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PROPERTIES OF CYCLODEXTRINS

PART II. PREPARATION OF A STABLE β-CYCLODEXTRIN HYDRATE AND DETERMINATION OF ITS WATER CONTENT AND ENTHALPY OF SOLUTION IN WATER FROM 15–30°

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ABSTRACT

The solubility of β-cyclodextrin (β-CD) in water has been measured by a refractive-index method at 15–30°. Evidence was obtained that the same, solid β-CD hydrate phase is present in this temperature range. The formula of the hydrate was shown to be $C_{42}H_{70}O_{35}\cdot(12.0 \pm 0.5)H_2O$. A method for preparation of this β-CD hydrate is described. The relations between refractive index and concentration of β-CD are given. The calculated enthalpy of solution $\Delta H_{sol} = 7.094 \pm 0.021$ kcal. mole^{-1}. This value will only slightly be influenced by the number of moles of water per mole of β-CD in the hydrated solid. The solubility of β-CD as a function of temperature can be described by

$$2.303 \log_{10} m = (3.88 \pm 0.02) - \frac{(3583 \pm 18)}{T}$$

where m = mole fraction of anhydrous β-CD at saturation, and T = absolute temperature.

INTRODUCTION

It has been shown by several investigators[1,2] that the composition of clathrates may vary over a range in which they are stable with respect to their decomposition into guest and host. Therefore, clathrates may be non-stoichiometric compounds, the geometry of the host lattice (A) and the guest (B) determining the limiting composition (A_nB) of the solid clathrate. In the case of cyclodextrin inclusion-compounds, both guest and water molecules are situated within the crystal structure[3].

A method to investigate the composition of these compounds is to analyse the ternary system water–cyclodextrin(CD)–host. A reliable starting point for analysing this system is the preparation and identification of a stable and reproducible CD-hydrate. In Part I of this series[4], it was shown that at least three different α-CD hydrate modifications had to be postulated at 20°. The formation of these hydrates is still a matter of chance, and α-CD is thus not the most favourable CD species for study of the ternary system CD–water–host. This paper deals with the preparation of a β-CD hydrate which appears to be stable between 15 and 30°. The

water content of this hydrate has been measured both by gas–liquid chromatography and elemental analysis. Its solubility and its enthalpy of solution have been determined by means of precise measurements of refractive index.

EXPERIMENTAL

Materials. — β-Cyclodextrin (β-CD) was prepared[5] and tested for purity[6] by literature procedures. Water, twice distilled, had n_D^{20} 1.33299 ±0.00002.

Apparatus. — Refractometer, Bausch and Lomb precision refractometer Type 33-45-03-01. Dry-box, Ateliers de Technochimie (Ivry/Seine, France), equipped with a Mettler analytical balance. The air within the dry-box was circulated continuously through concentrated sulphuric acid. Relative humidity, 2%. Vials, 10 and 20 ml with rubber stoppers (washed with hot β-CD solution and finally with twice-distilled water) and aluminium caps. Thermostat, Colora, equipped with a Lauda R10 electronic-relay box and a rotating disc. The vials were put into stainless-steel, screwcap containers and mounted on the rotating disc. Temperature, ±0.05°.

Preparation of β-CD hydrate crystals. — The crystals were prepared by dissolving 16 g of β-CD (stored over silica gel, and containing about 2% w/w of water) in 100 g of hot water. After storage of the solution for *ca.* 10 min at room temperature, rectangular crystals were formed. The crystals were isolated, and dried, first with filter paper, and then for 12 h over silica gel. After this, the crystals were equilibrated at 20° over a saturated solution of β-CD hydrate, containing the solid hydrate. One batch of crystals was equilibrated *in vacuo*, and another at 1 atm. The water content was determined both by a g.l.c. method[7] and by elemental analysis.

Solubility measurements by the refractive-index method. — Samples of β-CD were powdered, and dried in layers (< 0.2 cm) during 16 h at 90°/0.2 cm. Their water-content[7] was 0.2% w/w. The samples were weighed (dry-box) into the vials, water was added to obtain the composition required, and the vials were placed in the thermostat. After thermal equilibration, samples of the solutions were taken for measurement of refractive index. The mathematical expression fitting the data relating refractive index and concentration was obtained by means of a computer[4]. Insertion of the mean value of the refractive index of the saturated solutions (approached from the undersaturated and the oversaturated side) into this mathematical expression yielded the value of the solubility at the given temperature.

Solubility measurements by the oven-drying method. — In order to check the solubility data obtained by the refractive-index method, four independent determinations of the solubility of β-CD hydrate at 20° were made. Aliquot parts of saturated solutions of β-CD (approached from the undersaturated side) were weighed into vials, and evaporated at 80°/1 atm. The residue was dried at 90°/0.2 cm, and weighed, and its water content was determined by the g.l.c. method[7].

RESULTS

Water-content determination. — The water content[7] of both batches was

16.0 ±0.6% (w/w). The same result was obtained (within experimental error) by elemental analysis, indicating that the formula of the β-CD hydrate can be expressed by $C_{42}H_{70}O_{35}\cdot(12.0 \pm 0.5)H_2O$.

Solubility measurements. — In Fig. 1, the refractive-index data are plotted. The relations between the refractive index and the concentration are listed in Table I. From these relations and the refractive index of the saturated β-CD solutions, the solubility of β-CD was calculated. The results are listed in Table II.

TABLE I

RELATION BETWEEN REFRACTIVE INDEX AND CONCENTRATION OF β-CD IN WATER

Temperature	*Relations*[a]
15°	$c = -908.87 + 681.609\, n_D^{15}$
	$n_D^{15} = (1.33341 \pm 0.00002) + (0.001466 \pm 0.000024)c$
20°	$c = -926.79 + 695.271\, n_D^{20}$
	$n_D^{20} = (1.33299 \pm 0.00001) + (0.001438 \pm 0.000008)c$
25°	$c = -921.99 + 691.939\, n_D^{25}$
	$n_D^{25} = (1.33248 \pm 0.00001) + (0.001445 \pm 0.000009)c$
30°	$c = -925.17 + 694.610\, n_D^{30}$
	$n_D^{30} = (1.33192 \pm 0.00001) + (0.001439 \pm 0.000010)c$

[a] c = concentration in g of dry β-CD/100 g of solution.

TABLE II

REFRACTIVE INDEX OF THE SATURATED SOLUTION AND THE SOLUBILITY OF β-CD IN WATER

Temperature	n_D (*sat*)	*Solubility (g of dry β-CD/100 g of solution)*
15°	1.33517	1.199 ±0.014
20°	1.33511	1.476 ±0.006
25°	1.33507	1.792 ±0.009
30°	1.33509	2.198 ±0.012

Table III shows the results of the solubility measurements at 20° by the oven-drying method. From this Table, it can be seen that the solubility corresponds, within experimental error, with the value obtained by the refractive-index method. A plot of $\log_{10} m$ *versus* $1/T$ (where m is the mole fraction of β-CD in the saturated solution) yields a straight line (Fig. 2). This suggests that, in all cases, the same hydrate modification of β-CD is in equilibrium with its saturated solution.

Calculation of the enthalpy of solution. — From equation (1) and Table IV, the enthalpy of solution (ΔH_{sol}) has been calculated graphically.

$$2.303 \log_{10} m = \frac{-\Delta H_{sol}}{R} \cdot \frac{1}{T} + \text{constant} \qquad (1)$$

in which ΔH_{sol} = heat absorbed on dissolution per mole of solute dissolved in the

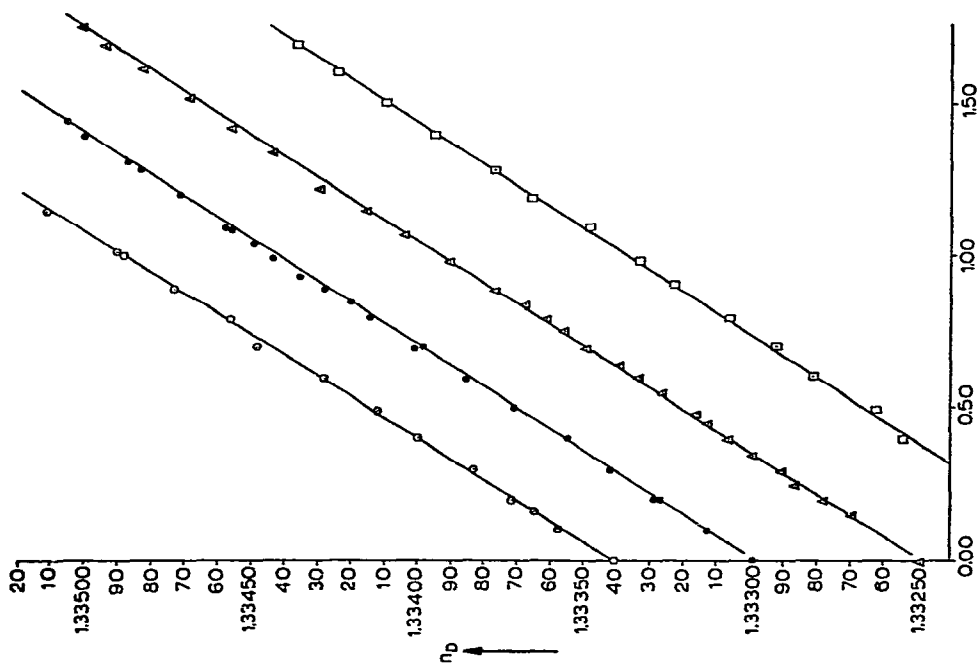

Fig. 1 Refractive index of β-CD solutions as a function of concentration
○ 15°, ● 20°, △ 25°, □ 30°.

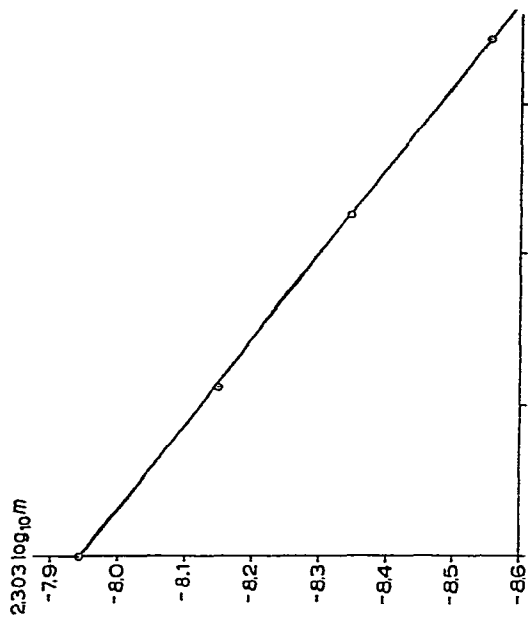

Fig. 2 Solubility of β-CD as a function of temperature.

TABLE III

DETERMINATION OF THE SOLUBILITY OF β-CD AT 20° BY THE OVEN-DRYING METHOD

Weight of saturated solution ±0.0001 g	*Weight of residue ±0.0001 g*	*Mean value of water % (w/w) of the residue ±0.1*	*Solubility of β-CD (g of dry β-CD/100 g of solution) ±0.005*
6.3883	0.0939		1.467
7.0812	0.1045		1.473
6.1249	0.0898	0.2	1.465
6.9955	0.1028		1.467
10.0555[a]	0.0001		—

[a]Blank (water).

TABLE IV

VALUES OF 2.303 $\log_{10} m$ OF β-CD IN A SATURATED SOLUTION AT DIFFERENT TEMPERATURES (m = MOLE FRACTION)

Temperature	*1/T (°K^{-1})*	*2.303 $\log_{10}$ m*
15°	$3.472.10^{-3}$	−8.557
20°	$3.413.10^{-3}$	−8.346
25°	$3.356.10^{-3}$	−8.148
30°	$3.300.10^{-3}$	−7.941

nearly saturated solution in kcal.mole^{-1}; R = gas constant = 1.98 kcal.mole^{-1}. degree^{-1}; T = absolute temperature; and m = mole-fraction of β-CD in the saturated solution.

From Fig. 2, it follows that ΔH_{sol} = (7.094 ±0.021) kcal.mole^{-1}, and so the solubility of β-CD in water from 15 to 30° can be described by equation (2).

$$2.303 \log_{10} m = (3.88 \pm 0.02) - \frac{3583 \pm 18}{T} \qquad (2)$$

DISCUSSION

The term ΔH_{sol} represents the heat absorbed per mole of solute dissolved in the nearly saturated solution. When calculating ΔH_{sol}, we have to consider the exact formulation of heats of solution from solubility data of hydrated non-electrolytes as given by Williamson[8] [equation (3)].

$$\Delta H_{sol} = \left(1 - \frac{xs}{55.51}\right) RT^2 \frac{ds}{\partial T} \left[\frac{\partial \ln f_2}{dn} + \frac{1}{s}\right] \qquad (3)$$

in which x = number of moles of water per mole of substance (CD) in the hydrated solid (CD·xH$_2$O); n = the molality of the solute [number of moles of unhydrated

solute (CD) in 1000 g of water]; s = the molality of the solute at saturation; and f_2 = the activity coefficient of the solute (CD) in solution.

The saturated or nearly saturated β-CD solution at 25° is a dilute solution: $f_2 \approx 1$, $(\partial \ln f_2)/\partial n \approx 0$, whereas $1/s = 62$. Thus $1/s \gg (\partial \ln f_2)/\partial n$ (*cf.* data[9] for D-glucose at 25°: $(\partial \ln f_2)/\partial n = -0.082$, but $1/s = 0.174$).

Since s at 25° $= 16.09.10^{-3}$, and $x = 12.0$, $(xs/55.51)$ is small with respect to 1, and equation (3) can be simplified, in this case, to give equation (4), which is the differential form of equation (1)

$$\Delta H_{sol} = RT^2 \frac{d\ln s}{dT} = RT^2 \frac{d\ln m}{dT} \quad (4)$$

The number of moles of water in the hydrated β-CD crystals will have little influence on the value found for ΔH_{sol}.

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REFERENCES

1 J. H. VAN DER WAALS, *Trans. Faraday Soc.*, 52 (1956) 184.
2 M. H. J. POWELL, *J. Chem. Soc.*, (1950) 298, 300, 468.
3 A. HYBL, R. E. RUNDLE, AND D. E. WILLIAMS, *J. Am. Chem. Soc.*, 87 (1965) 2779.
4 N. WIEDENHOF AND J. N. J. J. LAMMERS, *Carbohyd. Res.*, 4 (1967) 318.
5 D. FRENCH, A. O. PULLEY, AND W. J. WHELAN, *Stärke*, 15 (1963) 280.
6 J. N. J. J. LAMMERS, *Stärke*, 19 (1967) 70.
7 J. N. J. J. LAMMERS AND N. WIEDENHOF, *Stärke*, 19 (1967) 252.
8 A. T. WILLIAMSON, *Trans. Faraday Soc.*, 40 (1944) 421.
9 J. B. TAYLOR, *Trans. Faraday Soc.*, 53 (1957) 1198.

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SYNTHESIS OF *O*-GLYCOSYLTHREONINES

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ABSTRACT

A synthesis of *N*-(2,4-dinitrophenyl)-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-L-threonine methyl ester and *O*-(β-D-glucopyranosyl)-L-threonine methyl ester is described. Attempts at glycosylation of L-threonine derivatives by the Koenigs–Knorr method or by the orthoester method gave the corresponding 1,2-orthoacetates of D-glucose, which were converted into isomeric D-glucosides.

INTRODUCTION

Recent, rapid progress in the development of glycoprotein chemistry necessitates the synthesis and study of simple model compounds corresponding to portions of these biopolymers[1]. The identification of glycosidic bonds involving oxyamino acids as a key protein–carbohydrate linkage in a number of glycoproteins[2–7] has stimulated the synthesis of some *O*-glycosylserines[8–13]. However, the synthesis of an *O*-glycosylthreonine has only recently been accomplished[13a], although there is no doubt of the importance of this type of linkage in natural biopolymers. Moreover, in some glycoproteins[7], L-threonine is detected as the only oxyamino acid involved in the protein–carbohydrate glycosidic linkage.

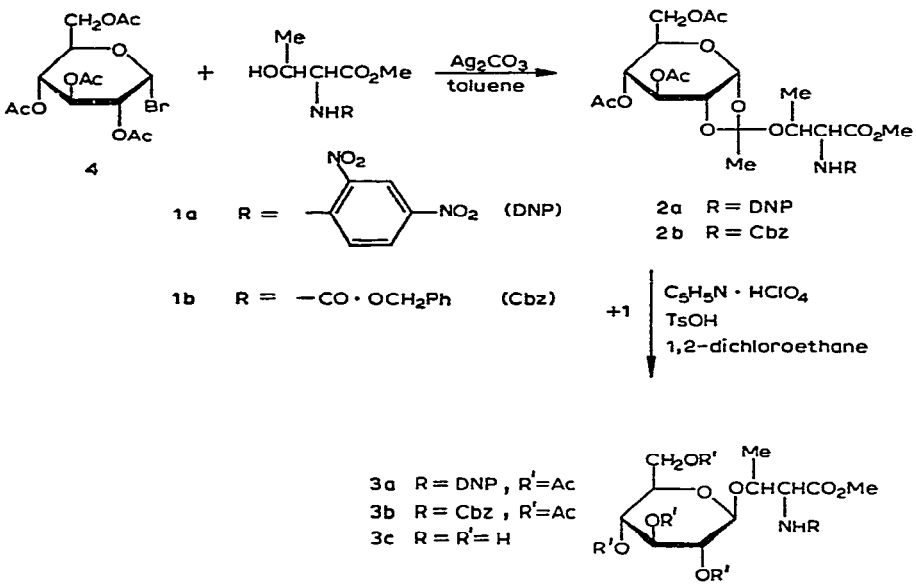
This paper deals with the first synthesis of *N*-(2,4-dinitrophenyl)-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-L-threonine methyl ester (**3a**) and *O*-(β-D-glucopyranosyl)-L-threonine methyl ester (**3b**).

RESULTS AND DISCUSSION

The first approach to the synthesis of *O*-glycosylthreonines involved attempts at glycosylation of *N*-(2,4-dinitrophenyl)-L-threonine (**1a**) with 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide (**4**) by the Koenigs–Knorr method (using silver carbonate as the acid acceptor) and also by the orthoester method[14]. In both cases, the orthoester **2a** was the sole product.

A successful synthesis was accomplished by a new, two-step procedure involving the orthoester method[15]. 3,4,6-Tri-*O*-acetyl-α-D-glucopyranose 1,2-[2-(2,4-dinitrophenyl)amino-2-methoxycarbonyl-1-methylethyl orthoacetate] (**2a**) and the

analogous *N*-benzyloxycarbonyl derivative (**2b**) were prepared, in *ca.* 70% yield, by application of the Koenigs–Knorr procedure for orthoester synthesis.



When compound **2a** in dry 1,2-dichloroethane containing a small proportion of L-threonine methyl ester **1a** and a mixture of toluene-*p*-sulphonic acid and pyridinium perchlorate as catalyst were heated in a sealed tube, the L-threonine glycoside **3a** was formed in 30–40% yield.

A more-convenient procedure avoids the isolation of the intermediate threonine orthoester **2a**, and the preparation of threonine glycoside **3a** involves preparation of the orthoester (**2a**), under the conditions of the Koenigs–Knorr reaction, and the further conversion into the threonine glycoside under the conditions[15] of orthoester "rearrangement".

Methanolysis of glucoside **3a** gave *N*-(2,4-dinitrophenyl)-L-threonine methyl ester and methyl D-glucoside, which were identified by paper chromatography. The threonine derivative had an unchanged optical rotation, proving that no racemisation had occurred during the glycosylation. The structure of the orthoester (**2a**) and the glucoside (**3a**) are supported by n.m.r. data. Compound **2a** gave signals at τ 8.23 (orthoester, *C*-methyl protons) and τ 7.92 (three *O*-acetyl groups). The presence of a signal at τ 8.23 indicates that compound **2a** is an *endo*-isomer[16,17]. The glucoside **3a** gave a signal at τ 7.93 (twelve protons of four *O*-acetyl groups) but no signal at τ 8.20. The signal for the anomeric proton has not been identified, but the marked stereospecificity of the orthoester method[14,15], which, in all known examples, produces only 1,2-*trans* isomers, suggests the β-D configuration for the glucoside **3a**.

The *N*-benzyloxycarbonyl-L-threonine D-glucoside **3b** was prepared analogously. Treatment of *N*-benzyloxycarbonyl-L-threonine methyl ester (**1b**) with

tetra-*O*-acetyl-α-D-glucopyranosyl bromide in the presence of silver carbonate gave the L-threonine orthoester **2b** which was converted directly, without purification, into compound **3b** (35–40%) by treatment with a small proportion of compound **1b** in the presence of catalyst as described above.

A preliminary investigation of compounds **3a** and **3b** shows that the stability of *O*-D-glucosyl derivatives of L-threonine in an acid medium is comparable with that of glucosides derived from aliphatic alcohols; the L-threonine orthoesters **2a** and **2b**, on the contrary, are very labile and are completely cleaved after treatment with 0.5N sulphuric acid in 90% acetone for 5 min at room temperature.

Deacetylation of compound **3b** was effected by treatment with 4% methanolic methylamine at room temperature, and subsequent, direct hydrogenolysis of the *N*-benzyloxycarbonyl group gave *O*-(β-D-glucopyranosyl)-L-threonine methyl ester, which was isolated as the hydrochloride.

EXPERIMENTAL

Paper chromatography was performed on paper "M" (Leningrad factory No. 2), and thin-layer chromatography (t.l.c.) on neutral alumina (activity, Brockmann grade III) and silica KSK with the solvent systems: chloroform–acetone, 95:5 (*A*); benzene–methanol, 85:15 (*B*); *tert*-pentyl alcohol–propyl alcohol–water, 8:2:3 (*C*); chloroform–acetone, 97:3 (*D*). Electrophoresis was performed in pyridine–acetate (pH 4.5) buffer at 40 volts/cm, for 15–20 min. The following spray reagents were used: silver nitrate, Bonner reagent, benzidine–chlorine reagent, hydroxylamine–ferric chloride, and (for t.l.c.) sulphuric acid and morin (on alumina). The solvent evaporation was performed *in vacuo* at <40°.

N-(*2,4-Dinitrophenyl*)-L-*threonine methyl ester* (**1a**). — *N*-(2,4-Dinitrophenyl)-L-threonine[18], m.p. 144.5–145°, was converted into the methyl ester **1a** by treatment with diazomethane. The product (95%) had m.p. 119.5–120° (from aqueous ethanol); $[\alpha]_D^{20}$ −63.3° (*c* 1.09, chloroform); ν_{max} 1068 (C–O–C), 1370 (OAc), 1345, 1528 (−NO_2), 1590 (C=C–C=C), 1732 (COOR), 1240, 3350 (NH), and 3435 cm^{-1} (OH) (Found: C, 44.04; H, 4.38; N, 14.30. $C_{11}H_{13}N_3O_7$ calc.: C, 44.15; H, 4.38; N, 14.04%).

*3,4,6-Tri-*O*-acetyl-α-D-glucopyranose 1,2-[2-(2,4-dinitrophenyl)amino-2-methoxycarbonyl-1-methylethyl orthoacetate*] (**2a**). — Compound **1a** (0.92 g, 3.1 mmoles) and silver carbonate (1.76 g, 6.2 mmoles) in dry toluene (30 ml) were boiled with vigorous stirring and slow distillation. When 5 ml of toluene had been distilled off, compound **4** (2.5 g, 6.1 mmoles) in toluene (30 ml) was added during 45 min, and distillation and addition of solvent were continued until the volume of distillate was 200 ml. The mixture was cooled and filtered, a drop of pyridine was added, and the mixture was evaporated *in vacuo*. The product **2a** (1.36 g, 70%), R_F 0.68, was isolated by t.l.c. (alumina, solvent *A*); $[\alpha]_D^{20}$ +28.2° (*c* 1.24, chloroform); ν_{max} 1050 (C–O–C), 1380 (OAc), 1345, 1530 (−NO_2), 1595 (C=C–C=C), 1750 (COOR), 1230, 3350 cm^{-1} (−NH) (Found: C, 48.62; H, 4.89; N, 6.51. $C_{25}H_{31}N_3O_{15}$ calc.: C, 48.94; H, 5.09; N, 6.85%).

N-(2,4-Dinitrophenyl)-O-(2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl)-L-threonine methyl ester (**3a**). — (*a*) A mixture of compounds **2a** (780 mg, 1.2 mmole) and **1a** (35 mg, 0.12 mmole), toluene-*p*-sulphonic acid (7 mg), and pyridine perchlorate (7 mg) in dry 1,2-dichloroethane (5 ml) was heated in a sealed tube in boiling xylene for 30 min. The cooled mixture was evaporated to dryness, 0.5N sulphuric acid in 90% aqueous acetone (4 ml) was added, and, after 10 min, the acid was neutralized with sodium hydrogen carbonate. The filtered solution was evaporated, and the residue was chromatographed (preparative t.l.c., alumina, solvent *A*) to give a product having R_F 0.6. Crystallisation from chloroform–ether–hexane gave compound **3a** (265 mg, 34%), m.p. 161–163°, $[\alpha]_D^{20}$ −8.9° (*c* 1.5, chloroform); ν_{max} 1050 (C–O–C), 1370 (OAc), 1345, 1530 (−NO_2), 1595 (C=C–C=C), 1755 (COOR), 1230, 3360 cm^{-1} (−NH) (Found: C, 48.81; H, 5.00; N, 6.72. $C_{25}H_{31}N_3O_{15}$ calc: C, 48.94; H, 5.09; N, 6.85%).

(*b*) A mixture of compound **1a** (1.38 g, 4.6 mmoles) and silver carbonate (2.64 g, 9.5 mmoles) in dry toluene (35 ml) was boiled with vigorous stirring and continuous distillation. When 5 ml of toluene had been distilled off, a solution of compound **4** (3.75 g, 9.1 mmoles) in dry toluene (50 ml) was added during 40 min, and slow distillation and addition of solvent were continued during 1 h. The mixture was cooled and filtered, and the filtrate was evaporated. Toluene-*p*-sulphonic acid (18 mg) and pyridine perchlorate (18 mg) were added, and the mixture was heated in a sealed tube and treated as described in the previous experiment. Preparative t.l.c. (alumina, 4-mm layer, solvent *A*) gave compound **3a** (200 mg, 41%), R_F 0.6. Crystallisation as above gave material having m.p. 160–162°, $[\alpha]_D^{20}$ −9.9° (*c* 1.6, chloroform).

