. N.m.r. spectra were measured on *ca.* 20% solutions, with a 6% solution of tetramethylsilane in chloroform as external reference, by using a Varian A-60 spectrometer under normal working conditions. Infrared spectra were measured as Nujol mulls or liquid films by using a Perkin–Elmer Infracord spectrometer. Methyl sulphoxide was distilled from calcium hydride under reduced pressure, prior to use.

Oxidation experiments using the Pfitzner–Moffatt reagent[6]

(*a*) *1-Deoxy-3,4:5,6-di-*O*-isopropylidene-*L*-fructose* (**1**).—Dicyclohexylcarbodiimide (8.25 g) was added to a stirred solution of 1,2:3,4-di-*O*-isopropylidene-L-rhamnitol[22] (2.85 g) in methyl sulphoxide (60 ml), and then anhydrous orthophosphoric acid (3 ml) was gradually added while a temperature of 25–30° was maintained. After 18 h, insoluble dicyclohexylurea was filtered off and washed with methyl sulphoxide and acetone. The combined filtrate and washings were diluted with chloroform (300 ml) and water (300 ml), and the solution was adjusted to pH 8 with 2M aqueous potassium carbonate. The chloroform layer was separated, the aqueous layer was extracted with chloroform (4 × 100 ml), and the combined extracts were washed with water (4 × 300 ml) and dried ($MgSO_4$). Removal of the chloroform afforded a mobile syrup which was fractionally distilled to give the title compound (**1**,4 g, 49.5%), b.p.

86°/0.2 mm, $[\alpha]_D^{25}$ *ca.* 0° (*c* 2, methanol) (Found: C, 59.1; H, 8.5. $C_{12}H_{20}O_5$ calc.: C, 59.0; H, 8.25%). The i.r. spectrum of the product showed negligible hydroxyl absorption but a strong C=O absorption at *ca.* 1740 cm^{-1}.

On being heated under reflux with toluene-*p*-sulphonylhydrazine (0.3 g) in dry methanol (50 ml) for 30 min, the title compound (0.3 g) afforded a *hydrazone* (**3**, 0.37 g) m.p. 163–164°, on concentration and cooling of the solution. (Found: C, 55.2; H,7.0. $C_{19}H_{28}N_2O_6S$ calc.: C, 55.3; H, 6.8%).

On reduction with sodium borohydride and acid hydrolysis, in the usual manner[23], the title compound yielded a mixture of two components having chromatographic properties indistinguishable from those of L-rhamnitol and 1-deoxy-D-glucitol.

(*b*) *Methyl 6-deoxy-2,3-*O*-isopropylidene-β-*D-ribo-*hexofuranosid-5-ulose* (**2**).—To a solution of methyl 6-deoxy-2,3-*O*-isopropylidene-β-D-allofuranoside[24] (0.44 g) in methyl sulphoxide (6 ml) and dry benzene (6 ml) containing pyridine (0.32 ml) and trifluoroacetic acid (0.16 ml), dicyclohexylcarbodi-imide (2.48 g) was added, and the solution was stirred for 18 h. at 30° . Ether (100 ml) was then added, followed by a solution of oxalic acid (1.1 g) in methanol (10 ml). After the evolution of gas had ceased, water (100 ml) was added, and insoluble material was filtered off. The organic layer was washed with dilute aqueous sodium hydrogen carbonate and water, and dried ($MgSO_4$). Removal of the solvents yielded a residue which gave the title compound (0.31 g, 71%), b.p. 80–82°/0.1 mm, $[\alpha]_D^{28}$—120° (*c* 0.8, chloroform), ν_{max} 1740 cm^{-1} (C=O), on fractional distillation (Found: C, 55.8; H, 7.8. $C_{10}H_{16}O_5$ calc.: C, 55.5, H, 7.5%). Reduction of the product with sodium borohydride and acid hydrolysis[23] gave a mixture of 6-deoxy-D-allose and 6-deoxy-L-talose (chromatographic identification).

The title compound (0.13 g) in methanol (25 ml) containing toluene-*p*-sulphonylhydrazine (0.19 g) was heated under gentle reflux for 30 min. The *hydrazone* (**4**, 40 mg), m.p. 152–153.5°, was deposited on cooling the solution at 0° (Found: C, 53.3; H, 6.2. $C_{17}H_{24}N_2O_6S$ calc.: C, 53.1; H. 6.3%).

Oxidation experiments using phosphorus pentaoxide–methyl sulphoxide[7]

(*a*) *1,2:5,6-Di-*O*-isopropylidene-α-*D-ribo-*hexofuranos-3-ulose* (**5**).—1,2:5,6-Di-*O*-isopropylidene-α-D-glucofuranose (20 g) was gradually added to a filtered solution of phosphorus pentaoxide (12 g) in methyl sulphoxide (240 ml), and the solution was stirred for 48 h at 50°. The cooled solution was dispersed in 0.1N potassium carbonate (200 ml), and the aqueous solution was extracted with chloroform (6 × 200 ml). Concentration of the dried ($MgSO_4$) chloroform extracts gave a mobile syrup (13 g) which on distillation gave the title compound (9 g), b.p. 104°/0.05 mm, $[\alpha]_D$ +105° (*c* 2, chloroform), ν_{max} 1740 cm^{-1} (C=O) {lit.[12], b.p. 97°/0.01 mm, $[\alpha]_D$ +107° (chloroform)}.

(*b*) *1-Deoxy-3,4:5,6-di-*O*-isopropylidene-*L*-fructose* (**1**, 0.34 g, 34%) b.p. 86—87°/0.2 mm, was obtained when 1,2:3,4-di-*O*-isopropylidene-L-rhamnitol (1 g) was oxidised in like manner with phosphorus pentaoxide (2.5 g) in methyl sulphoxide (20 ml) for 48 h at 25–30°. The derived toluene-*p*-sulphonylhydrazone had m.p. 163–164°.

(*c*) *Methyl 4,6-*O*-benzylidene-2-*O*-toluene-*p*-sulphonyl-α-*D-ribo-*hexopyranosid-3-*

ulose (0.65 g, 49%), m.p. 165-166°, $[\alpha]_D^{29}$ +48° (*c* 0.5, *N*,*N*-dimethylformamide), was obtained when methyl 4,6-*O*-benzylidene-2-*O*-toluene-*p*-sulphonyl-α-D-glucopyranoside (1.3 g) was treated with phosphorus pentaoxide (1.2 g) in methyl sulphoxide (15 ml) {lit.[2a], m.p. 165–167°, $[\alpha]_D^{25}$ +45° (*N*,*N*-dimethylformamide)}.

Oxidation experiments using acetic anhydride–methyl sulphoxide[7]

(*a*) *Methyl 6-deoxy-2,3-*O*-isopropylidene-β-*D-ribo-*hexofuranosid-5-ulose* (2).— Methyl 6-deoxy-2,3-*O*-isopropylidene-β-D-allofuranoside[24] (0.87 g, 4 mmoles) in methyl sulphoxide (12 ml) was treated with acetic anhydride (8 ml) for 24 h at 30°. Chloroform (150 ml) and water (150 ml) were then added, and the solution was brought to pH 8 by the addition of 2M potassium carbonate. The aqueous layer was separated and washed with chloroform (4 × 100 ml), and the combined organic extracts were washed with water (3 × 100 ml) and dried ($MgSO_4$). Removal of the solvent and distillation of the residue gave the title compound (0.7 g, 81%), b.p. 80–82°/0.1 mm, $[\alpha]_D^{28}$ −122° (*c* 0.8, chloroform), which was identical (i.r. spectrum) with the product previously obtained.

Analogous procedures applied to 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose and methyl 4,6-*O*-benzylidene-2-*O*-toluene-*p*-sulphonyl-α-D-glucopyranoside gave the corresponding carbonyl compounds (see above) in 55–60% and 49% yield, respectively.

(*b*) *1,2:5,6-Di-*O*-isopropylidene-3-*O*-toluene-*p*-sulphonyl-α-*D*-allofuranose* (7). — To a solution of 1,2:5,6-di-*O*-isopropylidene-α-D-allofuranose[16] (6, 1.2 g) in dry pyridine (10 ml) was added toluene-*p*-sulphonyl chloride (1.5 g) in dry pyridine (10 ml), and the solution was kept for 48 h at room temperature. Water (2 ml) was then added and, after 20 min, the solution was poured into ice–water (200 ml). The deposited material was filtered off and on recrystallisation (twice) from ethanol gave the title compound (1.4 g), m.p. 121°, $[\alpha]_D^{28}$ +88° (*c* 1, chloroform) (Found: C, 55.0; H, 6.4; S, 7.7. $C_{19}H_{26}O_8S$ calc.: C, 55.1; H, 6.3; S, 7.7%).

*3-Azido-3-deoxy-1,2:5,6-di-*O*-isopropylidene-α-*D*-glucofuranose* (8). —A solution of the foregoing sulphonate (3.5 g) in *N*,*N*-dimethylformamide (60 ml) containing suspended sodium azide (9 g) was heated under reflux for 4 h. Water (175 ml) was added to the cooled solution which was extracted with chloroform (6 × 150 ml), and the combined extracts were washed with water (4 × 300 ml) and dried ($MgSO_4$). Removal of the solvent and fractional distillation of the residue yielded the title compound (1.5 g), b.p. 106°/0.05 mm, $[\alpha]_D^{25}$ −36° (*c* 0.65, chloroform), ν_{max} 2100 cm^{-1} (azide) (Found: C, 50.8; H, 6.4; N, 14.7. $C_{12}H_{19}N_3O_5$ calc.: C, 50.5; H, 6.7; N, 14.7%).

*3-Acetamido-3-deoxy-1,2:5,6-di-*O*-isopropylidene-α-*D*-glucofuranose* (9).—A solution of the foregoing azide (2 g) in dry ether (70 ml) containing suspended lithium aluminium hydride (0.63 g) was heated under reflux for 1 h. Ethyl acetate (10.5 ml), and ether (50 ml) were then added to the cooled solution, followed by water (0.5 ml), and the mixture was heated under reflux for 10 min. Insoluble material was filtered off and washed thoroughly with ether, and the combined filtrate and washings were dried ($MgSO_4$) and concentrated. The residue (1.8 g) in dry methanol (6.7 ml) was treated with acetic anhydride (3.4 ml) for 30 min at room temperature. Toluene (40 ml) was then added, and the solution was concentrated. The addition of toluene and concentration of the solution was repeated twice and finally afforded a syrup which gave the title compound (1.8 g), m.p. 95–96°, $[\alpha]_D^{34}$ −36° (*c* 0.75, chloroform), ν_{max} 1650, 1560 cm^{-1} (NHAc) on crystallisation from chloroform–light petroleum (b.p. 60–80°) (Found: C, 56.0; H, 8.0; N, 4.7. $C_{14}H_{23}NO_6$ calc.: C, 55. 8; H, 7.7; N, 4.65%).

3-Acetamido-3-deoxy-β-D-glucopyranose.—Removal of the ketal groups from the foregoing compound **9** (0.5 g) was accomplished on heating under reflux on a boiling water-bath for 1 h with dilute sulphuric acid (10 ml) at pH 1.4. The cooled solution was neutralized with barium carbonate, filtered, and concentrated. The residue was dissolved in ethanol (25 ml), and the solution was filtered and reduced in volume to *ca.* 3 ml. Addition of a few drops of ethyl acetate caused a crystalline material (0.25 g) to be deposited; this was recrystallised from 85% aqueous ethanol to give the title compound (0.22 g), m.p. 200–201° (decomp.), $[\alpha]_D^{29}$ +51° (equil., *c* 1, water). Baer[18b] recorded m.p. 204–205° (decomp.), $[\alpha]_D$ +52.7° (equil., water) for this compound.

3-Acetamido-3-deoxy-1,2-O-isopropylidene-α-D-glucofuranose. — A solution of compound **9** (0.5 g) in 50% aqueous acetic acid (10 ml) was kept overnight at room temperature, and the disappearance of the starting material was monitored by using t.l.c. (acetone). The solution was concentrated, and water was distilled several times from the residue to remove traces of acetic acid. This gave the title compound (0.45 g), $[\alpha]_D^{24}$ −2° (*c* 1.5, chloroform), as a clear syrup. The n.m.r. spectrum (methanol–water, 3:1) showed the following characteristic signals: τ 8.23 (singlet, NAc), 8.73, 8.89 (singlets, CMe_2) with integrated areas in the ratio 1:1:1 [*cf.* 1,2-*O*-isopropylidene-α-D-glucofuranose[25]: τ 8.72, 8.87 (singlets, CMe_2)].

3-Amino-3-deoxy-D-xylose hydrochloride. — A solution of the foregoing compound (0.5 g) in ethanol (3.5 ml) was oxidised with N periodic acid (1.9 ml) for 15 min in the dark. The solution was neutralised with barium carbonate, insoluble material was filtered off and washed with ethanol, and the solution was concentrated. The syrupy residue (0.37 g) was dissolved in ethanol (3.5 ml) and reduced overnight with sodium borohydride (0.3 g). The solution was neutralized with 50% aqueous acetic acid, cations were removed on Amberlite IR-120 (H^+), and the eluate was concentrated, with repeated addition of methanol, to a syrup (0.36 g), $[\alpha]_D^{28}$ +7° (*c* 1, methanol), which could not be induced to crystallize. An aliquot part (0.145 g) of this residue was heated under reflux with 4% hydrochloric acid (3 ml) on a boiling water-bath for 18 h. Concentration of the solution, with several additions of methanol, gave a residue which failed to crystallize. This material was purified by passage down a column of Dowex-50 (H^+) and elution with 0.3N hydrochloric acid. Combination and evaporation of the appropriate fractions yielded a crystalline residue which gave the title compound (80 mg), m.p. 168–169° (decomp.), $[\alpha]_D^{23}$ +26 ±2° (equil., *c* 1.2, water), on recrystallisation from ethanol–acetone. {lit.[20], m.p. 169° (decomp.), $[\alpha]_D^{25}$ +29° (*c* 2, water)}.

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SUMMARY

Examples are provided which further demonstrate the value of the Pfitzner–Moffatt reagent and acid anhydride–methyl sulphoxide mixtures as oxidants in carbohydrate chemistry. Convenient and stereospecific syntheses of 3-acetamido-3-deoxy-D-glucose and 3-amino-3-deoxy-D-xylose have been developed from 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose.

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GALACTOMANNAN PEPTIDES OF *Trichophyton Mentagrophytes*

S. A. BARKER, O. BASARAB,
Department of Chemistry, The University, Birmingham 15 (Great Britain)
AND C. N. D. CRUICKSHANK
Medical Research Council Skin Unit, The Medical School, The University, Birmingham 15 (Great Britain)

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INTRODUCTION

The galactomannan peptide fraction isolated previously[1] from *Trichophyton mentagrophytes* by cetyltrimethylammonium bromide (Cetavlon) precipitation of its borate complex at pH 7.6–9.1, appeared homogeneous[2] on Sephadex G-25, G-50, and G-75, and in the ultracentrifuge. This fraction, having a molecular weight > 30,000, contained about 82% of carbohydrate (D-galactose and D-mannose in a ratio which varied from 1:4 to 1:7 according to the culture material) and 11% of protein (aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, valine, isoleucine, leucine, tyrosine, phenylalanine, and lysine). In a fraction isolated by deep culture in a medium containing D-glucose (4%), "Panmede" (1%) and acid-hydrolysed casein (2%), all of the galactose was present as terminal galactofuranose groups attached to mannose residues arranged in a highly branched structure. The linkages in the chain were mainly 1→2 and 1→4.

When skin-tested in guinea pigs sensitised with the whole mycelium, such galactomannan peptides give[2] both *immediate* skin reactions (wheal within 30 min after intradermal injection) and *delayed* skin reactions (reactions after 1 or 2 days). The mean reaction-diameter of such reactions is approximately proportional to the logarithm of the concentration of the galactomannan peptide injected.

Studies by Holborow and Loewi[3] on blood group substances showed that the carbohydrate part mediated the immediate reaction, and that the peptide part mediated the delayed hypersensitivity reactions. Immediate reactions to pure polysaccharides (dextrans and pneumococcus polysaccharides) have been demonstrated, but delayed reactivity has never been shown[4] to be associated with anticarbohydrate immunisation. In previous studies[2] on the galactomannan peptide fraction of *T. mentagrophytes*, we have demonstrated that periodate oxidation of the carbohydrate residues, followed by reduction of the resulting aldehyde groups, gave a 41% mean decrease in the immediate reaction while the delayed reaction remained practically unaffected. The proteolytic enzyme ficin caused the nitrogen content of the galactomannan peptide to fall from 1.2% to <0.05%. Tests of the treated glycopeptide showed a slight decrease (5%) in the immediate reactions and a major decrease (61%) in the delayed reactions.

We have now been able to resolve such galactomannan peptide fractions into two (and sometimes three) galactomannan peptides. The biological activity interrelationship between these glycopeptides, in terms of the carbohydrates and amino acids, is presented.

EXPERIMENTAL

Galactomannan peptides from deep culture of T. mentagrophytes. *(a) Isolation.*— The acetone-precipitated medium together with mycelium obtained as previously described[5] from a submerged culture (7–8 days) of *T. mentagrophytes* (NCTC D281) grown in a medium containing, *inter alia*, malt extract (4%) and "Panmede" (1%), was extracted with ethylene glycol. The extract was dialysed, and then fractionated by precipitation with Cetavlon[1] of the borate complex of the galactomannan peptides formed between pH 7–9.5.

The galactomannan peptides (100 mg) were fractionated on DEAE Sephadex A-50 (medium, Cl^-, 20 × 1.5 cm), using a linear gradient formed by mixing 300 ml of phosphate buffer (0.005M, pH 7.0) containing 0.02M sodium chloride with 300 ml of the same buffer containing 0.8M sodium chloride. After scanning (Fig. 1) with orcinol[6], ninhydrin[7], and for absorbance at 280 mμ, galactomannan peptides 1 (30 mg) and 2 (52 mg) were recovered by freeze-drying the combined, dialysed, 5-ml fractions.

(b) Sugar assay.— Both glycopeptides were oxidised with 0.01M sodium metaperiodate (5 ml) at room temperature for 4 min. Galactomannan peptides 1 (7.4 mg) and 2 (6.9 mg) gave formaldehyde[8] (210 and 89 μg) corresponding to 1.2 mg (16.2%) and 0.51 mg of galactofuranose (7.4%) respectively.

Application of the cysteine–sulphuric acid primary reaction[9], using absorbance at 430 mμ (at which, traces of xylose present absorb insignificantly) and inserting the above galactose figures in the hexose assay, showed that galactomannan peptides 1 and 2 contained 72.2 and 68% of mannose respectively corresponding to mannose/galactose ratios of 4.5:1 and 9.2:1.

The original mixture of the two glycopeptides, after hydrolysis with 2N sulphuric acid at 105° for 4 h and neutralization, gave a negative reaction with glucose oxidase[10] [confirmed by paper chromatography in ethyl acetate–pyridine–water (8:2:1)].

(c) Amino acid assay.— Galactomannan peptides 1 (3.00 mg) and 2 (1.64 mg) were hydrolysed separately with 6N hydrochloric acid (1 ml/mg) under nitrogen at 118° for 8 h. The diluted hydrolysate was repeatedly evaporated *in vacuo* and then dissolved in 2 ml of a leucine solution (0.0375mM in 0.1N hydrochloric acid). A control hydrolysis was performed on a standard amino acid mixture and each solution was analysed in a Technicon Autoanalyser. The corrected analyses for each glycopeptide are shown in Table I.

(d) Biological activity of intact and modified galactomannan peptides.— Batches of 3–6 guinea pigs, previously sensitised with the original mixture of galactomannan peptides of *T. mentagrophytes* mycelium suspended in Freund's complete adjuvant, were injected intradermally with 0.1 ml of each fraction (1 mg/ml physiological saline). Control animals not sensitised, as well as an animal injected with Freund's complete

TABLE I

COMPARISON OF AMINO ACID COMPOSITION OF GLYCOPEPTIDES (μmoles/mg)

Amino acid	*Galactomannan peptide I*	*Glycopeptide I*	*Galactomannan peptide 2*	*Glycopeptide II*	*Glycopeptide III*
Aspartic acid	0.012	0.010	0.034	0.045	0.038
Threonine	0.074	0.076	0.226	0.212	0.182
Serine	0.050	0.052	0.168	0.182	0.156
Glutamic acid	0.018	0.012	0.078	0.120	0.106
Proline	0.050	0.091	0.184	0.275	0.296
Glycine	0.032	0.043	0.126	0.110	0.144
Alanine	0.022	0.019	0.076	0.042	0.076
Valine	0.016	0.019	0.050	0.050	0.055
Isoleucine	0.008	0.005	0.028	0.020	0.021
Leucine	0.006	0.003	0.002	0.025	0.025
Tyrosine	—	0.003	0.024	0.007	0.013
Phenylalanine	0.004	0.003	0.102	0.015	0.013
Ammonia	0.038	0.024	—	0.072	0.102
Lysine	0.006	0.005	0.012	0.027	0.029
Histidine	0.006	0.005	0.010	0.007	0.009
Arginine	0.004	0.002	0.008	0.005	0.009
Cysteine		0.012		0.015	0.025
% Amino acids	3.24	3.62	11.32	11.71	12.18

adjuvant without mycelium, were also challenged. The periodate oxidised fractions referred to in Table II were obtained by oxidation as above to selectively destroy galactofuranose residues, the reaction being stopped with ethylene glycol (0.05 ml), and the oxidised glycopeptides then recovered by freeze-drying the dialysed solutions. The aldehyde groups present were removed by reduction with a weight of sodium borohydride equal to the total weight of glycopeptide. Carbon dioxide was bubbled through the solution. After 2 h, more sodium borohydride (half of the original weight) was added and then, after a further 30 min, the solutions were dialysed and freeze-dried. The methylated galactomannan peptide mixture was that previously reported[1]. In each case, the mean delayed reaction diameter was recorded (Table II) 24 h after injection.

After one week of rest, the same batches of animals were tested for immediate reactions. Some prick tests for immediate type reactivity were also performed on an infected human. After 20 min, the mean diameter (mm) for the original galactomannan peptide mixture was 5.5 ±0.3, galactomannan peptide 1, 4.1 ±0.1, and galactomannan peptide 2, 6.0 ±0.6. On repetition 2 days later on the other forearm of the same person, the diameters for galactomannan peptide 1 was 4.2 ± 0.2 and for galactomannan peptide 2, 5.5 ±0.1. The sterile physiological saline used to dissolve the substances pricked in both experiments gave no reaction.

In order to study further the effect of structure on biological activity, the original mixture of galactomannan peptides (22.9 mg) was heated with 0.01N hydrochloric acid (1 ml) at 85° for 2 h (conditions which had been previously shown[1] to remove

TABLE II

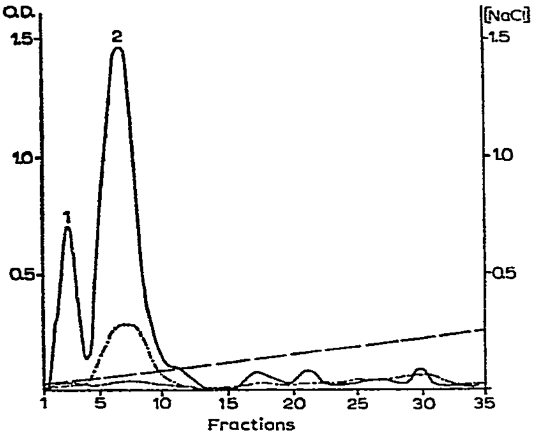
DELAYED – AND IMMEDIATE-TYPE REACTIVITY IN ANIMALS SENSITISED WITH GALACTOMANNAN PEPTIDE MIXTURE

Fraction tested	*Mean delayed reaction diameters (mm)*			
	Expt. 1	*Expt. 2*	*Expt. 3*	*Expt. 4*[a]
Galactomannan peptide 1	13.7 ±1.2	—	8.6 ±1.1	13.8 ±1.3
Galactomannan peptide 2	15.0 ±0.6	13.3 ±1.0	14.0 ±1.4	17.5 ±0.4
Galactomannan peptide original mixture	14.5 ±0.5	17.1 ±0.9	13.6 ±1.7	18.8 ±0.6

Fraction tested[b]	*Mean delayed reaction diameters (mm)*	*Mean immediate reaction diameters (mm)*
Galactomannan peptide original mixture	16.2 ±0.7	16.1 ±2.0
Methylated mixed galactomannan peptides	8.1 ±0.5	6.6 ±1.8
Galactomannan peptide 1	9.5 ±0.9	15.6 ±2.3
Oxidised-reduced galactomannan peptide 1	6.7 ±1.1	12.6 ±2.3
Galactomannan peptide 2	14.5 ±0.2	14.1 ±1.9
Oxidised-reduced galactomannan peptide 2	16.5 ±0.2	14.3 ±0.7

[a] Animals sensitised with galactomannan peptide 2.
[b] Animals sensitised with *T. mentagrophytes* mycelium.

galactofuranose residues). The nondialysable mannan peptides were recovered and tested (Table III).



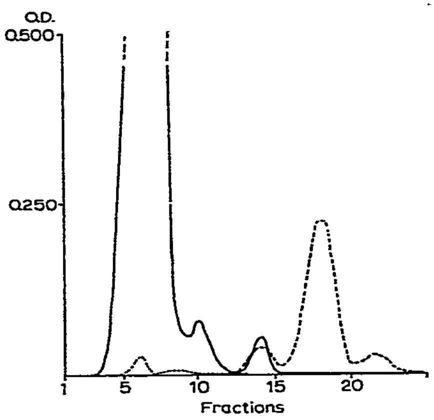


Fig. 1. Fractionation on DEAE Sephadex of *T. mentagrophytes* galactomannan peptides produced by deep culture. Fraction assayed with orcinol ————; ninhydrin— — — —; optical density at 280 mμ —·—·—·—·; salt concentration ————.

Fig. 2. Fractionation on Sephadex G-50 of pronase-treated galactomannan peptides. Fractions assayed with orcinol ————; ninhydrin ————.

The mixture of galactomannan peptides (12 mg) was incubated at 37° for 24 h with pronase P (1 mg) in 0.01M sodium bicarbonate buffer pH8 (5 ml) containing 0.01M calcium chloride as activator. A control without the protease was also incubated. After boiling both neutralised solutions at 100° for 10 min, they were fractionated separately on Sephadex G-50 (37 × 2 cm). A ninhydrin reactive peak corresponding to the cleaved peptides was eluted (5 ml fractions) in the gel filtration of the pronase-treated material between tubes 15–19 (Fig. 2). The residual galactomannan peptide was recovered by freeze-drying. The biological activity of the fractions is reported in Table III.

TABLE III

BIOLOGICAL ACTIVITY OF MODIFIED GALACTOMANNAN PEPTIDES[a]

Fraction tested	*Mean delayed reaction diameter (mm)*		*Mean immediate reaction diameter*
	Expt. 1	*Expt. 2*	
Original galactomannan peptides	12.6 ±1.2	15.5 ±0.6	10 ±0
Pronase treated galactomannan peptides	7.3 ±1.0	10.5 ±0.5	—
Control incubated galactomannan peptides	11.6 ±1.5	—	—
Mannan peptides (after treatment)	—	16.5 ±0.5	10 ±0

[a] Animals sensitised with *T. mentagrophytes* mycelium.

Galactomannan peptides from surface culture of T. mentagrophytes. (*a*) *Isolation.* — Acetone dried mycelium (74 g) of *T. mentagrophytes* grown in surface culture in a glucose–Panmede medium was extracted with ethylene glycol (400 ml × 4) for 16 h at room temperature and the combined dialysed extract was freeze-dried (5.2 g). Subsequent fractionation with Cetavlon–borate[2] gave 550 mg of glycopeptides precipitating at pH 7.5–9.1. This glycopeptide fraction was homogeneous on Sephadex G-100 from which it was eluted just after blue dextran (Pharmacia Uppsala, mol. wt. 2×10^6).

When fractionated (2.5 ml fractions) on DEAE Sephadex A-50 (medium, Cl^-, 40 × 1.4 cm) with a loading of 100 mg dissolved in 1 ml of phosphate buffer, the elution pattern observed at pH 7.0 indicated that more than two glycopeptides were present (Fig. 3). Variation of pH of the eluting buffer with a constant salt gradient as above showed that a third glycopeptide was best separated at pH 7.3. Repeated fractionation on DEAE Sephadex afforded homogeneous samples of glycopeptides I, II, and III.

(*b*) *Analyses.*— Sugar analysis of the surface culture glycopeptides was carried out as previously described for those from deep culture except that galactose was assayed using galactose oxidase* and the galactose figures inserted in the cysteine hexose assay. The percentages of galactose and mannose in the glycopeptides were as follows: I, 8.7; 84; II, 7.28; 71.5; III, 5.7; 82%. Results for amino acid analyses using the 24-h hydrolysis conditions previously described are given in Table IV.

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* As recommended by Seravac Laboratories

(*c*) *Biological activity.*—The three glycopeptides were tested in guinea pigs sensitised with *T. mentagrophytes* mycelium (Table IV).

TABLE IV

DELAYED- AND IMMEDIATE TYPE-REACTIVITY FOR GLYCOPEPTIDES PRODUCED BY SURFACE CULTURE

Fraction tested		*Mean delayed reaction diameter (mm)*	*Mean immediate reaction diameter (mm)*
Ethylene glycol extract		15.2 ±1.2	17.3 ±0.9
Glycopeptide I		10.0 ±0.8	14.7 ±0.7
Glycopeptide II	Preparation 1	12.0 ±1.0	15.5 ±1.5
	Preparation 2	13.0 ±0.6	15.3 ±0.5
Glycopeptide III		12.0 ±1.1	15.2 ±1.1

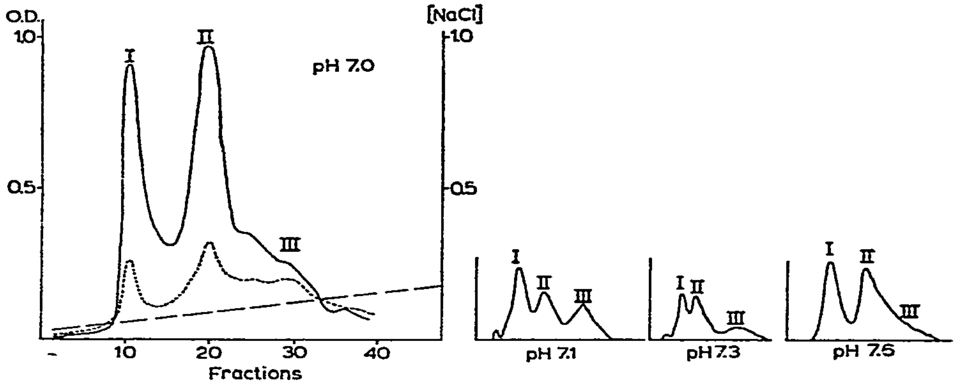

Fig. 3. Fractionation on DEAE Sephadex of *T. mentagrophytes* galactomannan peptides produced by surface culture. Fractions assayed with orcinol ————; optical density at 280 mμ ....; salt concentration — — — —.

DISCUSSION

The idea of using the carbohydrate-mediated immediate reaction and the protein-mediated delayed reaction as a biological method of control during structural studies of a glycopeptide is an attractive one. There is a continuing need for such controls in any chemical and enzymic degradation that supposedly operates on solely the carbohydrate or protein moieties. In this respect, the major loss of delayed activity after pronase treatment of the mixed deep culture galactomannan peptides (Table III) confirms earlier results obtained after treatment with ficin, another wide-spectrum protease. The dual loss of both delayed and immediate reactivity encountered with the methylated galactomannanpeptides is not unexpected since previous work showed that hydroxy amino-acids were modified during the methylation. The removal of galactofuranose residues did not alter either the delayed or immediate reactivity of the galactomannan peptides (Tables III) and galactomannan peptides 1 and 2 showed equal immediate-type reactivity (Table II) despite the fact that the former contains twice the number of galactofuranose residues per molecule. However, it was noticeable that delayed activity was dependent on the protein content of the individual galactomannan peptide, those containing 11.32–12.18% protein (2, II, and III) being slightly more active than those containing 3.24–3.64% protein (1 and I); see Tables II and IV.

Comparison of the amino acid composition of the individual glycopeptides (Table I) reveals several interesting features. The ratio of each amino acid in galactomannan peptide 1 to that in 2 is approximately 1:3 and the ratio of each amino acid to another in the same galactomannan peptide is similar for different galactomannan peptides. These same features hold between glycopeptide I and glycopeptide III (and, to a lesser extent, II). The most reasonable explanation is that galactomannan peptide 2 contains three peptide units of the same type as those in galactomannan peptide 1. There could be the same relationship between glycopeptides III and I with the same peptide unit to which more proline residues have been attached. This latter difference could arise because of two levels of proteolytic activity produced in deep agitated culture and still surface culture respectively, the peptides in the former being partially degraded. Proteolytic activity is an indispensable part of the pathogenicity of dermatophytes such as *T. mentagrophytes*. Such degradation could also be responsible for the variation of the glutamic acid/ammonia ratio in the individual glycopeptides. It is noteworthy that glycopeptides produced by surface culture, with their high proline contents, are noticeably more resistant to both pronase and ficin than are the glycopeptides produced by deep culture. Thus, the effect of ficin on the amino acid composition and delayed activity [14.8 (before)→13.6 (after)] of glycopeptide I was negligible and with glycopeptide II, apart from some small loss of threonine, serine, and glutamine, the resulting slight drop of protein content to 9.65% was insufficient to appreciably alter the delayed activity [18.6 (before)→16.2 (after)]. In both cases the proline content of the glycopeptide remained virtually constant. Proline was also not removed in the pronase treatment of glycopeptide II where the drop of protein content to 9.8% and drop in delayed activity [18.6 (before)→16.2 (after): 20 (before)→17 (after)] was almost identical. Glycopeptide III was likewise relatively resistant to pronase [12 (before)→8.7 (after)]. In all cases complete retention of immediate activity was evident.

ACKNOWLEDGMENT

We thank Professor M. Stacey, F.R.S., for his continued interest in this work.

SUMMARY

Glycopeptides from deep and surface cultures of *Trichophyton mentagrophytes* have been separated and their sugar and amino acid components assayed. Aspects of some structure-biological activity relationships are presented.

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FLUOROCARBOHYDRATES

PART XVI. THE SYNTHESIS OF 3-DEOXY-3-FLUORO-D-XYLOSE AND 3-DEOXY-3-FLUORO-β-D-ARABINOSE*

J. A. WRIGHT AND N. F. TAYLOR
Biochemistry Group, School of Biological Sciences, Bath University, Bath (Great Britain)

(Received July 19th, 1966)

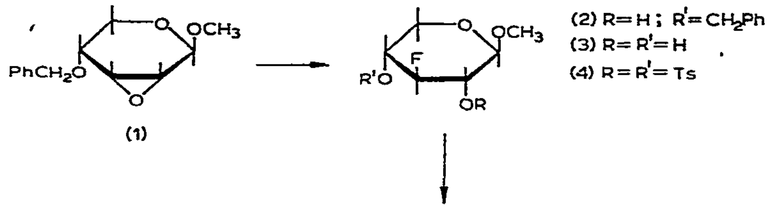
INTRODUCTION

Since our preliminary communication[1] the synthesis of a number of secondary fluorosugars has been reported. Thus, Codington, Doerr, and Fox[2] have synthesised 2-deoxy-2-fluoro-D-ribose from 2′-deoxy-2′-fluorouridine and another group[3] has reported the synthesis of 2-deoxy-2-fluoro-D-allose, 2-deoxy-2-fluoro-D-altrose, and 3-deoxy-3-fluoro-D-glucose by the action of hydrogen tetrafluoridoborate in hydrogen fluoride on suitably blocked 2,3-anhydro-D-allosides. We now report on a synthesis of 3-deoxy-3-fluoro-D-xylose and 3-deoxy-3-fluoro-β-D-arabinose.

RESULTS AND DISCUSSION

The successful scission of aliphatic epoxides[4] and steroid epoxides[5,6] by hydrogen fluoride has been know for some time and originally, as a model for such studies in the carbohydrate series, we examined the action of anhydrous hydrogen fluoride in dioxan on methyl 2,3-anhydro-4-*O*-benzyl-β-L-ribopyranoside[1]. While this work was in progress Cohen, Levy, and Bergmann[7] reported the use of potassium hydrogen fluoride (KHF_2)in ethane-1,2-diol for the scission of benzyl 2,3-anhydro-β-D-ribopyranoside to give benzyl 3-deoxy-3-fluoro-β-D-xylopyranoside which, on catalytic hydrogenation, gave 3-deoxy-3-fluoro-D-xylose. We have now examined the action of potassium hydrogen fluoride in ethanediol on methyl 2,3-anhydro-4-*O*-benzyl-β-D-ribopyranoside (**1**), (also in the L series) and found that much improved and less contaminated yields of the fluorohydrin (**2**) are obtained by this procedure than those obtained by the action of hydrogen fluoride in dioxan on compound (**1**). The structure of compound (**2**) was established by catalytic hydrogenation (palladium on charcoal) which removed the benzyl group to give the glycoside (**3**) as a crystalline material which did not reduce Fehling's solution or consume periodate and contained fluorine[8]. The retention of the fluorine under these conditions was expected as a result of previous work with 6-deoxy-6-fluoro-D-galactose[9]. Thin-layer chromatography[10] (t.l.c.) of compound (**3**) gave only one component (R_F, 0.4, ethyl acetate) and paper chromatography followed by treatment with periodate and benzidine did not reveal the presence of vicinal hydroxyl groups[11]. The glycoside (**3**) was further characterised as the di-*O*-

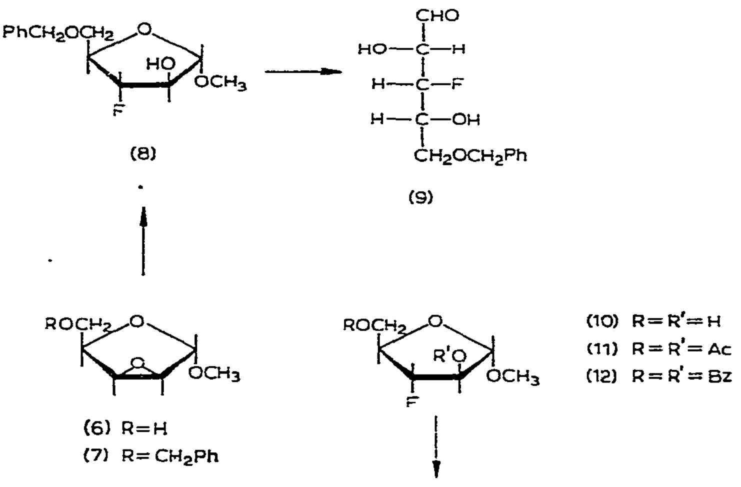
toluene-*p*-sulphonyl derivative (4). These results are in accordance with the expected *trans* scission[12] of the epoxide (1) and so the structure methyl 3-deoxy-3-fluoro-β-D-xylopyranoside was assigned to compound (3). Acid hydrolysis of compound (3) with 0.05M sulphuric acid gave rise to a reducing sugar as a syrup which was characterised as the 2,5-dichlorophenylhydrazone and assigned the structure 3-deoxy-3-fluoro-αβ-D-xylose (5).



(1)

(2) R=H; R'=CH$_2$Ph
(3) R=R'=H
(4) R=R'=Ts

3-Deoxy-3-fluoro-D-xylose (5)



(8)

(9)

(6) R=H
(7) R=CH$_2$Ph

(10) R=R'=H
(11) R=R'=Ac
(12) R=R'=Bz

3-Deoxy-3-fluoro-β-D-arabinose (13)

In order to examine the general applicability of this method of introducing fluorine into carbohydrates we have also examined the action of potassium hydrogen fluoride in ethane-1,2-diol on methyl 2,3-anhydro-5-*O*-benzyl-α-D-lyxofuranoside (7) obtained in good yields by the benzylation of methyl 2,3-anhydro-α-D-lyxofuranoside[13] (6). Refluxing compound (7) with potassium hydrogen fluoride in ethane-1,2-diol for 1 h gave a product which was shown by t.l.c. to contain two major components (R_F, 0.5 and 0.6, ethyl acetate–light petroleum, 1:1) one of which was shown to be starting material (R_F, 0.6) and the other a fluorohydrin. The latter compound was isolated by preparative t.l.c. as a syrup which gave the correct elemental analysis and showed characteristic bands at 3500 (OH) and 1040 cm^{-1} (C–F) in the infrared spectrum. The structure of the fluorohydrin (8) was established by hydrolysis with 0.125M sulphuric acid to give a reducing sugar (R_F, 0.37, ethyl acetate–light petroleum, 1:1) which did not crystallise but readily formed a crystalline phenylhydrazone derivative. The reducing sugar (9) was shown to consume 1 mol. of periodate[14] with the liberation of 0.7 mol. of formic acid[15]. Such a result is consistent with the structure of 5-*O*-benzyl-3-deoxy-3-fluoro-αβ-D-arabinose if we assume *trans* opening[12] of the epoxide ring in (7). On this basis, the structure methyl 5-*O*-benzyl-3-deoxy-3-fluoro-α-D-arabinofuranoside was assigned to the fluorohydrin (8). Catalytic hydrogenation of compound (8) with 5% palladium on charcoal removed the benzyl group to give a non-reducing glycoside (10) as a syrup (R_F, 0.2, ethyl acetate–light petroleum, 1:1) containing fluorine and characterised as the non-crystalline 2,5-diacetate (11) and the crystalline 2,5-dibenzoate (12). Acid hydrolysis of compound (10) with 0.1N sulphuric acid gave a reducing syrup (R_F, 0.15, ethyl acetate) which failed to crystallise spontaneously but immediately crystallised on seeding with a crystal of β-D-arabinose. On the basis of the mutarotation of the free sugar (13) ($[\alpha]_D^{20}$ −150°→−105°, water) it was assigned the structure 3-deoxy-3-fluoro-β-D-arabinose and further characterised as the crystalline 2,5-dichlorophenylhydrazone.

The biological activity of 3-deoxy-3-fluoro-D-arabinose and 3-deoxy-3-fluoro-D-xylose is now being investigated in these laboratories.

EXPERIMENTAL

Melting points were determined by an Electrothermal apparatus and are uncorrected. Paper chromatography was carried out on Whatman No. 1 paper using the organic phase of butanol–ethanol–water (4:1:5). Reducing sugars were detected with aniline hydrogen phthalate[16] and glycosides were detected with a 1% solution of sodium metaperiodate in acetone followed by benzidine[11]. Column chromatography was carried out with aluminium oxide for chromatographic absorption (British Drug Houses Ltd.). T.l.c. plates (40 × 20 cm) were prepared with Silica-gel G, according to Stahl (Shandon Scientific Co. Ltd.). After the solvent was removed from the developed plate it was sprayed with sulphuric acid–ethanol (1:1) and heated to 100° for ten min. All preparative t.l.c. plates (20 × 20 cm) were prepared with Silica-gel PF 254 (Shandon Scientific Co. Ltd.). After the solvent had been removed from the developed plate it was examined under ultraviolet light (254 mμ) and the different zones eluted.

The solvent systems used were light petroleum (b.p. 40–60°)–ethyl acetate, 4:1 (*A*), 1:1 (*B*) and ethyl acetate (*C*).

Microanalyses and fluorine determinations were carried out by Drs. Weiler and Strauss, Oxford.

Methyl 4-O-benzyl-3-deoxy-3-fluoro-β-D-xylopyranoside (2). — Methyl 2,3-anhydro-4-*O*-benzyl-β-D-ribopyranoside[18] (1) (2.0 g) in ethane-1,2-diol (40 ml) containing potassium hydrogen fluoride (4.0 g) was refluxed gently for 40 min. On cooling, the mixture was poured, with stirring, into a cold, saturated solution of sodium hydrogen carbonate. The neutral solution was extracted with ether (3 × 75 ml), dried (Na_2SO_4), and evaporated to dryness. T.l.c. (solvent *B*) of the resulting syrup, revealed two major components R_F, 0.5 and 0.65. The syrup was submitted to column chromatography and eluted with ether to yield starting material (0.6 g) (t.l.c. R_F, 0.65, solvent *B*). Elution with chloroform yielded a product (1.0 g) which crystallised on storage at room temperature and was recrystallised from ether–light petroleum (b.p. 40–60°) to give the title compound as large needles, m.p. 84°, $[\alpha]_D^{20}$ −50.5° (*c* 1.35, chloroform) (Found: C, 61.0; H, 6.6; F, 7.4. $C_{13}H_{17}FO_4$ calc.: C, 60.9; H, 6.6; F, 7.2%).

*Methyl 3-deoxy-3-fluoro-β-*D*-xylopyranoside* (3).—Methyl 4-*O*-benzyl-3-deoxy-3-fluoro-β-D-xylopyranoside (2) (1.0 g) in ethanol (20 ml) containing 5% palladium on charcoal (500 mg) was hydrogenated at room temperature and atmospheric pressure until the uptake of hydrogen ceased (2 h). The mixture was filtered and evaporated to dryness *in vacuo* to leave a syrup which crystallised on storage. Recrystallisation from methanol–ether gave the product, m.p. 105° (Found: C 44.0; H, 6.7; F, 11.1. $C_6H_{11}FO_4$ calc.: C, 43.4; H, 6.6; F, 11.4%).

The glycoside did not reduce Fehling's solution or consume periodate. Paper chromatography and detection with periodate–benzidine[11] did not reveal the presence of vicinal hydroxy groups thus indicating the 3-deoxy-3-fluoroxylose configuration for for the glycoside. The glycoside was further characterised as the 2,4-ditoluene-*p*-sulphonate (4), needles, m.p. 138°, $[\alpha]_D^{20}$ −35° (*c* 0.57, chloroform) (Found: C, 50.9; H, 4.7; S, 13.7; F, 4.0. $C_{20}H_{23}FO_8S_2$ calc.: C, 50.6; H, 4.9; S, 13.5; F, 4.0%).

*3-Deoxy-3-fluoro-αβ-*D*-xylose* (5). — Methyl 3-deoxy-3-fluoro-β-D-xylopyranoside (3) (1.0 g) was refluxed with 0.05M sulphuric acid (100 ml) for 1 h, allowed to cool, and neutralised with barium carbonate. After filtration, the solution was evaporated to dryness *in vacuo*, the residue was dissolved in ethanol and the filtered solution evaporated to dryness *in vacuo*. A portion (28.5 mg) of the resulting viscous syrup (R_F, 0.24, t.l.c. solvent *C*) was treated with 2,5-dichlorophenylhydrazine (92.5 mg) in methanol (15 ml). The methanol was evaporated and the residue was crystallised from aqueous methanol by allowing to stand in the refrigerator overnight. Recrystallisation from aqueous methanol gave the 2,5-dichlorophenylhydrazone, m.p. 75° (Found: C, 43.0; H, 4.6; N, 9.9; F, 6.5. $C_{11}H_{13}Cl_2FN_2O_3$ calc.: C, 42.5; H, 4.46; N, 9.6; F, 6.48%).

*Methyl 2,3-anhydro-5-O-benzyl-α-*D*-lyxofuranoside* (7).—To methyl 2,3-anhydro-α-D-lyxofuranoside[13] (6) (5.0 g) in anhydrous dimethylformamide (25 ml) were added silver oxide (7.0 g) and benzyl bromide (6 ml). The mixture was shaken for 24 h at room temperature then diluted with chloroform (250 ml) and water (250 ml). The chloroform layer was separated and filtered, pyridine (30 ml) was added, and the solution washed successively with water (6 × 100 ml), 2M hydrochloric acid (3 × 100 ml), saturated aqueous sodium hydrogen carbonate (100 ml), and water (100 ml). The chloroform extract was dried ($MgSO_4$), evaporated to dryness, and the resulting light brown oil distilled to give the product as a colourless oil (6.5 g), n_D^{20} 1.5192, b.p. 135° (bath)/0.01 mm, $[\alpha]_D^{20}$+23.4° (*c* 5.8, chloroform) (Found: C, 66.3; H, 6.65. $C_{13}H_{16}O_4$ calc.: C, 66.1; H, 6.7%).

*Methyl 5-O-benzyl-3-deoxy-3-fluoro-α-*D*-arabinofuranoside* (8). — Methyl 2,3-

anhydro-5-*O*-benzyl-α-D-lyxofuranoside (7) (3.0 g) and potassium hydrogen fluoride (6.0 g) in ethane-1,2-diol (60.0 ml) were refluxed gently for 1 h. When cool, the solution was poured into saturated aqueous sodium hydrogen carbonate (500 ml) with stirring and extracted with chloroform (3 × 100 ml) and the extract was dried ($MgSO_4$) and evaporated to dryness *in vacuo*. The resulting syrup on t.l.c. (solvent *B*) was found to contain two major components, R_F, 0.5 and 0.6, one of which was shown to be starting material (R_F, 0.6 and comparative i.r. spectrum). The mixture was submitted to preparative t.l.c. using two elutions with solvent *A* to yield a fluorine-containing syrup (0.9 g), $[\alpha]_D^{20}$ +87° (*c* 0.9, chloroform) with i.r. bands at 3500 (OH) and 1050 cm^{-1} (C–F) (Found: C. 61.2; H, 6.6; F, 7.4. $C_{13}H_{17}FO_4$ calc.: C, 61.0; H, 6.6; F, 7.2%).

5-O-Benzyl-3-deoxy-3-fluoro-αβ-D-arabinose (9). — Methyl 5-*O*-benzyl-3-deoxy-3-fluoro-α-D-arabinofuranoside (8) (1.0g) was dissolved in a mixture of dioxan (50.0 ml) and 0.25M sulphuric acid (50.0 ml) and the solution was refluxed for 3 h. The cold solution was neutralised with barium carbonate, filtered, and evaporated to dryness *in vacuo*. The residue was taken up in absolute ethanol (50.0 ml), filtered, and the filtrate evaporated to dryness *in vacuo* to yield a viscous colourless syrup (890 mg), R_F, 0.37 (t.l.c. solvent B), $[\alpha]_D^{20}$+107° (*c* 1.4, ethanol) (Found: C, 59.9; H, 6.6; F, 7.4. $C_{12}H_{15}FO_4$ calc.: C, 59.5; H, 6.2; F, 7.8%). The free sugar (250 mg) in water (2.5 ml) was treated with a solution of phenylhydrazine hydrochloride (175 mg) and sodium acetate (175 mg) in water (5.0 ml). A yellow oil separated which solidified on cooling. Recrystallisation from aqueous ethanol gave the phenylhydrazone, m.p. 72° (Found: C, 64.9; H, 6.3; N, 8.7; F, 6.0. $C_{18}H_{21}FN_2O_3$ calc.: C, 65.0; H, 6.3; N, 8.5; F, 5.7%).

Periodate oxidation of 5-O-benzyl-3-deoxy-3-fluoro-αβ-D-arabinose (9). — 5-*O*-Benzyl-3-deoxy-3-fluoro-αβ-D-arabinose (9) (42.8 mg) was dissolved in water (10.0 ml) and 0.05M sodium metaperiodate (10 ml). At intervals, 2 ml portions were added to 0.025M sodium arsenite (5 ml) and 20% aqueous potassium iodide (1 ml). Excess arsenite was titrated against standard iodine to the starch end-point. The periodate consumed was as follows:

Time (min)	5	10	15	30	24 (h)
$NaIO_4$ consumed (mol.)	0.77	0.87	0.9	0.92	1.00

5-*O*-Benzyl-3-deoxy-3-fluoro-αβ-D-arabinose (9) (50.3 mg) was dissolved in water (10.0 ml) and 0.05M sodium metaperiodate (10 ml). At intervals, 2 ml portions were removed and excess periodate was destroyed by the addition of ethane-1,2-diol (1.0 ml) The acid was determined[15] using 0.01M sodium hydroxide, and phenolphthalein–thymol blue (3:1) as an indicator. The results were as follows:

Time (h)	0.25	0.5	1.0	4.0	8.0
Acid liberated (mol.) .	0.60	0.62	0.65	0.73	0.73

The acid liberated was identified as formic acid by reduction to formaldehyde and characterisation with chromotropic acid[17].

Methyl 3-Deoxy-3-fluoro-α-D-arabinoside (10). — Methyl 5-*O*-benzyl-3-deoxy-3-

fluoro-α-D-arabinofuranoside (**8**) (1.0 g) in ethanol (25.0 ml) containing 5% palladium on charcaol (1.0 g) was hydrogenated at room temperature and atmospheric pressure until the uptake of hydrogen ceased (3 h). After filtration, the solution was evaporated to dryness *in vacuo* to leave a viscous non-reducing syrup (**10**), R_F, 0.19 (t.l.c., solvent *B*), $[\alpha]^{20}_{D}$ +107° (*c* 1.0, ethanol) (Found: C, 43.2; H, 6.8; F, 11.2. $C_6H_{11}FO_4$: calc.: C, 43.4; H, 6.6; F, 11.4%). The glycoside (**10**) was further characterised as the non-crystalline 2,5-diacetate (**11**) (Found: C, 48.4; H, 6.2; F, 7.6; $C_{10}H_{15}FO_6$: calc.: C, 48.0; H, 6.0; F, 7.6%) and the 2,5-dibenzoate (**12**) m.p. 81° (Found: C, 64.1; H, 5.3; F, 5.5. $C_{20}H_{19}FO_6$ calc.: C, 64.1; H, 5.1; F, 5.1%).

3-Deoxy-3-fluoro-β-D-arabinose (**13**). — Methyl 3-deoxy-3-fluoro-α-D-arabinofuranoside (**10**) (1.0 g) in 0.1N sulphuric acid (100 ml) was refluxed for 1 h then allowed to cool and neutralised with barium carbonate. The filtered solution was evaporated to dryness *in vacuo*, the residue was taken up in absolute ethanol, and the solution filtered and evaporated to dryness *in vacuo* to leave a viscous syrup (850 mg), R_F, 0.15 (t.l.c., solvent *C*). On addition of a crystal of β-D-arabinose the syrup crystallised. Recrystallisation from ethanol gave the title compound as colourless plates, m.p. 120°, $[\alpha]^{20}_{D}$ −150°→105° (*c* 1.0 water), (Found: C, 39.7; H, 6.1; F, 12.8. $C_5H_9FO_4$ calc.: C, 39.5; H, 5.9; *F*, 12.5%). To the fluorosugar (100 mg) in methanol (3.0 ml), 2,5-dichlorophenylhydrazine (130 mg) was added and the solution was evaporated to dryness. Trituration of the residue with ether–light petroleum (b.p. 40–60°) and recrystallisation from the same solvent system gave the 2,5-dichlorophenylhydrazone, m.p. 120°, (Found: C, 42.8; H, 4.3; N, 9.3; F, 5.9. $C_{11}H_{13}Cl_2FN_2O_3$ calc.: C, 42.5; H, 4.2; N, 9.6; F, 6.1%).

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SUMMARY

The action of potassium hydrogen fluoride (KHF_2) in ethane-1,2-diol on methyl 2,3-anhydro-4-*O*-benzyl-β-D-ribopyranoside (**1**) and methyl 2,3-anhydro-5-*O*-benzyl-α-D-lyxofuranoside (**7**) has been examined. In both cases the expected *trans* scission of the epoxide ring occurs to yield methyl 4-*O*-benzyl-3-deoxy-3-fluoro-β-D-xylopyranoside (**2**) and methyl 5-*O*-benzyl-3-deoxy-3-fluoro-α-D-arabinofuranoside (**8**) respectively. The structure of compound (**2**) was established by catalytic hydrogenation, which removed the benzyl group, to yield a crystalline glycoside (**3**) which did not consume periodate. Acid hydrolysis of methyl 3-deoxy-3-fluoro-β-D-xylopyranoside (**3**) yielded syrupy 3-deoxy-3-fluoro-αβ-D-xylose (**5**) characterised as the 2,5-dichlorophenylhydrazone.

Acid hydrolysis of compound (**8**) yielded a reducing sugar (**9**) characterised as the phenylhydrazone. Compound (**9**) consumed 1 mol. of periodate and liberated formic

acid which is consistent with the structure 5-*O*-benzyl-3-deoxy-3-fluoro-αβ-D-arabinose. Catalytic hydrogenation of the fluorohydrin (**8**) removed the benzyl group to give methyl 3-deoxy-3-fluoro-α-D-arabinofuranoside (**10**) which, on acid hydrolysis, yielded crystalline 3-deoxy-3-fluoro-β-D-arabinose (**13**). The β configuration was assigned to compound (**13**) on the basis of its mutarotation.

This method of introducing fluorine into carbohydrates affords reasonable yields of uncontaminated fluorohydrins and is considered to have general applicability.

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STRUKTURAUFKLÄRUNG DES Vi-ANTIGENS AUS *Citrobacter freundii* (*E. coli*) 5396/38

K. HEYNS UND G. KIESSLING
Chemisches Staatsinstitut, Institut für Organische Chemie, Universität Hamburg (Deutschland)

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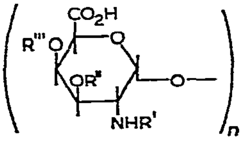
EINFÜHRUNG

Frühere Untersuchungen[1] haben gezeigt, daß das aus mehreren Mikroorganismen nach verschiedenen Methoden[2,3] erhältliche Vi-Antigen ein hochacetyliertes Polysaccharid ist. Es besteht im wesentlichen aus einer Aminohexuronsäure[1], die durch Vergleich mit authentischem Material als 2-Amino-2-desoxy-D-galaktopyranuronsäure (D-Galaktosaminuronsäure) identifiziert werden konnte[4]. In den letzten Jahren sind in Polysacchariden anderer Mikroorganismen zusätzlich Glucosaminuronsäure und Mannosaminuronsäure aufgefunden worden[5–8]. Genaue Strukturangaben liegen nur für das Staphylococcen–Polysaccharid–Antigen vor[8]. Versuche, die Struktur des Vi-Antigens durch Perjodatspaltung zu erschließen, haben ergeben, daß das Polysaccharid kein Perjodat verbraucht[1]. Dieser Befund führte zur Annahme, im Vi-Antigen seien die Monosaccharide durch glykosidische 1→3 Bindungen verknüpft.

In der vorliegenden Arbeit wird die Strukturaufklärung des Vi-Antigens auf dem klassischen Wege der Methylierung beschrieben. Dieses aufwendigere Verfahren führt auch hier zu einer sicheren Aussage der Bindungsart, weil die Hydrolysenprodukte des methylierten Polysaccharids charakteristische Bruchstücke des Vi-Antigens repräsentieren. Das Bauelement **1** des Vi-Antigens kann entweder in einer 1→3- oder einer 1→4-Verknüpfung innerhalb der Polysaccharidkette vorliegen. Durch direkte Methylierung des Vi-Antigens und anschließende Hydrolyse sollte es möglich sein, Aufschluß über die Struktur des Polysaccharids zu erhalten. Die Stellung der Methylgruppen in den Hydrolyseprodukten wäre dann ein unmittelbarer Beweis für die Verknüpfungsart der Monosaccharide im Polysaccharid. Die in dieser Richtung durchgefürthen Analysen sind zur Zeit noch nicht auswertbar, da die zur Identifizierung benötigten Methyläther der Galaktosaminuronsäure unbekannt sind.



(1)

1→3 Verknüpfung
R' = H, Ac
R'' = Saccharidkette
R''' = H, Ac

1→4 Verknüpfung
R' = H, Ac
R'' = H, Ac
R''' = Saccharidkette

Für die Strukturermittlung des Vi-Antigens war daher eine Reduktion der Carboxylgruppen unumgänglich. Durch diese Reaktion wird das Vi-Antigen in ein dem Chitin analog gebautes Poly-Galaktosamin verwandelt, so daß im Hydrolysat eines carboxylreduzierten und methylierten Vi-Antigens Methyläther des Galaktosamins[9] auftreten. Die Frage, um welche Methyläther es sich dabei handelt, wird durch die Reihenfolge von Methylierung und Reduktion bestimmt. Im Falle einer primären Methylierung mit nachfolgender Reduktion sollten im Hydrolysat Monomethyläther des Galaktosamins erscheinen, bei Methylierung mit voraufgegangener Reduktion sind dagegen Dimethyläther zu erwarten. Verläuft die Methylierung nicht quantitativ, so sind im Hydrolysat zusätzlich partiell und nicht methyliertes Galaktosamin zu finden.

TABELLE I

VERMUTETE HYDROLYSEPRODUKTE DES METHYLIERTEN, REDUZIERTEN VI-ANTIGENS

Behandlung	*Bindungstyp 1→3*		*Bindungstyp 1→4*		*Verzweigung*
	Terminal nicht red.	*Mittelst. +termin. reduz.*	*Terminal nicht red.*	*Mittelst. +termin. reduz.*	
1. Methylierung	340[a]	040	340	300	000
2. Reduktion	(000 300	(000)	(000 300	(000)	
3. Hydrolyse	040)[b]		040)		
1. Reduktion	346	046	346	306	006
2. Methylierung	(000 300	(000 040	(000 300	(000 300	
	040 006	006)	040 006	006)	(000)
3. Hydrolyse	340 306		340 306		
	046)		046)		

[a] 2-Amino-2-desoxy-D-galaktopyranose (000), 3-*O*-(300), 4-*O*-(040), b-*O*-(006), 3,4-di-*O*-(340), 3,6-di-*O*-(306), 4,6-di-*O*-(046), 3,4,6-tri-*O*-methyl (346) Derivat. [b] In Klammern gesetzte Produkte sind bei nicht quantitativ verlaufener Methylierung zu erwarten.

Aus Tab. I geht hervor, mit welchen Derivaten des Galaktosamins als Hydrolyseprodukten man rechnen muß, je nach Bindungstyp im Vi-Antigen, der Reihenfolge von Methylierung und Reduktion und je nachdem, ob das im Hydrolysat auftretende Monosaccharid im Polysaccharid terminal oder mittelständig angeordnet war. Der Tabelle ist zu entnehmen, daß die Frage der 1→3- oder 1→4- Verknüpfung im wesentlichen darauf hinausläuft, ob in den Hydrolysaten nach Reduktion und Methylierung Derivate des 3-*O*-Methyl- und/oder 4-*O*-Methyl-galaktosamins gefunden werden.

Eine Entscheidung darüber, ob in dem Polysaccharid eventuell Kettenverzweigungen vorliegen, ist nicht möglich, da die von Verzweigungsstellen erwarteten Substanzen uncharakteristisch sind und auch von anderen partiell oder nicht methylierten Stellen der Polysaccharidkette stammen könnten. Die nicht reduzierenden Molekülenden sollten dagegen charakteristische Methyläther liefern. Ihre Mengen sind aber wegen des hohen Molekulargewichts[10] des Vi-Antigens von *ca.* 1,700,000 äußerst gering. Nur bei einem stark verzweigten Polysaccharid wären daher analytisch faßbare Mengen aus Endgruppen zu erhalten.

ERGEBNISSE UND DISKUSSION

Für die Strukturanalyse wurden zwei nach verschiedenen Methoden[2,3] hergestellte Präparate verwendet, die allgemein als Vi-W und Vi-J bezeichnet werden[11]. Obwohl beide Produkte sich in ihrem physikalisch-chemischen Verhalten[3,11,12], in ihren serologischen und immunologischen Eigenschaften[11], sowie im Acetylgehalt[11] und der Vi-Phagen-Rezeptoreigenschaft[13] zum Teil merklich unterscheiden, sind sie doch im wesentlichen chemisch identisch[11]. Ihre Strukturanalyse führt zu dem gleichen Ergebnis.

Für die Ermittlung der glycosidischen Bindungsart wurden zwei Wege eingeschlagen. Sie unterscheiden sich nur durch die Reihenfolge von Methylierung und Reduktion. Da sich die beiden Antigenpräparate für eine direkte Methylierung oder Reduktion nicht eigneten, wurden sie, mit einer Ausnahme, zuvor in die Carbonsäureester übergeführt.

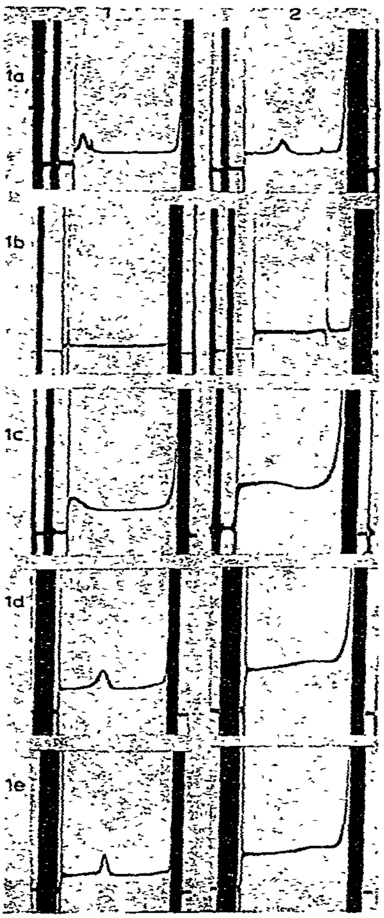
Vi-W wurde als freie Säure und Vi-J als Na-Salz eingesetzt. Die Verfahren werden je nach der Reihenfolge der Reaktionsschritte wie folgt abgekürzt:

Verfahren 1 *E*sterbildung, *M*ethylierung, *R*eduktion, *H*ydrolyse = Verfahren EMRH
Verfahren 2 *E*sterbildung, *R*eduktion, Methylierung, Hydrolyse = Verfahren ERMH
Verfahren 3 *M*ethylierung, *E*sterbildung, *R*eduktion, *H*ydrolyse = Verfahren MERH
(nur bei Vi-J angewandt).

Die Veresterung des Vi-Antigens ist für eine nachfolgende Methylierung wichtig, da nur der Ester in Dimethylformamid und Dimethylsulfoxid löslich und dann einer Methylierung im nichtwässrigen Medium nach Kuhn zugänglich ist. Auch für Reduktionen mit Natriumborhydrid mußten die Carboxylgruppen im Vi-Antigen verestert werden.

Vi-W ließ sich, in Methanol suspendiert, mit ätherischer Lösung von Diazomethan leicht und rasch umsetzen. Dabei bildet sich in 90% Ausbeute ein gelbes, amorphes Produkt (Vi-W Ester : Vi-W E), welches in Wasser, Methanol, Formamid, Dimethylformamid, Dimethylsulfoxid löslich ist. Etwa 10% des eingesetzten Vi-W blieb als in Wasser und Methanol unlöslicher grauer Rückstand zurück.

Der lösliche Vi-W Ester unterscheidet sich auffällig vom Vi-W. Während Vi-W im normalen Ultrazentrifugenlauf zwei Peaks aufweist, die bis zu ihrem Verschwinden im Zellbodensediment über mehrere Stunden klar zu erkennen sind, liefert die Lösung des Vi-W Esters unter gleichen Bedingungen keinen zur Auswertung geeigneten Peak. Erst nach einer Stunde ist sein Maximum gerade aus dem Meniskus aufgetaucht. Eine sichere Lokalisation dieses Maximums ist jedoch wegen der raschen Verflachung des Peaks nicht möglich und wird mit fortgesetzter Sedimentation immer schwerer. Auch bei einem Lauf in der Überschichtungszelle wird eine schnelle Verflachung des Peaks beobachtet. Der über zwei Stunden gut ausgebildete Peak ermöglichte die Berechnung eines mittleren Sedimentationskoeffizienten zu 0,9 S. Vi-J (Na-Salz) wurde zur Veresterung mit methanolischer Salzäure umgesetzt. Auch hier entstand ein hellgrauer methanol-unlöslicher Rückstand, der *ca.* 4% des eingesetzten Vi-J ausmacht. Der methanol-lösliche Anteil, der sich bei der Behandlung mit methanolischer Salzsäure gebildet hatte, ist ein hellgelbes bis farbloses Produkt. Das Verhalten dieses Vi-J Esters (Vi-J E) bei der Sedimentation in der Ultrazentrifuge ähnelt dem des Vi-W Esters. Aus einem Lauf in der Überschichtungszelle errechnet sich ein mittlerer Sedimentationskoefficient von 1.1 S



Vi-W 0.4%ig (Standardzelle)
1, 20′ 30″
2, 315′ 00″
schnelle Komponente : 2.49 S
langsame Komponente : 1.35 S

Vi-J 0.2%ig (Standardzelle)
1, 23′ 40″
2, 295′ 39″
schnelle Komponente : 2.6 S
langsame Komponente : 2.3 S

Vi-W E (methanol-löslich)
0.4%ig (Standardzelle)
1, 59′ 25″
2, 311′ 45″

Vi-W E methanol-löslich
0.4%ig
(Überschichtungszelle)
1, 2′ 15″
2, 190′ 15″
0.9 S

Vi-J E methanol-löslich
0.4%ig
(Überschichtungszelle)
1, 12′ 35″
2, 194′ 55″
1.1 S

Abb. 1. Sedimentationsläufe von Vi-W, Vi-J und der methanol-löslichen Anteile nach Veresterung.

In Abb. I sind die Sedimentationsläufe von Vi-W (1a) und Vi-J (1b) denen der zugehörigen Ester gegenübergestellt. Die im Vergleich zu Vi-W und Vi-J bedeutende Verringerung der Werte der Sedimentationskoeffizienten und die sehr viel raschere Verflachung der peaks in der Lösungen von Vi-W E und Vi-J E zeugen von den tiefgreifenden Veränderungen, die die Antigenpräparate durch die Umsetzung mit methanolischer Salzsäure oder Diazomethan erfahren haben. Zur Zeit läßt sich noch völlig übersehen, ob das vom Vi-W und Vi-J stark abweichende Verhalten des Vi-W E und Vi-J E in der Ultrazentrifuge nur auf die Veresterung de Carboxylgruppen und

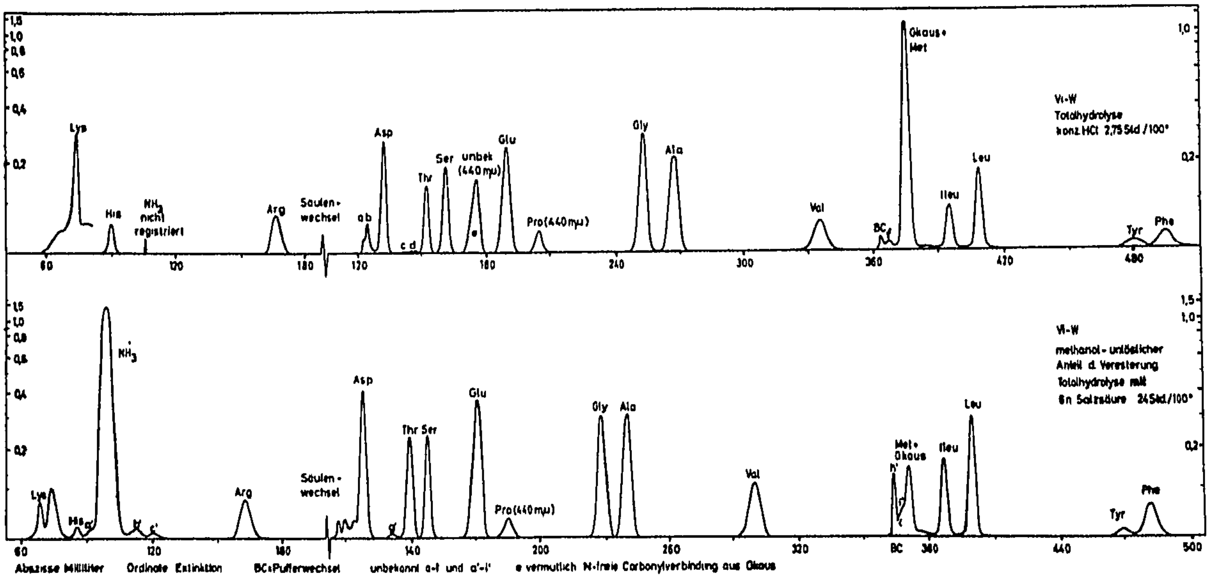


Abb. 2. Vergleich der Analysen der Totalhydrolysate von Vi-W und des methanol-unlöslichen Anteils nach Veresterung.

auf eine partielle Entacetylierung des Polysaccharids zurückzuführen ist. Vielmehr kann hierfür eine partielle Depolymerisation verantwortlich gemacht werden, wie später bei der Diskussion des Strukturmodells begründet werden soll.