Methanolysis of N-(2,4-dinitrophenyl)-O-(2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl)-L-threonine methyl ester (**3a**). — A solution of compound **3a** (98 mg) in 0.1N sulphuric acid in abs. methanol (5 ml) was refluxed for 35 min, cooled, neutralised ($BaCO_3$), and evaporated to dryness. Methyl D-glucoside was identified in the residue by paper chromatography (solvent *C*, R_F 0.6), and *N*-(2,4-dinitrophenyl)-L-threonine methyl ester {$[\alpha]_D^{20}$ −61° (*c* 1.0, chloroform)} was isolated from the residue (preparative t.l.c., alumina, solvent *B*, R_F 0.34).

N-Benzyloxycarbonyl-O-(2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl)-L-threonine methyl ester (**3b**). — A mixture of compound **1b** (1.1 g, 4.1 mmoles) and silver carbonate (2.2 g, 7.8 mmoles) in dry toluene (30 ml) was boiled with continuous, slow distillation. When 5–7 ml of solvent had been distilled off, compound **4** (3.0 mg, 7.3 mmoles) in dry toluene (25 ml) was added during distillation of solvent and treatment as above. The dry residue was dissolved in 1,2-dichloroethane (10 ml), and toluene-*p*-sulphonic acid (15 mg) and pyridinium perchlorate (15 mg) were added. The mixture was heated and treated as in the previous experiments. The chromatographically homogeneous product **3b** (890 mg, 37%) was isolated (preparative t.l.c., alumina, solvent *D*, R_F 0.35). After crystallisation from ether–light petroleum, the product had m.p. 55–56°, $[\alpha]_D^{20}$ −7.0° (*c* 1.15, chloroform) (Found: C, 53.97; H, 6.24; N, 2.51. $C_{27}H_{35}NO_{14}$ calc.: C, 54.27; H, 5.90; N, 2.34%).

O-(*β*-D-*Glucopyranosyl*)-L-*threonine methyl ester* (**3c**).— Compound **3b** (200 mg) was treated with 4% methanolic methylamine (5 ml) for 5 h at room temperature. The mixture was evaporated to dryness, and the residue was treated with dry ether (50 ml) containing 0.5 ml of abs. methanol. The filtered solution was evaporated, and the dry residue was hydrogenated in 75% aqueous methanol (1 ml) in the presence of 5% palladised barium sulphate (50 mg); the reaction was monitored by electrophoresis. The catalyst was removed by centrifugation, and compound **3c** (50 mg) was isolated by preparative paper electrophoresis, and converted into the salt by the addition of 0.1N hydrochloric acid in abs. methanol. The hydrochloride of compound **3c** had $[\alpha]_D^{20} - 5°$ (*c* 1.0, water) (Found: C, 44.50; H, 7.30; N, 4.50. $C_{11}H_{22}ClNO_8$ calc.: C, 44.74; H, 7.17; N, 4.74%).

REFERENCES

1 N. K. KOCHETKOV, V. A. DEREVITSKAYA, L. M. LIHOSHERSTOV, N. V. MOLODTZOV, AND S. G. KARA-MURZA, *Tetrahedron*, 18 (1962) 273.
2 K. TANAKA, M. BERTOLINI, AND W. PIGMAN, *Biochem. Biophys. Res. Commun.*, 16 (1964) 404.
3 B. ANDERSON, N. SENO, P. SAMPSON, J. RILEY, P. HOFFMAN, AND K. MEYER, *J. Biol. Chem.*, 239 (1964) PC2716.
4 N. K. KOCHETKOV, V. A. DEREVITSKAYA, AND S. G. KARA-MURZA, *Dokl. Akad. Nauk SSSR*, 163 (1965) 650.
5 N. K. KOCHETKOV, V. A. DEREVITSKAYA, AND S. G. KARA-MURZA, *Carbohyd. Res.*, 3 (1967) 403.
6 U. LINDAHL AND L. RODÉN, *J. Biol. Chem.*, 240 (1965) 2821.
7 J. ADAMS, *Biochem. J.*, 94 (1965) 368; 97 (1965) 345.
8 V. A. DEREVITSKAYA, M. G. VAFINA, AND N. K. KOCHETKOV, *Carbohyd. Res.*, 3 (1967) 377.
9 F. MICHEEL AND H. KÖCHLING, *Ber.*, 91 (1958) 673; 93 (1960) 2372.
10 J. K. N. JONES, M. B. PERRY, B. SHELTON, AND B. J. WALTON, *Can. J. Chem.*, 39 (1961) 1005.
11 B. LINDBERG AND B. G. SILWANDER, *Acta Chem. Scand.*, 19 (1965) 530.
12 J. R. VERCELLOTTI AND A. E. LUETZOW, *J. Org. Chem.*, 31 (1966) 825.
13a J. R. VERCELLOTTI, R. FERNANDEZ, AND C. J. CHANG, *Carbohyd. Res.*, 5 (1967) 97.
13 K. KUM AND S. ROSEMAN, *Biochemistry*, 5 (1966) 3061.
14 N. K. KOCHETKOV, A. YA. KHORLIN, AND A. F. BOCHKOV, *Tetrahedron Letters*, (1964) 289; *Tetrahedron*, 23 (1967) 693.
15 N. K. KOCHETKOV, A. F. BOCHKOV, AND V. SNYATKOVA, *Izv. Akad. Nauk SSSR, Ser. Khim.*, 12 (1967) 2684.
16 A. S. PERLIN, *Can. J. Chem.*, 41 (1963) 399.
17 R. U. LEMIEUX AND A. R. MORGAN, *Can. J. Chem.*, 43 (1965) 2199.
18 K. R. RAO AND H. A. SOBER, *J. Am. Chem. Soc.*, 76 (1954) 1328.

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THE MUCILAGE OF THE ROOTS OF *Clossostemon bruguieri* (MOGHAT)

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ABSTRACT

Moghat (the powdered, dried roots of *Clossostemon bruguieri*) was found to consist, to the extent of about 70%, of a mucilage mixture; this mixture could be separated into two mucilages, *A* and *B*. Mucilage *A* constitutes about 50% of the mucilage mixture, and is composed mainly of D-glucopyranose residues joined by (1 → 4)-linkages, as well as some L-rhamnose. Mucilage *B* constitutes the rest of the mucilage mixture, and is a glycogen-like substance mainly composed of D-glucopyranose residues joined by (1 → 4)-linkages, together with a small proportion of L-arabinose.

INTRODUCTION AND DISCUSSION

Moghat, the powdered, sundried roots of *Clossostemon bruguieri* (Family Sterculiaceae), is used in Egypt to make a hot beverage given to women after delivery, as a nutritive and demulcent drink. Its pharmacognosy was studied by Fahmy and El Key[1].

The aim of this work was to study the mucilages that constitute more than 70% of the dried roots. A mixture of two mucilages was isolated by extraction of moghat with 0.1M hydrochloric acid and precipitation with acetone. About 50% of this mixture was separated as an insoluble, copper complex, and was designated mucilage *A*. It is a pale-brown, amorphous substance, having $[\alpha]_D^{18}$ −200° and η_{sp} 0.24, that is sparingly soluble in water, but is soluble in aqueous alkali. Hydrolysis with acid followed by paper chromatography of the hydrolyzate indicated the presence of 77% of a glucose (shown to be D-glucose), 20% of a rhamnose (shown to be L-rhamnose), and no uronic acid. Complete methylation of mucilage *A* with methyl sulfate and sodium hydroxide, and then with methyl iodide and silver oxide, gave a product having $[\alpha]_D^{18}$ −175° which, on hydrolysis and paper chromatography, showed a large spot of a 2,3,6-tri-*O*-methylglucose (characterized as being the D enantiomorph) and a faint spot of a 2,3,4,6-tetra-*O*-methylglucose; this mixture of methylated sugars is characteristic of the hydrolyzate of methylated D-glucopyranose polymers having (1 → 4)-linkages. The type of L-rhamnose linkage has not yet been

established. Assay of end groups by periodate oxidation showed that the mucilage molecule is composed of chains of about 31 sugar residues.

Mucilage *B*, which constituted the remaining 50% of the mucilage mixture, gave a soluble, copper complex which, after removal of the insoluble, copper complex of mucilage *A*, was precipitated with ethanol. Mucilage *B* is a white, amorphous, water-soluble, glycogen-like substance which gives with iodine a red color that disappears after partial hydrolysis with saliva. It has $[\alpha]_D^{18}$ −220° and η_{sp} 0.17. Acid hydrolysis followed by paper chromatography indicated the presence of about 89% of a glucose (shown to be D-glucose), 9% of an arabinose (shown to be L-arabinose), and no uronic acid. The acetylated mucilage had $[\alpha]_D^{18}$ −160°. On methylation of mucilage *B* followed by hydrolysis, paper chromatography showed a large spot of a 2,3,6-tri-*O*-methylglucose (identified as the D enantiomorph) and faint spots of a 2,3-di- and a 2,3,4,6-tetra-*O*-methylglucose. Mucilage *B* is apparently composed of D-glucopyranose residues joined by α-D-(1 → 4)-linkages. The type of the L-arabinose linkages has not yet been established. Assay of end groups by periodate oxidation indicated the presence of about 18 D-glucopyranose residues in each chain.

EXPERIMENTAL

Isolation of the mucilage mixture. — Finely powdered, dried roots of moghat (5 kg) were packed in a cloth, and soaked in 0.1M hydrochloric acid (9 l) for 24 h. The aqueous extract was centrifuged at room temperature, and the clear, supernatant liquor was poured into acetone (3 ml per ml) to precipitate the mucilage mixture. Purification[2] was effected by dissolving the precipitate in M sodium hydroxide (500 ml), acidifying the solution with acetic acid, and precipitating the mucilages with acetone (3 ml per ml); yield, 3.5 kg.

Separation and purification of mucilage A. — The mucilage mixture (3.5 g) was dissolved in 2M ammonium hydroxide, the solution was rendered neutral with dilute hydrochloric acid, the suspension was centrifuged, and the clear, supernatant solution was treated with 25% aqueous cupric chloride solution. The resultant, insoluble, copper complex was removed by centrifugation, and washed successively with water, ethanol, 5% ethanolic hydrogen chloride (until the washings were free from copper ions), and ethanol (until free from chloride ions). The product was dried to constant weight under vacuum at 50°, giving 1.7 g of mucilage *A*. It was a pale-brown, amorphous substance, sparingly soluble in water, soluble in alkali, and partially hydrolyzed by saliva (Found: ash, 0.03; N, 0.03%). It had $[\alpha]_D^{18}$ −200° (*c* 0.4, M ammonia) and η_{sp} 0.24 (*c* 0.3, M ammonia). The molecular weight, based on viscosity measurements[3], was about 114,000.

Acid hydrolysis of mucilage A. — A portion (420 mg) of mucilage *A* was heated with 0.5M sulfuric acid (30 ml) for 12 h at 100° in a sealed tube. The resulting mixture was filtered to remove a small amount of flocculent material, and the filtrate was rendered neutral with 0.05M barium hydroxide. Barium sulfate was removed, and the solution was evaporated to dryness. The residue was extracted several times with

boiling ethanol, and the ethanol extracts were combined. de-ionized with a mixture of Amberlite IR-120 (H^+) and IRA-400 (OH^-) ion-exchange resins, and evaporated to a brown syrup (280 mg).

Chromatography and estimation of sugars in mucilage A. — A portion of the acid hydrolyzate was examined on a paper chromatogram by use of 5:1:4 butyl alcohol–ethanol–water, and *p*-anisidine hydrochloride as the spraying agent, which showed a main spot of a glucose and a small spot of a rhamnose. No uronic acid could be detected with the benzidine reagent or the Lefèvre–Tollens test[4]. After separation on the chromatogram, quantitative estimation of the sugars by the Somogyi method[5] indicated 77% of a glucose and 20% of a rhamnose. After separation on the chromatogram, the D-glucose {which had $[\alpha]_D^{18}$ +52.5° (equilibrium, water)} was oxidized to D-gluconic acid (m.p. and mixed m.p., 125°); and the L-rhamnose {which had $[\alpha]_D^{18}$ +8.0° (equilibrium, water)} was converted into the 2-benzoyl-2-phenylhydrazone[6] (m.p. and mixed m.p., 182°).

Acetylation of mucilage A. — The dry, powdered mucilage *A* (0.5 g) was warmed with pyridine (20 ml) for 2 h at 70°, and the mixture was cooled, and kept overnight at room temperature. Acetic anhydride (10 ml) was then added dropwise during 30 min, and the mixture was kept for 3 days at 52°. The resulting, clear solution was diluted with acetic acid (20 ml) and poured, with stirring, into ethanol. The precipitate was dried, and the product was reacetylated[7], giving 0.1 g of a white material, $[\alpha]_D^{18}$ −100° (*c* 0.4, chloroform).

Methylation of mucilage A. — The powdered mucilage *A* (2.5 g) was suspended in water (30 ml) and allowed to swell for 2 h at 5°. It was then methylated[8] ten times with methyl sulfate and sodium hydroxide solution under an atmosphere of nitrogen, the mixture was dialyzed, and the product was methylated twice with methyl iodide and silver oxide. The product (extracted with chloroform) was a brown, brittle substance (yield 1.26 g). It had $[\alpha]_D^{18}$ −175° (*c* 0.4, chloroform).

Methanolysis and hydrolysis of methylated mucilage A. — The methylated product (0.3 g) was heated for 12 h at 100° with methanolic hydrogen chloride (3%), the mixture was neutralized with cold, ethereal diazomethane[9], and then concentrated at room temperature, and the product was refluxed with 4% hydrochloric acid for 7 h. The acid was neutralized with silver carbonate, and the filtrate was de-ionized with a mixture of Amberlite IR-120 (H^+) and IRA-400 (OH^-) ion-exchange resins, and evaporated to a syrup (260 mg).

Chromatography and estimation of the hydrolysis products of methylated mucilage A. — Examination of the hydrolysis products on paper chromatograms, with the upper layer of 5:1:4 butyl alcohol–ethanol–water as the mobile phase and *p*-anisidine hydrochloride as the spray reagent, showed three spots, corresponding to a 2,3,6-tri-, a 2,3-di-, and a 2,3,4,6-tetra-*O*-methylglucose, in addition to a faint spot of a methylrhamnose. The product eluted from the first spot gave 2,3,6-tri-*O*-methyl-D-glucose, m.p. 121°, $[\alpha]_D^{18}$ +71° (equilibrium, water). From the third spot, the aniline derivative of a 2,3,4,6-tetra-*O*-methylglucose, m.p. and mixed m.p. 132°, was isolated.

Periodate oxidation of mucilage A. — A series of samples of the mucilage (50 mg)

were dissolved in M ammonia, and each solution was rendered neutral with dilute hydrochloric acid, made up to 5 ml with 3% aqueous sodium chloride, and oxidized with 0.37M periodate (2 ml) at room temperature. The excess of periodate was decomposed with ethylene glycol, and the formic acid liberated was titrated with 0.05M barium hydroxide. The results obtained (5-h intervals) indicated chains having about 31 sugar residues per end group.

Separation and purification of mucilage B. — After removal of the copper complex of mucilage *A* from the aqueous solution of the mixture of mucilages, the supernatant liquor was concentrated at 50° to 500 ml, and treated with ethanol (2.5 liters), giving a precipitate which was isolated by centrifugation. It was washed successively with alcohol, 5% ethanolic hydrogen chloride (until the washings were free from copper ions), and ethanol (until free from chloride ions). The product was dried to constant weight under vacuum at 50° (yield, 1.76 g).

Mucilage *B* was a white, amorphous, water-soluble substance. With iodine, it gave a red color which was not given if the mucilage was first treated with saliva. It had $[\alpha]_D^{18}$ +220° (*c* 0.4, M ammonia), and η_{sp} 0.17 (*c* 0.4, M ammonia). The average molecular weight was 82,000 (Found: ash, 0.02; N, 0.03%).

Acid hydrolysis of mucilage B. — Mucilage *B* (400 mg) was hydrolyzed with 0.5M sulfuric acid (30 ml) as for mucilage *A*, giving 260 mg of a syrup from the hydrolyzate.

Chromatography and estimation of sugars in the hydrolyzate of mucilage B. — The acid hydrolyzate of mucilage *B* was examined on paper chromatograms by use of 5:1:4 butyl alcohol–ethanol–water, and *p*-anisidine hydrochloride as the spray reagent; this revealed a main spot of a glucose and a small spot of an arabinose; no uronic acid could be detected with the benzidine reagent or the Lefèvre–Tollens test[4]. Quantitative determination of the sugars by the Somogyi method[5] indicated about 89% of a glucose and 9% of an arabinose. Another portion of the syrup was separated on paper chromatograms, and extracted therefrom with methanol. The first spot, which had $[\alpha]_D^{18}$ +52.5° (equilibrium, water), was converted into D-gluconic acid (m.p. and mixed m.p., 125°); the second spot, which had $[\alpha]_D^{18}$ +105° (equilibrium, water), was converted into L-arabinose 2-benzoyl-2-phenylhydrazone (m.p. and mixed m.p., 170°).

Acetylation of mucilage B. — The dry, powdered mucilage *B* (0.5 g) was acetylated in the same manner as for mucilage *A*, giving 0.38 g of a white powder, $[\alpha]_D^{18}$ +150° (*c* 0.4, chloroform).

Methylation of mucilage B. — Mucilage *B* (3 g) was suspended in water (35 ml), allowed to swell, and methylated completely with methyl sulfate followed by methyl iodide, exactly as for mucilage *A*. The product (extracted with chloroform) was a brown substance (2.1 g) having $[\alpha]_D^{18}$ +160° (*c* 0.5, chloroform).

Hydrolysis of methylated mucilage B, *and chromatography.* — The methylated product (0.3 g) was treated with methanolic hydrogen chloride (3%) as for methylated mucilage *A*, and the product was hydrolyzed, giving 250 mg of a syrup. A portion was examined on paper chromatograms; these indicated mainly a 2,3,6-tri-*O*-methyl-

glucose, together with small proportions of a 2,3,4,6-tetra-*O*-methylglucose and a 2,3-di-*O*-methylglucose. Another portion of the hydrolyzate was chromatographed on paper, and the separate fractions were extracted from the chromatogram; the first spot was 2,3,6-tri-*O*-methyl-D-glucose (m.p. and mixed m.p. 122°), $[\alpha]_D^{18}$ +71° (equilibrium, water). The second fraction was converted into the aniline derivative of 2,3,4,6-tetra-*O*-methylglucose (m.p. and mixed m.p. 132°).

Periodate oxidation of mucilage B. — Samples (50 mg) of mucilage *B* were oxidized by the method used for oxidizing mucilage *A*. Glycogen was used as the control, and the results showed that mucilage *B* is composed of about 18 sugar residues per end group.

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REFERENCES

1. M. Fahmy and M. El Keiy, *Repts. Pharm. Soc. Egypt*, 4 (1934) 1.
2. El S. Amin, *J. Chem. Soc.*, (1956) 828.
3. P. J. Flory and T. G. Fox. Jr., *J. Amer. Chem. Soc.*, 73 (1951) 1904.
4. K. U. Lefevre and B. Tollens, *Ber.*, 40 (1907) 4513.
5. M. Somogyi, *J. Biol. Chem.*, 160 (1945) 61, 69.
6. H. Zinner, J. Brock, B. Peter, and H. Schraukellis, *J. Prakt. Chem.*, 29 (1965) 101.
7. E. L. Hirst, J. K. N. Jones, and J. Raudier, *J. Chem. Soc.*, (1948) 1779.
8. E. B. Clinton, E. Bollow, and E. G. V. Percival, *J. Chem. Soc.*, (1952) 1055.
9. L. Hough, J. K. N. Jones, and W. H. Wadman, *J. Chem. Soc.*, (1952) 3393.

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THE ACID HYDROLYSIS OF GLYCOSIDES

XI. EFFECT OF pH ON THE HYDROLYSIS OF ACID DISACCHARIDES

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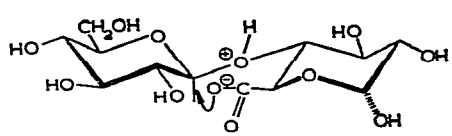
ABSTRACT

At pH values higher than 0.5, 2-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-D-xylose (**1**) is hydrolyzed faster than predicted. Above a pH of 3, pseudocellobiouronic acid (**2**) is hydrolyzed at rates that increase with increase in pH. The hydrolysis of the aldobiouronic acid **1** is probably influenced by both the un-ionized and ionized carboxyl groups. The hydrolysis of the pseudoaldobiouronic acid (**2**) possibly involves intramolecular catalysis through the carboxyl group.

INTRODUCTION

Capon and Ghosh[1] have shown that, at a pH of 4.8, 2-naphthyl β-D-glucopyranosiduronic acid is hydrolyzed 35 times as fast as 2-naphthyl β-D-glucopyranoside. They attributed this behavior to the fact that the rate measured is the sum of the rates for the ionized and un-ionized acids, an interpretation later adopted, in different context, by Saunders and Timell[2]. Subsequently, Smidsrød, Haug, and Larsen[3] studied the effect of pH on the hydrolysis of various alditol-containing disaccharides. Above pH 2, reduced pseudocellobiouronic acid (3-*O*-β-D-glucopyranosyl-L-gulonic acid) was hydrolyzed much more rapidly than cellobiitol. Reduced cellobiouronic acid was hydrolyzed at a rate lower than that for cellobiitol below a pH of 3, but at a higher rate at pH 4. The results were interpreted in terms of an intramolecular, acid catalysis *via* the carboxyl group, as originally suggested by Marchessault and Rånby[4].

It was considered of interest to adduce additional information on this subject, and the effect of pH on the rate of hydrolysis has accordingly been determined for 2-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-D-xylose (**1**) and 4-*O*-(β-D-glucopyranosyl)-D-glucuronic acid (pseudocellobiouronic acid) (**2**).



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RESULTS AND DISCUSSION

Compound **1** was obtained by partial, acid hydrolysis of a (4-*O*-methylglucurono)xylan[5]. "Pseudoaldobiouronic acid" (**2**) was synthesized, essentially according to the method of Johansson, Lindberg, and Theander[6]. Hydrolyses were conducted at 90°, at pH values ranging from 0.3 to 6. Polarimetric measurements were found to give inaccurate results, because of decomposition of compound **2** in weakly acidic solutions. The extent of hydrolysis was determined instead, from the amounts of D-xylose and D-glucose formed from the respective compounds. D-Glucose and 3-*O*-methyl-D-glucose were used as reference compounds for **1** and **2**, respectively, and the proportion of neutral sugars in the hydrolyzates was measured by gas–liquid chromatography (g.l.c.) of the (trimethylsilyl)ated sugars.

The rate of hydrolysis of aldobiouronic acid **1** at pH values below 5 is shown graphically in Fig. 1. At a pH of 4, the compound is hydrolyzed at a rate 32 times

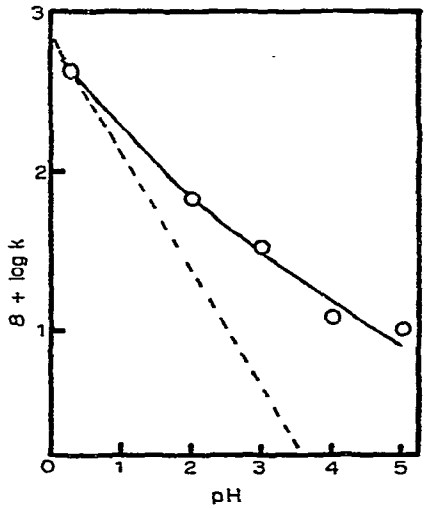

Fig. 1. Profile curve of pH versus rate, for hydrolysis of 2-*O*-(4-*O*-methyl-α-D-glucopyranosyluroni acid)-D-xylose (**1**) at 90°.

that predicted from the Zucker–Hammett hypothesis[7]. As first pointed out by Capon and Ghosh[1], and later confirmed by others[2], the inductive effects of both the unionized and ionized forms of the carboxyl group would be expected to influence the rate of hydrolysis, even at the low pH values used here. Intramolecular catalysis (involving the carboxylate anion, as suggested by Saunders and Timell[2]) could, however, also be operative.

The effect of pH on the hydrolysis of compound **2** is shown in Fig. 2. At pH 4, the rate of hydrolysis of **2** is 55 times that predicted. Unlike the results obtained previously[3], above a pH of 3, the rate of hydrolysis increased with pH. Intramolecular, nucleophilic catalysis by the carboxylate anion might offer an explanation for this

behavior. The same explanation might account for the facile cleavage of pectic acids under similar conditions[8,9].

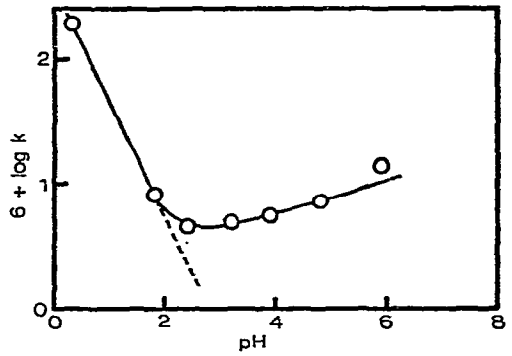

Fig. 2. Profile curve of pH *versus* rate, for hydrolysis of pseudocellobiouronic acid (2) at 90°.

It is clear from the present results that linkages on both the reducing and the nonreducing side of uronic acid residues in acidic polysaccharides can be preferentially cleaved under weakly acidic conditions. This result should afford a valuable new tool in study of the structure of such polysaccharides.

EXPERIMENTAL

Disaccharides. — Isolation and characterization of aldobiouronic acid **1** have been reported[5]. Pseudocellobiouronic acid **2**, which has now also been synthesized by a different route[10], was prepared from 1,2,3,2′,3′,4′,6′-hepta-*O*-acetyl-β-cellobiose[6] in a yield of 63%, $[\alpha]_D$ +16.8° (*c* 2, water).

The derived methyl ester heptaacetate[6] had m.p. 161–163°, $[\alpha]_D$ −21.5° (*c* 1, chloroform).

Anal. Calc. for $C_{27}H_{36}O_{19}$: C, 48.8; H, 5.46. Found: C, 48.9; H, 5.60.

Kinetic measurements. — The pH values were adjusted with the aid of 0.5M sulfuric acid, 0.1M acetate buffer, and 0.1M phosphate buffer. Compound **1** or **2** (15–20 mg) was weighed in a 5-ml beaker, and a known amount (1–9 mg) either of D-glucose (in the case of **1**) or of 3-*O*-methyl-D-glucose (in the case of **2**) was added. Buffer solution (2 ml) was introduced, and the pH value desired was obtained by dropwise addition of weak acid or base. The sugar solution was introduced into three ampoules (0.7 ml each) which were sealed and placed in a constant-temperature bath kept at 90°. At various time-intervals, the ampoules were removed and opened, and the reaction mixture was neutralized with a mixture of anion- and cation-exchange resins. The filtrate was evaporated to dryness, and the residue was dissolved in warm pyridine (1 ml); the solution was concentrated to 0.2 ml, and this was treated in a small vial for 5 min with hexamethyldisilazane (0.05 ml) and chlorotrimethylsilane (0.03 ml). The mixture was analyzed by g.l.c., by use of a column of 3% SE 52 on

Chromosorb W. Average values were calculated from the results of two or three experiments.

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REFERENCES

1. B. CAPON AND B. C. GHOSH, *Chem. Commun.*, (1965) 587.
2. M. D. SAUNDERS AND T. E. TIMELL, *Carbohyd. Res.*, 6 (1968) 12.
3. O. SMIDSRØD, A. HAUG, AND B. LARSEN, *Acta Chem. Scand.*, 20 (1966) 1026.
4. R. H. MARCHESSAULT AND B. G. RÅNBY, *Svensk Papperstidn.*, 62 (1959) 230.
5. N. ROY AND T. E. TIMELL, *Carbohyd. Res.*, 6 (1968) 483.
6. I. JOHANSSON, B. LINDBERG, AND O. THEANDER, *Acta Chem. Scand.*, 17 (1963) 2019.
7. L. ZUCKER AND L. P. HAMMETT, *J. Amer. Chem. Soc.*, 61 (1939) 2791.
8. P. ALBERSHEIM, *Biochem. Biophys. Res. Commun.*, 1 (1959) 253.
9. P. ALBERSHEIM, H. NEUKOM, AND H. DEUEL, *Arch. Biochem. Biophys.*, 90 (1960) 46.
10. P. SIPOS AND S. BAUER, *Tetrahedron Lett.*, (1967) 443.

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COMPARISON OF THE EFFICACY OF VARIOUS DIALKYLBORANES IN THE REDUCTION OF ACYLATED ALDONOLACTONES

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(Received December 18th, 1967)

ABSTRACT

In order to find the most suitable dialkylborane for use in the reduction of acylated aldono-1,4-lactones to the corresponding acylated furanoses, an investigation of the properties of bis(2,4,4-trimethylpentyl)borane, bis(2,3-dimethyl-2-butyl)borane, dicyclohexylborane, and disiamylborane was undertaken. It was found that, as the size of the alkyl group is increased, the extent of reduction decreases, and that an increase in temperature increases the rate of the reaction without markedly affecting the yield of aldose. Of the reagents studied, disiamylborane was found to be the most satisfactory.

INTRODUCTION

Several years ago, we described[1,2] the use of bis(3-methyl-2-butyl)borane (di-*sec*-isoamylborane; disiamylborane) to reduce acylated aldonolactones to the corresponding acylated aldoses. The procedure was developed as a general means of preparing furanose derivatives of carbohydrates, particularly hexoses, from the readily available 1,4-lactones. Subsequently, we reported the utilization of the acylated furanoses for preparation of a number of hexofuranosyladenine nucleosides and a heptofuranosyladenine nucleoside[3]. The reduction was based on the report of Brown and Bigley[4] that they had reduced butyrolactone and the γ-lactone of 4-hydroxyvaleric acid (γ-valerolactone) to the corresponding hydroxy aldehydes with disiamylborane. Brown and his co-workers were primarily interested in disiamylborane and other alkylboranes as reagents for effecting hydroboration reactions, and have described a considerable number of such reagents[5].

In the course of our work with disiamylborane, it became apparent that the reagent had a number of disadvantages. Although it can be obtained as a semisolid mass by evaporation of the solvent, this procedure is inadvisable, since the residue is hygroscopic and does not provide the sought-for requirement of a stable substance that can be prepared in quantity, stored for some time, and used as needed. Solutions

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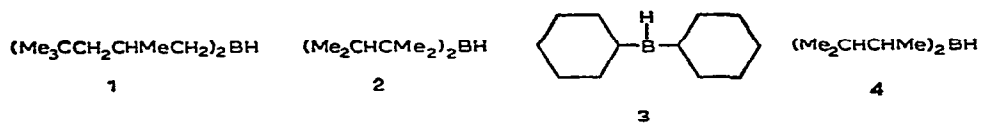
**Taken, in part, from the Ph.D. thesis of A. Chan, Jr.

of disiamylborane in tetrahydrofuran are reasonably stable if stored in a refrigerator, but the best results are obtained from freshly prepared material. In these laboratories, the reagent is either used at once or stored for not more than a week. Because of the advantages that a stable, solid reagent would offer, and because the method we have described[2] has been used in a number of laboratories for similar reductions[6,7], including the reduction of free aldonolactones to aldoses[8,9], we initiated an investigation of other dialkylboranes in an attempt to find a reagent having more desirable handling properties.

RESULTS AND DISCUSSION

Synthetic procedures. — By using procedures previously described[2,4,5], there were prepared, by treatment of the appropriate alkenes with borane, four dialkylboranes, namely, bis(2,4,4-trimethylpentyl)borane (**1**), bis(2,3-dimethyl-2-butyl)-borane (**2**), dicyclohexylborane (**3**), and disiamylborane (**4**). These compounds were



selected in order that possible steric effects resulting from differences in the bulkiness of the alkyl group might be studied. They were used for reducing 2,3,5,6-tetra-*O*-benzoyl-D-gulono-1,4-lactone (**5**) or 2,3,5,6-tetra-*O*-acetyl-D-galactono-1,4-lactone (**6**), or both, to the corresponding tetra-*O*-acylhexofuranoses. With all reagents except **1**, the reduction was studied at two temperatures, namely, 40–45° and 25–28° (the temperature of the reaction mixture on an unheated, magnetic stirrer). In each case, the course of the reduction was followed by periodically removing samples and determining, by the anthrone method[2], the amount of reducing sugar present.

Bis(2,4,4-trimethylpentyl)borane (**1**). — In Figure 1, data from the reduction of 2,3,5,6-tetra-*O*-benzoyl-D-gulono-1,4-lactone (**5**) with **1** at 28° are shown. It will be noted that a period of 18–20 h was required for completion of the reaction at that

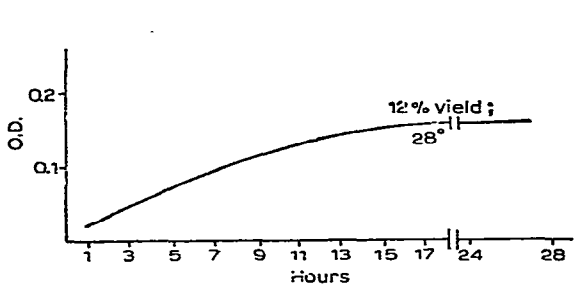


Fig. 1. Reduction of 2,3,5,6-tetra-*O*-benzoyl-D-gulono-1,4-lactone (**5**) with bis(2,4,4-trimethylpentyl)-borane.

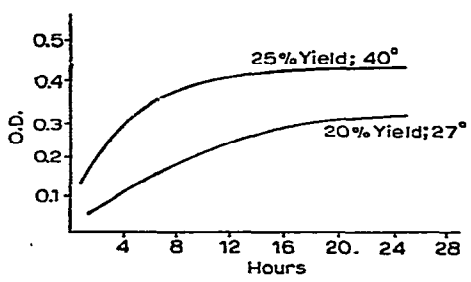


Fig. 2. Reduction of 2,3,5,6-tetra-*O*-benzoyl-D-gulono-1,4-lactone (**5**) with bis(2,3-dimethyl-2-butyl)borane.

temperature, and, more importantly, that the final yield of reducing sugar was only 12%. From these results, it is evident that, even if **1** were obtainable as a stable solid, it would not be a satisfactory reagent for the intended purpose.

Bis(2,3-dimethyl-2-butyl)borane (**2**). — The results of the reduction of lactone **5** with **2** at 27° and at 40° are shown in Figure 2. At the lower temperature, a period of about 22 h was required for completion of the reaction, but the yield of product was only 20%. At 40°, the reaction proceeded much more rapidly, as might be anticipated, and reached completion in 8 h. A yield of 25% of the benzoylated hexofuranose was obtained, but this somewhat higher yield is not significant. Again, because of the failure of this reagent to reduce the lactone in good yield, it is not a reagent of choice for this reaction.

Dicyclohexylborane (**3**). — The studies performed with **2** were repeated with dicyclohexylborane (**3**); the results are shown in Figure 3. 2,3,5,6-Tetra-*O*-benzoyl-D-gulono-1,4-lactone (**5**) was reduced at 28° and at 44°. At the lower temperature, the reaction required 8 h for completion, and a 61% yield of the reducing sugar was obtained. At 44°, the reaction required only 4 h to proceed to completion, but the yield (53%) of reducing sugar differed little from that at the lower temperature. Despite the fact that this reagent gave 2–2.5 times the yield given by **2**, and 5 times that given by **1**, it was nevertheless inferior to disiamylborane.

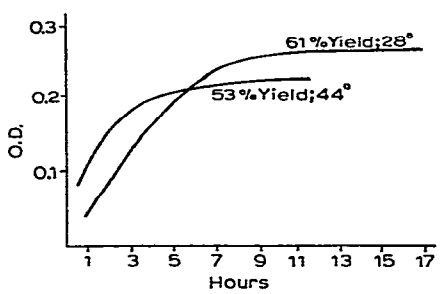


Fig. 3. Reduction of 2,3,5,6-tetra-*O*-benzoyl-D-gulono-1,4-lactone (**5**) with dicyclohexylborane.

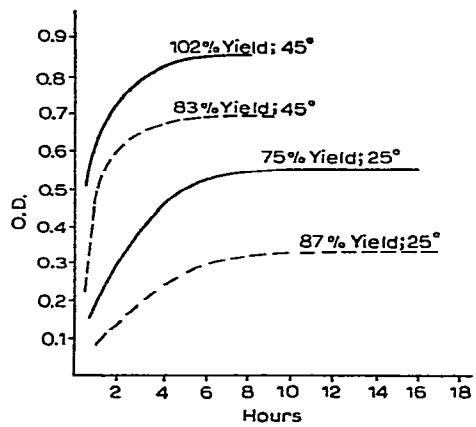


Fig. 4. Reduction of 2,3,5.6-tetra-*O*-benzoyl-D-gulono-1,4-lactone (**5**) (------) and 2,3,5,6-tetra-*O*-acetyl-D-galactono-1,4-lactone (**6**) (——) with disiamylborane.

Disiamylborane (**4**). — Figure 4 shows the results of reduction of 2,3,5,6-tetra-*O*-benzoyl-D-gulono-1,4-lactone (**5**) and 2,3,5,6-tetra-*O*-acetyl-D-galactono-1,4-lactone (**6**) with disiamylborane (**4**). With both lactones **5** and **6**, at 25°, the reaction reached completion in about 8 h. At the higher temperature, the time required for completion of the reaction decreased to about 4 h. The yields of 2,3,5,6-tetra-*O*-acetyl-D-galacto-

furanose were 75% at 25°, and 102% at 45°; the yields of 2,3,5,6-tetra-*O*-benzoyl-D-gulofuranose were 87% at 25°, and 83% at 45°. In other experiments, yields on reduction of lactone **5** amounted to 90–100%, and 80–90% yields were obtained[2] on reduction of the lactone **6**. It appears, therefore, that neither (a) alteration of the temperature nor (b) the use of benzoate protecting groups instead of acetate protecting groups, greatly affects the course of the reaction or the extent to which it proceeds.

From these results, it is apparent that the yield of reducing sugar decreases with increasing bulk of the alkyl group in the reductant, and that, of the reagents studied, disiamylborane is the most satisfactory for effecting this particular reduction. In fact, with this reagent, yields of product of at least 70% (and usually significantly higher) have been obtained consistently with a considerable variety of aldonolactones[2,3]. Lee and Nolan[10] referred to the use of bis(isopinocampheyl)borane in a reduction of acylated sugar lactones, but gave no experimental details. Based on the current studies, it can be predicted that this dialkylborane, which is prepared from 2-pinene[11], would be much less satisfactory than disiamylborane, since the alkyl group is considerably larger. It is possible that dialkylboranes having alkyl groups smaller than that in disiamylborane would prove to be very satisfactory reagents, and might, indeed, give yields approaching 100% in cases where disiamylborane usually yields 80–90% or somewhat less. However, 2-methyl-2-butene, from which disiamylborane is prepared, is a low-boiling liquid (b.p. 38°), and such lower alkenes, as 2-methylpropene, 2-butene, and 1-butene are gases. These substances have not been studied in our laboratories, because they presumably would not afford the sought-for solid, stable dialkylborane.

It appears, therefore, that disiamylborane, despite some disadvantages, is the most suitable reagent for conducting reductions of this nature under the conditions used. It is not impossible that conditions might be found under which other dialkylboranes would give results that are equally good, but reaction conditions involving different solvents, higher temperatures, and so on, have not yet been investigated.

Although the reduction of acylated lactones to acylated aldoses with disiamylborane is eminently satisfactory, preparation of the reagent is tedious. Unfortunately, in contrast to our initial experience[3], disiamylborane obtained from commercial sources has proved unsatisfactory. With commercially prepared disiamylborane, we have encountered a considerable number of unexpected and undesired side-reactions; for example we have isolated a large proportion of benzyl benzoate after reduction of a benzoylated lactone, together with carbohydrate derivatives that were not the expected product, and are as yet unidentified. No such behavior has ever been encountered when disiamylborane prepared in our laboratories was used. Giudici and Fluharty[8] reported side reactions with use of the commercial reagent and also with reagent that they prepared themselves. They attributed the difficulties to the presence of borane in the reagent, which could account for the observations, but they did not indicate how the borane was identified. In our preparations, stoichiometric amounts of borane and 2-methyl-2-butene are used, and the temperature is carefully maintained between $-5°$ and $-10°$. If it falls lower, additional reaction-time is

allowed. In any event, if undesirable side-reactions have occurred in any reductions performed with disiamylborane prepared in our laboratories, the extent has been insufficient to diminish the yields significantly. It might further be noted that we have tried to use commercial borane, available as a M solution in tetrahydrofuran, and have also found it to be unsatisfactory. It is likely that the contaminants in commercial borane are carried over into the disiamylborane in the commercial preparation of the latter compound. Up to the present, we have succeeded in simplifying the preparation of disiamylborane, as described[2], only in regard to purification of the solvents. Formerly, both tetrahydrofuran and diglyme were dried and distilled twice from lithium aluminum hydride, *in vacuo* and under nitrogen. These elaborate purification measures appear to be unnecessary. These solvents have been used, with no apparent deleterious effect, after drying over sodium and distilling. On a number of occasions, diglyme, dried over sodium, has been used successfully without distillation. However, if the solvents remain in contact with sodium for too long a time they become discolored, and distillation appears advisable.

EXPERIMENTAL

Preparation of diborane. — Diborane was prepared precisely as previously described[2]. To an aliquot of the borane in tetrahydrofuran, acetone was added, the product was hydrolyzed with water to produce boric acid, and the boric acid was titrated, after addition of D-mannitol, to the phenolphthalein end-point. In separate preparations, the concentration of borane varied from 1.6 to 2.5M.

Preparation of bis(2,4,4-trimethylpentyl)borane[12] **(1)**. — To 1.57 g (2.2 ml, 14 mmoles) of 2,4,4-trimethyl-2-pentene in a flask (equipped with a pressure-equalizing dropping-funnel, a magnetic stirrer, and a nitrogen inlet) was added dropwise 3.8 ml of 1.86M borane in tetrahydrofuran during 10 min. Stirring was continued for 96 h at room temperature.

Preparation of bis(2,3-dimethyl-2-butyl)borane[12] **(2)**. — To 1.18 g (1.8 ml, 14 mmoles) of 2,3-dimethyl-2-butene in a flask (equipped with a pressure-equalizing dropping-funnel and a nitrogen inlet) was added dropwise 3.9 ml of 1.8M borane in tetrahydrofuran during 10 min. Stirring was continued for 24 h at room temperature.

Preparation of dicyclohexylborane[12] **(3)**. — In a flask equipped with a pressure-equalizing dropping-funnel and a nitrogen inlet was placed 1.15 g (1.4 ml, 14 mmoles) of cyclohexene, and the flask was placed in a bath cooled to 0°. A solution of borane in tetrahydrofuran (4.13 ml, 1.7M) was added dropwise, and stirring was continued for 1 h at 0°.

Preparation of disiamylborane[2] **(4)**. — Disiamylborane was prepared as previously described. In a flask equipped with a pressure-equalizing dropping-funnel and a nitrogen inlet was placed 25 ml (0.25 mole) of 2-methyl-2-butene, and the flask was immersed in a bath kept at −5 to −10°. A solution of borane in tetrahydrofuran (50 ml, 2.5M) was added dropwise to the magnetically stirred mixture, and stirring was continued for 6 h. The solution was diluted to 100 ml with tetrahydrofuran, to yield a 1.25M solution of disiamylborane.

Reduction of tetra-O-acyl-aldono-1,4-lactones[2]. — To flasks, containing the dialkylboranes prepared as above and kept either at room temperature or in a water bath at 40° or 45°, was added the acylated lactone (1 or 2 g) dissolved in tetrahydrofuran. The amount of carbohydrate added was adjusted to maintain about a four-fold excess* of dialkylborane, and the volume of tetrahydrofuran in which the carbohydrate was dissolved was so adjusted that, on addition to the dialkylborane solution, a final concentration M in dialkylborane was attained. Thus, in the preparation of bis(2,4,4-trimethylpentyl)borane described above, 14 mmoles of the reagent was contained in 6.0 ml. To this was added 2 g (3.36 mmoles) of 2,3,5,6-tetra-*O*-benzoyl-D-gulono-1,4-lactone (5) in 8.0 ml of tetrahydrofuran. In each instance, a control reaction was conducted, for use as a blank, by adding to a flask containing the dialkylborane an identical volume of tetrahydrofuran containing no carbohydrate. Aliquots (0.5–1.0 ml) were removed periodically from each flask and transferred to a volumetric flask, usually of 50-ml capacity. The reaction was terminated by careful addition of a few drops of water, and the volume was brought to the mark with ethanol. A suitable aliquot (1 ml) of the diluted sample was used for determining the reducing-sugar content by a modified anthrone method[13], with 2,3,5,6-tetra-*O*-benzoyl-D-gulofuranose (or the D-*galacto* analog) as a standard to determine the final yield.

ACKNOWLEDGMENTS

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REFERENCES

1 P. KOHN, R. H. SAMARITANO, AND L. M. LERNER, *J. Am. Chem. Soc.*, 86 (1964) 1457.
2 P. KOHN, R. H. SAMARITANO, AND L. M. LERNER, *J. Am. Chem. Soc.*, 87 (1965) 5475.
3 P. KOHN, R. H. SAMARITANO, AND L. M. LERNER, *J. Org. Chem.*, 31 (1966) 1503.
4 H. C. BROWN AND D. B. BIGLEY, *J. Am. Chem. Soc.*, 83 (1961) 486.
5 H. C. BROWN, *Hydroboration*, W. A. Benjamin, Inc., New York, 1962.
6 E. WALTON, S. R. JENKINS, R. F. NUTT, M. ZIMMERMAN, AND F. W. HOLLY, *J. Am. Chem. Soc.* 88 (1966) 4524.
7 H. KUZUHARA AND H. G. FLETCHER, JR., *J. Org. Chem.*, 32 (1967) 2531.
8 T. A. GIUDICI AND A. L. FLUHARTY, *J. Org. Chem.*, 32 (1967) 2043.
9 G. NAKAMINAMI, M. NAKAGAWA, S. SHIOI, Y. SUGIYAMA, S. ISEMURA, AND M. SHIBUYA, *Tetrahedron Lett.*, (1967) 3983.
10 J. B. LEE AND T. J. NOLAN, *Tetrahedron*, 23 (1967) 2789.
11 H. C. BROWN AND G. ZWEIFEL, *J. Am. Chem. Soc.*, 83 (1961) 486.
12 H. C. BROWN AND A. W. MOERIKOFER, *J. Am. Chem. Soc.*, 84 (1962) 1478.
13 L. C. MOKRASCH, *J. Biol. Chem.*, 208 (1954) 55; W. E. TREVELYAN AND J. S. HARRISON, *Biochem. J.*, 50 (1952) 298.

*In recent reductions a two-fold excess of disiamylborane has been successfully used. Giudici and Fluharty[8] reported that stoichiometric amounts gave the best results.

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CONFORMATIONAL STUDIES ON SOME α-GLYCOPYRANOSE DERIVATIVES

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ABSTRACT

The conformations of eleven α-glycopyranosides and glycosylamine derivatives, and their acyl derivatives, were studied by n.m.r. spectral analysis in deuterium oxide and in methyl sulfoxide-d_6. In deuterium oxide at room temperature, the α-glycosylamines examined were found to exist as a rapidly equilibrating mixture of the two chair conformations, but, in methyl sulfoxide-d_6 at room temperature, they were found to exist mainly in the *1C*(D) [*C1*(L)] conformation. However, the α-glycosides and their acyl derivatives examined were found to exist mainly in the *C1*(D) [*1C*(L)] conformation in various solvents.

INTRODUCTION

Calculations of free energies[1] and Reeves' conformational instability factors[2] show that many of the hexopyranoses are more stable in the *C1* (D) [or *1C*(L)] conformation than in the corresponding alternative[1] one, namely, *1C*(D) [or *C1*(L)]. However, a previous paper[3] from this laboratory showed that (tetra-*O*-acetyl-α-D-mannopyranosyl)theophylline exists in the *1C*(D) conformation, in which the aglycon is in the equatorial orientation. Recently, some pyridinium α-D-glycosides[4] and tri-*O*-acetyl-β-D-xylopyranosyl chloride[5] have also been found to exist in this conformation.

In consideration of these findings, together with the conformational behavior of α-D-lyxopyranose, which has been reported[6] to exist as an equilibrium mixture of the two chair conformations, it was of interest to investigate the conformations of α-pyranosides of D-mannose and L-rhamnose. These sugars have the same steric arrangement of groups as lyxopyranose, at C-2, C-3, and C-4 of the pyranose ring, and are widely distributed as components of such biologically significant substances as glycosides, polysaccharides, and glycoproteins.

The present paper describes the conformations of α-pyranosides of D-mannose, L-rhamnose, and D-glucose, as revealed by n.m.r. spectral analysis.

METHODS AND MATERIALS

Unless otherwise stated, n.m.r. spectra were recorded with a Varian A-60

spectrometer at normal operating temperature (*ca.* 30°). Sodium 5,5-dimethylsilapentane-1-sulfonate (DSS) in deuterium oxide, and tetramethylsilane (TMS) in other solvents, were used as internal standards. Chemical shifts are expressed on the τ scale. Coupling constants, in Hz, are the directly measured line-spacings. Multiplet spacings were measured at a sweep width of 250 Hz. The concentration of the sample was about 10% or less. I.r. spectra were recorded in Nujol or potassium bromide with a Shimadzu AR-6 spectrophotometer.

Optical rotatory dispersion curves were obtained with an automatic o.r.d. recorder (Yanagimoto ORD-200).

The configuration of the compounds examined was established by optical rotatory and n.m.r. spectral data. The positions of substituents on purine bases were confirmed by comparing the u.v. spectra of the compounds with those of derivatives of known structure.

All materials described in the present work were synthesized, except for quercitrin, which was obtained by purification of commercially available material.

RESULTS

With most of the compounds examined, the signal of the anomeric proton was observed at lower magnetic field than the signals of other ring protons. This is due to the combined deshielding effect of the ring oxygen atom and the oxygen or nitrogen atom of the aglycon. The signal of H-1′ of 9-α-L-rhamnopyranosyladenine hydrochloride[7] (**1**) in deuterium oxide appears at τ 3.78 as a sharp doublet. The coupling constant ($J_{1',2'}$, 4.8 Hz), as estimated from the Karplus equation[8], is smaller than that expected

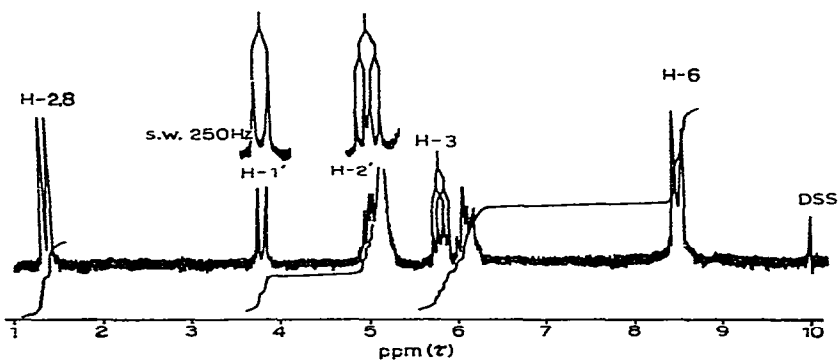

Fig. 1. N.m.r. spectrum of 9-α-L-rhamnopyranosyladenine hydrochloride (**1**).

for a *trans*-diaxial relationship between H-1′ and H-2′, and larger than that for a true *gauche*-diequatorial arrangement. The H-2′ signal appears at τ 5.00 as a quartet, partly overlapped by the HOD signal. The latter was moved to high magnetic field on heating, to give a well defined quartet for H-2′. A broad quartet at τ 5.79 was collapsed by irradiating at 46 Hz downfield, and the quartet was assigned, therefore,

to H-3′. A two-proton multiplet at high field is assigned to H-4′ and H-5′, but was not analyzed because of its complexity. The n.m.r. spectrum is shown in Fig. 1.