Die bei der Veresterung erhaltenen methanol-unlöslichen Rückstände wurden, nachdem ihre Proteinnatur erkannt worden war, totalhydrolysiert und nach Moore, Spackman, Stein[14] analysiert. Ein Vergleich dieser Analysen mit denjenigen von Totalhydrolysaten des Vi-Wu nd Vi-J ist besonders aufschlußreich. Vi-W enthält 4,3% Aminosäuren, der bei der Veresterung gebildete methanol-unlösliche Rückstand dagegen 26,7%. Durch die Behandlung des Vi-W mit Diazomethan ist offensichtlich die Hauptmenge des Proteins vom Polysaccharid abgelöst worden, und findet sich angereichert im unlöslichen Rückstand. Im Vi-J beträgt der Aminosäuregehalt 2,4%, im methanol-unlöslichen Rückstand dagegen 88%. Hier ist durch die Behandlung mit methanolischer Salzsäure eine noch weitergehende Trennung des Proteins vom Polysaccharid erfolgt.

TABELLE II

ANALYSE NACH MOORE, SPACKMAN, STEIN[14]

	Vi-W		*Vi-J*		*Vi-W Methanol-unlösl. Rückst. nach Veresterung*		*Vi-J Methanol-unlösl. Rückst. nach Veresterung*	
	µMol/100 mg	%	µMol/100 mg	%	µMol/100 mg	%	µMol/100 mg	%
Lys	2.29	0.32	1.04	0.15	3.8	0.55	8.27	0.64
His	0.71	0.10	0.39	0.06	9.7	1.51	2.85	0.22
NH_3	(10.38)	(2.96)	—	—	(119)	(20.2)	(10.6)	(8.16)
Arg	1.78	0.29	0.89	0.15	9.3	1.62	8.18	0.63
Asp	3.35	0.42	2.37	0.31	22.9	3.05	16.3	12.50
Thr	1.91	0.21	1.11	0.13	13.3	1.58	6.94	5.33
Ser	2.36	0.23	1.55	0.16	12.4	1.30	8.22	6.32
Glu	5.08	0.70	2.63	0.39	28.6	4.21	16.9	13.00
Pro	2.70	0.29	0.78	0.09	7.9	0.91	4.35	3.34
Gly	5.86	0.41	2.35	0.18	23.2	1.74	7.5	5.77
Ala	4.65	0.39	2.27	0.20	24.8	2.21	17.3	13.30
Val	2.00	0.22	0.85	0.10	13.4	1.57	6.31	4.86
Meth +Gkaus[c]	[a]	[a]	[a]	[a]	10.1	1.51	2.56	1.97
Ileu	1.25	0.15	0.59	0.07	8.4	1.10	5.74	4.41
Leu	2.84	0.35	1.62	0.21	17.2	2.26	10.53	8.10
Tyr	0.50	0.90	0.34	0.06	1.6	0.29	4.06	3.12
Phe	1.11	0.17	0.53	0.09	7.7	1.27	5.86	4.51
Summe		4.34[b]		2.35[b]		26.68[b]		88.02[b]

[a]Nicht berechnet; [b]ohne NH_3; [c]Gkaus=Galaktosaminuronsäure.

Auf der Tab. II ist die vollständige Aminosäureverteilung im Vi-W und Vi-J sowie in den bei der Veresterung erhaltenen unlöslichen Rückständen angegeben.

Abb. 2 zeigt eine Gegenüberstellung der Analysen des Vi-W und des nach Veresterung von Vi-W anfallenden methanol-unlöslichen Rückstandes. Sie vermittelt auch einen Eindruck von dem unterschiedlichen Verhältnis der Menge an Galaktosaminuronsäure zur Gesamtmenge an Aminosäuren in beiden Hydrolysaten.

Die Reduktion der veresterten Carboxylgruppen des Vi-Antigens wurde in wässriger Lösung mit Natriumborhydrid vorgenommen, obgleich die Ausbeuten hierbei im allgemeinen nur mäßig sind. Eine Anwendung des Verfahrens von Brown und Subba Rao[17] mit Diboran war nicht möglich, da die Antigenpräparate in den Lösungsmitteln Tetrahydrofuran und Diglyme nicht löslich sind.

Für die Methylierung des Vi-Antigens erwiesen sich vor allem zwei Methoden als brauchbar. Nach Haworth in der Modifikation von Levene[15] wurde Vi-J mit wässriger Natronlauge und Dimethylsulfat methyliert. Nach Kuhn, Baer, Seeliger[16] ließen sich in Dimethylformamid mit Bariumoxid, Bariumhydroxid-Oktahydrat und Methyljodid Vi-W und Vi-J umsetzen. Ungeeignet war die Methode nach Kuhn und Trischmann[17], weil die bei der Aufarbeitung gebildeten Mengen an Bariumsulfat das gesamte Antigenmaterial adsorbierten und einer weiteren Analyse entzogen.

Die Hydrolyse methylierter und reduzierter Vi-Antigen-Präparate mußte unter sorgfältig angepaßten Bedingungen durchgeführt werden. Einerseits war für die Spaltung der glykosidischen Bindungen wie bei Vi-J bzw. Vi-W[1] eine starke Säure erforderlich, andererseits mußte die Spaltung mit Rücksicht auf die Methoxylgruppen möglichst schonend erfolgen. Konzentrierte Salzsäure, welche zur Hydrolyse von Polysacchariden bevorzugt angewendet wird, da sie leicht flüchtig ist und sich somit gut aus den Hydrolysaten entfernen läßt, erwies sich als ungeeignet, da sie bei der Hydrolyse methylierter Vi-Antigen-Präparate Methoxylgruppen in beträchtlicher Menge abspaltet[17]. Croon, Herrström, Kull, und Lindberg[18] empfehlen daher, Ameisensäure oder Schwefelsäure zu verwenden und die Hydrolyse in zwei Stufen vorzunehmen.

An einem reduzierten, nicht methylierten Vi-Antigen Präparat (Vi-W ER) als Substrat wurde die Brauchbarkeit des Zweistufenverfahrens überprüft, wobei in der ersten Stufe 72%ige Schwefelsäure und in der zweiten Stufe 12%ige angewandt wurde. Hydrolysen-Dauer und -Temperatur wurden optimal dem Substrat angepaßt. Unter den hierbei gefundenen günstigsten Hydrolysebedingungen wurden alle Methyläther des Galaktosamins auf ihre Stabilität überprüft. Bei keiner Verbindung wurde Entmethylierung beobachtet. Es war daher zu erwarten, daß die Zweistufen-Hydrolyse mit Schwefelsäure auch bei methylierten und reduzierten Antigen-Präparaten wie Vi-(W,J) EMR, Vi-(W,J) ERM, Vi-J MER ohne Entmethylierung ablaufen würde.

Die Hydrolysate mit Schwefelsäure wurden nach Abstumpfung der Säure mit Natriumhydrogencarbonat und Verdünnung mit Wasser direkt der säulenchromatographischen Analyse zugeführt.

Alle Hydrolysate methylierter und reduzierter Vi-Antigen-Präparate wurden wegen der kleinen Mengen eingesetzten Materials der automatische säulenchromatographischen Analyse nach Moore, Spackman, Stein[14] unterworfen. Mit Hilfe eines zur Trennung von Methyläthern des Galaktosamins entwickelten Spezialpuffers (0.4N

Natriumcitrat mit konz. Salzsäure auf p_H 2.00 gebracht) war eine zuverlässige Aussage über die Zusammensetzung der Hydrolysate möglich. Kleine Störungen, die vorwiegend durch die begleitenden Aminosäuren Asp, Ser, Thr auftraten, konnten durch Erniedrigung des p_H auf 1.6[19] weitgehend ausgeschaltet werden.

Hydrolysate von Vi-Antigenen, die nach dem Verfahren EMRH oder MERH umgesetzt worden waren, enthielten als charakteristisches Produkt 3-*O*-Methylgalaktosamin neben freiem Galaktosamin. In den anderen Hydrolysaten, bei denen die Vi-Antigene nach dem Verfahren ERMH behandelt worden waren, wurde als entscheidende Substanz 3,6-Di-*O*-methylgalaktosamin gefunden; daneben 3- und 6-*O*-Methylgalaktosamin sowie Galaktosamin als Produkte der unvollständig verlaufenen Methylierung. In keinem der Analysengänge wurde das Auftreten von 4-*O*-Methyl- bzw. 4,6-Di-*O*-Methyl-galaktosamin beobachtet. Damit ist bewiesen, daß die D-Galaktosaminuronsäurebausteine im Vi-Antigen im wesentlichen in einer glykosidischen 1→4-Verknüpfung vorliegen.

Auf Tab. III sind die quantitativen Analysen der nach der Hydrolyse des methylierten Vi-Antigene erhaltenen partiell methylierten Galaktosamin-Derivate für die unterschiedlichen Analysengänge zusammengefaßt. Die Spalten 300 und 306 geben die Anteile der die Struktur klärenden Verbindungen 3-*O*-Methyl-und 3,6-Di-*O*-Methylgalaktosamin wieder. Auf Abb. 3 sind einige Kurven abgebildet, aus denen zu erkennen ist, wie die quantitative Analyse der Methyläther durchgeführt wurde.

Für das Vorliegen einer α-glykosidischen Verknüpfung sprechen der hohe posi-

TABELLE III

ANALYSEN VON HYDROLYSATEN METHYLIERTER UND REDUZIERTER Vi-ANTIGENE

				µg Galaktosamin bzw. -Methyläther				*Ausbeute in %, [a]berechnet als Galaktosamin. HCl*				
Nr.	*Vi*	*mg*	*Behandlung*	*300*	*306*	*000*	*006*	*300*	*306*	*000*	*006*	*Summe*
1	Vi-W	5.7	EMRH	81	–	18.4	—	1.34	–	0.32	—	1.66
3	Vi-W	5.0	ERMH	71	468	22	86.4	1.34	8.35	0.44	1.63	11.8
4	Vi-W	6.5	ERMH	75.6	120.4	126.8	244	1.10	1.80	1.96	3.54	8.4
5	Vi-W	10	ERMH	<5	448	37.6	18.4	0.05	3.99	0.38	0.17	4.5
7	Vi-J	4.7	EMRH	146	—	12.4	—	3.8	—	0.34	—	4.1
8	Vi-J	5.0	EMRH	224	—	242	—	5.47	—	6.31	—	11.8
9	Vi-J	5.0	EMRH	203	—	201.2	—	4.97	—	5.23	—	10.2
12	Vi-J	18.3	MERH	124	—	608	—	0.83	—	4.33	—	5.1
13	Vi-J	4.0	ERMH	227	94.4	68.4	120.8	6.96	2.72	2.24	3.74	15.6
14	Vi-J	4.3	ERMH	71	145.6	62.8	150	2.02	3.90	1.90	4.26	12.1
15	Vi-J	8.0	ERMH	96	109.8	147.9	163.8	1.47	1.58	2.41	2.50	7.9
17	Vi-J	16.0	ERMH	<5	132.8	76	68.4	0.04	0.96	0.62	0.51	2.1

[a] Diesen Berechnungen wurden die Mengen eingesetzter Vi-Antigene sowie die gefundenen Mengen Methyläther und folgende Formeln für Vi-W und Vi-J zugrunde gelgt: Vi-W: $C_8H_{11}O_8N$, M = 217; Vi-J : $C_{10}H_{12}O_7NNa$, M = 281.

tive Drehwert[3] des Vi-Antigens von +300° und seine ungewöhnliche Hydrolysenbeständigkeit[1]. Ob Kettenverzweigungen im Molekül vorkommen, kann aus den vorhandenen analytischen Daten nicht abgeleitet werden. Sicher wird der Verzweigungsgrad sehr gering sein.

Um ein anschauliches Bild von der Struktur eines Molekülausschnitts der Vi-Antigens zu erhalten, wurde mit Hilfe von Atomkalotten nach Stuart und Briegleb ein

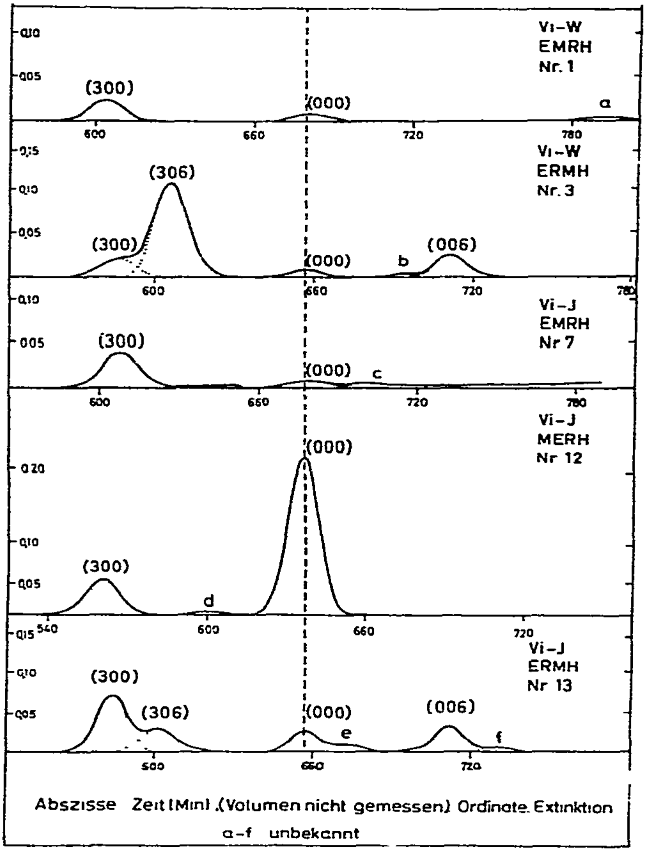


Abb. 3. Analysen von Hydrolysaten methylierter und reduzierter Vi-Antigene nach Spackman, Stein Moore im Citratpuffer pH 2.00.

Molekülmodell zusammengesetzt, dem α-1(→4) verknüpfte Einheiten von 2-Acetamido-2-desoxy-3-*O*-acetyl-α-D-galaktopyranuronsäure zu Grunde gelegt wurden. Dabei ergibt sich eine Struktur, in der die Pyranoseringe infolge der sterischen Hinderung der Substituenten an C-2, C-3 und C-5 in einer Zick-Zack-Form fixiert sind. Die Abb. 4 gibt einen aus diesem Kalottenmodell hergeleiteten Strukturvorschlag wieder.

Dieser Strukturvorschlag stellt nur einen Teil der Struktur des Gesamtantigens dar. Er beschränkt sich im wesentlichen auf die glykosidischen Bindungen zwischen den Monosacchariden. Bezüglich der Struktur des Gesamtantigens können noch keine sicheren Aussagen gemacht werden. Wahrscheinlich liegt das Vi-Antigen nicht als einzelne gerade bzw. geknäulte, eventuell schwach verzweigte Polysaccharidkette vor.

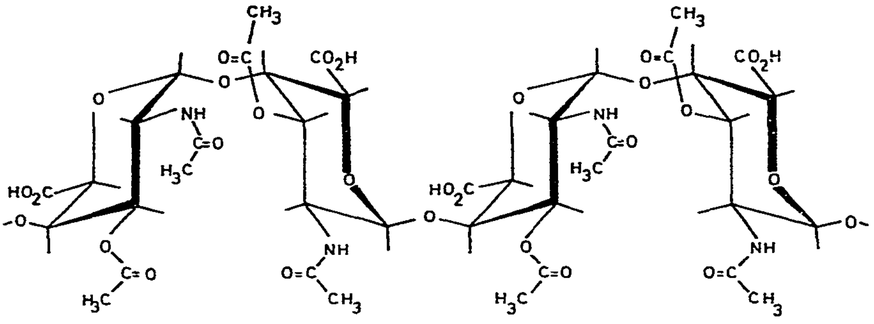


Abb. 4. Strukturvorschlag für Vi-J.

Das Verhalten des Vi-Antigens gegenüber verdünntem Alkali spricht nach Jarvis[10] vielmehr dafür, daß mehrere Polysaccharidketten vom Molekulargewicht ~100,000 zu Blatt- oder Bündelstrukturen vom Molekulargewicht ~1,700,000 durch Esterbindungen zusammengehalten werden. Auf Grund dieser Vorstellung läßt sich das Verhalten des vorgereinigten Vi-Antigens beim Refluxieren mit 1M Essigsäure als Hydrolyse von Esterbindungen deuten und somit als Depolymerisation des Gesamtmoleküls, ohne daß glykosidische Bindungen angegriffen werden. Eine Depolymerisation durch Lösung glykosidischer Bindungen würde im Widerspruch stehen zur auffallenden Resistenz[1] des Vi-Antigens gegenüber starken wäßrigen Mineralsäuren.

Auch die durch methanolische Salzsäure bewirkten Veränderungen des Vi-Antigens stehen mit einer voraufgegangenen Spaltung von Esterbindungen des Polysaccharids im Einklang. Daß auch die Einwirkung von Diazomethan auf Vi-W zu Produkten führt, die den aus Vi-J mit methanolischer Salzäure gebildeten ähneln, läßt sich verstehen, wenn man eine Diazomethan-katalysierte Umesterung annimmt[20]. Ob die im Vi-Antigen enthaltene kleine Menge an Protein, das zum größten Teil durch Diazomethan vom Polysaccharid abgelöst werden kann, ebenfalls Bindungen zwischen verschiedenen Polysaccharidketten des gleichen Moleküls vermittelt, bleibt zu klären.

EXPERIMENTELLER TEIL

Umsetzungen mit Vi-Antigen. — Für alle Umsetzungen wurden, wenn nicht anders vermerkt, Vi-W bzw. Vi-J in vergleichbaren Mengen eingesetzt.

Umsetzung mit Diazomethan. — 25.3 mg Vi-W wurden in 2 ml Methanol suspendiert und unter kräftigem Rühren mit 2 ml einer ätherischen Diazomethan-Lösung versetzt. Nach 10 Min. wurden nochmals 2 ml Diazomethan-Lösung zugegeben und nach weiteren 10 Min. wurde die Suspension zentrifugiert, wobei sich aus einer gelben Lösung ein graugelbes Sediment abschied. Die gelbe Lösung wurde abdekantiert und das Sediment in 2 ml Methanol suspendiert. Der größte Teil des Sediments löste sich dabei gelb auf. Nach Zentrifugation wurde wieder eine gelbe Lösung und eine kleinere Menge hellgraues Sediment erhalten. Die gesammelten gelben Lösungen wurden zur Trockne eingedampft : 28.5 mg eines gelben Lackes (Vi-W E). Methanol-unlöslicher Rückstand 2.1 mg (8%).

Umsetzung mit methanolischer Salzsäure. — 14.8 mg Vi-J wurden 2 Std. bei 50°/0.05 Torr über Phosphorpentoxid getrocknet und dann im Einschmelzrohr mit 2 ml einer 2%igen Lösung von trocknem Chlorwasserstoff in absolutem Methanol 3 Std. bei 65–70° aufbewahrt. Nach 2 Std. enthielt die gelegentlich geschüttelte Lösung nur noch kleine Mengen unlöslicher Flocken. Durch Zentrifugation der erkalteten Lösung und Eindampfen des klaren Überstandes wurden 14.3 mg eines hellgelben Lackes erhalten (Vi-J E). Methanol-unlösliches Sediment 0.8 mg (5,4%).

Methylierung nach Kuhn, Baer, und Seeliger[16]. — 5–10 mg Vi-W E bzw. Vi-J E wurden in 0.4–0.5 ml Dimethylformamid gelöst; Vi-W ER und Vi-J ER wurden dagegen in 0.1–0.2 ml Formamid gelöst und ihre klaren Lösungen mit der 2–4-fachen Menge Dimethylformamid verdünnt.

Die klaren Lösungen der Antigenpräparate wurden bei Raumtemperatur unter Stickstoff 3-4-mal, und zwar in Abständen von je 1–2 Std. mit Bariumoxid (x), Bariumhydroxid–Oktahydrat (y) und Methyljodid (z) versetzt, wie sich aus der Tabelle ergibt :

eingesetzt	*x (mg)*	*y (mg)*	*z (ml)*	*erhalten*
Vi-W E	1. 33	28	0.2	
	2. 34	38	0.2	
	3. 38	25	0.2	Vi-W EM
Vi-W ER	1. 69	—	0.2	
	2. 85	—	0.2	
	3. 51	—	0.2	Vi-W ERM
Vi-J E	1. 41	7	0.2	
	2. 28	17	0.2	
	3. 27	9	0.2	Vi-J EM
Vi-J ER	1. 27	10	0.2	
	2. 28	15	0.2	
	3. 30	9	0.2	
	4. 45	27	0.2	Vi-J ERM

Nach 3 bis 5 Std. wurde den Ansätzen 1,5 ml 2N Essigsäure zugesetzt. Dabei lösten sich die Bariumsalze auf, überschüssiges Methyljodid wurde am Rotationsverdampfer abgezogen, und die verbliebenen rotbraunen Lösungen wurden durch eine kleine

Austauschersäule mit Dowex 50 (H^+) 12 × 70 mm filtriert. Mit Wasser wurde bis zur Neutralität der Eluate gewaschen. Die gesammelten Eluate wurden zu braunen Sirupen eingedampft und diese nach Verdünnen mit 1 ml Wasser mehrere Male mit Chloroform extrahiert. Die zu Sirupen eingeengten wässrigen Phasen wurden dann in 1 ml Methanol gelöst und mit 2 ml ätherischer Diazomethanlösung nachverestert. Der Überschuß an Diazomethan wurde nach 30 Min. mit wenigen Tropfen Eisessig vernichtet und die gelblichen Lösungen zu Sirupen eingedampft.

Methylierung nach Haworth, modifiziert nach Levene[15]. — 22,2 mg Vi-J wurden unter Rühren in 2,2 ml Wasser gelöst und anschließend mit 1 ml Tetrachlorkohlenstoff unterschichtet. In kleinen Zeitabständen wurde diesem System unter Stickstoff insgesamt 1 ml 35%ige Natronlauge und 0,5 ml Dimethylsulfat zugesetzt. Der Ansatz wurde über Nacht und unter Stickstoff kräftig gerührt. Danach wurde von feinen Flocken abzentrifugiert. Der alkalische Überstand wurde mit Eisessig auf p_H 6 gebracht und diese Lösung gegen fliessendes destilliertes Wasser dialysiert. Das hellgelbe Dialyse-Retentat wurde unter Zusatz weniger Tropfen 6N Salzsäure zur Trockne gebracht und der Rückstand mehrere Male mit Äthanol abgedampft (Bezeichnung des erhaltenen Produktes : Vi-J M).

Reduktion. — Für die Reduktion wurden eingesetzt Vi-W E, Vi-J E, Vi-W EM, Vi-J EM und Vi-J ME.

Die Antigen-Präparate wurden gelöst in 1 ml Wasser und nach Zugabe von 10–15 mg Natriumborhydrid über Nacht bei 0–4° aufbewahrt. Danach wurden die farblosen bis schwach trüben Lösungen mit einigen Tropfen Eisessig angesäuert und zur Trockne gebracht. Der Rückstand wurde zur Entfernung der Borsäure zweimal mit Methanol abgedampft. (Bezeichnung der erhaltenen Produkte : Vi-W ER, Vi-J ER, Vi-W EMR, Vi-J EMR, Vi-J MER).

Hydrolyse. — Alle durch Veresterung, Methylierung und Reduktion modifizierten Vi-Antigen-Präparate wurden zur Hydrolyse in 0,12 ml 72%iger Schwefelsäure 8 Std. bei 45° aufbewahrt, anschliessend nach Verdünnen der Lösung mit 0,92 ml Wasser 4 St. bei 100° im Einschmelzrohr. Die abgekühlten Hydrolysate wurden mit 0,8N Natriumhydrogencarbonat-Lösung auf 2 ml aufgefüllt.

Automatische Analysen nach Moore, Spackman, Stein[14]. — (*a*) Besondere Ausrüstungen des automatischen Gerätes : 4 thermostatierte Säulen, je 1500 × 9 mm, mit dem sauren Ionenaustauscher 150 A (Bruchharz, Korndurchmesser 31-41 μ) der Fa. Beckman; Arbeitstemperatur 60°. Pumpen (für 2 Säulensysteme) : 4 LKB Miniflow Precision Micropumps Typ 4501; Nennleistung für 2 Pufferpumpen 34 ml/h, für 2 Ninhydrinpumpen 17 ml/h. Energieaufnahme je Pumpe 2 Watt. Photometer : LKB Multichannel Absorptiometer Typ 5901 A. Schreibgerät : Philips Recorder PR 3210 A. (*b*) Puffer : 0.4N Natriumcitrat mit Salzsäure auf p_H 2.00 eingestellt.

Von den Hydrolysaten der Antigen Präparate wurden jeweils 500 μl auf Methyläther des Galaktosamins analysiert. Die Auswertung erfolgte nach bekannten Verfahren[14] und mit Hilfe von Eichwerten authentischer Vergleichsubstanzen[19]. Die als Vergleichssubstanzen verwendeten Methyläther des Galaktosamins wurden nach bekannten Vorschriften[9] synthetisiert.

Analyse des Proteinanteils.—Hierfür wurden volgende Hydrolysate eingesetzt:

eingesetzte Produkte		Behandlung			für Analyse
Vi-W	21,4 mg	12N HCl	2 Std.	100°	1/20
Vi-J	5,1 mg	12N HCl	2 Std.	100°	1/5
Vi-W E methanol-unlöslich	1,84 mg	6N HCl	24 Std.	100°	1/1
Vi-J E methanol-unlöslich	1,3 mg	6N HCL	24 Std.	100°	2/2

Sendimentationsanalysen. — In der Ultrazentrifuge Fa. Beckman Spinco Modell E, Rotor An-D. 4,0 mg Vi-W E bzw. 4,3 mg Vi-J E in je 1 ml Acetatpuffer pH 4,66 für die Analyse in der Standardzelle und in der Überschichtungszelle, in beiden Fällen bei 59,780 UpM und 20°. Sedimentationskoeffizient für Vi-W E 0,9 S und für Vi-J E 1,1S, beide Werte aus den Analysen in der Überschichtungszelle.

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ZUSAMMENFASSUNG

Die Struktur des aus *Citrobacter freundii* 5396/38 isolierten Vi-Antigens (Vi-W, Vi-J) wurde auf zwei Wegen aufgeklärt: (1) durch Methylierung mit anschließender Reduktion, (2) durch Methylierung mit voraufgegangener Reduktion. In den Hydrolysaten der so umgesetzten Antigenpräparate wurden das 3-*O*-Methylgalaktosamin (nach 1) bzw. das 3,6-Di-*O*-methylgalaktosamin (nach 2) gefunden. Das Auftreten dieser Verbindungen und das Fehlen des 4-*O*-Methylgalaktosamins bzw. des 4,6-Di-*O*-methylgalaktosamins beweist die glykosidische 1→4 Verknüpfung der Monomeren.

Die Identifizierung dieser Substanzen mit authentischem Material und ihre quantitative Bestimmung erfolgte durch das gleichartige säulenchromatographische Verhalten bei der automatischen Analyse nach Moore, Spackman, und Stein unter Anwendung eines Spezialpuffers.

Aufgrund der Befunde wird ein Strukturmodell des Vi-Antigens entwickelt.

SUMMARY

The structure of the Vi-antigen isolated from *Citrobacter freundii* 5396/38 was elucidated by applying (1) methylation followed by reduction, and (2) reduction prior to methylation. Hydrolysates of the Vi-antigen material treated in these two ways contained 2-amino-2-deoxy-3-*O*-methyl-D-galactopyranose (3-*O*-methylgalactosamine)

(from 1) and 2-amino-2-deoxy-3,6-di-*O*-methyl-D-galactopyranose (3,6-di-*O*-methylgalactosamine) (from 2), respectively. The occurrence of these two compounds, as well as the absence of 2-amino-2-deoxy-4-*O*-methyl-D-galactopyranose and 2-amino-2-deoxy-4,6-di-*O*-methyl-D-galactopyranose, conclusively demonstrates 1→4 glycosidic bonds in the polysaccharide.

The products formed by hydrolysis were quantitatively determined and identified using authentic control substances in the amino acid analyser of Moore, Spackman, and Stein by means of a special buffer. From these results a model of the structure of the Vi-antigen is presented.

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2,3-THIONOCARBONATE AND 2,3-CARBONATE DERIVATIVES OF D-GLUCOPYRANOSIDES*

E. I. STOUT, W. M. DOANE, B. S. SHASHA, C. R. RUSSELL, AND C. E. RIST
*Northern Regional Research Laboratory**, Peoria, Illinois 61604 (U.S.A.)*

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Sugar derivatives containing the bis(*O*-thiocarbonyl) disulfide group and an adjacent hydroxyl group rearrange readily in pyridine to form cyclic thionocarbonates[1]. Although a number of systems in which the hydroxyl and bis(*O*-thiocarbonyl) disulfide groups are vicinal have been investigated, no information is available on the behavior of these groups when present in a *trans* configuration on a pyranoid ring. Such a system was explored because formation of a thionocarbonate might give a unique, *trans*-fused ring-structure.

Methyl 4,6-*O*-benzylidene-α-D-glucopyranoside (**1**) was xanthated and coupled to give mainly bis(methyl 4,6-*O*-benzylidene-2-*O*-thiocarbonyl-α-D-glucopyranoside) disulfide (**2**). Probably some of the 2,3'- and 3,3'-disulfides were also present, since xanthation of **1** gives about 85–90% of the 2-xanthate and 10–15% of the 3-xanthate[2]. Since the rearrangement reaction of **2** and its isomers should be similar, no further attempt was made to purify the product. Disulfide **2** readily dissolved in pyridine, and underwent rearrangement to give nearly equimolar amounts of **1**, carbon disulfide, free sulfur, and a crystalline product formulated as methyl 4,6-*O*-benzylidene-α-D-glucopyranoside 2,3-thionocarbonate (**3**). Therefore, the course of this reaction in the *trans* system is the same as that reported[1] for vicinal *cis*-diols on furanoid and pyranoid rings and for vicinal diols in acyclic sugars.

The structure proposed for **3** is consistent with its elemental analysis, molecular weight, and i.r. (thionocarbonate absorption[2] at 7.8 μ, Fig. 1A) and u.v. (thionocarbonate absorption[1] near 238 mμ) spectra. Since mild alkaline hydrolysis of **3** gave **1**, no inversion of configuration had occurred during rearrangement. Treatment of **3** with silver nitrate[3] gave the corresponding *trans*-2,3-carbonate (**4**) in 93% yield. This conversion provides a route to the previously unavailable[4] *trans*-carbonate. The utility of cyclic carbonates as protecting groups might thus be extended to *trans*-diols on pyranoid rings. The *trans*-cyclic carbonate exhibited carbonyl absorption at 5.45 and 5.55 μ in the infrared spectrum (Fig. 1B). This absorption appears to be characteristic for such a derivative, since five-membered cyclic carbonates fused *cis*

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to the pyranoid ring exhibit a single absorption maximum in this region, and larger-ring or linear carbonates absorb[5] near 5.7 μ.

For comparison of infrared spectra, a similar *trans*-fused system was prepared. *trans*-1,2-Cyclohexanediol was converted into the crystalline *trans*-thionocarbonate *via* the xanthate, and then into the *trans*-1,2-carbonate. The i.r. spectrum showed two maxima of nearly equal intensity at 5.45 and 5.51 μ (Fig. 1C).

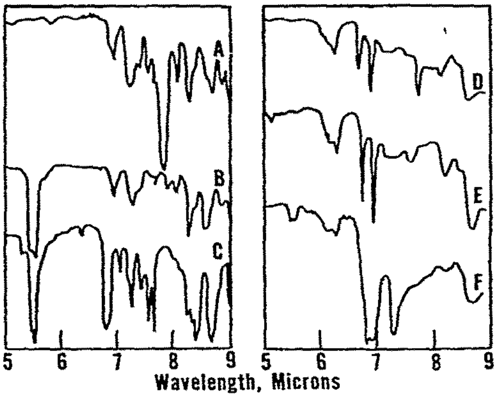

Fig. 1. Infrared spectra of methyl 4,6-*O*-benzylidene-α-D-glucopyranoside 2,3-thionocarbonate (**3**, A, film), methyl 4,6-*O*-benzylidene-α-D-glucopyranoside 2,3-carbonate (**4**, B, film), *trans*-1,2-cyclohexanediol carbonate (C, film), 6-*O*-tritylamylose 2,3-thionocarbonate (D, KBr), 6-*O*-tritylamylose (E, KBr), and 6-*O*-tritylamylose 2,3-carbonate (F, Nujol).

Although the mixture from the rearrangement showed only **1** and **3** by t.l.c., fractionation on a column of Adsorbosil* gave a minor fraction in addition to **1** and **3**. T.l.c. of this fraction showed three spots of almost equal intensity, with R_F values intermediate between those of **1** and **3**. T.l.c., sulfur, molecular weight, i.r., and u.v. analyses indicated that these components were the bis(methyl 4,6-*O*-benzylidene-α-D-glucopyranoside) 2,2′-, 2,3′-, and 3,3′-thionocarbonates (**5**).

Further support for these structures was obtained by conversion of **5** into the corresponding carbonates with silver nitrate. The three carbonates displayed carbonyl absorption only at 5.7 μ. Apparently, these intermolecular structures are products of reaction of **3** with **1** since a pyridine solution of **3**, when kept with **1**, gave **5**. T.l.c. of the solution after 7 days showed, in addition to **3** and **1**, three components having R_F values equal to those of **5**. When the mixture was treated with silver nitrate, it gave a product having carbonyl absorptions near 5.5 and 5.7 μ in the ratio of 1:2. On the other hand, a pyridine solution of *trans*-1,2-cyclohexanediol thionocarbonate was stable when kept in the presence of *trans*-1,2-cyclohexanediol.

To eliminate any influence which the fused benzylidene group might exert on formation of the *trans*-thionocarbonate (**3**), and to extend the utility of the reaction

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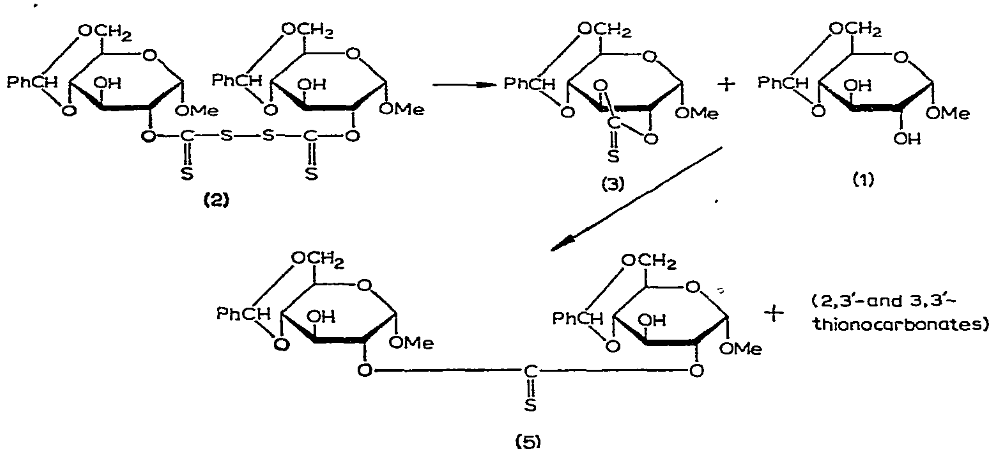
*Mention of firm names or trade products does not imply that they are endorsed or recommended by the U. S. Department of Agriculture over other firms or similar products not named.

to polymeric structures, 6-*O*-tritylamylose was examined. This product, which had a degree of substitution (D.S.) by the trityl group of 1.0, was difficult to xanthate to a D.S. greater than 0.2 when treated only with sodium hydroxide and carbon disulfide. However, when methyl sulfoxide was also added, a xanthate D.S. of *ca.* 1 was readily obtained. This product was oxidized to give 6-*O*-tritylamylose-bis(*O*-thiocarbonyl disulfide (tritylamylose xanthide), which dissolved completely on being kept in pyridine for 5 h at 25°. During this time, carbon disulfide was evolved and free sulfur was formed, indicating rearrangement of the xanthide group to thionocarbonate. The sulfur content of the tritylamylose derivative isolated from the pyridine after 8 h was in agreement with the formation of one thionocarbonate group from each xanthide group. The i.r. spectrum of this derivative (Fig. 1D) showed absorption at 7.8 μ for the thionocarbonate structure, whereas the starting 6-*O*-tritylamylose gave no maximum in this region (Fig. 1E). De-*O*-tritylation with methanolic hydrochloric acid solution, followed by hydrolysis with alpha amylase, gave a water-soluble product that showed a strong absorption at 233 mμ, presumably for the thionocarbonate group.



In further support of the thionocarbonate structure, treatment of the tritylamylose product with silver nitrate gave the corresponding carbonate, having no absorption maximum at 233mμ or 7.8 μ, but showing carbonyl absorption at 5.45 and 5.55μ (Fig. 1F). For some preparations, additional carbonyl absorption was observed near 5.7 μ. Thus, as with the monosaccharide derivative **3**, the *trans*-thionocarbonate group of the polysaccharide undergoes ring opening to form intermolecular thionocarbonates.

EXPERIMENTAL

Melting points were taken in a calibrated Thomas–Kofler apparatus. Optical rotations were measured at 5893 Å with a Rudolph polarimeter. I.r. spectra were determined either as films (or Nujol mulls) on silver chloride plates, or as potassium bromide discs, with a Perkin–Elmer Model 137 spectrophotometer. U.v. spectra were recorded with a Perkin–Elmer Model 202 spectrophotometer. T.l.c. was performed on Silica Gel G with chloroform–acetone (9.5:0.5 v/v) as eluent, and methanol containing 5% of sulfuric acid as detector. The amylose used was a commercial product (A. E. Staley Company, Decatur, Illinois). *alpha*-Amylase was a product of British Drug Houses. Adsorbosil was obtained from Applied Science Laboratories, Inc., State College, Pennsylvania.

Bis(methyl 4,6-O-benzylidene-2-O-thiocarbonyl-α-D-glucopyranoside) disulfide (**2**). — To a solution of methyl 4,6-*O*-benzylidene-α-D-glucopyranoside (**1**, 5 g) in *p*-dioxane (15 ml) were added 5N sodium hydroxide (3.35 ml) and carbon disulfide (15 ml). After the mixture had been stirred for 15 min, it was diluted with water (15 ml) and the pH was adjusted to 6 with aqueous acetic acid. The xanthate was then oxidatively coupled by addition of iodine, and the corresponding xanthide was extracted into ether. The extract was washed with water, dried with anhydrous sodium sulfate, and evaporated to dryness. T.l.c. revealed a major constituent having R_F 0.6 and a minor component near the origin. The latter was removed when the product was dissolved in carbon disulfide and filtered. The carbon disulfide was evaporated off, to give **2** (3.3 g), m.p. 118–120°, $\lambda_{\max}^{\mathrm{MeOH}}$ 238 mμ (ε 17,500) and 287 mμ (ε 7,800), $\lambda_{\max}^{\mathrm{film}}$ 8.1 and 9.4 μ [O(C=S) SS(C=S)O].

Anal. Calc. for $C_{30}H_{34}O_{12}S_4$: C, 50.4; H, 4.8; S, 17.9. Found: C, 50.4; H, 5.5; S, 17.8.

Methyl 4,6-O-benzylidene-α-D-glucopyranoside 2,3-thionocarbonate (**3**). — A solution of **2** (1.56 g) in pyridine (25 ml) was kept for 3 h at 25°. U.v. analysis of the crude product after evaporation of the pyridine showed 93% conversion into the thionocarbonate (**3**) based on the formation of equimolar quantities of **1**, **3**, carbon disulfide, and free sulfur. The product was adsorbed onto a column (22 cm × 1.8 cm) of Adsorbosil (24 g) and fractionated by successive elutions with 1:1 chloroform–hexane (200 ml), 2:1 chloroform–hexane (300 ml), 3:1 chloroform–hexane (130 ml), chloroform (130 ml), 9:1 chloroform–acetone (200 ml), 8:2 chloroform–acetone (100 ml), and 1:1 chloroform–acetone (100 ml). Fractions of approximately 60 ml each were collected, and evaporated under vacuum to constant weight.

Fractions 2–6 gave **3** (0.45 g), m.p. 132–135° (m.p. 137–138° after recrystallization from carbon disulfide), $[\alpha]_D^{25}$ 0° (*c* 3.0, chloroform), $\lambda_{\max}^{\mathrm{MeOH}}$ 238 mμ (ε 14,500), $\lambda_{\max}^{\mathrm{film}}$ 7.8 μ [O(C=S)O].

Anal. Calc. for $C_{15}H_{16}O_6S$: C, 55.6; H, 4.9; S, 9.9; mol. wt., 324. Found: C, 55.3; H, 5.1; S, 9.7; mol. wt., 317 (Rast, in camphor).

A higher yield of **3** was obtained on treatment of **2** (12 g) with pyridine (15 ml) for 4.5 h. The syrup that resulted on evaporation of the pyridine was dissolved in chloroform (400 ml) and stirred with Adsorbosil (165 g). After 10 min, the Adsorbosil was removed by filtration and extracted with several portions of chloroform. The filtrate and extracts were combined, and evaporated to a syrup. Crystallization from carbon disulfide gave 5.0 g (92%) of **3**, identical with the product recovered from the column.

Methyl 4,6-O-benzylidene-α-D-glucopyranoside 2,3-carbonate (**4**). — To a solution of **3** (100 mg) in acetone (5 ml) was added water (1 ml) containing silver nitrate (120 mg). An excess of barium carbonate was added to neutralize the acid formed. After 5 min, the suspension was centrifuged, and the supernatant liquor was filtered. The filtrate was evaporated and the residue washed with water. The syrup was then dissolved in ether and dried with sodium sulfate. Evaporation of the ether gave amorphous **4**, yield 88 mg (93%), m.p. 62–64°, $[\alpha]_D^{25}$ +69° (*c* 1.0, chloroform), λ_{max}^{film} 5.45 and 5.55 μ [O(C=O)O].

Anal. Calc. for $C_{15}H_{16}O_7$: C, 58.4; H, 5.2. Found: C, 58.4; H, 5.4.

Alkaline hydrolysis of **3** *and* **4**. — A solution of **3** (100 mg) in acetone (10 ml) was treated with a saturated aqueous solution of barium hydroxide (3 ml). The suspension was refluxed for 10 min and evaporated to dryness. The product was extracted with chloroform, and the chloroform extract evaporated to give crystalline **1**, yield 82 mg (94%); an authentic sample of **1** in admixture with this product showed no depression of melting point. Similar treatment of **4** gave **1** in 95% yield.

Bis(methyl 4,6-O-benzylidene-α-D-glucopyranoside) thionocarbonates (**5**) *and carbonates*. — (a) Fractions 8 and 9 collected from the column (see preparation of **3**) were evaporated to give an amorphous powder (0.09 g), m.p. 48–50°. On t.l.c., this product showed three spots of almost equal intensity, with R_F values between those of **1** and **3**. Identification of these components as the isomeric, intermolecular, thionocarbonate derivatives (**5**) of **1** followed from t.l.c. and the following analyses: λ_{max}^{MeOH} 233–236 mμ (ε 10,900), λ_{max}^{film} 7.8 μ [O(C=S)O].

Anal. Calc. for $C_{29}H_{34}O_{12}S$: C, 57.4; H, 5.6; S, 5.3; mol. wt., 606. Found: C, 57.6; H, 5.8; S, 5.1; mol. wt., 578 (Rast, in camphor).

When this substance was treated with silver nitrate by a procedure similar to that used for **3**, the product exhibited carbonyl absorption at 5.7 μ.

(b) A mixture of **3** (23 mg) and **1** (185 mg) in pyridine (1 ml) was kept for 7 days at room temperature. T.l.c. showed, in addition to **1** and **3**, three spots having R_F values corresponding to that of **5**. The pyridine was evaporated, and the product was treated with silver nitrate. I.r. spectroscopy revealed carbonyl absorption maxima near 5.5 and 5.7 μ in the ratio of 1:2.

Methyl 4,6-O-benzylidene-α-D-glucopyranoside (**1**) *from* **2**. — Evaporation of the solvents from fractions 13–18 (see preparation of **3**) gave crystalline **1**, 0.65 g (106%, based on the rearrangement), m.p. and mixed m.p. with authentic samples, 164–165°.

Carbon disulfide and elemental sulfur from **2**. — The carbon disulfide formed on rearrangement of **2** (1.56 g) in pyridine (25 ml) was collected and determined[3] to be 0.95 mole per mole of **2**. In the same experiment, the pyridine was evaporated and **1** and **3** were separated from the elemental sulfur by dissolving **1** and **3** in a small volume of methanol. The insoluble, elemental sulfur weighed 70 mg (1.0 mole per mole of **2**).

trans-*1,2-Cyclohexanediol thionocarbonate and carbonate*. — The bis(*O*-thiocarbonyl) disulfide derivative of *trans*-1,2-cyclohexanediol, prepared in a manner similar to that used for **2**, contained 32.9% of sulfur (theoretical, 33.5%). This product (1.125 g) was dissolved in pyridine (25 ml) and kept overnight. After the pyridine had been evaporated, the residue was extracted with water to remove the free diol, and then dissolved in ethanol and filtered to remove free sulfur. Evaporation of the filtrate gave the *trans*-thionocarbonate in 87% yield (based on the rearrangement), m.p. 110–110.5° (from ether). Corey *et al.*[6] prepared this thionocarbonate by a different route, and gave no m.p. The *trans*-thionocarbonate was converted into the corresponding carbonate in 65% yield by the silver nitrate procedure. The *trans*-1,2-carbonate gave λ_{max}^{film} 5.45 and 5.51 μ [O(C=O)O].

Anal. Calc. for $C_7H_{10}O_3$: C, 59.2; H, 7.0. Found: C, 59.2; H, 7.0.

The thionocarbonate was recovered unchanged from a mixture of the thionocarbonate and the diol in pyridine that had been kept for several days.

6-O-Tritylamylose xanthide. — 6-*O*-Tritylamylose was prepared according to Whistler and Hirase[7]. Determination of the trityl content by the method of Hearon, Hiatt, and Fordyce[8] gave a D.S. value of 1.0. To a suspension of 6-*O*-tritylamylose (5 g) in carbon disulfide (15 ml) was added 5N sodium hydroxide (5 ml). The mixture was triturated in a mortar, with slow addition of methyl sulfoxide (30 ml). A homogeneous, dark-red solution resulted after about 25 min. The mixture was then diluted with methyl sulfoxide (120 ml), and neutralized with aqueous acetic acid to give a clear, yellow solution. Upon crosslinking by treatment with a dilute solution of iodine, a light-brown precipitate formed; this was centrifuged, and washed successively with aqueous thiosulfate, water, ethyl alcohol, carbon disulfide, and ether. The yield of the yellowish powder, referred to as 6-*O*-tritylamylose xanthide, was 5.5 g. The product contained 13% of sulfur, corresponding to a xanthide D.S. of 0.97. Products containing 3–4% of sulfur were obtained when reactions were performed in the same way but without methyl sulfoxide.

6-O-Tritylamylose 2,3-thionocarbonate. — 6-*O*-Tritylamylose xanthide (1.5 g) was kept in anhydrous pyridine at 25°. The product swelled immediately on addition of pyridine, and dissolved completely in about 5 h. At the end of 8 h, the 6-*O*-tritylamylose thionocarbonate that formed was precipitated by pouring the solution into ether, and was washed with ethanol, carbon disulfide, and ether. The dried product contained 3% of sulfur, corresponding to a thionocarbonate D.S. of 0.39. The i.r. spectrum (KBr) showed absorption at 7.8 μ for the thionocarbonate group (Fig. 1D).

Detritylation and enzymic hydrolysis of 6-O-tritylamylose 2,3-thionocarbonate. — Detritylation was performed by stirring a suspension of the thionocarbonate derivative in methanol containing 5% of hydrochloric acid for 16 h at 25°. The suspension was filtered, and the product was washed successively with methanol, carbon disulfide, and ether. The dried product contained 4.6% of sulfur, corresponding to a thionocarbonate D.S. of 0.25. Since a portion dissolved in concentrated sulfuric acid gave no precipitate of triphenylmethanol on dilution with water, detritylation was considered to be complete. Enzymic hydrolysis of the amylose thionocarbonate was accomplished by treating an aqueous suspension of the thionocarbonate with alpha amylase at 60°. Samples of the supernatant liquor were periodically withdrawn and their u.v. spectra examined. During the first few minutes of the hydrolysis, a strong peak at 233 mμ and a relatively weak peak at 303 mμ were observed. The latter peak, presumably due to some xanthate ion, disappeared on standing or on addition of dilute acid, whereas the peak at 233 mμ remained.

6-O-Tritylamylose 2,3-carbonate. — Freshly prepared 6-*O*-tritylamylose thionocarbonate was dispersed in methyl sulfoxide and heated to 65°. The dispersion was treated dropwise with aqueous silver nitrate. The suspension was then filtered, and the precipitate was washed with water. The silver sulfide formed was removed by washing with aqueous sodium cyanide. The white precipitate obtained after washing with water, alcohol, and ether contained no sulfur. Carbonyl absorption was observed at 5.45 and 5.55 μ, with no absorption at 7.8 μ for the thionocarbonate group (Fig. 1F). For some preparations, additional carbonyl absorption was observed at 5.7 μ.

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SUMMARY

A unique sugar derivative, methyl 4,6-*O*-benzylidene-α-D-glucopyranoside 2,3-thionocarbonate, which contains a *trans*-fused ring-structure, was prepared in good yield by rearrangement of bis(methyl 4,6-*O*-benzylidene-2-*O*-thiocarbonyl-α-D-glucopyranoside) disulfide. The thionocarbonate was converted into the novel methyl 4,6-*O*-benzylidene-α-D-glucopyranoside 2,3-carbonate in 93% yield by treatment with silver nitrate. By following the same reaction sequence, 2,3-thionocarbonate and 2,3-carbonate groups were introduced into 6-*O*-tritylamylose. The rearrangement of bis(*O*-thiocarbonyl) disulfide derivatives provides a new route for the synthesis of carbohydrate thionocarbonates and carbonates previously unavailable.

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A KINETIC ANALYSIS OF THE REACTIVITIES OF THE HYDROXYL GROUPS IN FORMYLATION OF THE D-GLUCOPYRANOSYL RESIDUES IN DEXTRIN

STANLEY P. ROWLAND, MARY ANN F. BRANNAN, HERMANN J. JANSSEN, AND PAUL F. PITTMAN
Southern Regional Research Laboratory, Southern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture, New Orleans, Louisiana 70119 (U.S.A.)

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INTRODUCTION

Esterification of cellulose, starch, and other carbohydrates with carboxylic acids has evoked considerable interest both for the modification of physical properties and for investigative purposes[1]. However, delineation of the reactivities of the hydroxyl groups on C-2, C-3, and C-6 of the D-glucopyranosyl residues in esterifications has met with substantially less success than that achieved in etherifications, as the ethers are more stable[2].

From a study of the esterification of D-glucose with 90% formic acid, Tarkow and Stamm[3] concluded that the reaction occurs only with the hydroxyl groups on C-4 and C-6. All early investigators[4-6] of the homogeneous reaction of starch with formic acid reported formation of a product that approximated a monoester, and indicated either exclusive formylation of the 6-hydroxyl groups or preferential esterification of these primary hydroxyl groups, together with some reaction at the secondary hydroxyl groups[7]. Wolff and co-workers[8] clarified the reversibility of the formylation, and the dependence of the degree of substitution (D.S.) upon the concentration of the formic acid. Employing buffered periodate oxidation, Moe and co-workers[5] showed that the minor fraction of the formate groups was on C-2 and C-3. Whistler and Roberts[9] measured approximately 70% of the total formyl groups as involving the primary, 6-hydroxyl group in the monoformyl derivative of D. S. 1. These results were obtained by acetylation of the monoformate to complete substitution, with subsequent selective hydrolysis of the formyl groups for measurement of the free primary hydroxyl groups by reaction with chlorotriphenylmethane (trityl chloride) or *p*-toluenesulfonyl chloride. By measuring the unreacted primary hydroxyl groups by tritylation, Fedorova and Rogovin[10] showed that approximately half of the formyl groups were on the C-6 oxygen atoms after homogeneous or heterogenous formylation of cellulose catalyzed by phosphoric acid.

The difference in equilibrium constants in the acetylation of primary and secondary alcohols has been recognized as applying to carbohydrates, where the selectivity of the esterification of the primary hydroxyl groups is a function of the temperature, the reagent, and the catalyst. Malm and co-workers[11], employing partially acetylated cellulose which dissolved in the reaction mixtures, established that conditions which accelerate the reaction lower the reactivity of primary hydroxyl groups, relative to those of the secondary hydroxyl groups, from 16 to as low as 2.5. Fedorova and Rogovin[10] conducted the acetylation of regenerated cellulose in a homogeneous reaction by employing phosphoric acid as the solvent; they reported that 51–52% of the ester groups were on the primary hydroxyl groups. From a study of the homogeneous reactions of cellulose secondary acetates with aqueous acetic acid, Hiller[12] found the acetylation of primary hydroxyl groups to be favored, with concurrent deacetylation at the secondary positions. He calculated equilibrium constants and second-order velocity-coefficients for these hydrolyses. No distinction was made between the reactivities of the secondary hydroxyl groups on C-2 and C-3 in the investigations noted above.

The purpose of the present study was to explore the kinetics of a "simplified", homogeneous system for esterification of the D-glucopyranosyl residues, for clarification of the specificity of esterification of the hydroxyl groups on C-2, C-3, and C-6. Ninety percent formic acid, which served as the carboxylic acid, the catalyst, and the solvent for the reaction, was employed with dextrin, a soluble polymer of low, molecular weight containing (1→4)-linked α-D-glucopyranose residues.

EXPERIMENTAL

Formylation.—White dextrin (reagent grade) was dried in *vacuo* over phosphorus pentaoxide for 24 h, and formylated with 90.8% formic acid (reagent grade, nominally 90%) at 30 ± 0.1°. The reaction was conducted with 100.0 ml of formic acid per g of dextrin, a mole ratio[13] of formic acid/D-glucosyl residue of 385:1. The formic acid was equilibrated at 30° prior to introduction of the dextrin, which dissolved during the first 5 min. The reaction was stopped by adding 300 ml of cold 3:1 (v/v) methanol–isopropyl alcohol, and the precipitate was removed by filtration, washed with methanol–isopropyl alcohol and then with ether, and dried for 1 h at 100—105° before being weighed. The extent of formylation was measured by the modified Eberstadt method[14] of saponification, and the results were expressed on the basis of the weight of dried, formylated product. The product was stored over phosphorus pentaoxide in a desiccator.

The degree of polymerization of dextrin was measured by the method of Meyer and Gibbons[15], employing 3,5-dinitrosalicylic acid for determination of the reducing equivalent. Maltose served as the standard. The average degree of polymerization was found to be 16.

Formylation data are expressed in terms of degree of reaction (D.R.), which is calculated to be 1.0 when all of the hydroxyl groups of the D-glucose residue are substituted (*i.e.*, D. S. 1/3), and are based on an average equivalent weight of 163.1 for the D-glucose residue.

Tritylation. — One-gram samples of formylated dextrin were tritylated under anhydrous conditions[16] in 10 ml of pyridine for 2 h at 115–120°. The trityl chloride (m.p. 111–112°) was prepared by the Friedel–Crafts reaction[17], and the ratio of moles of trityl chloride to equivalents of primary hydroxyl group was in the range of 1.5–7.0; since the fraction of free primary hydroxyl groups was an unknown, this required a preliminary tritylation to establish the approximate magnitude. The reaction mixture was diluted with 2 volumes af acetone, and the product was precipitated (in a Waring Blendor) with 25 volumes of 1:1 (v/v) methanol–ethanol. The product was filtered off, washed, dried for 1 h at 100–105°, and stored in a desiccator. The analyses for trityl group were performed by the method of Hearon[16,18]. A D. R. of trityl groups = 0.33 was realized for dextrin at a molar ratio of trityl chloride/primary hydroxyl group = 4.0. The trityl content of formylated dextrin (D. R. of formate = 0.44) showed dependency of trityl D. R. upon the proportion of reagent. Since the increment in D. R. of tritylation was a constant function of the molar ratio of trityl chloride to primary hydroxyl group for both dextrin and formylated dextrin, the approach was as follows: (*a*) enough trityl chloride was used to provide a molar ratio of trityl chloride to primary hydroxyl group within the range of 1.5–7, (*b*) the ratio was revised on the basis of the measured D. R. of tritylation, and (*c*) the experimental value of D. R. of tritylation was plotted *versus* the molar ratio of trityl chloride to primary hydroxyl group, and interpolated to the D. R. at the molar ratio of 4.0.

RESULTS

The formylation of dextrin proceeds readily with 90% formic acid at 30°, reaching equilibrium at a D. R. of 0.44 (see Fig. 1). The apparent equilibrium-constants (K and K′) from pseudo-first-order and second-order treatments of kinetics for the overall formylation are 0.79 and 0.23, respectively, expressed in concentration units.

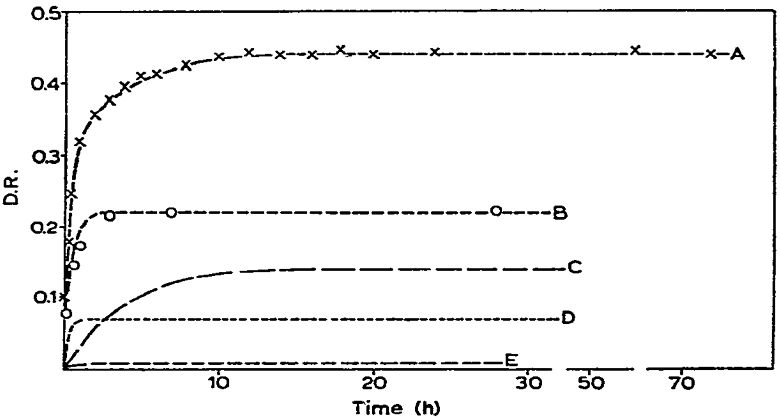

Fig. 1. Formylation of dextrin: X = total formylation: O = formylation of primary hydroxyl groups as measured by tritylation of the free primary hydroxyl groups; curve B is derived from the data of Fig. 2; curves C, D, and E represent kinetic analysis for individual, secondary hydroxyl groups.

As measured by tritylation of the free primary hydroxyl groups in the formylated dextrins, the formylation of the primary hydroxyl groups levels off rapidly at a D. R.

of 0.22. This portion of the reaction accounts for half of the total formylation, and is characterized by a K of 2.11. Fedorova and Rogovin[10] have indicated that neither loss nor redistribution of formyl (or acetyl) groups occurs under the conditions of tritylation.

Tritylation is a valuable, but not necessarily reliable, method for estimating the number of free primary hydroxyl groups; extensive studies have shown that the proportion of tritylation occurring at the primary hydroxyl group depends upon the conditions of reaction, the proportion of trityl chloride, and, possibly, even the nature of the compond; *i.e.*, tritylation is not specific for the primary hydroxyl group[18–20]. Since there is no basis on which to predict the deviation of the results, it is pertinent to compare our results on dextrin with those of Whistler and Roberts[9] on starch. The two carbohydrates differ only in molecular weight; Nickerson[6] has shown that they are formylated at similar, if not identical, rates. Whistler and Roberts[9] obtained measures of the fractions of primary hydroxyl group formylated from the direction opposite to that employed here: *i.e.*, they measured the primary hydroxyl groups which had originally been formylated. It is reasonable, therefore, to expect the true value to lie between the two sets of results, which are summarized in Fig. 2. The apparent fractions of formylated primary hydroxyl group measured from these different directions converge at the equilibrium D. R. which was reached in the formylation of dextrin. Below this D.R., considerable divergence is apparent. It is assumed that the midpoints between the two curves provide the best estimate of the fractions of primary hydroxyl group formylated in dextrin. These points were plotted, giving curve B of Fig. 1. This curve exhibits the characteristics of a pseudo-first-order reaction, yielding a pseudo-first-order rate-coefficient of 1.52 h^{-1} for the forward reaction. The equilibrium constant is unchanged from that noted above. That this portion of the bimolecular esterification reaction conforms to first-order kinetics is the consequence of such a massive excess of formic acid and water in the reagent that, for practical purposes, these

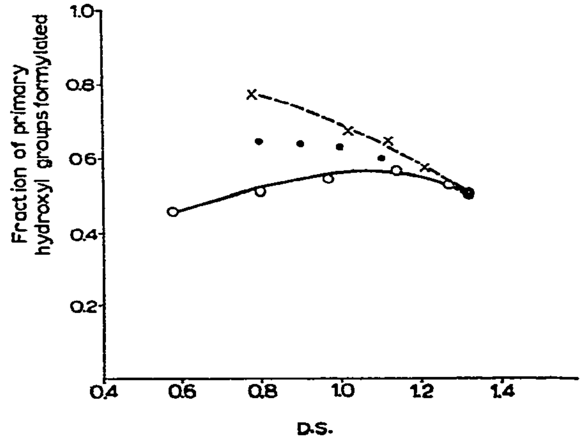

Fig. 2. Fraction of primary hydroxyl groups formylated, as a function of D. S.: ○ = data from this study; x = data of Whistler and Roberts[9]; • = midpoints.

components undergo no change in concentration throughout the course of the esterification. The reaction of each type of hydroxyl group in the D-glucosyl residues of dextrin is expected to appear as a pseudo-first-order reaction, although each is probably a bimolecular reaction involving a protonated carboxylate and the hydroxyl group[21].

The extent of formylation which appears attributable to the secondary hydroxyl groups (*i.e.*, the 2-and 3-hydroxyl groups of each D-glucopyranosyl residue plus the 4-hydroxyl group on the terminal D-glucopyranosyl group of each molecule) was calculated as the difference between total formylation and formylation of the primary hydroxyl group. Calculation of rate coefficients and apparent equilibrium-constants for the individual secondary hydroxyl group was made on the basis of simultaneous, independent, pseudo-first-order, reversible reaction. The total reaction at secondary hydroxyl groups, as defined above, was considered to be the sum of three components for the individual secondary hydroxyl groups, each of which is described by the differential equation,

$$-\,d[\mathrm{OH}]/d\theta = k_f\,[\mathrm{OH}] - k_b[\mathrm{F}]$$

where $[\mathrm{F}] = [\mathrm{OH}]_0 - [\mathrm{OH}]_t$, θ is time in h, k_f and k_b are the forward and backward rate-coefficients (expressed in concentration units), F and OH designate the formate and hydroxyl groups, and the subscripts refer to elapsed time.

An analog-computer analysis, using values determined earlier for the primary hydroxyl group, was performed by successive approximations to the experimental curve for total formylation. For this analysis, the differential equation was rearranged to the following expression:

$$-\,d[\mathrm{OH}]/d\theta = k_f\,(K[\mathrm{OH}] - [\mathrm{F}])/K$$

where K is the apparent equilibrium-constant from the pseudo-first-order treatment of the kinetics. Close duplication of the experimenal data was achieved, as is shown by the coincidence of curve A (from the computer analysis) with the data points, X,

TABLE I

KINETIC PARAMETERS FOR CURVES IN FIGURE I

Curve	*Apparent equilibrium-constant*[a]	*Apparent equilibrium-constant*[b]	*Velocity coefficient* (h^{-1})	*Hydroxyl group involved*
A	0.79	0.23	—	all
B	2.11	0.62	1.52	primary
C	0.75	0.22	0.12	secondary
D	0.29	0.085	0.96	secondary
E	0.48	0.14	1.59[c]	terminal 4-hydroxyl

[a] $K = \frac{[\mathrm{G{-}OCHO}]}{[\mathrm{G{-}OH}]}$; this K was employed in computer analysis. G is the D-glucopyranosyl residue.

[b] $K' = \frac{[\mathrm{G{-}OCHO}]\,[\mathrm{H_2O}]}{[\mathrm{G{-}OH}]\,[\mathrm{HCO_2H}]}$. [c] The value for this hydroxyl group cannot be considered meaningful, as the small proportion of this hydroxyl group permits large variations in the parameters without significant effect upon the total reaction-curve.

in Fig. 1. The approximation for the forward rate-coefficients and apparent equilibrium-constants of the secondary hydroxyl groups yielded curves C, D, and E. Only this one solution was obtainable from possible variations of parameters. For this computation, the relative, initial concentrations of hydroxyl groups were taken as 0.327 for each of the 2-, 3-, and 6-hydroxyl groups and 0.20 for the terminal 4-hydroxyl group. The sum of curves E, D, C, and B is shown as curve A; it is, essentially, the best curve passing through the experimental data. The rate coefficients and apparent equilibrium-constants which describe the curves of Fig. 1 are summarized in Table I.

Attempts to examine the kinetics of formylation of the secondary hydroxyl groups by protecting the primary hydroxyl group with a trityl group were unsuccessful, owing to cleavage of the trityl ether linkage during formylation. Likewise, hydrolysis of the benzylidene group occurred during formylation of the free 2- and 3-hydroxyl groups of methyl 4,6-*O*-benzylidene-α-D-glucopyranoside with 90% formic acid at 30°.

DISCUSSION

The parameters estimated (and summarized in Table 1) as characterizing the reactivities of the individual types of hydroxyl groups in the D-glucopyranosyl residues of dextrin clearly establish that esterification at the primary hydroxyl group is thermodynamically favored. The ratio of the equilibrium constants for esterification of the primary and the two secondary hydroxyl groups is 1.0/0.36/0.14, indicating significant differences between the individual secondary hydroxyl groups. The ratios of K_I/K_{II} for the hydroxyl groups of dextrin lie in a range generally comparable to those for simple primary alcohols (methanol, ethanol) as compared to secondary alcohols (such as isopropyl alcohol); *i.e.*, from[22] 1.0/0.27 to 1.0/0.45.

The primary hydroxyl group undergoes esterification more rapidly than either of the secondary hydroxyl groups (on C-2 or C-3). The ratio of apparent velocity-coefficients for esterification of the primary and two secondary hydroxyl groups of dextrin is 1.0/0.08/0.63, as compared to the corresponding ratio of 1.0/0.26 to 1.0/0.30 for k_I/k_{II} for a simple primary–secondary alcohol pair (ethanol/isopropyl alcohol)[23]. The ratio of velocity coefficients for the primary and combined secondary hydroxyl groups calculated by Hiller[12] for the esterification of partially acetylated celluloses with aqueous acetic acid, over a range of temperatures, was 1.0/0.03 to 1.0/0.13. It is evident that the kinetic analysis of the formylation of dextrin indicates that the two secondary hydroxyl groups are quite different in reactivity, and that the ratios of k_I/k_{II} for the hydroxyl groups of dextrin lie beyond the range indicated for k_I/k_{II} from data given in preceding studies of simple primary–secondary alcohols.

The kinetic analysis of the reaction at the secondary hydroxyl group indicates, qualitatively, the presence of one secondary hydroxyl group that reacts rapidly to a low level of equilibrium, and another secondary hydroxyl group that reacts slowly to a higher level of equilibrium. As far as we are aware, no experimental means is currently available for verifying this hypothesis, or for identifying the relationship between the hydroxyl groups at C-2 and C-3 and curves C and D of Fig. 1; however, it is pertinent to note that a consideration of the mechanism of esterification and the behavior of certain D-glucose derivatives suggests that it is the hydroxyl group at C-2 that undergoes the more rapid reaction to the lower level of equilibrium.

A significant difference between the hydroxyl groups at C-2 and C-3 develops from the lower inductive effect transmitted to the hydroxyl group at C-3 from the oxygen atoms at C-1. Thus, the hydroxyl group at C-2 is the more acidic, and the average state of its proton may be envisaged as incipient ionization toward the more nucleophilic, 3-hydroxyl group. The higher protonation of the 3-hydroxyl group may be accentuated in the formic acid, which is strongly acidic. As esterification involves the addition of the unprotonated alcohol to the protonated carboxylic acid[21,24], the reaction of the hydroxyl group at C-2 would be expected to occur more rapidly than that at other secondary hydroxyl groups. It follows, also, that the ester group at C-2 should be characterized by the more electrophilic carbonyl carbon atom which should be less stable toward nucleophilic attack and to removal of the ester group. Susceptibility at this position to preferential hydrolysis in an acidic medium is less predictable. Preferential esterification of D-glucose derivatives is indicated as occurring at the hydroxyl group at C-2 (not C-3) for benzoylation[25] and for (benzylthiocarbonyl)ation[26]. Ester groups on C-2 of D-glucose derivatives exhibit a facility for selective removal which also is consistent with the foregoing analysis[25,27,28].

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The authors are indebted to Dr. R. H. Barker for helpful discussions and suggestions.

SUMMARY

The kinetics of the homogeneous formylation of dextrin with 90% formic acid have been analyzed as the composite of four, simultaneous, independent, reversible reactions at the C-2, C-3, and C-6 hydroxyl groups of each D-glucopyranosyl residue and the C-4 hydroxyl group on the terminal D-glucopyranosyl group of each molecule. The velocity coefficient and the equilibrium constant for the primary hydroxyl group are higher than the values for the (secondary) C-2 and C-3 hydroxyl groups. Results of the kinetic analysis suggest that one secondary hydroxyl group is esterified rapidly to a low level of equilibrium, whereas the other secondary hydroxyl group is slowly esterified to a higher level of equilibrium. These results are shown to be qualitatively consistent with the behavior of other carbohydrates on esterification or de-esterification.