In the n.m.r. spectrum of **1** in methyl sulfoxide-d_6 containing a few drops of deuterium oxide (Fig. 2), the H-1′ signal appears at τ 4.00 as a doublet having a spacing of 8.0 Hz, which indicates the *trans*-diaxial orientation of H-1′ and H-2′.

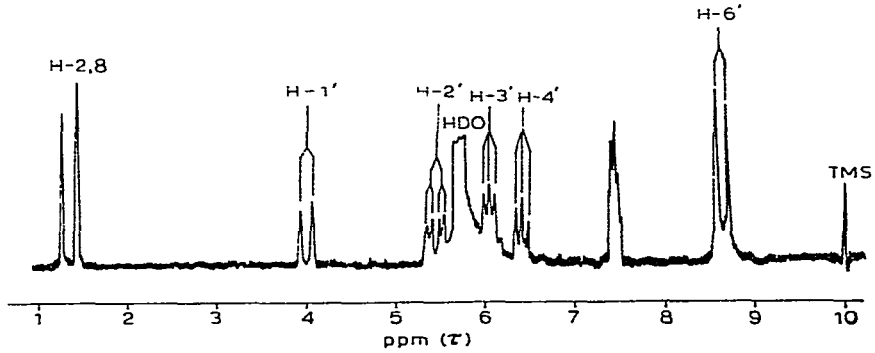

Fig. 2. N.m.r. spectrum of 9-α-L-rhamnopyranosyladenine hydrochloride (**1**) in methylsulfoxide-d_6 containing few drops of deuterium oxide.

A quartet at τ 5.43 is assigned to H-2′, and arises through unequal coupling with H-1′ and with H-3′. The H-3′ and H-4′ signals are observed at τ 6.05 and 6.42, respectively, as triplets. These assignments were confirmed by spin-decoupling experiments. Small values of $J_{2',3'}$ (2.9 Hz), $J_{3',4'}$ (3.6 Hz). and $J_{4',5'}$ (3.5 Hz) are characteristic of vicinal protons having a *gauche* relationship. In these assignments, several percent of an unfavored conformer might escape detection. In *N*,*N*-dimethylformamide as solvent, the H-1′ signal was observed at τ 3.70 as a doublet having $J_{1',2'} = 9.5$ Hz.

In a similar manner, eleven other α-glycosides and their acetyl derivatives were examined. Table I shows the chemical shifts and first-order coupling constants of the non-acetylated α-glycosides, as well as the presumed conformation, and Table II shows data for their acylated derivatives. The conformations of these acetyl derivatives were also confirmed by observing the chemical shifts of acetate-methyl signals in the axial and equatorial orientations, as shown in Table III.

DISCUSSION

α-D-Glycosylamines. — From the *J* values of methine and methylene protons (see Table I), it is found that the pyranoid molecules of **1**, 9-α-L-rhamnopyranosyladenine picrate (**2**), 7-α-L-rhamnopyranosyltheophylline (**3**), 9-α-D-mannopyranosyladenine hydrochloride (**6**), 9-α-D-mannopyranosyladenine picrate (**7**), and 7-α-D-mannopyranosyltheophylline[3] (**8**) exist, in methyl sulfoxide-d_6, preponderantly in the so-called alternative conformation[1] (*1C* for the D-series, *C1* for the L-series), in which the aglycons are oriented equatorially. From the fact that **1** and **8** in *N*,*N*-dimethylformamide, and **8** in pyridine-d_5, also exist in this conformation, the remaining α-glycosides also are probably much more stable in the so-called alternative chair

TABLE I

CHEMICAL SHIFTS AND FIRST-ORDER COUPLING CONSTANTS OF α-GLYCOPYRANOSIDES

Compound	*Solvent*	*Chemical shifts (τ)*						*Coupling constants (Hz)*					*Conformation presumed*
		H-1′	H-2′	H-3′	H-4′	H-5′	H-6′	$J_{1',2'}$	$J_{2',3'}$	$J_{3',4'}$	$J_{4',5'}$	$J_{5',6'}$	
1	D_2O	3.78d[a]	5.00q	5.79q	6.06	6.15	8.50d	4.8	3.0	7.0		6.5	*Cl* ⇌ *1C*
1	Me_2SO-d_6	4.00d	5.43q	6.05t	6.42t	6.10	8.64d	8.0	2.9	3.6	3.5	6.8	*Cl* (L)
2	Me_2SO-d_6	3.96d	5.42q	6.02t	6.37t	5.81	8.59d	8.0	3.0	3.5	3.5	6.8	*Cl* (L)
3	D_2O	3.70d	5.39q	5.85q	6.08	6.10	8.53d	6.0	3.3	5.6		6.2	*Cl* ⇌ *1C*
3	Me_2SO-d_6	3.82d	5.64q	6.06t			8.64d	8.2	3.1	3.2		6.7	*Cl* (L)
4	Me_2SO-d_6	4.40d	6.04t				8.83d	1.8	2.0			5.6	*1C* (L)
5	CD_3OD	4.65d	5.73q	6.18q			9.04d	2.0	3.4	8.5		5.6	*1C* (D)
6	D_2O	3.78d	5.09q	5.83				6.0	3.0				*Cl* ⇌ *1C*
6	Me_2SO-d_6	3.95d	5.58q	6.06t				8.3	3.0	3.2			*1C* (D)
7	Me_2SO-d_6	4.05d	5.46q	6.13t	6.43t			8.1	2.9	3.4	3.5		*1C* (D)
8[b]	D_2O	3.82d	5.46q	5.92q				6.2	3.5	5.0			*Cl* ⇌ *1C*
8	Me_2SO-d_6	3.88d	5.62q	6.05				9.0	3.0				*1C* (D)
8	pyridine-d_5	3.00d	4.70q	4.9				8.8	3.5				*1C* (D)
9[c]	D_2O	3.67d	5.81q					5.6	4.0				
9	Me_2SO-d_6	3.73c	6.06	6.06				4.0[d]					
10	Me_2SO-d_6	3.70c	6.09	6.09				5.0[d]					
11[e]	D_2O	3.33d	5.88q	6.20				5.3	4.8				
11	Me_2SO-d_6	3.45c	6.12	6.12				6.0[d]	7.0[d]	7.0[d]			

[a]d, doublet; t, triplet; q, quartet; c, complicated. [b]See ref. 3. [c]Measured with a 100-MHz instrument at 50°. [d] Half-width. [e]See ref. 22.

TABLE II

CHEMICAL SHIFTS AND FIRST-ORDER COUPLING CONSTANTS OF ACYLATED DERIVATIVES OF α-GLYCOPYRANOSIDES IN CHLOROFORM-*d*

Compound	*Chemical shifts* (τ)						*Coupling constants* (Hz)					*Conformation presumed*
	H-1'	*H-2'*	*H-3'*	*H-4'*	*H-5'*	*H-6'*	$J_{1',2'}$	$J_{2',3'}$	$J_{3',4'}$	$J_{4',5'}$	$J_{5',6'}$	
12	3.39d[a]	4.25q	4.53t	5.05t	5.77o	8.43d	8.0	3.5	3.5	3.5	7.0	*Cl* (L)
13	3.00d	3.86q	4.02t	4.55t	5.45o	8.24d	8.0	3.5	3.5	3.5	7.0	*Cl* (L)
14	4.54	4.38	4.46q	4.81t	6.06o	8.78d	1.0	2.0	10.0	10.0	6.0	*1C* (L)
15	4.33	4.38	4.73q	5.09t	6.68o	9.10d	2	2.5	9.0	9.0	6.0	*1C* (D)
16[b]	3.55d	4.00q	4.50t	4.94			8.5	3.0	3.5	3.5		*1C* (D)
16[c]		3.39q	3.88t	4.43t			8.3	3.2	3.5	3.5		*1C* (D)
17	3.46d	4.57q	3.74t	4.74t			5.7	9.0	9.0	9.0		*Cl* (D)
18	3.19d	4.55q	4.55				2.5	5.0				

[a]d, doublet; o, octet; q, quartet; t, triplet. [b]Measured in methyl sulfoxide-d_6. [c]Measured in pyridine-d_5.

TABLE III

CHEMICAL SHIFTS OF ACETYL METHYL PROTONS OF ACETYLATED α-GLYCOPYRANOSIDES IN CHLOROFORM-*d*

Compound	*Chemical shifts* (τ) *Axial* OAc	*Equatorial and primary* OAc *and* NAc
12	7.80 (3)[a], 7.81 (3)	8.04 (3)
14	7.78 (3)	7.93 (3), 7.95 (3)
15	7.88 (3)	8.03 (6)
16[b]	7.83 (6)	7.99 (3), 8.11 (3)
16[c]	7.86 (3), 7.92 (3)	7.95 (3), 8.00 (3)
17		7.91 (9), 7.96 (3), 8.13 (3)
18	7.81 (3)	7.91 (3), 7.93 (3), 8.00 (3)

[a]Proton intensity. [b]Measured in methyl sulfoxide-d_6. [c]Measured in pyridine-d_5.

conformation in these solvents. This conclusion is reasonable, because α-D-mannopyranose and α-L-rhamnopyranose (6-deoxy-α-L-mannopyranose) are stereochemical homomorphs (mirror images), except for the nature of the C-5 substituent.

The n.m.r. spectra of **1**, **3**, **6**, and **8**, measured in deuterium oxide instead of methyl sulfoxide-d_6, show J values of H-1′ that are larger than those expected for the *gauche* relationship and smaller than those anticipated for the *trans*-diaxial arrangement of vicinal protons. These observed J values are roughly intermediate between those expected for the *C1* and *1C* conformations. A possible shift of the aglycon from the true axial orientation in the so-called normal[1] chair conformation is ruled out, because such a distortion would cause the dihedral angle between H-1′ and H-2′ to increase, giving a J value smaller than that expected for the *gauche* relationship. Furthermore, consideration of $J_{1',2'}$ (4.8–6.2 Hz), $J_{2',3'}$ (3.0–3.5 Hz), and $J_{3',4'}$ (5.0–7.0 Hz) allows exclusion of the boat forms, because, on the basis of the Karplus equation[8], the boat forms possible would have given different J values.

If a pyranoid sugar is in rapid equilibrium between its two chair conformations, with a preponderant contribution from the favored one, the J values and chemical shifts observed are determined by the proportion of each conformer present, and are intermediate between those predicted for each separate conformer[9,10]. The spectrum of **8**, measured at 4°, gives signals essentially the same as those measured at room temperature. Much lower temperatures would be required for n.m.r. spectral observation of separate conformers, in the manner observed with cyclohexane [11–13]. Recently, separate conformers have been observed directly by Bhacca and Horton[14], by means of an n.m.r. spectral analysis at 220 MHz of 1,2,3,4-tetra-*O*-acetyl-β-D-ribopyranose, measured in acetone-d_6 over the temperature range −84° to +20°.

From the J values observed with **1**, **3**, **6**, and **8** in deuterium oxide, and with the use of $J_{1',2'} = 1.7$ Hz and 8.5 Hz for diequatorial[15] and diaxial orientations, respectively, for α-D-mannopyranose as a reference standard, the proportions of, and free-energy differences ($\Delta G°$) between, the *C1* and *1C* conformations can be estimated approximately. The possible influence of the aglycon and solvent on the J values

cannot, however, be excluded. As shown in Table IV, the $\Delta G°$ values are found to be in the range of 0.1–0.4 Kcal. mole^{-1}. Bhacca and Horton's observation[14] and other available evidence[15–18] strongly indicate that the energy barrier for *C1* ⇌ *1C* interconversion is not high enough to restrict rapid interconversion at room temperature.

TABLE IV

CALCULATED AND OBSERVED *J* VALUES AND FREE-ENERGY DIFFERENCES

Compound	*Proportion of conformers* (%)		*Calcd. J value (Hz)*			*Observed J value (Hz)*			$-\Delta G°$ (*Kcal. mole*$^{-1}$)[b]
	C1	1C	$J_{1',2'}$	$J_{2',3'}$	$J_{3',4'}$	$J_{1',2'}$	$J_{2',3'}$	$J_{3',4'}$	
1 (L)	45	55	4.8	3.3[a]	6.6	4.8	3.3	7.0	+0.1
3 (L)	60	40	5.8	3.3	5.4	6.0	3.3	5.6	−0.3
6 (D)	40	60	5.8	3.3	5.4	6.0	3.0		−0.3
8 (D)	35	65	6.1	3.3	5.1	6.2	3.5	5.0	−0.4

[a]This is an average value of $J_{2',3'}$ observed, and is used as a reference for the true *gauche* relationship of the vicinal protons. [b]Calculated from the equation $-\Delta G° = RT\ln K$, where $K = N_N/N_A$ (N_N = molar proportion of the normal [*C1* (D)] and N_A alternative [*1C* (D)] conformation or *1C* (L) and *C1* (L), respectively).

On the basis of these results, it is reasonable to conclude that **1**, **3**, **6**, and **8** exist preponderantly in the so-called alternative chair conformation[1] in methyl sulfoxide, *N*,*N*-dimethylformamide, and pyridine, and that these compounds exist in rapid conformational equilibrium in deuterium oxide at room temperature.

On the other hand, the *J* values and signal shapes observed with 9-α-D-glucopyranosyladenine[22] (**9**), 9-α-D-glucopyranosyladenine picrate (**10**), and 7-α-D-glucopyranosyltheophylline[22] (**11**) are difficult to explain in terms of a time-average between chair conformers, because the $J_{1',2'}$ values are anticipated to be in the 1–4 Hz range for either conformer. If these compounds exhibit rapid equilibrium between both conformers, the $J_{1',2'}$ values should be approximately equal to those anticipated for the separate *C1* and *1C* conformers. The averaged *J* values of the other ring-methine protons would have to be smaller than those for the *C1* conformer and larger than those for the *1C* conformer. The $J_{1',2'}$ values actually observed with **9** and **11** in deuterium oxide are found to be 5.6 and 5.3 Hz, respectively. These values are larger than those expected for both conformers in rapid equilibrium between the rigid *C1* and *1C* conformations. In n.m.r. spectra measured in methyl sulfoxide-d_6, the H-1′ signal appears as a broad peak having a half band-width in the range of 4–6 Hz. The complexity of the H-1′ signal might be due to virtual coupling. This conclusion is partly verified by the fact that a sharp doublet ($J_{1',2'}$ 5.6 Hz) is observed in the 100-MHz n.m.r. spectrum of **9**, measured in deuterium oxide at 50°.

On the basis of these facts, it appears probable that the pyranoid ring in **9**, **10**, and **11** exists in a distorted shape, although the effects of virtual coupling may influence the results observed. This problem requires further investigation.

O.r.d. analysis of these α-glycosides showed positive plain curves for the members of the D-series and negative plain ones for members of the L-series in the region of 300–700 nm, and no significant differences were observed between curves for the two chair conformations.

Acyl derivatives of α-D-glycosylamines. — With regard to the acyl derivatives of α-glycosylamines, the alternative conformation is favored, with various solvents, by 7-(2,3,4-tri-*O*-acetyl-α-L-rhamnopyranosyl)theophylline (**12**), 7-(2,3,4-tri-*O*-benzoyl-α-L-rhamnopyranosyl)theophylline (**13**), and 7-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)theophylline[3] (**16**). It must be recognised that a small percentage of the unfavored conformer may escape detection[14].

The n.m.r. spectra of 6-acetamido-9-(2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl)-purine (**17**) and 7-(2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl)theophylline (**18**) are noteworthy. With regard to **17**, the large values of $J_{2',3'}$, $J_{3',4'}$ and $J_{4',5'}$, and the chemical shifts of the five acetate-methyl signals, one of which is an acetamido group on the purine base, suggest that the molecule **17** is mainly in the *C1* (D) conformation. However, the $J_{1',2'}$ value (5.7 Hz) suggests a 1,2-dihedral angle of *ca.*, 30°, on the basis of the Karplus equation[8]. This angle is much smaller than that for the true *gauche* orientation. With regard to **18**, the shape of the H-1′ signal, and the total width of the H-2′ and H-3′ signals (*ca.* 6 Hz) is not in agreement with either of the rigid *C1* or *1C* conformations. Furthermore, one of the four signals of acetate-methyl groups appears at τ 7.81, in the region indicative of the axial orientation. These irregularities might arise through flattening of the pyranose ring, and virtual coupling may also be involved. On the basis of these facts, adenine and theophylline as aglycons differ in their influence on the shape of the pyranose ring. Details of this point require further study in terms of the total molecular conformation.

Factors influencing conformational inversion. — As a factor influencing conformational inversion, Lemieux and Morgan[4] studied the effect of a quaternized nitrogen atom in the axial orientation at the anomeric position. In our experiments, a tertiary nitrogen atom in the axial orientation was found to cause conformational inversion or conformational equilibration. Such α-glycopyranosides as *p*-nitrophenyl α-L-rhamnopyranoside (**4**), quercitrin (3′,4″,5,7-tetrahydroxyflavone-3-yl α-L-rhamnopyranoside) (**5**), *p*-nitrophenyl 2,3,4-tri-*O*-acetyl-α-L-rhamnopyranoside[23] (**14**), and quercitrin heptaacetate (**15**), together with sugar phosphates[20], are found to exist in the normal conformation. The C-1–N bond distance in α-glycosylamines is shorter than the C-1–O bond in α-glycosides. Therefore, the aglycons in α-glycosides[21] may have little influence on the conformation from the viewpoint of steric, mesomeric, and polar effects, but the C-1 substituent in α-glycosylamines may have a strong influence on the conformation, and may bring about conformational equilibrium or inversion. It is of considerable interest to note that the driving force is not sufficient to force conformational inversion in compound **17**, although the corresponding glycopyranosylpyridinium derivative was found to exist in the alternative conformation[4].

From a consideration of Reeves' instability factors[2], the alternative confor-

mation of the compounds examined has the anomeric effect in addition to having three axial substituents. The fact that the alternative conformation is favored in spite of many destabilizing factors indicates that the axial aglycon greatly destabilizes the normal conformation, probably by the reverse anomeric effect[4] and by steric hindrance between *syn*-axial hydrogen atoms and the axial aglycons.

The effect of solvents on the shape of the pyranose ring is clearly shown in this study. Methyl sulfoxide-d_6, pyridine-d_5, and *N*,*N*-dimethylformamide are considered to solvate[19] strongly the axial hydroxyl groups of pyranose derivatives, and may stabilize axial substituents sufficiently to give the alternative conformation.

EXPERIMENTAL

Chemical shifts and coupling constants other than those listed in Tables I, II and III are described in this section.

9-α-L-Rhamnopyranosyladenine hydrochloride (**1**). — This compound was prepared according to the procedure reported by Baker and Hewson[7].

9-α-L-Rhamnopyranosyladenine picrate (**2**). — This compound was prepared by treatment of **1** with alcoholic picric acid; m.p. 214–215° (dec.).

Anal. Calc. for $C_{17}H_{16}N_8O_{10}\cdot H_2O$: C, 40.0; H, 3.5; N, 22.0. Found: C, 39.9; H, 3.6; N, 22.0.

7-α-L-Rhamnopyranosyltheophylline (**3**). — Substance **13** was treated with methanolic ammonia to give a syrupy product; $[\alpha]_D^{18}$ −90° (*c* 0.4, water); $\lambda_{max}^{H_2O}$ 275 nm; n.m.r. data (D_2O): τ 1.69 (H-8, 1 proton), 6.47 and 6.67 (1-NMe and H-3,6); (Me_2SO-d_6): τ 1.63 (H-8, 1 proton), 6.54 and 6.73 (1-NMe and H-3,6); ν_{max}^{Nujol} 3100–3600 (OH), 1700 cm^{-1} (C=O).

p-*Nitrophenyl α-L-rhamnopyranoside* (**4**). — The glycoside was prepared according to the procedure described by Kamiya, Esaki, and Hama[23].

Quercitrin (**5**). — The glycoside was obtained commercially, and was recrystallized from ethanol.

9-α-D-Mannopyranosyladenine hydrochloride (**6**). — This compound was prepared by the procedure reported by Baker and Hewson[7] for the synthesis of **1**, except that 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl bromide was used. The product was amorphous, $[\alpha]_D^{19}$ +33.4° (*c* 0.8, water); $\lambda_{max}^{H_2O}$ 259 nm; n.m.r. data (D_2O): τ 1.51, 1.40 (2 protons, H-2, H-8), data (Me_2SO-d_6): τ 1.27, 1.46 (2 protons, H-2, H-8), o.r.d. data (Me_2SO-d_6): τ 1.27, 1.46 (2 protons, H-2, H-8); o.r.d. data (*c* 1.5, water) positive plain curve, [∅] +575, +250, +130, and +100° at 300, 400, 500, and 600 nm, respectively.

9-α-D-Mannopyranosyladenine picrate (**7**). — Treatment of **6** with methanolic picric acid gave fine, yellow crystals of **7**, m.p. 215–218°; n.m.r. data (Me_2SO-d_6): τ 1.54 and 1.35 (2 protons, H-2 and 8), 1.41 (2 protons, aromatic); ν_{max}^{Nujol} 3000–3500 (OH, NH), 1700, 1615, 1570, 1555 (C=C, C=N), 1515, 1335 (NO_2), 1720 cm^{-1}. (aromatic).

Anal. Calc. for $C_{17}H_{16}N_8O_{11}\cdot H_2O$: C, 38.78; H, 3.43; N, 21.28. Found: C, 38.53; H, 3.65; N, 21.07.

7-α-D-Mannopyranosyltheophylline (**8**). — The glycoside was prepared by the procedure reported in our previous paper[3].

9-α-D-Glucopyranosyladenine (**9**). — The glycoside was prepared by the procedure described in our previous paper[22]; fine needles, m.p. 158–164° (sintered), 259–261° (dec.); $[\alpha]_D^{21}$ +112.1° (*c* 1.2, water); $\lambda_{max}^{H_2O}$ 259 nm (ε 15.2 × 10³); n.m.r. data (Me_2SO-d_6): τ 1.84, 1.77 (2 protons, H-2, H-8); o.r.d. data (*c* 1.2, water): positive plain curve, [Ø] +1290, +770, +470, +300, and +220° at 300, 400, 500, 600, and 700 nm, respectively.

Anal. Calc. for $C_{11}H_{15}N_5O_5 \cdot 0.5H_2O$: C, 43.16; H, 5.27; N, 22.86. Found: C, 43.34; H, 5.26; N, 22.94.

9-α-D-Glucopyranosyladenine picrate (**10**). — Treatment of **9** with alcoholic picric acid gave fine needles, m.p. 216°; n.m.r. data (Me_2SO-d_6): τ 1.43, 1.54 (2 protons, H-2, H-8), 1.43 (aromatic).

7-α-D-Glucopyranosyltheophylline (**11**). — This compound was prepared by the procedure described in our previous paper[22].

7-(2,3,4-Tri-O-acetyl-α-L-rhamnopyranosyl)theophylline (**12**). — Substance **3** was acetylated with acetic anhydride and pyridine by the usual procedure to give a syrup; $[\alpha]_D^{18}$ −40° (*c* 1.0, chloroform); λ_{max}^{EtOH} 276 nm; ν_{max}^{Nujol} 1770, 1720 (C=O), 1230 cm^{-1} (OAc); n.m.r. data (chloroform-*d*): τ 2.06 (1 proton, H-8), 6.41 and 6.59 (1-NMe and H-3,6).

Anal. Calc. for $C_{19}H_{24}N_4O_9$: C, 50.44; H, 5.35; N, 12.39. Found: C, 50.16; H, 5.67; N, 12.20.

7-(2,3,4-Tri-O-benzoyl-α-L-rhamnopyranosyl)theophylline (**13**). — Condensation of 2,3,4-tri-*O*-benzoyl-α-L-rhamnopyranosyl bromide with *N*-chloromercuritheophylline was carried out by the usual procedure in boiling xylene to give fine crystals, m.p. 215–216°; $[\alpha]_D^{18}$ −4.5° (*c* 1.2, chloroform); ν_{max}^{Nujol} 1730, 1710 (C=O), 1675, 720 cm^{-1}; n.m.r. data (chloroform-*d*): τ 1.7–2.7 (16 protons, aromatic), 6.46 and 6.65 (1-NMe and H-3,6).

Anal. Calc. for $C_{34}H_{30}N_4O_9$: C, 63.94; H, 4.74; N, 8.77. Found: C, 63.75; H, 4.69; N, 8.71.

p-*Nitrophenyl 2,3,4-tri-O-acetyl-α-L-rhamnopyranoside* (**14**). — This glycoside was prepared by the procedure described by Kamiya, Esaki, and Hama[23].

Quercitrin heptaacetate (**15**). — Quercitrin was acetylated with a mixture of acetic anhydride and pyridine by the usual procedure.

7-(2,3,4,6-Tetra-O-acetyl-α-D-mannopyranosyl)theophylline (**16**). — The compound was prepared by the procedure described in our previous paper[3].

6-Acetamido-9-(2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl)purine (**17**). — Acetylation of **9** with a mixture of acetic anhydride and pyridine by the usual procedure gave rise to fine needles, m.p. 201–202°, $[\alpha]_D^{26}$ +120° (*c* 0.5, chloroform); n.m.r. data (chloroform): τ 1.57 and 1.98 (2 protons, H-2 and 8), 5.9 (NH, disappeared on addition of deuterium oxide).

Anal. Calc. for $C_{21}H_{25}N_5O_{10}$: C, 49.70; H, 4.97; N, 13.80. Found: C, 49.53; H, 5.12; N, 13.67.

7-(2,3,4,6-Tetra-O-acetyl-α-D-glucopyranosyl)theophylline (**18**). — Substance **11** was acetylated with a mixture of acetic anhydride and pyridine by the usual procedure to give fine needles, m.p. 147–148°; $[\alpha]_D^{26}$ +77.8° (*c* 0.45, chloroform); λ_{max}^{MeOH} 276 nm; n.m.r. data (chloroform-*d*): τ 2.06 (1 proton, H-8), 6.40 and 6.60 (1-NMe and H-3,6); ν_{max}^{Nujol} 1740, 1760 (C=O), 1230 (OAc), 770, 775 cm^{-1}.

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REFERENCES

1 E. L. Eliel, N. L. Allinger, S. J. Angyal, and G. A. Morrison, *Conformational Analysis*, Interscience Publishers, Inc., New York, 1965, p. 371.
2 R. E. Reeves, *J. Am. Chem. Soc.*. 71 (1949) 215; *Advan. Carbohyd. Chem.*, 6 (1951) 107.
3 K. Onodera, S. Hirano, F. Masuda, and N. Kashimura, *J. Org. Chem.*, 31 (1966) 2403.
4 R. U. Lemieux and A. R. Morgan, *Can. J. Chem.*, 43 (1965) 2205.
5 C. V. Holland, D. Horton, and J. S. Jewell, *J. Org. Chem.*, 32 (1967) 1818.
6 M. Rudrum and D. F. Shaw, *J. Chem. Soc.*, (1965) 52.
7 B. R. Baker and K. Hewson, *J. Org. Chem.*, 22 (1957) 959.
8 M. Karplus, *J. Chem. Phys.*, 30 (1959) 11; *J. Am. Chem. Soc.*, 85 (1963) 2870.
9 F. A. L. Anet, *J. Am. Chem. Soc.*, 84 (1962) 10531. F. Sweet and R. K. Brown, *Can. J. Chem.*, 45 (1967) 1007; C. B. Anderson and D. T. Sepp, *J. Org. Chem.*, 32 (1967) 607.
10 E. L. Eliel and M. H. Gianni, *Tetrahedron Letters*, (1962) 97.
11 F. R. Jensen, D. S. Noyce, C. H. Sederhol, and A. J. Berlin, *J. Am. Chem. Soc.*, 84 (1962) 386.
12 F. A. L. Anet, M. Ahmad, and L. D. Hall, *Proc. Chem. Soc.*, (1964) 145.
13 F. A. Bovey, F. P. Hodd, E. W. Anderson, and R. L. Kornegay, *Proc. Chem. Soc.*, (1965) 146.
14 N. S. Bhacca and D. Horton, *J. Am. Chem. Soc.*, 89 (1967) 5993.
15 R. U. Lemieux and J. D. Stevens, *Can. J. Chem.*, 44 (1966) 249.
16 R. U. Lemieux and J. D. Stevens, *Can. J. Chem.*, 43 (1965) 2059.
17 L. D. Hall, *Advan. Carbohyd. Chem.*, 19 (1964) 51.
18 S. Inouye, *Chem. Pharm. Bull.* (Tokyo), 14 (1966) 1112.
19 K. Onodera and S. Hirano, *Biochem. Biophys. Res. Commun.*, 25 (1966) 239.
20 R. U. Lemieux and S. Levine, *Can. J. Chem.*, 42 (1964) 1973.
21 K. Onodera, S. Hirano, and F. Masuda, *Tetrahedron Lett.*, (1966) 2189.
22 S. Kamiya, S. Esaki, and M. Hama, *Agr. Biol. Chem.* (Tokyo), 31 (1967) 379.

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BENZYLIDEN-VERBINDUNGEN DER PENTONSÄUREN UND PENTONSÄURE-LACTONE

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ABSTRACT

Di-*O*-benzylidene derivatives of pentonic acids may be readily prepared by oxidation of the di-*O*-benzylidene-*aldehydo*-pentoses with chromium trioxide in *N,N*-dimethylformamide or by saponification of the corresponding methyl esters. Treatment of ammonium D-xylonate with benzaldehyde and hydrochloric acid gives the di-*O*-benzylidene-D-xylonic acid, whereas the D-ribonate and D-lyxonate salts give the 3,5-*O*-benzylidene-pentono-1,4-lactones. With benzaldehyde and zinc chloride, the methyl pentonates variously give the di- and mono-*O*-benzylidene derivatives and the mono-*O*-benzylidenepentonolactones. The structures of the di-*O*-benzylidene derivatives obey the rules of Hann and Hudson. The *O*-benzylidenepentonolactones give the corresponding amides and hydrazides on treatment with ammonia and hydrazine hydrate, respectively; reduction with lithium aluminium hydride gives the *O*-benzylidenepentitols.

ZUSAMMENFASSUNG

Di-*O*-benzyliden-pentonsäuren lassen sich gut aus Di-*O*-benzyliden-*aldehydo*-pentosen durch Oxydation mit Chromtrioxyd in Dimethylformamid oder aus Di-*O*-benzyliden-pentonsäure-methylestern durch Verseifen mit Natronlauge darstellen. Die Di-*O*-benzyliden-D-xylonsäure erhält man auch aus Ammonium-D-xylonat mit Benzaldehyd und Salzsäure. D-Ribonate und D-Lyxonate ergeben bei der analogen Reaktion 3,5-*O*-Benzyliden-pentonsäure-γ-lactone. Die Umsetzungen der Pentonsäure-methylester mit Benzaldehyd und Zinkchlorid ergeben je nach Vertreter Di-*O*-benzyliden-pentonsäure-methylester, Mono-*O*-benzyliden-pentonsäure-methylester und *O*-Benzyliden-pentonsäure-lactone; zum Teil entstehen diese Verbindungen im Gemisch. Die Bildung der Di-*O*-benzyliden-pentonsäure-methylester erfolgt nach den Regeln von Hann und Hudson. Die *O*-Benzyliden-pentonsäure-lactone werden durch Behandeln mit Ammoniak in *O*-Benzyliden-pentonsäure-amide, mit Hydrazinhydrat in *O*-Benzyliden-pentonsäure-hydrazide und mit $LiAlH_4$ in Benzyliden-pentite übergeführt.

EINFÜHRUNG

Für Synthesen von Pentonsäure- und Pentit-Derivaten benötigten wir Di-*O*-

benzyliden-pentonsäuren (**2a-d**). Zur Darstellung dieser Verbindungen wird ein Weg von Gätzi und Reichstein[1] gewiesen, die Di-*O*-isopropyliden-*aldehydo*-D-arabinose mit Kaliumpermanganat in Wasser zu Di-*O*-isopropyliden-D-arabinonsäure oxydierten. Dementsprechend untersuchten wir zunächst die Oxydation der Di-*O*-benzyliden-*aldehydo*-pentosen (**1a-d**) zu Di-*O*-benzyliden-pentonsäuren (**2a-d**).

Von den Di-*O*-benzyliden-*aldehydo*-pentosen sind die der D-Ribose[2] (**1a**), der D-Xylose[3] (**1b**) und der D-Lyxose[4] (**1c**) bekannt. Sie wurden aus den zugehörigen 2,4:3,5-Di-*O*-benzyliden-pentose-dialkylthioacetalen durch Behandeln mit Quecksilber(II)-chlorid und Quecksilberoxyd in wäßrigem Aceton gewonnen; die beiden Benzyliden-Gruppen nehmen wie in den zugehörigen Dialkylthioacetalen die 2,4:3,5-Stellung ein. Die analoge Darstellung der bisher unbekannten 2,3:4,5-Di-*O*-benzyliden-*aldehydo*-D-arabinose (**1d**) aus dem 2,3:4,5-Di-*O*-benzyliden-D-arabinose-diäthylthioacetal[5] ist schwieriger, weil hier die beiden Benzyliden-Gruppen wesentlich lockerer gebunden sind und während der Reaktion mit Quecksilber(II)-chlorid leicht abgespalten werden. Diese Reaktion muß daher unter besonders milden Bedingungen durchgeführt werden. Wir erzielten die günstigsten Ergebnisse, wenn wir das in Aceton gelöste Quecksilber(II)-chlorid langsam unter Rühren bei 30° zu der Mischung von Di-*O*-benzyliden-D-arabinose-diäthylthioacetal und Quecksilberoxyd in wäßrigem Aceton zutropfen ließen und dann die Temperatur langsam bis zum Sieden des Acetons erhöhten. Die so gewonnene 2,3:4,5-Di-*O*-benzyliden-*aldehydo*-D-arabinose war nicht ganz rein, sie ließ sich jedoch mit einer Ausbeute von 72% in ein reines, kristallisiertes 2,4-Dinitrophenylhydrazon überführen.

Die Oxydation der Di-*O*-benzyliden-*aldehydo*-pentosen (**1a-d**) mit Kaliumpermanganat bei Gegenwart von Kaliumcarbonat in Aceton–Wasser (Reaktionszeit 16 Stunden bei 20°) lieferte nur unbefriedigende Ergebnisse[6]. Die 2,4:3,5-Di-*O*-benzyliden-Verbindungen der D-Ribonsäure (**2a**), D-Xylonsäure (**2b**) und der D-Lyxonsäure (**2c**) konnten nur mit Ausbeuten von 23–30% isoliert werden. Bei der Oxydation der 2,3:4,5-Di-*O*-benzyliden-*aldehydo*-D-arabinose (**1d**) trat eine starke Zersetzung auf, es ließ sich kein definiertes Oxydationsprodukt isolieren.

Bei Verwendung des Oxydationsmittels Chromtrioxyd in Benzol–Eisessig[7] (10 Minuten erhitzen auf 90–95°) waren die Ergebnisse fast die gleichen[6]. Völlig unbefriedigend verlief die Oxydation der Di-*O*-benzyliden-*aldehydo*-pentosen (**1a-d**) mit Chromtrioxyd in Pyridin[8]. Dabei wurde die in Pyridin gelöste Verbindung **1** zu dem frisch hergestellten Chromtrioxyd–Pyridin–Komplex gegeben und anschließend 8 Stunden unter Rühren auf 60° erwärmt; nach dem Aufarbeiten wurde die eingesetzte Verbindung **1** größtenteils zurückgewonnen, es gelang uns nicht, ein Oxydationsprodukt zu isolieren.

Zu geringen und sehr schwankenden Ausbeuten an Di-*O*-benzyliden-pentonsäuren führte auch die katalytische Oxydation[9] der *aldehydo*-Verbindungen **1a-c** mit Sauerstoff und Platin[6], was bei unseren Versuchen aber vielleicht darauf zurückzuführen ist, daß in unserem Laboratorium mit Thioalkoholen gearbeitet wird, wodurch die Katalysatoren leicht vergiftet werden.

Am besten eignete sich eine von Snatzke[10] für die Oxydation von Steroiden ange-

gebene Methode, bei der Chromtrioxyd in Dimethylformamid als Oxydationsmittel benutzt wird. In Anlehnung an dieses Verfahren läßt man die Di-*O*-benzyliden-*aldehydo*-pentose in Dimethylformamid mit Chromtrioxyd und Schwefelsäure 5 Stunden bei 20° stehen, erhitzt anschließend noch 5 Minuten auf 80° und reinigt di gebildete Di-*O*-benzyliden-pentonsäure über das Natriumsalz. Man gewinnt so die reinen, kristallisierten 2,4:3,5-Di-*O*-benzyliden-Verbindungen der D-Ribonsäure (**2a**), D-Xylonsäure (**2b**) und der D-Lyxonsäure (**2c**) mit Ausbeuten von 61–70%. Es ist wichtig, die im "Experimentellen Teil" angegebenen Mengen an Chromtrioxyd und Schwefelsäure genau einzuhalten, sonst liegen die Ausbeuten tiefer. Die 2,3:4,5-Di-*O*-benzyliden-*aldehydo*-D-arabinose (**1d**) läßt sich auch nach dieser Methode nicht zur entsprechenden D-Arabinonsäure **2d** oxydieren; es tritt Zersetzung ein, der dabei abgespaltene Benzaldehyd wird zur Benzoesäure oxydiert.

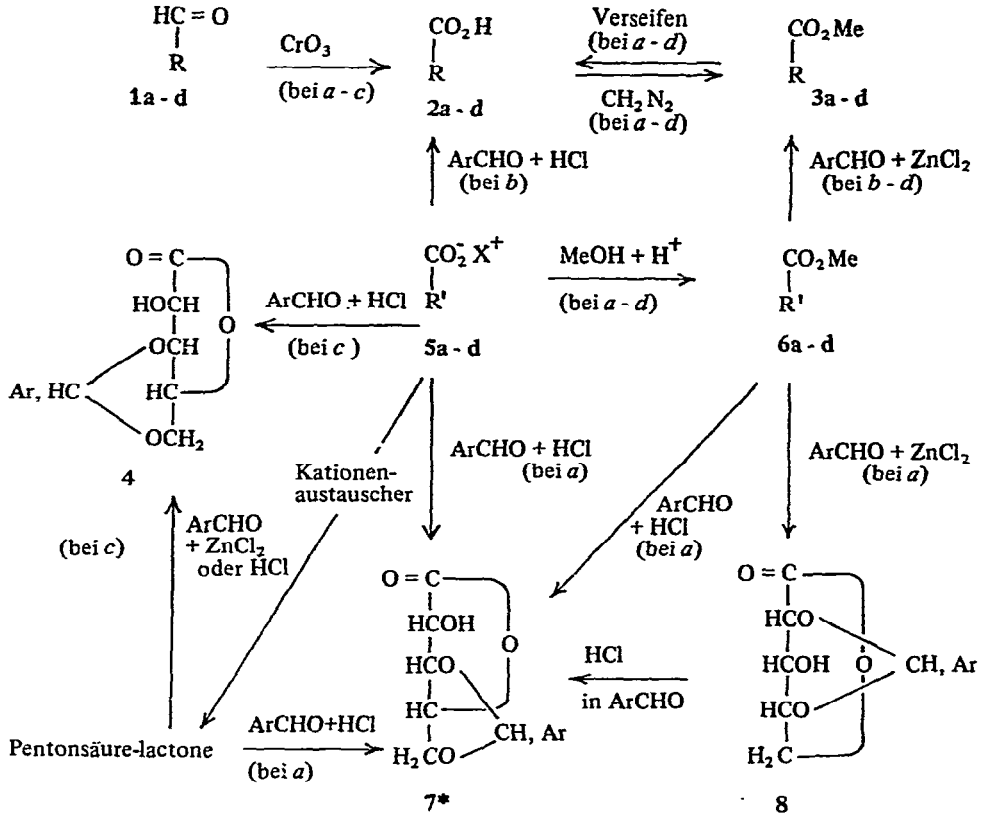
Alberda van Ekenstein und Lobry de Bruyn[11] erhielten bei der Umsetzung von Natrium-D-xylonat (**5b**) mit Benzaldehyd und konz. Salzsäure eine Di-*O*-benzyliden-D-xylonsäure; sie ist mit der oben genannten 2,4:3,5-Di-*O*-benzyliden-D-xylonsäure (**2b**) identisch. Beim Überarbeiten der Methode stellten wir fest, daß die Ausbeute an **2b** von der Art des eingesetzten Salzes abhängt. Die höchste Ausbeute erreicht man beim Einsatz des Ammonium-D-xylonates (55%), wesentlich tiefer liegen die Ausbeuten, wenn man das Kalium- (22%), Natrium- (19%) oder das Calcium-D-xylonat (3.4%) analog umsetzt. Die 2,4:3,5-Di-*O*-benzyliden-D-xylonsäure erhält man auch aus dem D-Xylonsäure-γ-lacton[12] durch Schütteln mit Benzaldehyd und konz. Salzsäure (52%).

Die D-Ribonate (**5a**) und die D-Lyxonate (**5c**) reagieren mit Benzaldehyd bei Gegenwart von konz. Salzsäure anders als die D-Xylonate; aus ihnen entstehen dabei das 3,5-*O*-Benzyliden-D-ribonsäure-γ-lacton (**7**) bzw. das 3,5-*O*-Benzyliden-D-lyxonsäure-γ-lacton (**4**). Die zu den D-Ribonaten und D-Lyxonaten gehörigen Metallionen haben hier keinen Einfluß auf die Reaktion, die erzielten Ausbeuten an Benzyliden-Verbindungen (**7**,**4**) waren beim Einsatz des Ammonium-, Kalium-, Natrium- oder des Calcium-pentonats gleich groß.

Wir waren bemüht, nach der genannten Methode von Alberda van Ekenstein und Lobry de Bruyn[11] auch eine Di-*O*-benzyliden-D-arabinonsäure darzustellen. Es gelang uns aber leider nicht, durch Behandeln von Salzen der D-Arabinonsäure mit Benzaldehyd und Salzsäure eine definierte Verbindung zu gewinnen.

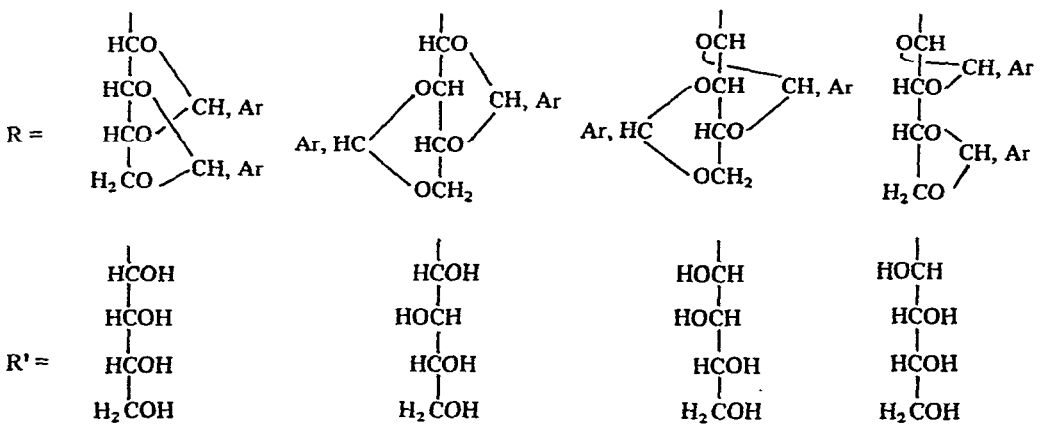
Für die Synthese von Di-*O*-benzyliden-pentonsäuren (**2**) kann auch folgender Weg eingeschlagen werden: Eine Pentose wird elektrolytisch zum Calcium-pentonat (**5**) oxydiert, daraus stellt man den Pentonsäure-methylester (**6**) her, der mit Benzaldehyd und Zinkchlorid zum Di-*O*-benzyliden-pentonsäure-methylester (**3**) kondensiert wird. Dessen alkalische Verseifung ergibt schließlich die Di-*O*-benzyliden-pentonsäure (**2**).

Die Oxydation der Pentosen zu den Calcium-pentonaten (**5**) führt man am besten elektrolytisch nach Kiliani[13] durch, wobei eine verbesserte Versuchsanordnung benutzt wird[14]. Die in dieser Arbeit benutzten Natrium-, Kalium- und Ammoniumpentonate wurden aus den entsprechenden, in Wasser gelösten Calcium-Salzen durch



Ar = C_6H_5
X = Ca/2, K, Na oder NH_4

a, D-Ribose-Reihe b, D-Xylose-Reihe c, D-Lyxose-Reihe d, D-Arabinose-Reihe



* Die Struktur der Verbindung ist nicht sicher.

Zugabe von Natrium-, Kalium- bzw. Ammoniumcarbonat, Abfiltrieren des Calciumcarbonates und Ausfällen mit Methanol gewonnen. Die D-Arabinonate kristallisieren alle gut. Das Ammonium-D-xylonat[15], Kalium-D-lyxonat[16] und das Kalium-D-ribonat[17] sind ebenfalls kristallisierte Verbindungen. Alle übrigen Ammonium-, Natrium- und Kalium-pentonate konnten durch Eindampfen der Lösungen nur als Sirupe isoliert werden, sie lassen sich nur schwer und unter großen Verlusten reinigen. Sie wurden daher als Rohprodukte für die Versuche verwendet. Die Darstellung der Verbindungen wird im "Experimentellen Teil" nur soweit beschrieben, wie sie für erfolgreich verlaufende Synthesen in Frage kamen.

Die Pentonsäure-methylester (**6**) erhält man in guten Ausbeuten durch Umsetzung der Calcium-pentonate (**5**) mit Methanol bei Gegenwart von konz. Schwefelsäure. Bei der Darstellung des D-Arabinonsäure-methylesters wird jedoch nur eine Ausbeute von 45% erreicht. Dieser Ester läßt sich günstiger aus dem Kalium-D-arabinonat und Methanol durch Einleiten von Chlorwasserstoff[18] gewinnen (79%).

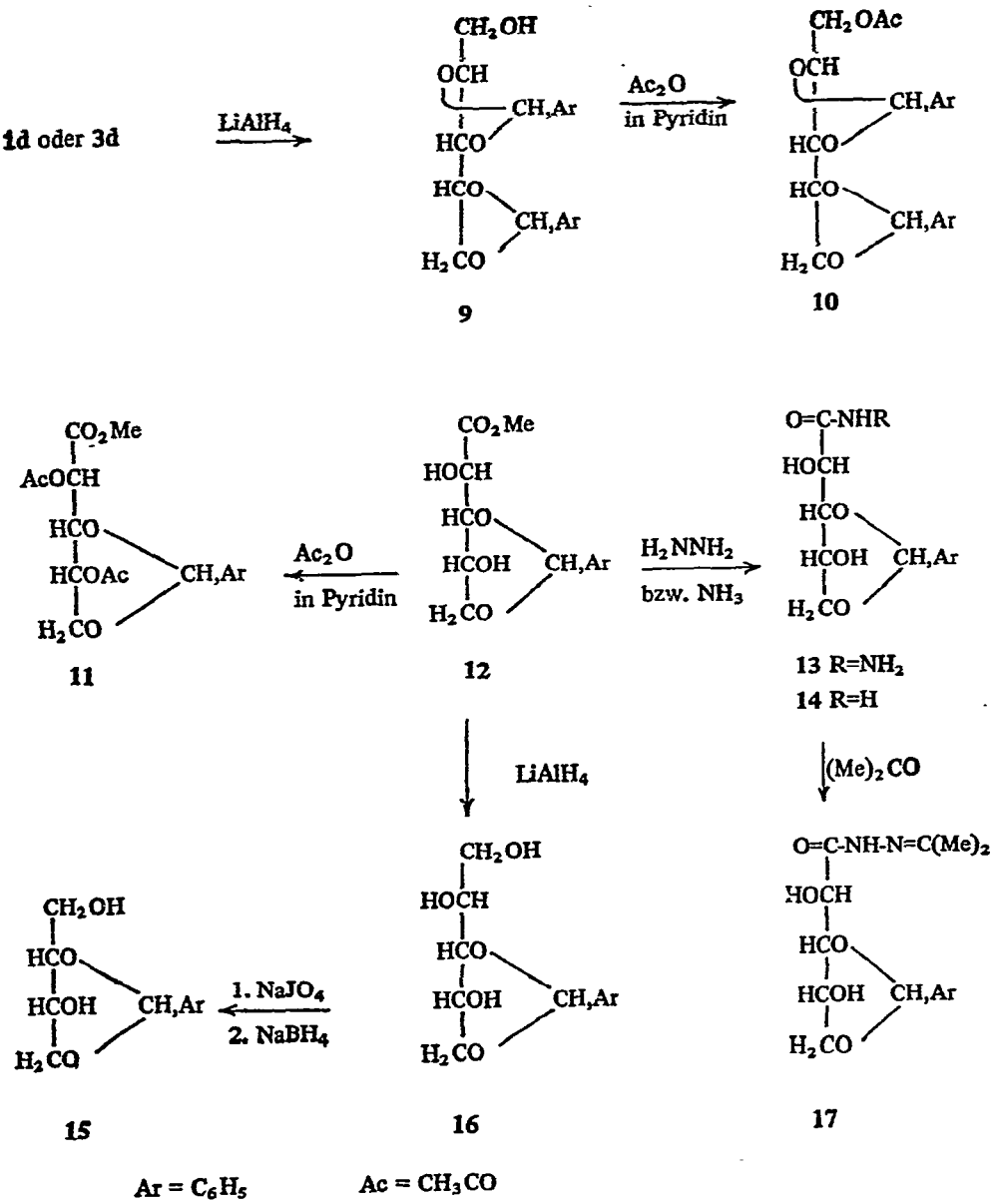
Der D-Xylonsäure-methylester (**6b**) liefert bei der Kondensation mit Benzaldehyd und Zinkchlorid den 2,4:3,5-Di-*O*-benzyliden-D-xylonsäure-methylester (**3b**). Die 2,4:3,5-Stellung der Benzyliden-Gruppen ergibt sich durch eine weitere Synthese der Verbindung **3b** aus 2,4:3,5-Di-*O*-benzyliden-D-xylonsäure (**2b**) und Diazomethan.

Nach der Umsetzung des D-Lyxonsäure-methylesters (**6c**) mit Benzaldehyd und Zinkchlorid erhält man zwei kristallisierte Verbindungen, den 2,4:3,5-Di-*O*-benzyliden-D-lyxonsäure-methylester (**3c**) und das 3,5-*O*-Benzyliden-D-lyxonsäure-γ-lacton (**4**). Letzteres läßt sich mit besserer Ausbeute aus dem Calcium-D-lyxonat oder D-Lyxonsäure-γ-lacton[12] durch Umsetzen mit Benzaldehyd in konz. Salzsäure gewinnen. Die Struktur des 2,4:3,5-Di-*O*-benzyliden-esters **3c** kann wieder durch eine weitere Darstellung aus 2,4:3,5-Di-*O*-benzyliden-D-lyxonsäure (**2c**) und Diazomethan bewiesen werden.