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IMPROVEMENTS IN THE PRODUCTION AND ISOLATION OF COLOMINIC ACID

S. A. BARKER, R. G. JONES, AND P. J. SOMERS
Department of Chemistry, The University, Birmingham 15 (Great Britain)

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INTRODUCTION

Colominic acid, a polymer of *N*-acetylneuraminic acid, has been obtained[1-7] from several strains of *Escherichia coli*. A common feature of the published methods of isolation is that they utilise nutrient media which contain polysaccharides or proteins (of yeast, casamino acids, and agar) rendering subsequent purification of the colominic acid difficult. This study shows that colominic acid can be produced in improved yield using a much cheaper and simpler fermentation medium. Furthermore, the purification of the colominic acid from the culture filtrate is assisted because the particular strain [016 (N.C.T.C. 9016)] of *E. coli* employed yields the polymer in the ionized rather than in the lactone form which permits direct precipitation by detergents without prior alkaline hydrolysis.

METHODS

Assays for *N*-acetylneuraminic acid were carried out by Warren procedure[8].

Optimum conditions for colominic acid production. — The concentration of some of the components of a synthetic medium consisting of glycine (0.5 g), $(NH_4)_2SO_4$ (0.2 g), $MgSO_4 . 7H_2O$ (0.01 g), K_2HPO_4 (0.2 g) and KH_2PO_4 (0.1 g) in 90 ml of water were varied whilst those of the others were kept constant in a series of experiments to determine their relative importance in colominic acid production. After sterilisation by autoclaving, sterile glucose solution (5%, 10 ml) was added. A subculture of *E. coli* O 16 (N.C.T.C. 9016) was made from a nutrient broth agar slope into the standard glucose medium and 5 ml of this actively growing culture was inoculated after 17 h into each of the various growth media (50 ml) which were incubated and shaken at 37° for 10 h. Aliquot parts (3 ml) were removed at intervals and analysed. The optical density (OD) at 650 mμ using a 10 mm path length was determined, the cells were removed by centrifugation, and an aliquot part (1 ml) was hydrolysed with 0.1N HCl for 1 h at 80°. Aliquot parts (0.2 ml) of the supernatant were analysed[8] for *N*-acetylneuraminic acid (NANA).

A similar experiment was carried out with variation of some of the constituents of a medium consisting of succinic acid (0.6 g), ammonium sulphate (0.2 g), glycine (0.01 g), L-glutamic acid (0.025 g), magnesium sulphate (0.02 g), ferrous sulphate

(0.5 mg), and potassium dihydrogen phosphate (0.47 g) adjusted to pH 5.5 with potassium hydroxide and diluted to 100 ml; the results are given in Table I. If acetic acid (0.6 g/100 ml) was substituted for succinic acid (0.6 g/100 ml), there was no measurable growth whereas if citric acid (0.6 g/100 ml) was used the O.D.$_{650\,\mathrm{m}\mu}$ reached only 0.19 and there was no detectable colominic acid in the supernatant. Addition of 1 ml of a vitamin solution containing nicotinic acid (1 mg), aneurine hydrochloride (1 mg), biotin (10 μg), and *p*-aminobenzoic acid (1 mg) to 50 ml of the succinate medium gave a maximum growth corresponding to O.D.$_{650\,\mathrm{m}\mu}$ 4.02 (control 4.17) and a maximum NANA/ml concentration of 222 μg (control 231 μg). If ferrous sulphate was omitted, the growth was slowed so that the maximum production of colominic acid (231 μg NANA/ml in both cases) was at 8 h instead of 7 h; the maximum growth, corresponding to O.D.$_{650\,\mathrm{m}\mu}$ 3.90, was slightly less than when ferrous sulphate was present (4.17). The addition of zinc acetate (0.006 g/100 ml) enhanced growth in the early stages but retarded it in the later stages (8h, O.D.$_{650\,\mathrm{m}\mu}$ 3.78, control 3.91). However, production of colominic acid was much impaired, being 166 μg NANA/ml (control 206 μg, both after 8 h). Calcium chloride (1 mg/100 ml) had no effect on cell growth or colominic acid production.

TABLE I

COLOMINIC ACID PRODUCTION WITH VARIOUS GLUCOSE AND SUCCINIC ACID MEDIA

Glucose media						
Glycine concentration (g)	0	0.1	0.2	0.3	0.4	0.5
Maximum growth (O.D.$_{650\,\mathrm{m}\mu}$)	1.62	1.90	1.73	1.99	2.43	2.40
Maximum titre (NANA μg/ml)	54	64	62	82	94	89
Ammonium sulphate (g)	0	0.01	0.02	0.05	0.1	0.2
Maximum growth (O.D.$_{650\,\mathrm{m}\mu}$)	2.02	2.21	2.69	2.83	2.80	3.08
Maximum titre (NANA μg/ml)	78	73	81	87	92	98
Dipotassium hydrogen phosphate (g)	0.2	0.4	0.6	1.0	2.0	4.0
Potassium dihydrogen phosphate (g)	0.1	0.2	0.3	0.5	1.0	2.0
Maximum growth (O.D.$_{650\,\mathrm{m}\mu}$)	3.30	2.75	3.05	3.63	2.75	1.25
Maximum titre (NANA μg/ml)	97	82	84	72	52	52
Succinic acid media						
Succinic acid (g)	0.1	0.2	0.3	0.4	0.5	0.6
Maximum growth (O.D.$_{650\,\mathrm{m}\mu}$)	1.01	1.60	2.1	2.87	3.64	4.12
Maximum titre (NANA μg/ml)	35	62	106	141	184	201
Glutamic acid (g)	0	0.01	0.025	0.05		
Maximum growth (O.D.$_{650\,\mathrm{m}\mu}$)	3.68	3.85	4.12	4.04		
Maximum titre (NANA μg/ml)	171	185	202	203		

When the succinate medium was adjusted to different initial pH values, the level of colominic acid production after 9 h in μg NANA/ml was 198 (pH 5.5), 149 (pH 6.0), and 122 (pH 6.5). Using the succinate media (pH 5.5), maximum production of colominic acid was much lower at 27° (72 μg NANA/ml) than at 37° (195 μg NANA/ml).

If, in the succinate medium, half of the succinate (0.3 g/100 ml) was replaced by glucose (0.3 g/100 ml) the maximum colominic acid titre was lowered from 189 μg to 92 μg NANA/ml. All hydrolyses involving succinate medium were in 0.1N HCl for 2 h at 85°.

Optimum conditions for colominic acid precipitation. — A culture of *E. coli* O 16 grown on the standard glucose medium to maximum growth was centrifuged and the supernatant adjusted to pH 7 with KOH (0.5N). Additions of benzylcetyldimethylammonium chloride (2%) were made to aliquots (1 ml) of the supernatant and the extent of precipitation was determined from a constant total volume of 2 ml at 25°. After 30 min, the precipitate was removed by centrifugation and resuspended in HCl (0.1N, 2 ml). The NANA contents[8] of the original supernatant and the resuspended precipitate were determined after acid hydrolysis (0.1N HCl, 80°, 1 h); the results are given in Table II.

TABLE II

EFFECT OF DETERGENT CONCENTRATION ON COLOMINIC ACID PRECIPITATION

Detergent (%)	*NANA ex precipitate (μg/ml)*	*NANA ex supernatant (μg/ml)*	*Precipitation of colominic acid (%)*
0	0	47.1	0
0.02	6.7	40.4	14.3
0.04	39.5	8.2	83.1
0.06	41.4	6.5	86.2
0.08	38.5	8.8	81.5
0.10	32.1	12.5	72.0
0.12	31.2	15.9	66.2
0.14	26.3	21.1	55.5
0.16	19.2	26.9	41.6
0.18	18.3	27.1	39.4
0.20	15.8	31.7	33.2

Conditions for dissociation of the detergent–colominic acid complex. — Culture filtrate (10 l) of *E. coli* O 16 prepared using the standard glucose medium was neutralised to pH 7 with 2N KOH. An aqueous solution of benzylcetyldimethylammonium chloride (2%, 256 ml) was added with stirring to 8.6 l of the neutral culture filtrate. A sample (500 ml) was removed from the suspension after 30 min and the precipitate was recovered by centrifugation (3000 r.p.m., 30 min) the rest being recovered separately.

The sample precipitate was dispersed in M NaCl (5 ml) by vigorous stirring. After 30 min, a red-purple precipitate (P_1) and a clear purple supernatant (S_1) were obtained by centrifugation (5000 r.p.m., 60 min). The precipitate P_1 was resuspended in M NaCl (5 ml) and a second precipitate (P_2) and supernatant (S_2) were recovered. The precipitate P_2 was resuspended in M NaCl. Aliquots (0.25 ml) of the supernant and resuspended precipitates were diluted to 5 ml with H_2SO_4 (0.1N). After hydrolysis at 80° for 1 h, and neutralization with N NaOH, aliquot parts (0.2 ml) were assayed for

NANA: P_1, 4 mg; S_1, 36.4 mg; P_2, 1.4 mg; S_2 4.2 mg. Benzylcetyldimethylammonium chloride exhibits four peaks in the range 250–275 mμ which were also shown by P_1 and P_2 but not by S_1 or S_2.

Isolation of colominic acid. — The detergent precipitate corresponding to 8 l of culture filtrate from the previous experiment was suspended in M NaCl (50 ml) and vigorously stirred. The bulk of the insoluble matter was removed by centrifugation and the supernatant was further clarified on a sintered glass filter No. 5. The clear filtrate (2 × 25 ml) was fractionated (Fig. 1) on Sephadex G-25 (250 ml, 2.54 × 50 cm) by elution with water and the colominic acid fractions were passed, without concentration, down a column of CM-Sephadex (H^+, 44 ml, 1.5 × 25 cm). All of the colominic

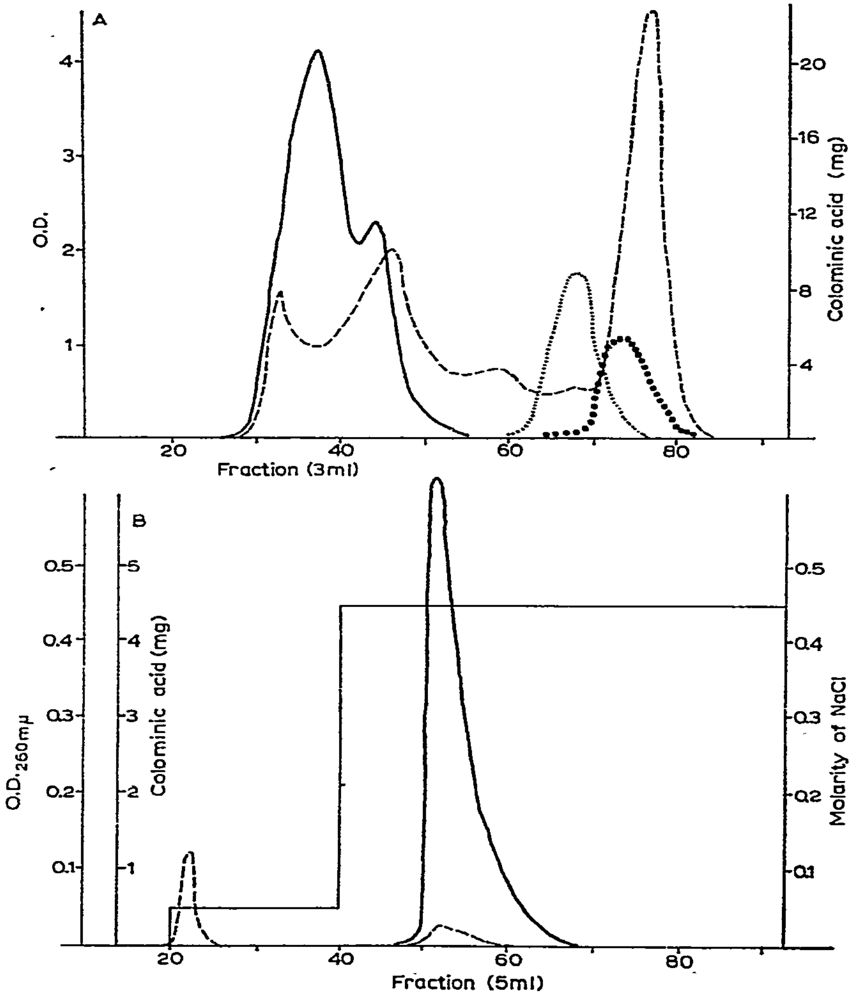

Fig. 1. Purification of colominic acid on (A) Sephadex G-25 and (B) DEAE Sephadex. ——— Colominic acid, – – – – O.D. 260 mμ, ... O.D. 570 mμ, in ninhydrin assay, •••• O.D. 570 mμ, without treatment.

acid was eluted with water and this solution, after concentration to 5 ml, was applied to DEAE Sephadex A-50 (Cl^-, 100 ml, 2 × 32 cm). After a water wash (100 ml), elution was carried out with a stepwise gradient of NaCl solution (0.05M, 200 ml, 0.45M, 250 ml). Analysis of the resulting fractions (Fig. 1) located colominic acid in fractions 50–70 which, after concentration to 25 ml, were desalted on Sephadex G-25 and freeze-dried to a white powder (150 mg).

Characterisation of colominic acid.—A solution of colominic acid (17.6 mg; 11.3% moisture, from *E. coli* O 16) in 0.1N HCl (200 ml) was divided into two portions one being hydrolysed at 80° and the other at 85°. A standard NANA solution (99 µg/ml, 0.1N HCl) was also heated at 85°. The results of assays for NANA are given in Table III.

TABLE III

ACID HYDROLYSIS OF COLOMINIC ACID

Colominic acid O 16		*Colominic acid 235*		*Colominic acid O 16*		*NANA*
Time (min)	*NANA (µg/ml) liberated 80°*	*Time (min)*	*NANA (µg/ml) liberated 80°*	*Time (min)*	*NANA (µg/ml) liberated 85°*	*(µg/ml) 85°*
0	0	0	4.3	0	0	99
11	5.0	10	3.5	60	31.0	90.3
20	7.3	20	13.8	120	48.1	86.5
33	11.9	36	18.7	180	54.0	78.7
57	23.4	45	25.1	240	55.6	72
70	26.8	60	34.7	300	51.1	65.5
90	31.8	90	36.5			
102	37.5	120	35.6			
122	41.5					

After correcting for moisture content and destruction of NANA at 85° in 0.1N HCl, the colominic acid was found to contain 92.6% of anhydroNANA assuming no internal esterification. Results for the hydrolysis of colominic acid from *E. coli* K 235 L + O (40 µg per 1.05 ml 0.1N HCl) heated at 80° are presented for comparison. The isolation of crystalline NANA from a larger scale hydrolysis using conventional procedures[9] gave a product having $[\alpha]_D^{21}$ −33.3° (*c* 1.0, water) (Found: C, 42.55; H, 6.20; N, 4.37 $C_{11}H_{19}NO_9$ calc.: C, 42.74; H, 6.19; N, 4.53%).

An aqueous solution of colominic acid (50 mg/5 ml) from *E. coli* K 235 L + O was prepared and its optical rotation determined. The solution was rapidly titrated to, and maintained at, pH 11.0 with NaOH (2N) and the optical rotation redetermined immediately and at intervals. The specific rotations were corrected for the dilution. A parallel experiment was performed with an aqueous solution of the colominic acid (47.9 mg/5 ml) isolated from *E. coli* O 16 (See Table IV).

The rate of sedimentation in a solution of colominic acid (0.947%) was made in phosphate buffer (pH 7.0, 0.1 M) at 59,780 r.p.m. and determined in a Spinco Model E Ultracentrifuge. The sedimentation coefficient was S_W^{20} 1.26 ±0.01 Svedberg units.

A graph of the boundary sedimentation distance against time was best fitted by a linear relationship.

TABLE IV

EFFECT OF ALKALI ON THE OPTICAL ROTATION OF COLOMINIC ACID

Colominic acid source	*Aqueous solution*	*Time (min) at pH 11*					
		1	*10*	*30*	*60*	*240*	*1440*
E. coli K 235 L + O	−56.7	− 8.2	+ 8.5	+10.3	+10.9	+10.8	+10.5
E. coli O 16	+13.4	+13.4	+13.4	—	+13.5	+13.4	+13.3

DISCUSSION

Several media composed of natural constituents have been described for the production of colominic acid in *E. coli* cultures. Barry and Goebel[1] used a medium composed of casamino acids, glucose, yeast extract, disodium hydrogen phosphate, and potassium dihydrogen phosphate maintained at pH 7 during fermentation with the continuous addition of alkali. Later[2], dialysates of the casamino acids and yeast extract were used in the same medium. Kimura and Turimi[6] used solid media containing yeast extract, peptone, and agar in addition to mineral salts. All these procedures entail cumbersome and elaborate methods in the preparation of the medium.

A preliminary experiment showed that, using *E. coli* O group 16 (N.C.T.C. 9016) and the standard glucose–mineral salt medium previously described, as much, if not more, colominic acid (95 μg NANA/ml) was produced than in a typical natural medium (84.5 μg NANA/ml) composed of glycine (0.5 g), $MgSO_4 \cdot 7H_2O$ (0.01 g), KH_2PO_4 (0.1 g), K_2HPO_4 (0.2 g), yeast extract (0.2 g), and peptone (1 g) in 100 ml. Maximum growth, as indicated by optical density, was considerably greater in the synthetic medium 3.01 (natural 1.78). It was of interest that the pH rose in the natural medium (from 6.6 to 8.35) and fell in the synthetic (from 6.4 to 3.9).

The relative importance of the constituents of the glucose–mineral salt media was assessed in a series of experiments. Increasing the glycine concentration from 0 to 0.5 g/100 ml increased colominic acid production by *ca.* 35%. Increasing the ammonium sulphate concentration from 0 to 0.2 g/100 ml increased growth by 33% and colominic acid production by 25%. The effect of glycine and ammonium sulphate may be interrelated and is more complex than if they acted solely as nitrogen sources; their effect on the pH of the medium as growth proceeds is also important.

In view of the apparent rapid pH change and the use of pH control by Barry and Goebel[1], an investigation of the effect of increasing the medium buffering capacity was made. Cell growth was decreased at higher concentrations of phosphate buffer although, at the highest levels, logarithmic growth was still proceeding. Colominic acid production was not increased at the higher levels of phosphate buffer.

A major improvement was effected by using succinic acid as the carbon source instead of glucose. In a direct comparison of the glucose–mineral salt medium and succinate–mineral salt medium previously described and using the same conditions of hydrolysis to assay the colominic acid, the former gave a maximum of 90 μg compared with 206 μg NANA/ml with the latter.

Surprisingly, the colominic acid produced in the culture medium could be precipitated with benzylcetyldimethylammonium chloride without pretreatment with alkali. The colominic acid from *E. coli* K 235 L + O, kindly supplied by Dr. Goebel, was in the lactone form and did not precipitate until after incubation with alkali at room temperature. It was noteworthy that the lactone form ($[\alpha]_D$ −56.7°) of Goebel's colominic acid, after such incubation, showed almost the same rotation (+10.5°) as that (+13.3°) of the colominic acid described herein.

The most economical way of removing the detergent from the colominic acid complex was to extract the precipitate with M NaCl. In this way, most (99%) of the detergent remained insoluble. Fractionation on Sephadex G-25, Carboxymethyl Sephadex, and DEAE Sephadex sufficed to provide highly purified colominic acid which, significantly, was more stable to acid than was the lactone form. In assessing the NANA content, the importance of making a correction for the destruction of NANA under the same conditions was illustrated. Examination of the polymer in the ultracentrifuge gave a sedimentation constant of S_w^{20} 1.26 which for an ideal spherical molecule represents a molecular weight of 10,000–12,000. Analysis by the Archibald approach-to-equilibrium method gave a molecular weight of 9,000–10,000.

ACKNOWLEDGMENTS

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SUMMARY

A novel synthetic medium has been devised for producing high yields of colominic acid from *E. coli* O 16. Precipitation with a cationic detergent provided a rapid method of isolation, and subsequent dissociation of the complex with NaCl followed by gel filtration and anion exchange fractionation resulted in a preparation with $[\alpha]_D$ +13° and a molecular weight of 10,000–12,000, containing at least 91% of *N*-acetylneuraminic acid. This colominic acid has been shown to be devoid of internal ester linkages in contrast to previous preparations.

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SYNTHESIS AND PROPERTIES OF SOME SERINE GLYCOSIDES

V. A. DEREVITSKAYA, M. G. VAFINA, AND N. K. KOCHETKOV
Institute for Chemistry of Natural Products, U.S.S.R. Academy of Sciences, Moscow (U.S.S.R.)

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INTRODUCTION

Recent investigations of glycoproteins clearly demonstrate that fragments containing *O*-glycosylated hydroxy amino-acids play an important role in the structure of these biopolymers. Glycosidic bonding between a carbohydrate moiety and the serine and/or threonine residues of peptide chains has been found in mucins of submaxillary glands[1], protein–chondroitin complexes[2], blood-group substances[3], and some other glycoproteins. A detailed study of the properties of this type of linkage, particularly the stability under different conditions, using simple model compounds, is desirable. The *O*-2-amino-2-deoxyglucoside[4,5] and the *O*-xyloside[6] of serine have been synthesized, but detailed data on their properties have not yet been published. We now report on the synthesis and properties of a number of serine glycosides. The compounds selected were derivatives of D-glucopyranosyl-, D-galactopyranosyl-, and D-galactofuranosyl-serine.

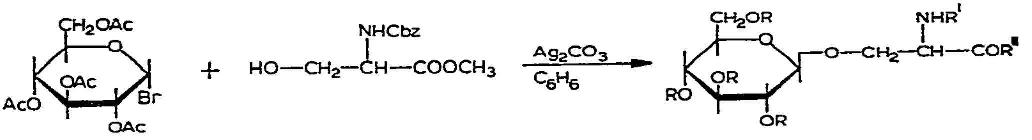
RESULTS AND DISCUSSION

N-Benzyloxycarbonyl-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-L-serine methyl ester (**1a**) was obtained in approximately 40% yield by Königs–Knorr glycosylation of *N*-benzyloxycarbonyl-serine methyl ester in benzene using silver carbonate as the hydrogen bromide acceptor. Glycosylation in the presence of lead carbonate resulted only in the formation of the orthoacetate (**2**).

No racemisation took place during the Königs-Knorr reaction since only L-serine and methyl D-glucoside were detected after methanolysis of compound (**1a**).

The derivative of D-serine (D-**1a**) was prepared (10% yield) by the Königs–Knorr glycosylation of *N*-benzyloxycarbonyl-DL-serine with subsequent separation of the diastereoisomers by crystallization. Due to the high lability in alkali of the glycosidic bonds in compounds (**1a**) and (D-**1a**), the successful deacetylation of the tetraacetates is possible only under mild conditions. The best results were achieved by the treatment of compounds (**1a**) and (D-**1a**) with triethylamine in methanol at room temperature. *N*-Benzyloxycarbonyl-*O*-(β-D-glucopyranosyl)-L-serine methyl ester (**1b**) and its D-isomer (D-**1b**) were thus obtained in high yields. Deacetylation by methanolic barium methoxide gave lower yields.

The stereospecificity of the glycosylation was established when compound (**1b**), incubated with emulsin in standard conditions, underwent complete cleavage. No trace of α anomer was detected.



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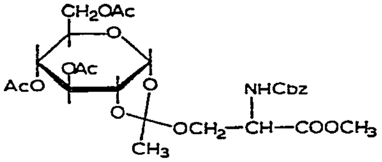


1a R = Ac;R' = Cbz;R" = OCH_3
1b R = H;R' = Cbz;R" = OCH_3
1c R = H;R' = H;R" = OCH_3
1d R = H;R' = H;R" = OH
1e R = H;R' = Cbz;R" = $NHCH_3$
1f R = H;R' = H;R" = $NHCH_3$
1g R = H;R' = $COCH_2NHCbz$;R" = $NHCH_3$
1h R = H;R' = $COCH_2NH_2$;R" = $NHCH_3$
1i R = H;R' = Cbz;R" = OH
1j R = H;R' = Cbz;R" = $NHNH_2$

The elimination of the benzyloxycarbonyl group from compound (**1b**) to give *O*-(β-D-glucopyranosyl)-L-serine methyl ester (**1c**) was carried out by hydrogenolysis in the presence of palladium–barium sulphate in 50% aqueous methanol containing an equivalent of acid to prevent[7] possible destruction of the product. Usually the hydrogenolysis was performed in the presence of oxalic acid because of the more convenient properties of the products. Hydrochloric acid was also used. Since an unprotected amino group exerts a slight stabilising influence on *O*-glycosidic bonds as compared with the *N*-benzyloxycarbonyl derivative, hydrolysis of the ester grouping of compound (**1c**) proceeds smoothly on treatment with alkali in aqueous methanol giving rise to *O*-(β-D-glucopyranosyl)-L-serine (**1d**) in high yield.

The simplest route to *N*-benzyloxycarbonyl-*O*-(β-D-glucopyranosyl)-L-serine methylamide (**1e**), a model peptide of serine, is the aminolysis of compound (**1a**) by the action of methylamine in methanol; simultaneous deacetylation takes place. Direct glycosylation of *N*-benzyloxycarbonyl-L-serine methylamide gave a less satisfactory result. *O*-(β-D-Glucopyranosyl)-L-serine methylamide (**1f**) was obtained by hydrogenolysis of compound (**1e**) under standard conditions.

The use of compound (**1f**) as a starting material in peptide synthesis gave structurally closer models for the relevant fragment of natural glycoproteins. By condensation of compound (**1f**) with *N*-benzyloxycarbonyl-glycine in the presence of dicyclohexylcarbodiimide (DCC), under conditions[7] which exclude aminoacylation of the unprotected hydroxyl groups, *N'*-benzyloxycarbonyl-*O*-(β-D-glucopyranosyl)-*N*-glycyl-L-serine methylamide (**1g**) was obtained, which when subjected to hydrogenolysis in the presence of oxalic acid, gave *O*-(β-D-glucopyranosyl)-*N*-glycyl-L-serine methylamide (**1h**), isolated as the crystalline oxalate.

In order to investigate the influence of the free carboxyl group of a serine derivative with a protected amino group, the synthesis of *N*-benzyloxycarbonyl-*O*-(β-D-glucopyranosyl)-L-serine (**1i**) was undertaken. The preparation of compound (**1i**) by hydrolysis of the methyl ester (**1b**) is difficult, since the stabilities of ester groupings and the *O*-glycosidic bond under standard conditions of hydrolysis are similar. Incubation of compound (**1b**) at pH 8–9 results in complete cleavage of the *O*-glycosidic bond and only a very small amount of compound (**1i**) was obtained. However, by brief treatment of compound (**1b**) with 0.5N sodium hydroxide, a rapid hydrolysis of the ester group took place, thus stabilising the *O*-glycosidic bond and affording compound (**1i**) in 40% yield.

Attempts to prepare compound (**1i**) *via* *N*-benzyloxycarbonyl-*O*-glucosyl-L-serine hydrazide (**1j**) failed. The hydrazide (**1j**) was obtained by hydrazinolysis of the methyl ester (**1b**). Treatment of compound (**1j**) with *N*-bromosuccinimide[8] gave only a small amount of compound (**1i**). The main product of the reaction was a neutral substance with an i.r. band at 1775 cm^{-1} characteristic of a lactone, and which consumed 1 mol. of periodate. Reduction of the oxidation products with sodium borohydride and subsequent acid hydrolysis and repeated reduction gave only glycerol and serine. These data accord with the seven-membered lactone structure (**3**).

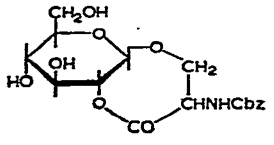
The sequence of reactions described above was used for syntheses of some galactopyranosides of serine. Starting from *N*-benzyloxycarbonyl-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-L-serine methyl ester (**4a**), *N*-benzyloxycarbonyl-*O*-galactopyranosyl-L-serine methylamide (**4b**), *O*-galactopyranosyl-L-serine methylamide (**4c**), and *N*-benzyloxycarbonyl-*O*-galactopyranosyl-*N*-glycyl-L-serine methylamide (**4d**) were prepared.

The preparation of *O*-galactofuranosides of serine for a study of the influence of ring size on *O*-glycoside stability was achieved by application of a new method[9] of glycosylation by orthoesters, since the appropriate acetobromo-D-galactofuranoses are not accessible. *N*-Benzyloxycarbonyl-*O*-(2,3,5,6-tetra-*O*-acetyl-β-D-galactofuranosyl)-L-serine methyl ester (**5a**) was obtained by glycosylation of *N*-benzyloxycarbonyl-L-serine methyl ester with 3,5,6-tri-*O*-acetyl-D-galactofuranose 1,2-(methyl orthoacetate) (**6**) in boiling nitromethane in the presence of a catalytic amount of mercuric bromide.

Deacetylation of compound (**5a**) with triethylamine in methanol led to *N*-benzyloxycarbonyl-*O*-(β-D-galactofuranosyl)-L-serine methyl ester (**5b**) which was hydrogenolysed to *O*-(β-D-galactofuranosyl)-L-serine methyl ester, isolated as the oxalate (**5c**).

Stability of the glycosidic bond in serine glycosides. — *(a) In acid media.* Preliminary experiments showed that the cleavage of the serine glycosides in acid media is normal and results in the formation of monosaccharides and serine derivatives.

The rate of hydrolysis was ascertained by paper-chromatographic determination of the liberated monosaccharide.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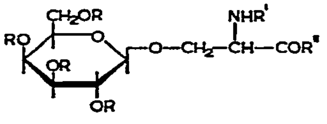
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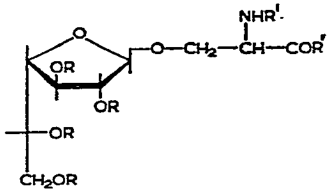
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(4a) R = Ac;R′ = Cbz;R″ = OCH_3
(4b) R = H;R′ = Cbz;R″ = $NHCH_3$
(4c) R = H;R′ = H;R″ = $NHCH_3$
(4d) R = H;R′ = $COCH_2NHCbz$;R″ = $NHCH_3$
(5a) R = Ac;R′ = Cbz;R″ = OCH_3
(5b) R = H;R′ = Cbz;R″ = OCH_3
(5c) R = H;R′ = H;R″ = OCH_3

Treatment of serine glycosides at pH 1–7 and 37° showed that the glycosidic bond is completely stable during 24 h; no trace of monosaccharide could be detected. Hence, the stability of the glycosidic bond under these conditions corresponds to the stability normally expected for glycosides. Under more drastic conditions (100°) substantial hydrolysis occurs. Data on the cleavage of the serine glycosides at pH 1.5 and in 0.4N hydrochloric acid (Table I) show these glycosides to be slightly more stable in acid medium as compared with other glycosides (see data for methyl β-D-glucopyranoside in Table I), but this difference is insignificant. The glycoside of serine methyl ester (**1b**) is a little more labile than is the methylamide derivative (**1e**), although the influence of a free carboxylic function seems to be rather small. The influence of substituents on the amino group of the serine moiety on the stability is more significant; the stability of the glycosidic bond increases in the series: $NHCbz < NHCOCH_2NH_2 \leqslant NH_2$. Galactopyranosyl-serines are less stable in acidic media than are the glucopyranosides, and the galactofuranosides, as expected, are much more labile.

(*b*) *In alkaline media.* Preliminary experiments showed that glycosides of serine are cleaved in alkaline media with the formation of monosaccharides and α-aminoacrylic acid derivatives. The latter compound was isolated in the case of *N*-benzyloxycarbonyl-*O*-D-glucopyranosyl-L-serine methyl ester (**1b**) and identified by comparison with *N*-benzyloxycarbonyl-α-aminoacrylic acid prepared from the *O*-toluene-*p*-sulphonate of *N*-benzyloxycarbonyl-L-serine methyl ester. Hence, in alkaline media glycosides of serine undergo β-elimination.

For the quantitative determination of glycosidic bond stability in an alkaline medium, the amount of monosaccharide released or α-aminoacrylic acid derivative formed may be ascertained. The data in Table II show that the stability of *O*-glycosides of *N*-benzyloxycarbonyl-L-serine increases in the series: methyl ester (**1b**) < methylamide (**1e**) < acid (**1i**) as expected from the ease of β-elimination. The cleavage of the

TABLE I

STABILITY OF GLYCOSIDES OF L-SERINE DERIVATIVES IN ACID AT 100°

Substance	*Hydrolysis* (%)		
	0.4N *HCl*		*pH 1.5*
	3h	*6h*	*24 h*
β-D-Glucopyranosides of:			
N-benzyloxycarbonyl-L-serine methyl ester (**1b**)		94	43
N-benzyloxycarbonyl-L-serine methylamide (**1c**)	56	85	35
N-benzyloxycarbonyl-L-serine (**1i**)		86	23
L-serine methylamide (**1f**)	48	65	stable
L-serine (**1d**)		50	stable
N'-benzyloxycarbonyl-*N*-glycyl-L-serine methylamide (**1g**)	75	93	49
N-glycyl-L-serine methylamide (**1h**)		77	27
Methyl β-D-glucopyranoside		100	
β-D-Galactopyranosides of:			
N-benzyloxycarbonyl-L-serine methylamide (**4b**)	74		49
L-serine methylamide (**4c**)	54		34
N'-benzyloxycarbonyl-*N*-glycyl-L-serine methylamide (**4d**)	90		73
β-D-Galactofuranoside of L-serine methyl ester (**5c**)	48[a]		100

[a] At 37°.

TABLE II

INFLUENCE OF SUBSTITUENTS AT THE CARBOXYLIC GROUP OF O-GLUCOSYL-L-SERINE DERIVATIVES ON STABILITY IN ALKALINE MEDIA (37°, 24 h)

Substance	*pH*	*Degradation* (%)
β-D-Glucopyranosides of:		
N-benzyloxycarbonyl-L-serine methyl ester (**1b**)	8	48
	9	40
	11	15
N-benzyloxycarbonyl-L-serine methylamide (**1e**)	8	traces
	9	15
	11	95
N-benzyloxycarbonyl-L-serine (**1i**)	11	stable

glycosidic bond of the methyl ester (**1b**) in an alkaline medium is in competition with ester hydrolysis and the glycosidic bond of the resulting acid (**1i**) proved to be much more stable.

The rate of cleavage of the methyl ester (**1b**) at different pH values is represented in Fig. 1. As can be seen, cleavage of the glycosidic bond at pH 11 rapidly ceases because the competing hydrolysis of ester grouping predominates and the acid (**1i**) formed has a much more stable glycosidic bond. At lower pH values, the competing ester hydrolysis becomes less important and, at pH 8–9, cleavage of the glycosidic bond proceeds more quickly.

Data on the stability of other glycosides of serine is shown in Table III. It is quite clear that the substitution of the amino group exerts a strong influence on the stability of the glycosidic bond, which increases, in alkaline media, in the series: NHCbz<NHCOCH$_2$NHCbz<NHCOCH$_2$NH$_2$$\leqslant$NH$_2$. This sequence corresponds to the increase of electrodonating power of the substituted amino group preventing the nucleophilic attack of hydroxide and, consequently, lowering the extent of elimination.

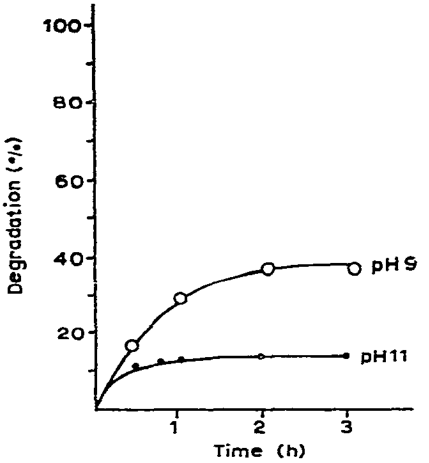

Fig. 1. The rate of cleavage of *N*-benzyloxycarbonyl-*O*-(β-D-glucopyranosyl)-L-serine methyl este at different pH values.

Apparently, the stability of the glycosidic bond depends on the combined influences of the substituents on the amino and carboxyl groups of serine and the most stable compound should be *O*-(β-D-glucopyranosyl)-L-serine. In fact, this substance remained unaltered on incubation with 0.1N sodium hydroxide at 20° for one week. The data in Table III also show that the structure of the monosaccharide unit has relatively little influence on glycosidic bond stability in alkaline media since galactosides are only slightly more stable than are glucosides. Unlike acid hydrolysis, ring size is of no importance, since galactofuranosides are as stable in alkaline media as are galactopyranosides.

The cleavage of glycosidic bonds is rapidly accelerated with increase in temperature, especially in the range 80–90° (Table IV). However, at elevated temperatures the destruction of a monosaccharide and of α-aminoacrylic acid derivatives proceeds faster, and quantitative data on glycosidic bond stability as a function of temperature were not obtained.

EXPERIMENTAL

Paper chromatography (p.c.) was performed on paper "M" (Leningrad factory No. 2) and thin-layer chromatography (t.l.c.) on alumina and silica using the solvent

TABLE III

STABILITY OF GLYCOSIDES OF L-SERINE DERIVATIVES AT pH 11, 37°, 24 h

Substance	*Degradation (%)*
β-D-Glucopyranosides of:	
N-benzyloxycarbonyl-L-serine methylamide (**1e**)	95
L-serine methylamide (**1f**)	0
N'-benzyloxycarbonyl-N-glycyl-L-serine methylamide (**1g**)	62
N-glycyl-L-serine methylamide (**1h**)	0
L-serine (**1d**)	0[a]
β-D-Galactopyranosides of:	
N-benzyloxycarbonyl-L-serine methylamide (**4b**)	72
L-serine methylamide (**4c**)	0
N'-benzyloxycarbonyl-N-glycyl-L-serine methylamide (**4d**)	53
β-D-Galactofuranoside of:	
N-benzyloxycarbonyl-L-serine methyl ester (**5b**)	0

[a] 0.1N sodium hydroxide, 8 days.

TABLE IV

STABILITY OF N-BENZYLOXYCARBONYL-O-(β-D-GLUCOPYRANOSYL)-L-SERINE METHYLAMIDE AT DIFFERENT TEMPERATURES, pH 8.

Temperature (°)	*Time (h)*	*Degradation (%)*
37	192	22
80	12	37
90	12	75
100	1.5	70

systems: chloroform–acetone, 14:1(*A*); ethyl acetate–methanol, 9:1 (*B*), 17:3 (*C*); and butanol–water–acetic acid, 4:4:1 (*D*). Electrophoresis was performed in pyridine–acetate (pH 4.5) and citrate–phosphate (pH 8) buffers at 30–35 V/cm for 20 min. The following spray reagents were used: benzidine–chlorine reagent, ninhydrin, hydroxylamine–ferric chloride, silver nitrate, bromophenol blue, and (for t.l.c.) sulphuric acid.

N-*Benzyloxycarbonyl*-O-*(2,3,4,6-tetra*-O-*acetyl-β-*D-*glucopyranosyl)*-L-*and* D-*serine methyl ester* (**1a**). — *N*-benzyloxycarbonyl-L-serine methyl ester (2.53 g) and silver carbonate (5.52 g) were boiled in dry benzene (60 ml) with slow distillation. When 20 ml of benzene had distilled off, 2,3,4,6-tetra-*O*-acetyl-D-glucopyranosyl bromide (8.22 g) in benzene (100 ml) was added and distillation and addition of solvent was continued until the volume of distillate was 350–450 ml, the reaction mixture volume being 50–60 ml. The mixture was filtered, evaporated *in vacuo*, and the residue chromatographed on a column of silica gel by elution with a benzene–chloroform gradient. Crystallization of the product from ether–hexane gave compound (**1a**) as colourless crystals (2.33 g, 40%), m.p. 93°, $[\alpha]_D^{20}+16°$ (*c* 1, chloroform), R_F 0.35 (p.c., solvent *A*) (Found: C, 53.3; H, 5.6; N, 2.7. $C_{26}H_{33}NO_{14}$ calc.: C, 53.5; H, 5.7; N, 2.4%).

The reaction with *N*-benzyloxycarbonyl-DL-serine methyl ester was performed analogously. The mixture of diastereoisomers (2.4 g) was dissolved in dry benzene (20 ml), hexane was added to turbidity and, after storage overnight in the refrigerator, compound (**D-1a**) separated. Recrystallization from ether–hexane gave colourless needles (580 mg, 10%), m.p. 96–97°, $[\alpha]_D^{20}$ −27° (*c* 2, chloroform) (Found: C, 53.9; H, 5.9; N, 2.5. $C_{26}H_{33}NO_{14}$ calc.: C, 53.5; H, 5.7; N, 2.4%).

N-*Benzyloxycarbonyl-*O-*(2,3,4,6-tetra-*O-*acetyl-β-D-galactopyranosyl)-L-serine-methyl ester* (**4a**). — This compound was synthesized as above from *N*-benzyloxycarbonyl-L-serine methylester (2.52 g) and 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl bromide (8.82 g) in benzene in the presence of silver carbonate (5.52 g). Chromatography of the product on alumina (elution with light petroleum→benzene→ether) gave the title compound (3.1 g, 54%), as a colourless syrup, $[\alpha]_D^{20}$ + 5° (*c* 2, chloroform), R_F 0.35 (t.l.c. alumina, solvent *A*) (Found: C, 53.7; H, 5.9; N, 2.4. $C_{26}H_{33}NO_{14}$ calc.: C, 53.5; H, 5.7; N, 2.4%).

*3,4,6-Tri-*O-*acetyl-1,2-*O-*(2′-benzyloxycarbonylamino-2′-carbomethoxy)-ethylorthoacetyl α-D-glucopyranose* (**2**). — The condensation between *N*-benzyloxycarbonyl-DL-serine methyl ester (0.253 g) and 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide in benzene in the presence of lead carbonate (1.135 g), as described above gave, after chromatography on alumina (gradient elution with benzene–chloroform), compound (**2**) as a colourless syrup (0.34 g, 58%), R_F 0.45 (t.l.c., Silicagel G, solvent *A*) (Found: C, 54.1 ;H , 5.8; N, 2.7. $C_{26}H_{33}NO_{14}$ calc.: C, 53.5; H, 5.7; N, 2.4%).

N-*Benzyloxycarbonyl-*O-*(β-D-glucopyranosyl)-L-(and D-) serine methyl ester* (**1b**). — A solution of compound (**1a**) (200 mg) in 10% methanolic triethylamine (10 ml) was left overnight at room temperature. The solution was then evaporated *in vacuo* with the addition of methanol to remove triethylamine. Chromatography of the residue on silica gel gave the amorphous compound (**1b**) (0.136 mg, 94%), $[\alpha]_D^{20}$ −10° (*c* 2, methanol), R_F 0.35 (t.l.c., solvent *B*) (Found: C, 52.3; H, 6.1; N, 3.5. $C_{18}H_{25}NO_{10}$ calc.: C, 52.00; H, 6.1; N, 3.4%).

The derivative of D-serine, prepared analogously, was a solid, $[\alpha]_D^{20}$ −3° (*c* 2, methanol) (Found: C, 51.9; H, 6.1; N, 3.3. $C_{18}H_{25}NO_{10}$ calc.: C, 52.0; H, 6.1; N, 3.4%).

Treatment of the L and D isomers (5 mg) with β-glucosidase (5 mg) in acetate buffer (pH 5.2, 1 ml) afforded, after two days (37°), only glucose and *N*-benzyloxycarbonyl-L-serine methyl ester as revealed by p.c. and t.l.c. (solvent *A*).

O-*(β-D-Glucopyranosyl)-L-serine methyl ester* (**1c**). — Compound (**1b**) (54 mg) was hydrogenated in 50% aqueous methanol (5 ml) in the presence of 5% palladised barium sulphate (60 mg) and N hydrochloric acid (0.1 ml) until the reaction was complete (electrophonetic control, pH 4.5). Evaporation of the filtrate and reprecipitation of the residue by acetone from methanol gave amorphous compound (**1c**) (as the hydrochloride) (84%), $[\alpha]_D^{20}$ −18° (*c* 2, water) (Found: C, 37.7; H, 6.4; Cl, 11.1; N, 4.7. $C_{10}H_{20}ClNO_8$ calc.: C, 37.8; H, 6.3; Cl, 11.2; N, 4.4%).

The derivative of D-serine, prepared analogously, was an amorphous solid, $[\alpha]_D^{20}$ + 6° (*c* 2, water) (Found: C, 37.5; H, 6.4; Cl. 10.8; N, 4.7%).

O-(*β*-D-*Glucopyranosyl*)-L-*serine* (**1d**). — A solution of compound (**1c**) (hydrochloride, 0.41 g) in 0.3N sodium hydroxide in aqueous methanol (9:1, 20 ml) was left for 15 h. The mixture was freed from alkali with IRC-50 (H^+) and then filtered through a column of the same resin. Evaporation of the eluate *in vacuo* gave compound (**1d**) (0.34 g, 80%), m.p. 225° (from aqueous methanol), $[\alpha]_D^{20}$ $-19°$ (*c* 2, water) (Found: C, 40; H, 6.43; N, 5.24. $C_9H_{17}NO_8$ calc.: C, 39.98; H, 6.29; N, 5.24%).

N-*Benzyloxycarbonyl*-O-(*β*-D-*glucopyranosyl*)-L-*serine methylamide* (**1e**).—Compound (**1a**) (1.67 g) in 4% methanolic methylamine (100 ml) was left for 10 h at room temperature. The solution was then evaporated and the residue was reprecipitated from methanol by ether and subjected to chromatography on cellulose. The product eluted first was rechromatographed on silica gel (gradient elution with chloroform–acetone) affording the methylamide (**1e**) (0.59 g, 50%), m.p. 165–166° (from ether–acetone), $[\alpha]_D^{20}$ $-2°$ (*c* 2, methanol), R_F 0.13 (t.l.c. Silicagel G, solvent *B*) (Found: C, 51.7; H, 6.3; N, 6.25. $C_{18}H_{26}N_2O_9$ calc.: C, 52.2; H, 6.3; N, 6.8%).

The methylamide of the D-serine derivative, prepared as above, had m.p. 178–180°, $[\alpha]_D^{20}$ $+6°$ (*c* 2, methanol) (Found: C, 52.0; H, 6.4; N, 6.5%).

N-*Benzyloxycarbonyl*-O-(*β*-D-*galactopyranosyl*)-L-*serine methylamide* (**4b**). — Treatment of compound (**4a**) (1.3 g) according to the above procedure gave the title compound (0.615 g, 66%) as colourless crystals, m.p. 202–203° (from ethanol), $[\alpha]_D^{20}$ $+ 14°$ (*c* 2.1, water), R_F 0.11 (t.l.c. silica, solvent *B*) (Found: C, 51.9; H, 6.2; N, 7.0; $C_{18}N_{26}N_2O_9$ calc.: C, 52.2; H, 6.3; N, 6.8%).

O-(*β*-D-*Glucopyranosyl*)-L-*serine methylamide* (**1f**). — Hydrogenation of the benzyloxycarbonyl derivative (**1e**) (50 mg) in the presence of oxalic acid (7.7 mg) and 5% palladised barium sulphate (25 mg) in 50% aqueous methanol as described above, gave hygroscopic crystals of compound (**1f**) (oxalate, 31 mg, 77%) $[\alpha]_D^{20}$ $- 11°$ (*c* 2, water) (Found: C, 41.0; H, 6.8; N, 8.03. $C_{11}H_{22}N_2O_9 \cdot 0{,}5\ CH_3OH$ calc.: C, 40.5; H, 6.8; N, 8.21%)

O-(*β*-D-*Galactopyranosyl*)-L-*serine methylamide* (**4c**). — Hydrogenation of the benzyloxycarbonyl derivative (**4b**) (100 mg) as above in the presence of oxalic acid gave crystalline compound (**4c**) (oxalate, 61 mg, 78%), m.p. 128–129°, $[\alpha]_D^{20}$ $\pm 0°$ (*c* 1, water) (Found: C, 41.2; H, 6.7; N, 8.4. $C_{11}H_{22}N_2O_9$ calc.: C, 40.6; H, 6.5; N, 8.6%).

N′-*Benzyloxycarbonyl*-O-(*β*-D-*glucopyranosyl*)-N-*glycyl*-L-*serine methylamide* (**1g**). — Methylamide (**1e**) (50 mg) was hydrogenated as above. The supernatant liquid after centrifugation was evaporated, the residue was dissolved in 90% aqueous pyridine (5 ml), *N*-benzyloxycarbonylglycine (25.3 mg) and dicyclohexylcarbodiimide (37.4 mg) were added. After 2 days in the refrigerator, the precipitate was removed, the filtrate was evaporated, and the residue subjected to chromatography on silica (gradient elution with chloroform–acetone). Lyophilisation of an aqueous solution of the product gave the amorphous dipeptide (**1g**) (40 mg, 65%), $[\alpha]_D^{20}$ $+3°$ (*c* 2, methanol), R_F 0.50 (t.l.c. Silicagel-G, solvent *H*) (Found: C, 50.9; H, 6.3; N, 8.6. $C_{20}H_{29}N_3O_{10}$ calc.: C, 51.0; H, 6.2; N. 8.9%).

N′-*Benzyloxycarbonyl*-O-(*β*-D-*galactopyranosyl*)-N-*glycyl*-L-*serine methylamide* (**4d**). —This compound was prepared by the above method from the benzyloxycarbonyl derivative (**4b**) (200 mg) and *N*-benzyloxycarbonylglycine (212 mg) in the presence of dicyclohexylcarbodiimide (150 mg) and obtained as colourless crystals (192 mg, 86%), m.p. 144–145° (from ethanol), $[\alpha]_D^{20}$ ±0° (*c* 2, methanol) (Found: C, 51.0; H, 6.2; N, 9.0. $C_{20}H_{29}N_3O_{10}$ calc.: C, 51.0; H, 6.2; N, 8.9%).

O-(*β*-D-*Glucopyranosyl*)-N-*glycyl*-L-*serine methylamide* (**1h**). — The benzyloxycarbonyl derivative (**1g**) (100 mg) was hydrogenated as above in the presence of oxalic acid (10.6 mg). The oxalate (**1h**), after precipitation by acetone, trituration with dry acetone, and washing with ether was an amorphous compound (61.5 mg, 79%), $[\alpha]_D^{20}$ −7° (*c* 2, water) (Found: C, 40.1; H, 6.9; N, 10.0·$C_{13}H_{24}N_3O_{10}\cdot CH_3OH$. calc.: C, 40.6 H, 6.8; N, 10.1%).

N-*Benzyloxycarbonyl*-O-(*β*-D-*glucopyranosyl*)-L-*serine* (**1i**). — The methyl ester (**1b**) (100 mg) was treated with 0.5N sodium hydroxide (1.6 ml) with shaking for 2 min and then neutralized with 50% acetic acid to pH 4.5. The solution was subjected to chromatography on DEAE-cellulose (linear gradient elution with pyridine–acetate buffer, 0.01M→0.1M, pH 4.5). Evaporation of the fraction giving a positive test with the benzidine–chlorine reagent gave the pyridinium salt of compound (**1i**) (isolated as the hydrate, 40%), $[\alpha]_D^{20}$ −3° (*c* 2, 50% aqueous pyridine). (Found: C, 51.2; H, 5.9; N, 5.5%. $C_{22}H_{28}N_2O_{10}\cdot 1.5H_2O$ calc.: C, 51.1; H, 5.9; N, 5.7%).

N-*Benzyloxycarbonyl*-O-(*β*-D-*glucopyranosyl*)-L-*serine hydrazide* (**1j**). — To the solution of methyl ester (**1b**) (0.875 g) in ethanol (30 ml), hydrazine (0.135 ml) was added and the mixture was left overnight at 20°. Evaporation of the mixture and crystallization of residue from ethanol gave the hydrazide (**1j**) (0.526 g, 60%), m.p. 178–180°, $[\alpha]_D^{20}$ − 9° (*c* 2, water) (Found: C, 47.1; H, 6.3; N, 9.9. $C_{17}H_{25}N_3O_9\cdot H_2O$ calc.: C, 47.1; H, 6.3; N, 9.7%).

Lactone of N-*benzyloxycarbonyl*-O-(*β*-D-*glucopyranosyl*)-L-*serine* (**3**). — The hydrazide (**1j**) (100 mg) was slowly added to a solution of *N*-bromosuccinimide (82 mg) in ethanol (2 ml) and the mixture was stirred for 10 min. After centrifugation, the supernatant liquid was subjected to the preparative paper electrophoresis resulting in the isolation of acid (**1i**) (15 mg, 15%) and lactone (**3**) (30 mg, 44%), $[\alpha]_D^{20}$ +4° (*c* 2, 50% aqueous pyridine).

N-*Benzyloxycarbonyl*-O-(*2,3,5,6-tetra*-O-*acetyl*-*β*-D-*galactofuranosyl*)-L-*serine methyl ester* (**5a**). — The orthoester (**6**) (2.30 g) and *N*-benzyloxycarbonylserine methyl ester (2.0 g) were boiled in nitromethane (20 ml) with a slow distillation and simultaneous addition of the solvent to keep constant the volume of the reaction mixture. After distillation of 10 ml of nitromethane, mercuric bromide (0.14 g) was added, the mixture was boiled for 3 h, and left overnight. Pyridine (0.1 ml) was added, the solvents were evaporated, and the residue was dissolved in chloroform (10 ml). A mixture of ether (30 ml) and light petroleum (100 ml) was then added yielding an oil which was chromatographed on alumina (gradient elution with carbon tetrachloride–ether) to give amorphous compound (**5a**) (0.64 g, 17.3%), $[\alpha]_D^{20}$ −17.4° (*c* 1.1, chloroform) (Found: C, 53.5; H, 5.8; N, 2.6. $C_{26}H_{33}NO_{14}$ calc.: C, 53.5; H, 5.7; N, 2.4%).

O-*β*-D-*Galactofuranosyl*-L-*serine methyl ester* (**5c**).—The substance (**5a**) (0.57 g) was treated as described above for compound (**1b**), followed by chromatography on silica (gradient elution with chloroform–acetone) to give amorphous compound (**5b**) (0.305 g, 76%), $[\alpha]_D^{20}$ −37°(*c* 1.8, methanol).

Hydrogenation of compound (**5b**) (235 ml) in the presence of oxalic acid as described above gave rise (after reprecipitation by dry acetone from methanol) to the oxalate (**5c**) (130 mg, 70%), $[\alpha]_D^{20}$ −57° (*c* 1, water) (Found: C, 40.9; H, 6.3; N, 4.3. $C_{11}H_{20}NO_{10}$ calc.: C, 40.5; H, 6.2; N, 4.3%).

Reaction of N-benzyloxycarbonyl-O-(β-D-glucopyranosyl) L-serine methyl ester with sodium methoxide. — The benzyloxycarbonyl derivative (**1b**) (200 mg) was dissolved in 0.1N methanolic sodium methoxide (10 ml) and the solution was stored for 2 h. The presence of glucose in the mixture was revealed by p.c. and t.l.c. Water (5 ml) was added to the mixture and, after 30 min, the methanol was evaporated. The residue was neutralized and crystallization of the separated oil gave rise to *N*-benzyloxycarbonyl-α-aminoacrylic acid[10] (40 mg, 40%), m.p. 106°, which was identical with an authentic sample synthesised by treatment of *N*-benzyloxycarbonyl-*O*-toluene-*p*-sulphonyl-DL-serine with sodium hydroxide in aqueous formamide.

Determination of glycosidic bond stability. — The determination was carried out in the following buffers: M acetate–hydrochloric acid, pH 1.5; 0.4M citrate–phosphate, pH 9.0; 0.4 carbonate–hydrogen carbonate, pH 11; and 0.1N sodium hydroxide. The course of reaction was monitored by quantitative determination of the glucose (Method A) and α-aminoacrylic acid derivative liberated (Method B).

Method A. Aliquot portions (0.02 ml) of the solution of 0.06 mmole of the substance under investigation in 0.4 ml of buffer were heated in sealed capillaries for a given time at different temperatures and then quantitatively transferred to a paper sheet and developed in solvent *D*. The chromatogram was sprayed with butanolic aniline hydrogen phthalate and heated at 105° for 15 min. The coloured zones were excised, extracted with 50% acetic acid for 2 h and the optical density at 380 mμ was measured. The quantity of glucose was calculated using a calibration curve; the accuracy of the method was *ca.* 10%.

Method B. A solution of 0.04 mmole of the substrate in 2 ml of buffer was heated in a thermostat. Aliquot portions (0.1 ml) ware diluted with 50% aqueous ethanol to 3 ml and the optical density of the released α-aminoacrylic acid derivative was measured at 224 mμ.

SUMMARY

Some *O*-D-glucopyranosyl-, *O*-D-galactopyranosyl-, and *O*-D-galactofuranosyl-L-serine derivatives have been synthesised by the Königs–Knorr and orthoester methods of glycosylation. The stability of the glycosidic bond in acid and alkali has been determined in order to demonstrate the influence of substituents on the amino and carboxyl groups of the serine moiety.

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DERIVATE DER ZUCKER-THIOACETALE

XXXIX. MITTEILUNG[1]. ISOPROPYLIDEN-VERBINDUNGEN DER D-XYLOSE-THIOACETALE

H. ZINNER UND J. MILBRADT
Institut für Organische Chemie der Universität, Rostock (D.D.R.)

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Beim Behandeln von D-Xylose-diäthylthioacetal mit Aceton und wasserfreiem Kupfersulfat oder Diphosphorpentoxyd erhielten Dalley und McIlroy[2] ein sirupöses Di-*O*-isopropyliden-D-xylose-diäthylthioacetal, in dem sich die beiden Isopropyliden-Gruppen in 2,4:3,5-Stellung befinden sollen. Nach der üblichen Ringbezeichnung für cyclische Acetale und Ketale[3] wären hier ein βC- und ein β-Ring vorhanden, die sechsgliedrig sind. Die bisherigen Erfahrungen haben aber gezeigt, daß Zuckeralkohole und Aldose-dialkylthioacetale mit Ketonen bevorzugt fünfgliedrige αT- und α-Ketal-Ringe bilden. Dieser Widerspruch veranlaßte uns, die Darstellung der *O*-Isopropyliden-D-xylose-dialkylthioacetale und ihre Strukturen näher zu untersuchen.

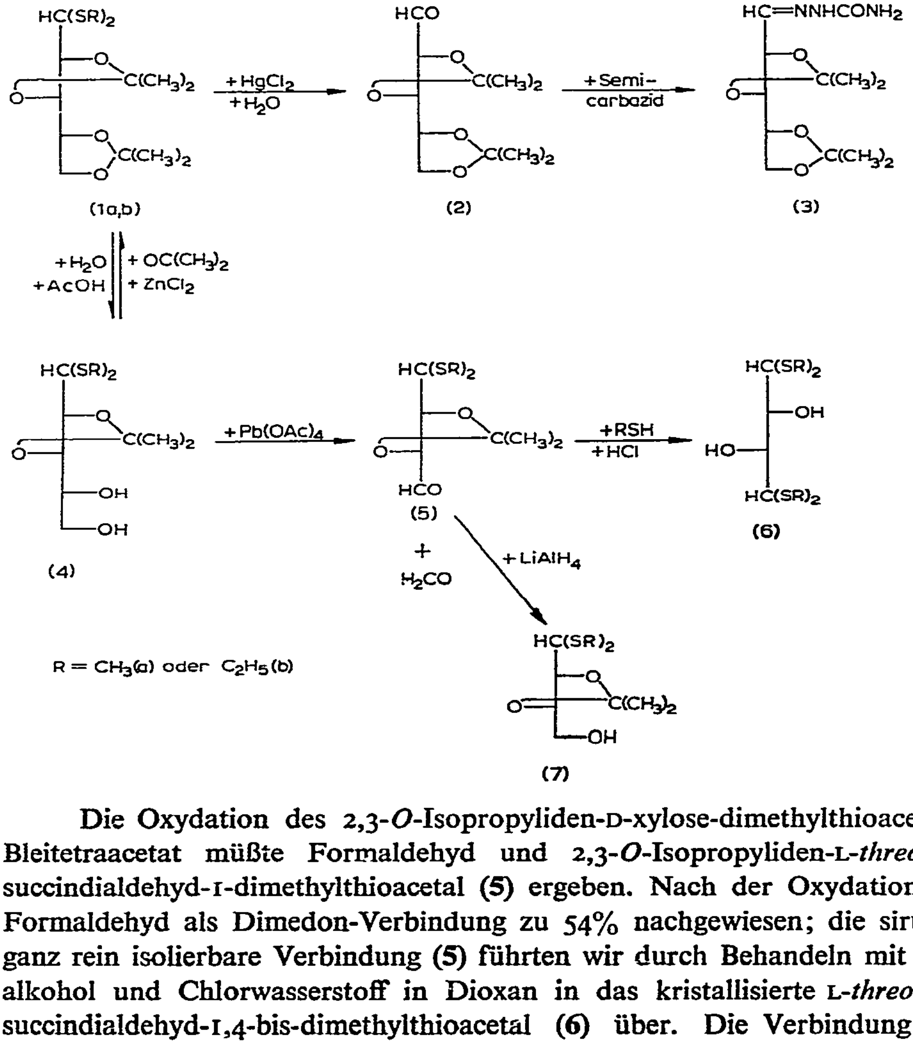
Wir kondensierten das Dimethylthioacetal und das Diäthylthioacetal der D-Xylose mit Aceton bei Gegenwart von Schwefelsäure und Kupfersulfat und erhielten das kristallisierte 2,3:4,5-Di-*O*-isopropyliden-D-xylose-dimethylthioacetal (**1a**) bzw. das entsprechende, sirupöse Diäthylthioacetal (**1b**). Letzteres zeigte etwa die gleichen Eigenschaften wie das von Dalley und McIlroy beschriebene Di-*O*-isopropyliden-D-xylose-diäthylthioacetal[2].

Aus den Verbindungen **1a** und **1b** erhält man durch Abspalten von Thioalkohol die 2,3:4,5-Di-*O*-isopropyliden-*aldehydo*-D-xylose (**2**), die ein gut kristallisiertes Semicarbazon (**3**) bildet. Dieses zeigt etwa den gleichen Schmelzpunkt und den gleichen absoluten Betrag der spezifischen Drehung wie das von Bourne, McSweeny und Wiggins[4] auf anderem Wege (siehe unten) dargestellte 2,3:4,5-Di-*O*-isopropyliden-L-xylose-semicarbazon. Damit ist die 2,3:4,5-Stellung der beiden Isopropyliden-Gruppen in den Verbindungen **1a** und **1b** gesichert.

Die genannten Autoren[4] stellten das 2,3:4,5-Di-*O*-isopropyliden-L-xylose-semicarbazon über mehrere Stufen aus dem 1,2:3,4:5,6-Tri-*O*-isopropyliden-L-idit dar. Die partielle Hydrolyse dieser Verbindung ergab 1,2:3,4-Di-*O*-isopropyliden-L-idit und 3,4-*O*-Isopropyliden-L-idit. Die erstgenannte Verbindung lieferte bei der Oxydation mit Natriumperjodat (Verbrauch 0.98 Mol.) Formaldehyd und 2,3:4,5-Di-*O*-isopropyliden-*aldehydo*-L-xylose, die in das Semicarbazon übergeführt wurde. Die Struktur des 3,4-*O*-Isopropyliden- und damit auch die des 1,2:3,4-Di-*O*-isopropyliden-L-idits und die der 2,3:4,5-Di-*O*-isopropyliden-*aldehydo*-L-xylose konnte durch Oxydation mit Natriumperjodat bewiesen werden; die 3,4-*O*-Isopropyliden-Verbindung verbrauchte dabei 2.12 Mol. des Oxydationsmittels und lieferte 1.90 Mol.

Formaldehyd. Bei der Überprüfung der Untersuchungen von Bourne, McSweeny und Wiggins[4] konnten wir die angegebenen Strukturen voll bestätigen.

Ein weiterer Strukturbeweis für das 2,3:4,5-Di-*O*-isopropyliden-D-xylose-dimethylthioacetal (**1a**) ist durch eine partielle Hydrolyse der Verbindung zum 2,3-*O*-Isopropyliden-D-xylose-dimethylthioacetal (**4**) und dessen Oxydation mit Bleitetraacetat möglich. Zur Hydrolyse wird die Di-*O*-isopropyliden-Verbindung **1a** eine Stunde in 80-proz. Essigsäure auf 40° erwärmt. Man erhält ein Gemisch von nicht hydrolysierter Verbindung **1a** und Verbindung **4**. Letztere läßt sich durch Chromatographie an Aluminiumoxyd von ersterer abtrennen und nach einer Destillation im Hochvakuum als reiner Sirup gewinnen.



Die Oxydation des 2,3-*O*-Isopropyliden-D-xylose-dimethylthioacetals (**4**) mit Bleitetraacetat müßte Formaldehyd und 2,3-*O*-Isopropyliden-L-*threo*-dihydroxy-succindialdehyd-1-dimethylthioacetal (**5**) ergeben. Nach der Oxydation wurde der Formaldehyd als Dimedon-Verbindung zu 54% nachgewiesen; die sirupöse, nicht ganz rein isolierbare Verbindung (**5**) führten wir durch Behandeln mit Methylthio-alkohol und Chlorwasserstoff in Dioxan in das kristallisierte L-*threo*-Dihydroxy-succindialdehyd-1,4-bis-dimethylthioacetal (**6**) über. Die Verbindung zeigte etwa den gleichen Schmelzpunkt und die gleiche, allerdings negative spezifische Drehung wie die entsprechende, schon bekannte Verbindung der D-Reihe[5].

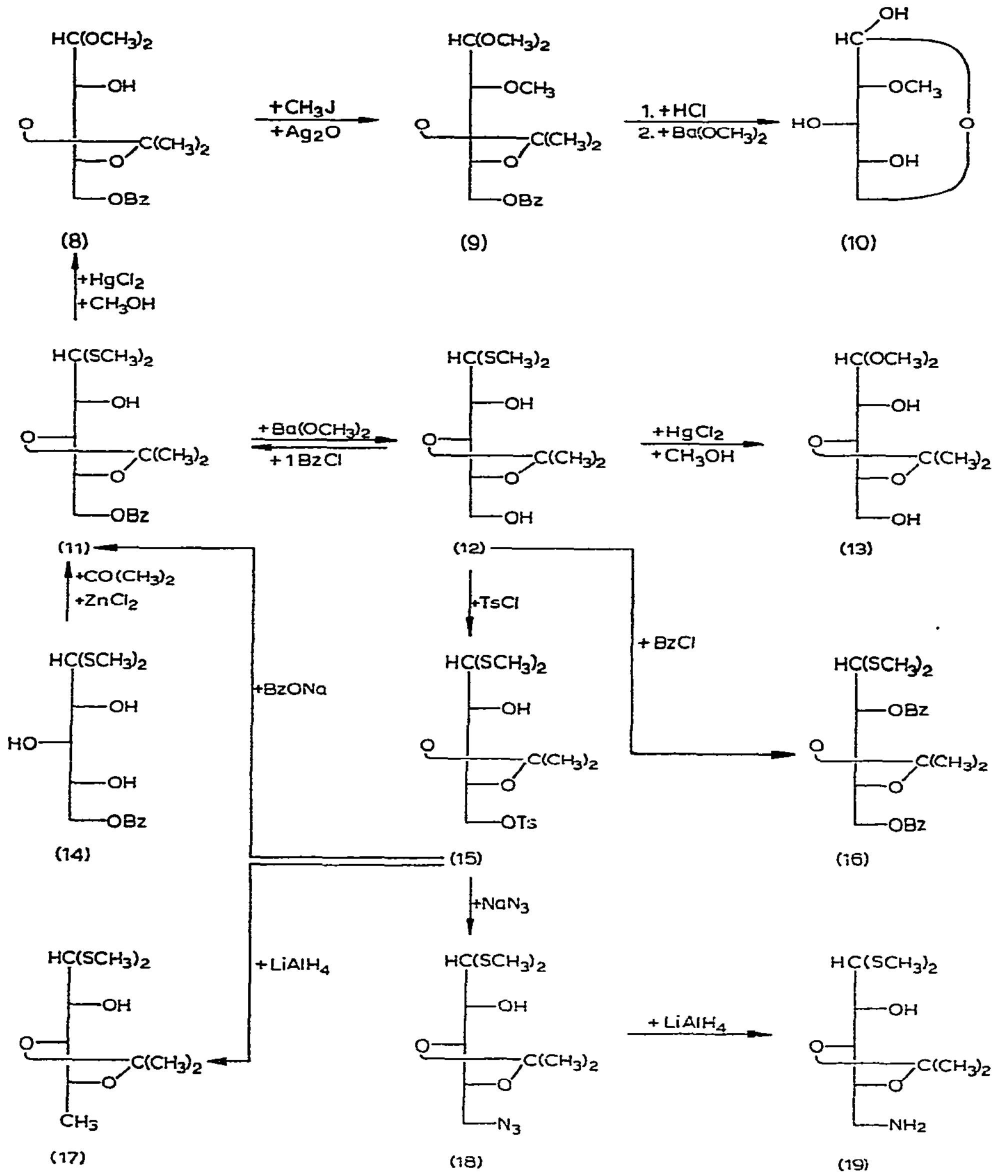
Durch Reduktion des 2,3-*O*-Isopropyliden-L-*threo*-dihydroxysuccindialdehyd-1-dimethylthioacetals (**5**) mit Lithiumaluminiumhydrid in Äther und anschließende Destillation im Hochvakuum gewinnt man das sirupöse, aber reine 2,3-*O*-Isopropyliden-L-threose-dimethylthioacetal (**7**).

Wie oben angegeben wurde, entsteht bei der Kondensation des D-Xylose-dimethylthioacetals mit Aceton bei Gegenwart von Schwefelsäure und Kupfersulfat das 2,3:4,5-Di-*O*-isopropyliden-D-xylose-dimethylthioacetal (**1a**). Wenn man für die Reaktion nur Kupfersulfat oder Zinkchlorid als Kondensationsmittel benutzt, so erhält man ein Gemisch von vier Verbindungen: 3,4-*O*-Isopropyliden-(**12**), 4,5-*O*-Isopropyliden-(**20**) und 2,3:4,5-Di-*O*-isopropyliden-D-xylose-dimethylthioacetal (**1a**) sowie ein Di-*O*-isopropyliden-D-xylose-dimethylthioacetal, das nur in sehr geringer Menge (1.6%) entsteht und dessen Struktur wir deswegen noch nicht aufklären konnten. Wahrscheinlich befinden sich die beiden Isopropyliden-Gruppen in 2,4:3,5-Stellung. Durch fraktionierte Kristallisation und Säulenchromatographie an Aluminiumoxyd gelang es uns, die vier Verbindungen voneinander zu trennen.

Zunächst soll die Struktur des 3,4-*O*-Isopropyliden-D-xylose-dimethylthioacetals (**12**) bewiesen werden. Die Verbindung hat zwei freie Hydroxyl-Gruppen, sie bildet ein Di-benzoat (**16**); beim Behandeln mit Bleitetraacetat in Benzol entsteht aus ihr kein Formaldehyd. Damit ist eine 2,3-Stellung des Isopropyliden-Restes ausgeschlossen.

Die 3,4-*O*-Isopropyliden-Verbindung **12** kann auch aus dem 5-*O*-Benzoyl-D-xylose-dimethylthioacetal[6] (**14**) durch Kondensation mit Aceton bei Gegenwart von Zinkchlorid zum 5-*O*-Benzoyl-3,4-*O*-isopropyliden-D-xylose-dimethylthioacetal (**11**) und dessen katalytische Entbenzoylierung mit Bariummethylat in Methanol synthetisiert werden. Da die Benzoyl-Gruppe die 5-Stellung blockierte, kann sich der Isopropyliden-Rest in **12** nicht in 2,5-, 3,5- oder 4,5-Stellung befinden; es bleibt jetzt nur noch eine 2,4- oder 3,4-Struktur übrig. Das 3,4-*O*-Isopropyliden-D-xylose-dimethylthioacetal (**12**) kann durch partielle Benzoylierung mit Benzoylchlorid in Pyridin wieder in das 5-*O*-Benzoyl-3,4-*O*-isopropyliden-D-xylose-dimethylthioacetal zurückgeführt werden. Die partielle Tosylierung mit *p*-Toluolsulfonsäurechlorid in Pyridin ergibt das 3,4-*O*-Isopropyliden-5-*O*-*p*-toluolsulfonyl-D-xylose-dimethylthioacetal (**15**). Daraus gewinnt man durch Austausch der Tosylat-Gruppe mit Natriumbenzoat in Dimethylformamid ebenfalls das 5-*O*-Benzoyl-3,4-*O*-isopropyliden-D-xylose-dimethylthioacetal (**11**). Diese Reaktion ist ein Beweis dafür, daß die Tosyl-Gruppe in **15** und damit auch die Benzoyl-Gruppe in **11** die 5-Stellung einnimmt und daß keine Umlagerung stattgefunden hat, denn der Austausch verläuft nach einem S_N2-Mechanismus unter Walden-Inversion; hätte die Tosylat-Gruppe nicht am symmetrischen C-5, sondern an einem der asymmetrischen C-Atome gesessen, könnte kein D-Xylose-Derivat entstanden sein.

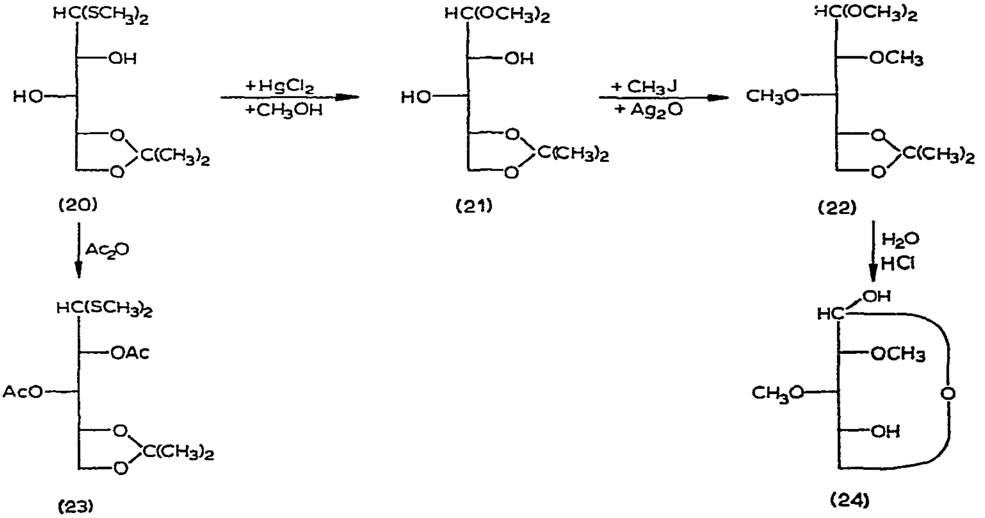
Daß die Isopropyliden-Gruppe in **11** und damit auch in **12** die 3,4-, nicht aber die 2,4-Stellung einnimmt, ergibt sich schließlich aus folgender Reaktionsreihe: Das 5-*O*-Benzoyl-3,4-*O*-isopropyliden-D-xylose-dimethylthioacetal (**11**) wird mit Quecksilber(II)-chlorid, Quecksilberoxyd und Methanol in das entsprechende Dimethylacetal **8** übergeführt und dieses zum 5-*O*-Benzoyl-3,4-*O*-isopropyliden-2-*O*-methyl-D-xylose-dimethylacetal (**9**) methyliert, aus dem man zunächst mit Salzsäure den Ketal-Ring und die Dimethylacetal-Gruppierung und anschließend mit Bariummethylat in Methanol den Benzoyl-Rest abspaltet; man erhält so die bekannte, kristallisierte 2-*O*-Methyl-D-xylose[7,8] (**10**).



Die Struktur des 3,4-*O*-Isopropyliden-D-xylose-dimethylthioacetals (**12**) wird auch durch die Umsetzung mit Quecksilber(II)-chlorid, Quecksilberoxyd und Methanol gestützt. Es bildet sich dabei nicht das Methyl-3,4-*O*-isopropyliden-D-xylopyranosid, sondern das 3,4-*O*-Isopropyliden-D-xylose-dimethylacetal (**13**). Die Bildung des Dimethylacetals wird verständlich, wenn man bedenkt, daß im Methyl-3,4-*O*-isopropyliden-D-xylopyranosid die Verschmelzung eines Fünfringes mit einem Sechsring vorliegen würde, was eine gewisse Deformation des Sechsringes bedingt[9]. Daher entsteht nicht das Xylopyranosid, sondern das spannungsfreie 3,4-*O*-Isopropyliden-dimethylacetal (**13**). Befände sich in der Verbindung **12** die Isopropyliden-Gruppe nicht in 3,4-, sondern in 2,4-Stellung, so wäre bei der genannten Umsetzung ein Methyl-2,4-*O*-isopropyliden-D-xylopyranosid zu erwarten, in dem zwei Sechsringe ohne Deformation verschmolzen wären. Ein Analogiebeispiel hierfür ist die Reaktion von 2,4-*O*-Benzyliden-D-ribose-thioacetalen mit Methanol, Quecksilber(II)-chlorid und Quecksilberoxyd, wo in guter Ausbeute das Methyl-2,4-*O*-benzyliden-$\alpha\beta$-D-ribopyranosid gebildet wird[10]. Es sei noch erwähnt, daß man 3,4-*O*-Isopropyliden-dimethylacetal (**13**) auch aus dem oben genannten 5-*O*-Benzoyl-3,4-*O*-isopropyliden-D-xylose-dimethylacetal (**8**) durch Entbenzoylierung mit Bariummethylat in Methanol erhält.

Mit dem 3,4-*O*-Isopropyliden-5-*O*-*p*-toluolsulfonyl-D-xylose-dimethylthioacetal (**15**) führten wir noch einige Synthesen durch. Die Hydrierung der Verbindung mit Lithiumaluminiumhydrid führt zum 5-Desoxy-3,4-*O*-isopropyliden-D-xylose-dimethylthioacetal (**17**). Beim Erhitzen von **15** mit Natriumazid in Dimethylformamid wird die Tosylat- gegen eine Azido-Gruppe ausgetauscht, das dabei gewonnene 5-Azido-5-desoxy-3,4-*O*-isopropyliden-D-xylose-dimethylthioacetal (**18**) läßt sich mit Lithiumaluminiumhydrid zum 5-Amino-5-desoxy-3,4-*O*-isopropyliden-D-xylose-dimethylthioacetal (**19**) reduzieren.

Für das oben genannte 4,5-*O*-Isopropyliden-D-xylose-dimethylthioacetal (**20**) ist die Struktur noch zu beweisen. Wir behandelten die Verbindung mit Methanol, Quecksilber(II)-chlorid und Quecksilberoxyd; dabei entstand nicht ein Methyl-*O*-isopropyliden-D-xylosid, sondern das 4,5-*O*-Isopropyliden-D-xylose-dimethylacetal (**21**).



Bei der Oxydation mit Bleitetraacetat in Benzol verbraucht die Verbindung in 16 Stunden 0.93 Mol. des Oxydationsmittels; Formaldehyd wird dabei nicht gebildet. Nach diesem Ergebnis könnte der Isopropyliden-Rest in der oxydierten Verbindung nur die 4,5- oder die 2,5-Stellung einnehmen.

Nach den bisherigen Erfahrungen ist aber die Bildung eines γ-Ringes (2,5-Stellung) unwahrscheinlich, wenn die Möglichkeit zum Entstehen von α-Ringen besteht. Die 4,5-Struktur der Verbindung **20** dürfte damit sehr wahrscheinlich sein.

Wir versuchten auch, die Struktur des 4,5-*O*-Isopropyliden-D-xylose-dimethylacetals (**21**) durch Methylieren zu stützen. Die Methylierung der Verbindung führte zum sirupösen, aber reinen 4,5-*O*-Isopropyliden-2,3-di-*O*-methyl-D-xylose-dimethylacetal (**22**). Daraus ließen sich der Ketal-Ring und die Dimethylacetal-Gruppierung durch Hydrolyse mit Salzsäure entfernen. Die dabei zu erwartende 2,3-Di-*O*-methyl-D-xylose (**24**) wurde in der Literatur[11] als schwer kristallisierende Verbindung mit einem Schmelzpunkt von 79–80° und einer spezifischen Drehung von +23° (nach vier Stunden, in Wasser) beschrieben. Die von uns gewonnene 2,3-Di-*O*-methyl-D-xylose kristallisierte bisher noch nicht. Die sirupöse Substanz zeigt eine spezifische Drehung von +17.2° in Wasser, sie ist demnach noch nicht rein.

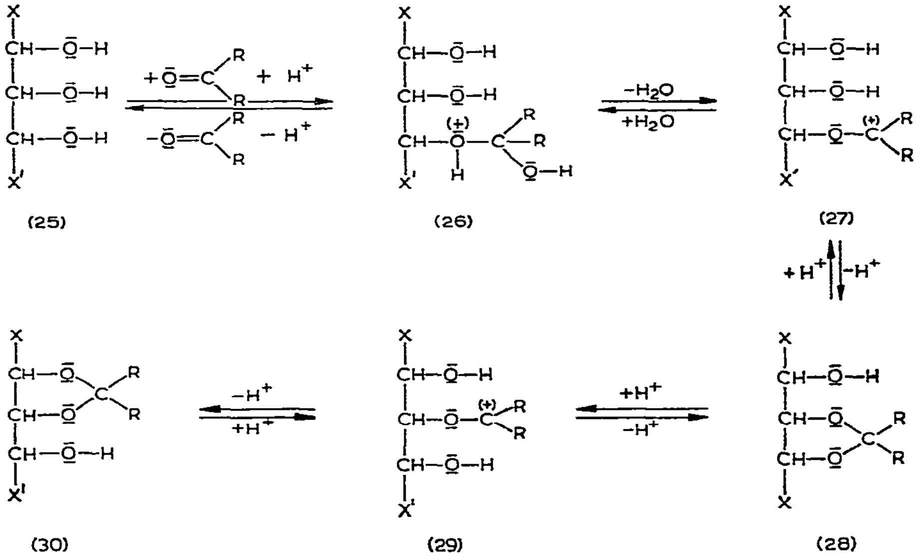
Zur weiteren Charakterisierung führten wir das 4,5-*O*-Isopropyliden-D-xylose-dimethylthioacetal durch Acetylieren mit Acetanhydrid und Pyridin in das 2,3-Di-*O*-acetyl-4,5-*O*-isopropyliden-D-xylose-dimethylthioacetal (**23**) über.

Die Bildung der cyclischen Ketale aus einer Polyhydroxy-Verbindung, einem Keton und einem sauren Katalysator verläuft über mehrere Zwischenstufen. Man nimmt an[12], daß die Polyhydroxy-Verbindung (**25**) zunächst das Keton und ein Proton addiert; das gebildete Additionsprodukt (**26**) spaltet nun Wasser ab und liefert ein Carbenium-Ion (**27**). Dieses liefert schließlich durch Abgabe eines Protons das cyclische Ketal (**28**). Der Prozeß ist reversibel, die Hydrolyse des cyclischen Ketals zur Polyhydroxy-Verbindung verläuft also rückläufig über die gleichen Zwischenstufen. Die Ringöffnung im cyclischen Ketal (**28**) unter dem Einfluß eines Protons muß aber nicht unbedingt zum gleichen Carbenium-Ion (**27**) führen, aus dem es ursprünglich entstanden war; die Ringöffnung kann auch in der Weise stattfinden, daß das Carbenium-Ion (**29**) entsteht. Dieses hat nun nicht nur die Möglichkeit, das cyclische Ketal (**28**) zurückzubilden, es kann auch mit der oberen Hydroxyl-Gruppe in Reaktion treten, wobei das isomere cyclische Ketal (**30**) entsteht. Nach diesem Mechanismus muß es prinzipiell möglich sein, ein Ketal, das man im zugehörigen Keton gelöst hat, mit einem sauren Katalysator in ein isomeres Ketal umzulagern.

Eine solche Umlagerung stellten wir beim 4,5-*O*-Isopropyliden-D-xylose-dimethylthioacetal (**20**) fest. Wenn man die Verbindung in Aceton bei Gegenwart von Zinkchlorid eine Stunde bei 20° stehen läßt, erhält man nach dem Aufarbeiten das 3,4-*O*-Isopropyliden-D-xylose-dimethylthioacetal (**12**) mit einer Ausbeute von 90% als reine Verbindung. Eine Di-*O*-isopropyliden-Verbindung konnten wir nicht als Nebenprodukt isolieren.

Läßt man das 4,5-*O*-Isopropyliden-D-xylose-dimethylthioacetal (**20**) nicht eine, sondern 24 Stunden in Aceton bei Gegenwart von Zinkchlorid stehen, so isoliert man anschließend 41% 3,4-*O*-Isopropyliden- (**12**) und außerdem 20% Di-*O*-isopropyliden-D-xylose-dimethylthioacetal, in dem wahrscheinlich, wie oben bereits erwähnt, eine 2,4:3,5-Struktur der beiden Ketal-Ringe vorliegt.

Eine Umlagerung des schon bestehenden Ketal-Ringes und die Bildung eines zweiten Ketal-Ringes finden auch statt, wenn man Aceton und Zinkchlorid 24 Stunden auf 3,4-*O*-Isopropyliden-D-xylose-dimethylthioacetal (**12**) einwirken läßt. Wir erhielten dabei das 2,3:4,5-Di-*O*-isopropyliden-D-xylose-dimethylthioacetal (**1a**) mit einer Ausbeute von 90%.



Die leichte Bildung eines α-Ketal-Ringes zeigt sich bei der Umsetzung des 2,3-*O*-Isopropyliden-D-xylose-dimethylacetals (**4**) mit Aceton und Zinkchlorid; nach zweistündiger Reaktionszeit kann man das 2,3:4,5-Di-*O*-isopropyliden-D-xylose-dimethylthioacetal (**1a**) mit einer Ausbeute von 78% als reine Verbindung isolieren. Umlagerungen finden hier offenbar nicht statt, denn außer **1a** ließen sich keine anderen Isopropyliden-Verbindungen isolieren.

Die vorliegenden Untersuchungsergebnisse zeigen, daß α-Ketal-Ringe schneller gebildet werden als αT-Ketal-Ringe, sich aber unter dem Einfluß saurer Katalysatoren leicht in αT-Ketal-Ringe umlagern. Diese sind stabiler als α-Ketal-Ringe, denn wenn in einer Verbindung (z. B. 2,3:4,5-Di-*O*-isopropyliden-D-xylose-dimethylthioacetal) beide Ringtypen vorliegen, wird der α-Ketal-Ring bei der Hydrolyse mit Säure zuerst abgespalten. Zwischen Bildungstendenz und Stabilität besteht also keine Parallele.

EXPERIMENTELLER TEIL

*Darstellung der O-Isopropyliden-*D*-xylose-dialkylthioacetale.* — Bei den unten unter (a) bis (i) aufgeführten Umsetzungen werden folgende Verbindungen isoliert: 2,3:4,5-Di-*O*-isopropyliden-D-xylose-dimethylthioacetal (**1a**), Nadeln aus *n*-Hexan, Schmp.

88°, $[\alpha]_D^{20}$ −54.6° (*c* 1.31, Chloroform). ($C_{13}H_{24}O_4S_2$ Ber.: C, 50.61; H, 7.84. Gef.: C, 50.52; H, 7.73%).

2,3:4,5-Di-*O*-isopropyliden-D-xylose-diäthylthioacetal (**1b**), Sirup, der bei 10^{-4} Torr und 80–85° Badtemperatur destilliert wurde, $[\alpha]_D^{20}$ −51.6° (*c* 1.50, Chloroform), $[\alpha]_D^{20}$ −66.4° (*c* 1.54, Aceton), n_D^{22} 1.4960. Lit.[2], $[\alpha]_D^{18}$ −62.3, (*c* 2.62, Aceton), n_D^{20} 1.5004. ($C_{15}H_{28}O_4S_2$ Ber.: C, 53.54; H, 8.39. Gef.: C, 53.33; H, 8.56%).

2,3,4,5-Di-*O*-isopropyliden-D-xylose-dimethylthioacetal, Nadeln aus Benzin (Siedepunkt 110–120°), Schmp. 175°, $[\alpha]_D^{20}$ −32.8° (*c* 1.26, Chloroform). ($C_{13}H_{24}O_4S_2$ Ber.: C, 50.61; H, 7.48. Gef.: C, 50.86; H, 7.70%).

2,3-*O*-Isopropyliden-D-xylose-dimethylthioacetal (**4**), Sirup, der bei 10^{-4} Torr und 140–145° Badtemperatur destilliert wurde, $[\alpha]_D^{20}$ −68.0° (*c* 1.15, Chloroform), n_D^{22} 1.5310. ($C_{10}H_{20}O_4S_2$ Ber.: C, 44.75; H, 7.51. Gef.: C, 44.71; H, 7.62%).

3,4-*O*-Isopropyliden-D-xylose-dimethylthioacetal (**12**), Nadeln aus Benzol-*n*-Hexan, Schmp. 109°, $[\alpha]_D^{20}$ +42.1° (*c* 1.20, Chloroform). ($C_{10}H_{20}O_4S_2$ Ber.: C, 44.75; H, 7.51. Gef.: C, 44.49; H, 7.46%).

4,5-*O*-Isopropyliden-D-xylose-dimethylthioacetal (**20**), Sirup, der bei 10^{-4} Torr und 130–135° Badtemperatur destilliert wurde, $[\alpha]_D^{20}$ +11.2° (*c* 2.22, Chloroform), n_D^{20} 1.5312. ($C_{10}H_{20}O_4S_2$ Ber.: C, 44.75; H, 7.51. Gef.: C, 44.50; H, 7.53%).

(*a*) *Umsetzung von* D-*Xylose-dimethylthioacetal mit Aceton, Kupfersulfat und Schwefelsäure.* Zu einer Aufschlämmung von 2.28 g (0.01 Mol) D-Xylose-dimethylthioacetal[13] und 5.0 g wasserfreiem Kupfersulfat in 25 ml Aceton gibt man eine unter Kühlen hergestellte Lösung von 0.5 ml konz. Schwefelsäure in 25 ml Aceton, schüttelt 20 Stdn. bei 20°, neutralisiert dann unter Eiskühlung mit einer konz. Ammoniak-Lösung, filtriert den Kupferkomplex ab, trocknet das Filtrat mit Natriumsulfat, dampft ein und kristallisiert das zurückbleibende 2,3:4,5-Di-*O*-isopropyliden-D-xylose-dimethylthioacetal (**1a**) um. Ausbeute 2.10 g (68%).

(*b*) *Umsetzung von* D-*Xylose-diäthylthioacetal mit Aceton, Kupfersulfat und Schwefelsäure.* 2.56 g (0.01 Mol) D-Xylose-diäthylthioacetal[13] werden behandelt, wie unter (*a*) angegeben. Das nach dem Aufarbeiten erhaltene sirupöse 2,3:4,5-Di-*O*-isopropyliden-D-xylose-diäthylthioacetal (**1b**) wird im Hochvakuum destilliert. Ausbeute 2.18 g (65%).

(*c*) *Umsetzung von* D-*Xylose-dimethylthioacetal mit Aceton und Zinkchlorid oder Kupfersulfat.* Man schüttelt eine Lösung von 22.8 g (0.1 Mol) D-Xylose-dimethylthioacetal[13] in 300 ml Aceton bei 20° entweder 2 Stdn. mit 30.0 g wasserfreiem Zinkchlorid oder 120 Stdn. mit 50.0 g wasserfreiem Kupfersulfat. Bei Verwendung von Zinkchlorid gibt man dann 300 ml Äther und eine Lösung von 40.0 g Kaliumcarbonat in 40 ml Wasser hinzu, schüttelt kräftig durch, filtriert das Zinkcarbonat ab, wäscht portionsweise mit 300 ml Äther nach, trocknet das vereinigte ätherische Filtrat mit Natriumsulfat und destilliert den Äther ab. Bei Verwendung von Kupfersulfat wird nach beendeter Reaktion das Kupfersulfat abfiltriert, mit 100 ml Aceton nachgewaschen und das vereinigte Filtrat ebenfalls eingedampft. Den Rückstand (nach diesem oder jenem Verfahren gewonnen) löst man in 100 ml heißem Benzol, läßt die Lösung 3 Tage bei + 6° stehen, saugt das auskristallisierte 3,4-*O*-Isopropyliden-D-xylose-dimethylthioacetal (**12**) ab und wäscht mit 20 ml Benzol nach. Nach dem Umkristallisieren wird die reine Verbindung **12** mit einer Ausbeute von 9.11 g (34%) gewonnen. Das vereinigte benzolische Filtrat versetzt man mit 15 ml Äthanol, dampft ein, löst den Rückstand in 50 ml Benzol, filtriert die Lösung durch eine Säule (Durchmesser 3.5 cm), die auf eine Länge von 35 cm mit Aluminiumoxyd gefüllt ist, wäscht mit 150 ml Benzol und dann mit 150 ml Äthanol nach. Man fängt zunächst die ersten 200 ml und dann getrennt davon die restlichen 150 ml der durchgelaufenen Flüssigkeit auf. Erstere dampft man ein, extrahiert den Rückstand mit 40 ml siedendem *n*-Hexan und filtriert das zurückbleibende 2,3,4,5-Di-*O*-isopropyliden-D-xylose-dimethylthioacetal heiß ab, das nach dem Umkristallisieren mit einer Ausbeute von 0.50 g (1.6%) gewonnen wird. Beim Abkühlen des heißen Filtrates kristallisiert das 2,3:4,5-Di-*O*-isopropyliden-D-xylose-dimethylthioacetal aus, es wird nach dem Umkristallisieren mit einer Ausbeute von 7.20 g (23%) gewonnen. Die oben genannten 150 ml der durch die Säule gelaufenen Flüssigkeit werden eingedampft; das dabei zurückbleibende 4,5-*O*-Isopropyliden-D-xylose-dimethylthioacetal (**20**) erhält man nach einer Destillation im Hochvakuum mit einer Ausbeute von 2.35 g (9%).

(*d*) *Umsetzung von 4,5-O-Isopropyliden-*D*-xylose-dimethylthioacetal* (**20**) *mit Aceton und Zinkchlorid.* Man schüttelt 2.68 g (0.01 Mol) **20** in 30 ml Aceton mit 3.0 g wasserfreiem Zinkchlorid 24 Stdn. bei 20° und arbeitet auf, wie unter (*c*) beschrieben, dabei wird jedoch nur der zehnte Teil der unter (*c*) angegebenen Lösungsmittel und Reagenzien verwendet. Es werden 1.10 g (41%) reines 3,4-*O*-Isopropyliden-D-xylose-dimethylthioacetal (**12**) isoliert. Das benzolische Filtrat, das man nach dem Absaugen des rohen **12** erhält, wird eingedampft, das zurückbleibende 2,3,4,5-Di-*O*-isopropyliden-D-xylosedimethylthioacetal umkristallisiert. Die Ausbeute an reiner Substanz beträgt 0.63 g (20%).

(*e*) *Umsetzung von 3,4-O-Isopropyliden-*D*-xylose–dimethylthioacetal* (**12**) *mit Aceton und Zinkchlorid.* Man schüttelt 2.68 g (0.01 Mol) **12** in 30 ml Aceton mit 3.0 g wasserfreiem Zinkchlorid 24 Stdn. bei 20°, gibt 30 ml Äther und eine Lösung von 4.0 g Kaliumcarbonat in 4.0 ml Wasser hinzu, schüttelt durch, filtriert das Zinkcarbonat ab, wäscht mit 30 ml Äther nach, trocknet die ätherischen Extrakte, dampft ein und kristallisiert den Rückstand um. Man gewinnt 2.78 g (90%) reines 2,3:4,5-Di-*O*-isopropyliden-D-xylose-dimethylthioacetal (**1a**).

(*f*) *Umsetzung von 2,3-O-Isopropyliden-*D*-xylose-dimethylthioacetal* (**4**) *mit Aceton und Zinkchlorid.* 2.68 g (0.01 Mol) **4** werden behandelt, wie unter (*e*) angegeben, man schüttelt jedoch nur 2 Stdn. Die Ausbeute an reinem 2,3:4,5-Di-*O*-isopropyliden-D-xylose-dimethylthioacetal beträgt 2.40 g (78%).

(*g*) *Umlagerung von 4,5-O-Isopropyliden-*D*-xylose-dimethylthioacetal* (**20**). 2.68g (0.01 Mol) **20** werden in 30 ml Aceton mit 3.0 g wasserfreiem Zinkchlorid 1 Stde. bei 20° geschüttelt. Dann arbeitet man auf, wie unter (*c*) angegeben; man verwendet jedoch nur den zehnten Teil der Lösungsmittel und Reagenzien. Es werden 2.41 g (90%) reines 3,4-*O*-Isopropyliden-D-xylose-dimethylthioacetal (**12**) gewonnen.

(*h*) *Partielle Hydrolyse von 2,3:4,5-Di-O-isopropyliden-*D*-xylose-dimethylthioacetal*

(**1a**). Man erwärmt 3.08 g (0.01 Mol) **1a** mit 25 ml 80-proz. Essigsäure im Wasserbad 1 Stde. auf 40° und verfährt, wie früher zur Darstellung der 2,3-*O*-Cyclohexyliden-D-arabinose-thioacetale angegeben[1]. Das Rohprodukt wird im Hochvakuum destilliert. Die Ausbeute an reinem 2,3-*O*-Isopropyliden-D-xylose-dimethylthioacetal (**4**) beträgt 1.16 g (43%).

(*i*) *Entbenzoylierung von 5-O-Benzoyl-3,4-O-isopropyliden-*D*-xylose-dimethylthioacetal* (**11**). Man löst 3.73 g (0.01 Mol) **11** (siehe unten) in 100 ml Methanol, gibt 20 ml 0.1N $Ba(OCH_3)_2$ in Methanol hinzu, läßt 16 Stdn. bei 20° stehen, neutralisiert mit 0.1N H_2SO_4, schüttelt mit etwas Bariumcarbonat und Aktivkohle, filtriert, dampft ein und kristallisiert den Rückstand um. Man gewinnt 2.58 g (96%) reines 3,4-*O*-Isopropyliden-D-xylose-dimethylthioacetal (**12**).

*2,3:4,5-Di-O-isopropyliden-*aldehydo-D-*xylose* (**2**). — Man erwärmt 0.01 Mol **1a** oder **1b** mit 60 ml Aceton, 6.0 ml Wasser, 6.0 g Quecksilber(II)-chlorid und 6.0 g Quecksilberoxyd 5 Stdn. unter Rühren auf 40°, filtriert, wäscht den Rückstand mit 30 ml Aceton, dampft das vereinigte Filtrat bei Gegenwart von 1.0 g Quecksilberoxyd ein, extrahiert den Rückstand dreimal mit je 30 ml heißem Chloroform, schüttelt die Lösung mit 30 ml N KJ sowie mit 10 ml Wasser, trocknet über Natriumsulfat, dampft im Vakuum ein und destilliert den Rückstand bei 10^{-4} Torr und 70–90° Badtemperatur. Ausbeute 1.38 g (60%), farbloser Sirup, $[\alpha]_D^{20}$ $-22.8°$ (*c* 2.09 Methanol), n_D^{20} 1.4538. Die Verbindung ist nicht ganz analysenrein. Für die entsprechende L-Verbindung werden angegeben[4] $[\alpha]_D^{19}$ $+25.2°$ (*c* 3.9, Methanol), n_D^{16} 1.4545.

*2,3:4,5-Di-O-isopropyliden-*D*-xylose-semicarbazon* (**3**). — 0.218 g (0.001 Mol) **2** werden in 2.0 ml absol. Äthanol mit 0.10 g Semicarbazid 3 Stdn. unter Rückfluß gekocht. Nach dem Erkalten gibt man bis zur Trübung Wasser hinzu, läßt bei 0° stehen, filtriert die ausgeschiedene Kristalle ab und kristallisiert aus Benzol-*n*-Hexan um. Ausbeute 0.154 g (54%), Nädelchen, Schmp. 179°, $[\alpha]_D^{20}$ $-30.5°$ (*c* 1.19, Chloroform). Für die entsprechende L-Verbindung werden angegeben[4] Schmp. 175–176°, $[\alpha]_D^{18}$ $+29.4°$ (*c* 2.7, Chloroform). ($C_{12}H_{21}N_3O_5$ Ber.: C, 50.17; H, 7.37; N, 14.62. Gef.: C, 50.23; H, 7.69; N, 14.68%).

L-threo-*Dihydroxy-succindialdehyd-1,4-bis-dimethylthioacetal* (**6**). —Man oxydiert 2.68 g (0.01 Mol) **4** mit Bleitetraacetat und setzt das dabei gebildete 2,3-*O*-Isopropyliden-L-*threo*-dihydroxy-succindialdehyd-1-dimethylthioacetal (**5**) mit Methylthioalkohol und Chlorwasserstoff in Dioxan um, wie früher für die Darstellung der entsprechenden D-Verbindung beschrieben[5]. Ausbeute 1.68 g (61%), Nadeln aus *n*-Hexan, Schmp. 118°, $[\alpha]_D^{20}$ $-4.4°$ (*c* 1.92, Methanol). Für die entsprechende D-Verbindung werden angegeben[5] Schmp. 116°, $[\alpha]_D^{22}$ $+5.5°$ (*c* 1.72, Methanol).

*2,3-O-Isopropyliden-*L*-threose-dimethylthioacetal* (**7**).—Man löst 2.68 g (0.01 Mol) **4** in 100 ml Benzol, gibt 5.0 g gepulvertes Bleitetraacetat hinzu, rührt 20 Min. bei 20°, filtriert das ausgefallene Bleisalz ab, wäscht das Filtrat dreimal mit je 30 ml gesättigter Natriumhydrogencarbonat-Lösung sowie einmal mit 20 ml Wasser, trocknet mit Natriumsulfat und dampft zu einem Sirup ein, der aus rohem 2,3-*O*-Isopropyliden-L-*threo*-dihydroxysuccindialdehyd-1-dimethylthioacetal (**5**) besteht. Der Sirup wird in 100 ml Äther mit 0.5 g Lithiumaluminiumhydrid 5 Stdn. unter Rückfluß gekocht.

Dann zersetzt man das überschüssige Hydrid unter Kühlen mit 20 ml Wasser, saugt den Hydroxydniederschlag ab, dampft das Filtrat ein und destilliert den Rückstand bei 10^{-4} Torr und 110–120° Badtemperatur. Ausbeute 1.65 g (69%), Sirup, $[\alpha]_D^{23}$ −56.4° (*c* 1.41, Chloroform), n_D^{23} 1.5183. ($C_9H_{18}O_3S_2$ Ber.: C, 45.34; H, 7.61. Gef.: C, 45.26; H, 7.70%).

*5-O-Benzoyl-3,4-O-isopropyliden-*D*-xylose-dimethylacetal* (**8**).— 3.73 g (0.01 Mol) **11** (siehe unten) werden mit 60 ml Methanol, 6.0 g Quecksilber(II)-chlorid und 6.0 g Quecksilberoxyd behandelt, wie früher für die Darstellung des 4,5-*O*-Cyclohexyliden-D-arabinose-dimethylacetals angegeben[1]. Das sirupöse Rohprodukt wird bei 10^{-4} Torr und 140–145° Badtemperatur destilliert. Ausbeute 1.77 g (52%), Sirup, $[\alpha]_D^{20}$ +31.5° (*c* 1.87, Chloroform), n_D^{20} 1.5038. ($C_{17}H_{24}O_7$ Ber.: C, 59.98; H, 7.11. Gef.: C, 59.94; H, 7.18%).

*5-O-Benzoyl-3,4-O-isopropyliden-2-O-methyl-*D*-xylose-dimethylacetal* (**9**).— Man löst 3.40 g (0.01 Mol) **8** in 50 ml Dimethylformamid, fügt 3.0 ml Methyljodid und 4.0 g Silberoxyd hinzu, schüttelt 12 Stdn. bei 20°, saugt die Silberverbindungen ab, wäscht mit 10 ml Dimethylformamid nach, versetzt das Filtrat mit einer Lösung von 0.2 g Natriumcyanid in 100 ml Wasser, extrahiert die Lösung zweimal mit je 80 ml Äther, wäscht den vereinigten Äther-Extrakt zweimal mit je 30 ml Wasser, trocknet mit Natriumsulfat, destilliert den Äther ab und destilliert den Rückstand bei 10^{-4} Torr und 125–130° Badtemperatur. Ausbeute 2.76 g (78%), Sirup, $[\alpha]_D^{20}$ +24.2° (*c* 1.66, Chloroform), n_D^{20} 1.4914. ($C_{18}H_{26}O_7$ Ber.: C, 61.00; H, 7.39; OCH_3, 26.27. Gef.: C, 61.12; H, 7.12; OCH_3, 26.01%).

*2-O-Methyl-*D*-xylose* (**10**).— Man kocht 0.35 g (0.001 Mol) **9** mit 2.0 ml Methanol und 0.5 ml N HCl 3 Stdn. unter Rückfluß, verdünnt mit 20 ml Methanol, neutralisiert durch Schütteln mit 2.0 g eines Anionenaustauschers (Wofatit L), filtriert und dampft zu einem Sirup ein. Dieser wird mit 10 ml Methanol und 2.0 ml 0.1N $Ba(OCH_3)_2$ in Methanol entbenzoyliert, wie zur Darstellung von **12** unter (*i*) angegeben. Den Rückstand kristallisiert man aus Methanol–Benzol um. Ausbeute 0.082 g (50%), Blättchen, Schmp. 137–138°, $[\alpha]_D^{20}$ −23.5° (nach 3 Min.)→ +35.2° (nach 24 Stdn., *c* 1.05, Wasser); Lit.[7], Schmp. 132–133°, $[\alpha]_D$ −23.9° → + 35.90 (nach 20 Stdn., *c* 3.62, Wasser); Lit.[8], Schmp. 135–137°, $[\alpha]_D^{16}$ −23°→ +35° (nach 24 Stdn., *c* 3.50, Wasser).

*5-O-Benzoyl-3,4-O-isopropyliden-*D*-xylose-dimethylthioacetal* (**11**). — (*a*) 3.32 g (0.01 Mol) 5-*O*-Benzoyl-D-xylose-dimethylthioacetal[6] werden mit 30 ml Aceton und 3.0 g wasserfreiem Zinkchlorid 3 Stdn. bei 20° geschüttelt. Dann gibt man 50 ml Äther und eine Lösung von 4.0 g Kaliumcarbonat in 4.0 ml Wasser hinzu, schüttelt kräftig durch, filtriert das Zinkcarbonat ab, wäscht mit 50 ml Äther nach, trocknet das vereinigte Filtrat mit Natriumsulfat, destilliert den Äther ab und kristallisiert den Rückstand mehrmals aus *n*-Hexan um. Ausbeute 1.30 g (35%).

(*b*) Man löst 2.68 g (0.01 Mol) **12** in 20 ml Pyridin, läßt bei — 10° unter Rühren im Verlauf von 30 Min. eine Lösung von 1.55 g (0.011 Mol) Benzoylchlorid in 5.0 ml Pyridin zutropfen, rührt 6 Stdn. bei —10°, läßt 16 Stdn. bei 20° stehen, gießt dann in 150 ml Wasser, filtriert nach einigen Stdn. das Rohprodukt ab und kristallisiert nach dem Trocknen aus *n*-Hexan um. Ausbeute 2.60 g (70%).

(*c*) Eine Lösung von 4.23 g (0.01 Mol) **15** (siehe unten) in 60 ml Dimethylformamid kocht man mit 4.32 g (0.03 Mol) Natriumbenzoat 2 Stdn. unter Rückfluß, kühlt auf 20°, fügt 60 ml Wasser hinzu, schüttelt die Lösung dreimal mit je 50 ml Äther, wäscht die vereinigten Äther-Extrakte zweimal mit je 30 ml Wasser, trocknet mit Natriumsulfat, dampft ein und kristallisiert aus *n*-Hexan um. Ausbeute 2.80 g (75%), Nadeln, Schmp. 90°, $[\alpha]_D^{20}$ +31.3° (*c* 1.83, Chloroform). ($C_{17}H_{24}O_5S_2$ Ber.: C, 54.81; H, 6.49. Gef.: C, 54.52; H, 6.53%).

*3,4-O-Isopropyliden-*D*-xylose-dimethylacetal* (**13**). — (*a*) 2.68 g (0.01 Mol) **12** werden mit 60 ml Methanol, 6.0 g Quecksilber(II)-chlorid und 6.0 g Quecksilberoxyd behandelt, wie früher für die Darstellung des 4,5-*O*-Cyclohexyliden-D-arabinose-dimethylacetals angegeben[1]. Das Rohprodukt wird aus wenig Äther umkristallisiert. Ausbeute 1.11 g (47%).

(*b*) 3.40 g (0.01 Mol) **8** werden mit 100 ml Methanol und 20 ml 0.1N $Ba(OCH_3)_2$ in Methanol entbenzoyliert, wie zur Darstellung von **12** unter (*i*) angegeben. Den Rückstand kristallisiert man aus wenig Äther um. Ausbeute 2.23 g (94%), Prismen, Schmp. 80°, $[\alpha]_D^{20}$ +45.6° (*c* 1.54, Chloroform). ($C_{10}H_{20}O_6$ Ber.: C, 50.83; H, 8.53. Gef.: C, 50.56; H, 8.54%).

*3,4-O-Isopropyliden-5-O-p-toluolsulfonyl-*D*-xylose-dimethylthioacetal* (**15**). — Man löst 2.68 g (0.01 Mol) **12** in 20 ml Pyridin, läst unter Rühren bei —10° eine Lösung von 2.10 g (0.011 Mol) *p*-Toluolsulfonsäurechlorid in 10 ml Pyridin zutropfen, rührt 6 Stdn. bei —10°, läßt 16 Stdn. bei 20° stehen, gießt in 150 ml Wasser und kristallisiert das ausgefallene Rohprodukt aus viel *n*-Hexan um. Ausbeute 3.51 g (83%), Nadeln, die Verbindung schmilzt bei 102°, erstarrt dann wieder und schmilzt erneut bei 109°, $[\alpha]_D^{20}$ +28.3° (*c* 1.01, Chloroform). ($C_{17}H_{26}O_6S_3$ Ber.: C, 48.31; H, 6.20. Gef.: C, 48.37; H, 6.47%).

*2,5-Di-O-benzoyl-3,4-O-isopropyliden-*D*-xylose-dimethylthioacetal* (**16**). — Man gibt zu einer Lösung von 2.68 g (0.01 Mol) **12** in 20 ml Pyridin bei 0° unter Rühren tropfenweise eine Lösung von 3.09 g (0.022 Mol) Benzoylchlorid in 5.0 ml Pyridin, läßt 16 Stdn. bei 20° stehen, gießt unter Rühren in 500 ml Wasser, nimmt den ausgefallenen Sirup in 30 ml Chloroform auf, wäscht mit 30 ml 2-proz. Kaliumhydrogensulfat- und mit 30 ml gesättigter Natriumhydrogencarbonat-Lösung sowie mit 20 ml Wasser, schüttelt mit Natriumsulfat und Aktivkohle, filtriert, dampft ein und trocknet den zurückbleibenden Sirup bei 0.1 Torr und 65°. Ausbeute 4.24 g (89%), Sirup, $[\alpha]_D^{20}$ +25.5° (*c* 1.79, Chloroform), n_D^{20} 1.5602. ($C_{24}H_{28}O_6S_2$ Ber.: C, 60.48; H, 5.92. Gef.: C, 60.68; H, 5.93%).

*5-Desoxy-3,4-O-isopropyliden-*D*-xylose-dimethylthioacetal* (**17**). — Eine Lösung von 4.32 g (0.01 Mol) **15** in 30 ml Benzol und 80 ml Äther wird unter Rühren tropfenweise mit einer Lösung von 1.5 g Lithiumaluminiumhydrid in 50 ml Äther versetzt und 8 Stdn. unter Rückfluß gekocht. Dann gibt man unter Kühlen 5.0 ml Wasser hinzu, saugt den Hydroxydniederschlag ab, dampft das Filtrat ein und destilliert den Rückstand bei 10^{-4} Torr und 100–105° Badtemperatur. Ausbeute 1.15 g (46%), Sirup, $[\alpha]_D^{20}$ —2.1° (*c* 3.71, Chloroform), n_D^{20} 1.5035. ($C_{10}H_{20}O_3S_2$ Ber.: C, 47.58; H, 7.99. Gef.: C, 47.43; H, 7.59%).

*5-Azido-5-desoxy-3,4-O-isopropyliden-*D*-xylose-dimethylthioacetal* (**18**) — 4.23 g (0.01 Mol) **15** werden in 60 ml Dimethylformamid mit 1.95 g (0.03 Mol) Natriumazid 2 Stdn. unter Rückfluß gekocht. Nach dem Abkühlen gibt man 60 ml Wasser hinzu, extrahiert die Lösung dreimal mit je 50 ml Äther, wäscht die vereinigten Extrakte zweimal mit 30 ml Wasser, trocknet mit Natriumsulfat, dampft ein und kristallisiert den Rückstand aus *n*-Hexan um. Ausbeute 1.79 g (61%), Nadeln, Schmp. 76°, $[\alpha]_D^{20}$ $+106.6°$ (*c* 0.69, Chloroform). ($C_{10}H_{19}N_3O_3S_2$ Ber.: C, 40.93; H, 6.53; N, 14.32. Gef.: C, 40.63; H, 6.88; N, 14.49%).

*5-Amino-5-desoxy-3,4-O-isopropyliden-*D*-xylose-dimethylthioacetal* (**19**).—2.93 g (0.01 Mol) in 100 ml Äther gelöstes **18** werden mit 1.5 g Lithiumaluminiumhydrid hydriert, wie zur Darstellung von **17** angegeben; man kocht jedoch nur 2 Stdn. unter Rückfluß und kristallisiert den Rückstand aus *n*-Hexan um. Ausbeute 1.98 g (74%), Nadeln, Schmp. 66° $[\alpha]_D^{20}$ $+28.6°$ (*c* 0.62, Chloroform). ($C_{10}H_{21}NO_3S_2$ Ber.: C, 44.91; H, 7.92; N, 5.24. Gef.: C, 44.78; H, 8.02; N, 5.52%).

*4,5-O-Isopropyliden-*D*-xylose-dimethylacetal* (**21**). — 2.68 g (0.01 Mol) **20** werden behandelt, wie oben zur Darstellung von **13** unter (*a*) angegeben. Das sirupöse Rohprodukt wird bei 10^{-4} Torr und einer Badtemperatur von 125–130° destilliert. Ausbeute 1.20 g (51%), Sirup, $[\alpha]_D^{20}$ $+18.9°$ (*c* 2.16, Chloroform), n_D^{20} 1.4578. ($C_{10}H_{20}O_6$. Ber.: C, 50.83; H, 8.53. Gef.: C, 51.05; H, 8.23%).

*4,5-O-Isopropyliden-2,3-di-O-methyl-*D*-xylose-dimethylacetal* (**22**).—2.36 g (0.01 Mol) **21** werden mit 6.0 ml Methyljodid und 8.0 g Silberoxyd in 100 ml Dimethylformamid methyliert, wie oben zur Darstellung von **9** beschrieben. Das Rohprodukt wird bei 2 Torr und 100–110° Badtemperatur destilliert. Ausbeute 1.88 g (71%), Öl, $[\alpha]_D^{22}$ $+22.3°$ (*c* 1.35, Chloroform), n_D^{22} 1.4370. ($C_{12}H_{24}O_6$ Ber.: C, 54.53; H, 9.15; OCH_3, 46.97. Gef.: C, 54.25; H, 8.97; OCH_3, 46.59%).

*2,3-Di-O-acetyl-4,5-O-isopropyliden-*D*-xylose-dimethylthioacetal* (**23**).— Man läßt eine Mischung von 2.68 g (0.01 Mol) **20**, 3.0 ml Acetanhydrid und 10 ml Pyridin 16 Stdn. bei 20° stehen, gießt in 150 ml Wasser, nimmt den ausgefallenen Sirup in 30 ml Chloroform auf, wäscht die Chloroform-Lösung je zweimal mit 30 ml 2-proz. Kaliumhydrogensulfat- und mit 30 ml gesättigter Natriumhydrogencarbonat-Lösung sowie mit 20 ml Wasser, trocknet mit Natriumsulfat, dampft ein und destilliert den Rückstand bei 10^{-4} Torr und 110–120° Badtemperatur. Ausbeute 2.82 g (80%), Sirup, $[\alpha]_D^{20}$ $-7.6°$ (*c* 2.39, Chloroform), n_D^{20} 1.4960. ($C_{14}H_{24}O_6S_2$ Ber.: C, 47.70; H, 6.86. Gef.: C, 47.45; H, 6.76%).

ZUSAMMENFASSUNG

Bei der Kondensation von D-Xylose-dimethylthioacetal mit Aceton bei Gegenwart von Kupfersulfat und Schwefelsäure bildet sich das 2,3:4,5-Di-*O*-isopropyliden-D-xylose-dimethylthioacetal. Wird als Kondensationsmittel nur Kupfersulfat oder Zinkchlorid eingesetzt, so entsteht ein Gemisch von 3,4-*O*-Isopropyliden-, 4,5-*O*-Isopropyliden-, 2,3:4,5-Di-*O*-isopropyliden- und 2,3,4,5-Di-*O*-isopropyliden-D-xylose-dimethylthioacetal (Struktur unsicher). Aus dem Gemisch lassen sich die einzelnen Verbindungen durch fraktionierte Kristallisation und Chromatographie an Aluminiumoxyd abtrennen. Die 2,3:4,5-Di-*O*-isopropyliden-Verbindung kann partiell zum 2,3-*O*-Isopropyliden-D-xylose-dimethylthioacetal hydrolysiert werden. Die 4,5-*O*-Isopropyliden-Verbindung wird beim einstündigen Behandeln mit Zinkchlorid in Aceton in die 3,4-*O*-Isopropyliden-Verbindung umgelagert. Beim Verlängern der Reaktionszeit auf 24 Stunden entsteht neben letztgenannter Verbindung das 2,3,4,5-Di-*O*-isopropyliden-Derivat. Aus dem 3,4-*O*-Isopropyliden-dimethylthioacetal erhält man beim Behandeln mit Zinkchlorid in Aceton das 2,3:4,5-Di-*O*-isopropyliden-dimethylthioacetal. Die Strukturen der Verbindungen werden durch Überführen in zugehörige Dialkylacetale, Oxydationen mit Bleitetraacetat und Methylierungen bewiesen. Besonders vom 3,4-*O*-Isopropyliden-D-xylose-dimethylthioacetal werden Derivate dargestellt.

SUMMARY

When D-xylose dimethyl dithioacetal was condensed with acetone in the presence of copper sulphate and sulphuric acid the 2,3:4,5-di-*O*-isopropylidene derivative was obtained. When copper sulphate or zinc chloride was used as catalyst, a mixture of the 3,4-*O*-, 4,5-*O*-, 2,3:4,5-di-*O*-, and 2,3,4,5-di-*O*-isopropylidene derivatives was formed. The components of the mixture were isolated by crystallisation and chromatography on alumina. The 2,3:4,5-di-*O*-isopropylidene derivative was partially hydrolysed to 2,3-*O*-isopropylidene-D-xylose dimethyl dithioacetal. The 4,5-*O*-isopropylidene derivative rearranged into the 3,4-isomer by treatment for 1 h with zinc chloride in acetone. Extension of the reaction time to 24 h gave the 2,3,4,5-di-*O*-isopropylidene derivative in addition to the latter compound. Treatment of the 3,4-*O*-isopropylidene derivative with zinc chloride in acetone gave 2,3:4,5-di-*O*-isopropylidene-D-xylose dimethyl dithioacetal. The structures of the above derivatives was established by the application of oxidation with lead tetra-acetate and methylation. Derivatives of the 3,4-*O*-isopropylidene compound were obtained.

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THE STRUCTURE OF BLOOD GROUP SUBSTANCE-A: GENERAL MOLECULAR ARCHITECTURE AND NATURE OF THE CARBOHYDRATE–PEPTIDE LINKAGE

N. K. KOCHETKOV, V. A. DEREVITSKAYA, AND S. G. KARA-MURZA
Institute for Chemistry of Natural Products, U.S.S.R Academy of Sciences, Moscow (U.S.S.R.)

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INTRODUCTION

Although numerous investigations concerning the blood group substances (BGS) have made clear some important details of the structure of these complex glycoproteins, especially the nature of immunochemical determinants, *i.e.* terminal carbohydrate units[1,2], the general molecular architecture remains uncertain. The concept of BGS structure as a peptide backbone to which are attached relatively short carbohydrate chains has, to date, no strong experimental support although it seems reasonable on biochemical and genetic grounds[3–6]. We now report on blood group substance-A (BGS-A) from pig stomach linings.

The high molecular weight of BGS and the complexity of its structure cause serious difficulties in the traditional approach to structure determination, *i.e.* partial hydrolysis followed by isolation and structural determination of low-molecular weight fragments. We have developed a new approach which includes analytical gel-filtration and which has permitted definite conclusions to be drawn about the general molecular architecture of BGS including the nature of the linkages between the carbohydrate and the peptide chains*.

MATERIALS AND METHODS

Blood group substance-A (BGS-A) — The biopolymer was isolated from pig stomach linings and purified according to Kabat's method[7].

Gel-filtration. — Sephadex G-25, G-50, G-100, and G-200 were used. The gel-filtration on Sephadex G-200 was performed in 0.01N acetate buffer (pH 5) and on the other types of Sephadex in 0.1N acetic acid. Agar gel was prepared as follows: the 7% gel from commercial agar was squeezed through a rough sieve, washed with N NaOH and water, filtered, and dried with hot acetone. This preparation was used for obtaining gels with required specific concentrations of water followed by crushing and twice rubbing through a 60 mesh sieve with subsequent removal of fine particles by decantation.

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*During and after the preparation of this article, two papers concerning the same subject were published: J. B. ADAMS, *Biochem. J.*, 94 (1965) 368; K. O. LLOYD, E. A. KABAT, E. J. LAYUG, AND F. GRUEZA, *Biochemistry*, 5 (1966) 1489.

Analytical methods. (a) Neutral monosaccharides[8]. A 0.1% solution of anthrone in H_2SO_4 (1.5 ml) was added to an aliquot part (0.1–0.5 ml) of the solution to be examined, the mixture was shaken, heated for 20 min at 90°, and the optical density at 608 mμ was measured.

(*b*) *Fucose*. The method of Dische and Shettles[9] was modified as follows: 2.5 ml of diluted H_2SO_4 (3.3:1) was added to the aliquot part (0.2 ml) and the solution was heated on a boiling water bath for 10 min. After cooling, a 3% solution of cysteine hydrochloride (0.1 ml) was added and after 4 h at room temperature in the dark, the optical densities at 396 mμ and 430 mμ were measured and the difference $\Delta\varepsilon$ ($\varepsilon_{396} - \varepsilon_{430}$) was calculated.

(*c*) *Hexosamines*. A modified Elson–Morgan method[10] was used: 4N HCl (0.5 ml) was added to the aliquot part (0.5 ml) and the solution was heated at 110° in a sealed tube for 2 h. After neutralization with 2N NaOH (phenolphthalein), a 1% solution of acetylacetone in 0.5M Na_2CO_3 (1 ml), Erhlich reagent (1 ml), and ethanol (1 ml) were added. The mixture was warmed at 60–70° for 5–10 min and the optical density at 540 mμ was measured.

(*d*) *Total amino-group content*[11]. A 4% solution of $NaHCO_3$ (0.2 ml) and a 0.1% solution of 2,4,6-trinitrobenzenesulfonic acid (0.2 ml) were added to the aliquot part (0.2 ml) and the solution was warmed at 40° for 2 h. N HCl (0.2 ml) and water (2.2 ml) were then added and the optical density at 340 mμ was measured.

(*e*) *Total amino acid content (excluding proline and diamino acids)*. The fractions to be examined, corresponding to one definite peak on a gel-filtration curve, were combined, evaporated to dryness and the residue was heated in a sealed tube with 6N HCl at 105° for 20 h. The hydrolysate was evaporated to dryness *in vacuo*, and the residue was dissolved in water and re-evaporated. The dry residue was dissolved in citrate buffer (pH 5.28), adsorbed on a column (1.2 × 15 cm) of Dowex 50X8 (Na^+) and eluted with the same buffer. The first 20 ml of eluate was collected. The determination of the amino group content in the aliquot was performed as in (*d*) using valine as a standard.

(*f*) *Histidine*[12]. To the aliquot part (0.5–1.0 ml) of the fraction, a 4% solution of $NaHCO_3$ (1 ml) was added followed by a solution of diazotized 5-amino-1-*H*-tetrazole (0.1 ml) (prepared from 70 mg of 5-amino-1-*H*-tetrazole in 3 ml of water). After 20 min, water (3 ml) was added and the optical density at 480 mμ was measured.

Acid hydrolysis of BGS. (*a*) A solution of 10–15 mg of BGS per ml of dilute HCl (pH 1.5) was heated in a sealed tube on a boiling water bath for 3 h. The hydrolyzate was neutralised with 2N $(BaOH)_2$ and 2 ml portions were subjected to gel-filtration. The fractions were analysed for various components as described above.

(*b*) A solution of BGS in 0.4N HCl (10–15 mg per ml) was heated at 90° and 2 ml portions were withdrawn at intervals, and subjected to analytical gel-filtration as described above.

Cleavage of BGS by alkali in the presence of sodium borohydride. BGS (200 mg) was dissolved in 0.1N NaOH, $NaBH_4$ (40 mg) was added and the mixture was stored at 17°, 20°, or 25° during 6–15 days[13,14]. After acidification with 2N acetic acid, aliquot parts (0.5 ml) were subjected to gel-filtration. The cleavage of BGS by alkali in the absence of $NaBH_4$ was performed analogously.

The amino acid composition in BGS after cleavage by alkali. BGS (46.1 mg) was dissolved in 0.1M NaOH (3 ml) and of two 1 ml aliquot parts, one was immediately neutralized with N HCl and evaporated. To the other $NaBH_4$ (10 mg) was added and, after storage at 17° for 5 days, the solution was evaporated. Both residues were subjected to total hydrolysis (5.7N HCl, 20 h, 102°) and the amino acid content was assayed with an amino acid analyser.

Cleavage of BGS in sodium carbonate solution. BGS (50 mg) in 0.05M Na_2CO_3 (5 ml, pH 10.8) was heated[14] on a boiling water bath for 4–10 min, neutralised with N acetic acid and subjected to gel-filtration.

Isolation of the high molecular weight fragments after alkaline cleavage of BGS. The preparations required for subsequent degradation of the peptide part of BGS were obtained as follows: BGS (250 mg) was treated with 0.1N NaOH (5 ml) for 6 days at 20° in the absence and presence of $NaBH_4$ (50 mg) and, in each case, the mixture was subjected to preparative gel-filtration on a column (coarse, 3.03 × 20 cm) of Sephadex G-50. The high molecular weight fractions (as revealed by analyses with the anthrone method) were lyophilised. The yield of polymeric material was 100–150 mg.

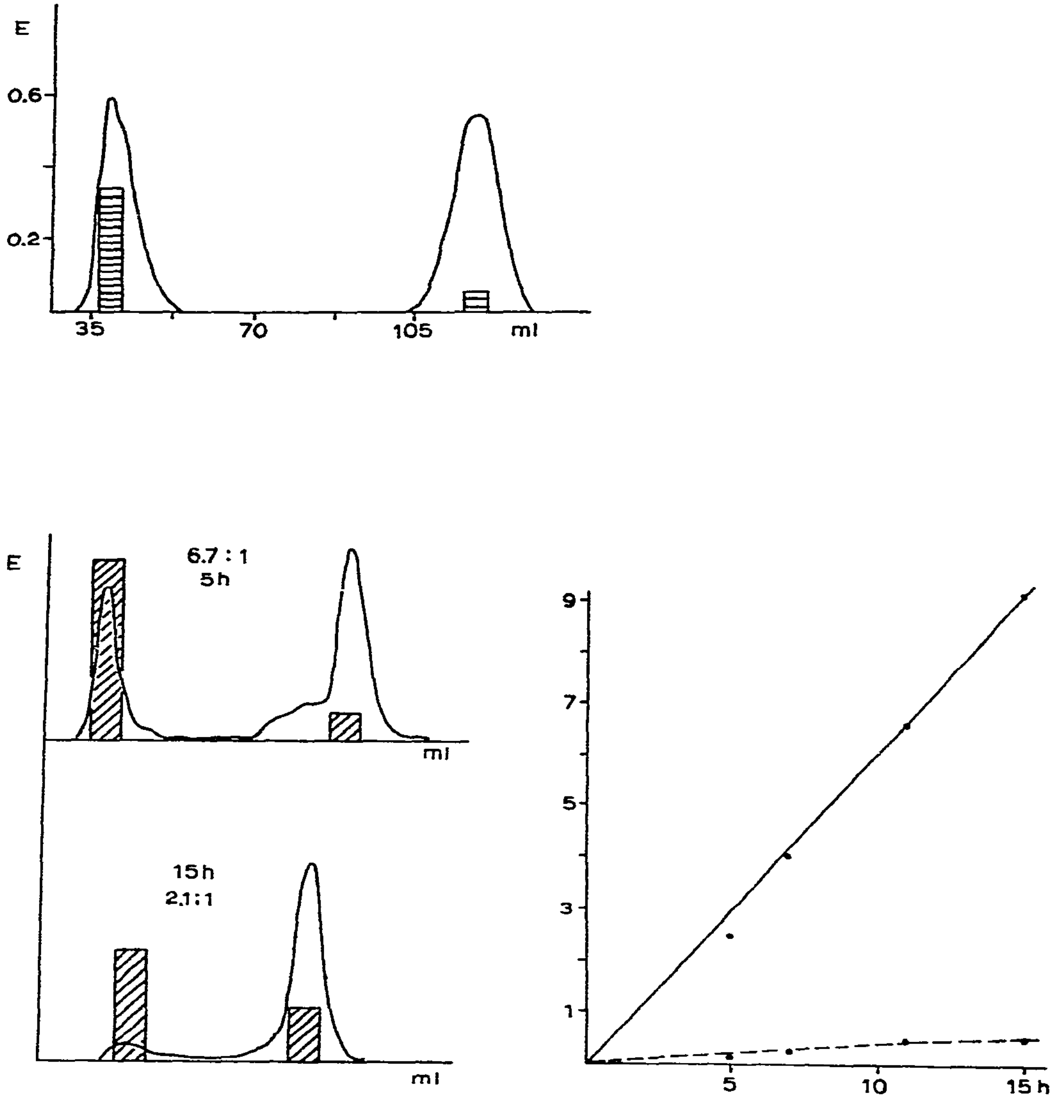
Cleavage of the peptide part of BGS. (*a*). The above polymeric material (90 mg) was dissolved in 0.1N acetic acid (3 ml), bromine water was added until a colour remained, and the excess of bromine was immediately removed by the addition of 0.02M $Na_2S_2O_3$. The mixture was brought to pH 11 with 2N NaOH and, after 30 min, acidified with acetic acid and subjected to gel-filtration.

(*b*) The above polymeric material (30 mg) in dilute HCl (pH 1.5, 3 ml) was heated at 100° for 3 h then neutralised and subjected to gel-filtration.

RESULTS AND DISCUSSION

Monitoring of the course of biopolymer degradation by analytical gel-filtration.

Data obtainable by the usual method of biopolymer structure elucidation, *i.e.* degradation and determination of the structure of low-molecular weight fragments, seem, in the case of certain complex biopolymers, unsuitable for the establishment of the general picture of biopolymer molecular architecture. The main difficulties are connected with the isolation and structure determination of series of overlapping fragments which require large amounts of biopolymer. In order to overcome the weak points of this classical method, a new approach has been developed which avoids the investigation of individual fragments and which consists of monitoring polymer degradation by analysis of the composition and molecular weight of all the fragments formed. For this purpose, the biopolymer is subjected to partial degradation by an appropriate method and aliquot parts of the reaction mixture are analysed by gel-filtration. The use of different gels permits the separation of fragments according to their molecular weight and permits a distinction between the low-molecular weight fragments split off and the macromolecular "core" of the polymer. Quantitative determination of the components in the fractions obtained allows the fragmentation pattern to be built up. A knowledge of the specificity of the degradation method used and the data on changes of composition and molecular weights of the fragments released during the reaction enables a general picture of the biopolymer molecular



Gel-filtration of the products obtained by hydrolysis of BGS-A. Fig. 1. Dilute HCl (pH 1.5), 3h, 100°, Sephadex G-200 (2.16 × 63 cm); Fig. 2, 0.4N HCl, 90°, Sephadex G-25 (1.8 × 65 cm); ——— reaction with anthrone, ▨ relative amino acid contents (schematic). Fig. 3. Relative velocities of splitting of sugars and amino acids from BGS-A on hydrolysis with 0.4N HCl at 90°; relation between quantities of carbohydrates (———) and amino acids (– – – –) in low molecular weight and polymer fractions.

architecture to be established. All analyses in this approach may be performed on a microscale.

We have applied such an approach to BGS using mild acid and alkaline hydrolysis for the degradation of sugar chains, carbohydrate–peptide bonds, and peptide chains.

Acid hydrolysis of BGS-A. The acid hydrolysis of BGS has been carefully studied[1] and the structure of the terminal units of the carbohydrate chains established[15]. Reinvestigation of the acid hydrolysis of BGS using the analytical gel-filtration technique[16] permits a new important conclusion to be made. The separation pattern (Fig. 1) of the products of a mild acid hydrolysis of BGS-A (pH 1.5, 100°, 3 h) using Sephadex G-200 established the following points: (*a*) The polymeric core of the macromolecule was not significantly degraded and possibly remains unaltered. Since it is not retained by Sephadex G-200, the core of BGS-A has a very high molecular weight, and fractionation in 6M urea proved the absence of aggregation of fragments. (*b*) Intermediate fractions, which should contain the fragments of intermediate molecular weight, were absent (the low molecular weight fractions contained only monosaccharides and lower oligosaccharides as established by gel-filtration on Sephadex G-25). (*c*) The monosaccharides were approximately evenly distributed between the core and the low molecular fragments, whereas *the amino acids remained almost entirely in the core.* (*d*) The content of galactose and hexosamines in the core and the low molecular fractions was comparable and that, apparently, these monosaccharides are distributed rather uniformly in the carbohydrate part of BGS-A, whereas most of the fucose was present in the fragments of low molecular weight, thus confirming the terminal position of this monosaccharide in the carbohydrate chains.

Thus, no substantial degradation of the peptide part of BGS occurs during mild acid hydrolysis, since it remains completely in the core. Under more vigorous conditions (0.4N HCl, 90°, 5 and 15 h) degradation of the peptide chain occurs, but even in this case, the release of monosaccharides proceeds much more rapidly than that of amino acids, as can be seen from the patterns in Fig. 2. A comparison of the velocities of cleavage of the monosaccharides and amino acids under the above conditions is shown in Fig. 3.

Alkaline degradation of BGS-A. The lability of BGS in solutions with pH > 7, even in very mild conditions (*i.e.* treatment with sodium carbonate solution), is well known[14]. However, this peculiarity has not yet been utilised in the elucidation of structure. Obviously, this susceptibility of BGS may be a consequence of the presence of alkali-labile carbohydrate–peptide bonds including, for example, *O*-glycosidic bonds of serine and/or threonine residues, or *O*-acylglycosidic bonds involving dibasic amino acid residues.* If this is so, *O*-glycosidic bonds of hydroxy amino-acids and dibasic acids in media with pH > 7 should cleave with the formation of carbohydrate fragments and liberation, within the peptide chain, of modified serine, threonine, or dibasic acid residues. An investigation of simple *O*-glycosides of serine[17]

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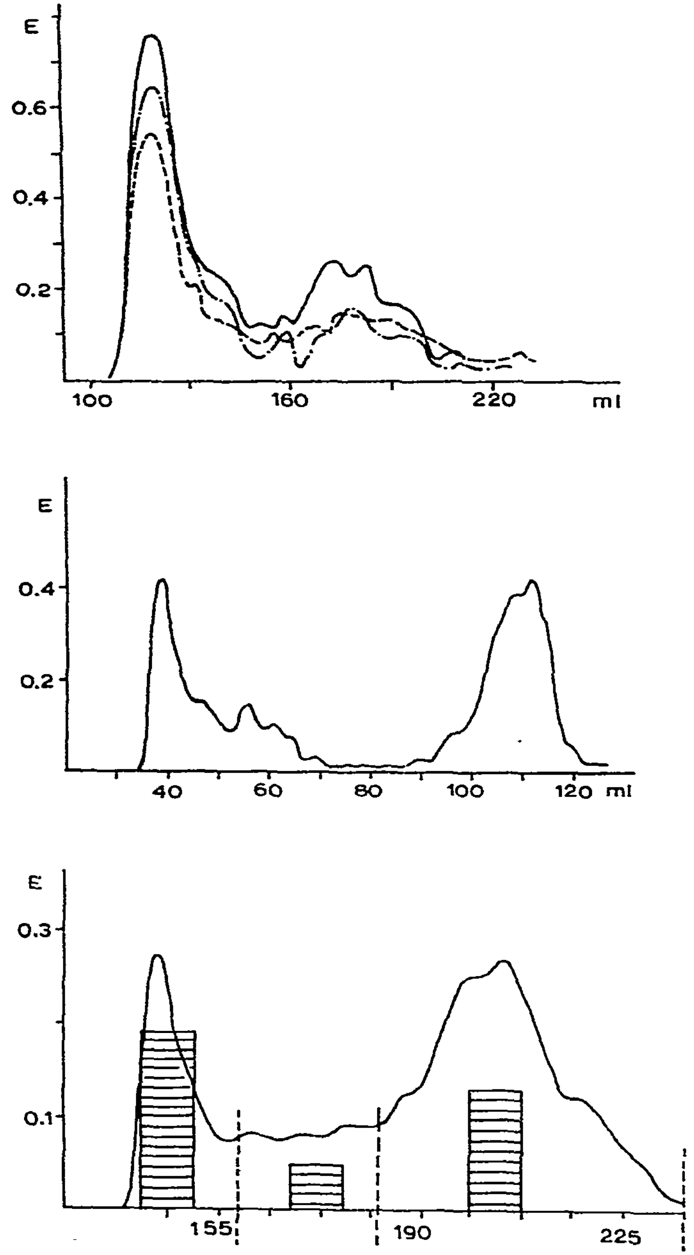
* The possibility that oligosaccharide chains contain some alkali-labile bonds cannot be excluded.



Gel-filtration of products obtained by treatment of BGS-A with 0.1N NaOH + 1% NaBH4: Fig. 4. 6 days, 17°, Sephadex G-25 (2 × 117 cm); Fig 5. 6 days, 17°, Sephadex G-100 (1.8 × 66 cm); Fig. 6. 15 days, 25°, Sephadex G-25 (2 × 130 cm); ——— reaction with anthrone, – – – –Elson–Morgan reaction, –·–·– Dische and Shettles reaction, ▤ relative amino acid contents (schematic).

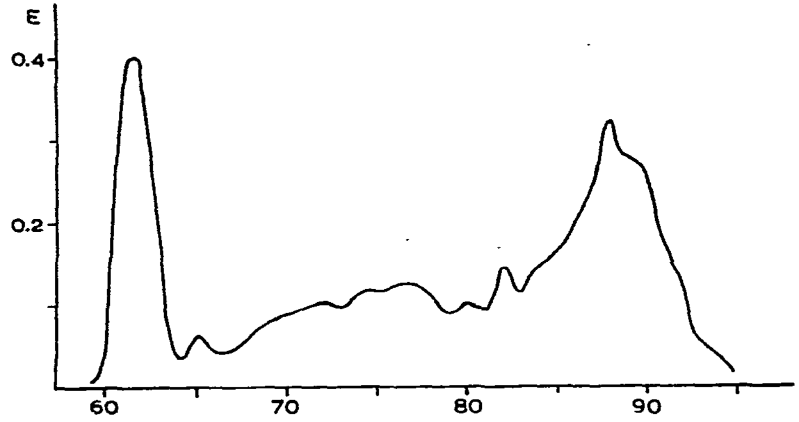
showed that, at pH > 7, these compounds are highly labile and undergo β-elimination with the formation of α-aminoacrylic acid derivatives. When alkaline degradation is carried out in the presence of sodium borohydride, the α-aminoacrylic acid residues (formed from serine) are reduced to alanine. This approach permitted a demonstration of the presence of *O*-glycosidic bonds in mucins of submaxillary glands[18], and, recently[19,20], in some other glycoproteins.

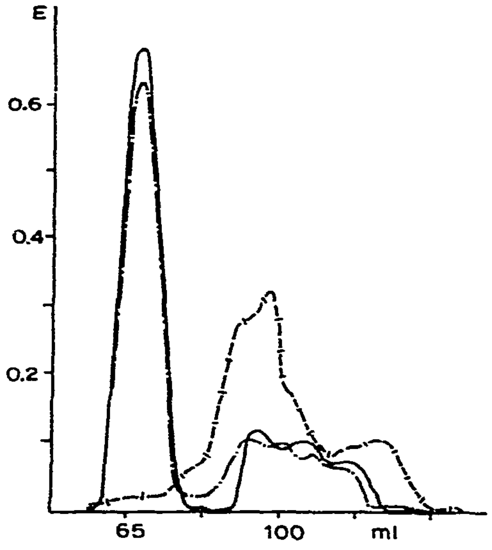
Treatment of BGS-A with 0.1N sodium hydroxide in the presence of 1% sodium borohydride with subsequent total hydrolysis of the resulting modified biopolymer caused a characteristic change in the amino acid composition (see Table I); the serine and threonine contents were decreased whereas the contents of the other amino acids remained constant and that of alanine was increased. These results constitute unambiguous evidence[21] of the presence of *O*-glycosidic bonds between hydroxy amino acids and the carbohydrate chains in BGS-A. This type of bond is, without doubt, the main one involved in the carbohydrate–peptide linkages. It should be mentioned that a part of the carbohydrate content remained attached to the polypeptide chain even after rather drastic acid and alkaline hydrolysis, possibly indicative of other types of carbohydrate–peptide linkage, for example *N*-acyl-glycosylamine bonds involving aspartic and glutamic acids; this type of bond has been detected in various glycoproteins.