Der 2,4:3,5-Di-*O*-benzyliden-D-ribonsäure-methylester (**3a**) läßt sich gut aus 2,4:3,5-Di-*O*-benzyliden-D-ribonsäure (**2a**) mit Diazomethan darstellen. Durch Umsetzung des D-Ribonsäure-methylesters mit Benzaldehyd und einem Katalysator entsteht der Ester **3a** nicht. Setzt man bei dieser Reaktion Zinkchlorid als Katalysator ein, so erhält man das 2,4-*O*-Benzyliden-D-ribonsäure-δ-lacton (**8**). Bei Verwendung von konz. Salzsäure als Katalysator entsteht jedoch das 3,5-*O*-Benzyliden-D-ribonsäure-γ-lacton (**7**). Die lenkende Wirkung des Katalysators ist die gleiche, wenn man an Stelle des Esters **3a** das D-Ribonsäure-lacton[19] mit Benzaldehyd umsetzt. Mit Zinkchlorid erhält man wieder das δ-Lacton **8**, mit konz. Salzsäure das γ-Lacton **7**. Wie oben schon erwähnt, entsteht das γ-Lacton auch aus dem Calcium-D-ribonat (**5a**) durch Behandeln mit Benzaldehyd und Salzsäure. Konzentrierte Salzsäure bewirkt eine Umlagerung des δ-Lactons **8**; wenn man die in Benzaldehyd gelöste Verbindung **8** mit Salzsäure einige Stunden schüttelt, wird sie nahezu quantitativ in das γ-Lacton **7** umgelagert.

Bei der Reaktion des D-Arabinonsäure-methylesters (**6d**) mit Benzaldehyd und Zinkchlorid entstehen nebeneinander 3,5-*O*-Benzyliden-D-arabinonsäure-methylester (**12**) und 2,3:4,5-Di-*O*-benzyliden-D-arabinonsäure-methylester (**3d**).

In der letztgenannten Verbindung nehmen die beiden Benzyliden-Gruppen die gleiche Stellung ein wie im 2,3:4,5-Di-*O*-benzyliden-D-arabinose-diäthylthioacetal[5] und in der daraus darstellbaren 2,3:4,5-Di-*O*-benzyliden-*aldehydo*-D-arabinose (**1d**);



denn die Reduktion dieser Verbindung mit Lithiumaluminiumhydrid führt zum 2,3:4,5-Di-*O*-benzyliden-D-arabinit (**9**), der auch bei der analogen Reduktion des 2,3:4,5-Di-*O*-benzyliden-D-arabinonsäure-methylesters entsteht. Zur Charakterisierung führten wir den 2,3:4,5-Di-*O*-benzyliden-D-arabinit in das 1-Acetat (**10**) über.

Die Struktur des 3,5-*O*-Benzyliden-D-arabinonsäure-methylesters (**12**) ergibt sich aus einer Reihe von Umsetzungen. Die Verbindung hat zwei freie Hydroxylgruppen; beim Acetylieren entsteht der 2,4-Di-*O*-acetyl-3,5-*O*-benzyliden-D-arabinonsäure-methylester (**11**). Die Methylester-Gruppierung reagiert in der üblichen Weise mit Hydrazinhydrat und mit Ammoniak unter Bildung eines Säurehydrazids (**13**) bzw. eines Säureamids (**14**). Durch Reduktion mit Lithiumaluminiumhydrid wird der 3,5-*O*-Benzyliden-D-arabinonsäure-methylester in den 3,5-*O*-Benzyliden-D-arabinit (**16**) übergeführt, dessen Struktur sich aus der Oxydation mit Natriumperjodat ergibt. Die Verbindung **16** verbraucht nach 24 Stunden 1.1 Mol Perjodat; dabei entstehen Formaldehyd und 2,4-*O*-Benzyliden-D-erythrose, die durch Reduktion mit Natriumborhydrid in den bekannten 2,4-*O*-Benzyliden-D-erythrit (**15** = 1,3-*O*-Benzyliden-L-erythrit[20]) übergeführt werden kann.

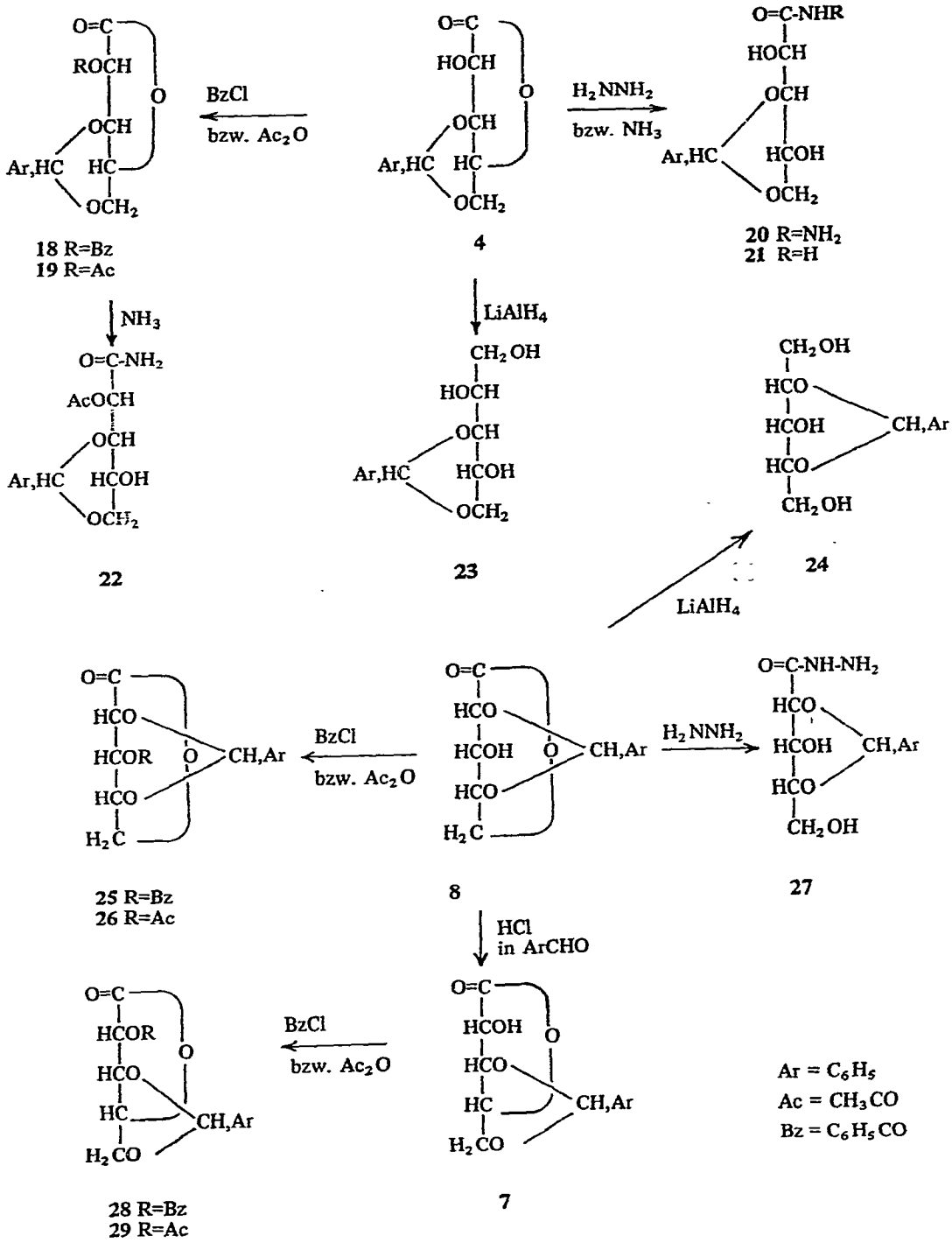
Es sei noch erwähnt, daß sich das 3,5-*O*-Benzyliden-D-arabinonsäurehydrazid (**13**) außerordentlich glatt mit Aceton unter Bildung von Aceton-[(3,5-*O*-benzyliden-D-arabinonyl)-hydrazon] (**17**) umsetzt; die Ausbeute an reiner Verbindung beträgt 94%.

Für das 3,5-*O*-Benzyliden-D-lyxonsäure-γ-lacton (**4**) und das 2,4-*O*-Benzyliden-D-ribonsäure-δ-lacton (**8**) ist die Struktur leicht zu beweisen; die beiden Verbindungen lassen sich durch Reduktion mit Lithiumaluminiumhydrid in den bekannten 3,5-*O*-Benzyliden-D-lyxit (**23** = 1,3-*O*-Benzyliden-D-arabinit[21]) bzw. in den bekannten 2,4-*O*-Benzyliden-ribit[2,22] (**24**) überführen. In beiden Lactonen kann eine freie Hydroxyl-Gruppe durch Benzoylieren oder Acetylieren nachgewiesen werden, man erhält so das 2-*O*-Benzoyl- (**18**) und das 2-*O*-Acetyl-3,5-*O*-benzyliden-D-lyxonsäure-γ-lacton (**19**) bzw. das 3-*O*-Benzoyl- (**25**) und das 3-*O*-Acetyl-2,4-*O*-benzyliden-D-ribonsäure-δ-lacton (**26**).

Die beiden Lactone (**4** und **8**) reagieren in der üblichen Weise mit Hydrazinhydrat unter Bildung von 3,5-*O*-Benzyliden-D-lyxonsäure-hydrazid (**20**) bzw. 2,4-*O*-Benzyliden-D-ribonsäure-hydrazid (**27**). Die Reaktion des 3,5-*O*-Benzyliden-D-lyxonsäure-γ-lactons mit Ammoniak führt zum 3,5-*O*-Benzyliden-D-lyxonsäureamid (**21**). Das 2-*O*-Acetyl-3,5-*O*-benzyliden-D-lyxonsäure-γ-lacton (**19**) führten wir analog in das 2-*O*-Acetyl-3,5-*O*-benzyliden-D-lyxonsäure-amid (**22**) über.

Im Gegensatz zum 3,5-*O*-Benzyliden-D-lyxonsäure-γ-lacton (**4**) reagiert das 3,5-*O*-Benzyliden-D-ribonsäure-γ-lacton (**7**) weder mit Ammoniak zum entsprechenden Säureamid noch mit Hydrazinhydrat zum Säurehydrazid. Auch die Reduktion mit Lithiumaluminiumhydrid zum 3,5-*O*-Benzyliden-D-ribit gelang uns nicht. Unter den üblichen Bedingungen wurde das Ausgangsmaterial (**7**) zurückgewonnen, unter schärferen Bedingungen trat Zersetzung ein. Die freie Hydroxyl-Gruppe in **7** reagiert jedoch mit Benzoylchlorid und Acetanhydrid in Pyridin unter Bildung von 2-*O*-Benzoyl- (**28**) bzw. 2-*O*-Acetyl-3,5-*O*-benzyliden-D-ribonsäure-γ-lacton (**29**).

Das unterschiedliche chemische Verhalten der beiden 3,5-*O*-Benzylidenpentonsäure-γ-lactone (**4** und **7**) dürfte zumindest zum Teil auf sterische Effekte zurückzuführen sein. Wie aus Modellbetrachtungen hervorgeht, sind im Ribonsäure-Derivat (**7**) der 1,3-Dioxan-Ring und der Lacton-Ring trans-verknüpft; das System



18 R=Bz
19 R=Ac

4

20 R=NH2
21 R=H

22

23

24

25 R=Bz
26 R=Ac

8

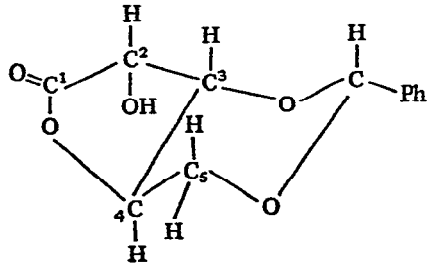
27

28 R=Bz
29 R=Ac

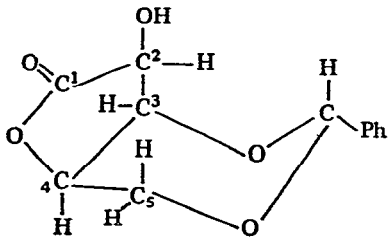
7

ist ähnlich dem *trans*-Dekalin weitgehend starr im Gegensatz zum *cis*-verknüpften Lyxonsäure-Derivat (4).

Bei den Reaktionen zur Öffnung des Lacton-Ringes durch Hydrieren oder durch Anlagern von Ammoniak erfolgt im Übergangszustand ein Wechsel der Hybridisierung des Carbonylkohlenstoffs von sp^2 nach sp^3. Durch diesen Wechsel wird im Lacton-Ring eine zusätzliche Pitzer-Spannung hervorgerufen. Im Falle des Lyxonsäure-Derivates (4) wird diese zusätzliche Spannung wegen des Vorliegens eines flexiblen Ring-Systems kleiner sein als beim Ribonsäure-Derivat (7) mit starrem Ring-System. Es ist daher verständlich, daß das 3,5-*O*-Benzyliden-D-lyxonsäure-γ-lacton (4) reaktionsfähiger ist als das entsprechende Ribonsäure-γ-lacton (7).



(7)



(4)

Die Struktur des 3,5-*O*-Benzyliden-D-ribonsäure-γ-lactons (7) konnten wir wegen des fehlenden Reaktionsvermögens mit Lithiumaluminiumhydrid und mit Ammoniak experimentell nicht beweisen. Da aber allgemein bei Gegenwart von Salzsäure ausschließlich γ-Lactone gebildet werden[23] und sich das 2,4-*O*-Benzyliden-D-ribonsäure-δ-lacton (8) zu 7 umlagern läßt, ist anzunehmen, daß diese fragliche Verbindung ein γ-Lacton ist; die Benzyliden-Gruppe könnte dann in 7 die 3,5- oder die 2,3-Stellung einnehmen, wobei die letztere aus energetischen Gründen unwahrscheinlicher ist. Als gesichert kann jedoch die vorläufig angenommene Struktur des 3,5-*O*-Benzyliden-D-ribonsäure-γ-lactons (7) nicht angesehen werden.

Die Regeln von Hann und Hudson[24,25] gestatten Aussagen über die Bildungstendenz von Acetal-Ringen, die bei der Umsetzung von Zuckeralkoholen mit einem Aldehyd bei Gegenwart eines Katalysators auftreten. In letzter Zeit konnte gezeigt werden, daß diese Regeln nicht nur für die cyclische Acetal-Bildung der Zuckeralkohole, sondern auch für die der Aldose-dialkylthioacetale[3,4,26,27] und die der Aldose-dialkylacetale[5] Gültigkeit haben. Es wurde weiterhin behauptet[5], daß die Regeln für die cyclische Acetal-Bildung aller der Zucker-Derivate zutreffen müssen, die eine gestreckte, nicht cyclische Form aufweisen. Die oben beschriebenen Umsetzungen der Pentonsäure-methylester (**6b-d**) mit Benzaldehyd zu den Di-*O*-benzylidenpentonsäure-methylestern (**3b-d**) sind ein weiteres Beispiel für die Verallgemeinerung der Regeln von Hann und Hunson; denn die Benzyliden-Gruppen befinden sich in den Estern **3b-d** in den gleichen Stellungen wie in den zugehörigen Pentose-dialkylthioacetalen[3,4,27] und den Pentose-dialkylacetalen[5].

EXPERIMENTELLER TEIL

Alle Schmelzpunkte wurden auf dem Mikroheiztisch nach Boëtius bestimmt.

2,3:4,5-Di-O-benzyliden-aldehydo-D-*arabinose* (**1d**). — Man löst 4.33 g (0.01 Mol) 2,3:4,5-Di-*O*-benzyliden-D-arabinose-diäthylthioacetal[5] in 50 ml Aceton, fügt 5.0 ml Wasser und 6.0 g Quecksilberoxyd hinzu, läßt bei 30° unter Rühren im Verlaufe von etwa 30 Min. eine Lösung von 5.0 g Quecksilber(II)-chlorid in 50 ml Aceton zutropfen, rührt dann noch 1 Stde. bei 30°, 1 Stde. bei 40°, 1 Stde. bei 50° und 1.5 Stdn. unter Rückfluß, filtriert, wäscht mit 100 ml warmem Aceton nach, dampft die vereinigten Filtrate bei Gegenwart von 1.0 g Quecksilberoxyd ein, extrahiert den Rückstand viermal mit je 30 ml Chloroform, wäscht die vereinigten Filtrate zweimal mit je 40 ml 15-proz. Kaliumjodid-Lösung sowie zweimal mit je 50 ml Wasser, trocknet mit Natriumsulfat, schüttelt mit Aktivkohle, filtriert und dampft zu einem Sirup ein. Ausb. 2.06 g (63%), $[\alpha]_D^{20}$ *ca.* 0° (*c* 3.84, Chloroform). Die Verbindung ist nicht analysenrein.

2,3:4,5-Di-O-benzyliden-aldehydo-D-*arabinose-2,4-diniotrophenylhydrazon.* — Die vorstehende Verbindung (0.82 g; 0.0025 Mol) und 2,4-Dinitrophenylhydrazin (0.50 g; 0.0025 Mol) werden in 50 ml Methanol 1.5 Stdn. unter Rückfluß gekocht. Das ausgefallene Rohprodukt wird aus Aceton–Methanol umkristallisiert. Ausb. 0.91 g (72%), gelbe Nadeln, Schmp. 159–160°, $[\alpha]_D^{22}$ +7.2° (*c* 1.11, Chloroform) ($C_{25}H_{22}N_4O_8$ Ber.: C, 59.28; H, 4.37; N, 11.06. Gef.: C, 59.46; H, 4.52; N, 11.02%).

Darstellung der Pentonate (**5**). — Eine Mischung von 30 g (0.2 Mol) einer Pentose, 600 ml Wasser, 3.2 g Calciumbromid und 18 g Calciumcarbonat wird elektrolysiert, wie früher für die Darstellung des Calcium-D-galaktonates[14] beschrieben. Nach einer aufgenommenen Strommenge von 10.7 Ampère·Stunden und negativ ausfallender Fehlingscher Probe wird das Reaktionsgemisch auf 70° erwärmt, das überschüssige Calciumcarbonat abfiltriert und mit 150 ml warmem Wasser gewaschen; die vereinigten Filtrate werden im Vakuum bei 40° Badtemperatur auf 100 ml eingeengt. Das Calcium-D-arabinonat kristallisiert aus und wird aus Wasser umkristallisiert. Die auf 100 ml eingeengte, D-Lyxonat, D-Xylonat bzw. D-Ribonat enthaltende Lösung wird jeweils in 600 ml Methanol eingerührt; die ausgefällten Salze werden abfiltriert und gründlich mit Methanol gewaschen. Nach nochmaligem Umfällen aus Wasser–Methanol und Trocknen bei 78° im Vakuum erhält man die Calciumpentonate als reine, bromidfreie Verbindungen.

Calcium-D-ribonat. — Ausb. 20.4 g (55%), amorphes Pulver, $[\alpha]_D^{20}$ +4.7° (*c* 3.56, Wasser). In der Literatur[28] werden keine Eigenschaften angegeben. ($C_{10}H_{18}CaO_{12}$ Ber.: Ca, 10.82. Gef.: Ca, 11.14%).

Calcium-D-xylonat. — Ausb. 27.0 g (73%), amorphes Pulver, $[\alpha]_D^{20}$ +10.5° (*c* 4.31, Wasser). Lit.[29] für Calcium-D-xylonat-dihydrat, $[\alpha]_D^{20}$ +12.4° (*c* 2, Wasser). ($C_{10}H_{18}CaO_{12}$ Ber.: Ca, 10.82. Gef.: Ca, 10.77%).

Calcium-D-lyxonat. — Ausb. 25.9 g (70%), amorphes Pulver, $[\alpha]_D^{20}$ −5.2° (*c* 1.39, Wasser). ($C_{10}H_{18}CaO_{12}$ Ber.: Ca, 10.82. Gef.: Ca, 11.09%).

Calcium-D-arabinonat-pentahydrat. — Ausb. 39.6 g (86%), Nadeln, Schmp.

160–165° (Zers.), $[\alpha]_D^{20}$ −5.4° (*c* 1.79, Wasser). Lit.[30], Schmp. 99–101°, $[\alpha]_D^{21}$ −6.8° (*c* 2.645, Wasser). ($C_{10}H_{28}CaO_{17}$ Ber.: Ca, 8.71. Gef.: Ca, 9.25%).

*Ammonium-*D*-xylonat.* — Man löst 3.70 g (0.01 Mol) Calcium-D-xylonat in 40 ml Wasser, gibt unter Rühren bei 40° eine Lösung von 1.06 g (0.011 Mol) Ammoniumcarbonat in 5 ml Wasser hinzu, läßt 4 Stdn. bei 20° stehen, filtriert das Calciumcarbonat ab, schüttelt das Filtrat mit Aktivkohle, filtriert, dampft das Filtrat im Vakuum bei 40° zu einem Sirup ein und läßt durch Stehenlassen im Eisschrank kristallisieren. Die Kristallmasse wird mit 20 ml Methanol verrieben, abfiltriert, mit Methanol nachgewaschen und im Vakuum über Diphosphorpentoxyd getrocknet. Ausb. 2.45 g (67%), Prismen, Schmp. 118–122°, $[\alpha]_D^{20}$ +19.1° (*c* 2.06, Wasser). Lit.[15], Schmp. 120–122°, $[\alpha]_D^{27}$ +6.2°.

*Kalium-*D*-arabinonat.* — Man löst 4.60 g (0.01 Mol) Calcium-D-arabinonat-pentahydrat unter Erwärmen in 80 ml Wasser, fügt unter Rühren 1.52 g (0.011 Mol) Kaliumcarbonat in 10 ml Wasser hinzu und arbeitet auf wie zuvor angegeben. Die im Vakuum bei 40° auf 25 ml eingeengte Lösung läßt man unter Rühren in 200 ml Methanol eintropfen. Das in Blättchen ausfallende Kalium-D-arabinonat wird bei 78° im Vakuum getrocknet. Ausb. 3.23 g (79%), Schmp. 218–220° (Zers.), $[\alpha]_D^{20}$ −5.7° (*c* 2.95, Wasser). Lit.[31], Schmp. 220° (Zers.), $[\alpha]_D^{20}$ −5.0 ±1° (*c* 5, Wasser).

Darstellung der Di-O-benzyliden-pentonsäuren (**2a-d**). — (*a*) Man löst 3.26 g (0.01 Mol)* einer Di-*O*-benzyliden-*aldehydo*-pentose[2–4] in 80 ml Dimethylformamid, gibt eine Lösung von 2.0 g Chromtrioxyd und 1.6 ml konz. Schwefelsäure in 80 ml Dimethylformamid hinzu, läßt 5 Stdn. bei 20° stehen, erwärmt 5 Min. auf 80°, kühlt auf 20° ab, gießt in einen Scheidetrichter, der je 140 ml Äther und Wasser enthält, schüttelt durch, extrahiert die wäßrige Schicht noch viermal mit je 40 ml Äther, wäscht die vereinigten Äther-Extrakte zweimal mit je 100 ml Wasser und schüttelt sie dann mit 200 ml 5-proz. Natriumcarbonat-Lösung. Die wäßrige Schicht, welche das Natriumsalz der Di-*O*-benzyliden-pentonsäure enthält, wird zweimal mit je 40 ml Chloroform extrahiert (die Chloroform-Extrakte werden verworfen), dann mit 80 ml Chloroform** unterschichtet und unter Umschütteln bis zu einem pH-Wert von 2–3 mit 2N HCl versetzt. Man trennt die Chloroform-Schicht ab, extrahiert die wäßrige Phase noch dreimal mit je 40 ml Chloroform**, wäscht die vereinigten Chloroform-Extrakte zweimal mit 100 ml Wasser**, trocknet über Natriumsulfat, destilliert das Chloroform (zuletzt im Vakuum) ab und kristallisiert den Rückstand um.

(*b*) Ammonium-D-xylonat (1.83 g; 0.01 Mol) wird mit 15 ml Benzaldehyd und 2.0 ml konz. Salzsäure 8 Stdn. bei 20° geschüttelt. Anschließend versetzt man das Reaktionsgemisch bis zu einem pH-Wert von 8 mit 5-proz. Natriumcarbonat-Lösung, schüttelt zum Entfernen des Benzaldehyds dreimal mit je 50 ml Äther und arbeitet die wäßrige Phase auf, wie unter (*a*) beschrieben.

(*c*) Man erwärmt 1.78 g (0.005 Mol) eines Di-*O*-benzyliden-pentonsäure-

*Die 2,4:3,5-Di-*O*-benzyliden-*aldehydo*-D-lyxose wurde als kristallisiertes Monohydrat (0.01 Mol = 3.44 g) eingesetzt.

**Für die Darstellung der 2,4:3,5-Di-*O*-benzyliden-D-xylonsäure nimmt man die doppelte Menge warmes Chloroform und verwendet warmes Wasser (60°) zum Waschen.

methylesters (siehe unten) mit 45 ml 10-proz. methylalkoholischer Kalilauge 90 Min. unter Rückfluß, dampft die Lösung im Vakuum bei 40° ein, nimmt den Rückstand in 50 ml Wasser auf und schüttelt mit 30 ml Chloroform durch. Der Chloroform-Extrakt wird verworfen. Die wäßrige Schicht wird mit 40 ml Chloroform** unterschichtet und unter Umschütteln bis zu einem pH-Wert 2-3 mit 2N HCl versetzt. Man arbeitet dann weiter auf, wie oben unter (*a*) angegeben.

(*d*) D-Xylonsäure-γ-lacton[12] (1.48 g; 0.01 Mol) wird mit 15 ml Benzaldehyd und 1.5 ml konz. Salzsäure 8 Stdn. bei 20° geschüttelt. Anschließend versetzt man das Reaktionsgemisch bis zu einem pH-Wert von 8 mit 5-proz. Natriumcarbonat-Lösung und schüttelt zum Entfernen des Benzaldehyds dreimal mit je 50 ml Äther. Die wäßrige Phase unterschichtet man mit 80 ml Chloroform, fügt bis zu einem pH-Wert von 2-3 2N HCl hinzu und arbeitet auf, wie oben unter (*a*) angegeben. Die Eigenschaften und Analysen der einzelnen Verbindungen sind aus Tabelle I zu ersehen.

Darstellung der Pentonsäure-methylester (**6a-d**). — (*a*) Man rührt 3.70 g (0.01 Mol) eines Calcium-pentonates (**5**) mit 50 ml Methanol und 0.7 ml konz. Schwefelsäure 8 Stdn. bei 65°, filtriert dann das abgeschiedene Calciumsulfat ab, wäscht dreimal mit je 30 ml warmem Methanol, schüttelt das vereinigte Filtrat mit 10 g eines Anionenaustauschers (Wofatit L) bis zur neutralen Reaktion der Lösung, filtriert den Austauscher ab, wäscht mit Methanol nach und dampft im Vakuum bei 40° zu einem Sirup ein, der im Hochvakuum bei 20–30° getrocknet wird.

(*b*) In eine Aufschlämmung von 20.4 g (0.1 Mol) Kalium-D-arabinonat in 60 ml Methanol leitet man bis zur Sättigung einen kräftigen Strom von Chlorwasserstoff, rührt dann 45 Min., erhitzt auf 60°, filtriert das ausgefallene Kaliumchlorid ab, wäscht mit 100 ml warmem Methanol nach, engt das Filtrat auf 50 ml ein, filtriert den ausgefallenen Ester ab, trocknet über Kaliumhydroxyd und kristallisiert um. Die einzelnen Methylester sind in Tabelle I aufgeführt.

Darstellung der Di-O-benzyliden-pentonsäure-methylester (**3a-d**). — (*a*) Zu einer Lösung von 3.42 g (0.01 Mol) einer Di-*O*-benzyliden-pentonsäure in 250 ml Methanol (für die 2,4:3,5-Di-*O*-benzyliden-D-xylonsäure nimmt man 450 ml Methanol) fügt man unter Eiskühlung soviel einer ätherischen Diazomethan-Lösung hinzu, bis die gelbe Farbe bestehen bleibt, läßt 2 Stdn. stehen, destilliert das Lösungsmittel ab und kristallisiert den Rückstand um.

(*b*) Man schüttelt 1.80 g (0.01 Mol) eines Pentonsäure-methylesters mit 15 ml Benzaldehyd, 2.0 g wasserfreiem Zinkchlorid und 1.0 g Natriumsulfat 4 Stdn. bei 20°, fügt anschließend 100 ml Äther hinzu, wäscht die ätherische Lösung zweimal mit je 100 ml Wasser (bei der Umsetzung des D-Lyxonsäure-methylesters enthält das Waschwasser das 3,5-*O*-Benzyliden-D-lyxonsäure-γ-lacton), einmal mit 80 ml 5-proz. Natriumhydrogencarbonat-Lösung und schließlich dreimal mit je 100 ml Wasser. Dann destilliert man den Äther ab, gibt zum Rückstand 100 ml Wasser und destilliert im Vakuum bei 40° Badtemperatur den Benzaldehyd mit Wasser ab. Diese Wasserdampfdestillation wird wiederholt, bis der Benzaldehyd übergetrieben ist. Den Rückstand nimmt man in 100 ml Chloroform auf, trocknet mit Natriumsulfat, destilliert das Chloroform ab und kristallisiert den Rückstand um.

TABELLE I

EIGENSCHAFTEN UND ANALYSEN DER DI-*O*-BENZYLIDEN-PENTONSÄUREN (**2a-d**), PENTONSÄURE-METHYLESTER (**6a-d**), DI-*O*-BENZYLIDEN-PENTONSÄURE-METHYLESTER (**3a-d**), DES 3,5-*O*-BENZYLIDEN-D-ARABINONSÄURE-METHYLESTERS (**12**) UND DES 2,4-DI-*O*-ACETYL-3,5-*O*-BENZYLIDEN-D-ARABINONSÄURE-METHYLESTERS (**11**).

Verbindung	*Ausbeute %*	*Umkristall. aus*	*Kristallform*	*Schmp. bzw.*	$n_D^{21}[\alpha]^{20}$	*Formel*	*Analyse*	
							C	*H*
2,4:3,5-Di-*O*-benzyliden-D-ribonsäure (**2a**)	nach (*a*) 64, nach (*c*) 60	Chloroform–Petroläther	Nädelchen	189.5–190.5°	−48.8° *c* 2.84, DMF	$C_{19}H_{18}O_6$	Ber. 66.64 Gef. 66.29	Ber. 5.30 Gef. 5.47
2,4:3,5-Di-*O*-benzyliden-D-xylonsäure (**2b**)	nach (*a*) 70, nach (*b*) 55 nach (*c*) 73 nach (*d*) 52	Chloroform–Petroläther	Nädelchen	198.5–200.0°[a]	−21.3°[a] *c* 1.05, DMF	Lit.[11]		
2,4:3,5-Di-*O*-benzyliden-D-lyxonsäure (**2c**)	nach (*a*) 61, nach (*c*) 64	Aceton–Wasser	Nadeln	164–165°	−32.1° *c* 1.67, DMF	$C_{19}H_{18}O_6$	Ber. 66.64 Gef. 66.60	Ber. 5.30 Gef. 5.30
2,3:4,5-Di-*O*-benzyliden-D-arabinonsäure (**2d**)	nach (*c*) 78	Chloroform–Petroläther	Blättchen	119.5–122°	−13.2° *c* 2.39, DMF	$C_{19}H_{18}O_6$	Ber. 66.64 Gef. 66.62	Ber. 5.30 Gef. 5.47
D-Ribonsäure-methylester (**6a**)	nach (*a*) 75		Sirup	1.4892	−5.1° *c* 3.30, Wasser	$C_6H_{12}O_6$	Ber. 39.99 Gef. 40.21	Ber. 6.71 Gef. 6.95
D-Xylonsäure-methylester (**6b**)	nach (*a*) 91		Sirup	1.4876	+15.2° *c* 3.21, Wasser	$C_6H_{12}O_6$	Ber. 39.99 Gef. 40.31	Ber. 6.71 Gef. 6.53
D-Lyxonsäure-methylester (**6c**)	nach (*a*) 74		Sirup	1.4915	+20.5° *c* 1.74, Wasser	$C_6H_{12}O_6$	Ber. 39.99 Gef. 40.05	Ber. 6.71 Gef. 6.84
D-Arabinonsäure-methylester (**6d**)	nach (*b*) 79	Methanol	Nadeln	140–141°[b]	+12.6° *c* 2.20, Wasser[b]	Lit.[32]		
2,4:3,5-Di-*O*-benzyliden-D-ribonsäure-methylester (**3a**)	nach (*a*) 96	Aceton–Wasser	Nadeln	135.5–137.5°	−44.4° *c* 2.33, Chlf.	$C_{20}H_{20}O_6$	Ber. 67.39 Gef. 67.29	Ber. 5.66 Gef. 5.95
2,4:3,5-Di-*O*-benzyliden-D-xylonsäure-methylester (**3b**)	nach (*a*) 96 nach (*b*) 41	Aceton–Wasser	Nadeln	191.5–194.5°	+8.8° *c* 1.68, Chlf.	$C_{20}H_{20}O_6$	Ber. 67.39 Gef. 67.31	Ber. 5.66 Gef. 5.77
2,4:3,5-Di-*O*-benzyliden-D-lyxonsäure-methylester (**3c**)	nach (*a*) 95 nach (*b*) 24	Aceton–Wasser	Nadeln	182–182.5°	−35.9° *c* 1.84, Chlf.	$C_{20}H_{20}O_6$	Ber. 67.39 Gef. 67.25	Ber. 5.66 Gef. 5.83
2,3:4,5-Di-*O*-benzyliden-D-arabinonsäure-methylester (**3d**)	nach (*a*) 95 nach (*c*) 18	Methanol	Nadeln	89.5–91°	−16.5° *c* 1.68, Chlf.	$C_{20}H_{20}O_6$	Ber. 67.39 Gef. 67.41	Ber. 5.66 Gef. 5.88
3,5-*O*-Benzyliden-D-arabinonsäure-methylester (**12**)	nach (*c*) 5	Wasser	Nadeln	154–157.5°	−42.9° *c* 2.33, DMF	$C_{13}H_{16}O_6$	Ber. 58.19 Gef. 58.16	Ber. 6.01 Gef. 6.10
2,4-Di-*O*-acetyl-3,5-*O*-benzyliden-D-arabinonsäure-methylester[c] (**11**)	65	Äthanol–Wasser	Blättchen	110–111°	−22.3° *c* 2.18, DMF	$C_{17}H_{20}O_8$	Ber. 57.95 Gef. 57.84	Ber. 5.72 Gef. 5.82

[a]Lit.[11]: Schmp. 199°, $[\alpha]_D$ −22° (*c* 0.32, Methanol). [b]Lit.[32]: Schmp. 148°, $[\alpha]_D$ +6° (*c* 5.56, Wasser). [c]Die Verbindung wird aus **12** durch Acetylieren mit Acetanhydrid in Pyridin gewonnen.

(*c*) D-Arabinonsäure-methylester (1.80 g; 0.01 Mol) wird zunächst behandelt, wie zuvor unter (*b*) angegeben. Den Rückstand, der nach dem Abdestillieren des Benzaldehyds mit Wasserdampf übrigbleibt, extrahiert man dreimal mit je 30 ml heißem Wasser (80°) und filtriert. Aus dem Filtrat erhält man durch Eindampfen im Vakuum bei 40° den 3,5-*O*-Benzyliden-D-arabinonsäure-methylester, den man durch Umkristallisieren aus Wasser unter Verwendung von Aktivkohle reinigt. Der bei der Extraktion mit heißem Wasser unlösliche Anteil besteht aus dem 2,3:4,5-Di-*O*-benzyliden-D-arabinonsäure-methylester, der aus Methanol umkristallisiert wird. Die einzelnen Di-*O*-benzyliden-pentonsäure-methylester sind in Tabelle I aufgeführt.

Darstellung der O-*Benzyliden-pentonsäure-lactone.* — (*a*) Man schüttelt 1.80 g (0.01 Mol) eines Pentonsäure-methylesters mit 15 ml Benzaldehyd und 2.0 g wasserfreiem Zinkchlorid 4 Stdn. bei 20°. Dann arbeitet man auf, wie oben für die Darstellung von **3** unter (*b*) beschrieben. Das *O*-Benzyliden-D-lyxonsäure-γ-lacton kristallisiert beim Stehenlassen aus dem Waschwasser aus, wird abfiltriert und unter Verwendung von Aktivkohle umkristallisiert.

(*b*) Man schüttelt 1.48 g (0.01 Mol) eines Pentonsäure-lactons[12,19] mit 15 ml Benzaldehyd und 2.0 g wasserfreiem Zinkchlorid 7 Stdn. bei 20° und arbeitet das *O*-Benzyliden-D-ribonsäure-δ-lacton auf, wie oben zur Darstellung von **3** unter (*b*) angegeben. Das *O*-Benzyliden-D-lyxonsäure-γ-lacton (**4**) fällt aus, wenn man 50 ml Äther und 50 ml Wasser hinzugibt; **4** wird abgesaugt, mit 100 ml Wasser, 50 ml Äther und 50 ml Petroläther gewaschen und unter Verwendung von Aktivkohle umkristallisiert.

(*c*) Man schüttelt 1.85 g (0.005 Mol) eines Calcium-pentonates (**5**) mit 15 ml Benzaldehyd und 2 ml konz. Salzsäure 15 Stdn. bei 20°, fügt dann 50 ml Äther hinzu, neutralisiert unter Umrühren mit 5-proz. Natriumcarbonat-Lösung, filtriert das dabei ausfallende Rohprodukt ab, wäscht mit 50 ml Äther und mit 50 ml Petroläther und kristallisiert um.

(*d*) Man schüttelt 1.48 g (0.01 Mol) eines D-Pentonsäure-lactons 7 Stdn. bei 20° mit 15 ml Benzaldehyd und 1.5 ml konz. Salzsäure. Dann arbeitet man auf, wie zuvor unter (*c*) angegeben.

(*e*) Man schüttelt 1.80 g (0.01 Mol) eines D-Pentonsäure-methylesters mit 15 ml Benzaldehyd und 2 ml konz. Salzsäure 4 Stdn. bei 20°. Das Aufarbeiten erfolgt, wie unter (*c*) angegeben.

(*f*) Man schüttelt eine Lösung von 2.36 g (0.01 Mol) 2,4-*O*-Benzyliden-D-ribonsäure-δ-lacton in 30 ml Benzaldehyd und 2.5 ml konz. Salzsäure 8 Stdn. bei 20° und arbeitet wie unter (*c*) auf. Die einzelnen Vertreter sowie ihre *O*-Acetyl- und *O*-Benzoyl-Derivate zeigt Tabelle II.

Darstellung der O-*Benzyliden-pentonsäure-amide.* — Man löst 1.18 g (0.005 Mol) eines *O*-Benzyliden-pentonsäure-lactons (**4**, **19**) oder 1.34 g (0.005 Mol) 3,5-*O*-Benzyliden-D-arabinonsäure-methylester (**12**) in 75 ml Methanol. In die Lösung leitet man unter Eiskühlung 15 Min. Ammoniak (4–6 Blasen pro Sek.) ein, läßt 16 Stdn. bei 20° verschlossen stehen, dampft dann im Vakuum ein und kristallisiert

TABELLE II

EIGENSCHAFTEN UND ANALYSEN DER *O*-BENZYLIDEN-PENTONSÄURE-LACTONE UND IHRER *O*-ACETYL- UND *O*-BENZOYL-DERIVATE, DER *O*-BENZYLIDEN-PENTONSÄURE-AMIDE, *O*-BENZYLIDEN-PENTONSÄURE-HYDRAZIDE, *O*-BENZYLIDEN-PENTITE UND DES 2,3:4,5-DI-*O*-BENZYLIDEN-D-ARABINITS.

Verbindung	*Ausbeute %*	*Umkristall. aus*	*Kristallform*	*Schmp.*	$[\alpha]_D^{20}$	*Formel*	*Analyse*		
							C	*H*	*N*
2,4-*O*-Benzyliden-D-ribonsäure-δ-lacton (8)	nach (*a*) 12 nach (*b*) 22	Chloroform–Benzin	Nadeln	160–164°	−70.2° *c* 1.23, DMF	$C_{12}H_{12}O_5$	Ber. 61.02 Gef. 60.88	5.12 5.12	
3,5-*O*-Benzyliden-D-ribonsäure-γ-lacton (7)	nach (*c*) 52 nach (*d*) 70 nach (*e*) 60 nach (*f*) 96	Aceton–Petroläther	Nadeln	233–235.5°	−174.1° *c* 2.26, DMF	$C_{12}H_{12}O_5$	Ber. 61.02 Gef. 61.06	5.12 4.77	
3,5-*O*-Benzyliden-D-lyxonsäure-γ-lacton (4)	nach (*a*) 19 nach (*b*) 31 nach (*c*) 48 nach (*d*) 69	Aceton–Petroläther	Nadeln	203.5–206°	+31.6° *c* 2.31, DMF	$C_{12}H_{12}O_5$	Ber. 61.02 Gef. 61.20	5.12 5.32	
3-*O*-Acetyl-2,4-*O*-benzyliden-D-ribonsäure-δ-lacton (26)	70	Methanol–Wasser	Blättchen	143–145.5°	−71.2° *c* 1.56, Chlf.	$C_{14}H_{14}O_6$	Ber. 60.42 Gef. 60.63	5.07 5.03	
2-*O*-Acetyl-3,5-*O*-benzyliden-D-ribonsäure-γ-lacton (29)	70	Äthanol	Nadeln	180.5–182.5°	−158.4° *c* 2.87, DMF	$C_{14}H_{14}O_6$	Ber. 60.42 Gef. 60.31	5.07 5.42	
2-*O*-Acetyl-3,5-*O*-benzyliden-D-lyxonsäure-γ-lacton (19)	71	Äthanol	Prismen	136.5–138.5°	+141.4° *c* 2.44, Chlf.	$C_{14}H_{14}O_6$	Ber. 60.42 Gef. 60.44	5.07 5.34	
3-*O*-Benzoyl-2,4-*O*-benzyliden-D-ribonsäure-δ-lacton (25)	70	Äthanol	Blättchen	181–182.5°	−75.4° *c* 1.72, Chlf.	$C_{19}H_{16}O_6$	Ber. 67.05 Gef. 66.85	4.74 4.83	
2-*O*-Benzoyl-3,5-*O*-benzyliden-D-ribonsäure-γ-lacton (28)	70	Aceton–Wasser	Nadeln	188–190°	−241.3° *c* 1.27, Chlf.	$C_{19}H_{16}O_6$	Ber. 67.05 Gef. 67.01	4.74 4.97	

2-*O*-Benzoyl-3,5-*O*-benzyliden-D-lyxonsäure-γ-lacton (**18**)	72	Aceton–Wasser	Nadeln	181–183°	+145.8° *c* 1.92, Chlf.	$C_{19}H_{16}O_6$	Ber. 67.05 Gef. 67.32	4.74 4.96	
3,5-*O*-Benzyliden-D-lyxonsäure-amid (**21**)	73	Äthanol	Nadeln	180–183°	+3.8° *c* 2.74, DMF	$C_{12}H_{15}NO_5$	Ber. 56.89 Gef. 56.86	5.97 5.78	5.53 5.66
2-*O*-Acetyl-3,5-*O*-benzyliden-D-lyxonsäure-amid (**22**)	63	Aceton–Petroläther	Nadeln	184°	+1.9° *c* 1.67, DMF	$C_{14}H_{17}NO_6$	Ber. 56.94 Gef. 57.07	5.80 5.93	4.74 5.01
3,5-*O*-Benzyliden-D-arabinonsäure-amid (**14**)	72	Aceton–Petroläther	Nadeln	200–203°	−66.5° *c* 2.09, DMF	$C_{12}H_{15}NO_5$	Ber. 56.89 Gef. 56.88	5.97 6.02	5.53 5.68
2,4-*O*-Benzyliden-D-ribonsäure-hydrazid (**27**)	66	Propanol–*n*-Hexan	Nadeln	126.5–130°	−5.8° *c* 1.68, DMF	$C_{12}H_{16}N_2O_5$	Ber. 53.72 Gef. 53.50	6.01 6.28	10.44 10.37
3,5-*O*-Benzyliden-D-lyxonsäure-hydrazid (**20**)	68	Äthanol	Nadeln	181.5–183°	+10.5° *c* 1.66, DMF	$C_{12}H_{16}N_2O_5$	Ber. 53.72 Gef. 53.80	6.01 6.14	10.44 10.45
3,5-*O*-Benzyliden-D-arabinonsäure-hydrazid (**13**)	70	Propanol–*n*-Hexan	Nadeln	186.5–190°	−72.2° *c* 1.16, DMF	$C_{12}H_{16}N_2O_5$	Ber. 53.72 Gef. 53.50	6.01 6.01	10.44 10.48
2,4-*O*-Benzyliden-ribit (**24**)	60	Chloroform	Nadeln	133–135°[a]	±0°[a] *c* 1.98, Methanol	Lit.[22]			
1,3-*O*-Benzyliden-D-arabinit (**23**)	75	Aceton–Petroläther	Nadeln	150.5–152°[b]	−7.3°[b] *c* 1.37, Pyridin	Lit.[21]			
3,5-*O*-Benzyliden-D-arabinit (**16**)	75	Aceton–Petroläther	Nadeln	149.5–151.5°	−56.6° *c* 1.29, Pyridin	$C_{12}H_{16}O_5$	Ber. 59.97 Gef. 59.89	6.71 6.56	
2,3:4,5-Di-*O*-benzyliden-D-arabinit (**9**)	76 bzw 68	Aceton–Benzin	Prismen	101–105°	+2.5° *c* 2.63, Pyridin	$C_{19}H_{20}O_5$	Ber. 69.49 Gef. 69.28	6.14 6.14	
1-*O*-Acetyl-2,3:4,5-di-*O*-benzyliden-D-arabinit (**10**)	90	Äthanol–Wasser	Nädelchen	49–51°	−4.4° *c* 2.27, Chlf.	$C_{21}H_{22}O_6$	Ber. 68.09 Gef. 68.03	5.99 6.04	

[a] Lit.[22]: Schmp. 133.5–134.5°, $[\alpha]_D^{27}$ 0 ±0.2° (*c* 2, Äthanol). [b] Lit.[21]: Schmp. 151–152°, $[\alpha]_D^{20}$ −7.6° (*c* 2, Pyridin).

den Rückstand unter Verwendung von Aktivkohle um. Die einzelnen Verbindungen sind in Tabelle II aufgeführt.

Darstellung der O-Benzyliden-pentonsäure-hydrazide. — Man kocht 1.18 g (0.005 Mol) eines *O*-Benzyliden-pentonsäure-lactons (**4**, **8**) oder 1.34 g (0.005 Mol) 3,5-*O*-Benzyliden-D-arabinonsäure-methylester (**12**) in 30 ml Methanol mit 6.0 ml 85-proz. Hydrazinhydrat 3 Stdn. unter Rückfluß, dampft dann im Vakuum bei 40° Badtemperatur ein und kristallisiert den Rückstand unter Verwendung von Aktivkohle um. In Tabelle II sind die einzelnen Vertreter aufgeführt.

Darstellung der O-Benzyliden-pentite. — 2,3:4,5-Di-*O*-benzyliden-D-arabinonsäure-methylester (**3d**) (1.78 g; 0.005 Mol) oder rohe 2,3:4,5-Di-*O*-benzyliden-*aldehydo*-D-arabinose (**1d**) (1.63 g; 0.005 Mol) oder 1.18 g (0.005 Mol) eines *O*-Benzyliden-pentonsäure-lactons (**8**, **4**) oder 3,5-*O*-Benzyliden-D-arbinonsäure-methylester (**12**) (1.34 g; 0.005 Mol.) werden in 150 ml Dioxan-Äther (2:1) mit 0.8 g $LiAlH_4$ 6 Stdn. unter Rückfluß gerührt. Dann kühlt man auf 0° ab, fügt langsam 5 ml Wasser hinzu, neutralisiert unter Kühlen mit 3N HCl, filtriert die anorganischen Bestandteile ab, wäscht mit 150 ml warmem Aceton nach, dampft die vereinigten Filtrate ein und kristallisiert den Rückstand um. Einzelne *O*-Benzyliden-pentite sind in Tabelle II aufgeführt.

Aceton-[(3,5-O-benzyliden-D-arabinonyl)-hydrazon] (**17**). — Man kocht 1.34 g (0.005 Mol) 3,5-*O*-Benzyliden-D-arabinonsäure-hydrazid (**13**) in 20 ml Aceton 10 Min. unter Rückfluß, läßt abkühlen und kristallisiert das ausgefallene Rohprodukt aus Aceton–Benzin (Sdp. 90–110°) um. Ausb. 1.45 g (94%), Blättchen, Schmp. 214–216°, $[\alpha]_D^{20}$ −31.2° (*c* 1.23, Dimethylformamid) ($C_{15}H_{20}N_2O_5$ Ber.: C, 58.43; H, 6.54; N, 9.09. Gef.: C, 58. 13; H, 6.42; N, 9.24%).

2,4-O-Benzyliden-D-erythrit (**15**). — 3,5-*O*-Benzyliden-D-arabinit (0.60 g; 0.0025 Mol) wird in 40 ml Wasser gelöst und mit einer Lösung von 0.54 g Natriumperjodat in 20 ml Wasser und 0.13 g Natriumhydrogencarbonat versetzt. Man läßt 6 Stdn. bei 20° stehen, dampft die Lösung ein, fügt 20 ml absol. Äthanol hinzu, dampft wieder zur Trockene ein, extrahiert den Rückstand dreimal mit je 50 ml heißem Essigester, wäscht die vereinigten Extrakte zweimal mit je 70 ml Wasser, trocknet mit Natriumsulfat und dampft die Lösung ein. Die zurückbleibende rohe 2,4-*O*-Benzyliden-D-erythrose löst man in 70 ml Äthanol, gibt 1.0 g Natriumborhydrid in 10 ml Wasser hinzu, läßt 4 Stdn. bei 20° stehen, dampft die Lösung bei 40° Badtemperatur im Vakuum ein, extrahiert den Rückstand dreimal mit je 50 ml heißem Essigester, wäscht die vereinigten Extrakte zweimal mit je 50 ml 10-proz. Natriumsulfat-Lösung, trocknet mit Natriumsulfat, dampft ein und kristallisiert den Rückstand aus Chloroform um. Ausb. 0.45 g (85%), Blättchen, Schmp. 135.5–137°, $[\alpha]_D^{20}$ −14.4° (*c* 0.51, Chloroform). Lit.[20], Schmp. 135–137°, $[\alpha]_D$ −14° (*c* 0.5, Chloroform).

LITERATUR UND ANMERKUNGEN

1 K. GÄTZI UND T. REICHSTEIN, *Helv. Chim. Acta*, 21 (1938) 914.
2 H. ZINNER UND H. SCHMANDKE, *Ber.*, 94 (1961) 1304.
3 H. ZINNER, G. REMBARZ, H.-W. LINKE UND G. ULBRICHT, *Ber.*, 90 (1957) 1761; H. ZINNER, K.-H. ROHDE UND A. MATTHEUS, *Ann.*, 677 (1964) 160.