Data obtained from the acid hydrolysis of BGS-A support the conclusion that *O*-glycosidic bonds form the main type of carbohydrate–peptide linkage; under the conditions chosen (pH 1.5, 100°, 3 h) the model serine glycosides[17] are stable.

The patterns of separation of the products of the alkaline degradation of BGS in the presence of sodium borohydride (6 days at 20°) on Sephadex G-25 and G-100 are represented in Figs. 4 and 5. The following conclusions may be drawn. (*a*) Almost all of the amino acids remain in the core of the biopolymer. The degradation of the peptide part of BGS may be observed only under more vigorous conditions (see Fig. 6). (*b*) The monosaccharides and lower oligosaccharides are almost absent from the reaction mixture. In other words, the carbohydrate fragments split off have rather high molecular weights (established by comparison of the gel-filtration patterns on Sephadex G-25 and G-100). (*c*) The molecular weights of the carbohydrate fragments formed are similar and clearly different from that of the core since there is no intermediate fraction on gel-filtration on Sephadex G-100. (*d*) All the components of macromolecular fraction have high molecular weights, although the gel-filtration of this fraction on 7% agar-gel shows a marked polydispersity (Fig. 7). (*e*) Galactose and hexosamines are split off in a regular manner thus proving the approximately uniform distribution of the monosaccharides in the carbohydrate part of BGS.

The same conclusion may be drawn from results obtained by application of the analytical gel-filtration technique to the degradation of BGS-A with sodium carbonate solution (Fig. 8). The macromolecular fragments generated in this case were proved, by gel-filtration on Sephadex G-100, to have higher molecular weights than in the former case. Consequently, the biopolymer is less extensively ruptured than in the alkaline degradation.





Gel-filtration of products obtained by treatment of BGS-A with 0.1N NaOH + 1% NaBH$_4$, 6 days, 17°, 7% agar gel (3 × 133 cm) (Fig. 7); 0.05M Na$_2$CO$_3$, 3 min, 100°, Sephadex G-25 (1.2 × 66 cm) (Fig. 8); ——— reaction with anthrone, -·-·- Dische and Shettles reaction, +—+—+— reaction with Ehrlich's reagent.

Cleavage of the peptide part of BGS-A

As seen from the above results, the degradation of BGS by acid or alkali occurs with retention of the macromolecular framework (core) of the biopolymer

TABLE I

CHANGE IN THE AMINO ACID COMPOSITION OF BGS-A AFTER DEGRADATION
(0.1N NaOH + 1% $NaBH_4$, 6 days at 17°)

Amino acid	*Contents of amino acid in μM*		
	BGS	*Degraded BGS*	*Difference in μM*
Asparatic acid	0.241	0.245	—
Threonine	1.988	1.567	−0.421
Serine	0.999	0.609	−0.390
Proline	1.218	1.199	—
Glycine	0.316	0.314	—
Alanine	0.563	0.833	+0.270
Glutamic acid	0.352	0.346	—

which contains practically all of the peptide portion of BGS. Thus, one may conclude that a peptide chain forms the skeleton of the macromolecule. In order to verify this idea a specific method of degradation of the peptide chain of BGS is required. As is well known, the structural study of BGS lacks a suitable method of this sort. The cleavage of peptide bonds takes place only under very vigorous acid or alkaline conditions which also split almost all of the glycosidic bonds resulting in extensive degradation of the biopolymer. The proteolysis of BGS has, to date, been of little value. The action of ficin and papain results in only a few ruptures of the peptide chain[22]. The protease from the allantoic fluid of chick embryos infected by Asian influenza virus[23] is much more effective, but the enzyme has not yet been isolated in a pure state with the appropriate activity.

We have used a chemical approach for the degradation of the peptide chain of BGS-A based on the reactions of peptides containing unsaturated amino acid residues[24,25] (*i.e.* α-aminoacrylic and related acids) as studied on model compounds by Patchornik *et al.*[26]. The introduction of an enamine grouping into the BGS peptide chain can be easily achieved by the treatment with base. This results in the elimination of the carbohydrate chains form the serine and threonine residues and the conversion of these hydroxy amino acids into unsaturated α-amino acids with retention of the intact peptide chain. In order to decrease the number of these unsaturated groupings, the alkaline treatment of BGS-A was carried out in the presence of small amounts of sodium borohydride which reduced some of the enamine units. The modified polymer, isolated by preparative gel-filtration, was treated with bromine with subsequent adjustment to pH 10.8 or subjected to mild acid hydrolysis. The results of gel-filtration of the mixture formed after bromine treatment are represented in Fig. 9. As can be seen, such degradation causes a disappearance of the fragments of high molecular weight and the formation of a mixture of fragments of intermediate molecular weight showing polydispersity; the monosaccharides and lower oligosaccharides were absent. Thus, unlike the acid and alkaline degradations, the macromolecular framework of BGS is destroyed. That cleavage takes place at the enamine links of the modified BGS was established in experiments with unmodified BGS. Under similar conditions, very little degradation occurred and this was possibly associated with the attack of histidine residues by bromine.

For the estimation of the amino acid contents in the fractions obtained, the degradation of modified BGS by means of weak acid is more convenient. The results of gel-filtration of the reaction mixture after such a treatment are represented in Fig. 10. The analysis of amino acid contents was performed by means of the histidine determination.

The gel-filtration pattern (Fig. 10) shows that, in contrast to the acid and alkaline degradations, the distribution of amino acids is uniform and establishes that the degradation described above is connected with rupture of the peptide chain which formed the macromolecular skeleton of BGS.

It should be mentioned that degradation of the peptide chain of modified BGS by mild acid hydrolysis, in contrast to bromine degradation, leads to partial rupture of glycosidic bonds in the carbohydrate part of the biopolymer and, therefore, low molecular fractions appear containing the monosaccharides and lower oligosaccharides.

It seems that this new method for the degradation of the peptide chain of BGS, including the removal of the carbohydrate chain and cleavage of the resulting enamine links, may be of value for other glycoproteins containing *O*-glycosidic carbohydrate–peptide bonds. Moreover, this approach may also be of interest in protein chemistry although, in this case, special activation of serine and threonine hydroxyl groups will be necessary.

CONCLUSIONS

Acid and alkaline degradations of BGS with the analytical gel-filtration technique show quite clearly that, contrary to previous conclusions[27], a peptide chain forms the skeleton of the macromolecule. The macromolecular fragment obtained after acid hydrolysis contains nearly all the amino acids whereas peptide chain degradation leads to disintegration of the macromolecular framework without the appearance of low-molecular substances. This conclusion is supported by the application of hydrazinolysis (known to be a non-specific method for the degradation of peptide chains) to BGS. Extensive degradation of the macromolecule occurs with formation of carbohydrate fragments of rather high molecular weight together with hydrazides of amino acids and lower peptides*, as shown in Fig. 11.

The carbohydrate part of BGS-A consists of oligo- and/or polysaccharide chains attached to the central peptide chain by *O*-glycosidic bonds although other types of peptide–carbohydrate bonding may be present because carbohydrate still remains attached to the macromolecular fraction after vigorous acid hydrolysis which causes some degradation of the peptide part of the biopolymer. The size of the carbohydrate chains is not known. It follows, from our data and the results of Kabat *et al.*[28],

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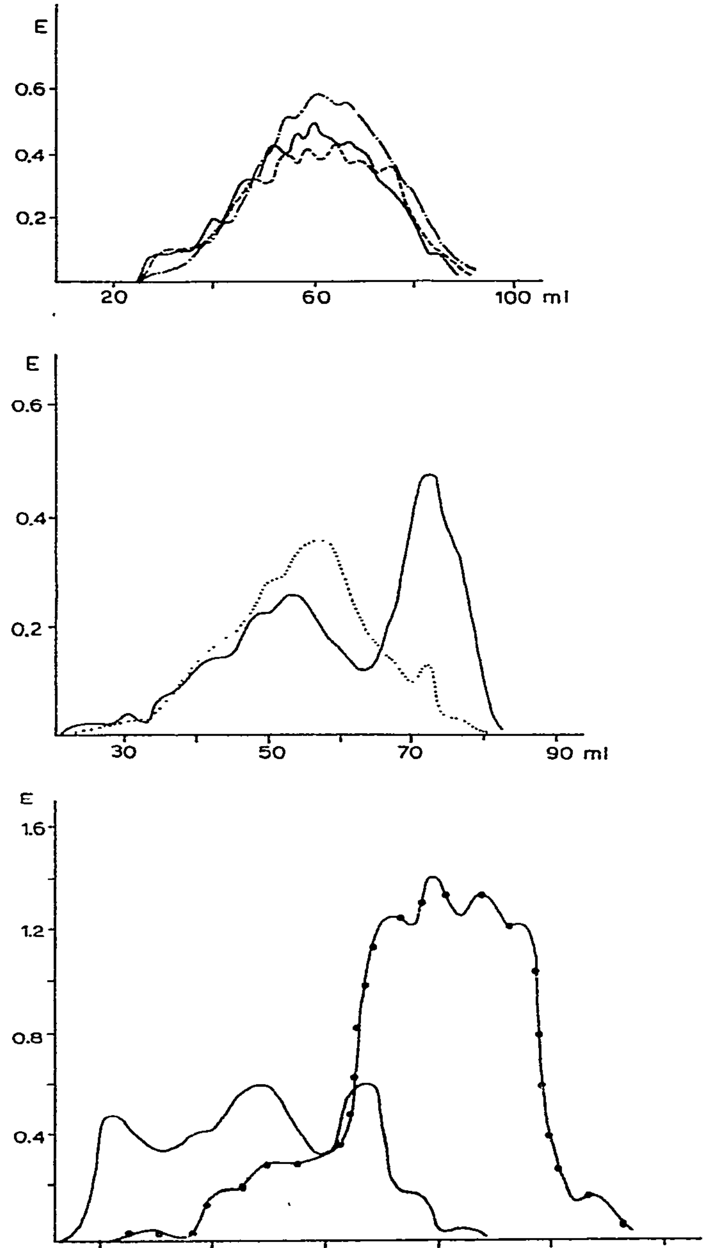
*Details of this part of the investigation will be published elsewhere.



Gel-filtration of products obtained by treatment (see text) of modified BGS-A with bromine water and alkali, Sephadex G-100 (1.2 × 70 cm) (Fig. 9); by treatment with HCl (pH 1.5), 3 h, 100°, Sephadex G-100 (1.2 × 70cm) (Fig. 10); by treatment of BGS-A with anhydrous hydrazine, 15 h, 60°, Sephadex G-25 (1.8 × 65 cm); ——— reaction with anthrone, - - - - - Elson–Morgan reaction, -·-·-· Dische and Shettles reaction, ... histidine content, —•—•— reaction with TNBS.

that these chains should be rather long and Morgan's assumption[4] of chains containing 7–10 monosaccharide units seems to be unlikely. There are no chemical data on the differences of the size and structure of the carbohydrate chains and they may not be similar but fucose occupies mainly terminal positions.

The general molecular architecture of BGS is similar to that of other animal glycoproteins. The data presented herein provide the first experimental support of the hypothesis suggested by Morgan based on genetic and biochemical considerations[3–6]. Gibbons and Roberts[29] and Schiffman *et al.*[10] considered the lability of BGS toward alkali as incompatible with this suggestion. However, as can be seen from the above discussion, this lability is in complete agreement with the proposed structure of BGS-A

The data presented in this article are concerned only with BGS-A isolated from pig stomach linings, but the fact that the change of the specificity of blood group substances can be effected by alteration of the terminal carbohydrate residues suggests that a similar structure is likely for other blood group substances.

SUMMARY

The molecular architecture of blood group substance-A (BGS), isolated from hog stomach linings, was investigated by chemical degradation and subsequent analytical gel-filtration of the products formed. Acid hydrolysis, alkaline degradation, and a new method of peptide chain cleavage at the sites of enamine groups were studied. The data obtained confirm the hypothesis that BGS comprises a peptide chain with carbohydrate moieties as side chains. *O*-Glycosidic bonds involving serine and threonine residues constitute the main type of carbohydrate–peptide linkage.

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D-*arabino*-HEXOSULOSE BIS(ACYLHYDRAZONES) AND 2-ACYLHYDRAZONE 1-ARYLHYDRAZONES

H. El Khadem and M. A. E. Shaban
Chemistry Department, Faculty of Science, Alexandria University, Alexandria, Egypt (U.A.R.)

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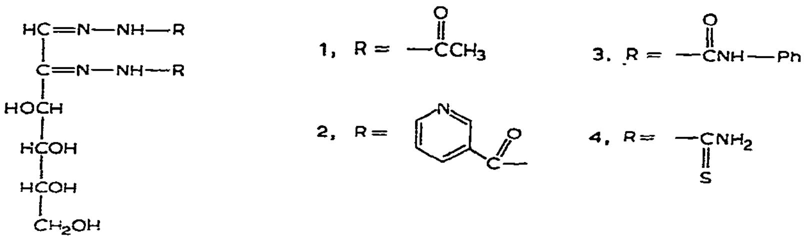
Hexosulose bis(benzoylhydrazones) have recently[1] been converted into 1-acylamido-1,2,3-triazole enol acylates by oxidizing their tetra-*O*-acetyl derivatives with iodine and mercuric oxide. We have now prepared a number of bis(acylhydrazones) other than the benzoyl and substituted benzoyl derivatives, as well as some 2-acylhydrazone 1-arylhydrazones, to study their reactions.

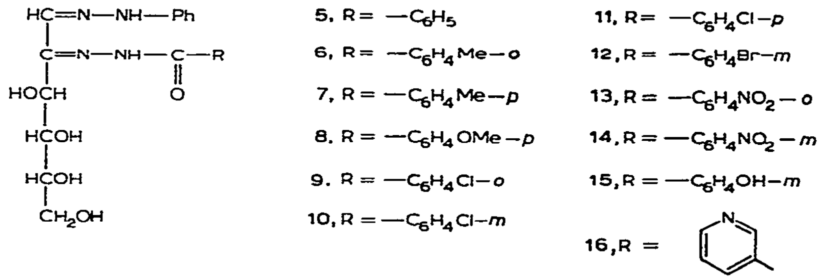
Of the bis(acylhydrazones), we prepared D-*arabino*-hexosulose bis(acetylhydrazone) (**1**), bis(nicotinoylhydrazone (**2**), bis(4-phenylsemicarbazone) (**3**), and bis(thiosemicarbazone) (**4**), from D-*arabino*-hexosulose and the corresponding acylhydrazine or semicarbazide. Compound (**3**) had been prepared by Stroh[2], who reported a melting point of 233–235°; our product, however, melted at 245°. The acetates of these bis(acylhydrazones) could not be isolated in crystalline form, probably because these hydrazones, like the bis(semicarbazones)[3], yielded a mixture of acetylated products. Like the bis(benzoylhydrazones)[1], these bis(acylhydrazones) are colorless. They probably exist in the enol form, as evidenced by the characteristic colorations they give with ferric chloride, and this might explain why they fail to give the formazan reaction[4] (to be expected from the hydrazone residue on C-1 in the keto form).



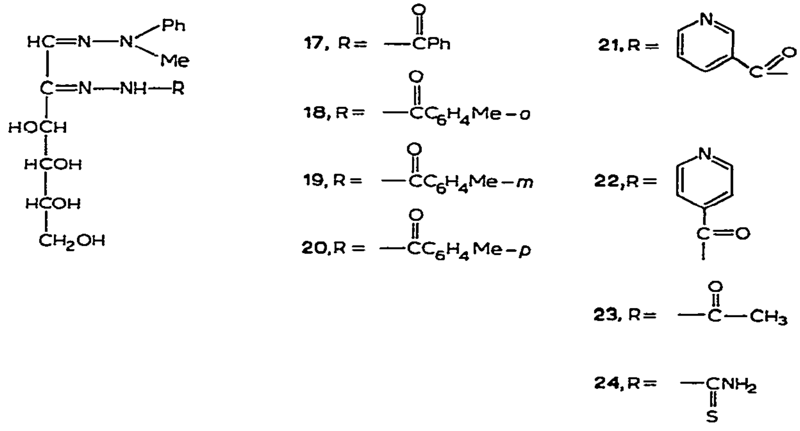
We have also prepared a number of D-*arabino*-hexosulose 2-acylhydrazone 1-phenylhydrazones (**5–16**) from D-*arabino*-hexosulose 1-phenylhydrazone[5] and the corresponding acylhydrazine. All of these mixed osazones, like the arylosazones, are yellow. They fail, however, to give crystalline acetates, and could not be converted into the 1-substituted 1,2,3-triazole with iodine and mercuric oxide. They react with copper sulfate, but, instead of being cyclized to the osotriazoles (like the arylosazones), are hydrolyzed to the glucosulose.

Like the acylosazones, these mixed osazones readily gave the formazan reaction.

Their acylhydrazone residue also seems to be in the enol form, as evidenced by the coloration they give with ferric chloride, but in this case, the enolized acylhydrazone residue on C-2 does not prevent occurrence of the formazan formation (which takes place by coupling on C-1). Unlike the arylosazone acetates, which give anhydro derivatives on deacetylation[6], the amorphous acetates obtained from these mixed osazones afford the starting osazones on saponification.



In addition, we have prepared another type of D-*arabino*-hexosulose 2-acylhydrazone 1-arylhydrazone (**17–24**) from D-*arabino*-hexosulose 1-(2-methyl-2-phenylhydrazone)[7] and acylhydrazines. These mixed osazones are also yellow, and give characteristic colorations with ferric chloride, suggesting the enolization of their respective acylhydrazone residue. Their crystalline acetates displayed the expected resistance toward oxidation (to enol acetates).



EXPERIMENTAL

Microanalyses were performed by A. Bernhardt, Mulheim, Germany. Infrared absorption spectra were recorded on a Unicam SP 200 spectrophotometer, and ultraviolet absorption spectra on a Unicam SP 800.

D-arabino-*Hexosulose bis(acetylhydrazone)* (**1**). — A solution of D-*arabino*-

hexosulose (1 g) in methanol (10 ml) was treated with acetylhydrazine (0.7 g) and a few drops of glacial acetic acid. The mixture was kept for 48 h at 0°, and the bis-(hydrazone) that separated was filtered off, washed, and dried (0.9 g). It crystallized from water–methanol in colorless plates, m.p. 215–216° (dec.); $[\alpha]_D^{20}$ −18.1° (*c* 1.7, water); λ_{max}^{EtOH} 296 mμ, log ε 4.49; λ_{min} 225 mμ, log ε 3.62; ν_{max}^{KBr} 1610 (C=N), 1675, 1695 (CONH), 3290 cm^{-1} (OH).

Anal. Calc. for $C_{10}H_{18}N_4O_6$: C, 41.4; H, 6.2; N, 19.3. Found: C, 41.6, H, 6.3; N, 19.2.

D-arabino-*Hexosulose bis(nicotinoylhydrazone)* (**2**). — A solution of D-*arabino*-hexosulose (1 g) in methanol (10 ml) was treated with nicotinoylhydrazine (1.5 g) as before, and the separated bis(hydrazone) (1 g) was filtered off, washed, and crystallized from water–methanol in colorless needles, m.p. 200–202° (dec.); $[\alpha]_D^{20}$ −52.8° (*c* 0.5, pyridine); λ_{max}^{EtOH} 251, 320 mμ, log ε 1.87, 4.36; λ_{min} 217, 286 mμ, log ε 3.86, 4.18; ν_{max}^{KBr} 1595 (C=N), 1665, 1685 (CONH), 3360 cm^{-1} (OH).

Anal. Calc. for $C_{18}H_{20}N_6O_6 \cdot 0.5\ H_2O$: C, 50.8; H, 4.9; N, 19.8. Found: C, 50.7; H, 5.0; N, 19.6.

D-arabino-*Hexosulose bis(4-phenylsemicarbazone)* (**3**). — A solution of 4-phenylsemicarbazide hydrochloride (2.2 g) in methanol (10 ml) was treated with powdered sodium acetate (0.5 g) and the mixture was filtered. To the filtrate, a solution of D-*arabino*-hexosulose (1 g) in methanol (10 ml) was added, and the mixture was kept overnight at room temperature. Compound **3** (0.8 g) was filtered off, washed, and crystallized from aqueous methanol in needles, m.p. 244–245° (dec.); $[\alpha]_D^{20}$ −114.9° (*c* 0.2, pyridine); ν_{max}^{KBr} 1600 (C=N), 1640, 1665 (CONH), 3300 cm^{-1} (OH).

Anal. Calc. for $C_{20}H_{24}N_6O_6 \cdot 0.5\ H_2O$: C, 53.1; H, 5.5; N, 18.6. Found: C, 53.2; H, 5.7; N, 18.9.

D-arabino-*Hexosulose bis(thiosemicarbazone)* (**4**). — A solution of D-*arabino*-hexosulose (1 g) in methanol (10 ml) was treated with a solution of thiosemicarbazide (1 g) in 50% aqueous methanol (10 ml) and a few drops of glacial acetic acid, and the mixture was kept for 24 h at 0°. The product (0.9 g) was filtered off, washed, and crystallized from methanol in yellow needles, m.p. 230–232° (dec.); $[\alpha]_D^{20}$ −27.2° (*c* 0.4, pyridine); ν_{max}^{KBr} 1500 (C=S), 1612 (C=N), 3220 cm^{-1} (OH).

Anal. Calc. for $C_8H_{16}N_6O_4S_2$: C, 29.6; H, 4.9; N, 25.9. Found: C, 29.8; H, 5.0; N, 25.8.

D-arabino-*Hexosulose 2-benzoylhydrazone 1-phenylhydrazone* (**5**). — A solution of D-*arabino*-hexosulose 1-phenylhydrazone (1 g) in water (15 ml) was treated with a solution of benzoylhydrazine (0.6 g) in methanol (5 ml) and a few drops of glacial acetic acid. After 24 h at room temperature, the product was filtered off, washed, and dried (1.2 g). It crystallized from ethanol in yellow, prismatic needles, m.p. 208–210° (dec.); $[\alpha]_D^{20}$ +54.2° (*c* 1.25, pyridine); λ_{max}^{EtOH} 204, 242, 278, 385 mμ, log ε 4.31, 4.23, 4.10, 4.34; λ_{min} 219, 265, 317 mμ, log ε 4.07, 4.07, 3.61; ν_{max}^{KBr} 1603 (C=N), 1665 (CONH), 3380 cm^{-1} (OH).

Anal. Calc. for $C_{19}H_{22}N_4O_5$: C, 59.1; H, 5.7; N, 14.5. Found: C, 59.1; H, 6.0; N, 14.5.

D-arabino-*Hexosulose 1-phenylhydrazone 2-*o*-toluoylhydrazone* (**6**). — A solution of D-*arabino*-hexosulose 1-phenylhydrazone (1 g) in water (15 ml), when treated with *o*-toluoylhydrazine (0.7 g), as above, gave compound **6** (0.9 g). It crystallized from aqueous ethanol in yellow, prismatic needles, m.p. 195° (dec.); $[\alpha]_D^{20}$ $-50.3°$ (*c* 0.78, pyridine); λ_{max}^{EtOH} 203, 245, 275, 376 mμ, log ε 4.64, 4.51, 4.37, 4.61; λ_{min} 222, 267, 318 mμ, log ε 4.35, 4.36, 3.80; ν_{max}^{KBr} 1603 (C=N), 1670 (CONH), 3400 cm^{-1} (OH).

Anal. Calc. for $C_{20}H_{24}N_4O_5 \cdot 0.5\ H_2O$: C, 58.7; H, 6.1; N, 13.7. Found: C, 59.1; H, 6.5; N, 13.5.

D-arabino-*Hexosulose 1-phenylhydrazone 2-*p*-toluoylhydrazone* (**7**). — D-*arabino*-Hexosulose 1-phenylhydrazone (1 g) was dissolved in water (20 ml) and treated with *p*-toluoylhydrazine (0.7 g), as above. The product (1.5 g) was filtered off, and crystallized from ethanol in yellow, prismatic needles, m.p. 214–215° (dec.); $[\alpha]_D^{20}$ $+75.1°$ (*c* 1.17, pyridine); λ_{max}^{EtOH} 204, 248, 276, 385 mμ, log ε 4.34, 4.23, 4.20, 4.31; λ_{min} 221, 265, 319 mμ, log ε 4.06, 4.19, 3.59; ν_{max}^{KBr} 1603 (C=N), 1665 (CONH), 3400 cm^{-1} (OH).

Anal. Calc. for $C_{20}H_{24}N_4O_5$: C, 60.0; H, 6.0; N, 14.0. Found: C, 60.4; H, 6.3; N, 14.0.

D-arabino-*Hexosulose 2-(*p*-methoxybenzoyl)hydrazone 1-phenylhydrazone* (**8**). — This was obtained by treating D-*arabino*-hexosulose 1-phenylhydrazone (1 g) with (*p*-methoxybenzoyl)hydrazine (0.7 g), by the procedure given above. The product (1.5 g) crystallized from ethanol in yellow, prismatic needles, m.p. 210–212° (dec.); $[\alpha]_D^{20}$ $+90.2°$ (*c* 0.78, pyridine); λ_{max}^{EtOH} 203, 283, 380 mμ, log ε 4.44, 4.27, 4.39; λ_{min} 225, 259, 324 mμ, log ε 4.15, 4.29, 3.81; ν_{max}^{KBr} 1255 (OMe), 1605 (C=N), 1660 (CONH), 3360 cm^{-1} (OH).

Anal. Calc. for $C_{20}H_{24}N_4O_6$: C, 57.8; H, 5.8; N, 13.5. Found: C, 57.9; H, 6.0; N, 13.6.

D-arabino-*Hexosulose 2-(*o*-chlorobenzoyl)hydrazone 1-phenylhydrazone* (**9**). — A solution of D-*arabino*-hexosulose 1-phenylhydrazone (1 g) in water (15 ml) was treated with (*o*-chlorobenzoyl)hydrazine (0.7 g) as above. The product (0.8 g) crystallized from aqueous ethanol in yellow needles, m.p. 168° (dec.); $[\alpha]_D^{20}$ $+20.0°$ (*c* 1.04, pyridine); λ_{max}^{EtOH} 203, 243, 275, 375 mμ, log ε 4.12, 4.18, 4.05, 4.13; λ_{min} 226, 263, 316 mμ, log ε 4.06, 4.04, 3.27; ν_{max}^{KBr} 1600 (C=N), 1630 (CONH), 3350 cm^{-1} (OH).

Anal. Calc. for $C_{19}H_{21}ClN_4O_5 \cdot H_2O$: C, 52.0; H, 5.2; N, 12.8. Found: C, 52.4; H, 5.5; N, 13.0.

D-arabino-*Hexosulose 2-(*m*-chlorobenzoyl)hydrazone 1-phenylhydrazone* (**10**). — This was prepared, as above, from D-*arabino*-hexosulose 1-phenylhydrazone (1 g) and (*m*-chlorobenzoyl)hydrazine (0.7 g). It crystallized from aqueous ethanol in yellow, prismatic plates, m.p. 205° (dec.); $[\alpha]_D^{20}$ $+93.5°$ (*c* 0.28, pyridine); λ_{max}^{EtOH} 204, 241, 279, 376 mμ, log ε 4.88, 4.62, 4.59, 4.77; λ_{min} 226, 260, 318 mμ, log ε 4.54, 4.53, 4.50; ν_{max}^{KBr} 1600 (C=N), 1660 (CONH), 3350 cm^{-1} (OH).

Anal. Calc. for $C_{19}H_{21}ClN_4O_5 \cdot 0.5\ H_2O$: C, 53.1; H, 5.1; N, 13.0. Found: C, 53.2; H, 5.3; N, 13.1.

D-arabino-*Hexosulose 2-(*p*-chlorobenzoyl)hydrazone 1-phenylhydrazone* (**11**). —

D-*arabino*-Hexosulose 1-phenylhydrazone (1 g), treated with (*p*-chlorobenzoyl)-hydrazine (0.7 g) as above, gave compound **11** (1.3 g). It crystallized from ethanol in yellow, prismatic needles, m.p. 218–220° (dec.); $[\alpha]_D^{20}+78.8°$ (*c* 1.24, pyridine); λ_{max}^{EtOH} 204, 244, 278, 384 mμ, log ε 4.21, 4.09, 3.92, 4.22; λ_{min} 223, 274, 318 mμ, log ε 3.91, 3.92, 3.48; ν_{max}^{KBr} 1600 (C=N), 1660 (CONH), 3400 cm^{-1} (OH).

Anal. Calc. for $C_{19}H_{21}ClN_4O_5$: C, 54.2; H, 5.0; N, 13.3. Found: C, 54.3; H, 5.4; N, 13.1.

D-arabino-*Hexosulose 2-(*m*-bromobenzoyl)hydrazone 1-phenylhydrazone* (**12**). — D-*arabino*-Hexosulose 1-phenylhydrazone (1 g) was treated with (*m*-bromobenzoyl)-hydrazine (0.8 g) as above. The product (1 g) crystallized from aqueous ethanol in yellow, prismatic needles, m.p. 208–210° (dec.); $[\alpha]_D^{20}+58.6°$ (*c* 0.46, pyridine); λ_{max}^{EtOH} 205, 285, 384 mμ, log ε 4.40, 4.00, 4.27; λ_{min} 268, 322 mμ, log ε 3.95, 3.54; ν_{max}^{KBr} 1600 (C=N), 1663 (CONH), 3370 cm^{-1} (OH).

Anal. Calc. for $C_{19}H_{21}BrN_4O_5 \cdot H_2O$: C, 47.2; H, 4.8; N, 11.6. Found: C, 47.0; H, 4.7; N, 11.4.

D-arabino-*Hexosulose 2-(*o*-nitrobenzoyl)hydrazone 1-phenylhydrazone* (**13**). — A solution of D-*arabino*-hexosulose 1-phenylhydrazone (1 g) in water (10 ml), treated with (*o*-nitrobenzoyl)hydrazine (0.7 g), gave **13** (0.8 g). It crystallized from aqueous methanol in yellow, prismatic needles, m.p. 164–165° (dec.); λ_{max}^{EtOH} 204, 244, 368 mμ, log ε 4.37, 4.30, 4.32; λ_{min} 225, 313 mμ, log ε 4.18, 3.74; ν_{max}^{KBr} 860, 1350, 1540 (NO_2), 1605 (C=N), 1688 (CONH), 3400 cm^{-1} (OH).

Anal. Calc. for $C_{19}H_{21}N_5O_7 \cdot 0.5\ H_2O$: C, 51.8; H, 5.0; N, 15.9. Found: C, 52.0; H, 5.3; N, 15.9.

D-arabino-*Hexosulose 2-(*m*-nitrobenzoyl)hydrazone 1-phenylhydrazone* (**14**). — Treatment of D-*arabino*-hexosulose 1-phenylhydrazone (1 g) with (*m*-nitrobenzoyl)-hydrazine (0.7 g), as above, gave **14** (0.9 g). It crystallized from ethanol in yellow needles, m.p. 195–196° (dec.); $[\alpha]_D^{20}+70.5°$ (*c* 0.39, pyridine); λ_{max}^{EtOH} 204, 221, 384 mμ, log ε 4.29, 4.28, 4.26; λ_{min} 209, 318 mμ, log ε 4.25, 3.63; ν_{max}^{KBr} 890, 1355, 1536 (NO_2), 1600 (C=N), 1665 (CONH), 3380 cm^{-1} (OH).

Anal. Calc. for $C_{19}H_{21}N_5O_7$: C, 52.9; H, 5.0; N, 16.2. Found: C, 52.6; H, 5.3; N, 16.6.

D-arabino-*Hexosulose 2-(*m*-hydroxybenzoyl)hydrazone 1-phenylhydrazone* (**15**). — D-*arabino*-Hexosulose 1-phenylhydrazone (1 g) and (*m*-hydroxybenzoyl)hydrazine (0.6 g) were treated as above, to give **15** (0.8 g). It crystallized from aqueous ethanol in yellow needles, m.p. 167–168° (dec.); $[\alpha]_D^{20}+70.0°$ (*c* 0.41, pyridine); λ_{max}^{EtOH} 204, 246, 283, 380 mμ, log ε 4.76, 4.48, 4.42, 4.61; λ_{min} 235, 267, 323 mμ, log ε 4.56, 4.39, 4.03; ν_{max}^{KBr} 1600 (C=N), 1650 (CONH), 3350 cm^{-1} (OH).

Anal. Calc. for $C_{19}H_{22}N_4O_6 \cdot H_2O$: C, 54.3; H, 5.7; N, 13.3. Found: C, 54.2; H, 6.2; N, 13.7.

D-arabino-*Hexosulose 2-nicotinoylhydrazone 1-phenylhydrazone* (**16**). — A solution of D-*arabino*-hexosulose 1-phenylhydrazone (1 g) in water (15 ml), treated with nicotinoylhydrazine (0.6 g) as above, gave **16** (0.7 g). It crystallized from ethanol in yellow, prismatic needles, m.p. 196–198° (dec.); $[\alpha]_D^{20}+92.0°$ (*c* 0.33, pyridine);

$\lambda_{\max}^{\text{EtOH}}$ 203, 280, 383 mμ, log ε 4.37, 4.03, 4.40; $\lambda_{\min}$ 265, 318 mμ, log ε 4.01, 3.69; $\nu_{\max}^{\text{KBr}}$ 1600 (C=N), 1660 (CONH), 3330 cm^{-1} (OH).

Anal. Calc. for $C_{18}H_{21}N_5O_5 \cdot H_2O$: C, 55.8; H, 5.4; N, 18.1. Found: 55.3; H, 5.7; N, 18.5.

D-arabino-*Hexosulose 2-benzoylhydrazone 1-(2-methyl-2-phenyl)hydrazone* (**17**). — A solution of D-*arabino*-hexosulose 1-(2-methyl-2-phenyl)hydrazone (0.2 g) in methanol (10 ml) was treated with benzoylhydrazine (0.1 g) and a few drops of glacial acetic acid. The mixture was heated on the water bath for 10 min and then allowed to cool; compound **17** separated out (0.2 g). It crystallized from aqueous methanol in yellow needles, m.p. 188°; $[\alpha]_D^{20}$ $-69.3°$ (*c* 1.45, pyridine); $\lambda_{\max}^{\text{EtOH}}$ 235, 275 mμ, log ε 4.02, 3.90; $\lambda_{\min}$ 260, 310 mμ, log ε 3.88, 3.40; $\nu_{\max}^{\text{KBr}}$ 1600 (C=N), 1665 (CONH), 3370 cm^{-1} (OH).

Anal. Calc. for $C_{20}H_{24}N_4O_5 \cdot 0.5\ H_2O$: C, 58.7; H, 6.1; N, 13.7. Found: C, 58.9; H, 6.3; N, 13.8.

*Tetra-*O*-acetyl-*D-arabino-*hexosulose 2-benzoylhydrazone 1-(2-methyl-2-phenylhydrazone).* — A solution of **17** (0.2 g) in dry pyridine (2 ml) was treated with acetic anhydride (5 ml), and kept overnight at room temperature with occasional shaking. The mixture was poured into ice-cold water, and the acetate that separated was filtered off, washed, and dried (0.2 g). It crystallized from aqueous methanol in yellow plates, m.p. 131–132°; $[\alpha]_D^{20}$ $-34.2°$ (*c* 1.1, ethanol); $\lambda_{\max}^{\text{EtOH}}$ 285, 370 mμ, log ε 4.00, 3.98; $\lambda_{\min}$ 260, 320, log ε 3.60, 3.40; $\nu_{\max}^{\text{KBr}}$ 1600 (C=N), 1690 (CONH), 1750 cm^{-1} (COO).

Anal. Calc. for $C_{28}H_{32}N_4O_9$: N, 9.9. Found: N, 9.9.

D-arabino-*Hexosulose 1-(2-methyl-2-phenyl)hydrazone 2-*o*-toluoylhydrazone* (**18**). — D-*arabino*-Hexosulose 1-(2-methyl-2-phenyl)hydrazone (0.2 g) was treated with *o*-toluoylhydrazine (0.12 g) as above, to give **18** (0.2 g). It crystallized from dilute methanol in yellow needles, m.p. 144–145°; $[\alpha]_D^{20}$ $-47.6°$ (*c* 0.5, ethanol); $\lambda_{\max}^{\text{EtOH}}$ 246, 363 mμ, log ε 4.22, 4.28; $\lambda_{\min}$ 222, 310 mμ, log ε 4.08, 3.47; $\nu_{\max}^{\text{KBr}}$ 1600 (C=N), 1660 (CONH), 3440 cm^{-1} (OH).

Anal. Calc. for $C_{21}H_{26}N_4O_5 \cdot 0.5\ H_2O$: C, 59.6; H, 6.4; N, 13.2. Found: C, 60.2; H, 6.7; N, 13.0.

D-arabino-*Hexosulose 1-(2-methyl-2-phenyl)hydrazone 2-*m*-toluoylhydrazone* (**19**). — This was obtained, as above, from D-*arabino*-hexosulose 1-(2-methyl-2-phenyl)-hydrazone (0.2 g) and *m*-toluoylhydrazine (0.12 g). The product (0.2 g) crystallized from dilute methanol in yellow, prismatic needles, m.p. 160–161°; $\lambda_{\max}^{\text{EtOH}}$ 205, 244, 273, 366 mμ, log ε 4.42, 4.19, 4.22, 4.21; $\lambda_{\min}$ 224, 257, 313 mμ, log ε 4.11, 4.16, 3.50; $\nu_{\max}^{\text{KBr}}$ 1595 (C=N), 1650 (CONH), 3470 cm^{-1} (OH).

Anal. Calc. for $C_{21}H_{26}N_4O_5 \cdot 0.5\ H_2O$: C, 59.6; H, 6.4; N, 13.2. Found: C, 59.3; H, 6.6; N, 12.7.

D-arabino-*Hexosulose 1-(2-methyl-2-phenyl)hydrazone 2-*p*-toluoylhydrazone* (**20**). — D-*arabino*-Hexosulose 1-(2-methyl-2-phenyl)hydrazone (0.2 g) in methanol (10 ml) was treated with *p*-toluoylhydrazine (0.1 g), as above, to give **20** (0.2 g). It crystallized from aqueous methanol in yellow needles, m.p. 186–187°; $[\alpha]_D^{20}$ $-16.6°$ (*c* 0.4, ethanol);

$\lambda_{\max}^{\mathrm{EtOH}}$ 248, 314 mμ, log ε 4.24, 3.51; $\lambda_{\min}$ 224, 274 mμ, log ε 4.07, 4.27; $\nu_{\max}^{\mathrm{KBr}}$ 1610 (C=N), 1660, (CONH), 3480 cm^{-1} (OH).

Anal. Calc. for $C_{21}H_{26}N_4O_5 \cdot H_2O$: C, 58.5; H, 6.5; N, 13.0. Found: C, 58.3; H, 6.7; N 12.8.

*Tetra-O-acetyl-*D-arabino*-hexosulose 1-(2-methyl-2-phenyl)hydrazone 2-*p*-toluoylhydrazone.* — This was obtained by the action of acetic anhydride on **20** (0.2 g) in dry pyridine (2 ml) yield, 0.2 g. It crystallized from aqueous methanol in yellow, prismatic needles, m.p. 155°.

Anal. Calc. for $C_{29}H_{34}N_4O_9$: C, 59.8; H, 5.8; N, 9.6. Found: C, 59.8; H, 6.1; N, 9.7.

D-arabino*-Hexosulose 1-(2-methyl-2-phenyl)hydrazone 2-nicotinoylhydrazone* (**21**). — D-*arabino*-Hexosulose 1-(2-methyl-2-phenyl)hydrazone (0.2 g) and nicotinoylhydrazine (0.1 g) gave **21** (0.1 g), which crystallized from aqueous methanol in yellow needles, m.p. 176–178°; $[\alpha]_{\mathrm{D}}^{20}$ $-69.16°$ (*c* 0.4, pyridine); $\lambda_{\max}^{\mathrm{EtOH}}$ 278, 370 mμ, log ε 4.10, 4.37; $\lambda_{\min}$ 260, 315 mμ, log ε 4.06, 3.66; $\nu_{\max}^{\mathrm{KBr}}$ 1598 (C=N), 1666 (CONH), 3350 cm^{-1} (OH).

Anal. Calc. for $C_{19}H_{23}N_5O_5 \cdot H_2O$: C, 54.4; H, 6.0; N, 16.7. Found: C, 54.3; H, 5.9; N, 16.6.

D-arabino*-Hexosulose-2-isonicotinoylhydrazone-1-(2-methyl-2-phenyl)hydrazone* (**22**). — D-*arabino*-Hexosulose 1-(2-methyl-2-phenyl)hydrazone (0.2 g) was treated with isonicotinoylhydrazine (0.1 g) as above. The product (0.12 g) crystallized from methanol in yellow, prismatic needles, m.p. 188–190°; $[\alpha]_{\mathrm{D}}^{20}$ $-29.8°$ (*c* 0.3, pyridine); $\lambda_{\max}^{\mathrm{EtOH}}$ 278, 360 mμ, log ε 4.10, 4.32; $\lambda_{\min}$ 260, 312 mμ, log ε 4.00, 4.62; $\nu_{\max}^{\mathrm{KBr}}$ 1595 (C=N), 1660 (CONH), 3260 cm^{-1} (OH).

Anal. Calc. for $C_{19}H_{23}N_5O_5$: C, 56.9; H, 5.7; N, 17.5. Found: C, 56.4; H, 5.7; N, 17.4.

D-arabino*-Hexosulose 2-acetylhydrazone1-(2-methyl-2-phenyl)hydrazone* (**23**). — A solution of D-*arabino*-hexosulose 1-(2-methyl-2-phenyl)hydrazone (0.2 g) in methanol (10 ml) was treated with acetylhydrazine (0.1 g) as above, and the product (0.15 g) was crystallized from aqueous methanol in yellow needles, m.p. 185–186°; $[\alpha]_{\mathrm{D}}^{20}$ $-50.9°$ (*c* 0.7, pyridine); $\lambda_{\max}^{\mathrm{EtOH}}$ 244, 352 mμ, log ε 3.04, 3.19; $\lambda_{\min}$ 218, 304 mμ, log ε 2.75, 2.49; $\nu_{\max}^{\mathrm{KBr}}$ 1600 (C=N), 1652 (CONH), 3350 cm^{-1} (OH).

Anal. Calc. for $C_{15}H_{22}N_4O_5 \cdot 0.5\ H_2O$: C, 51.9; H, 6.6; N, 16.1. Found: C, 51.7; H, 6.7; N, 16.4.

D-arabino*-Hexosulose 1-(2-methyl-2-phenyl)hydrazone 2-thiosemicarbazone* (**24**). — A solution of D-*arabino*-hexosulose 1-(2-methyl-2-phenyl)hydrazone (0.2 g) in methanol (10 ml) was treated with a solution of thiosemicarbazide (80 mg) in 50% aqueous methanol (5 ml) as above. The product (0.2 g) crystallized from methanol in yellow needles, m.p. 201–203° (dec.); $[\alpha]_{\mathrm{D}}^{20}$ $-27.6°$ (*c* 0.4, pyridine); $\lambda_{\max}^{\mathrm{EtOH}}$ 254, 302 mμ, log ε 4.40, 4.15; $\lambda_{\min}$ 226, 274 mμ, log ε 4.07, 3.96; $\nu_{\max}^{\mathrm{KBr}}$ 1500 (C=S), 1600 (C=N), 3160 cm^{-1} (OH).

Anal. Calc. for $C_{14}H_{21}N_5O_4S$: C, 47.3; H, 5.9; N, 19.7. Found: C, 47.5; H, 6.1; N, 19.6.

SUMMARY

D-*arabino*-Hexosulose bis(acylhydrazones) (**1–4**), 2-acylhydrazone 1-phenylhydrazones (**5–16**), and 2-acylhydrazone 1-(2-methyl-2-phenyl)hydrazones (**17–24**) were prepared from D-*arabino*-hexosulose or its hydrazone and acylhydrazines. The properties of these osazones were compared with those of arylosazones and bis(benzoylhydrazones).

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ANOMERIC FORM OF D-GLUCOSE PRODUCED DURING ENZYMOLYSIS

F. W. PARRISH AND E. T. REESE
Pioneering Research Division, U. S. Army Natick Laboratories, Natick, Mass. 01760 (U. S. A.)

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INTRODUCTION

The initial configuration of sugars resulting from enzymic hydrolysis of polysaccharides has always been determined by means of optical rotation. In a classic example, maltose produced by the action of β-amylase on starch was found to have a low optical rotation, *i.e.*, it was in the β-form[1]. The products from α-amylase had higher rotations, *i.e.*, were of the α-configuration[1]. In a similar way β-glucosidases were considered enzymes which released β-D-glucose from glucosides, whereas α-glucosidases released α-D-glucose. Indeed, the anomeric nature of the product was considered to imply that the same configuration occurred in the glycoside. This, of course, preceded the discovery by Kuhn[1] of inversion of configuration by β-amylase.

Application of gas–liquid chromatography (g.l.c.) to sugar analysis has shown that not only can simple sugars be determined, but that their anomeric forms are separable[2]. This technique appeared useful for the determination of anomeric configuration of sugars released by the action of carbohydrases. We now report application of g.l.c. to systems producing glucose, *i.e.*, glucosidases and exo-glucanases, enzymes whose characteristics we are currently examining.

METHODS

Enzymes

A. Exo-β-1,3-glucanases [*EC 3.2.1* -]

1. *Sporotrichum pruinosum* QM 826 enzyme was produced by growth on cellulose in shake culture[3]. Solutions of the enzymes, precipitated with acetone from the culture filtrates, were passed through diethylaminoethyl Sephadex (DEAE-Sephadex) at pH 7.0, the desired fraction coming through without any hold-back. Over 98% of the cellulase and all of the β-1,6-glucanase were removed. A small amount of amylase remained, but this was of no importance in the present tests.

2. *Basidiomycete sp.* QM 806 enzyme was produced by growth on starch in shake culture[3], and precipitated with acetone. This ppt. was nearly free of β-glucanases, other than the exo-β-1,3-glucanase, but contained a little amylase. It was used without further treatment.

B. Exo-α-glucanases–glucamylase–α-1,4-glucan-glucohydrolase [EC 3.2.1.3]

1. *Aspergillus niger* "Diazyme", 455DU/gram, Miles Chemical Company, Elkhart, Indiana.

2. *Endomyces sp.* "Matulase", Matsutani Chemical Company (Japan).

These enzymes were used without further treatment.

C. β-Glucosidases [EC 3.2.1.21]

1. Almond emulsin (gift of Dr. N. K. Richtmyer, National Institutes of Health).

2. *Aspergillus niger* QM 877 enzyme was produced by growth on dextran hydrolyzate. The β-glucosidase was freed from α-glucosidase by fractionation on DEAE-Sephadex.

3. *Aspergillus luchuensis* QM 873 enzymes were obtained by growth on starch in shake cultures[3]. No attempt was made to separate the β-glucosidase from exo-β-1,3-glucanase, amylase, and α-glucosidase.

D. α-Glucosidases [3.2.1.20]

1. *Aspergillus niger* QM 877 as above. The α-glucosidase fraction was used.

2. *Paecilomyces varioti* QM 10a was grown on starch. The maltase was freed of α-amylase and of glucamylase by separation on DEAE-Sephadex.

3. *Penicillium parvum* QM 1878 was grown on dextran hydrolyzate. The maltase was freed of nearly all amylase by passage through a DEAE-Sephadex column. The activity of this enzyme is slightly greater on isomaltose than on maltose.

E. Invertase–β-fructofuranosidase [EC 3.2.1.26]

A dry preparation, free of melibiase (Nutritional Biochem. Co.) was used.

PROCEDURES

A. Enzymolysis. — A concentrated enzyme solution (0.1 ml) was added to a 5% solution of substrate in 0.025M citrate buffer at pH 4.5 (0.4 ml). During incubation at 40°, samples (0.2 ml) were removed, cooled in dry ice, and freeze dried. Alternatively the samples were dried quickly by blowing air over them (usually about 5 min).

B. Analysis. — Dry (CaH_2) pyridine (0.1 ml), hexamethyldisilazane (0.2 ml), and trimethylchlorosilane (0.1 ml) were added to each dry sample. After 30 min the solvent was removed *in vacuo*, and the residue was treated with hexane (0.2 ml). A portion (5 μl) of the extract was applied to a column (6 ft × 1/4 in) of SE 30 (3%) on Chromosorb W at 150°, in conjunction with a hydrogen flame detector. Alternatively, a temperature gradient was used. The stream splitter at the column exit deflected most of the solvent from the detector, after which the splitter was rendered inoperative by closing one exit. This procedure gave higher sensitivity and increased resolution. The ratio of β-D-glucose to α-D-glucose (R β/α) was determined from the relative peak areas.

Optical rotation, coupled to concurrent measurements of extent of hydrolysis, followed the principles of Weill et *al*[4]. and was recorded on a Bendix Automatic Polarimeter Type 143A at 27°.

RESULTS

Any method for determining the anomeric form in which glucose is released requires a highly active enzyme and a highly susceptible substrate to minimize the effect of mutarotation. The enzymes that yield β-D-glucose caused most difficulty, in part because the equilibrium mixture β/α is high (1.76 from optical rotation data[2]; 1.51 from g.l.c.[2]; our value based on g.l.c. peak areas is 1.35). The result varies with different detectors, and standard mixtures were used for calibration. This, however, should be compensated somewhat by the lower rate of mutarotation of β-form to α-form[1]. A list of substrates that we have found suitable for our work is included in Table I. For exo-α-glucanases, starch was unsatisfactory, maltotriose of doubtful value, but maltodextrin mixture (maltotetraose and higher mol. wt. oligosaccharides) gave good results. For exo-β-1,3-glucanases, the tetrasaccharide obtained from laminaran was better than the trisaccharide, but laminaran also gave satisfactory results. For β-glucosidase, no substrate was very good. For α-glucosidases, maltotriose gave the best results.

Minimal reaction time is important, since longer incubation periods give R β/α values approaching the equilibrium value, and it is then difficult to determine whether inversion has occurred (Table I). Because of mutarotation, it is necessary to stop the reaction as soon as possible by freeze drying. Although requiring several h, this is done under conditions minimizing mutarotation. The small sample, when dried quickly with a stream of air or by freeze-drying, gave satisfactory results (Table I).

The purity of the enzymes is not of great importance. It is necessary that exo-β-1,3-glucanase be free of β-glucosidase, and *vice versa*, and similarly, that exo-α-glucanase and α-glucosidase be free of one another. That is, there should be but one enzyme present capable of acting on the substrate used. The enzymes used passed this test (except perhaps *A. luchuensis* where no purification was attempted). Six preparations examined for mutarotase activity all proved to be negative.

The trimethylsilyl ethers of fructose and of the glucose anomers were well separated by isothermal g.l.c. the retention times, relative to that of α-D-glucose, for β-D-fructose and β-D-glucose being 0.73 and 1.58, respectively. With a constant temperature (150°) during elution, pyridine, trimethylsilylating reagents, and citrate formed early peaks which interfered with the α-peak in some chromatographic systems. These were eliminated by replacing the pyridine with hexane or by the stream-splitting technique. In the g.l.c. of the hydrolyzate of sucrose, D-fructose appears before α-D-glucose, and sometimes interferes. This interference was eliminated by temperature-programming, a technique which also eliminated the pyridine and citrate problems.

Both exo-β-1,3-glucanases gave glucose as the α-D-anomer, and both exo-α-

TABLE I

RETENTION AND INVERSION OF CONFIGURATION BY CARBOHYDRASES

Enzyme	*Substrate*	*Time (min)*	*R β/α D-Glucose*
None	α-D-glucose, dry	0	0.05
None	α-D-glucose in solution at room temperature	5	0.48
None	α-D-glucose, in solution at 40°	10	1.05
None	α-D-glucose, at equilibrium	4000	1.35
Exo-β-1,3-glucanases			
Sporotrichum pruinosum QM 826	laminarane tetrasaccharide	10	0.39
	laminarane tetrasaccharide	30	0.93
	laminaran	10	0.75
Basidiomycete sp. QM 806	laminarane tetrasaccharide	10	0.46
	laminaran	10	0.59
β-Glucosidases			
Aspergillus niger QM 877	laminarane trisaccharide	6	1.59
Aspergillus luchuensis QM 873	salicin	5	1.55
Almond emulsin (Richtmyer)	salicin	4	1.60
Exo-α-glucanases (glucamylase)			
Aspergillus niger (Diazyme 455)	maltodextrins	5[a]	4.5
	maltodextrins	5[b]	3.8
Endomyces sp. (Matulase)	maltodextrins	5[a]	4.1
	maltodextrins	5[b]	4.0
α-Glucosidases			
Aspergillus niger QM 877	maltotriose	10	0.57
Paecilomyces varioti QM 100	maltotriose	6	0.54
Penicillium parvum QM 1878	maltotriose	10	0.0
β-Fructofuranosidase			
Invertase, powder NBC (melibiose-free)	sucrose	1	0.44
		10	0.50

[a] Freeze-dried.

[b] Blown dry.

glucanases produced glucose as the β-D anomer (Table I, II). Thus, all exo-glucanases brought about inversion in configuration.

Three β-glucosidases, when used on susceptible substrates, seem to liberate β-D-glucose, but the values R β/α are not much above the equilibrium value (1.35). We believe that values within 0.2 of the equilibrium value (*i.e.*, 1.35 ± 0.2) are of questionable significance, and further attention is being given to the β-glucosidases. The three α-glucosidases clearly produced α-D-glucose from maltotriose. This substrate is more susceptible to these enzymes than is maltose, and has, in addition, the advantage that the aglycon is maltose not D-glucose. Invertase released D-*glucose* in the α-D form.

TABLE II

INVERSION OF CONFIGURATION BY EXO-GLUCANASES (OPTICAL-ROTATION METHOD)

Enzyme	*Substrate*	*α due to D-glucose*[a]
Exo-β-(1,3)-glucanase	laminaran	
Basidiomycete sp. QM 806		+0.042
Sporotrichum pruinosum QM 826		+0.039
Exo-α-glucanases	maltodextrin	
Diazyme 455		+0.017
Matulase		+0.023
Calculated values, for:		
all glucose is α-D-glucose		+0.056
glucose is an equilibrium mixture		+0.026
all glucose is β-D-glucose		+0.009

[a] Optical rotation at 50% hydrolysis of substrate. The time required was less than 15 min at room temperature.

DISCUSSION

Determination of configuration. — G.l.c. has been found satisfactory for the measurement of α-D-glucose and β-D-glucose released by enzyme action. The two forms are well separated, and interference by the buffer and by pyridine can be eliminated. The high equilibrium ratio generally makes it easier to detect the α than the β form.

Two optical-rotation methods have been used for determining configuration. One depends on the abrupt change in rotation (to the equilibrium value) brought about by addition of ammonia to the hydrolyzate. Whereas others[5,6] have used successfully this method, we have observed changes of such small magnitude that we hesitate to draw conclusions from the data. The other method[4] is based on simultaneous determination of the glucose produced and optical-rotation. The rotation observed is, of course, made up of contributions from all components present. In g.l.c. each specific component is determined separately.

Glucosidases versus *exo-glucanases.* — The present work is part of a study to emphasize the differences between glucosidases and exo-glucanases. As they have in common the ability to hydrolyze dimers and trimers of D-glucose, they have both been considered "glucosidases". Yet they are clearly different in their action on long-chain polymers.

The present work uses g.l.c. to confirm earlier results (using optical rotation) showing inversion of configuration by exo-α-glucanases (glucamylases) and retention by α-glucosidase and by β-glucosidase (the results are less satisfactory with β-glucosidases.) We show for the first time that inversion accompanies the action of exo-β-1,3-glucanases, *i.e.* that the glucan having only β-D-linkages yields α-D-glucose. Li, Flora, and King[5] have obtained inversion by an exo-β-glucanase of broad specificity, an enzyme associated with the hydrolysis of β-1,4-glucans. It seems fair to generalize that glucosidases differ from exo-glucanases in that the former act to retain configuration, whereas the latter act in such a way as to invert it. In line with this generalization, pancreatic maltase is a true α-glucosidase, whereas the Takadiastase "maltase" (which produces inversion) appears to be an exo-α-glucanase[6].

Yeast invertase hydrolyzes[5] sucrose by transferring the fructosyl moiety to water[7]. This leaves the glucosyl unit attached to the bridge oxygen atom in the α-D-form. The present data substantiate this explanation.

Throughout all the work on configurational changes brought about by enzymes, the assumption is made that the resulting product is entirely of one form, *i.e.*, all α-D- or all β-D. Certainly in transfer reactions (*i.e.*, glucosidases), all the products retain configuration, so although the assumption is probably correct, the data do not exclude the possibility that a very small percentage of product is of the unexpected form.

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SUMMARY

G.l.c. offers a satisfactory method for determining the configuration of the D-glucose formed by enzymic hydrolysis of glucosides and of glucans. Exo-glucanases both α and β, give inversion; glucosidases, both α and β, give retention of configuration.

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SYNTHESIS OF D-GALACTOSE-*6-t* AND D-GALACTOSIDES-*6-t*

G. AVIGAD
Department of Biological Chemistry, The Hebrew University of Jerusalem (Israel)

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Syntheses of tritium-labelled D-galactose, including D-galactose-*1-t*[1] and D-galactose-*4-t*[2], have been described in the literature. It was also suggested[3] that D-galactose-*3-t* might be prepared by the reduction of D-*xylo*-hexopyranosid-3-uloses[4,5] with tritiated borohydrides, as for the preparation of D-glucose-*3-t*[6]. Several procedures may be proposed for the synthesis of D-galactose-*6-t*. One is to use tritiated borohydride for the reduction of ethyl (methyl, α-D-galactopyranosid)uronate[7] and subsequent hydrolysis. Another is to employ[8,9] D-glucose-*6-t* for the preparation of UDP-glucose-*6-t* [uridine 5-(D-glucosyl-*6-t* dihydrogen pyrophosphate)] and then to convert it into the galactoside-*6-t* in the UDP-glucose-4-epimerase (EC 5.1.3.2) system[10].

In the relatively simple procedure for the synthesis of D-galactose-*6-t* and D-galactosides-*6-t* described in this communication, advantage is taken of the observation that the enzyme galactose oxidase (D-galactose: oxygen oxidoreductase, EC 1.1.3.9) oxidizes many D-galactosides at C-6, with the formation of a carbonyl group[11,12]. This product can subsequently be reduced by tritiated borohydride to yield the D-galactoside-*6-t*. A multitude of galactosides, which are substrates for galactose oxidase[11,12], may thus be labelled with tritium.

A convenient way for the preparation of D-galactose-*6-t* is to start with *o*-nitrophenyl β-D-galactopyranoside (**1**) as the substrate for galactose oxidase. After labelling of **1** with tritium, it is hydrolyzed with β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) to give D-galactose-*6-t*. The course of hydrolysis of **1** may be followed spectrophotometrically[13], thereby providing a method which permits the handling of highly radioactive preparations on a small scale and with minimal manipulation. The aldehyde obtained from galactoside **1** by the action of galactose oxidase[11,12] is not cleaved by β-galactosidase. This affords a simple method of measuring the amount of residual substrate in the oxidized system as well as the reformation of **1** after the reduction of the aldehyde by borohydride.

The following description presents examples for the preparation of D-galactose-*6-t* and of raffinose containing a D-galactose-*6-t* residue. The specific activity (about 100 mcuries/mmole) of the tritiated sodium borohydride available commercially enabled the preparation of galactosides having activities of up to about 20 mcuries/mmole. The use of tritiated borohydrides of even higher specific activity might similarly provide very highly radioactive D-galactosides-*6-t*, which may be needed for various metabolic, enzymic, and other biological studies.

EXPERIMENTAL

Materials and Methods. — Tritiated sodium borohydride (New England Nuclear Corp., Boston, Mass.) and *o*-nitrophenyl β-D-galactopyranoside (Koch–Light Laboratories Ltd., Colnbrook, Bucks.) were commercial products. Galactose oxidase was prepared from *Dactylium dendroides* cultures[12,14]. Partially purified β-galactosidase was obtained from *Escherichia coli* ML 308 extracts[13]. Millipore filters (Millipore Filter Corp., Bedford, Mass) were used for microfiltration. Crystalline catalase and hexokinase (Boehringer and Sons, Mannheim) were employed.

Paper chromatography of sugars was conducted on Whatman No. 1 paper with butyl alcohol–ethanol–water (5:2:2) as solvent. Thin-layer chromatography was performed with propyl alcohol–ethyl acetate–water (6:3:2) or propan-2-ol–ethyl acetate–water (65:23:12) as the solvents on plates of Cellulose-D(Camag AG, Muttenz, Switzerland). Reducing sugars and ketoses were revealed by the *o*-aminobiphenyl[15] and urea–phosphoric acid[16] spray reagents, respectively.

Quantitative determination of D-galactosides and D-galactose in solution was performed with the galactose oxidase–peroxidase reagent[12,17]. Aldohexose contents were determined colorimetrically with the Somogyi[18] and *o*-aminobiphenyl[15] reagents, ketoses were assayed with the resorcinol reagent[19], and total sugar contents by the phenol–sulphuric acid reaction[20]. Absorbance was measured in the Zeiss Model PMQ II spectrophotometer, and the kinetics of change in absorbance were followed in the Gilford Model 2000 absorbance recorder by using cells of 1.0-cm light path. Hydrolysis of **1** by β-galactosidase was measured at 420 mμ in 10 mM phosphate buffer (pH 7.5) containing mM magnesium chloride at 30°. A unit of β-galactosidase is that amount which liberates 1 μmole of *o*-nitrophenol/min under these conditions. A unit of galactose oxidase is as defined by Avigad *et al.*[12].

Tritium was measured in the Packard Tri-Carb Model 3003 scintillation spectrometer by using a scintillator solution similar to that of Knoche and Bell[21], with 17% counting efficiency.

Preparation of tritiated D-*galactose and location of the tritium at C-6.* — A reaction system of 37.82 ml contained 1.17 mmoles of phosphate buffer (pH 7.0); 252 μmoles of **1**; catalase, 160 units; and galactose oxidase, 1500 units. After incubation of the mixture on a rotary shaker for 24 h at 30°, it was found, by assay of residual substrate with β-galactosidase, that 42% of **1** was oxidized. A freshly prepared solution (1 ml) of 5% (w/v) tritiated sodium borohydride (about 0.3 μcuries/μmole) was added to 30 ml of the reaction system. After 1 h at room temperature, 2N hydrochloric acid (1.5 ml) was added to destroy excess of borohydride, and then aqueous sodium hydroxide was added to bring the pH to 7.5. The solution, containing 185 μmoles of **1**, was hydrolyzed by incubation for 1 h with 24 units of β-galactosidase and mM magnesium chloride. D-Galactose (324 mg) was subsequently added, and the solution was passed through a column (1.8 × 16 cm) of an Amberlite MB-3 mixed-bed resin, which was then washed with water. The colorless filtrate (132 ml) was clarified by passage through an HA Millipore filter, and concentrated to 1.0 ml under reduced pressure, with repeated cycles of evaporation of added water and methanol[1,8]. A methanol–ethanol (1:1) mixture was added to 80% v/v. The D-galactose crystals, deposited at 4°, were collected by centrifugation, washed with ethanol, and dried in vacuum. Yield, 276 mg (1.53 mmoles); m.p. 164–166°; $[\alpha]_D$ +80° (*c* 1.0, water, equilibrium). The specific radioactivity was 8.0 × 10^3 decomp./min/μmole.

To establish the location of the tritium at C-6 of the tritiated D-galactose, C-6 was converted by periodate oxidation into formaldehyde, which was then treated with dimedone (5,5-dimethyl-1,3-cyclohexanedione)[22,23] as follows: 102 μmoles of D-galactose-*t* was oxidized in a solution (6.9 ml) containing sodium periodate (0.6 mmoles) and sodium hydrogen carbonate (2 mmoles). After 1 h in the darkness at room temperature, 0.2 ml of 0.2M sodium arsenite, 1.4 ml of 2N acetic acid solution, and 1.0 ml of 1M acetate buffer (pH 4.6) were added. An 8% solution (w/v; 1 ml) of dimedone in ethanol was then added, and the solution was boiled in a water bath for 10 min. The resulting crystalline precipitate (which was deposited overnight) was collected on an HA Millipore filter, washed with water, and dried *in vacuo*. Yield, 19.8 mg (77.3 μmoles); m.p. 188–190° (sublimes at 140°); specific radioactivity, 8.05 × 10^3 decomp./min/μmole.

Preparation of D-*galactose*-6-t *of high specific activity.* — A reaction system of 5.0 ml contained 60 μmoles of phosphate buffer (pH 7.5); 31.8 μmoles of galactoside **1**; catalase, 40 units; and galactose oxidase, 210 units. After 19 h of shaking at 30°, 49% of **1** was found to be oxidized according to the β-galactosidase assay. Tritiated sodium borohydride (4.1 mg) (about 20 μcuries/μmole) was added to a 4.5-ml sample of the reaction mixture. After 1 h at room temperature, the pH was adjusted to 3.0 with 0.1N hydrochloric acid, and then to 7.5 with sodium hydroxide. The solution (7.1 ml), which contained 26.5 μmoles of **1**, was incubated for 1 h with 6 units of β-galactosidase and mM magnesium chloride and then transferred through an Amberlite MB-3 column (1.2 × 12 cm). The effluent, on concentration, was found to contain only D-galactose (18 μmoles; activity, 6.55 × 10^6 decomp./min/μmole) according to chromatographic, colorimetric, and enzymic assays. A portion of this D-galactose-*6-t* was further purified by quantitative chromatography and elution from sheets of Whatman No. 3 MM paper. The activity of this sample was 6.65 × 10^6 decomp./min/μmole.

Preparation of tritiated raffinose. — A reaction system of 7.61 ml contained 99 μmoles of phosphate buffer (pH 7.5); 100.4 μmoles of raffinose; catalase, 95 units; and galactose oxidase, 420 units. After 16 h incubation at 30°, 65% of the galactoside was found to be oxidized[12]. Tritiated sodium borohydride (20.2 mg, *ca.* 0.8 μcuries/μmole) were added, and the mixture was incubated for 1 h at room temperature. After the addition of 5 μl of formaldehyde, the solution was added to a column (2.1 × 11.5 cm) of Darco G-60–Celite 545 (1:1), which had been washed previously with 20% aqueous ethanol and water. The charcoal column was washed with about 60 ml of water to remove salts, and the raffinose was subsequently eluted with 100 ml of 20% aqueous ethanol. This solution was clarified by passage through a GS Millipore filter, and then concentrated under reduced pressure to 1.0 ml. Yield, 81 μmoles of raffinose-*t* (2.88 × 10^5 decomp./min/μmole), as assayed colorimetrically and by galactose oxidase. The trisaccharide was chromatographically homogeneous. Ethanol was added to 75% v/v, and the raffinose pentahydrate crystals, which appeared after 48 h at 4°, were collected on a GS Millipore filter and dried *in vacuo*. Yield: 27.7 mg (46.6 μmoles); m.p. 80°; $[\alpha]_D$ +106° (*c* 0.5, water); specific activity, 2.98 × 10^5 decomp./min/μmole.

In order to establish that the tritium resides in the galactoside residue of the raffinose-*t*, the following experiment was performed. A sample of the tritiated trisaccharide was hydrolyzed in N hydrochloric acid for 3 h at 96°. After neutralization, a sample of the hydrolyzate, containing the equivalent of 5 μmoles of raffinose, was incubated in a 1.0-ml reaction system with 50 mM Tris buffer (pH 7.2); 10 mM magnesium chloride; 13 mM adenosine 5-triphosphate, and 6 units of yeast hexokinase[24]. After 30 min at 30°, 0.5M aqueous barium acetate (0.1 ml) and cold ethanol (4.5 ml) were added. After 15 min at 0°, the precipitate was collected on a GS Millipore filter and washed with cold 80% aqueous ethanol. The filtrate was passed through a small Amberlite MB-3 column and concentrated under reduced pressure. It contained only D-galactose, as determined by chromatographic, enzymic, and colorimetric methods. Its specific activity was 2.8 × 10^5 decomp./min/μmole. The precipitates containing the barium salts of the D-glucose and D-fructose phosphate esters were only negligibly radioactive. In a similar experiment, the raffinose-*t* hydrolyzate–hexokinase system was passed through a Dowex-1(Cl^-) column to adsorb the phosphate esters, and the D-galactose-*t*, which was found in the effluent, had an activity of 3.08 × 10^5 decomp./min/μmole*.

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SUMMARY

The aldehyde obtained by oxidation of *o*-nitrophenyl β-D-galactopyranoside with D-galactose oxidase was reduced with tritiated sodium borohydride. The labelled glycoside was subsequently hydrolyzed with β-D-galactosidase to yield D-galactose-6-*t*. Raffinose-*t*, labelled in the galactose residue, was prepared by a similar procedure. It is suggested that this method may be of general application to galactosides which can serve as substrates for galactose oxidase.

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*The use of galactose oxidase in a procedure for tritium-labelling of galactoside residues in ceruloplasmin was recently described by A. G. MORELL, C. J. A. VAN DEN HAMER, I. H. SCHEINBERG, AND G. ASHWELL, *J. Biol. Chem.*, 241 (1966) 3745. In our laboratory we could apply a similar method to mark terminal galactoside moieties in galactolipids and several polysaccharides.

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LINKAGE ANALYSIS OF CARBOHYDRATES BY USING SACCHARINIC ACID FORMATION

S. A. Barker, A. R. Law, P. J. Somers, and M. Stacey
Department of Chemistry, The University, Birmingham 15 (Great Britain)

(Received August 9th, 1966)

INTRODUCTION

Early workers[1] demonstrated that there was a high degree of specificity in the nature of the saccharinic acids formed during the alkaline degradation of polymeric carbohydrates by lime water. Thus, (1→4)-linked aldoses (lactose and maltose) gives primarily isosaccharinic acids [3-deoxy-2-*C*-(hydroxymethyl)pentonic acids][2], whereas the more alkali-labile, (1→3)-linked disaccharides derived from D-glucose and D-fructose were converted into acidic products in which th emetasaccharinic acids (3-deoxyhexonic acids)[3] appeared to be preponderant. The (1→2)-linked disaccharides were relatively stable to alkali[4]. Surprisingly, a mixture of meta- and iso-saccharinic acids was obtained[5] from melibiose, in contrast to the "α"-D-glucosaccharinic acid (2-*C*-methyl-D-*ribo*-pentonic acid **1**) isolated from D-gluc oseitself. In these studies, although the major products were characterised by isolation or by paper chromatography, accurate, sensitive methods of assaying the individual saccharinic acids were not available, and so the early promise of using saccharinic acid formation for linkage analysis was not fulfilled. We now describe such methods and their application to this problem.

METHODS

Determination of isosaccharinic and metasaccharinic acids. — Since isosaccharinic acids give formylpyruvic acid on periodate oxidation, they were assayed under the conditions devised by Warren[6] for *N*-acetylneuraminic acid, which is also believed to liberate formylpyruvic acid on oxidation with 0.0667M sodium periodate in 3M phosphoric acid for 20 min at 20°. After removal of excess of periodate with sodium arsenite, any formylpyruvic acid was condensed with 2-thiobarbituric acid by heating for 15 min at 100°, the chromophore was extracted into cyclohexanone, and the absorbance was determined at λ_{max} 549 mμ. The results obtained in the range 0-0.30 μmoles of "α"-D-glucoisosaccharinolactone are given in Table I. "α"-D-Glucometasaccharinolactone and 2-deoxy-D-*arabino*-hexose, assayed (Table I) under the same conditions, give a chromophore having λ_{max} 532 mμ, similar to that reported by Warren[6] from malondialdehyde.

TABLE I

CALIBRATIONS[a] OF THE PERIODATE–THIOBARBITURIC ACID ASSAYS

"α"-D-Isosaccharinic acid[6]		*"α"-D-Glucometasaccharinic acid*[6]		*2-Deoxy-*D-arabino-*hexose*[6]		*"α"-D-Isosaccharinic acid*[7]	
µMoles	*Absorbance (549 mµ)*	*µMoles*	*Absorbance (532 mµ)*	*µMoles*	*Absorbance (532 mµ)*	*µMoles*	*Absorbance (549 mµ)*
0.0378	0.550 0.545	0.0414	0.461 0.481	0.0305	0.959 1.071	0.0378	0.457
0.0756	1.124 1.152	0.0828	0.882 0.880	0.0610	2.380 2.280	0.0756	0.962
0.1134	1.599 1.646	0.1242	1.564 1.450	0.0915	3.380 3.400	0.1134	1.468
0.1512	2.200 2.280	0.1656	2.240 2.150	0.1220	4.55 4.39	0.1512	2.000
0.2264	3.290 3.310	0.2484	3.20 3.15	0.1830	6.81 6.90	0.2264	2.930
0.3024	4.350 4.390			0.2440	8.96 9.04		

[a] All aliquots of reference acids were originally present in 0.04N barium hydroxide (0.2 ml).

To assay isosaccharinic acid in the presence of metasaccharinic acid, the milder conditions used by Aminoff[7] to assay *N*-acetylneuraminic acid were employed with minor modifications. To standard solutions (0.5 ml) containing isosaccharinic acid (0–20 µg), a solution (0.25 ml) of 0.025M periodic acid in 0.125N sulphuric acid was added. The solutions were kept at room temperature for 30 min, 2% (w/v) sodium arsenite in 0.5N hydrochloric acid (0.2 ml) was added, and the mixtures were shaken to remove the liberated iodine. Aqueous 2-thiobarbituric acid (0.1M, 2ml), adjusted to pH 9.0 with sodium hydroxide, was added, and the mixture was heated for 7.5 min at 100°. After rapid cooling of the solution to room temperature, butanol [5 ml, containing 5% (v/v) of 12N hydrochloric acid] was added, and the red chromophore was extracted. Any precipitate was centrifuged down, and the absorbance of the supernatant solution at 549 mµ was determined (Table I).

The results obtained in both assays with identical aliquots (200–298 µg/ml) of several compounds, including a sample of maltose (200 µg/ml) degraded with 0.04N barium hydroxide for 48 h at 25°, are given in Table II. Lactic acid and sodium pyruvate gave no reaction in either assay.

Determination of "α"-D-glucosaccharinic acid (**1**). — In order to simulate the conditions of alkaline degradation, solutions of sodium pyruvate (0 to 0.45 µmoles) and acid **1** (0 to 0.45 µ moles) in 0.04N barium hydroxide (0.3 ml) were placed in centrifuge tubes, and the volumes were made up to 1.4 ml with water. A 0.1-ml portion of

0.025M periodic acid in 0.125N sulphuric acid was added to each tube, and all but four tubes were kept for 20 min at room temperature. These four tubes, containing the same amount of acid **1**, were kept for various times (Table III). Aqueous ethylene glycol (1.1 mg/ml, 0.25 ml) was added to each tube, the suspension was centrifuged down, and the supernatant solution was withdrawn. Sodium dihydrogen phosphate (1 ml of a 0.1M solution, adjusted to pH 7.4 with sodium hydroxide) was then added, together with reduced nicotinamide adenine dinucleotide ($NADH_2$) (3.9 mg/ml of water; 0.1 ml), and the absorbances at 340 mμ were recorded. Lactate dehydrogenase (0.05 ml of Koch-Light enzyme, diluted ten-fold) was added, and the absorbances at 340 mμ were determined after 15 min. A decrease in absorbance occurred due to the oxidation of $NADH_2$ to nicotinamide adenine dinucleotide by the pyruvate originally present, or by that produced from periodate oxidation of acid **1**. The results are given in Table III.

TABLE II

REACTIVITY IN DIFFERENT PERIODATE–THIOBARBITURIC ACID ASSAYS

Compound	*Assayed at* λ_{max} (mμ)	*Absorbance under conditions of* *Warren assay*[6]	*Modified Aminoff assay*[7]
N-Acetylneuraminic acid	549	0.801	0.799
"α"-D-Isosaccharinic acid	549	1.14	1.13
"α"-D-Glucometasaccharinic acid	532	0.912	0.049
2-Deoxy-D-*arabino*-hexose	532	1.178	0.890
Maltose, degraded with barium hydroxide	549	0.615	0.605

TABLE III

LACTIC ACID DEHYDROGENASE CALIBRATED WITH PERIODATE-OXIDISED "α"-D-GLUCOSACCHARINIC ACID (**1**)

Sodium pyruvate		*Periodate-oxidised acid* **1**					
μMoles	*Δ absorbance (340 mμ)*	*Time (min)*	*μMoles*	*Δ absorbance (340 mμ)*	*Time (min)*	*μMoles*	*Δ absorbance (340 mμ)*
0.0910	0.192	20	0.0689	0.143	10	0.4132	0.791
0.1365	0.278	20	0.1033	0.217	30	0.4132	0.806
0.1820	0.396	20	0.1378	0.288			
0.2730	0.569	20	0.2066	0.415	45	0.4132	0.807
0.3640	0.755	20	0.2756	0.552	60	0.4132	0.789
0.4550	0.920	20	0.4132	0.807			

Various amounts of sodium pyruvate and lactic acid in 0.04N barium hydroxide were assayed alone, and in admixture, by using a final, constant volume (0.4 ml) in the standard assay with lactate dehydrogenase. The absorbances at 340 mμ were 0.534 (30 μg of pyruvate), 0.532 (30 μg of pyruvate + 45 μg of lactate), 0.690 (40 μg of pyruvate), 0.698 (40 μg of pyruvate + 45 μg of lactate), 0.852 (50 μg of pyruvate), 0.857 (50 μg of pyruvate + 30 μg of lactate).

Degradation of oligosaccharides. — Solutions of a range of carbohydrates [200 μg/ml, except melibiose (400 μg/ml), and isomaltotriose (300 μg/ml)] in 0.04N barium hydroxide were kept at 25°, no precautions being taken to exclude oxygen. Aliquots (0.2 ml) were assayed by the Warren[6] periodate–thiobarbituric acid assay and, where applicable (the nigeran trisaccharides), also by the Aminoff[7] method. Other aliquots (0.2 ml) were taken for assay of saccharinic acid, whether free, or bound as its 6-*O*-glycosyl derivative. Where appropriate, D-glucose liberated during the degradation, or after hydrolysis with 2.04N sulphuric acid (0.25 ml) for 3.5 h at 100°, was monitored by the D-glucose oxidase assay[8] on centrifuged, neutralised aliquots (0.25 ml). Formaldehyde, liberated after periodate oxidation, was determined by the chromotropic acid method[9]. Aliquots (0.4 ml) of alkali-degraded melibiose were neutralised with 0.125N sulphuric acid (0.1 ml) and oxidised with 0.025M periodic acid (0.1 ml). After 20 min at room temperature, aqueous lead dithionate (20% w/v, 0.2 ml) was added. The precipitate was removed by centrifugation, and chromotropic acid reagent (4.5 ml) was added to 0.5 ml of the supernatant solution. The tubes were kept for 30 min at room temperature in the dark, then heated for 30 min at 100°, and cooled, and the absorbances at 570 mμ were determined.

The results are presented in Tables IV, V, and VI. Other compounds were investigated as controls. The products assayed (expressed as μmoles/μmole of original compound) were from sophorose (iso- and meta-saccharinic acid, <0.0310 at 20 h and 49 h), cellobiose (isosaccharinic acid, 0.278, 24 h; 0.368, 44 h), gentiobiose (iso- and meta-saccharinic acid <0.0214 at 20 h and 49 h), $\alpha\alpha$-trehalose (metasaccharinic acid <0.0278 at 24 h and 44 h), raffinose (iso- and meta-saccharinic acid < 0.0325 at 20.5 h and 51 h), and D-galactose (iso- and meta-saccharinic acid <0.0124 at 20.5 h and 51 h).

DISCUSSION

Linkage analysis of a polymeric carbohydrate by determination of the type of saccharinic acid arising from the reducing end of the polymer, by a sequential "peeling" reaction promoted by alkali, has long been an attractive possibility. Examination of the theoretical products of periodate oxidation of isosaccharinic acids suggested that, like *N*-acetylneuraminic acid, they should give formylpyruvic acid detectable by the Warren assay[6]. Malondialdehyde, arising from 2-deoxy-D-*arabino*-hexose, has been reported to react in this assay and has been isolated, as its bis-(2,4-dinitrophenylhydrazone), from periodic acid oxidation of the 6-*O*-methyl-D-glucometasaccharinic acids[10]. Calibration of the Warren reagents against iso- and meta-saccharinic acids showed that less formylpyruvic acid was obtained from *N*-acetylneuraminic acid than from an isosaccharinic acid, and that 2-deoxy-D-*arabino*-hexose gave more malondialde-

TABLE IV

ALKALINE DEGRADATION OF D-GLUCOSE AND (1→4)-LINKED OLIGOSACCHARIDES

Compound degraded	*Compound assayed (μmoles per μmole of compound degraded)*	*Degradation time (h)*								
		4	*8*	*24*	*32*	*48*	*56*	*72*	*80*	*96*
D-Glucose	D-glucose	0.813	—	0.558	—	0.414	—	0.337	—	0.238
	D-glucosaccharinic acid	0.112	—	0.333	0.396	0.535	0.612	0.668	—	0.685
	iso- or meta-saccharinic acids	$<$0.0135 at 20.5 h; 0.0068 at 52 h								
Maltose	D-glucosaccharinic acid	0.035	0.100	0.236	0.322	0.456	0.495	0.512	0.550	0.589
	D-glucoisosaccharinic acid	0.128	0.171	0.252	0.304	0.298	0.296	0.306	0.291	0.273
Maltotriose	D-glucosaccharinic acid	0	0	0.129	0.259	0.372	0.434	0.440	0.475	0.516
	D-glucoisosaccharinic acid	0.073	0.141	0.335	0.392	0.558	0.560	0564	0.550	0.550
Maltopentaose	D-glucosaccharinic acid	0	0	0.117	0.182	0.298	0.356	0.364	0.455	0.517
	D-glucoisosaccharinic acid	0.041	0.120	0.418	0.504	0.667	0.808	0.924	0.962	1.081
Lactose	D-galactosaccharinic acid	0.030	0.069	0.214	0.256	0.355	0.475	0.535	0.612	0.672
	D-glucoisosaccharinic acid	0.034	0.082	0.193	0.232	0.257	0.258	0.240	0.244	0.246
Lactulose	D-galactosaccharinic acid	0.150	0.189	0.326	0.415	0.520	0.575	0.670	0.724	0.735
	D-glucoisosaccharinic acid	0.231	0.308	0.352	0.377	0.394	0.377	0.377	0.358	0.348

TABLE V

ALKALINE DEGRADATION OF (1→3)- AND (1→6)-LINKED OLIGOSACCHARIDES

Compound degraded	*Compound assayed (μmoles per μmole of compound degraded)*	*Degradation time (h)*								
		4	*8*	*24*	*32*	*48*	*56*	*72*	*80*	*96*
Turanose	saccharinic acid	0.171	0.287	0.485	0.506	0.585	0.604	0.660	0.710	0.726
	metasaccharinic acid	0.496	0.518	0.535	0.553	0.577	0.625	0.600	0.548	0.578
Laminaritriose	saccharinic acid	0.050	0.126	0.310	0.328	0.429	0.453	0.515	0.515	0.546
	metasaccharinic acid	0.607	0.855	0.925	0.946	1.008	1.041	1.122	1.047	1.040
Laminaritetraose	saccharinic acid	0.050	0.117	0.281	0.300	0.366	0.425	0.482	0.490	0.499
	metasaccharinic acid	0.959	1.278	1.39	—	1.69	1.76	1.85	1.86	1.79
		4	*7*	*23*	*31*	*46.5*	*55*	*71*	*78.5*	*95*
Isomaltose	6-*O*-α-D-glucosyl-D-glucosaccharinic acid	0.064	0.124	0.360	0.445	0.571	0.650	0.725	0.741	0.737
	iso- or meta-saccharinic acids	0.0427 at 8 h;		0.0254 at 24 h						
Isomaltotriose	6-*O*-α-D-isomaltosyl-D-glucosaccharinic acid	0.013	0.069	0.258	0.321	0.473	0.530	0.565	0.585	0.580
Melibiose	6-*O*-α-D-galactosyl-D-glucosaccharinic acid	0.115	0.237	0.454	0.531	0.664	0.698	0.766	0.780	0.796
	formaldehyde	0	0	0	0	0	0	0	0	0
	iso- or meta-saccharinic acids	0.0427	0.0427	0.0344	0.0214	0.0254	0.0214	0.0214	—	—

TABLE VI

ALKALINE DEGRADATION OF OLIGOSACCHARIDES HAVING MIXED LINKAGES

Compound degraded	*Compound assayed (μmoles per μmole of compound degraded)*	*Degradation time (h)*								
		4	*8*	*24*	*32*	*48*	*56*	*72*	*80*	*96*
Panose	6-*O*-α-D-glucosyl-D-glucosaccharinic acid	0.069	—	0.364	—	0.403	—	0.465	—	0.490
	isosaccharinic acid	0.126	—	0.314	—	0.346	—	0.340	—	0.330
Trisaccharide *B*	saccharinic acid	0	0	0.126	0.239	0.345	0.350	0.372	0.410	0.435
	isosaccharinic acid	0.082	0.167	0.320	—	-0346	0.364	0.333	0.352	0.352
	metasaccharinic acid	0.032	0.062	—	—	0.196	—	—	0.246	0.243
Trisaccharide *A*	saccharinic acid	0.119	0.170	0.390	0.422	0.441	—	0.485	0.485	0.510
	isosaccharinic acid	0.063	0.113	0.258	0.286	0.290	0.332	0.308	—	0.296
	metasaccharinic acid	0.354	0.436	0.422	—	—	0.427	0.479	0.533	0.496

hyde than did a metasaccharinic acid. By using the milder conditions of the Aminoff[7] assay, negligible malondialdehyde was produced from the metasaccharinic acid, and this feature enabled the isosaccharinic acid to be determined specifically in any mixture with the metasaccharinic acid. After periodate oxidation of "α"-D-glucosaccharinic acid, virtually quantitative recovery of pyruvic acid was obtained, as determined by a specific assay with lactic acid dehydrogenase[11] (Table III).

The advent of sensitive, quantitative methods for assay of the three major saccharinic acids obtained from D-glucose polymers suggested the possibility of linkage analysis, on a microgram scale, on the following basis. D-Glucose (Table IV), gentiobiose [β-D-G*p*-(1→6)-D-G*p*], melibiose [α-D-Gal*p*-(1→6)-D-G*p*], isomaltose [α-D-G*p*-(1→6)-D-G*p*] (Table V), and sophorose [β-D-G*p*-(1→2)-D-G*p*], under the chosen conditions of alkaline degradation, produced negligible amounts of assayable meta- and iso-saccharinic acids. Since metasaccharinic acid, but no isosaccharinic acid, is produced from laminaritriose [β-D-G*p*-(1→3)-β-D-G*p*-(1→3)-D-G*p*], laminaritetraose, and turanose [α-D-G*p*-(1→3)-D-Fru] (Table V), and isosaccharinic acid, but no metasacharinic acid, is produced from maltose [α-D-G*p*-(1→4)-D-G*p*], maltotriose, and maltopentaose (Table IV), it may be concluded that formation of a metasaccharinic acid is diagnostic of a (1→3)-linkage, and that an isosaccharinic acid is diagnostic of a (1→4)-linkage.

D-Glucose yielded "α"-D-glucosaccharinic acid (**1**) under the alkaline degradation conditions. When this sugar is produced in a "peeling" reaction of a (1→3)- or (1→4)-linked disaccharide or trisaccharide, it will obviously contribute to, and probably accounts for all of, the acid **1** detected by the assay method using periodate oxidation followed by determination of free pyruvic acid. Indeed, the molar yield of acid **1** only decreases slightly in going from maltose (0.589 moles), through maltotriose (0.516 moles), to maltopentaose (0.517 moles), and, furthermore, acid **1** is not detectable during the first 8h of the degradation of maltotriose and maltopentaose. By contrast the proportions of isosaccharinic acid from the di-, tri-, and penta-saccharides at 96 h were 1:1.9:3.95, closely approximating to the theoretical yields of 1:2:4. Isosaccharinic acid is produced from lactulose more quickly, and in greater proportion, than from lactose. Glucosylsaccharinic acids originating from a (1→3)- or (1→4)-linked polymer would not give pyruvic acid on periodate oxidation, whereas a 6-*O*-D-galactosyl-D-glucosaccharinic acid would give pyruvic acid. The latter appears[11] to be the major product of alkaline degradation of melibiose [α-D-Gal*p* (1→6)-D-G*p*], since no formaldehyde can be detected after periodate oxidation of the degradation mixture, whereas free acid **1**, metasaccharinic acid, and isosaccharinic acid, all give formaldehyde after periodate oxidation. Little free D-glucose (max. 10%) was detectable during the alkaline degradation of isomaltose. Furthermore, on acid hydrolysis of the equilibrium mixture from degraded isomaltose, D-glucose and acid **1** were produced in roughly equimolar amounts. Hence, the diagnosis of a terminal, reducing (1→6)-link rests on the production of a compound giving a positive reaction in the periodate–pyruvic acid determination, a negative reaction to the periodate–formaldehyde assay, and the virtual absence of free iso- and meta-saccharinic acids during alkaline degradation. With isomaltotriose, a 6-*O*-isomaltosyl-"*α*"-D-glucosaccharinic acid appears to be formed, since less pyruvic acid is found in the periodate-oxidised degradation mixture than in that from isomaltose. Such "stopping" reactions[12] are not a prominent feature of the degradations of (1→4)- and (1→3)-linkages, since, on a molar basis, maltose gives less isosaccharinic acid than do maltotriose and maltopentaose, and laminaritriose gives less metasaccharinic acid than does laminaritetraose. As would be expected, panose [α-D-G*p*-(1→6)-α-D-G*p*-(1→4)-D-G*p*)] gives almost as much isosaccharinic acid as maltose. However, with the isomeric nigeran trisaccharides, isosaccharinic acid production from *A* [α-D-G*p*-(1→4)-α-D-G*p*-(1→3)-D-G*p*] is comparable with that from maltose (mole per mole), whereas that from *B* [α-D-G*p*-(1→3)-α-D-G*p*-(1→4)-D-G*p*] is greater. This may reflect the different nature of the leaving groups, D-glucose and nigerose, respectively, during isosaccharinic acid formation. The alkaline degradation of a (1→3)-linked residue proceeds much faster than for a (1→4)-linked compound. The order of liberation of acids in the initial stages of degradation of *A* is metasaccharinic > isosaccharinic acid, and for *B* is isosaccharinic acid > metasaccharinic acid. The rate of production of acid **1** from *B* shows a pronounced lag compared with that from *A*.

The presence of a reducing, terminal (1→2)-link in a D-glucose polymer confers marked stability to alkaline degradation, and hence prevents further "peeling". When sophorose is treated with periodate, a substituted hydroxymalondialdehyde is formed. This product (or free hydroxymalondialdehyde), under the acidic conditions of the assay, forms a chromophore with 2-thiobarbituric acid having λ_{max} at 450 mμ.

Nonreducing oligosaccharides, such as αα-trehalose and raffinose, did not produce sufficient saccharinic acids, under the degradation conditions, to interfere with the diagnostic use of the assays proposed for linkage analysis.

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SUMMARY

Methods for assaying isosaccharinic, metasaccharinic, and saccharinic acids on a microgram scale are described; these may be used to monitor the production of such acids during the alkali-mediated, "peeling" reaction of oligosaccharides. These methods of assay, coupled with determination of the formaldehyde produced following periodate oxidation of the equilibrium degradation mixture, are proposed for use in linkage analysis of oligosaccharides containing hexoses or hexuloses.

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STRUCTURE OF A GALACTAN FOUND IN THE ALBUMEN GLAND OF *Biomphalaria glabrata**

JOÃO BATISTA CHAVES CORRÊA, ALEXANDER DMYTRACZENKO, AND JOSÉ HAZENCLEVE DUARTE
Instituto de Bioquímica da Universidade Federal do Paraná, and Divisão de Bioquimica do Instituto de Biologia e Pesquisas Tecnológicas, P.O.Box 939, Curitiba, Paraná (Brazil)

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INTRODUCTION

Galactans having various structures are found inthe plant and animal kingdoms [1-6]. A synthetic galactan was recently studied by Dutton and Unrau[7]. Galactans from different species of mollusc have been intensively investigated recently. The albumen gland and egg of *Helix pomatia* have long been known to contain a polysaccharide which has been characterized[9-13] as a galactan. This polysaccharide is highly branched and contains both D- and L-galactopyranose residues linked β-(1→3) and β-(1→6)[14]. Galactans have been isolated from South Indian, common gastropods[15], *e.g.*, from the uterus of fully mature *Pila*, and from the albumen gland of *Viviparus* and *Ariophanta*. Since these galactans involve a variety of structures, it was considered worthwhile to investigate the structure of galactans from new sources. *Biomphalaria glabrata* is the intermediary host of *Schistosoma mansoni* cercariae, and its feminine sexual apparatus contains an albumen gland whose function is not clear. Duarte *et al.*[16] isolated several polysaccharides from the total soft part of this mollusc, but difficulties were encountered in the separation of the galactan from other polysaccharides.

We now describe a structural investigation of a galactan isolated from the dissected albumen gland of *B. glabrata*.

EXPERIMENTAL

General experimental conditions. — Intact and hydrolysed polysaccharides were assayed by the galactose-oxidase test[17]. Paper chromatography (descending) was performed on Whatman No. 4 paper with the following solvent systems (all v/v): (*a*) benzene–butyl alcohol–pyridine–water (1:5:3:3, upper layer), and (*b*) propyl alcohol–ethyl acetate–water (70:10:20). Detection was effected with silver nitrate[18] and 0.5% ninhydrin in water-saturated butanol. Disaccharide fractions, after reduction with sodium borohydride, were subjected to paper ionophoresis, using a molybdate–sulphuric acid mixture[19]; paper ionophoresis of the polysaccharide was perfor-

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*Following Opinion 735 of the International Commission on Zoological Nomenclature, published in May 1965, the generic name *Biomphalaria* is used as a senior synonym of *Australorbis*.

med for 4 h at 100 volts/cm in 0.2M borate buffer (pH 8.5). A Nujol mull of 2–5 mg of polysaccharide was used for determination of the i.r. spectrum with a Beckman IR-8 Infrared Spectrophotometer. Optical rotations were determined on aqueous solutions (Schmidt Haench polarimeter with a sodium lamp), at polysaccharide concentrations of 0.5–1.0 g/100 ml. Uronic acid was determined by the carbazole method[20], and sulphate by the benzidine procedure[21]. Osazone preparation was performed according to the method of Hassid and McCready[22]. Total nitrogen was assayed by the micro-Kjeldahl procedure[23]. Total carbohydrate was determined by the phenol–sulphuric acid method[24].

Purification of the polysaccharide from the albumen gland of B. glabrata. — Specimens of *B. glabrata*, with a maximum shell diameter of 15 mm, were collected from several creeks around Curitiba, Paraná, Brazil. After 24 h of observation, the snails that were not shedding cercariae were starved for 48 h. They were then killed by immersion, for about 60 sec in water at 70–75°, and removed from their shells. Only the snails that showed no tissue parasites were subsequently used. The albumen glands were dissected and immediately inactivated by storage at 80° for at least 10 min. After homogenization of the albumen glands in a Van Potter–Elvehjem homogenizer, the volume was made up to 100 ml. Homogenate containing 21 mg of total nitrogen per 100 mg of neutral carbohydrate was treated with proteolytic enzyme (0.2 mg of proteinase of *Bacillus subtilis*/ml of the homogenate) for 15 h at 37°, and then centrifuged at 18,000 *g* for 10 min. The precipitate was discarded, and ethanol (3 vol.) was added to the supernatant solution. The resulting precipitate was dissolved in 100 ml of distilled water (Fraction *A*) and submitted (3 times) to the Sevag deproteinisation method[25]. The combined epiphases of this process were treated with ethanol (3 vol.), and the resulting precipitate was dissolved in 80 ml of distilled water (Fraction *B*). This fraction was further purified by treatment with cetyltrimethylammonium bromide (Cetavlon)[26–28]. The precipitate obtained at pH 7 was discarded. The supernatant solution from this fraction afforded a precipitate at pH 8.5 (Fraction *C*, which, in this paper, is referred to as the polysaccharide). A further precipitate, obtained at pH 12, contained only glucose, and was kept at 0° for later analysis. The polysaccharide purification was monitored by using u.v. absorption spectra (Beckman DU Spectrophotometer) (Fig. 1). In each step of the purification, tests were also made for total carbohydrate and nitrogen (Table I).

TABLE I

PURIFICATION OF THE POLYSACCHARIDE

Fraction	*Homogenate*	*A*	*B*	*C*[a]
Mg of total nitrogen per 100 mg of neutral carbohydrate	21	1.7	0.5	—

[a] Too small to be determined.

Partial and total acid hydrolysis of the polysaccharide. — The optimal time for hydrolysis of the neutral carbohydrate was determined with N sulphuric acid (10 mg/2 ml) at 100° in sealed tubes under nitrogen. The hydrolysate was neutralized with barium hydroxide, and the pH was adjusted to approximately 5. The hydrolysate was passed through a column of Dowex 50W-X8 (200-400 mesh, H^+) coupled to a column of Amberlite IR-4B (25-50 mesh, OH^-). Partial hydrolysis was performed on the polysaccharide (300 mg) with Dowex 50W-X12 (200–400 mesh, H^+) in a sealed tube for 10 h at 110°. The partial hydrolysates were treated with ethanol (3 vol.) and the precipitate was again subjected to acid hydrolysis with 0.1N sulphuric acid for 7 h at 100°. The hydrolysates were combined and fractionated on charcoal–Celite[29].

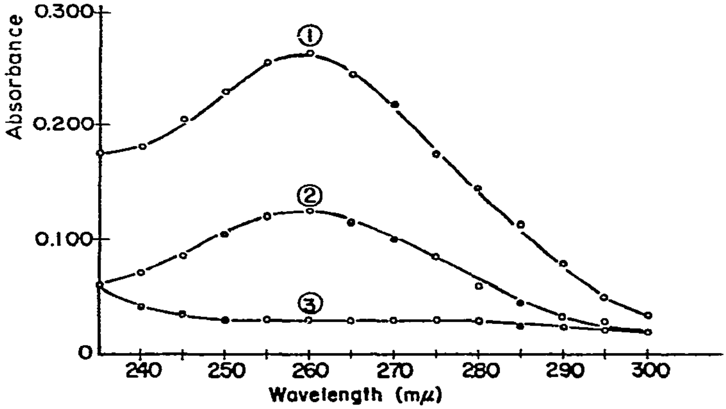

Fig. 1. U. v. spectra of fractions obtained during polysaccharide purification. (1) Fraction *A*, proteolytic enzyme; (2) Fraction *B*, Sevag method; (3) Fraction *C*, cetavlon precipitation (pH 8.5).

Periodate oxidation and Smith degradation of the polysaccharide. — A sample of the polysaccharide (30 mg) was subjected to periodate oxidation with 2 ml of 0.2M sodium periodate at *ca.* 2°, in the dark. Periodate consumption (up to 120 h) was estimated by the Fleury and Lange method[30], and formic acid by potentiometric titration[31]. Another sample (30 mg) was submitted to Smith degradation[32,33]. After periodate oxidation, the polyaldehyde was reduced with borohydride, and then hydrolysed with N sulphuric acid for 3 h at 100°. Borate ions were removed by successive treatments with methanol and evaporation, after removal of cations with Dowex 50W-X8 (200–400 mesh, H^+)[34]. Fractionation of the Smith degradation products was effected with ion-exchange resins[35] and preparative paper chromatography [solvent (*a*), Whatman No. 3MM paper]. Hexose was determined by the phenol–sulfuric acid method. The isolated polyhydric alcohols were determined by the chromotropic acid method[36], and also analysed by g.l.c. after acetylation. Standards of glycerol triacetate (b.p. 259°) and erythritol tetra-acetate (m.p. 85°) were used. G.l.c. was performed on an F and M 700 instrument, using nitrogen as carrier gas, with a column (6 × 1500 mm) of polyethylene glycol succinate (15% on Diatoport S) at 190°. The results are shown in Table II.

TABLE II

PRODUCTS FROM SMITH DEGRADATION OF THE POLYSACCHARIDE

Sample (mg)	*Moles of formic acid per residue of galactose*	*Moles of periodate per residue of galactose*	*Ratio, formic acid periodate*	*Ratio, glycerol: galactose*
30	0.62	1.17	1:1.9	1:0.9

Barry degradation of the polysaccharide. — Successive Barry degradations[37] were made on the polysaccharide and, after each degradation, samples were hydrolysed and analysed by paper chromatography [solvent (*a*)]. The first periodate oxidation was made with 200 mg of polysaccharide and 10 ml of 0.2M sodium periodate. The results are shown in Table III.

TABLE III

ANALYSIS OF PRODUCTS FROM BARRY DEGRADATIONS OF THE POLYSACCHARIDE

Barry degradation number	*Periodate oxidation number*	*Sample (mg)*	*Moles of formic acid per residue of galactose*	*Moles of periodate per residue of galactose*	*Ratio, formic acid: periodate*
	First	200	0.62	1.17	1:1.9
First	Second	104	0.44	1.00	1:2.3
Second	Third	50	0.49	1.08	1:2.2

RESULTS AND DISCUSSION

The major step in the purification (Table I) of the polysaccharide involved the use of Cetavlon, since this precipitated a mixture of nucleic acid (Fig. 1) and other impurities at pH 7, and left in solution a galactan, which was easily precipitated by borate buffer at pH 8.5. After hydrolysis and paper chromatography, no evidence could be obtained for the presence of sugars other than galactose. The presence of this hexose was confirmed by preparation of the osazone. Neither uronic acid nor sulphate was detectable by specific tests. The absence of nitrogen, nucleic acid (no absorption at 260 mμ, Fig. 1), and amino acids (no ninhydrin reaction) in fraction *C*, together with other data, indicated a high degree of chemical homogeneity, and, in the absence of evidence to the contrary, the results of chemical degradations may be regarded as applying to a single type of complex molecule.

Products of partial hydrolysis, containing 70% of reducing carbohydrate, were fractionated on charcoal–celite[29]. Only galactose was identified in the aqueous fraction. Disaccharides (major fraction) and oligosaccharides (small fraction) were also obtained. Before and after reduction with sodium borohydride, the reducing disaccharide (ratio of total to reducing sugar, 2:1) was found to contain two components, in comparable amounts, on examination by paper chromatography. Bourne *et al.*[38] have used paper ionophoresis, in the presence of molybdate, to distinguish between disaccharide alcohols. The unknown disaccharide alcohols showed ionophoretic migrations identical with those of standard compounds containing (1→3)- and (1→6)-linkages, and it may be concluded that the disaccharides contain these linkages in equimolecular proportions. Weinland[12] recorded similar results for the galactan from the eggs of *H. pomatia.*

The completely hydrolysed polysaccharide, when analysed with galactose oxidase, which is specific for D-galactopyranose and certain carbohydrate derivatives having the same configuration with free positions at C-6 and C-4, was found to contain 64% of D-galactopyranose and 36% of undetermined hexose, presumably L-galactopyranose (*ca.* 2:1 ratio of D- to L-galactopyranose). Since, as shown above, *ca.* 50% of the galactose was linked through position 6, and treatment of the intact polysaccharide with galactose oxidase resulted in reaction of 27% of the galactopyranose residues, it appears that D- and L-galactopyranose residues were present as end groups to the extent of 27% and 23%, respectively (*ca.* 1:1 ratio of D- to L-galactopyranose). May and Weinland[13] found a higher ratio for the D and L forms in the galactan from the eggs of *H. pomatia.*

The infrared spectrum of the polysaccharide, examined in the 1000–700 cm^{-1} region, had a band of moderate intensity at 890 cm^{-1}, characteristic of β-D linkages[39], and a band at 760 cm^{-1} that may be attributed to a ring-breathing vibration[40].

The polysaccharide had $[\alpha]_D^{20} + 19.5°$ (water), which changed to *ca.* $+56°$ after 40% of hydrolysis; this behaviour is indicative of the β-configuration for the mixture of D- and L-forms.

The presence of (1→6)-linkages in the polysaccharide was corroborated by data from periodate oxidation, in which a ratio of *ca.* 1:2 for formic acid liberated to periodate consumed was obtained (Table II). Since galactose residues involved in (1→3)-linkages do not consume periodate, their presence was not excluded. Paper chromatography of the Smith degradation products revealed components that migrated as glycerol and galactose. After separation of these products on an ion-exchange resin, the fraction containing the polyhydric alcohol was acetylated, and the product was identified by g.l.c. as glycerol triacetate. The hexose eluted from the same column was identified as galactose. Quantitative determination of these products gave a ratio of 1:0.9 for glycerol to galactose (Table II). The formation of glycerol and galactose, in a ratio of *ca.* 1:1, in the Smith degradation establishes the presence of (1→3)- and (1→6)-linkages in approximately equal proportions. No threitol (which would result from 4-linked galactose) was detected. Various structures may be proposed to explain these results. Thus, the galactan could involve a back-bone of (1→3)-linked galactopyranose residues, to which are attached side chains of single galactopyranose residues linked (1→6) or *vice versa.* Baldwin and Bell[10] investigated the methylation products of the galactan of the albumen gland of *Helix pomatia* and deduced a similar structure.

Successive Barry degradations were applied to the periodate-oxidised polysaccharide (Table III). The first degradation afforded a yellow, water-insoluble precipitate, corresponding to approximately half (104 mg) of the original sample (200 mg). The second degradation yielded a yellow, water-soluble product, corresponding to a quarter (50 mg) of the original sample; this product was precipitated with difficulty by ethanol (3 vol.). The original sample and the residual yellow precipitates afforded the same ratio (*ca.* 1:2) of formic acid released to periodate comsumed. Hydrolysis of the yellow precipitates yielded only galactose. Treatment of the oxidized polysaccharide with phenylhydrazine acetate and mild hydrolysis afforded a rapid fragmentation of the terminal units (as osazones), and the residual polysaccharide, the chain lengths of which were then 50% shorter than in the original, could be recovered. New, terminal, nonreducing units could then be oxidized by periodate, and further degradation was possible. This degradative pattern suggests that the galactan contains the same number of nonreducing end-groups as inner residues of the branched-chain structure. For each pair of terminal residues, one is linked to C-3 and the other to C-6 of the same inner residue of the polysaccharide chain (see Fig. 2). Thus, it may be concluded that the neutral polysaccharide from the albumen gland of *B. glabrata* contains D- and L-galactopyranose residues linked β-(1→3) and β-(1→6) (in equal proportions), in a highly branched structure, probably similar to the structure established by O'Colla[14] for the galactan of another gastropod (Fig. 2).

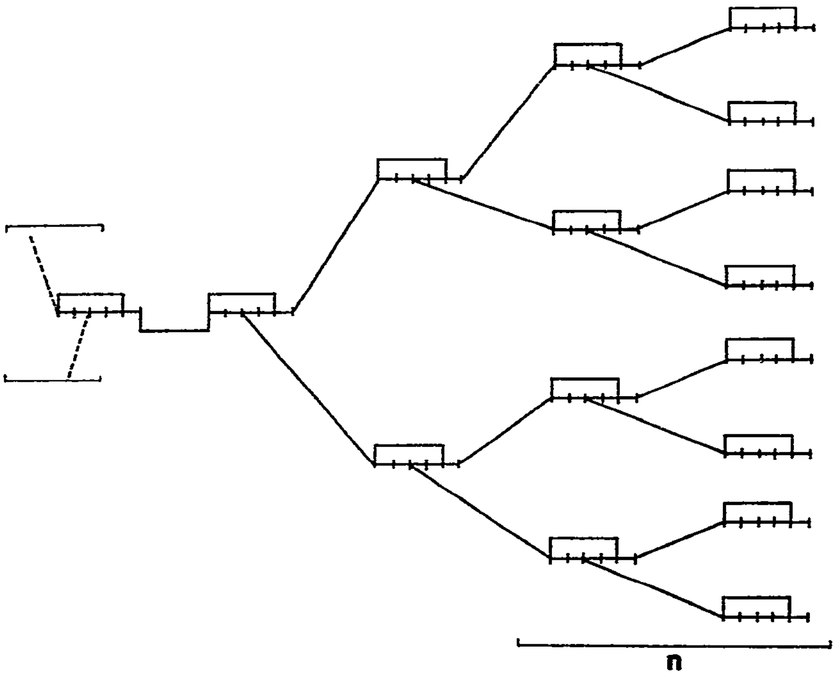

Fig. 2. Section of the pattern proposed for the polysaccharide isolated from the albumen gland of *B. glabrata*.

ACKNOWLEDGMENTS

We are indebted to the National Institute of Allergy and Infectious Diseases, U. S. Public Health Service, for financial support (Research Grant No. AI-06074), to Professor O. Romanus, Escola de Química da Universidade Federal do Paraná, for gas–liquid chromatographic data, and to Dra. Déa Amaral, Instituto de Bioquímica da Universidade Federal do Paraná, for the gift of galactose oxidase.

SUMMARY

A neutral galactan, found in the albumen gland of *B. glabrata*, has been studied by acid fragmentation, by periodate oxidation, and by Smith and Barry degradations. The galactan has a highly branched structure and contains D- (64 %) and L-galactopyranose residues (36%) linked β-(1→3) and β-(1→6), in approximately the same proportion. The release, on mild hydrolysis with acid, of two disaccharides, 3-*O*-galactopyranosylgalactopyranose and 6-*O*-galactopyranosylgalactopyranose, is discussed in relation to the occurrence of these sugar sequences in the polysaccharide structure.

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ACETAL EXCHANGE REACTIONS

M. E. Evans*, F. W. Parrish, and L. Long, Jr.
U. S. Army Natick Laboratories, Natick, Mass. 01760 (U.S.A.)

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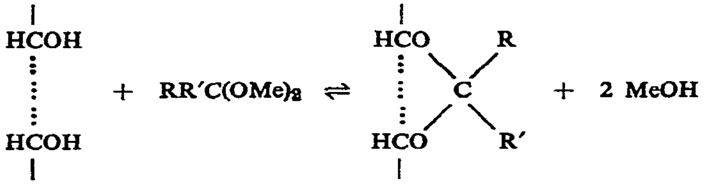
INTRODUCTION

Although 1,1-dimethoxyethane and 2,2-dimethoxypropane have been used to prepare ethylidene and isopropylidene derivatives of sugars[1], other dimethyl acetals do not appear to have been used except with a few nucleosides [2,3]. We now** report the use of acetal exchange for preparing acetals of methyl α-D-glucopyranoside. Further investigations, to be reported later, indicate that the method is widely applicable.

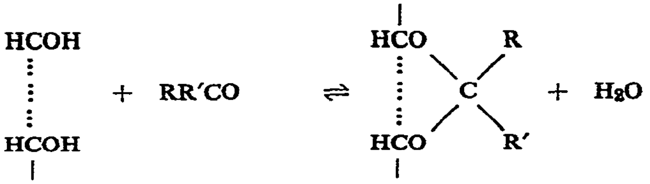
RESULTS AND DISCUSSION

The equilibrium involved in acetal exchange may be formulated as in equation *A*.



. . . . (*A*)

[Hampton[2b] indicated that the diol and the carbonyl compound are the primary reactants, with the dimethyl acetal acting as a desiccant:



. . . . (*B*)

$$RR'C(OMe)_2 + H_2O \rightarrow RR'CO + 2\ MeOH \quad \ldots\ldots\ldots\ldots \quad (C)$$

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*Present address: Chemistry Department, University of Alberta, Edmonton, Alberta, Canada.

**A preliminary account of part of this work has been published, see ref. 4.

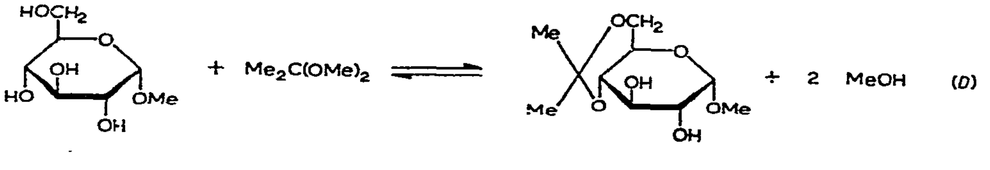
Since reaction *C* is an efficient scavenger for water (see the experimental section), equilibrium *B* should be displaced until no diol is left; this does not happen. Furthermore, addition of methanol to the reaction decreases the yield of cyclic acetal; this is readily accounted for by reaction *A*, but is difficult to explain by reactions *B* and *C*.] Equilibrium *A* appears to lie well to the right, except where the cyclic acetal is strained. In the work now presented, no attempt was made to displace the equilibrium by removing methanol from the mixture, although we have since found that this is possible.

Reaction of methyl α-D-glucopyranoside with various dimethyl acetals and an acid catalyst in *N*,*N*-dimethylformamide gives 4,6-acetals of the sugar together with minor products. Unchanged starting material is recovered readily, and overall recovery of carbohydrate material is nearly quantitative; these factors make the method attractive where valuable starting materials are involved. Aldehyde self-condensation products, often a source of inconvenience in conventional acetalation reactions, are eliminated, since little free aldehyde is present during acetal exchange. Small proportions of acid catalyze the reaction, and neutralization is effected simply with dry, basic, ion-exchange resin. Residual dimethyl acetal is removed by evaporation or by chromatography on silica gel.

Reaction of methyl α-D-glucopyranoside with 2,2-dimethoxypropane and *p*-toluenesulfonic acid in *N*,*N*-dimethylformamide gives unchanged glucoside, methyl 4,6-*O*-isopropylidene-α-D-glucoside (**1**), and in low yield, methyl 2,3:4,6-di-*O*-isopropylidene-α-D-glucoside (**2**). From treatment of methyl α-D-glucopyranoside with acetone, Ohle and Spencker[5] obtained no isopropylidene derivative, and Jones[6] isolated only a low yield.

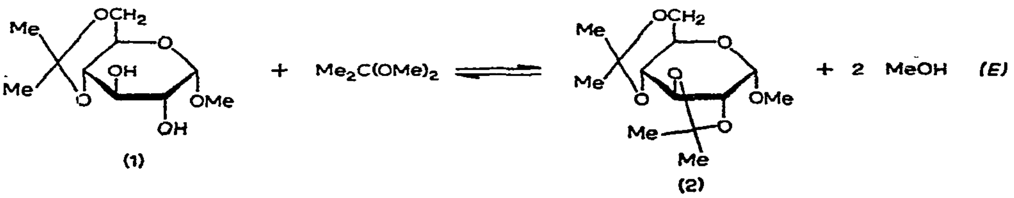
Evidence for the structure of **2** as methyl 2,3:4,6-di-*O*-isopropylidene-α-D-glucoside is given in ref. 4 and in the experimental section. This compound provides the first example of acid-catalyzed formation of a cyclic acetal bridging vicinal, *trans*-hydroxyl groups on a pyranoid ring. In a preliminary publication[4], we suggested that the low yield of **2** might be accounted for by initial formation of the 4,6-*O*-isopropylidene ring, and that this holds the molecule in a conformation that hinders formation of the 2,3-acetal ring. Treatment of methyl 4,6-*O*-isopropylidene-α-D-glucoside with 2,2-dimethoxypropane–*p*-toluenesulfonic acid–*N*,*N*-dimethylformamide, however, gives **2** in 14–19% yield, depending on the relative proportions of monoacetal and acetalating reagents used. In view of this observation, we consider the following explanation more probable than the earlier one.

Equilibrium (*D*) lies well to the right, as shown by the high yield of **1**; equilib-

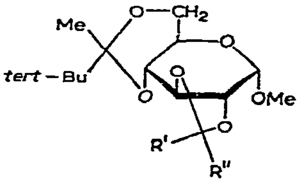


(1)

rium (*E*) lies to the left, owing to the strained conformation of **2**. With methyl α-D-glucopyranoside as the starting material, the high concentration of methanol produced in the mixture by reaction (*D*) displaces reaction (*E*) to the left; with methyl 4,6-*O*-isopropylidene-α-D-glucoside as the starting material, a much lower concentration of methanol is present and reaction (*E*) proceeds further to the right. Support for this explanation was obtained by treating the 4,6-acetal with 2,2-dimethoxypropane–*p*-toluenesulfonic acid–*N*,*N*-dimethylformamide in the presence of two molecular proportions of added methanol; in this experiment, the yield of diacetal was only 1.5%, whereas, in a parallel experiment in which no methanol was added, the yield was 14%.



Reaction of methyl α-D-glucopyranoside with 2,2-dimethoxy-3,3-dimethylbutane and *p*-toluenesulfonic acid in *N*,*N*-dimethylformamide gives methyl 4,6-*O*-(1-*tert*-butylethylidene)-α-D-glucoside, methyl 2,3:4,6-di-*O*-(1-*tert*-butylethylidene)-α-D-glucosides, and unchanged glucoside. Two diacetals, probably diastereoisomeric at the 2,3-methylidene carbon atom, were apparent in t.l.c., but we were unable to separate these by preparative t.l.c.; the slower-moving isomer was, however, obtained crystalline and was freed of the faster-moving isomer by recrystallization. Examination of the mixture by n.m.r. showed, *inter alia*, singlets at 59 c.p.s.* (*tert*-Bu), 76, 79, and 84.5 c.p.s. (Me). Since methyl 4,6-O-(1-*tert*-butylethylidene)-2,3-di-*O*-methyl-α-D-glucoside gives singlets at 57 c.p.s. (*tert*-Bu) and 83 c.p.s. (Me), the signals at 76 and 79 c.p.s. probably arise from the methyl groups attached to the 2,3-methylidene carbon atoms in **3** and **4**. The spectrum of the pure, slower-moving isomer shows no signal at 79 c.p.s., whereas, in the spectra of faster-moving fractions, this signal is enhanced at the expense of that at 76 c.p.s.



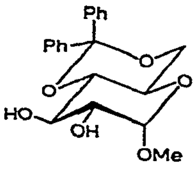
3 (R' = *tert*-Bu, R'' = Me)
4 (R' = Me, R'' = *tert*-Bu)

The 1-*tert*-butylethylidene mono- and di-acetals decompose during chromatography on silica gel, unless a base, such as triethylamine, is present in the eluant.

---

*All n.m.r. spectra were measured with a Varian A 60 spectrometer, using *ca.* 25% solutions in carbon tetrachloride, with tetramethylsilane (TMS; 0.0 c.p.s.) as an internal standard.

Treatment of methyl α-D-glucopyranoside with acetophenone (methyl phenyl ketone) dimethyl acetal and *p*-toluenesulfonic acid in *N*,*N*-dimethylformamide gives only methyl 4,6-*O*-(1-phenylethylidene)-α-D-glucoside and unchanged glucoside. With benzophenone (diphenyl ketone) dimethyl acetal, reaction is slow, and several days are necessary for the dimethyl acetal to dissolve; methyl 4,6-*O*-(diphenylmethylidene)-α-D-glucoside (5), methyl 6-*O*-(methoxydiphenylmethyl)-α-D-glucopyranoside (6), and unchanged glucoside are obtained. The 4,6-acetal is obtained in low yield; this fact and formation of the mixed acetal (6) may be due to the unfavorable[7], axial phenyl group in 5. If this explanation is correct, the phenyl group in methyl 4,6-*O*-(1-phenylethylidene)-α-D-glucoside is probably equatorial. Foster *et al.*[7] have shown that the phenyl group in methyl 4,6-*O*-benzylidene-α-D-glucoside is equatorially disposed.



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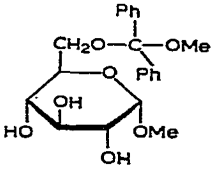

(6)

Treatment of 6 with dry hydrogen chloride in chloroform gives 5 and methyl α-D-glucopyranoside (chromatographic evidence only). Compound 6 is cleaved during chromatography on silica gel, unless an eluant containing a base is used.

Each of the monoacetals (derived from pinacolone, acetophenone, and benzophenone) was methylated, hydrolyzed with acid, and then treated with 2,2-dimethoxypropane and *p*-toluenesulfonic acid. In each case, the product was indistinguishable from that obtained by methylation of methyl 4,6-*O*-isopropylidene-α-D-glucoside.

Jones[6] reported that, in periodate oxidation of methyl 4,6-*O*-isopropylidene-α-D-glucoside, when the optical rotation of the solution is at its minimum value, the periodate uptake is 1 mole/mole. We investigated this oxidation at the natural pH of sodium periodate (pH 4) and at pH 7; in both cases, the compound consumes 2 moles/mole, with no discontinuity in the uptake–time curves. With the less acid-labile methyl 4,6-*O*-(1-phenylethylidene)-α-D-glucoside, at pH 4, the uptake–time curve shows a discontinuity at 1 mole/mole; borohydride reduction when the uptake is 1 mole/mole affords a product that gives analytical data consistent with those calculated for the expected diol. The periodate uptake–time curves are shown in Fig. 1.

The relative acid-stabilities of the monoacetals were assessed by hydrolysis of their permethyl ethers in 75% acetic acid at room temperature. The isopropylidene and 1-*tert*-butylethylidene derivatives are cleaved at indistinguishable rates, and hydrolysis is complete within 90 min. The 1-phenylethylidene and diphenylmethylidene compounds are more stable, and require 3 days and 4 days, respectively, for hydrolysis. Hampton[2b] has found a similar order of stability. Methyl 6-*O*-(methoxydiphenylmethyl)-2,3,4-tri-*O*-methyl-α-D-glucoside is cleaved within 10 min; compound **6** gives a mixture of hydrolysis and ring-closure products.

EXPERIMENTAL

General. — Methyl α-D-glucopyranoside was freed of D-glucose by passage through Dowex-1 (OH^-) resin and subsequent recrystallization from water. Dimethoxy diphenylmethane (Aldrich Chemicals), 2,2-dimethoxypropane (Eastman Organic Chemicals), and *N,N*-dimethylformamide (spectroscopic grade; Matheson, Coleman, and Bell) were used as supplied. *p*-Toluenesulfonic acid was used as the monohydrate. Where indicated, solutions were neutralized by shaking them for at least 2 h with excess Rexyn AG1 (OH^-) resin (in methanol). Column chromatography was performed on Brinkmann silica gel (0.05–0.2 mm) with a loading of 1–2%, and thin-layer chromatography was done on Brinkmann silica gel GF_{254}. Eluants are noted in parentheses; solvent 1 refers to 3:3:1 benzene–chloroform–isopropyl alcohol. Evaporations were performed below 40°.

Acetophenone dimethyl acetal. — A solution of acetophenone (12 g) and trimethyl orthoformate (16 g) in methanol (80 ml) containing Dowex 50W-(H^+) resin (1 g) was protected from moisture and boiled under reflux for 5 h. The solution was filtered into a flask containing a little AG1 resin (OH^-), and evaporated. The product was distilled from the AG1 resin at 85–87°/15 mm, to give acetophenone dimethyl acetal (13.2 g, 80%), which showed a single peak, distinct from that of acetophenone, in gas–liquid chromatography. The n.m.r. spectrum of the product showed a 5-proton multiplet at 537–555 c.p.s., a 6-proton singlet at 185 c.p.s., and a 3-proton singlet at 87 c.p.s. No C=O stretching was apparent in the infrared spectrum of the product.

Reactions of methyl α-D-glucopyranoside with various acetals. — *(1) With 2,2-dimethoxypropane.* Methyl α-D-glucopyranoside (3.88 g) was added to a stirred solution of *p*-toluenesulfonic acid (0.05 g) and 2,2-dimethoxypropane (4.0 g) in *N,N*-dimethylformamide (30 g); the glucoside dissolved within a few min. After 24 h, the solution was neutralized with AG1 resin, filtered, diluted with water (150 ml), and extracted with hexane for 2 h. The dried (K_2CO_3) extract was evaporated below 30°, and the product was purified by column chromatography (1:1 hexane–ether) to give **2** (0.10 g, 1.8%), m. p. 83–85°. The analytical sample had m.p. 85° (from hexane) and $[\alpha]_D^{25}$ +99° (*c* 2.0, benzene).

Anal. Calc. for $C_{13}H_{22}O_6$: C, 56.9; H, 8.1; mol. wt. 274. Found: C, 57.1; H, 8.0; mol. wt. (osmometry) 271, (mass spectrometry) 274.

After evaporation of the aqueous phase, xylene (2 × 100 ml) was evaporated from the product. Extraction with boiling dichloromethane (2 × 100 ml) left a residue of methyl α-D-glucopyranoside (0.68 g, 17.5 %), m.p. 165°. Evaporation of the extract gave **1** (3.7 g, 79%), m.p. 82–83.5°, $[\alpha]_D^{25}$ +105° (*c* 4.7, water). The product could not be recrystallized satisfactorily, but examination by t.l.c. (solvent 1) showed traces of only two contaminants of low R_F values. Jones[6] gave m.p. 84–86°, $[\alpha]_D$ +94° (*c* 5.0, water), and also noted difficulty in recrystallization.

(*2*) *With (1,1-dimethoxyethyl)benzene (acetophenone dimethyl acetal)*. The reaction was conducted essentially as described in (*1*). Unchanged D-glucoside (10%) was recovered, and methyl 4,6-*O*-(1-phenylethylidene)-α-D-glucoside (90%) was obtained. The latter compound was the only product detected at any stage in the reaction; m.p. 162°, $[\alpha]_D^{25}$ +150° (*c* 1.2, chloroform).

Anal. Calc. for $C_{15}H_{20}O_6$: C, 60.8; H, 6.8. Found: C, 60.7; H, 6.9.

(*3*) *With dimethoxydiphenylmethane (benzophenone dimethyl acetal)*. The reaction was performed essentially as described in (*1*), except that a reaction time of several days was used since the dimethoxydiphenylmethane dissolved only slowly. Unchanged glucoside (63%) was recovered. The dichloromethane extract contained two products which were separated by column chromatography; eluted first (with 0.25% of triethylamine in ethyl acetate) was **5** (14%), a foam, $[\alpha]_D^{25}$ +101° (*c* 4.5, acetone).

Anal. Calc. for $C_{20}H_{22}O_6$: C, 67.0; H, 6.2. Found: C, 66.9; H, 6.5.

Eluted second (with 0.25% of triethylamine and 5% of isopropyl alcohol in ethyl acetate) was **6** (18%), m.p. 118–119°, $[\alpha]_D^{25}$ +84° (*c* 0.7, acetone).

Anal. Calc. for $C_{21}H_{26}O_7$: C, 64.6; H, 6.7. Found: C, 64.7; H, 6.7.

(*4*) *With 2,2-dimethoxy-3,3-dimethylbutane (pinacolone dimethyl acetal)*. The reaction was performed essentially as described in (1). The hexane extract was fractionated by column chromatography; eluted first (with 0.25% of triethylamine in 2:1 hexane–ether) was a mixture of two compounds, probably the diastereoisomeric acetals **3** and **4**.

Anal. Calc. for $C_{19}H_{34}O_6$: C, 63.7; H, 9.6. Found: C, 63.8; H, 9.6.

Eluted second (with 0.25% of triethylamine in ethyl acetate) was methyl 4,6-*O*-(1-*tert*-butylethylidene)-α-D-glucoside (61%), m.p. 174–175°, $[\alpha]_D^{25}$ +100° (*c* 0.34, acetone).

Anal. Calc. for $C_{13}H_{24}O_6$: C, 56.5; H, 8.8. Found: C, 56.7; H, 8.7.

The dichloromethane extract yielded more (13%) of the 4,6-acetal, and the insoluble residue was methyl α-D-glucopyranoside (7%).

A sample of the mixture of diacetals **3** and **4** was applied to each of five 20 × 20 cm plates covered with a 1-mm thick layer of silica gel (Brinkmann, for thin-layer chromatography). After ten developments with hexane containing 0.5% of isopropyl alcohol and 0.25% of triethylamine, the appropriate regions of the plates were divided into six horizontal bands which were removed and individually eluted with dichloromethane containing 0.25% of triethylamine. Separation was incomplete, but the slower-moving component was obtained crystalline and, after recrystallization, had m.p. 114° and was free from the faster-moving isomer in t.l.c. (two developments with 2% of isopropyl alcohol in hexane).

Anal. Calc. for $C_{19}H_{34}O_6$: C, 63.7; H, 9.6. Found: C, 64.2; H, 9.7.

*Methylation of methyl 4,6-*O*-(diphenylmethylidene)-α-D-glucoside* (**5**). — A mixture of the title compound (0.36 g), silver oxide (1.4 g), methyl iodide (1.2 g), and *N,N*-dimethylformamide (10 ml) was stirred for 12 h at room temperature. Water (1 ml) was added and the mixture was stirred for a further 1 h. The silver salts were filtered off and washed with methanol (20 ml) and *N,N*-dimethylformamide (20 ml).

The filtrate and washings were combined, diluted with water (150 ml), and extracted with hexane for 16 h. Evaporation of the dried (K_2CO_3) extract gave methyl 2,3-di-*O*-methyl-4,6-*O*-(diphenylmethylidene)-α-D-glucoside (0.36 g, 93%), m.p. 106–110°. The analytical sample had m.p. 113–114° (from ethanol) and $[\alpha]_D^{25}$ +110° (*c* 1.3, chloroform).

Anal. Calc. for $C_{22}H_{26}O_6$: C, 68.4; H, 6.8. Found: C, 68.2; H, 6.9.

The following compounds were prepared similarly:

(*a*) Methyl 4,6-*O*-(1-*tert*-butylethylidene)-2,3-di-*O*-methyl-α-D-glucoside, m.p. 71–72°, $[\alpha]_D^{25}$ +92° (*c* 1.0, chloroform).

Anal. Calc. for $C_{15}H_{28}O_6$: C, 59.2; H, 9.3. Found: C, 59.4; H, 9.5.

(*b*) Methyl 2,3-di-*O*-methyl-4,6-*O*-(1-phenylethylidene)-α-D-glucoside, a syrup, $[\alpha]_D^{25}$ +150° (*c* 1.2, chloroform).

Anal. Calc. for $C_{15}H_{20}O_6$: C, 60.8; H, 6.8. Found: C, 60.7; H, 6.9.

(*c*) Methyl 4,6-*O*-isopropylidene-2,3-di-*O*-methyl-α-D-glucoside, m.p. 84°, $[\alpha]_D^{25}$ +120° (*c* 1.0, chloroform), lit.[6] m.p. 80°.

Anal. Calc. for $C_{12}H_{22}O_6$: C, 55.0; H, 8.5. Found: C, 54.8; H, 8.5.

Characterization of methyl 6-O-(methoxydiphenylmethyl)-α-D-glucopyranoside (**6**). — The title compound (1.04 g) was methylated by the procedure described above, and the product was purified by column chromatography (5% of isopropyl alcohol and 0.25% of triethylamine in hexane) to give metyl 6-*O*-(methoxydiphenylmethyl)-2,3,4-tri-*O*-methyl-α-D-glucoside (0.97 g, 84%), a syrup. This was dissolved in 75% (v/v) acetic acid (25 ml), and the solution was kept 12 h at 25°. The solvents were removed, and the products were separated by column chromatography. Eluted first (with dichloromethane) was benzophenone; eluted second (with ethyl acetate) was methyl 2,3,4-tri-*O*-methyl-α-D-glucoside. The latter compound was dissolved in pyridine (25 ml) and treated for 12 h at 70° with an excess of trityl chloride. The pyridine was evaporated off, and the product was purified by column chromatography (1% of triethylamine in 1:1 ether–hexane) to give methyl 2,3,4-tri-*O*-methyl-6-*O*-trityl-α-D-glucoside (1.34 g, 70%), m.p. and mixed m.p. 107–108°, $[\alpha]_D^{25}$ +96° (*c* 0.63, acetone). The i.r. and n.m.r. spectra were indistinguishable from those of the authentic compound.

Anal. Calc. for $C_{29}H_{34}O_6$: C, 72.8; H, 7.2. Found: C, 72.5; H, 7.4.

Hydrolysis of various methylated acetals. — A sample (0.01 g) of each acetal listed was dissolved in 75% (v/v) acetic acid (0.25 ml) and the solution was kept at room temperature. The hydrolysis was followed by t.l.c. (solvent 1), the times required for the disappearance of the starting acetals being listed in Table I.

A sample (0.5–1.0 g) of each of compounds **8**–**10** was hydrolyzed in 75% (v/v) acetic acid (10–20 ml) until no starting material was left. After evaporation of the solvents, toluene (2 × 10 ml) was evaporated from the residue. The ketone from compounds **9** and **10** was removed by column chromatography (dichloromethane eluted the ketone, and ethyl acetate, the sugar). Treatment of each product with 2,2-dimethoxypropane and *p*-toluenesulfonic acid gave compound **7**, indistinguishable by mixed m.p., and infrared and n.m.r. spectra, from the product of methylation of methyl 4,6-*O*-isopropylidene-α-D-glucoside.

TABLE I

TIMES TO COMPLETE HYDROLYSIS OF ACETALS

Compound	*Formula No.*	*Time*
Methyl 2,3-di-*O*-methyl-4,6-*O*-R′,R″-methylidene-α-D-glucoside		
R′ = R″ = Me	7	90 min
R′ = Me, R″ = *tert*-Bu	8	90 min
R′ = Me, R″ = Ph	9	3 days
R′ = R″ = Ph	10	4 days
Methyl 6-*O*-(methoxydiphenylmethyl)-2,3,4-tri-*O*-methyl-α-D-glucoside		10 min

*Graded hydrolysis of methyl 2,3:4,6-di-*O*-isopropylidene-α-D-glucoside* (**2**). — A sample (0.083 g) of the title compound was dissolved in glacial acetic acid (3.3 ml) and the solution was kept for 12 h at 25–30°. After removal of the acid at 30°/0.3 mm, toluene (3 × 20 ml) was evaporated from the residue. Extraction with warm dichloromethane (20 ml) left a deposit of methyl α-D-glucopyranoside (0.018 g, 30%), and evaporation of the extract gave methyl 4,6-*O*-isopropylidene-α-D-glucoside (0.05 g, 70%). Both products were identified by mixed melting points and by comparison of their infrared and n.m.r. spectra with those of the authentic compounds.

Hydrolysis of 2,2-dimethoxypropane. — A small crystal of *p*-toluenesulfonic acid was added to a suspension of water (0.020 g) in 2,2-dimethoxypropane (0.309 g); the water dissolved immediately. The n.m.r. spectrum was measured, and the integrated intensities of the signals from the methyl groups $Me_2C(OMe)_2$ and Me_2CO were found to be in the ratio 1.67:1. Calculated for complete removal of water from the system, this ratio is 1.67:1.

Periodate oxidations. — Compound **1** (0.06 mmole) in ethanol (0.4 ml) was treated with 0.133M aqueous sodium periodate (1.2 ml), and the solution was kept in the dark at 27°. At intervals, samples (0.1 ml) were withdrawn and diluted to 150 ml, and the periodate uptake was estimated from the absorbance at 222 mμ according to the method af Aspinall and Ferrier[9]. A similar experiment was performed with methyl 4,6-*O*-(1-phenylethylidene)-α-D-glucoside.

The experiment with the isopropylidene compound was repeated, but using 0.133M aqueous sodium periodate adjusted to pH 7 by addition of 5N sodium hydroxide solution.

The results are shown in Fig. 1.

Methyl 4,6-*O*-(1-phenylethylidene)-α-D-glucoside (0.179 g) in ethanol (5 ml) was treated with sodium periodate (0.321 g) in water (15 ml), and the solution was kept in the dark for 20 h at room temperature. Potassium borohydride (0.54 g) was added, and, after 12 h, the solution was neutralized by addition of carbon dioxide. Continuous extraction with ether, followed by evaporation of the extract, gave a syrup that was purified by elution from silica gel (0.25% of triethylamine in ethyl acetate).

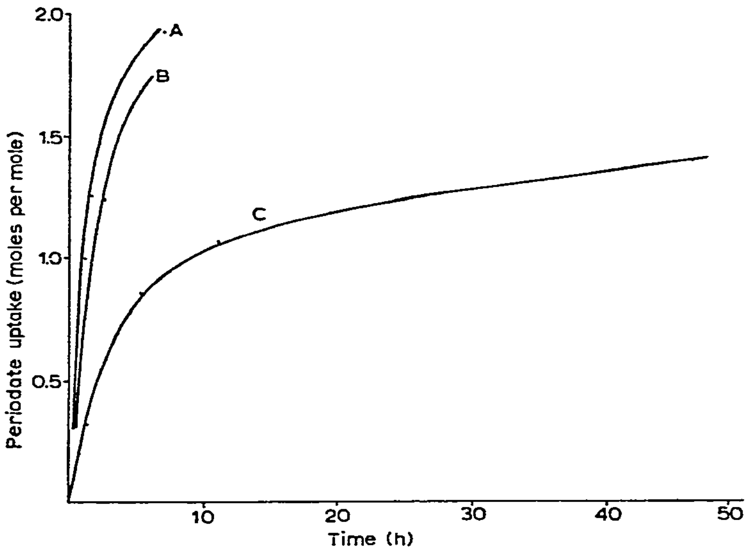

Fig. 1. Periodate oxidations. A. Methyl 4,6-*O*-isopropylidene-α-D-glucoside (1) at pH 7. B. Methyl 4,6-*O*-isopropylidene-α-D-glucoside (1) at pH 4. C. Methyl 4,6-*O*-(1-phenylethylidene)-α-D-glucoside at pH 4.

The product (0.176 g) showed a single spot in t.l.c. (10% of isopropyl alcohol in ethyl acetate).

Anal. Calc. for $C_{15}H_{22}O_6$: C, 60.4; H, 7.4. Found: C, 60.2; H, 7.5.

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SUMMARY

The dimethyl acetals derived from acetone, pinacolone, acetophenone, and benzophenone undergo acid-catalyzed acetal exchange with methyl α-D-glucopyranoside. The major products are 4,6-acetals of the sugar; the minor products include methyl 2,3:4,6-di-*O*-isopropylidene-α-D-glucoside (using the acetone acetal), and isomeric methyl 2,3:4,6-di-*O*-(1-*tert*-butylethylidene)-α-D-glucosides (using the pinacolone acetal). These compounds are all examples of a five-membered acetal ring fused *trans* to a pyranoid ring. Treatment of methyl α-D-glucopyranoside with benzophenone dimethyl acetal gives methyl 6-*O*-(methoxydiphenylmethyl)-α-D-glucopyranoside in addition to the 4,6-acetal. The 4,6-acetals derived from the four ketones exhibit a wide range of sensitivity to hydrolysis by 75% (v/v) acetic acid.

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SYNTHESIS OF α-L-IDOPYRANOSYL, (α-L-IDOPYRANOSYLURONIC ACID), α-D-MANNOPYRANOSYL, AND (α-D-MANNOPYRANOSYLURONIC ACID) PHOSPHATES*

P. PERCHEMLIDES**, T. OSAWA***, E. A. DAVIDSON†, AND R. W. JEANLOZ††

Department of Biochemistry, Duke University, Durham, N.C. 27706, and Laboratory for Carbohydrate Research, Departments of Biological Chemistry and Medicine, Harvard Medical School at the Massachusetts General Hospital, Boston, Mass. 02114 (U.S.A.)

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INTRODUCTION

The linkage between the L-iduronic acid and the 2-amino-2-deoxy-D-galactose moieties of dermatan sulfate (chondroitin sulfate B) has been shown[3,4] to be α-L-(1→3). A recent report by Jacobson and Davidson[5] of the presence, in rabbit skin, of a dehydrogenase and an epimerase acting successively upon uridine 5-(α-D-glucopyranosyl dihydrogen pyrophosphate) to give, first, uridine 5-(α-D-glucopyranosyluronic acid dihydrogen pyrophosphate), and then uridine 5-(β-L-idopyranosyluronic acid dihydrogen pyrophosphate), implies that the biosynthesis of dermatan sulfate proceeds with inversion. In order to study the biosynthesis of this polysaccharide, the preparation of various glycosyl phosphates possessing the L-*ido* configuration and the D-*manno* configuration was investigated.

Two procedures for the preparation of β-L-idopyranosyl phosphate were investigated. In the first, 2,3,4,6-tetra-*O*-acetyl-α-L-idopyranosyl bromide was treated with silver diphenyl phosphate, or with diphenyl hydrogen phosphate in the presence of triethylamine; however, the products of the reaction did not contain derivatives of L-idosyl phosphate. In the second, penta-*O*-acetyl-α-L-idopyranose was treated with

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*The synthesis of L-idopyranosyl and (L-idopyranosyluronic acid) phosphates was performed independently in both laboratories. Because of the similarity of our results, publication is made jointly. The study of D-mannopyranosyl and (D-mannopyranosyluronic acid) phosphates was conducted at Duke University, and that of 2,3,4,6-tetra-*O*-acetyl-α-L-idopyranosyl bromide at Massachusetts General Hospital. This is publication No. 420 of the Robert W. Lovett Memorial Group for the Study of Crippling Diseases, Harvard Medical School at the Massachusetts General Hospital, Boston, Massachusetts. This work was supported by research grants from the National Institute of Arthritis and Metabolic Diseases (Grants A-2903-06 and A-4315-04 to E. A. Davidson, and AM-03564-04 to R. W. Jeanloz), National Institutes of Health, United States Public Health Service. Preliminary communications have been presented[1,2].

**Duke University. Present address: Laboratory for Carbohydrate Research, Massachusetts General Hospital, Boston.

***Fulbright Fellow, Massachusetts General Hospital. Present address: Tokyo Medical and Dental University, Tokyo.

† Duke University.

††Massachusetts General Hospital.

crystalline anhydrous phosphoric acid according to MacDonald[6], and the product was then deacetylated. α-L-Idopyranosyl phosphate was isolated as its crystalline di(cyclohexylammonium) salt. Oxidation of the amorphous L-idopyranosyl phosphate afforded (α-L-idopyranosyluronic acid) phosphate, isolated as the crystalline di(cyclohexylammonium) salt.

A similar sequence of reactions was conducted with penta-*O*-acetyl-α-D-mannopyranose.

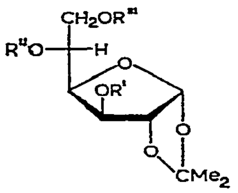
RESULTS AND DISCUSSION

The transformation of 1,2-*O*-isopropylidene-α-D-glucofuranose (**1**) into 1,2-*O*-isopropylidene-β-L-idopyranose (**2**) was accomplished either through the intermediate 1,2-*O*-isopropylidene-5,6-di-*O*-*p*-tolylsulfonyl-α-D-glucofuranose (**3**) according to Vargha[7], or through the intermediate 6-*O*-benzoyl (**4**)[8] and 6-*O*-benzoyl-5-*O*-*p*-tolylsulfonyl (**5**) derivatives[9,10]. Vargha's procedure[7] for the preparation of 3,5-di-*O*-acetyl-6-*O*-benzoyl-1,2-*O*-isopropylidene-β-L-idofuranose (**6**) from **5** was improved by replacement of the potassium acetate–acetic anhydride mixture by a suspension of dry Dowex-1(OAc$^-$) in acetic anhydride. Alkaline treatment of **6**, as described by Vargha[7], afforded **2** (increase of the time of hydrolysis to 4 h improved the yield to 85%). Hydrolysis of **2**, followed by acetylation according to Stoffyn and Jeanloz[4], gave 2,3,4-tri-*O*-acetyl-1,6-anhydro-β-L-idopyranose (**7**). Acetolysis of **7**, under conditions similar to those used for the preparation of the D isomer[11], led to the formation of crystalline penta-*O*-acetyl-α-L-idopyranose (**8**); the yield of this compound could be improved by anomerization of the product in the mother liquors. Assignment of the α-L configuration to **8** was made on the basis of the similarity of the numerical value (and opposite sign) of the optical rotation of **8** to that of the α-D isomer[11].

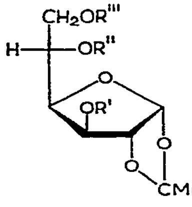
The n.m.r. spectrum of **8** confirmed the α-L configuration and established the conformation as CA [C(*aaaae*)][12]. The H-1 signal appeared as a doublet[13–15] at τ 3.87 with $J_{1,2}$ 2.0 c.p.s., indicating an equatorial–equatorial coupling of the protons on C-1 and C-2. Additional evidence for the CA conformation was obtained from the region of the acetoxy protons; two resonances appeared at relatively low field (τ 7.88, 7.89), and one at higher field (τ 7.93), with intensities in the approximate ratios of 3:1:1. The first two resonances are indicative of 4 axial acetoxy groups (C-1, C-2, C-3, and C-4), whereas the last indicates 1 equatorial acetoxy group (C-6).

Reaction of **8** with hydrobromic acid in glacial acetic acid gave crystalline 2,3,4,6-tetra-*O*-acetyl-α-L-idopyranosyl bromide (**9**). Assignment of the α-L configuration was based on the high, negative optical rotation, $[\alpha]_D^{15}$ $-120°$. This assignment is in agreement with the rule[16] of greater stability of the anomer having the halogen atom on C-1 *trans* to the hydroxymethyl group on C-5.

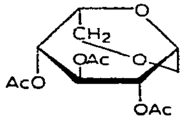
Reaction of **9** with silver acetate in dry benzene occurred with retention of configuration, since **8** was isolated, in accordance with Tipson's *trans* rule[17]. In the presence of methanol and silver carbonate, however, **9** gave methyl 2,3,4,6-tetra-*O*-acetyl-β-L-idopyranoside (**10**). Catalytic deacetylation of **10** gave methyl β-L-idopyranoside. Although no ortho ester could be isolated in the reaction, the possibility of its formation in small proportion cannot be excluded, because of the *trans* relationship of the halogen atom to the acetoxy group on C-2, and because of the strongly alkaline reagent (see Pacsu[18]); up to now, glycosides have only been obtained from acylated glycosyl halides having the halogen atom and the 2-acyloxy group *trans* when no acid acceptor was present[19]. In addition, the formation of 2,3,4,6-tetra-*O*-acetyl-L-idopyranose (**11**) was observed.



	R'	R''	R'''
1	H	H	H
3	H	Ts	Ts
4	H	H	Bz
5	H	Ts	Bz



	R'	R''	R'''
2	H	H	H
6	Ac	Ac	Bz



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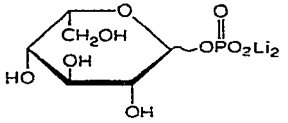

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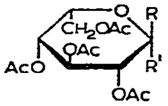
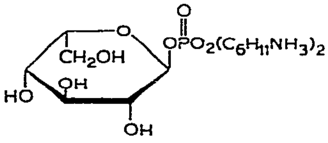


8 $R=OAc$; $R'=H$
9 $R=Br$; $R'=H$
10 $R=H$; $R'=OCH_3$
11 $R,R'=H,OH$



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Condensation of **9** with diphenyl hydrogen phosphate in the presence of triethylamine[20], or with silver diphenyl phosphate[21], did not afford definite products, either before or after alkaline hydrolysis*. Further work along this line was not pursued.

Phosphorylation of the pentaacetate **8**, according to MacDonald's procedure[6], gave the amorphous lithium salt of L-idopyranosyl phosphate (**12**) in a yield of about 55%. Conversion into the cyclohexylammonium salt resulted in crystalline di(cyclohexylammonium) α-L-idopyranosyl phosphate (**13**), but in a yield of only 60%. The α-L anomeric assignment for **13** ($[\alpha]_D$ −32°) was based on a comparison with the

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*These experiments were performed by Dr. N. Baggett.

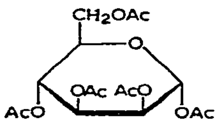
optical rotation of di(cyclohexylammonium) α- and β-D-glucopyranosyl phosphate, ($[\alpha]_D$ +64° and +7°, respectively)[22], of syrupy methyl α- and β-L-idopyranoside ($[\alpha]_D$ −98° and +61°)[23], and of **8**, respectively. The n.m.r. spectrum of **13** also supports an α-L assignment.

The phosphorylation of penta-*O*-acetyl-α-D-mannopyranose (**14**)[24] was performed in a manner similar to that used for the L-idose derivative, and gave the amorphous lithium salt of α-D-mannopyranosyl phosphate (**15**) in a yield of 50%. Its molecular rotation, [M], +13,150, was in agreement with the reported value[25], [M], +13,300.

Evidence that MacDonald's procedure[6] gives a mixture of anomers has been presented by O'Brien[26], who, starting from 2-acetamido-1,3,4,6-tetra-*O*-acetyl-2-deoxy-α-D-glucopyranose, isolated both anomers of 2-acetamido-2-deoxy-D-glucopyranosyl phosphate. The ratio of anomers isolated depends not only on the configuration of the starting material, but also on the relative acid-lability of the resulting products. The greater acid-lability of the β-D-anomers results from the equatorial orientation of the phosphate group, which is relatively unshielded and therefore more prone to attack[27].

Failure to obtain, from **12**, crystalline di(cyclohexylammonium) α-L-idopyranosyl phosphate (**13**) in a yield higher than 60% suggests that the phophorylation of **8** gives both anomers of **12**. Electrophoresis of the remaining mother liquor indicated that it contained mainly a hexosyl phosphate, probably β-L-idopyranosyl phosphate. N.m.r. spectroscopy (see below) and oxidation of **12** (see below) provided evidence for the presence of the β-L anomer.

The most likely explanation for the results of the various phosphorylation reactions is the formation of a carboxonium ion intermediate. The CA conformation of **8** permits ready neighboring-group participation, because of the *trans*-diaxial orientation of the acetoxy groups on C-2 and C-1. Anchimeric assistance (for example, by the 2-acetoxy group) could give a carboxonium ion (**16**). A second displacement by



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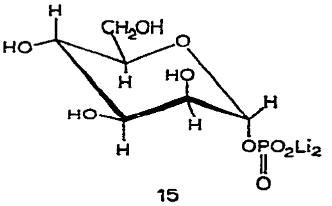

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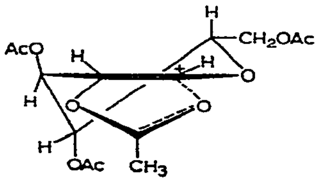

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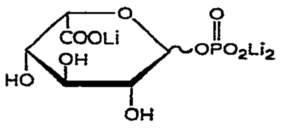

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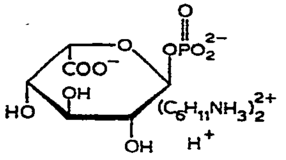

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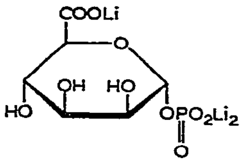

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the incoming phosphate ion would then result in retention of configuration. The presence of the β-L anomer, in addition to the α-L anomer, in the reaction product may be explained by the instability of the CA conformation of **8**, which results in an equilibrium of the CA [C(*aaaae*)] and CE [C(*eeeea*)] conformations during phosphorylation. In the latter conformation, the bonds to all secondary alcohol groups are equatorial, and the phosphorylation follows a course analogous to that postulated below for penta-*O*-acetyl-β-D-glycopyranoses.

In the case of penta-*O*-acetyl-β-D-gluco-(and galacto-)pyranose[6] and of 2-acetamido-1,3,4,6-tetra-*O*-acetyl-2-deoxy-β-D-gluco-(and galacto-)pyranose[28], phosphorylation with anhydrous phosphoric acid proceeded predominantly with inversion at the anomeric center. This result may be explained by postulating cleavage of the 1-acetoxy group to yield a carboxonium ion intermediate which then reacts with the phosphate ion to give the thermodynamically more stable α-D-phosphate. A reaction of the Walden-inversion type may also be involved, wherein the departing acetate anion shields the anomeric carbon atom, thus favoring the formation of the α-D-glycosyl phosphate.

The two mechanisms proposed would also explain the apparent course of phosphorylation of 2-acetamido-1,3,4,6-tetra-*O*-acetyl-2-deoxy-α-D-glucopyranose[26].

The phosphorylation of **14** appeared to proceed exclusively with retention of configuration; this observation was substantiated by the results of n.m.r. spectroscopy (see below). As with **8**, neighboring-group participation would result in ready displacement of the 1-acetoxy group, with formation of an intermediate carboxonium ion. A second displacement by the incoming phosphate group would then lead to retention of configuration. Since non-acidic conditions were observed during this reaction, as with the preparation of the L-*ido* compound, it is unlikely that any significant amount of the β-D anomer of **15** surviving the phosphorylation procedure would have escaped detection. These findings support the postulate that formation of β-L-idopyranosyl phosphate resulted from the phosphorylation of **8** in the CE conformation.

In order to study the configuration and conformation of **13**, its n.m.r. spectrum was compared with that of other glycosyl phosphates of known configuration (see Table I). Appearance of the H-1 signal as two doublets was found to be characteristic of all anomerically pure glycosyl phosphates studied, and was apparently due to additional splitting of the anomeric proton by phosphorus (see Fig. 1). Compound **13** showed $J_{1,2}$ 3.5 c.p.s., instead of the anticipated 2 or 8 c.p.s. for the CA or CE conformation, respectively. This result may be due to existence of **13** in a chair-conformation equilibrium or in a non-chair conformation. The spectrum of **12** showed three doublets in the region of the anomeric proton, and a greater area of the center doublet. These observations, as well as the p.p.m. values of the doublets compared to those of **13**, suggest that **12** is an anomeric mixture.

Equatorial-equatorial coupling between the C-1 and C-2 protons of **14** was indicated by appearance of the H-1 signal as a doublet at τ 3.85 with $J_{1,2}$ 1.5 c.p.s. A similar observation was made by Lemieux and Stevens[29]. Resonances in the region of the acetoxy protons were observed at τ 7.83, 7.91, 7.93, and 7.98, with intensities in the ratios 2:1:1:1. The low-field value indicates an axial orientation of acetoxy groups (C-1 and C-2), whereas the other values indicate an equatorial orientation of 3 acetoxy groups (C-3, C-4, and C-6). The $J_{1,2}$ value of 1.5 c.p.s. for both doublets of α-D-mannopyranosyl phosphate (**15**) (see Table I) indicates an equatorial orientation of the protons on C-1 and C-2, and supports an α-D configuration.

TABLE I

CHEMICAL SHIFTS (p.p.m.)[a] AND COUPLING CONSTANTS (c.p.s.), IN DEUTERIUM OXIDE, OF THE ANOMERIC PROTON OF SOME HEXOPYRANOSYL PHOSPHATES

Phosphate	*Attachment of the protons at C-1 and C-2*	*H-1*[a]	$J_{1,2}$[b]	J_{H-1}, *P*[c]	*Conformation*
α-D-Glucopyranosyl	*e-a*	6.03	3.0, 3.0	7.5, 7.5	CA
2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl[d]	*a-a*		10.5, 12.0	7.9	CA
α-D-Galactopyranosyl	*e-a*	6.03	2.5, 3.0	7.5, 8.0	CA
α-D-Mannopyranosyl (15)	*e-e*	5.91	1.5, 1.5	8.5, 8.5	CA
α-L-Idopyranosyl (13)	*e-e* and/or *a-a*	5.86	3.75, 3.50	7.5, 7.5	$CA \rightleftharpoons CE$
"Crude"L-idopyranosyl (12)	anomeric mixture	5.87,6.08	2.5, 3.5, 3.5	7.5, 8.5, 7.5, 7.5	
2-Acetamido-2-deoxy-α-D-galactopyranosyl	*e-a*	5.98	3.0, 3.0	7.5, 7.5	CA
(α-D-Glucopyranosyluronic acid)	*e-a*	6.01	3.0, 3.0	7.5, 8.0	CA
(α-D-Mannopyranosyluronic acid)	*e-e*		1.5, 1.5	8.5, 8.5	CA

[a] Chemical shift values are given in parts per million (p.p.m.) measured from the resonance value of an external, tetramethylsilane standard to the center of the doublets for the anomeric proton.
[b] The first value refers to the downfield doublet.
[c] Distance between first peak of each doublet and second peak of each doublet, respectively.
[d] This sample (non-crystalline) was measured in $CDCl_3$.

Oxidation of the amorphous lithium salt of **12** was performed in the presence of Adams or platinum-on-carbon catalysts[30]. Essentially the same results were ob-

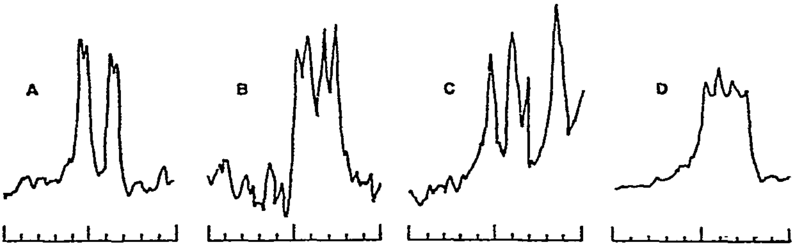

Fig. 1. Nuclear magnetic resonance spectra (partial, in the region of the anomeric proton) at 60 Mc.p.s. of α-D-mannopyranosyl phosphate (A), (α-D-glucopyranosyluronic acid) phosphate (B), 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl phosphate (C), and α-L-idopyranosyl phosphate (D). Spectra A, B, and D were observed in D_2O, and spectrum C in $CDCl_3$. Each division of the scale represents 5 c.p.s.

served either in the presence or absence of potassium carbonate. Although **12** underwent very rapid oxidation at C-6, with resulting formation of uronic acid, keto sugar formation was the principal course of the reaction. After the platinum-on-carbon oxidation, (L-idopyranosyluronic acid) phosphate (**17**) was isolated (in a yield of 25%) as the amorphous lithium salt; this was transformed into the amorphous barium and cyclohexylammonium salts. From the latter, the crystalline α-D anomer was obtained as the di(cyclohexylammonium) salt (**18**). The mother liquor from this crystallization contained about half of the total material, gave a positive test for (glycosyluronic acid) phosphate, and showed a specific optical rotation of approximately + 13°. The presence of (glycosyluronic acid) phosphate in the mother liquors was also shown by electrophoresis. These observations suggest the presence of (β-L-idopyranosyluronic acid) phosphate, resulting from the presence of both anomeric forms in the crude starting-material.

The anomeric assignment made for the crystalline di(cyclohexylammonium) salt (**18**) was, in part, based on the observation that its optical rotation (− 16°) bore the same sign and was of the same order of magnitude as the optical rotation of crystalline **13** (− 32°). In addition, the mother liquor, which apparently contained mainly the other anomer, had a positive rotation.

Oxidation of α-D-mannopyranosyl phosphate (**15**) in the presence of platinum-on-carbon catalyst, in a manner similar to that applied to L-idopyranosyl phosphate, gave (α-D-mannopyranosyluronic acid) phosphate (**19**) in a yield of 25%.

Formation of the keto groups resulting from the oxidation of axially attached, secondary hydroxyl groups was observed in the oxidation of both **12** and **15**. The results of the catalytic oxidation of the closely related *muco*-inositol[30] and benzyl α-D-xylopyranoside[31] suggested that the hydroxyl group in **12** most likely to be oxidized is that located on C-4. It is of interest that, although the keto compound was degraded, probably by carbon–carbon cleavage, the level of uronic acid remained constant throughout the oxidation. The isolation of **18** suggests the presence of a CE conformation, as a CA conformation would favor formation of ketose, with subsequent carbon–carbon cleavage. It is possible, however, that formation of the carboxyl group precludes further oxidation.

EXPERIMENTAL

General

Melting points were taken on a hot stage and correspond to "corrected melting point". Specific rotations were determined with a Rudolph photoelectric polarimeter, Model 200, or with a Cary Model 60 spectropolarimeter, at the wavelength of the sodium D line.

The chloroform used was A.R. grade and contained approximately 0.75% of ethanol. Infrared spectra were recorded for potassium bromide discs with a Perkin–Elmer spectrophotometer Model 237. Chromatography was performed on "Silica Gel Davison", from the Davison Co., Baltimore, Maryland 21201 (grade 950, 60–

200 mesh), which was used without pretreatment. When deactivation by contact with moist air occurred, reactivation was conducted 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 ratio of weight of substance to weight of absorbent was 1:50–100. The ratio of weight of substance (in g) to volume of fraction of eluent (in ml) was 1:100. The ratio of diameter to length of the column was 1:20. Evaporations were conducted *in vacuo*, with the bath temperature below 45°. Volumes of volatile solvent smaller than 20 ml were evaporated under a stream of dry nitrogen.

Hexoses, uronic acids, and ketoses were determined by the anthrone[32], carbazole[33] (or orcinol[34]), and *o*-phenylenediamine[35] methods, respectively. In the study of the oxidation, values obtained by the carbazole method were corrected for the L-idopyranosyl phosphate present, and the values obtained by the *o*-phenylenediamine method were corrected for both the L-idopyranosyl phosphate and the L-iduronic acid present; a hydrolyzate of 1,2-*O*-isopropylidene-β-L-idofuranurono-6,3-lactone was used as the standard for L-iduronic acid.

Spots on paper chromatograms were revealed by the silver nitrate dip procedure[36], the Hanes–Isherwood spray reagent[37], the *o*-phenylenediamine dihydrochloride spray test[35], and the hydroxamate test[38], as means of detecting reducing sugars, inorganic and organic phosphate, ketoses, and lactones, respectively.

Electrophoresis of 50–200 μg of material was performed on Schleicher and Schuell No. 598 paper strips (2.5 × 18.5 in) in 0.05M sodium barbiturate buffer (pH 10.0) at 650 volts for 2 h. The electrophoretograms were dried at 85° before revelation of the spots.

The n.m.r. spectra of the acetylated glycopyranoses were determined by Mr. R. Pitcher, Varian Associates, Inc., Pittsburgh, Pa., with a Varian HR-100 n.m.r. spectrometer, on 15–20% solutions in deuterochloroform containing 2% of tetramethylsilane (τ 10.00) as the internal standard. The n.m.r. spectra of the glycopyranosyl phosphates were determined by Mr. S. Justice, of the Research Triangle Institute, Durham, N. C., with a Varian A-60 n.m.r. spectrometer, on 15–20% solutions in deuterium oxide. The solutions were prepared after lyophilization of the materials from a solution in deuterium oxide. An external, capillary tube containing tetramethylsilane (0.00 p.p.m.) was used as the reference standard.

The microanalyses were performed by Galbraith Analytical Laboratories, Knoxville, Tennessee, by Dr. M. Manser, Zurich, Switzerland, and by Dr. L. Nagy, Cambridge, Massachusetts.

3,5-Di-O-acetyl-6-O-benzoyl-1,2-O-isopropylidene-β-L-idofuranose (6). — Dry Dowex-1 X-10(CH_3COO^-)ion-exchange resin (200–400 mesh, 700 g), was gradually added, with constant stirring, to acetic anhydride (1.5 l). After the mixture had been cooled, 48 g of 5[9,10] was added in small portions to the stirred mixture, the mixture was refluxed for 20 h and filtered hot, and the resin washed with acetic anhydride (300 ml). Peak positions were read directly from precalibrated Varian chart paper. All spectra were determined at 35–40°. After evaporation of the filtrate, the residual syrup was dissolved in ether, and the solution was washed with water, dried with sodium sulfate, decolorized (Norit A), and evaporated. The residue was crystallized from absolute ethanol to give 31 g of **6** (75%), m.p. 121°.

Penta-O-acetyl-α-L-idopyranose (**8**). — Compound **7** (2.72 g) was added to 50 ml of 2:1:0.03 acetic anhydride–glacial acetic acid–sulfuric acid. After being kept at room temperature for 24 h, the solution was poured onto a mixture of ice and sodium hydrogen carbonate (or sodium acetate), stirred for 4 h, and extracted with chloroform. The extract was dried with sodium sulfate and evaporated; the resulting syrup was dissolved in benzene and chromatographed on silica gel. Elution with 9:1 benzene–ether gave a crystalline product, which was recrystallized from absolute ethanol (1.1 g). Anomerization of the dried product from the mother liquor with 4:2:0.1 acetic anhydride–glacial acetic acid–sulfuric acid for 16 h at room temperature gave 0.60 g of crystalline product; a second isomerization gave 0.25 g; the total yield was 1.95 g (50%), m.p. 95–96°, $[\alpha]_D^{25}$ $-57°$ (*c* 1, chloroform).

Anal. Calc. for $C_{16}H_{22}O_{11}$: C, 49.23; H, 5.68. Found: C, 49.35, 49.08; H, 5.80, 5.79.

Sorkin and Reichstein[11] reported m.p. 90–92° and $[\alpha]_D^{25}$ $+54.3°$ (*c* 1.565, chloroform) for the α-D anomer.

2,3,4,6-Tetra-O-acetyl-α-L-idopyranosyl bromide (**9**)*. — Syrupy **8**, obtained by acetolysis of 2,3,4-tri-*O*-acetyl-1,6-anhydro-β-L-idopyranose (0.94 g) as described above, was dissolved in 25 ml of a 30% solution of hydrobromic acid in glacial acetic acid at 0°; crystals began to appear after a few min. After being kept at room temperature for 2 h, the solution was diluted with 1,2-dichloroethane (15 ml), poured into ice-water (50 ml), and extracted with chloroform. The organic layer was washed with water, dried with calcium chloride, and evaporated. The crystalline residue was recrystallized from 1,2-dichloroethane–ether–pentane to give 1.70 g (52%) of plates, m.p. 126–127°, $[\alpha]_D^{25}$ $-120°$ (*c* 0.75, chloroform).

Anal. Calc. for $C_{14}H_{19}BrO_9$: C, 40.89; H, 4.66; Br, 19.44. Found: C, 40.75; H, 4.68; Br, 19.43.

Reaction of 2,3,4,6-tetra-O-acetyl-α-L-idopyranosyl bromide (**9**) *with silver acetate.* — To a solution of **9** (0.1 g) in abs. benzene (10 ml) was added dry silver acetate (0.2 g). After being heated under reflux for 5 h, with stirring, the mixture was filtered, and the filtrate was evaporated to a syrup which was dissolved in 1,2-dichloroethane and chromatographed on silica gel. Elution with 1,2-dichloroethane–ether (9:1) gave crystalline fractions. After recrystallization from ethanol–hexane, 45 mg (47%) of penta-*O*-acetyl-α-L-idopyranose (**8**) was obtained, m.p. 94–95°, $[\alpha]_D^{25}$ $-45°$ (*c* 0.32, chloroform). The product showed no depression of the m.p. in admixture with the **8** previously described.

Reaction of 2,3,4,6-tetra-O-acetyl-α-L-idopyranosyl bromide (**9**) *with methanol.* — To a solution of crude **9** (0.14 g) in methanol (15 ml) was added silver carbonate (0.2 g), and the mixture was shaken overnight at room temperature. After filtration

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*This product was first obtained by Dr. N. Baggett.

through a pad of Celite, the solution was evaporated to dryness. The residue was dissolved in benzene and chromatographed on silica gel. Benzene–ether (2:1) eluted crystalline fractions (72 mg) which gave, after recrystallization from 1,2-dichloroethane–hexane, 30 mg (27%) of methyl 2,3,4,6-tetra-*O*-acetyl-β-L-idopyranoside (**10**), m.p. 116–117°, $[\alpha]_D^{26}$ +42° (*c* 0.95, chloroform).

Anal. Calc. for $C_{15}H_{22}O_{10}$: C, 49.72; H, 6.12; CH_3CO, 65.19. Found: C, 49.62; H, 6.13; CH_3CO, 66.70.

A sample was deacetylated with sodium methoxide in methanol. After removal of the sodium ions with Dowex-50, the sample was chromatographed on Whatman paper No. 1 (reference compounds, methyl α-L-idopyranoside and methyl β-L-idopyranoside, prepared from the crystalline 4,6-*O*-benzylidene derivatives[23]). After development with 3:1:1 *tert*-pentyl alcohol–isopropyl alcohol–water and with butyl alcohol saturated with 1% ammonia, the spot of deacetylated **9** had the same R_F as the spot of methyl β-L-idopyranoside.

Elution with 1:1 benzene–ether gave 38 mg of crystalline material which was recrystallized from 1,2-dichloroethane–hexane to give 34 mg (30%) of product, m.p. 125–127°, $[\alpha]_D^{23}$ −2° (*c* 0.32, methanol). The elementary analysis corresponded to that for a tetra-*O*-acetylhexose. A solution of this product in *p*-dioxane reduced an alkaline solution of iodine. The infrared spectrum exhibited absorption maxima at 3460, 1745, 1435, 1380, 1270, 1225, 1150, 1115, 1055, 950, 915, and 835 cm^{-1}.

Anal. Calc. for $C_{14}H_{10}O_{10}$: C, 48.28; H, 5.79. Found: C, 48.37; H, 5.25.

α-L-*Idopyranosyl phosphate* (**13**). — Finely powdered **8** (500 mg) was placed in the top section of a Thunberg tube equipped with a side arm, and powdered, anhydrous phosphoric acid (600 mg) was placed in the bottom section. After the tube and its contents had been thoroughly dried *in vacuo* over magnesium perchlorate for 3 days, the side arm was connected to a vacuum pump and the contents of the lower section were heated at 60°. After the phosphoric acid had melted, the two substances were mixed, and the reaction was allowed to proceed for 2 h; vigorous evolution of gases occurred. The melt was dissolved in purified tetrahydrofuran (3 ml), and the solution was added to ice-cold N lithium hydroxide (30 ml). The solution was kept for 18 h at room temperature and then for 2 h at 0°. Lithium phosphate was removed by centrifugation, and the supernatant liquor was freed of lithium ions by passage through a column (2.2 × 15 cm) of Dowex-50 X-10 (Cl^-) (200–400 mesh). The acidic portion of the eluate was collected in cyclohexylamine (1.1 ml), and the resulting solution was chromatographed on a column (2.2 × 20 cm) of Dowex-1 X-10 (Cl^-) (200–400 mesh). The column was washed with water until the effluent gave a negative anthrone test. Subsequently, the column was eluted with 0.05N lithium chloride in 0.003N hydrochloric acid. The fractions giving a positive anthrone reaction were combined, the pH was adjusted to 8.0 with 0.1N lithium hydroxide, and the solution was lyophilized. After the material had been thoroughly dried *in vacuo* over magnesium perchlorate, the lithium chloride was removed by dissolution in abs. methanol (1 ml) and precipitation with dry acetone (10 ml) to give 200 mg (55%), $[\alpha]_D^{25}$ −27.5° (*c* 1, water).

Anal. Calc. for $C_6H_{11}Li_2O_9P \cdot 0.5\ H_2O$: C, 25.62; H, 4.30; P, 11.02. Found: C, 25.81; H, 4.30; P, 10.25.

The presence of traces of inorganic phosphate was shown by chromatography on Whatman No. 1 paper with 7:1:2 isopropyl alcohol–ammonia–water[39], and by colorimetric analysis[40].

Conversion of the lithium salt (75 mg) into the barium form was accomplished by dissolution in water (8 ml) and addition of conc. ammonia to alkalinity, followed by the addition of magnesium acetate (50 mg). After filtration, the solution was passed through a column of Dowex-50 (H^+). Dilute barium hydroxide solution was added to the effluent until it was alkaline, and the solution was concentrated to 10 ml. After addition of ethanol (30 ml), the solution was kept for 2 days in a refrigerator, and then centrifuged. The insoluble material was dissolved in water (10 ml), the solution was centrifuged, ethanol (20 ml) was added to the supernatant liquor, and the suspension was centrifuged. This procedure was repeated 3 times, to give 48 mg of amorphous powder, $[\alpha]_D^{22}$ −15.6° (*c* 0.32, water).

Anal. Calc. for $C_6H_{11}BaO_9P$: C, 18.22; H, 2.84; P, 7.83. Found: C, 18.10; H, 2.89; P, 7.44.

Colorimetric determination by the anthrone method gave an idose content of 47.1% (calc. 45.6%), crystalline 2,3,4-tri-*O*-acetyl-1,6-anhydro-*β*-L-idopyranose being used as the standard.

Conversion into the cyclohexylammonium salt was accomplished by passing an aqueous solution of the lithium salt through Dowex-50 W-X8 (cyclohexylammonium$^+$, 200–400 mesh). (The resin had been prepared by percolating the H^+ form with a 20% solution of cyclohexylamine in water, and then with distilled water.) Absence of lithium ions was verified by a negative flame-test. The effluent was lyophilized, and the residue was dried by additions of ethanol, followed by evaporation. The material was then dissolved in the minimum volume of water, and abs. ethanol was gradually added to turbidity. After 24 h in the cold, the crystalline di(cyclohexylammonium) salt **13** was obtained in a yield of 50–60% (relative to the amount of lithium salt taken), m.p. 145–150°, $[\alpha]_D^{25}$ −32° (*c* 0.41, water).

Anal. Calc. for $C_{18}H_{39}N_2O_9P$: C, 47.16; H, 8.50; P, 6.70. Found: C, 46.71; H, 8.41; P, 5.84.

Electrophoresis of the mother liquor showed a major component, an idopyranosyl phosphate, contaminated with a small proportion of inorganic phosphate.

Free L-idopyranosyl phosphate was also prepared, care being taken to avoid acidic conditions that might cause hydrolysis of this phosphate. Thus, after hydrolysis of the acetate groups with N lithium hydroxide and removal of the precipitated lithium phosphate, the solution was passed through a column of Dowex-50 in the cyclohexylammonium form. Chromatography of the anthrone-positive effluent was performed on a column of Dowex-1. Water, and then 0.05N and 0.075N lithium chloride, eluted free hexose, inorganic phosphate, and hexopyranosyl phosphate, respectively. Removal of the lithium chloride followed the procedure previously described. The chloride-free L-idopyranosyl phosphate was dissolved in water, and the solution was filtered and lyophilized. The residue was dried over magnesium perchlorate in a desiccator; $[\alpha]_D^{25}$ $-30.5°$ (*c* 2, water). The yield of crystalline di(cyclohexylammonium) salt from this sample was essentially identical to that reported above.

α-D-*Mannopyranosyl phosphate* (**15**). — Reaction of penta-*O*-acetyl-*α*-D-mannopyranose[24] with phosphoric acid was performed under conditions similar to those described for penta-*O*-acetyl-*α*-L-idopyranose. The product was isolated under non-acidic conditions, as described for the idose derivative, and an amorphous product in a yield of *ca.* 50% was obtained. Electrophoresis revealed only traces of inorganic phosphate. The material was hygroscopic; it was dried for 12 h at 110–120° *in vacuo*; $[\alpha]_D^{25}$ $+46.3°$ (*c* 1, water). Posternak and Rosselet[25] reported $[\alpha]_D^{25}$ $+33.7°$ (*c*, 3.01, water) for the anhydrous barium salt.

Anal. Calc. for $C_6H_{12}LiO_9 \cdot H_2O$: C, 25.37; H, 4.97. Found: C, 26.01; H, 5.05.

(*α*-L-*Idopyranosyluronic acid*) *phosphate* (**18**). — Preliminary experiments on the oxidation of **12** in the presence of platinum black showed a rapid disappearance of anthrone-positive material, and the rapid appearance of carbazole-positive material (uronic acids) and of *o*-phenylenediamine-positive material (ketoses). The formation of uronic acid reached a plateau at about 10%, whereas the ketoses slowly disappeared. These results were confirmed by chromatography on Whatman No. 1 paper with the solvent system 10:6:0.16 isobutyric acid–N ammonia–0.1M ethylenedinitrilotetraacetic acid.

The lithium salt of L-idopyranosyl phosphate (**12**) (240 mg) was dissolved in water (32 ml), the reaction vessel was immersed in a constant-temperature bath at 50°, and air-dried platinum-on-carbon catalyst (725 mg) was added. Oxygen was introduced in a stream below the surface of the solution at a rate sufficient to keep the catalyst well suspended. The course of the reaction was followed by the anthrone method; the oxidation was complete after 12 h. After the catalyst had been filtered off, the pH of the filtrate was adjusted to 9–10 with dilute lithium hydroxide, and the solution was lyophilized. The residue was dissolved in water (5–6 ml), and the solution passed through a Dowex-50 AG 50W-X8 (cyclohexylammonium$^+$) (2 × 15 cm, 200–400 mesh), column. Orcinol-positive material was collected, and passed through a Dowex-1 AG 1-X8, (Cl^-) (2 × 20 cm, 200–400 mesh), column. The column was washed with water until all anthrone-positive material had been removed. Elution with 0.075N lithium chloride gave a small peak that was positive to the anthrone and phosphate tests and negative to the orcinol test, correponding to starting material. Elution with 0.1N lithium chloride gave a small peak, which was positive to the *o*-phenylenediamine test, and probably corresponded to a keto sugar. Elution with 0.2N lithium chloride gave a large peak which was negative to the anthrone test and positive to the orcinol and phosphate tests. After the pH of the effluent containing the last peak had been adjusted with dilute lithium hydroxide to approximately 9, the solution was lyophilized, and then evaporated to complete dryness *in vacuo* over magnesium perchlorate. Lithium chloride was removed as previously described. Paper electrophoresis of the remaining material (about 65 mg) revealed the presence of (hexosyluronic acid) phosphate and traces of inorganic phosphate.

The oxidation at 45° of the lithium salt of L-idopyranosyl phosphate (**12**) (80 mg) in the presence of platinum black (prepared from 50 mg of platinic oxide) at pH 8.0–9.0 for 11 h, followed by filtration of the platinum salt, passage through a column (2.5 × 17 cm) of Dowex-1 (Cl^-) and elution with a rising concentration-gradient of lithium chloride solution gave two fractions; these contained hexose and hexuronic acid, respectively. After the pH of each fraction had been adjusted to 8.5 with lithium hydroxide, the solution was concentrated, and acetone or methanol was added. The precipitate was centrifuged off and dissolved in water, and the precipitation was repeated. The recovery of starting material from the first fraction gave 40 mg, and the second fraction, containing the hexuronic acid, gave 14 mg.

Conversion of the lithium salt (65 mg) into the cyclohexylammonium salt, followed by lyophilization and drying, gave 110 mg of crude material which was dissolved in the minimum volume of boiling, absolute ethanol, and the solution was kept at room temperature, giving crystals. Recrystallization from the same solvent gave 30 mg, m.p. 135–138°, $[\alpha]_D^{25}$ – 15.8° (*c* 0.657, water). The infrared spectrum exhibited absorption maxima at 3520, 2950, 2870, 1680, 1570, 1535, 1485, 1450, 1395, 1370, 1345, 1310, 1220, 1195, 1130, 1120, 1090, 1065, 1040, 990, 975, 950, 900, 845, and 770 cm^{-1}.

Anal. Calc. for $C_{18}H_{37}N_2O_{10}P \cdot H_2O$: C, 44.08; H, 8.01; P, 6.32. Found: C, 44.53, 44.27; H, 8.10, 8.26; P, 5.43.

Evaporation of the first mother-liquor gave 50 mg of amorphous material, $[\alpha]_D^{25}$ + 12.9° (*c* 1.43, water), which probably contained a large proportion of the β-L anomer. Both the crystalline and the amorphous material had the same electrophoretic mobility, and only traces of inorganic phosphate were detected in them.

(α-D-Mannopyranosyluronic acid) phosphate (**19**). — Oxidation of the lithium salt of **15** (155 mg), dissolved in water (20 ml), was performed in the presence of platinum-on-carbon catalyst (300 mg) for 8 h at 50°. Isolation followed the procedure used for the preparation of **12**, and gave 40 mg of amorphous lithium salt, $[\alpha]_D^{25}$ + 19.1° (*c* 0.943, water). On paper electrophoretograms, the material behaved as a (hexosyluronic acid) phosphate, and traces of inorganic phosphate were detected.

Anal. Calc. for $C_6H_8Li_3O_{10}P \cdot 3.5\ H_2O$; C, 20.30; H, 4.26; P, 8.73. Found: C, 20.13, 19.97; H, 4.04, 4.10; P, 7.50.

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SUMMARY

Penta-*O*-acetyl-α-L-idopyranose, obtained from 2,3,4-tri-*O*-acetyl-1,6-anhydro-β-L-idopyranose, was directly phosphorylated with anhydrous phosphoric acid, and

then deacetylated, to give an anomeric mixture of L-idopyranosyl phosphates from which the α-L anomer was crystallized as the di(cyclohexylammonium) salt. The same method, applied to penta-*O*-acetyl-α-D-mannopyranose, produced only α-D-mannopyranosyl phosphate. The conformation of the starting material and of the reaction intermediates, as well as the presence of both anomers of L-idopyranosyl phosphate, were established by n.m.r. spectroscopy. A reaction mechanism for the phosphorylation is presented. This mechanism is based primarily on the relative orientation of substituents at C-1 and C-2.

Oxidation of the amorphous L-idopyranosyl phosphate and of α-D-mannopyranosyl phosphate, when platinum-on-carbon or platinum black was used as the catalyst, gave (in low yield) an anomeric mixture of (L-idopyranosyluronic acid) phosphates (from which the α-L anomer was isolated as its crystalline cyclohexylammonium salt) and (α-D-mannopyranosyluronic acid) phosphate, respectively. Ketose formation was observed in both cases, and must be due to the oxidation of axially attached, secondary hydroxyl groups.

Attempts to prepare L-idopyranosyl phosphate from 2,3,4,6-tetra-*O*-acetyl-α-L-idopyranosyl bromide were unsuccessful. Reaction of the bromide with silver acetate gave penta-*O*-acetyl-α-L-idopyranose, whereas treatment with methanol in the presence of silver carbonate gave methyl 2,3,4,6-tetra-*O*-acetyl-β-L-idopyranoside.

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KERATAN SULFATE FRACTIONS FROM BOVINE AND HUMAN TISSUES*

J. A. CIFONELLI, A. SAUNDERS**, AND J. I. GROSS†
La Rabida-University of Chicago Institute, and the Department of Biochemistry, University of Chicago, Chicago, Illinois 60649 (U.S.A.)

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Kaplan and Meyer[1] have reported that the concentration of keratan sulfate in human costal cartilage varies with age. Similar variations with age have been reported for cartilage of chicken, dogfish, and squid[2]. It has been claimed that keratan sulfate is absent in newborn human costal cartilage; it has been found, however, in embryonic chick vertebral cartilage[2].

Various degrees of sulfation have been noted for most acid mucopolysaccharides, Meyer *et al.*[3] found that the chondroitin sulfate fractions from bovine corneal stroma ranged in degree of sulfation from approximately 0.2 to 1.0 molar equivalents per repeating disaccharide unit. Keratan sulfate containing more than 1 mole equivalent of sulfate per disaccharide repeating unit has been isolated from shark[4,5] and human cartilage[6], and keratan sulfate fractions containing slightly less, as well as containing more, than one mole equivalent of sulfate per disaccharide repeating unit were isolated from bovine corneal stroma[7].

The present study describes the distribution of sulfate content of keratan sulfate fractions isolated from various adult and embryonic mammalian tissues.

EXPERIMENTAL

Methods and materials. — Methods for the determination of uronic acid, hexosamine, carbohydrate, nitrogen, 6-deoxyhexose, and total sulfate have been described previously[6]. Sialic acid was determined by the method of Svennerholm[8].

Hydrolyzates were prepared by heating keratan sulfate preparations in N HCl for 3 h and deacidifying the solutions with Dowex-3 (CO_3^{2-}).

Paper chromatography was performed in 2-methyl-2-butanol–2-propanol–water (4:1:1.5) for 36–48 h. Visualization of spots was obtained by staining with a silver nitrate reagent[9].

Electrophoresis of keratan sulfate fractions was conducted in 0.1M pyridine–formate buffer, pH 3.0, at 8 volts/cm, for 1.0–1.5 h, using Sepraphore III as support

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**Present address: Department of Pathology, Stanford University School of Medicine.

†Deceased.

medium. Visualization of substances was accomplished by spraying the strips with 1% acridine orange solution.

G.l.c. was performed on an F & M Model 400 Biomedical Gas Chromatograph. Deacidified hydrolyzate samples were converted into the trimethylsilyl derivatives and chromatographed on a 4-ft column containing 2.5% SE-30 (obtained from F & M Scientific Co., Avondale, Pa.).

The Technicon amino acid autoanalyzer was used for the determination of 2-amino-2-deoxygalactose in hydrolyzates prepared by heating samples with 6N HCl for 20 h, and removing the acid by evaporation *in vacuo*.

Animal tissues were obtained from a local slaughter house and kept frozen until used. Bovine nasal septa were obtained from 2-year old animals slaughtered for market. Human intervertebral tissues, kindly provided by Dr. H. Fahrni, University of Vancouver, Vancouver, B.C. and Dr. S. Glagov, Department of Pathology, University of Chicago, were obtained at autopsy and were kept frozen until used. Cross-contamination of annulus and cartilage was avoided by excluding tissue from the region in which these are joined.

Isolation of mucopolysaccharides. — Freshly excised tissues were defatted and dehydrated by passage through several changes of acetone. The dried tissues were cut into small pieces and to each gram of tissue was added 0.15M acetate buffer, pH 5.0 (10 ml), 0.45M NaCl (5 ml), cysteine hydrochloride (0.3 mg), Versene (0.6 mg) and papain (twice crystallized) (2 mg). Digestion was carried out for 8–12 h at 55–60°. After dialysis overnight against tap water, the digest was centrifuged, and the sediment was discarded. Ethanol (one-fourth the volume) and acid-washed Norit (approximately 0.5 g per g of tissue) were added to the supernatant fractions. After being stirred for several min, the mixture was centrifuged, and the polysaccharide adsorbed to charcoal was eluted by stirring for 0.5 h at 2° with 30% ethanol containing 0.5% NH_4OH. Acid-washed Norit appeared to have a stronger affinity for keratan sulfate than for other acid mucopolysaccharides present in the extracts, and elution with ethanol–ammonia was required for complete recovery of the polysaccharide. When peptization of charcoal occurred, the ethanol and ammonia were removed *in vacuo*. The pH of the conc. solution was adjusted to 3–4 with acetic acid, and the solution was kept at room temperature for several h. A clear supernatant solution could then be obtained by centrifugation or filtration through Celite. The clarified solutions were applied to Dowex-1 X2, Cl^- (200–400 mesh) columns for fractionation[10] with graded concentrations of NaCl, as indicated in Tables I and II.

Preparations of acid mucopolysaccharides from extracts of bovine nasal septa differed from others in that chondroitin sulfate was removed by precipitation with 1.25 vol. of ethanol prior to resin-column chromatography[11]. Such extracts were kindly furnished by Dr. Lennart Rodén.

RESULTS

Properties of the keratan sulfate fractions. — Although some color was prod-

TABLE I

KERATAN SULFATE FRACTIONS ISOLATED FROM ADULT HUMAN INTERVERTEBRAL TISSUES AFTER COLUMN CHROMATOGRAPHY ON DOWEX-1

Tissue[a]	*NaCl molarity*	*Molar ratio of sulfate to hexosamine*	*Keratan sulfate (%)*[c]
Nucleus pulposus, normal	2.0	(–)[b]	25
	3.0	1.21	65
	4.0	1.60	10
Annulus, normal	2.0	(–)[b]	15
	3.0	1.16	70
	4.0	1.45	15
Intervertebral cartilage, normal	2.0	(–)[b]	25
	3.0	1.14	55
	4.0	1.43	20
Nucleus pulposus, degenerate	2.0	(—)[b]	15
	3.0	1.28	50
	4.0	1.44	35
Annulus, degenerate	2.0	(–)[b]	20
	3.0	1.11	70
	4.0	1.52	10

[a] Normal tissues were isolated from pooled autopsy material obtained from subjects ranging in age from 20–65 years. Degenerate tissues were obtained as pooled material from patients aged 39 and 63 years. [b] Sulfate content not determined, since these fractions represent mixtures containing approximately equal amounts of keratan sulfate and chondroitin sulfate. [c] Recoveries are estimated to the nearest 5% and are based on hexosamine content. Keratan sulfate content of the mixture fractions were based on the 2-amino-2-deoxyglucose and galactose concentrations.

uced by the keratan sulfate fractions with the carbazole reagent[12], the spectra were not characteristic of uronic acid. Analyses performed with the Technicon amino acid autoanalyzer indicated that all preparations examined, except those from corneal stroma, contained approximately 10% of the total hexosamine as 2-amino-2-deoxygalactose. This is in accordance with previous findings[6]. The proportions of sialic acid and 6-deoxyhexose were found to be variable, ranging from 0% in corneal stroma preparations to 3 or 4% in preparations from other tissues. Paper chromatography of hydrolyzates of keratan sulfate from intervertebral tissues showed galactose and 2-amino-2-deoxyglucose as principal components, along with much smaller amounts of material moving at a speed similar to those of fucose and mannose. Further evidence for the presence of these constituents was obtained by gas chromatography.

On the basis of nitrogen analyses, preparations from corneal stroma were protein-free, whereas preparations from other tissues contained 6–8% of protein. These results agreed with amino acid analyses previously reported[6]. The values of the optical rotations ranged from $[\alpha]_D - 9°$ to $+ 8°$, the rotations of the preparations from corneal stroma generally being positive, and the rotations of the preparations from intervertebral tissues or cartilage being more negative. Infrared spectra of several of the samples were similar to those reported for keratan sulfate[13].

TABLE II

KERATAN SULFATE FRACTIONS ISOLATED FROM BOVINE TISSUES AFTER COLUMN CHROMATOGRAPHY ON DOWEX-1

Tissue	*NaCl molarity*	*Molar ratio of sulfate to hexosamine*	*Keratan sulfate (%)*[b]
Adult			
Corneal stroma	2.0	0.90	40
	3.0	1.17	55
	4.0	1.22	5
Nucleus pulposus	2.0	(–)[c]	10
	3.0	1.14	50
	4.0	1.43	40
Nasal septum cartilage	1.0	0.46	10
	1.5	0.72	40
	2.0	0.90	30
	3.0	0.97	10
	4.0	1.09	10
Embryonic[a]			
Annulus	2.0	(–)[c]	15
	3.0	1.30	70
	4.0	1.42	15
Nucleus pulposus	2.0	(–)[c]	20
	3.0	1.30	60
	4.0	1.51	20

[a] Tissues obtained from an embryo 72 cm long.
[b] Recoveries estimated as indicated in Table I.
[c] Mixtures containing both keratan sulfate and chondroitin sulfate as described in Table I.

Chromatography of extracts on Dowex-1 columns gave the following results: (1) the fractions eluted with 1.0 and 1.5M NaCl contained only chondroitin sulfate, except for preparations from nasal septum cartilage that had been previously fractionated, (2) the fractions eluted with 2.0M NaCl were mixtures of chondroitin sulfate and keratan sulfate, except those from corneal stroma and nasal septum cartilage which contained only keratan sulfate, and (3) the fractions eluted with 3.0 and 4.0M NaCl consisted only of keratan sulfate. Keratan sulfate fractions, which were eluted by the various salt concentrations, did not differ in composition, except for sulfate content. The electrophoretic mobility of the preparations was proportional to the sulfate content.

Keratan sulfate from human tissues. — Table I presents the results of the fractionation of extracts prepared from various portions of human intervertebral discs. Fractions, that contained glycoprotein or chondroitin sulfate and were eluted with NaCl at concentrations up to 1.5M are not included. Keratan sulfate was present in fractions eluted by 2.0, 3.0, and 4.0M NaCl. The fractions eluted by 2M NaCl contained in all instances approximately equal amounts of chondroitin sulfate and keratan sulfate. The fractions eluted by 3M NaCl contained the major fraction of the keratan sulfate present in each tissue, and appeared to be slightly over-sulfated*. The fractions eluted by 4M NaCl contained generally only 10 to 20% of the total keratan sulfate, except for a fraction from degenerate nucleus pulposus which comprised 35% of the total. These preparations possessed high sulfate contents, ranging from 1.43 to 1.60 molar equivalents per equivalent of hexosamine, and no difference in degree of sulfation was noted for the degenerate tissues as compared to normal tissues. It is of interest that intervertebral cartilage showed much the same mucopolysaccharide composition as the other portions of the intervertebral disc.

Keratan sulfate from bovine tissues. — Fractions from adult and embryonic bovine tissues showed variations in sulfate content similar to those found in human intervertebral preparations. Table II indicates that the fractions, eluted by 2M salt from both embryonic and adult bovine intervertebral tissues, were similar to those from human sources and contained both chondroitin sulfate and keratan sulfate.

In the case of bovine corneal stroma, all of the chondroitin sulfate appeared in the fractions eluted with 0 to 2.0M NaCl. Accordingly, the fraction eluted by 2.0M salt contained predominantly keratan sulfate having a relatively low sulfate content. The lack of fractions from bovine nasal septum showing a significant content of chondroitin sulfate is due to the precipitation with ethanol, of the chondroitin sulfate present in the extracts before fractionation on Dowex. It is of interest that relatively large amounts of keratan sulfate were eluted from Dowex-1 with concentrations of salt up to 1.5M. This difference in elution patterns is consistent with the observation that sulfate contents of the nasal cartilage samples are generally lower.

Another interesting property shown by certain keratan sulfate fractions has been reported by Antonopoulos *et al.*[14]; they observed the tendency of cetylpyridinium complexes of keratan sulfate to redissolve on addition of excess cetylpyridinium chloride. This phenomenon was observed with the under-sulfated keratan sulfate fractions from nasal cartilage and corneal stroma eluted with salt concentrations up to 2M from Dowex-1 columns (Table II). In contrast, the fractions eluted with 3M and 4M NaCl formed, with cetylpyridinium chloride, complexes which showed little tendency to redissolve, even in a large excess of precipitating reagent.

In view of the presence of low-sulfated keratan sulfate in bovine corneal stroma and bovine nasal septa, such material was sought in the fractions obtained from bovine nucleus pulposus and eluted with 2M salt. When mixtures were rechromatographed on Dowex-1, no substantial separation of chondroitin sulfate and keratan sulfate was achieved. However, when these mixtures were first treated with 0.5N NaOH for 4 h at room temperature, the results presented in Table III were obtained. Although the mixture was not completely resolved, a large proportion of the total keratan sulfate was eluted by 3.0M NaCl. This fraction showed a sulfate content similar to that of the fraction obtained in the original fractionation by elution with 3.0M salt.

Keratan sulfate content of embryonic bovine tissues. — Table IV illustrates the

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*The terms over- and under-sulfated are used to denote in keratan sulfate, a content of sulfate groups, larger or smaller, than one mole of sulfate per disaccharide repeating unit.

keratan sulfate and total acid mucopolysaccharide contents in several tissues of bovine embryos. An increase in keratan sulfate of nucleus pulposus is apparent during embryonic development, the concentrations ranging from approximately 4% of the total acid mucopolysaccharides in 27-cm embryo to 15% in an embryo of 72-cm length. Results from annulus and intervertebral cartilage are shown only for the larger-size embryo, since the dissection of these tissues from the smaller embryo was not feasible. The annulus contained somewhat less keratan sulfate than was isolated from the nucleus pulposus, in agreement with the findings for human tissue (Table II).

One interesting finding is that keratan sulfate occurred in appreciable amounts in bovine embryonic intervertebral cartilage and represented 4% of the total acid mucopolysaccharide content. In contrast, cartilage of spinal processes or ribs contained 1% or less of keratan sulfate. The keratan sulfate produced in the embryonic intervertebral tissues could be resolved into several fractions having various sulfate contents similar to those from adult tissues (Table II). The total acid mucopolysaccharide contents of the more developed bovine embryonic intervertebral tissues were similar to those of adult human tissues, but the latter ones contained relatively more keratan sulfate.

DISCUSSION

Fractionation of various tissue extracts on Dowex-1, by the procedure described by Schiller *et al.*[10] for the separation of acid mucopolysaccharides, gave keratan sulfate fractions showing sulfate to hexosamine ratios ranging from approximately 0.5 to 1.6 (Tables I and II). All tissues examined, both from embryonic and adult mammalian sources, yielded keratan sulfate preparations with some degree of over-sulfation. The most highly sulfated keratan sulfate preparations were obtained from intervertebral

TABLE III

FRACTIONATION ON DOWEX-1 OF KERATAN SULFATE–CHONDROITIN SULFATE MIXTURE FROM NUCLEUS PULPOSUS AFTER ALKALINE TREATMENT[a]

NaCl molarity	*Components*[b]	*Recovery*[c] (%)	*Molar ratio of sulfate to hexosamine*	*Molar ratio of nitrogen to hexosamine*
0.0 and 1.0	Glycoprotein	5	0	
1.5	Chondroitin sulfate	17	1.06	1.90
2.0	Mixture	36	1.04	1.95
3.0	Keratan sulfate	37	1.14	2.05
4.0	Keratan sulfate	3		

[a] The fraction eluted with 2.0M salt, as shown in Table II, was prepared as a 0.5% solution in 0.5N NaOH and kept for 4 h at 24°. After dialysis and concentration, the solution was applied to Dowex-1 X2 (Cl^- 200–400 mesh) for fractionation. [b] The mucopolysaccharides of the mixture shown in the Table, as well as of the original starting material, consisted of approximately one-third CS and two-thirds keratan sulfate. The 1.5M preparation had a uronic acid to hexosamine ratio of 0.98 and fractions 3.0M and 4.0M had ratios <0.05. The glycoprotein fraction had a ratio of hexosamine to neutral sugar of 0.95. [c] Based on hexosamine content.

tissues; no under-sulfated fractions were found in these tissues (Table III). Embryonic sources gave keratan sulfate fractions showing sulfate contents which were similar to those from adult tissues. In contrast, bovine nasal septa and corneal stroma tissues yielded keratan sulfate which was predominantly under-sulfated.

These results indicate that the salt concentrations required for elution of keratan sulfate from Dowex-1 are related to the sulfate contents of the preparations, the fractions having the highest sulfate concentrations requiring salt concentrations up to 4M for elution. The unexpectedly high-salt concentrations needed for elution of keratan sulfate from the resin columns, though not fully understood, may involve different factors: for example molecular size polydispersity with the occurrence of fractions having relatively low molecular weights, as described by Anseth and Laurent[15], or the presence of regions with high sulfate densities in which contiguous hexosamine and galactose units are sulfated, as shown by Bhavanandan and Meyer[16]. Such factors may also account for the characteristic property shown by keratan sulfate–cetylpyridinium complexes to redissolve in excess complexing reagent, as reported by Antonopoulous *et al.*[14]. This behavior was observed for the keratan sulfate fractions eluted with concentrations of salt up to 2M, but only to a slight extent or not at all for those eluted with higher salt concentrations.

TABLE IV

KERATAN SULFATE CONTENT OF CALF EMBRYOS

Embryo size in cm	*Tissue*	*Keratan sulfate (%)*[a]	*Total mucopolysaccharide content (%)*[b]
7	Whole embryo	<1	1
27	Nucleus pulposus	4	15
72	Nucleus pulposus	15	20
	Annulus	10	10
	Vertebral cartilage	4	18
	Spinal cartilage	1	—
	Rib cartilage	<1	—

[a] Calculated on basis of total mucopolysaccharide content.
[b] Calculated from dry weight of tissue.

Mixed fractions containing both chondroitin sulfate and keratan sulfate, the latter amounting to approximately 10% of the total keratan sulfate, were invariably obtained from intervertebral tissue extracts during elution from Dowex-1 with 2M salt solution. Whether these represent complexes linked by covalent bonding, as suggested previously[17,18], or just simple mixtures is not clear from the data. It was found, however, that more complete separation of components on Dowex-1 occurred when the mixed fractions had undergone a preliminary treatment with alkali.

The keratan sulfate content of bovine intervertebral tissues was found to increase during embryonic development, and represented approximately 15% of the total acid mucopolysaccharide content by the third trimester (Table IV). In contrast, rib and spinal cartilage from the same source showed a keratan sulfate content amounting to 1% or less of the total acid mucopolysaccharide content. The latter results are in agreement with the findings of Kaplan and Meyer[1] that rib cartilage from human embryonic source is low in keratan sulfate. Similar findings on human fetal and rabbit embryonic cartilage have been reported by Matthews[19].

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SUMMARY

Keratan sulfate fractions having various sulfate contents have been obtained from different tissue sources. Highly sulfated fractions of keratan sulfate have been obtained from embryonic and adult bovine intervertebral tissues, as well as from adult human intervertebral tissues. Under-sulfated keratan sulfate fractions were isolated only from bovine corneal stroma and from bovine nasal-septum cartilage.

All tissues produced fractions showing relatively wide variations of sulfate content. Increase in keratan sulfate content of bovine intervertebral tissues was noted during embryonic development, although this concentration was one-half or less that of adult bovine tissues. Acid mucopolysaccharide content of embryos in the last trimester corresponded to those found in adult bovine intervertebral tissues.

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REACTIONS OF 2-AMINO-2-DEOXY-D-GLUCOSE WITH BENZOYLACETALDEHYDE AND 1-PHENYL-1,3-BUTANEDIONE

A. GÓMEZ SÁNCHEZ, M. GÓMEZ GUILLÉN,

Cátedra de Química Orgánica, Universidad de Sevilla, and Instituto de Química "Alonso Barba", C.S.I.C., Seville (Spain)

AND U. SCHEIDEGGER

Research Laboratory, Varian AG, Zürich (Switzerland)

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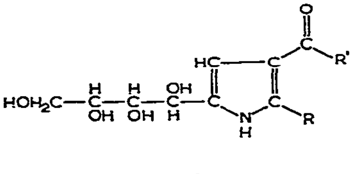
INTRODUCTION

The reaction of 2-amino-2-deoxy-D-glucose with β-dicarbonyl compounds[1] gives pyrrole derivatives of the general formula **1**. When the reaction is performed using 2,4-pentanedione[2,3] in an anhydrous, basic medium, 2-deoxy-2-[2-(4-oxo-2-pentenyl)amino]-D-glucose (**2**) is obtained in high yield. This substance cyclizes readily yielding 3-acetyl-5-(D-*arabino*-tetrahydroxybutyl)-2-methylpyrrole (**1**, R = R′ = Me), 3-acetyl-2-methylpyrrole, and D-erythrose[3]. On this basis, it has been considered[1,3] that enamines of type **3**, or a tautomeric form[4], may be the first intermediates in the formation of the (tetrahydroxybutyl)pyrroles (**1**). In order to get a further insight into the mechanism of these transformations, we have studied the reactions of 2-amino-2-deoxy-D-glucose with other highly enolizable β-dicarbonyl compounds, and this paper describes the results obtained with benzoylacetaldehyde and 1-phenyl-1,3-butanedione (benzoylacetone). β-Oxoaldehydes are more enolic than are the analogous β-diketones, and are considered to exist mainly in their hydroxymethyleneketone forms[5] (**4** in the case of benzoylacetaldehyde); 1-phenyl-1,3-butanedione contains 94% of enol form(s) in ethanol solution[6].



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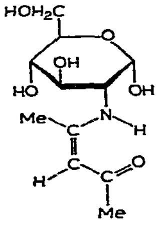

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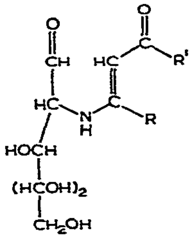

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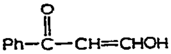


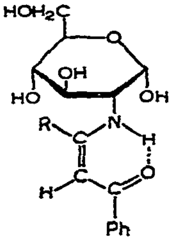
$Ph-\overset{O}{\overset{\|}{C}}-CH{=}CHOH$

4

RESULTS AND DISCUSSION

The reaction of 2-amino-2-deoxy-D-glucose with benzoylacetaldehyde and

1-phenyl-1,3-butanedione in methanol, in the presence of triethylamine, took place readily yielding 2-deoxy-2-[1-(3-oxo-3-phenyl-1-propenyl)amino]-D-glucose (**5**) and 2-deoxy-2-[2-(4-oxo-4-phenyl-2-butenyl)amino]-D-glucose (**6**), respectively. The former compound could also be obtained in high yield by merely treating an aqueous solution of the amino sugar with benzoylacetaldehyde at room temperature. Support for the structures of these substances are presented in the following discussion of their properties.



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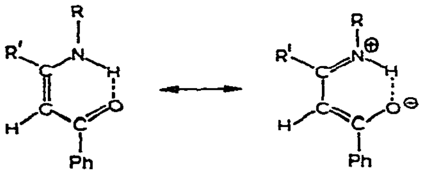
6 R=Me



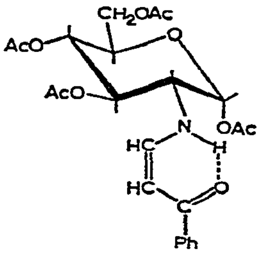
7a 7b

Compounds **5** and **6** had high, positive, optical rotations suggestive of α-D-anomeric configurations. Their i.r. spectra showed several strong bands in the double-bond region, those at the highest frequencies [1631 cm^{-1} and 1603 cm^{-1} in compounds **5** and **6**, respectively] being assigned as carbonyl stretching frequencies perturbed by chelation and by high contributions of the ionic forms (**7b**), (R = sugar residue) to the ground states of the molecules. Similar shifts of the carbonyl bands to lower frequencies have been observed[3] in the 2,4-pentanedione derivative **2**, in the intramolecularly bonded *cis*-forms of simple β-amino-α,β-unsaturated ketones[7] (**7**, R = H, alkyl), and in the enol forms of β-diketones[8].

The enamines **5** and **6** gave positive Fehling's and ferric chloride tests, presumably because of their hydrolysis to the parent β-dicarbonyl compounds and 2-amino-2-deoxy-D-glucose. However, these substances seem to be more resistant to hydrolytic cleavage than the derivative (**2**) of 2,4-pentanedione. The optical rotation of the benzoylenamine **5** in aqueous solution did not change with time; hydrolyis of the 1-phenyl-1,3-butanedione derivative (**6**) took place slowly in aqueous solution at room temperature, and could be followed polarimetrically and by the separation of 1-phenyl-1,3-butanedione. Under the same conditions, the derivative (**2**) of 2,4-pentanedione is rapidly destroyed, partly by hydrolysis and partly by transformation into compound **1'** (R = R' = Me) and 3-acetyl-2-methylpyrrole[3]. Hydrolysis of compounds **5** and **6** with dilute hydrochloric acid gave 2-amino-2-deoxy-D-glucose hydrochloride; in the case of compound (**6**), 1-phenyl-1,3-butanedione was also isolated.

Acetylation of the enamines **5** and **6** gave the tetra-*O*-acetyl derivatives **8** and **9**, respectively. The α-D-anomeric configurations of these substances were also indicated by their high, positive, optical rotations. Their i.r. spectra (carbon tetrachloride solutions) showed carbonyl bands at 1636 and 1606 cm^{-1} respectively, attributable, as in the parent compounds **5** and **6**, to the intramolecularly bonded ketoenamine system. Compounds **8** and **9**, in both the solid state and in solution, showed broad absorptions

(3300 to 3100 cm^{-1}) that may be assigned as stretching modes of the intramolecularly bonded NH group. A similar NH absorption is shown by the tetra-*O*-acetyl derivative (**10**) of compound **2**; this band remained unnoticed when the spectrum was taken under lower conditions of resolution and sensitivity[3]. The broadening of these bands



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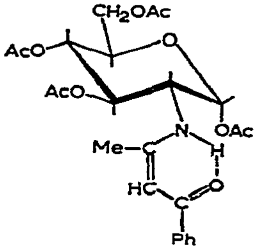

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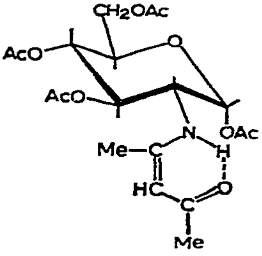


10

is similar to that experienced by the OH bands of the enol forms of β-diketones[8]. Weak, broad bands shown by the acetyl derivatives (**8**–**10**) at *ca.* 3500 cm^{-1} are considered to be overtones of the carbonyl stretching vibrations rather than due to OH or NH groups. Figure 1 shows the i.r. absorption of the acetyl derivative (**9**) in the NH stretching region and in the double-bond region.

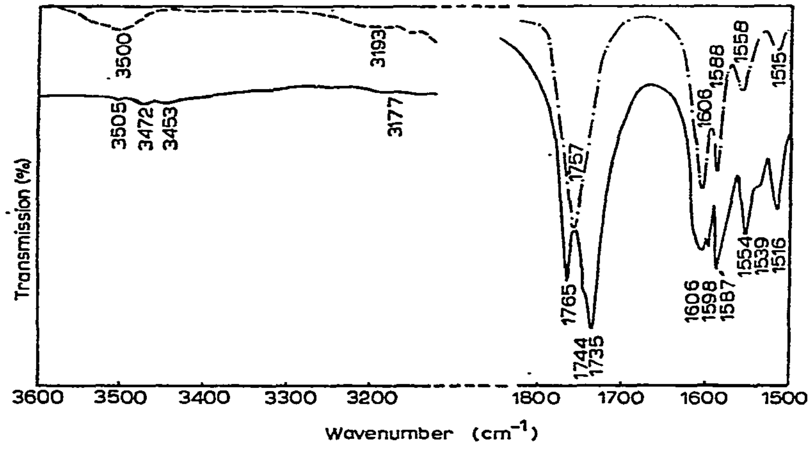


Fig. 1. Infrared absorption of 1,3,4,6-tetra-*O*-acetyl-2-deoxy-2-[2-(4-oxo-4-phenyl-2-butenyl)amino]-α-D-glucopyranose (**9**).
——— In Nujol; —·— in $CHCl_3$; – – – – in CCl_4.

Catalytic (sodium methoxide) deacetylation of compound **8** regenerated the parent substance (**5**). Treatment of the acetyl derivatives (**8**–**10**) with hydrochloric acid in acetone gave good yields of 1,3,4,6-tetra-*O*-acetyl-2-amino-2-deoxy-α-D-glucopyranose hydrochloride; this substance can be easily prepared from the 2,4-pentanedione derivative (**10**) in *ca.* 80% yield (*ca.* 35% from 2-amino-2-deoxy-D-glucose hydrochloride).

Data of the p.m.r. spectra of the acetyl derivatives are (**8**–**10**) included in Table I.

TABLE I

CHEMICAL SHIFTS (δ, p.p.m.) AND COUPLING CONSTANTS (Hz) OF COMPOUNDS 8–10 AT 100 MHz IN $CDCl_3$[a]

Substance	*H-1*	*H-2*	*H-3*	*H-4*	*H-5 and 2 H-6*	*OAc*	*N–H*	*H-1'*	*H-2'*	*Aromatic protons*
8	6.24d[b] $J_{1,2}$ 3.9	3.54sx $J_{2,3}$ 10.0	5.36t $J_{3,4}$ 9.8	5.11t $J_{4,5}$ 9.6	3.95—4.45m	2.02, 2.05 2.09, 2.30	10.08t $J_{NH,2}$ 10.0 $J_{NH,1'}$ 12.0	6.81q $J_{1',2'}$ 8.0	5.74d	7.4m 7.85m
9	6.21d $J_{1,2}$ 3.9	3.99sx $J_{2,3}$ 9.8	5.39t $J_{3,4}$ 9.8	5.15t $J_{4,5}$ 9.8	3.95—4.50m	2.01, 2.05 2.11[c], 2.35	11.31 $J_{NH,2}$ 10.2		5.69	7.4m 7.8m
10	6.16d $J_{1,2}$ 3.9	3.89sx $J_{2,3}$ 9.7	5.31t $J_{3,4}$ 9.7	5.10t $J_{4,5}$ 9.7	3.95—4.50m	1.97, 2.00 2.02, 2.05 2.10, 2.30	10.66d $J_{NH,2}$ 11.0		4.98	

[a] The spectrometer was locked on the signal of the tetramethylsilane internal reference. [b] Signal multiplicities are indicated as following: d (doublet), t (triplet), q (quartet), sx (sextet), and m (complex multiplet); the absence of any indication implies that a singlet was observed. [c] Signal of intensity six.

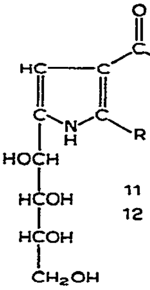
The absorptions of the enamine portion of these compounds are very similar to those of the intramolecularly bonded *cis*-form (**7**, R = H, alkyl, or aryl) of the enamines derived from β-dicarbonyl compounds and simple amines[9]. The p.m.r. spectrum of the benzoylacetaldehyde derivative (**8**) (Fig. 2) is representative, and will be commented on in some detail. The broad triplet at low field (δ 10.08 p.p.m.) is assigned to the N-H proton. The large paramagnetic shift is due to the presence of the strong, intramolecular hydrogen-bond, and the broadening to the ^{14}N-quadrupole relaxation. The multiplicity of the signal is attributed to the nearly equal couplings with H-2 of the sugar ring ($J_{NH,2}$ 10.0 Hz) and with the vinyl proton H-1′ ($J_{NH,1'}$ 12.0 Hz). This proton gives rise to the double doublet at δ 6.81 p.p.m., the second splitting being produced by the coupling ($J_{1',2'}$ 8.0 Hz) with the other vinyl proton (H-2′), which appears as a doublet at δ 5.74 p.p.m. The value of the coupling constant between the olefinic protons indicates[10] their *cis* disposition. The doublet ($J_{1,2}$ 3.9 Hz) at δ 6.24 p.p.m. is due to the anomeric proton H-1. The chemical shift of this proton, as compared with those of the anomeric protons in 2-acetamido-1,3,4,6-tetra-*O*-acetyl-2-deoxy-α and β-D-glucopyranose (δ 6.18 and 5.73 p.p.m., respectively)[11], and the value of $J_{1,2}$ indicate the equatorial disposition of H-1, and that the dihedral angle formed by H-1 and H-2 is of the order of 60°[12]. Therefore, compound **8** has the α-D-anomeric configuration. Further confirmation of this conclusion is provided by the signal at δ 2.30 p.p.m. due to the axial acetoxyl group at C-1 [11,12a]. The acetoxyl groups in equatorial positions of the pyranose ring and the C-6 acetoxyl group appear between δ 2.02 – 2.09 p.p.m.[11,12a]. The sextet at δ 3.54 p.p.m. is due to H-2, which is equally coupled ($J_{2,3} = J_{NH,2} = 10.0$ Hz) with H-3 and the N-H proton, and differently ($J_{1,2}$ 3.9 Hz) with H-1. The triplet at δ 5.36 p.p.m. may be assigned to H-3 ($J_{2,3}$ 10.0 Hz; $J_{3,4}$ 9.8 Hz), and the triplet at δ 5.11 p.p.m. to H-4 ($J_{4,5}$ 9.6 Hz). The protons on C-5 and C-6 give rise to a complex ABC spin system in the region δ 3.95 – 4.45 p.p.m.

In the p.m.r. spectra of compounds **9** and **10**, the vinyl protons (H-2′) appear as singlets, the N-H signals as doublets, and there are additional bands at δ *ca.* 2.0 p.p.m. due to the additional methyl groups in the *N*-substituents. The absorptions of the sugar portions remain essentially the same as in compound **8**; the only appreciable difference is the downfield shifts of the H-2 signals in **9** and **10** as compared with (**8**).

The spectral assignments, which are in agreement with the integral values of the spectra, were confirmed by double-resonance experiments. Irradiation of H-1 in compounds **9** and **10** removed $J_{1,2}$, and resulted in collapse of the H-2 sextet to a triplet. When H-2 was irradiated, the H-1 doublet collapsed to a singlet, the N-H doublet to a broad singlet, and the H-3 triplet to a doublet, thereby removing $J_{1,2}$, $J_{NH,2}$, and $J_{2,3}$, respectively. Application of the irradiating field to the centre of the H-3 triplet also simplified the H-2 multiplet. Irradiation of N-H in the enamine **8** caused the collapse of the H-1′ quartet to a doublet, and of the H-2 sextet to a quartet; a similar decoupling experiment with compound **9** resulted in the collapse of the H-2 sextet to a quartet.

Heating of the enamines **5** and **6** in aqueous ethanol, or in a mixture of triethylamine and methanol, caused cyclisation to 4-benzoyl-2-(D-*arabino*-tetrahydroxybutyl)-

pyrrole (**11**) and 3-benzoyl-5-(D-*arabino*-tetrahydroxybutyl)-2-methylpyrrole (**12**), respectively. The former compound could be obtained directly by heating a mixture of 2-amino-2-deoxy-D-glucose hydrochloride and the equivalent amount of sodium carbonate with benzoylacetaldehyde in water–ethanol; the 1-phenyl-1,3-butanedione derivative (**12**) was also prepared by allowing the β-diketone and the amino sugar to stand in aqueous acetone at room temperature.



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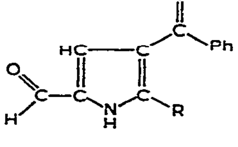
12 R=Me



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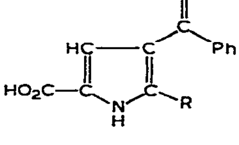

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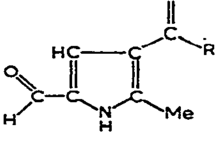


17 R=Me
18 R=OEt

The (tetrahydroxybutyl)pyrroles **11** and **12** have properties similar to those of compounds having analogous structures previously described[1,3]. Their acetylation gave the corresponding tetra-*O*-acetyl derivatives, and, in the oxidation with sodium periodate or lead tetra-acetate, 4-benzoyl-2-pyrrolecarboxaldehyde (**13**) and 4-benzoyl-5-methyl-2-pyrrolecarboxaldehyde (**14**), respectively, were obtained in high yields. These substances could be transformed into the corresponding 2-pyrrolecarboxylic acids, (**15**) and (**16**), by oxidation with silver oxide–sodium hydroxide. Decarboxylation of 4-benzoyl-2-pyrrolecarboxylic acid (**15**) gave known[13] 3-benzoylpyrrole.

The reaction between 2-amino-2-deoxy-D-glucose and benzoylacetaldehyde in dilute aqueous solution at pH 9-10 (*i.e.*, under conditions similar to those used in the reaction of the amino sugar and 2,4-pentanedione in the analytical method of Elson and Morgan[14]) yielded 4-benzoyl-2-(D-*arabino*-tetrahydroxybutyl) pyrrole (**11**) and 3-benzoylpyrrole. The same result was obtained by heating enamine **5** at pH 9–10.

The p.m.r. spectra of pyrrolecarboxaldehydes **13** and **14**, and of the known[3,15] 4-acetyl-5-methyl-2-pyrrolecarboxaldehyde (**17**), and 4-(ethoxycarbonyl)-5-methyl-2-pyrrolecarboxaldehyde (**18**) are summarised in Table II. The analyses of the spectra of the tri-substituted pyrroles (**14**), (**17**), and (**18**) are straightforward. The exchange of the NH protons with deuterium oxide removed $J_{1,3}$, and caused the H-3 doublet to collapse to a singlet. The CHO proton appeared as a singlet indicating that, as in other 2-pyrrolecarboxaldehydes[16] and in 2-furaldehyde[17], there is no coupling between this proton and H-3. The assignment of signals in the spectrum of 4-benzoyl-2-pyrrolecarboxaldehyde (**13**) is based on analogy with the spectra of compounds, **14**, **17** and **18**, and on the assumption that the CHO proton couples with H-5 but not with H-3 [16,17]. These assignments were confirmed by deuteration and by double-resonance experiments as follows. Treatment of the sample (**13**) with deuterium oxide changed the H-3 multiplet into a doublet (whose spacing was assigned as $J_{3,5}$), and the CHO signal

TABLE II

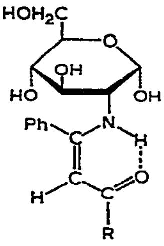
CHEMICAL SHIFTS (δ, p.p.m.) AND COUPLING CONSTANTS (Hz) OF 2-PYRROLECARBOXALDEHYDES AT 100 MHz IN $CDCl_3$[a]

Substance	*H-1*	*H-3*	*H-5*	*CHO*	*5-Me*	*Other signals*	$J_{1,3}$	$J_{1,5}$	$J_{3,5}$	$J_{CHO,5}$
4-Benzoyl-2-pyrrolecarboxaldehyde (13)	11.00[b]	7.45m[c,d]	7.70m	9.61[e]		Ph 7.54m, 7.85m	2.5	3.0	1.4	1.4
4-Benzoyl-5-methyl-2-pyrrolecarboxaldehyde (14)	11.15[b]	7.20d		9.42	2.70	Ph 7.52m, 7.81m	2.4			
4-Acetyl-5-methyl-2-pyrrolecarboxaldehyde (17)	11.10[b]	7.31d		9.43	2.66	4-MeCO 2.45	2.4			
4-(Ethoxycarbonyl)-5-methyl-2-pyrrolecarboxaldehyde (18)	11.16[b]	7.59d		9.41	2.64	Et 1.36t, 4.31q	2.6			

[a] The spectrometer was locked on the signal of the tetramethylsilane internal reference. [b] Very broad. [c] Signal multiplicities are indicated as follows: d (doublet), t (triplet), q (quartet), and m (multiplet); the absence of any indication implies that a singlet was observed. [d] Signal partly overlapping the phenyl signal at δ 7.54. [e] Broad singlet.

into a doublet (whose spacing was taken as $J_{CHO,5}$), and sharpened considerably the H-5 multiplet, which became an unresolved triplet. Irradiation, under these conditions, of the CHO proton resulted in collapse of this triplet to a doublet, whose spacing was equal to $J_{3,5}$, and did not affect the H-3 signal. Irradiation of H-5 caused the CHO and H-3 doublets both to become sharp singlets. The H-1 in all these pyrroles absorbed at same position as the H-1 of 2-pyrrolecarboxaldehyde, (δ 11.08 p.p.m.)[18]. Also, the values of $J_{1,3}$ in compounds **13**, **14**, **17**, and **18**, and $J_{1,5}$ and $J_{CHO,5}$ in **13** are of the same order of magnitude as those observed in other pyrrole derivatives[16].

The reaction between a primary amine and an unsymmetrical β-dicarbonyl compound could give rise to two enamines. Thus, the products formed in the reactions of 2-amino-2-deoxy-D-glucose with benzoylacetaldehyde and 1-phenyl-1,3-butanedione might have had structures **19** and **20**, respectively, instead of **5** and **6**. The results previously presented are inconsistent with the former possibility. Substances such as **19** and its corresponding tetra-*O*-acetyl derivative would show the spectroscopic properties of α,β-unsaturated aldehydes. Particularly, the p.m.r. spectrum of the tetra-*O*-acetyl derivative of **19** would be very different from the one observed (Table I, Fig. 2).



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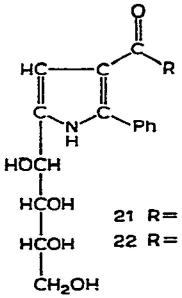
20 R = Me



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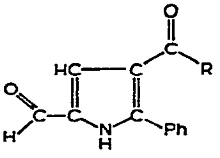

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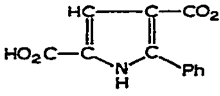

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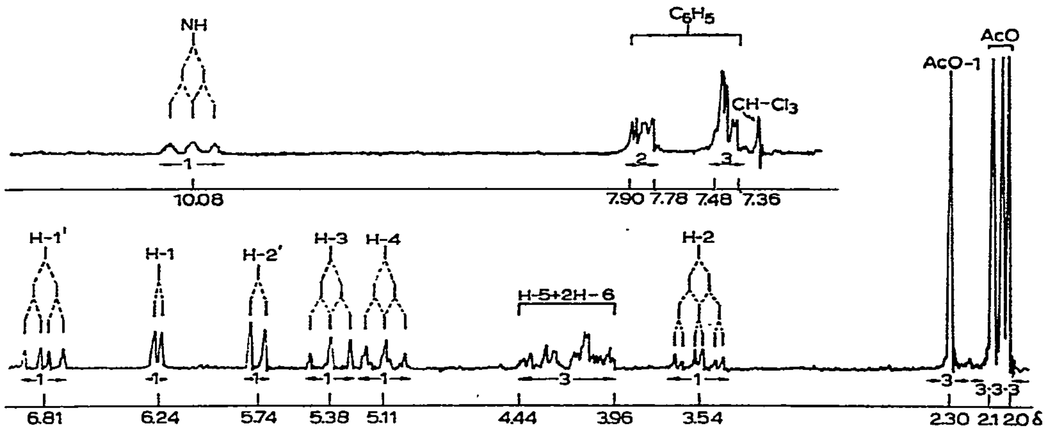

Fig. 2. The p.m.r. spectrum of 1,3,4,6-tetra-*O*-acetyl-2-deoxy-2-[1-(3-oxo-3-phenyl-1-propenyl)-amino]-α-D-glucopyranose (**8**) at 100 MHz in deuteriochloroform. Numbers below peaks give intensities of integrated peaks.

Cyclisation of enamine **19** would have given 5-(D-*arabino*-tetrahydroxybutyl)-2-phenyl-3-pyrrolecarboxaldehyde (**21**). Periodate oxidation of this compound would have afforded 2-phenyl-3,5-pyrroledicarboxaldehyde (**23**), instead of the isomeric 4-benzoyl-3-pyrrolecarboxaldehyde (**13**). The p.m.r. spectrum of the tri-substituted pyrrole (**23**) would be very different from the one observed (Table II), which clearly corresponds to a di-substituted pyrrole. Oxidation of aldehyde **23** would have given 2-phenyl-3,5-pyrroledicarboxylic acid (**25**), the decarboxylation of which cannot afford 3-benzoylpyrrole. The 1-phenyl-1,3-butanedione derivatives (**6**), (**12**), and (**14**) show the properties of homologous compounds of **5**, **11**, and **13**, respectively, rather than those expected for the isomeric ketones **20**, **22**, and **24**. Also, the p.m.r. spectrum of the periodate-oxidation product (**14**) (Table II) is better interpreted in terms of the proposed formulation than with the structure 4-acetyl-5-phenyl-2-pyrrolecarboxaldehyde (**24**). Therefore 2-amino-2-deoxy-D-glucose seems to react preferentialy with the carbonyl group of benzoylacetaldehyde and 1-phenyl-1,3-butanedione that is further away from the phenyl ring. Similar results have been obtained in the reactions of these β-dicarbonyl compounds with simple primary amines [9,19].

EXPERIMENTAL

Melting points are uncorrected. Concentrations and evaporations of solvents were carried out *in vacuo* at a temperature not over 50°. Identification of compounds was based on mixed melting points, and comparison of their chromatographic mobilities and i.r. spectra. Drying of organic solutions was effected with sodium sulphate, unless otherwise indicated. Paper chromatography was carried out on Whatman No. 1 paper by the horizontal technique (unless otherwise stated), with butyl alcohol–ethanol–water–ammonia (40:10:49:1, organic phase). Detection reagents used were (*a*) silver nitrate–sodium hydroxide[20] for monosaccharides and polyhydroxylic compounds; (*b*) aniline hydrogen phthalate[21] for reducing sugars; and (*c*) chromatographic Ehrlich reagent[3] for pyrroles; R_F values were measured at 20–25°. Optical rotations at 5461 Å were determined with a Bendix–Ericsson Type 143C polarimeter. 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 Beckman IR-7; the following abbreviations are used: s, strong; m, medium; w, weak; v, very; sh, shoulder; b, broad. The p.m.r. spectra were measured with a Varian HA-100 spectrometer. The samples were used as solutions in deuteriochloroform, with tetramethylsilane ($\delta = 0$) as the internal reference. Deuteration was effected by shaking the prepared sample with one drop of deuterium oxide.

*2-Deoxy-2-[1-(3-oxo-3-phenyl-1-propenyl)amino]-*D*-glucose* (**5**). — Benzoylacetaldehyde[22] (4.4 g, 0.03 moles) was added to a solution of 6.5 g (0.03 moles) of 2-amino-2-deoxy-D-glucose hydrochloride and 1.59 g (0.015 mole) of sodium carbonate in 15 ml of water. Separation of compound **5** started immediately. After several hours, the crystalline mass was filtered off and recrystallized from ethanol; yield, 7.3 g (79%), m.p. 168–170°, $[\alpha]_D^{21}$ +82° (*c* 1, water); R_F 0.81 (descending technique); λ_{max} (water)

247 mμ (ε 9460); ν_{max} (Nujol) 3279 b-m (OH, NH), 1631 b-s (C=O), 1600 sh-w (phenyl ring), 1585 w, 1520 sh-s, 1504 s (phenyl ring) cm^{-1}.

Anal. Calc. for $C_{15}H_{19}NO_6$: C, 58.24, H, 6.19; N, 4.53. Found: C, 57.96; H, 6.37; N, 4.82.

Compound 5 can also be obtained by heating under reflux 2-amino-2-deoxy-D-glucose hydrochloride (215 mg, 1 mmole) and benzoylacetaldehyde (500 mg, 3 mmoles) in a mixture of triethylamine (0.3 ml) and methanol (5 ml) for 10 min. The solvents were distilled off, and the residual syrup was washed with ether. Crystallization from methanol yielded 48 mg (15%) of product, m.p. 166–169°, identical with that described above.

Hydrolysis of compound 5. — A solution of compound 5 (3.1 g, 0.01 mole) in 0.5N hydrochloric acid (150 ml) was refluxed for 2.5 h. The reaction mixture was concentrated to *ca.* 70 ml, and extracted with ether (2 × 50 ml). Evaporation of the aqueous layer left a crystalline, dark residue (183 mg), m.p. 190–200° (dec.), which, when examined chromatographically [descending, spray reagents (*a*) and (*b*)], showed only the presence of 2-amino-2-deoxy-D-glucose. A solution of this material in the smallest amount of water was passed down a column of Amberlite IR-120 (H$^+$). The column was washed with water (500 ml), and then with 0.2N hydrochloric acid until the eluate gave a negative Fehling's test. The acid eluate was concentrated to *ca.* 5 ml and refrigerated. Crystalline 2-amino-2-deoxy-D-glucose hydrochloride (1.46 g, 68%), m.p. 190° (dec.), was collected, washed with ethanol, and identified with an authentic specimen.

1,3,4,6-Tetra-O-acetyl-2-deoxy-2- [1-(3-oxo-3-phenyl-1-propenyl)amino]-α-D-glucopyranose (8). — A solution of compound (5) (0.93 g 3 mmoles) in pyridine (5 ml) was treated with acetic anhydride (5 ml) for 48 h at 0°. The mixture was poured onto ice, and the crystalline solid was collected and washed thoroughly with water. Several recrystallizations from carbon tetrachloride gave the product (0.55 g, 38%), m.p. 180–181°, $[\alpha]_{5461}^{24}$ +286° (*c* 0.5, chloroform); ν_{max} (chloroform) 3472 vw (C=O overtone), 1764 s (OAc), 1639 s (ketone C=O), 1592 s (phenyl ring), 1558 m, 1504 m (phenyl ring) cm^{-1}; ν_{max} (*) (carbon tetrachloride) 3515 vw and 3492 vw (C=O overtone), 3195 b-w (intramolecularly bonded NH), 1763 s and 1755 s (OAc), 1622 m (ketone C=O), 1589 m (phenyl ring) cm^{-1}; p.m.r. spectrum, see Fig. 2 and Table I.

Anal. Calc. for $C_{23}H_{27}NO_{10}$: C, 57.85; H, 5.70; N, 2.93. Found: C, 57.68; H, 5.89; N, 2.97.

A sample (0.1 g) of this tetra-*O*-acetyl derivative was deacetylated by treatment with 0.5% methanolic sodium methoxide (1 ml) for 24 h. The mixture was neutralized with dilute hydrochloric acid and evaporated. The residual solid gave, after recrystallization from ethanol, compound 5, identical with the sample described above.

Hydrolysis of compound 8. — A solution of 0.48 g (1 mmole) of compound 8 in boiling acetone (3 ml) was treated with 5N hydrochloric acid (0.25 ml), and the mixture was refluxed for several minutes. Refrigeration of the reaction mixture afforded 1,3,4,6-tetra-*O*-acetyl-2-amino-2-deoxy-α-D-glucopyranose hydrochloride (0.125 g, 33%) m.p. 188–190°, $[\alpha]_D^{15}$ +125° (*c* 1, water) [lit.[23], m.p. 180°, $[\alpha]_D^{20}$ +130° (water)]. Its i.r. spectrum (in potassium bromide) was identical to that reported in the literature[23].

Anal. Calc. for $C_{14}H_{22}ClNO_9$: C, 43.81; H, 5.78; N, 3.65. Found: C, 44.03; H, 5.87; N, 3.80.

4-Benzoyl-2-(D-arabino-*tetrahydroxybutyl*)*pyrrole* (**11**). — (*a*) A suspension of 2-deoxy-2-[1-(3-oxo-3-phenyl-1-propenyl)amino]-D-glucose (**5**) (3.1 g, 0.01 mole) in 40 ml of ethanol–water (1:1) was gently heated until dissolution, and then under reflux for 6 h. On storage in the refrigerator, crystallization took place. Recrystallization from ethanol gave the product, 1.7 g (58%), m.p. 197–200°, $[\alpha]_{5461}^{23}$ −48° (*c* 0.5, pyridine); R_F 0.69; λ_{max} (water) 255.5, 295 sh mμ (ε 11,980, 6,600); ν_{max} (Nujol) 3425 s, 3333 s, and 3226 s (OH, NH), 1618 s (C=O), 1597 sh-m (phenyl ring), 1560 m (pyrrole ring), 1506 m (phenyl ring) cm^{-1}.

(*b*) A suspension of compound **5** (0.62 g, 2 mmoles) in methanol (9 ml) containing triethylamine (1 ml) was heated under reflux for 6 h. Refrigeration of the reaction mixture gave the product (172 mg, 30%), m.p. 198–200°.

(*c*) 2-Amino-2-deoxy-D-glucose hydrochloride (1.1 g, 5 mmoles) and sodium carbonate (0.27 g, 25 mmoles) were dissolved in water (4.5 ml), and ethanol (4 ml) and benzoylacetaldehyde (0.74 g, 5 mmoles) were added. The resulting solution was heated under reflux for 30 min. After several hours at room temperature, the crystalline mass (m.p. 183–190°) was collected and washed with ether. Recrystallization from water and then from ethanol gave 0.42 g (29%) of the product, m.p. 197–200°.

Anal. Calc. for $C_{15}H_{17}NO_5$: C, 61.84; H, 5.88; N, 4.81. Found: C, 61.96; H, 5.98; N, 4.93.

In an analytical oxidation, there was a consumption of 2.85 mol. of sodium metaperiodate (calc., 3.0 mol.)

2-(D-arabino-*Tetra-acetoxybutyl*)*-4-benzoylpyrrole.* — Acetylation of 4-benzoyl-2-(D-*arabino*-tetrahydroxybutyl)pyrrole (**11**), as indicated before for compound **5**, gave the product (87%), which, after recrystallization from ethanol–water (1:2), had m.p. 107–108°; $[\alpha]_{5461}^{24}$ −90° (*c* 0.5, chloroform); ν_{max} (chloroform) 3559 vw (C=O overtone), 3436 w (free NH), 3247 w (bonded NH), 1742 s (OAc), 1634 s (ketone C=O), 1603 sh-m (phenyl ring), 1575 m (pyrrole ring), 1506 m (phenyl ring) cm^{-1}.

Anal. Calc. for $C_{23}H_{25}NO_9$: C, 60.12; H, 5.48; N, 3.04. Found: C, 60.35; H, 5.80; N, 2.96.

4-Benzoyl-2-pyrrolecarboxaldehyde (**13**). — Compound **11** (874 mg, 3 mmoles), suspended in a mixture of water (90 ml) and ether (90 ml), was treated, under vigorous stirring, with sodium metaperiodate tri-hydrate (3.2 g, 12 mmoles) for 4 h. The layers were separated, and the aqueous fraction was extracted with ether (25 ml). The combined ethereal fractions were dried, and the solvent was distilled off. Recrystallization of the crystalline residue from ethanol–water afforded the title compound (532 mg, 89%), m.p. 126–128°, λ_{max} (ethanol) 204, 244.5, 297 mμ (ε 17,900, 32,000, 25,600); ν_{max} (chloroform) 3559 vw (C=O overtone), 3436 m (free NH), 3257 m (bonded NH), 2841 w and 2725 w (CHO), 1667 s (aldehyde C=O), 1642 s (ketone C=O), 1600 m (phenyl ring), 1577 w, 1558 s (pyrrole ring) cm^{-1}; p.m.r. spectrum, see Table II.

Anal. Calc. for $C_{12}H_9NO_2$: C, 72.35; H, 4.55; N, 7.03. Found: C, 72.16; H, 4.73; N, 6.90.

4-Benzoyl-2-pyrrolecarboxylic acid (**15**). — A suspension of silver oxide in water was prepared by adding 3.2 ml of N sodium hydroxide to a solution of 0.37 g (2.2 mmoles) of silver nitrate in water (1 ml). 3-Benzoyl-2-pyrrolecarboxaldehyde (0.20 g, 1 mmole) was added, and the mixture was heated for 1 h at 100°. The cooled reaction mixture was filtered, and the solid residue was washed several times with hot water. The filtrate and the washings were mixed and acidified (Congo Red) with dilute nitric acid. The solid was collected and washed with water. Recrystallization from ethanol–water gave the product (170 mg, 79%), m.p. 224–225° (dec.); λ_{max} (ethanol) 205.6, 237.5, 288 mμ (ε 38,500, 48,000, 14,700); ν_{max} (Nujol) 3247 s, 2710 m, 2618 m and 2538 m (CO_2H), 1695 s (CO_2H), 1616 s (ketone C=O), 1577 sh-w, 1550 s and 1522 m (pyrrole ring), 1499 m (phenyl ring) cm^{-1}.

Anal. Calc. for $C_{12}H_9NO_3$: C, 66.97; H, 4.22; N, 6.51; neutralization equivalent, 215. Found: C, 66.66; H, 4.49; N, 6.34; neutralization equivalent, 219.

3-Benzoylpyrrole. — 4-Benzoyl-2-pyrrolecarboxylic acid (**15**) (100 mg) was heated at 210–230° (bath temperature) in a tube fitted with a "cold finger". The sublimate was recrystallized from light petroleum–ether yielding the title compound (22.4 mg, 28%), m.p. 97–99° (lit.[13], 96–97°), R_F 0.94; λ_{max} (ethanol) 248, 279 sh mμ (ε 15,200, 8,860); ν_{max} (chloroform) 3460 s (free NH), 3289 m (bonded NH), 1634 s (C=O), 1605 s (phenyl ring), 1575 m (pyrrole ring), 1495 m (phenyl ring) cm^{-1}.

Anal. Calc. for $C_{11}H_9NO$: C, 77.17; H, 5.30; N, 8.18. Found: C, 77.47; H, 5.09; N, 8.07.

Treatment of compound **5** *at pH 9–10.* — A solution of compound **5** (1.55 g, 5 mmoles) in 30 ml of a sodium carbonate–sodium hydrogen carbonate buffer (pH 9.5) was heated under reflux for 30 min. Refrigeration of the reaction mixture afforded a crystalline solid (1.09 g, m.p. 173–178°) which, when examined chromatographically [spray reagents (*a*) and (*c*)], showed the presence of 4-benzoyl-2-(D-*arabino*-tetrahydroxybutyl)pyrrole (**11**) (R_F 0.68) and 3-benzoylpyrrole (R_F 0.91). This material was extracted with acetone (10 ml). Recrystallization of the insoluble portion from ethanol gave compound **11** (0.52 g, 36%), m.p. 196–198°, identical to the preparation described above. Evaporation of the acetone extract afforded 0.17 g (20%) of 3-benzoylpyrrole, m.p. 97–98°, identical to the sample described above.

Reaction between 2-amino-2-deoxy-D-glucose and benzoylacetaldehyde at pH 9–10. — 2-Amino-2-deoxy-D-glucose hydrochloride (2.16 g, 0.01 mole) and benzoylacetaldehyde (1.48 g, 0.01 mole) were dissolved in 70 ml of a sodium carbonate–sodium hydrogen carbonate buffer (pH 9.5.) The pH was adjusted to pH 9.1 by addition of 2N sodium hydroxide, and the resulting solution was heated to 100° for 45 min and cooled. The crystalline solid that separated (0.44 g, m.p. 185–190°) was collected and washed with water. Paper chromatography [spray reagents (*a*) and (*c*)] of this material showed the presence of compound **11** (R_F 0.69) and benzoylpyrrole (R_F 0.94). The solid was continuously extracted with ether. The insoluble fraction gave, after recrystallization from water, 4-benzoyl-2-(D-*arabino*-tetrahydroxybutyl)pyrrole (**11**) (0.35 g, 12%), m.p. 197–200°.

Evaporation of the ether solution left a small, dark residue. Thin-layer chromatography [silica-gel G (Merck); light petroleum–ether (2:1), spray reagent sulfuric acid–water (1:1)] of this material showed the presence of 3-benzoylpyrrole (R_F 0.22) in addition to two unidentified substances of R_F 0.36 and 0.85.

*2-Deoxy-2-[2-(4-oxo-4-phenyl-2-butenyl)amino]-*D*-glucose* (**6**). — A mixture of 2-amino-2-deoxy-D-glucose hydrochloride (2.15 g, 0.01 mole), 1-phenyl-1,3-butanedione (4.86 g, 0.03 moles), triethylamine (3 ml), and methanol (45 ml) was heated until dissolution occurred, and then under reflux for 10 min. Refrigeration of the reaction mixture afforded the title compound (1.57 g, 49%), m.p. 137–141°. Several recrystallizations from methanol gave the analytical sample (0.8 g, 25%), m.p. 152–154°, $[\alpha]_D^{25}$ +212° (*c* 0.5, methanol), R_F 0.77; λ_{max}(ethanol) 246, 251, 263 sh mμ (ε 16,200, 16,360, 7,300); ν_{max}(Nujol) 3584 m and 3165 s (OH, NH), 1603 s (C=O and phenyl ring), 1585 s, 1538 s cm^{-1}.

Anal. Calc. for $C_{16}H_{21}NO_6$: C, 59.43; H, 6.55; N, 4.33. Found: C, 59.02; H, 6.71; N, 4.26.

Hydrolysis of compound **6**. — Compound **6** (3.23 g, 0.01 mole) was heated in 0.1N hydrochloric acid (120 ml) at 100° for 1 h. The reaction mixture was extracted with ether, and the extract was dried and evaporated. The residue (0.95 g, 59%), m.p. 53–56°, was identified as 1-phenyl-1,3-butanedione. The aqueous fraction was passed down a column of Amberlite IR-120 (H^+). The column was eluted with water until the effluent was colourless, and then with 0.2N hydrochloric acid until the eluate gave a negative Fehling's test. Concentration of the acid eluate afforded 2-amino-2-deoxy-D-glucose hydrochloride (0.48 g, 22%), m.p. 191° (dec.), identified by comparison with an authentic sample.

*1,3,4,6-Tetra-*O*-acetyl-2-deoxy-2-[2-(4-oxo-4-phenyl-2-butenyl)amino]-α-*D*-glucopyranose* (**9**). — Acetylation of compound **6**, as indicated above for **5**, gave the title compound (66%). After crystallization from methanol, it had m.p. 193–195°, $[\alpha]_{5461}^{24}$ +260° (*c* 0.25, chloroform); ν_{max} (*) (Nujol), 3505 vw and 3453 vw (C=O overtone), 3177 b-w (intramolecularly bonded NH), 1765 s, 1744 sh-s and 1735 s (OAc), 1606 s (ketone C=O), 1598 s (phenyl ring), 1587 s, 1554 s, 1539 sh-m, 1516 m cm^{-1}; ν_{max} (*) (carbon tetrachloride), 3500 b-w, 3193 b-w; ν_{max} (*) (chloroform), 1557 b-s, 1606 s, 1588 s, 1558 m, 1515 w cm^{-1}, see also Fig. 1; p.m.r. spectrum, see Table I.

Anal. Calc. for $C_{24}H_{29}NO_{10}$: C, 58.65; H, 5.95; N, 2.85. Found: C, 58.70; H, 6.16; N, 2.83.

Hydrolysis of compound **9**. — Compound **9** was hydrolyzed, as indicated before for compound **8**, yielding 1,3,4,6-tetra-*O*-acetyl-2-amino-2-deoxy-α-D-glucopyranose hydrochloride (79%), m.p. 175–178° (dec.), identified with the sample described above.

3-Benzoyl-5-(D-arabino-*tetrahydroxybutyl)-2-methylpyrrole* (**12**). — (*a*) Compound **6** (0.32 g, 1 mmole) was refluxed in a mixture of methanol (4.5 ml) and triethylamine (0.3 ml) for 5 h. Crystallization of product occurred during this time. The reaction mixture was refrigerated, and the crystalline mass was collected; yield, 0.225 g (74%), m.p. 161–164°. After recrystallization from water and then from methanol, the analytical sample had m.p. 170–171°; $[\alpha]^{22}_{5461}$ $-58°$ (*c* 0.5, pyridine), R_F 0.78; λ_{max}(ethanol) 242, 315 mμ (ε 14,920, 6,280); ν_{max}(Nujol) 3236 b-s (OH, NH), 1616 sh-s (C=O), 1597 s (phenyl ring), 1567 s (pyrrole ring), 1515 w cm^{-1}.

(*b*) A solution of 2-amino-2-deoxy-D-glucose hydrochloride (4.3 g, 0.02 moles) and sodium carbonate (1.06 g, 0.01 moles) in water (32 ml) was treated with 1-phenyl-1,3-butanedione (3.24 g, 0.02 moles) dissolved in acetone (50 ml). The pH of the resulting solution was adjusted to 7.5 by addition of dilute hydrochloric acid, and left at room temperature for three days. Evaporation of solvents afforded a material that was washed thoroughly with ether and crystallized from water. The product (1.03 g, 16%) had m.p. 158–162°. An additional recrystallization from methanol gave the pure substance, m.p. 170–171°.

Anal. Calc. for $C_{16}H_{19}NO_5$: C, 62.94; H, 6.27; N, 4.59. Found: C, 63.10; H, 6.51; N, 4.32.

The compound consumed 3.0 mol. of sodium metaperiodate (calc., 3.0 mol.).

5-(D-arabino-*Tetra-acetoxybutyl*)*-3-benzoyl-2-methylpyrrole*. — Acetylation of compound (**12**), as indicated for compound **5**, gave the product (69%), which, after recrystallization from light petroleum–ether, had m.p. 125–127°, $[\alpha]^{19}_{5461}$ $-115°$ (*c* 0.5, chloroform); ν_{max} (chloroform) 3597 sh-w (C=O overtone), 3436 m (free NH), 3257 sh-w (bonded NH), 1751 s (OAc), 1637 s (ketone C=O), 1600 m (phenyl ring), 1575 m (pyrrole ring), 1534 sh-w cm^{-1}.

Anal. Calc. for $C_{24}H_{27}NO_9$: C, 60.88; H, 5.75; N, 2.96. Found: C, 61.02; H, 5.91; N, 2.72.

4-Benzoyl-5-methyl-2-pyrrolecarboxaldehyde (**14**). — (*a*) 3-Benzoyl-5-(D-*arabino*-tetrahydroxybutyl)-2-methylpyrrole (**12**) (0.31 g, 1 mmole), suspended in a mixture of water (30 ml) and ether (30 ml), was treated with sodium metaperiodate trihydrate (1.1 g, 4 mmoles) for 2 h under vigorous stirring. The layers were separated, and the aqueous fraction was extracted with ether (30 ml). The combined ethereal fractions were dried, and the solvents was removed by distillation. The product (167 mg, 79%) had m.p. 133–134° which did not change after a recrystallization from ethanol–water (1:2).

(*b*) Lead tetra-acetate (4.43 g, 0.01 mole) was added to a suspension of compound **12** (0.61 g, 2 mmoles) in benzene (60 ml) containing a few drops of acetic acid. The mixture was heated at 40–50° for *ca.* 5 h under vigorous stirring. The solid was filtered off and extracted with benzene (2 × 20 ml). The filtrate and the benzene extracts were combined, washed with 10% sodium carbonate (2 × 20 ml), and dried ($MgSO_4$). Removal of the solvent left the product (0.34 g, 79%), m.p. 129–133°. Recrystallization from ethanol–water (1:2) gave the analytical sample, m.p. 133–134°; λ_{max}(ethanol), 242, 286, 304 mμ (ε 14,020, 9,780, 11,600); ν_{max} (chloroform) 3521 sh-w

(C=O overtone), 3425 w (free NH), 3236 s (bonded NH), 2841 w and 2695 w (CHO), 1658 s (aldehyde C=O), 1631 s (ketone C=O), 1603 m (phenyl ring), 1563 s (pyrrole ring), 1502 s (phenyl ring) cm^{-1}; p.m.r. spectrum, see Table II.

Anal. Calc. for $C_{13}H_{11}NO_2$: C, 73.22; H, 5.20; N, 6.57. Found: C, 72.98; H, 5.44; N, 6.85.

4-Benzoyl-5-methyl-2-pyrrolecarboxylic acid (**16**). — Oxidation of compound **14** with alkaline silver oxide, as indicated for the preparation of 4-benzoyl-2-pyrrolecarboxylic acid (**15**), gave the title compound. After two recrystallizations from methanol–water (1:1), the product (57%) had m.p. 204–206° (dec.); λ_{max} (ethanol) 239, 245 sh, 303 mμ (ε 32,400, 29,400, 8000); ν_{max} (Nujol) 3247 s and 2558 sh-m (CO_2H), 1695 s (CO_2H), 1631 s (ketone C=O), 1603 m (phenyl ring), 1567 s (pyrrole ring), 1506 s (phenyl ring) cm^{-1}.

Anal. Calc. for $C_{13}H_{11}NO_3$: C, 68.11; H, 4.84; N, 6.11; neutralization equivalent, 229. Found: C, 68.44; H, 5.14; N, 6.10; neutralization equivalent, 246.

1,3,4,6-Tetra-O-acetyl-2-amino-2-deoxy-α-D-glucopyranose hydrochloride from 2-amino-compound **10**. — 1,3,4,6-Tetra-*O*-acetyl-2-deoxy-2-[2-(4-oxo-2-pentenyl)amino]-α-D-glucopyranose[3] (**10**) (4.29 g, 0.01 mole) was dissolved in 15 ml of boiling acetone and treated with 2 ml of 5N hydrochloric acid. The mixture was heated under reflux for 2 min. On cooling, 1,3,4,6-tetra-*O*-acetyl-2-amino-2-deoxy-α-D-glucopyranose hydrochloride rapidly crystallized. The suspension was diluted with ether (15 ml) and shaken. The solid fraction was collected, washed with ether, and recrystallized from methanol. Yield, 3.0 g (79%); m.p. 188–190°. The preparation was identical to that obtained from compound **8**.

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SUMMARY

2-Amino-2-deoxy-D-glucose reacts with benzoylacetaldehyde and 1-phenyl-1,3-butanedione to yield 2-deoxy-2-[1-(3-oxo-3-phenyl-1-propenyl)amino]-D-glucose (**5**) and 2-deoxy-2-[2-(4-oxo-4-phenyl-2-butenyl)amino]-D-glucose (**6**), respectively. Acetylation of these substances affords 1,3,4,6-tetra-*O*-acetyl-2-deoxy-2-[1-(3-oxo-3-phenyl-1-propenyl)amino]-α-D-glucopyranose (**8**) and 1,3,4,6-tetra-*O*-acetyl-2-deoxy-2-[2-(4-oxo-4-phenyl-2-butenyl)amino]-α-D-glucopyranose (**9**), whose structures are established on the basis of their i.r. and p.m.r. spectra, and hydrolysis to 1,3,4,6-tetra-*O*-acetyl-2-amino-2-deoxy-α-D-glucopyranose hydrochloride. Cyclisation of enamines **5** and **6**

yields 4-benzoyl-2-(D-*arabino*-tetrahydroxybutyl)pyrrole (**11**) and 3-benzoyl-5-(D-*arabino*-tetrahydroxybutyl)-2-methylpyrrole (**12**) respectively. Treatment of compound **5** at pH 9–10 gives the (tetrahydroxybutyl)pyrrole (**11**) and 3-benzoylpyrrole. Oxidation of pyrroles **11** and **12** with sodium periodate (or lead tetraacetate) yields 4-benzoyl-2-pyrrolecarboxaldehyde (**13**) and 4-benzoyl-5-methyl-2-pyrrolecarboxaldehyde (**14**), respectively, which can be subsequently oxidized to the corresponding 2-pyrrolecarboxylic acids (**15**) and (**16**). 1,3,4,6-Tetra-*O*-acetyl-2-amino-2-deoxy-α-D-glucopyranose hydrochloride can be readily prepared by hydrolysis of 1,3,4,6-tetra-*O*-acetyl-2-deoxy-2-[2-(4-oxo-2-pentenyl)amino]-α-D-glucopyranose (**10**).

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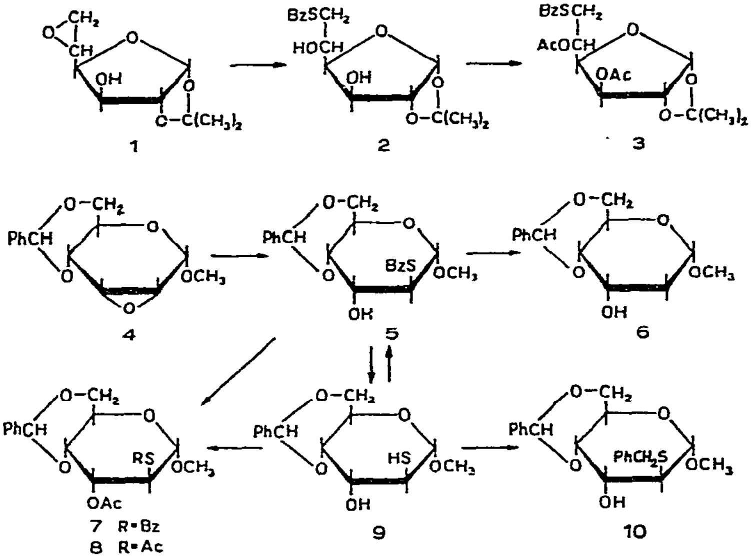
Notes

Syntheses with anhydro sugars

Part V. Synthesis of thio sugars by reaction of sugar epoxides with pyridinium thiolbenzoate

The thiolbenzoate anion has been used for nucleophilic displacement of both halo[1,2] and ester groups[3], and is a valuable agent in syntheses of thio sugars. Pyridinium thiolbenzoate can be applied to this purpose in another type of reaction, namely, epoxide-ring cleavage. Although not yet applied in a great number of cases, the method seems to be generally applicable and is of special advantage for preparation of thio sugars having the sulfur atom in a secondary position.

5,6-Anhydro-1,2-*O*-isopropylidene-α-D-glucofuranose (**1**), thiolbenzoic acid, and pyridine, in equivalent amounts, react at room temperature to yield 6-*S*-benzoyl-



1,2-*O*-isopropylidene-6-thio-α-D-glucofuranose (**2**) which is identical with the product obtained by treatment of 1,2-*O*-isopropylidene-6-*O*-*p*-tolylsulfonyl-α-D-glucofuranose with potassium thiolbenzoate in *N*,*N*-dimethylformamide[4]. Subsequent acetylation yields the di-*O*-acetyl derivative (**3**) obtained previously[5] by benzoylation of 3,5-di-*O*-acetyl-1,2-*O*-isopropylidene-6-thio-α-D-glucofuranose.

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Methyl 2,3-anhydro-4,6-*O*-benzylidene-α-D-allopyranoside (**4**), when melted with pyridinium thiolbenzoate, yields methyl 2-*S*-benzoyl-4,6-*O*-benzylidene-2-thio-α-D-altropyranoside (**5**). The structure of compound **5** was proved by Raney nickel desulfurization, which yielded the known methyl 4,6-*O*-benzylidene-2-deoxy-α-D-*ribo*-hexopyranoside[6] (**6**), as well as by catalytic debenzoylation with sodium methoxide in methanol which afforded a product (**2**) identical with that obtained by debenzylation of methyl 2-*S*-benzyl-4,6-*O*-benzylidene-2-thio-α-D-altropyranoside[7]. The last-named compound (**10**) could be obtained by benzylation of compound **9**, whereas *S*-benzoylation gave back compound **5**. Remarkably, compound **5** and its *O*-acetyl derivative (**7**) showed a negative optical rotation, whereas the di-*O*,*S*-acetyl compound (**8**) shows the normal positive value.

EXPERIMENTAL

6-S-Benzoyl-1,2-O-isopropylidene-6-thio-α-D-glucofuranose (**2**). — An intimate mixture of 5,6-anhydro-1,2-*O*-isopropylidene-α-D-glucofuranose (**1**, 1.0 g), thiolbenzoic acid (0.7 g), and pyridine (0.4 g) reacted with spontaneous warming and liquefaction during a few minutes. After about 1 h, ethanol (1 ml) was added to the viscous yellow mass, followed by sufficient water to initiate crystallization. Separation of the crude, crystalline product (**2**, 1.5 g, m.p. 100–103°) was achieved by further addition of water. Recrystallized twice from ethanol, the substance had m.p. 120–121°, $[\alpha]_D^{20}$ +3.0° (*c* 2.0, chloroform) (Found: C, 56.55; H, 6.01; S, 9.53. $C_{16}H_{20}O_6S$ calc.: C, 56.54; H, 5.92; S, 9.41%).

3,5-Di-O-acetyl-6-S-benzoyl-1,2-O-isopropylidene-6-thio-α-D-glucofuranose (**3**). — The 6-*S*-benzoyl derivative (**2**, 0.5 g), dissolved in a mixture of pyridine (2.5 ml) and acetic anhydride (1.0 ml), was left overnight at room temperature and then poured into 100 ml of ice water. The resulting sirup slowly solidified to yield crude product **3** (0.3 g), which, after two recrystallizations from ethanol, had m.p. 116–117°, $[\alpha]_D^{20}$ + 20.0° (*c* 5.0, chloroform).

Methyl 2-S-benzoyl-4,6-O-benzylidene-2-thio-α-D-altropyranoside (**5**). — The anhydro derivative (**4**, 10.4 g), thiolbenzoic acid (10.8 g), and pyridine (3.2 g) were mixed thoroughly and heated at 150–160° to complete liquefaction. The brown-red, viscous liquid was kept at this temperature for 10 min and then left to cool. Following dissolution in ether (*ca.* 150 ml), the mixture afforded crude product **5** (7.2 g, m.p. 154–159°), which, after two recrystallizations from ethanol, had m.p. 163–165°, $[\alpha]_D^{20}$ —25.2° (*c* 4.0, chloroform) (Found: C, 62.82; H, 5.59; S, 7.41. $C_{21}H_{22}O_6S$ calc.: C, 62.98; H, 5.67; S, 7.28%).

*Methyl 4,6-O-benzylidene-2-deoxy-α-D-*ribo*-hexopyranoside* (**6**). — Compound **5** (3.2 g), suspended in ethanol (320 ml), was treated with one tablespoonful of freshly prepared Raney nickel. The mixture was refluxed for 1 h, the catalyst was filtered off, and the solution was treated with charcoal. Concentration and cooling gave a crystalline mass smelling of benzaldehyde. Paper-chromatographic analysis (1-butanol–

acetic acid–water, 10:1:3, descending technique) revealed the presence of two carbohydrate derivatives (R_F 0.48 and 0.90) which both gave, with the diphenylamine–aniline–phosphoric acid reagent[8], a red-violet color typical for monosaccharide derivatives having a $-CH_2$-grouping adjacent to the carbonyl function[8]. The mixture could be readily fractionated by extraction of its chloroform solution by water. The faster-moving substance remained in the chloroform phase and crystallized after addition of ethanol to the concentrated chloroform solution to give compound **6** (0.7 g), m.p. 116–117°, $[\alpha]_D^{20}$ + 150.9° (*c* 2.1, chloroform); lit.[6], m.p. 117–119° and 126–128°, $[\alpha]_D$ + 152° (chloroform).

Identification of the second 2-deoxyaldose derivative, presumably methyl 2-deoxy-α-D-*ribo*-hexopyranoside, was abandoned.

Methyl 4,6-O-benzylidene-2-thio-α-D-altropyranoside (**9**). — The S-benzoyl derivative (**5**, 1.6 g) was added at room temperature to absolute methanol (20 ml) containing sodium methoxide (0.56 g). After 10 min, when all of the substance had dissolved, the solution was treated with a slight excess of glacial acetic acid and evaporated *in vacuo* until the first crystals appeared. Complete separation of the product (1 g) was brought about by cautious addition of water with continuous mixing. Recrystallization from ethanol gave compound **9**, m.p. 170–172°, $[\alpha]_D^{20}$ + 77.4° (*c*, 2.3 chloroform); lit.[7], m.p. 168–169°, $[\alpha]_D$ + 80.5° (chloroform).

Methyl 2-S-benzyl-4,6-O-benzylidene-2-thio-α-D-altropyranoside (**10**). — To a suspension of methyl 4,6-*O*-benzylidene-2-thio-α-D-altropyranoside (**9**, 0.37 g) in acetone (5 ml), benzyl chloride (0.3 ml) and 5% aqueous sodium hydroxide (1.5 ml) were added. The mixture was vigorously shaken for 30 min and then poured into water (100 ml); shaking was continued until the oily substance solidified. Recrystallization from ethanol afforded compound **10** (0.3 g), m.p. 139–140°, $[\alpha]_D^{20}$ + 97.6° (*c* 2.5, chloroform); lit.[7], m.p. 136–137°, $[\alpha]_D$ + 91° (chloroform).

Methyl 3-O-acetyl-2-S-benzoyl-4,6-O-benzylidene-2-thio-α-D-altropyranoside (**7**). — The *S*-benzoyl derivative (**5**, 1.0 g) was acetylated in the usual manner in pyridine (5 ml) and acetic anhydride (2 ml) to yield, after recrystallization of the crude product from ethanol, compound **7** (0.6 g), m.p. 130–131°, $[\alpha]_D^{20}$ −12.3° (*c* 4.0, chloroform) (Found: C, 62.25; H, 5.48; S, 7.30; $C_{23}H_{24}O_7S$ calc.; C, 62.14; H, 5.44; S, 7.21%).

Methyl 3-O-acetyl-2-S-acetyl-4,6-O-benzylidene-2-thio-α-D-altropyranoside (**8**).— Methyl 4,6-*O*-benzylidene-2-thio-α-D-altropyranoside (**9**, 1.0 g), when acetylated as described in the preceding paragraph, afforded the di-*O*-acetyl derivative (**8**, 0.8 g), m.p. 94–95°, $[\alpha]_D^{20}$ +75.8° (*c* 4.0, chloroform) (Found: C, 56.61; H, 5.72; S, 8.54. $C_{18}H_{22}O_7S$ calc.: C, 56.53; H, 5.79; S, 8.38%).

Methyl 2-S-benzoyl-4,6-O-benzylidene-2-thio-α-D-altropyranoside (**5**) *by partial benzoylation of compound* **9**. — To a solution of substance **9** (0.3 g) in acetone (12 ml), potassium carbonate (0.3 g) and benzoyl chloride (0.3 ml) were added. The mixture was shaken vigorously for 15 min and then left overnight. Part of the substance separated in spherical crystal aggregates; the remainder was obtained upon addition of water to the filtered reaction mixture from which acetone had been partly distilled off. Recrystallization from ethanol gave compound **5** (0.2 g), m.p. 163–165°, $[\alpha]_D^{20}$ —23.0° (*c* 1.8, chloroform).

Department of Biochemistry,
Charles University,
Albertov 2030, Praha 2 – Nové Město,
(Czechoslovakia)

JAN KOCOUREK*

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*Present address: The American National Red Cross, Blood Program Research Laboratory, Washington, D. C. 20006 (U.S.A.)

Proton magnetic resonance of crystalline α-D-glucose

INTRODUCTION

Nuclear magnetic resonance has been used to study the position of hydrogen atoms in crystals, and particularly to determine certain aspects of hydrogen atom behaviour which are inaccessible to diffraction studies, such as re-orientation of the ammonium ion in ammonium chloride. We commenced this study of α-D-glucose when the position of hydrogen atoms in the crystal was not known, in the hope that it might assist in determining the arrangement of protons involved in hydrogen bonding. While the work was in progress, Brown and Levy[1] published the results of neutron diffraction experiments which fixed the proton positions. By and large this work is in agreement with ours, but there is a small discrepancy which is discussed later.

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Department of Biochemistry,
Charles University,
Albertov 2030, Praha 2 – Nové Město,
(Czechoslovakia)

JAN KOCOUREK*

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RESULTS AND DISCUSSION

The proton magnetic resonance line of crystalline α-D-glucose differs from the more familiar high resolution spectra obtained from the same material in solution in that there is only a single broad and featureless peak. The width of this peak measured between points of maximum slope is approximately 10.3 gauss (G), which may be expressed as approximately 44,000 c.p.s. in the units commonly used in high resolution n.m.r. In solution, the molecules tumble freely and rapidly, so that all but the very small isotropic nuclear magnetic and molecular magnetic interactions are averaged to zero and a sharp line spectrum characteristic of the molecule is obtained. In the solid, these isotropic interactions are completely overwhelmed by the much larger anisotropic interactions originating from the magnetic fields due to the magnetic dipole moments of all protons in the crystal; hence a much broader signal is observed. A comparison of spectra of this type is most conveniently made by reference to the second moment (or "moment of inertia" of the absorption line about its "centre of gravity") expressed in units of G^2.

Van Vleck[2] has shown that, given the position co-ordinates of the atoms in a crystal, the second moment of the nuclear magnetic resonance line is strictly calculable, and is related to the inverse sixth power of the interatomic spacings in the crystal. For powder samples of carbohydrates, the expression for the second moment is given by equation (1)

$$\overline{\Delta H_2^2} = C_{\mathrm{HH}} \sum_{i,j} r_{ij}(\mathrm{H,H})^{-6} + C_{\mathrm{HO}} \sum_{i,k} r_{ik}(\mathrm{H,O})^{-6} + C_{\mathrm{HC}} \sum_{k,l} r_{kl}(\mathrm{H,C})^{-6} \tag{1}$$

where $\overline{\Delta H_2^2}$ is the second moment, r_{ij} (H,H) is the distance between the ith and jth protons, r_{ik} (H,O) is the distance between the ith proton and the kth oxygen nucleus and r_{kl} (H,C) is the distance between the kth proton and the lth carbon nucleus; C_{HH}, C_{HO}, and C_{HC} are constants appropriate to the nuclear interactions indicated by the subscript. In normal carbohydrates the most abundant isotopes of oxygen and carbon are ^{16}O (>99.9%) and ^{12}C (>98.9%) which have zero nuclear spin and consequently do not contribute to the second moment. For this reason the second and third terms of equation (1) become negligible and the expression reduces to equation (2). Thus, the experimental second moment of the proton magnetic resonance

$$\overline{\Delta H_2^2} = C_{\mathrm{HH}} \sum_{i,j} r_{ij}(\mathrm{H,H})^{-6}. \tag{2}$$

absorption line can be used as a useful measure with which to compare the second moment calculated from a hypothetical model of the hydrogen positions within a carbohydrate crystal.

In their original X-ray diffraction study, McDonald and Beevers[3] showed that α-D-glucose crystallizes in the space group $P2_12_12_1$ with cell dimensions a = 10.36Å, b = 14.84Å, c = 4.97Å. They found also that the molecular packing in the crystal was dictated by intermolecular hydrogen bonds and suggested that the three dimensional hydrogen bond network consisted in part of two roughly spiral chains perpendicular to the [100] plane and two other chains, which were almost linear, perpendicular to the [001] plane. Their data, however, did not allow the hydrogen atoms to be located with any precision, so to a first approximation they were placed midway between the appropriate oxygen atoms, although each proton involved in a hydrogen bond will be closer to the oxygen atom to which it is covalently linked.

The asymmetric position of the proton in the oxygen–oxygen bridge imparts a sense to the hydrogen bond chain. It was believed initially that the sensitivity of the n.m.r. second moment to the interproton distance would provide useful information on this property and also on the possible motions of such protons in the crystal. Unfortunately, calculations of the second moment for two models of the hydrogen bond chains yielded values of 15.70 G^2 and 15.73 G^2 which are not sensibly different.

Before this work was completed, Brown and Levy[1] completed a new structural analysis with neutron diffraction data. A redetermination of the cell parameters[4] yielded the result $a = 10.3662\,(0.0009)$Å, $b = 14.8506\,(0.0016)$Å, $c = 4.9753\,(0.0003)$Å (standard error in brackets). This analysis confirmed the work of McDonald and Beevers and, furthermore, has provided proton co-ordinates to a precision comparable with those of the heavier atoms.

The calculated second moment based on the new coordinates of Brown and Levy with cell dimensions obtained by Ellison, Levy, and Brown[4] is 14.99 G^2 of which 5.48 G^2 is contributed by the hydrogen bonded protons. This may be compared with the observed second moment (averaged over five spectra) of 14.31 G^2.

The standard deviation in the observed second moment is 0.13 G^2, and the residual instrumental errors are estimated to be less than 0.27 G^2. Hence, the error in observed second moment is probably less than 0.30 G^2 (the r.m.s of the two foregoing errors). The standard errors in the cell dimensions quoted by Ellison *et al.*[4] lead to an error of 0.01 G^2 in the calculated second moment. The difference between the observed and calculated second moments is slightly greater than twice the sum of the errors in these two quantities and is probably significant.

The infra-red spectrum and specific rotation of the specimen indicate that it is substantially pure α-D-glucose. The three most likely impurities, however, are β-D-glucose, α-D-glucose monohydrate and water. The powder second moment of β-D-glucose is 16.6 G^2 (based on the co-ordinates given by Ferrier[5]) so no significant change in the second moment could be anticipated from small amounts of this substance. The influence of α-D-glucose monohydrate[6] would be greater because of the water molecules present in the crystal but again it would increase the value of the observed second moment above that of pure α-D-glucose. As there was no narrow absorption line discernible in the spectra and because of the previous treatment of the sample it is reasonable to assume that the amount of adsorbed water present was negligible ($<0.1\%$).

The difference of 0.68 G^2 between the observed and calculated second moments, therefore, is probably best explained by the large thermal and zero point vibrations of the hydrogen atoms, as these could average out the dipolar magnetic fields at the various sites. Furthermore it is concluded that for many monosaccharides little additional information may be obtained from broadline n.m.r. which is not also provided by the appropriate diffraction data.

EXPERIMENTAL

The sample of analytical reagent grade α-D-glucose (NBC) was dried *in vacuo*, at 30°, over P_2O_5 for 24 h before sealing in an evacuated glass tube. It had $[\alpha]_D^{20}$ +111.6°, (*c* 3, water); lit.[7] $[\alpha]_D^{20}$ +112.2° (*c* 4, water).

The n.m.r. spectra were obtained at a frequency of 27 Mc/sec using a Pound-Knight marginal oscillator. All spectra were taken at room temperature and fixed frequency, the magnetic field being scanned over 50 gauss. Signal to noise ratios of approximately 100:1 were obtained in the spectra.

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Division of Forest Products, C.S.I.R.O., B. J. POPPLETON
South Melbourne (Australia)
Division of Chemical Physics, C.S.I.R.O., C. K. COOGAN
Chemical Research Laboratories,
Melbourne (Australia)

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The formation of D-*arabino*-hexosulose bis(semicarbazone) and bis(benzoylhydrazone)

D-*arabino*-Hexosulose bis(benzoylhydrazone) (**1**) has recently been used in preparing 1-substituted triazoles[1]. Like the bis(semicarbazone)[2], it was prepared from the osone, which is difficult to obtain. We have now attempted to prepare compound **1** and D-*arabino*-hexosulose bis(semicarbazone) (**2**) by other methods; these have shed some light on their mechanism of formation.

It is known that arylosazones are obtained through the intermediate formation of Amadori compounds, and that higher yields of osazones are obtained when these compounds are used instead of the corresponding sugar[3]. We have therefore tried to synthesize the above bis(hydrazones) from the Amadori compounds: 1-deoxy-1-*p*-toluidino-D-fructose (**3**) and 1-*p*-anisidino-1-deoxy-D-fructose (**4**). The reaction took place readily, and **2** and **1** were obtained in 14 and 21% yields, respectively.

When the reaction was carried out by using D-glucose and aniline, or *p*-toluidine, instead of the corresponding Amadori compounds, the yield increased to 28% for **2**. This unexpected result led us to believe that, unlike arylosazones[4], the above bis(hydrazone) is formed by a mechanism not involving the intermediate formation of Amadori compounds and yet catalyzed by the presence of aromatic amines. The highest yields of bis(hydrazones) were obtained when D-glucose semicarbazone or D-glucose benzoylhydrazone were respectively refluxed with semicarbazide, or benzoylhydrazine, in ethanolic acetic acid containing aniline or *p*-toluidine. The reaction was complete in four to eighteen h, and the yields amounted to 40% for **2** and 34% for **1**.

It was, therefore, concluded that, as with formation of arylosazones, the first step in the reaction is the formation of the hydrazone; but that this then reacts with the aromatic amine without formation of an Amadori compound. In other words, the mechanism of formation of bis(acylhydrazones) differs from that of arylosazones.

EXPERIMENTAL

D-arabino-*Hexosulose bis(semicarbazone)* (**2**).–(a); *From* **3**. A solution of **2** (5 g), semicarbazide hydrochloride (2 g), and sodium acetate (2.4 g) in 2N acetic acid (5 ml) and ethanol (50 ml) was refluxed for 1 h; **2** was filtered off after 24 h, and crystallized from dilute ethanol; yield, 0.8 g; m.p. and mixed m.p.[2] 215°.

Anal. Calc. for $C_8H_{16}N_6O_6$: C, 32.87; H, 5.52; N, 28.75. Found: 32.83; H, 5.84; N, 28.90.

(b) *From* **4**. A solution of **4** (5 g), semicarbazide hydrochloride (1.95 g), and sodium acetate (2.25 g) in 2N acetic acid (5 ml) and ethanol (50 ml) was treated as above; yield, 0.75 g; m.p. and mixed m.p. 215°.

Anal. Found: C, 32.68; H, 5.70; N, 28.62.

(c) *From* D-*glucose*. A solution of D-glucose (5 g), *p*-toluidine (7 g) or aniline

(3 g), semicarbazide hydrochloride (6.15 g), and sodium acetate (7.5 g) in 2N acetic acid (5 ml), water (8 ml), and ethanol (50 ml) was refluxed for 4 h. The product (2.3 g) had m.p. and mixed m.p. 125°.

(d) *From* D-*glucose semicarbazone.* A solution of D-glucose semicarbazone (5 g), *p*-toluidine (7 g) or aniline (3 g), semicarbazide hydrochloride (2.4 g), and sodium acetate (2.9 g) in water (8 ml), 2N acetic acid (5 ml), and ethanol (50 ml) was refluxed for 4 h. The product (2.5 g) had m.p. and mixed m.p. 215°.

D-arabino-*Hexosulose bis(benzoylhydrazone)* (**1**).–*From* **3**. A solution of **3** (2.5 g) and benzoylhydrazine (2.5 g) in 2N acetic acid (5 ml) and ethanol (50 ml) was refluxed for 8 h and concentrated. Compound **1** crystallized from ethanol; yield, 0.8 g; m.p. and mixed m.p.[1] 188°.

Anal. Calc. for $C_{20}H_{22}N_4O_6$: C, 57.97; H, 5.35; N, 13.52. Found: C, 57.52; H, 5.72; N, 13.54.

(b) *From* **4**. A solution of **4** (2.5 g) and benzoylhydrazine (2.5 g) in 2N acetic acid (5 ml) and ethanol (50 ml) was treated as above. The product (0.8 g) had m.p. and mixed m.p. 188°.

(c) *From* D-*glucose benzoylhydrazone.* A solution of D-glucose benzoylhydrazone (2.5 g), benzoylhydrazine (1.2 g), and *p*-toluidine (1.5 g) or aniline (1.25 g) in 2N acetic acid (5 ml), water (5 ml), and ethanol 35 ml) was refluxed for 18 h. The product (1.2 g) had m.p. and mixed m.p. 188°.

Faculty of Science,
Alexandria University,
Alexandria (U.A.R.)

H. EL KHADEM
G. H. LABIB
M. A. NASHED

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Preliminary Communication

Stereoselective ring-opening of β-D-mannopyranose 1,2-(alkyl orthoacetates)

D-Mannopyranosyl residues are common in the carbohydrate prosthetic groups of glycoproteins, structural studies on which suffer from a dearth of well-characterized oligosaccharides. With a view to synthesizing oligosaccharides containing D-mannose, particularly those having α-D-(1→2) linkages, the ring opening of β-D-mannopyranose 1,2-(alkyl orthoacetates) was investigated.

Earlier work has been directed toward the ring opening of the 1,2-(alkyl orthoacetates) of 3,4,6-tri-*O*-acetyl-β-D-mannopyranose[1] and 3,4,6-tri-*O*-acetyl-α-D-glucopyranose[2,3]. In order to avoid any embarassment in later conversions because of acetyl migration, the present studies were made with 3,4,6-tri-*O*-benzyl-β-D-mannose 1,2-(methyl orthoacetate) (**1**), m.p. 78–81°, $[\alpha]_D$ + 12.1° (*c* 1.65)*, which was prepared from the corresponding triacetate (**2**).

The acid-catalyzed ring opening of **1** (0.4M) in dichloromethane containing *p*-toluenesulfonic acid (0.04M), excluding water or any alcohol, showed complete rearrangement within 20 min at 46.5°, with almost exclusive formation of methyl α-D-glycoside derivatives. The reaction was performed in the n.m.r. sample tube, and followed by observing the decrease of orthoacetate C–Me signals at τ 8.35 and 8.56 with concomitant appearance of an *O*-acetyl peak at τ 7.91. The final, equilibrium mixture was analyzed by column chromatography on silica gel, whereby methyl 2-*O*-acetyl-3,4,6-tri-*O*-benzyl-α-D-mannoside (**3**), $[\alpha]_D$ +27.9° (*c* 2.24), and methyl 3,4,6-tri-*O*-benzyl-α-D-mannoside (**4**), $[\alpha]_D$ + 59.7° (*c* 1.85), were isolated in 82% and 7% yield, respectively. A small proportion of the β-D anomer (**5**) of **4** was identified by t.l.c. No other products were observed. A similar reaction, with 0.012M mercuric bromide as catalyst in nitromethane[3], was slower, but otherwise gave the same results.

The presence of methanol in the reaction mixture appeared to have little or no effect upon the direction of the ring opening, but there was more extensive loss of the 2-acetoxy group. Treatment of **1** (0.4 mmole) with methanol (0.4 mmole) and

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*The reactions were followed by t.l.c. on silica gel G (E. Merck, Darmstadt, Germany) with 1:1 chloroform–ethyl acetate as eluent and detection of the components by sulfuric acid. The products were purified either by recrystallization, usually from ether–petroleum ether, or by chromatography on silica gel (0.05–0.2 mm), with successive elution by dichloromethane, ethyl acetate, and methanol. The melting points (Fisher–Johns apparatus) are uncorrected. The n.m.r. spectra were obtained in chloroform or chloroform-*d*, with a tetramethylsilane external standard, by using a Varian A-60 spectrometer. All optical rotations were observed in dichloromethane.

p-toluenesulfonic acid (0.4 mmole) in dichloromethane (1 ml) for 10 min at 46.5° gave an equilibrium mixture containing 74% of **3**, 20% of **4**, and 6% of **5**. Similar treatment of 3,4,6-tri-*O*-acetyl-α-D-glucose 1,2-(alkyl orthoacetate) has previously[2] been shown to result in the complete removal of the 2-acetoxy group from the products, which were a mixture of 3,4,6-tri-*O*-acetyl-D-glucose and the corresponding alkyl α-(and β)-D-glycosides[2]. The stereoselectivity of the ring opening was not so pronounced in this D-glucose series as is described above for **1**. Almost exclusive formation of **4** was achieved by heating **1** in 2% methanolic HCl for 24 h or longer under reflux. Similarly, aqueous acetic acid hydrolyzed **1** to 3,4,6-tri-*O*-benzyl-D-mannose, m.p. 98–9°, $[\alpha]_D$ +22.7° (*c* 1.92), which was characterized by oxidation, with periodate, to the known 2,3,5-tri-*O*-benzyl-D-arabinose, m.p. 77–80°, undepressed on admixture with an authentic sample.

The acid-catalyzed rearrangements of the 1,2-(cyclohexyl orthoacetate) and 1,2-(isopropyl orthoacetate) of 3,4,6-tri-*O*-benzyl-β-D-mannose proceed with equal stereoselectivity, to give the corresponding α-D-mannoside. Studies are continuing to extend the reaction to other orthoesters.

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Department of Biochemistry,
University of Iowa,
Iowa City, Iowa 52240 (U. S. A.)

NEAL E. FRANKS
REX MONTGOMERY

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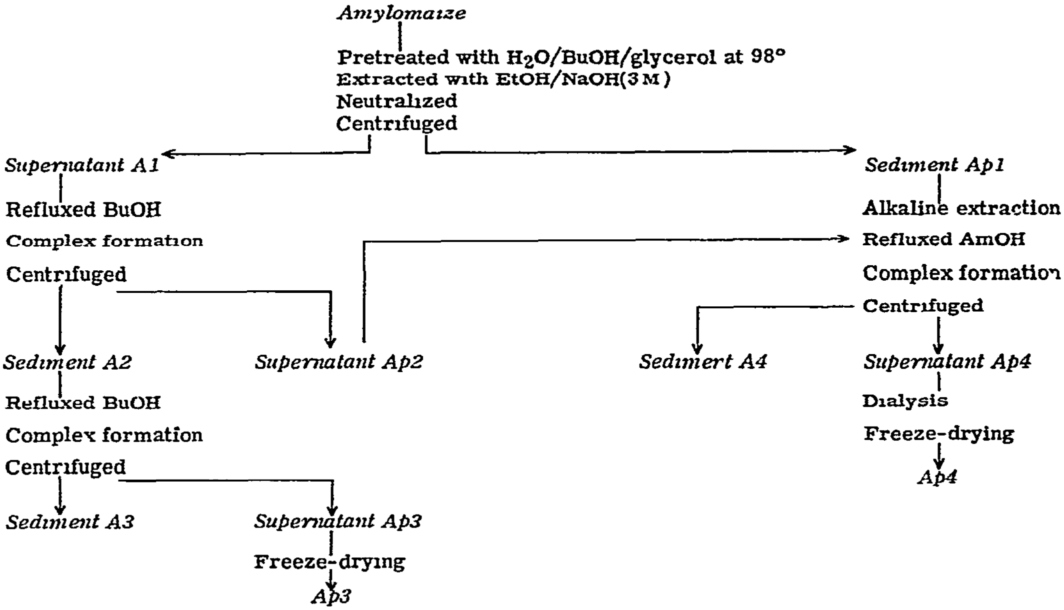
ERRATA

Carbohydrate Res., 3 (1966) 7–13

Page 8. The scheme should read:



Carbohydrate Res., 3 (1966) 102–110

The subtitle of the article should read:

Part XV. The enzymic synthesis of some *O*-D-glucosylpentose disaccharides

Page 110 The heading of Table II should read.

Properties of *O*-D-glucosyl-D-xyloses