4 H. ZINNER, W. REHDER UND H. SCHMANDKE, *Ber.*, 95 (1962) 1805.
5 H. ZINNER, B. RICHARD, M. BLESSMANN UND M. SCHLUTT, *Carbohyd. Res.*, 2 (1966) 197.
6 Auf die Beschreibung dieser Darstellungsmethode wurde im „Experimentellen Teil" verzichtet, weil sich die Di-*O*-benzyliden-pentonsäuren durch Oxydation der Di-*O*-benzyliden-*aldehydo*-pentosen mit Chromtrioxyd in Dimethylformamid bei Gegenwart von Schwefelsäure mit wesentlich besseren Ausbeuten darstellen lassen.
7 D. H. RAMMLER UND C. A. DEKKER, *J. Org. Chem.*, 26 (1961) 4615.
8 E. WALTON, J. O. RODIN, C. H. STAMMER, F. W. HOLLY UND K. FOLKERS, *J. Am. Chem. Soc.*, 80 (1958) 5168.
9 K. HEYNS UND H. PAULSEN, *Advan. Carbohydrate Chem.*, 17 (1962) 169.
10 G. SNATZKE, *Ber.*, 94 (1961) 729.
11 W. ALBERDA VAN EKENSTEIN UND C. A. LOBRY DE BRUYN, *Rec. Trav. Chim.*, 18 (1899) 306; zit. nach Beilstein, 1. Werk, Band 19, Seite 454.
12 J. U. NEF, *Ann.*, 403 (1914) 204.
13 H. KILIANI, *Ber.*, 65 (1932) 1269; 66 (1933) 117.
14 H. ZINNER, H. BRANDNER UND G. REMBARZ, *Ber.*, 89 (1956) 800.
15 HSI CH'OU FANG, *Iowa State Coll. J. Sci.*, 6 (1932) 423; zit. nach *Chem. Zentr.*, (1933) I, 1012.
16 K. MAURER UND A. MÜLLER, *Ber.*, 63 (1930) 2069.
17 R. J. DIMLER UND K. P. LINK, *J. Biol. Chem.*, 150 (1943) 345.
18 N. SPERBER, H. E. ZAUGG UND W. M. SANDSTROM, *J. Am. Chem. Soc.*, 69 (1947) 915.
19 M. STEIGER, *Helv. Chim. Acta*, 19 (1936) 189.
20 A. B. FOSTER, A. H. HAINES, J. HOMER, J. LEHMANN UND L. F. THOMAS, *J. Chem. Soc.*, (1961) 5005.
21 W. T. HASKINS, R. M. HANN UND C. S. HUDSON, *J. Am. Chem. Soc.*, 65 (1943) 1663.
22 D. J. J. POTGIETER UND D. L. MACDONALD, *J. Org. Chem.*, 26 (1961) 3934.
23 H. S. ISBELL UND H. L. FRUSH, *Methods Carbohydrate Chem.*, 2 (1963) 16.
24 R. M. HANN UND C. S. HUDSON, *J. Am. Chem. Soc.*, 66 (1944) 1909.
25 S. A. BARKER UND E. J. BOURNE, *Advan. Carbohydrate Chem.*, 7 (1952) 137.
26 H. ZINNER UND E. WITTENBURG, *Ber.*, 94 (1961) 1298.
27 C. F. HUEBNER, P. A. PANKRATZ UND K. P. LINK, *J. Am. Chem. Soc.*, 72 (1950) 4811.
28 K. LADENBURG, M. TISHLER, J. W. WELLMAN UND R. D. BABSON, *J. Am. Chem. Soc.*, 66 (1944) 1217.
29 H. S. ISBELL, *J. Res. Natl. Bur. Std.*, 14 (1935) 307, 315; zit. nach Beilstein *3. Ergänzungswerk*, Band 3, Seite 983.
30 W. G. OVEREND, M. STACEY UND L. F. WIGGINS, *J. Chem. Soc.*, (1949) 1358.
31 N. K. RICHTMYER UND C. S. HUDSON, *J. Am. Chem. Soc.*, 64 (1942) 1609.
32 J. DUBOURG UND P. NAFFA, *Bull. Soc. Chim. France*, (1959) 1353.

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1,2:3,4-DI-*O*-ISOPROPYLIDENE-α-D-*galacto*-HEXODIALDO-1,5-PYRANOSE AND ITS 6-ALDEHYDROL*†

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ABSTRACT

Oxidation of 1,2:3,4-di-*O*-isopropylidene-α-D-galactopyranose (**1**) with methyl sulfoxide–*N*,*N*′-dicyclohexylcarbodiimide–pyridinium phosphate (Pfitzner–Moffatt reagent) furnishes a preparative route to the title aldehyde **2**. In aqueous solution, **2** exists entirely in the form of its 6-aldehydrol (**8**). In common with a number of other *aldehydo* sugar derivatives, compound **2** exhibits zero spin-coupling between the aldehyde proton and the proton vicinal to it. The aldehyde **2** was characterized as the crystalline 6-oxime (**3**) and 6-(*p*-nitrophenyl)hydrazone (**7**). Compounds **2**, **3**, and **7** are important as potential intermediates in chain-extension reactions leading to 6-amino-6,8-dideoxy-octoses related to lincomycin.

INTRODUCTION

The addition of acetylenic or vinylic Grignard reagents to suitably protected *aldehydo* sugars is a useful method for chain extension in the sugar series[1–6], and similar additions to *keto* sugar derivatives furnish a route to branched-chain sugars[7]. The versatility of the ethynyl and vinyl groups in numerous synthetic transformations provides potential access to a wide variety of modified sugars[1–6]. A program in this laboratory has emphasized the development of high-yielding procedures, based on addition of Grignard reagents, for chain-extension and chain-branching reactions, together with effective methods for separation of the diastereoisomeric products formed. Structural characterization of the separated epimers has been achieved by n.m.r. spectroscopy and by degradation to known, crystalline derivatives[4–6].

Although the addition of unsaturated Grignard reagents to *aldehydo* (and *keto*) sugar derivatives can be effected in high yield[4–7], the number of useful preparative routes to the carbonyl derivatives required is limited. Isopropylidene acetals and

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perbenzoic esters of *aldehydo* sugars can be obtained by way of dialkyl dithioacetals of aldoses, and addition of ethynylmagnesium bromide to the aldehyde group has been studied with such acetals[1,4] and esters[5]. Cleavage of suitable, protected glycols furnishes a further route to protected *aldehydo* sugars. In this laboratory, addition of unsaturated Grignard reagents has been effected with 2,3-*O*-isopropylidene-D-glyceraldehyde[1,2,6] and with 1,2-*O*-isopropylidene-α-D-*xylo*-pentodialdo-1,4-furanose[1,2], derived by glycol cleavage from 1,2:5,6-di-*O*-isopropylidene-D-mannitol and 1,2-*O*-isopropylidene-α-D-glucofuranose, respectively. Protected *keto* sugars are readily obtained on a preparative scale by the methyl sulfoxide–acetic anhydride procedure for oxidation of "isolated" secondary alcohols to ketones[8]. However, attempts to prepare a protected *aldehydo* sugar by treating an "isolated" primary alcohol derivative with methyl sulfoxide–acetic anhydride were unsuccessful. Instead of the desired aldehyde, the (methylthio)methyl ether of the alcohol was the principal product[3].

The reagent of Pfitzner and Moffatt[9] (methyl sulfoxide–*N*,*N*′-dicyclohexylcarbodiimide–pyridinium phosphate) can be used for oxidation of nucleosides to the 5′-*aldehydo* derivatives[9], and for oxidation of "isolated" secondary alcohol groups to ketones in sugar derivatives[8,10]. In this laboratory, the use of the Pfitzner–Moffatt oxidant for preparing *aldehydo* sugar derivatives[1] from "isolated" primary alcohol derivatives has presented technical difficulties in preparative-scale work, because separation of the product aldehyde from side-products and excess reagents is not readily achieved.

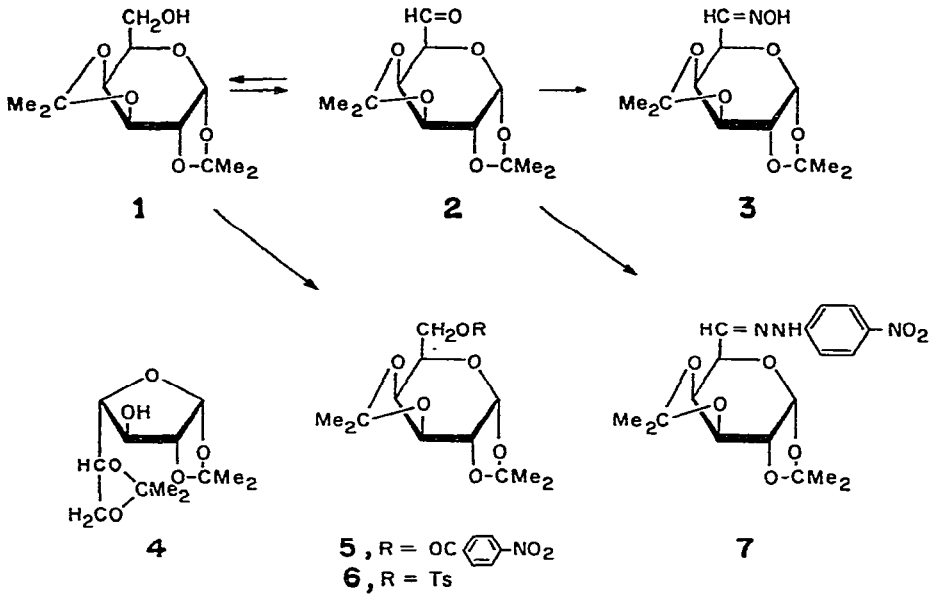
The present report describes the use of the Pfitzner–Moffatt oxidant for preparative conversion of 1,2:3,4-di-*O*-isopropylidene-α-D-galactopyranose (**1**) into the corresponding 6-aldehyde **2**. This aldehyde was characterized by formation of crystalline derivatives, and by n.m.r. spectroscopic studies on the free aldehyde **2**, its derived aldehydrol (**8**), and related *aldehydo* sugar derivatives.

DISCUSSION

Acetonation of D-galactose by the conventional procedure[11] with copper(II) sulfate and sulfuric acid as catalysts gave 1,2:3,4-di-*O*-isopropylidene-α-D-galactopyranose (**1**) as a distilled syrup. It has been pointed out by DeJongh and Biemann[12] that, prepared by this procedure, **1** contains a small proportion (< 3%) of a side product, which they isolated by g.l.c. and formulated on the basis of mass-spectrometric evidence as 1,2:5,6-di-*O*-isopropylidene-α-D-galactofuranose (**4**). T.l.c. of the distilled product isolated in the present work revealed the presence of a small proportion of a side product having a mobility greater than that of **1**. Careful fractional distillation gave pure **1**, b.p. 128–129°/0.8 torr, that was chromatographically homogeneous; the side product boiled at 89–91°/1 torr. Unless the acetal **1** was separated in this way from the low-boiling side-product, much difficulty was experienced in isolating the aldehyde **2** derived by oxidation of **1**.

The purified acetal **1** gave a crystalline *p*-nitrobenzoate (**5**). The *p*-toluenesulfo-

nate (6) of 1 had a specific rotation in agreement with literature values[13], and its m.p. corresponded to that reported[14,15] for a high-melting dimorph of 6.



Oxidation of the pure acetal 1 with the Pfitzner–Moffatt reagent gave the crude aldehyde 2 as a syrup in about 80% yield. Careful control of the isolation procedure was required in order to ensure maximum separation of 2 from the reagents and decomposition products. Although the crude aldehyde 2 gave satisfactory results in reactions with unsaturated Grignard reagents[16], t.l.c. revealed that, in addition to 2, this material contained several side-products, including one that gave a positive Schiff test. The aldehyde 2 could be obtained chromatographically homogeneous and analytically pure by careful fractional distillation of the crude product.

The pure aldehyde 2 gave positive Schiff and Tollens reactions, and showed characteristic absorptions for the aldehyde group in its i.r. spectrum. Reduction of 2 with aqueous sodium borohydride regenerated the parent alcohol 1, characterized as its 6-*p*-toluenesulfonate (6). Treatment of 2 with (*p*-nitrophenyl)hydrazine hydrochloride or with hydroxylamine hydrochloride, in aqueous alcohol containing sodium acetate, did not lead to crystalline derivatives, but, when pyridine was used as the acid acceptor and catalyst, the crystalline oxime (3) and (*p*-nitrophenyl)-hydrazone (7) of the aldehyde 2 were readily obtained.

The n.m.r. spectrum of the aldehyde 2 in chloroform-*d* (Fig. 1) shows the signal of the aldehyde proton (H-6) at τ 0.43. This signal is observed as a singlet, indicating that the vicinal coupling ($J_{5,6}$) is approximately zero. Other *aldehydo* sugar derivatives isolated in this laboratory [1,4–6,17] show the signal of the aldehyde proton at similar field, and the coupling of the aldehyde proton with the proton vicinal to it is either very small or zero (see Table I), except in the case of β-unsaturated aldehyde derivatives[1], where a large (7 Hz) vicinal coupling is observed.

The magnitude of the vicinal coupling for the saturated aldehyde derivatives indicates that the dihedral angle of the aldehyde proton and the proton vicinal to

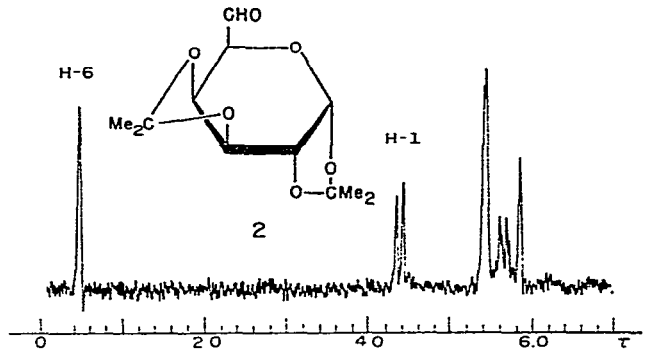
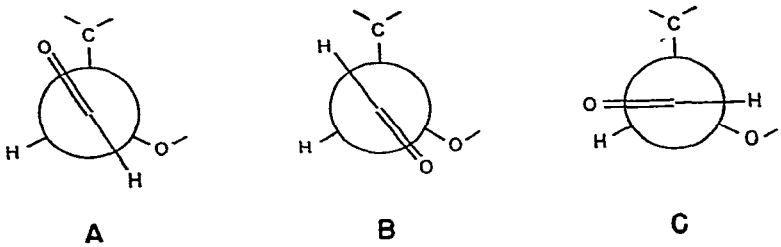


Fig. 1. The low-field portion of the 60-MHz n.m.r. spectrum of 1,2:3,4-di-*O*-isopropylidene-α-D-*galacto*-hexodialdo-1,5-pyranose (2) in chloroform-*d*.

it is approximately 90°, if it can be assumed that the Karplus relationship is valid for such systems. This arrangement would require the carbonyl group to be almost eclipsed by a carbon atom (rotamer A) or an oxygen atom (rotamer B) attached to the vi-



cinal carbon atom; the carbonyl group is definitely not eclipsed by the vicinal hydrogen atom (rotamer C), because this arrangement would lead to a large, vicinal coupling-constant. The favored rotamer state for simple aldehydes is considered[20] to be that in which the carbonyl group is eclipsed by a large substituent. The large, vicinal couplings observed with β-unsaturated aldehydes is consistent with the presumed planarity of the conjugated π-system.

In aqueous solution, the aldehyde **2** exists entirely as the aldehydrol (**8**). The n.m.r. spectrum of **8** (see Fig. 2) shows the H-6 signal at τ 4.95 as a one-proton, wide doublet, $J_{5,6}$ 7.1 Hz, and no aldehyde-proton signal is observed. The resonance of the aldehydrol proton is observed at much higher field than that of the parent aldehyde. The chemical shift of this signal is in the "anomeric proton region", and this observation emphasizes that the aldehydrol form should receive consideration as a possible contributor to tautomeric equilibria whenever free sugars in aqueous solution are examined by n.m.r. spectroscopy.

The large, vicinal coupling observed for the aldehydrol is consistent with a favored rotamer state that has the vicinal protons antiparallel (rotamer D). Similar

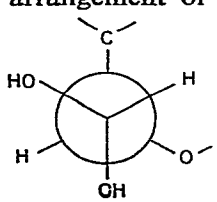
TABLE I

N.M.R. SPECTRAL DATA FOR *aldehydo* SUGAR DERIVATIVES AND RELATED COMPOUNDS[a]

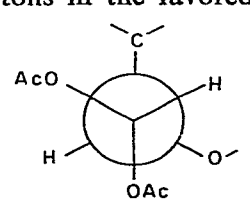
Compound	*Chemical shift of aldehyde[b] proton, τ*	*Coupling of aldehyde[b] proton with vicinal proton, Hz*	*References*
Saturated aldehydes			
4-Acetamido-4,5-dideoxy-2,3-*O*-isopropylidene-*aldehydo*-L-xylose	0.67	0	17
3-*O*-Benzyl-1,2-*O*-isopropylidene-α-D-*xylo*-pentodialdo-1,4-furanose	0.35	3	1,18
2,3:4,5-Di-*O*-isopropylidene-L-arabinose	0.35	0	4[c]
1,2:3,4-Di-*O*-isopropylidene-α-D-*galacto*-hexodialdo-1,5-pyranose (2)	0.43	0	*c*
2,3-*O*-Isopropylidene-D-glyceraldehyde	0.35	1.7	1,6
2,3,4,5-Tetra-*O*-benzoyl-L-arabinose	0.30	0	5
Unsaturated aldehydes			
trans-2,3-Dideoxy-4,5:6,7-di-*O*-isopropylidene-L-*arabino*-hept-2-enose	0.38	7	1
trans-2,3-Dideoxy-4,5-*O*-isopropylidene-D-*glycero*-pent-2-enose	0.55	7	1
Aldehydrols (and acetates)			
1,2:3,4-Di-*O*-isopropylidene-α-D-*galacto*-hexodialdo-1,5-pyranose 6-aldehydrol[d] (8)	4.95	7.1	*c*
1,1-Di-*O*-acetyl-2,3,4,5-tetra-*O*-benzoyl-L-arabinose aldehydrol	2.80	7	5
1,1-Di-*O*-acetyl-2,3-*O*-iospropylidene-D-glyceraldehyde aldehydrol	3.20	4.7	6
Dithioacetals			
2,3,4,5-Tetra-*O*-acetyl-L-arabinose diethyl dithioacetal	6.13	7.8	19
2,3,4,5-Tetra-*O*-acetyl-L-arabinose diphenyl dithioacetal	5.80	5.6	19
2,3,4,5-Tetra-*O*-benzoyl-L-arabinose diethyl dithioacetal	5.68	6.9	5

[a]For solutions in chloroform-*d*. [b]Proton of the aldehyde, aldehydrol, or dithioacetal group. [c]Present work. [d]In deuterium oxide.

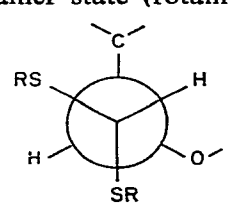
large vicinal couplings have been noted for aldehydrol acetates of sugars, and also for some dialkyl dithioacetals of aldoses (see Table I), again indicating the antiparallel arrangement of protons in the favored rotamer state (rotamers E and F). These



D



E



F

rotamer states (D, E, and F) are the expected, favored conformers, because they correspond to maximum staggering of large groups. In certain systems, interactions with groups on carbon atoms more distant than the vicinal position may influence the favored conformation[21].

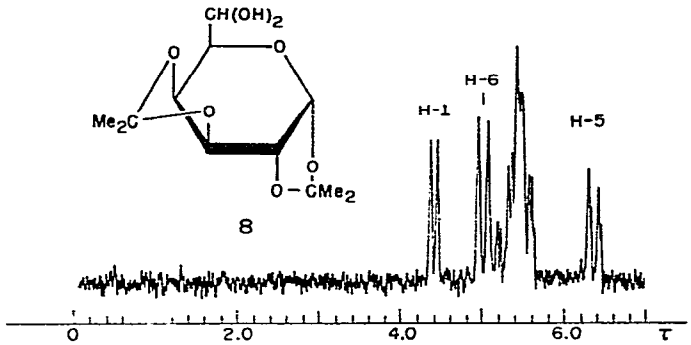


Fig. 2. The low-field portion of the 60-MHz n.m.r. spectrum of 1,2:3,4-di-*O*-isopropylidene-α-D-*galacto*-hexodialdo-1,5-pyranose 6-aldehydrol (**8**) in deuterium oxide.

When the aldehyde **2** is converted into the 6-aldehydrol **8**, a considerable upfield shift of the H-5 signal is observed (see Figs. 1 and 2), with the result that, in **8**, the H-5 signal gives rise to a sharp quartet outside the envelope of signals for H-2, 3, and 4. Since the $J_{5,6}$ coupling is evident from the H-6 doublet, it is possible to measure $J_{4,5}$ (1.7 Hz) directly; the value observed is close to that determined[22,24] for other, related derivatives. This upfield shift of the resonance of the vicinal proton, that occurs when the aldehyde is hydrated, may, in some instances, provide a useful method for obtaining additional conformational information about a ring system.

In moist chloroform, the aldehyde **2** is hydrated to only a small extent.

EXPERIMENTAL

General methods. — Melting points were determined with a Thomas–Hoover apparatus and are uncorrected. Specific rotations were determined in a 2-dm polarimeter tube. Infrared spectra were measured with a Perkin–Elmer Model 137 "Infracord" infrared spectrometer. N.m.r. spectra were measured at 60 MHz with a Varian A-60 n.m.r. spectrometer. Chemical shifts are given on the τ-scale, and spectra were measured at ~30°. Unless otherwise mentioned, spectra were measured for solutions (15–20%) in chloroform-*d*, with tetramethylsilane (τ = 10.00) as the internal standard. Microanalyses were performed 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. T.l.c. was effected on plates coated with a 250-μm layer of Silica Gel G (E. Merck, Darmstadt, Germany), activated at 110°, as the adsorbent. The developers used were: (*A*) 9:1 benzene–methanol; (*B*) 2:3:2:2:1

hexane–benzene–ethyl acetate–tetrahydrofuran–*tert*-butyl alcohol; and (*C*) 7:1:1:1 benzene–chloroform–acetone–methanol. Indication was effected with sulfuric acid, iodine vapor, or Schiff reagent.

*Preparation of 1,2:3,4-di-*O*-isopropylidene-α-*D*-galactopyranose* (**1**). — D-Galactose (20 g) was acetonated with the use of copper(II) sulfate and sulfuric acid as the condensing agents[11]. The resultant oil was found by t.l.c. to contain a major component having R_F 0.23 (*A*) and a minor component having R_F 0.72 (*A*). Fractional distillation of the oil gave the minor component as the first fraction, b.p. 89–91°/1 torr, R_F 0.72 (*A*), 0.90 (*B*), 0.70 (*C*). The pure acetal **1** distilled at 128–129°/0.8 torr as a colorless, chromatographically homogeneous oil; yield 24 g (80%), $[\alpha]_D^{20}$ −59° (*c* 1.4, chloroform) (lit.[11] $[\alpha]_D$ −55° in chloroform); R_F 0.23 (*A*), 0.83 (*B*), 0.36 (*C*); λ_{max}^{film} 2.88 (OH), 7.21, 7.28 μm (CMe_2).

If the low-boiling side-product present in the crude **1** was not removed, the aldehyde **2** derived by oxidation of **1** proved to be very difficult to purify*.

The *6*-p-*toluenesulfonate* (**6**) of **2**, prepared by the usual method[23], was obtained from ethanol as colorless plates in 70% yield; m.p. 99–100°, $[\alpha]_D^{20}$ −66° (*c* 1.0, chloroform); R_F 0.76 (*A*), 0.86 (*B*), 0.79 (*C*); n.m.r. data: τ 2.19, 2.68 (2-proton doublets, A_2B_2 system, $J_{A,B}$ 8.2 Hz, aryl), τ 4.54 (1-proton doublet, $J_{1,2}$ 4.8 Hz, H-1), τ 5.40 (1-proton quartet[23], $J_{2,3}$ 2.1 Hz, $J_{3,4}$ 7.6 Hz, H-3), τ 5.64–5.97 (5-proton multiplet, H-2, 4, 5, 6, 6'), τ 7.57 (3-proton singlet, Me of Ts), τ 8.50, 8.67, 8.69, 8.70 (3-proton singlets, CMe_2); X-ray powder diffraction data: 7.69 w, 6.78 s (1,1), 5.82 w, 5.39 s (1,1), 4.91 w, 4.62 s (1,1), 4.43 w, 4.21 m, 4.10 w, 3.91 vw, 3.78 vw, 3.72 vw, 3.37 m, 3.25 vw.

Various melting points have been recorded for this substance: m.p.[13] 89–91°; m.p.[25] 91–92°; m.p.[14] 102–103°; and m.p.[15] 104–105°. It is probable that there are at least two polymorphs of **6** that have been isolated.

*1,2:3,4-Di-*O*-isopropylidene-6-*O*-p-nitrobenzoyl-α-*D*-galactopyranose* (**5**). —*p*-Nitrobenzoyl chloride (1 g) was added portionwise to a solution of **1** (1 g) in dry pyridine (20 ml), and the solution was kept for 48 h at room temperature. Water was then added[24], and the solvent was evaporated. The residue was dissolved in benzene (30 ml), and the solution was washed successively with cold, 10% sulfuric acid solution, 5% sodium hydrogen carbonate solution, and water. The dried (calcium sulfate) solution was evaporated, and the residue was crystallized from ethanol to give **5** as pale-yellow plates; yield 1.3 g (82%), m.p. 116.5–117.5°, $[\alpha]_D^{20}$ −57° (*c* 1.0, chloroform); R_F 0.80 (*A*), 0.84 (*B*), 0.83 (*C*), λ_{max}^{KBr} 5.79 (C=O), 6.21 (aryl), 6.54, 7.38 (NO_2), 7.24, 7.31 μm (CMe_2); n.m.r. data: τ 1.79 (4-proton singlet, aryl), τ 4.45 (1-proton doublet, $J_{1,2}$ 4.8 Hz, H-1), τ 5.24–5.85 (6-proton multiplet, H-2,3,4,5,6,6'), τ 8.50, 8.51, 8.63 (3-, 3-, and 6-proton singlets, CMe_2); X-ray powder diffraction data:

**Note added in proof.* The side product was identified as 1,2:3,4-di-*O*-isopropylidene-β-L-arabinopyranose by elemental analysis and by comparison of its n.m.r. spectrum with that published for this compound[23]. It was determined that this side product arose because the commercial D-galactose used contained L-arabinose as an impurity.

9.19 vs (1), 7.71 w, 6.99 w, 6.45 w, 5.60 m, 5.23 w, 4.95 s, 4.69 w. 4.40 s, 4.27 vw, 4.08 s, 3.77 w, 3.59 w, 3.52 vw, 3.28 w.

Anal. Calc. for $C_{19}H_{23}NO_9$: C, 55.74; H, 5.66; N, 3.42. Found: C, 55.79; H, 5.57; N, 3.50.

1,2:3,4-Di-O-*isopropylidene*-α-D-galacto-*hexodialdo-1,5-pyranose* (**2**). — To a solution of **1** (10 g) in methyl sulfoxide (35 ml) and benzene (10 ml) were added pyridine (2 ml), phosphoric acid (1 ml), and *N,N'*-dicyclohexylcarbodiimide (25 g). The mixture was stirred for 5 h at room temperature, and filtered to remove *N,N'*-dicyclohexylurea, and a solution of oxalic acid (20 g) in methanol (50 ml) was added to the filtrate. The resulting suspension was stirred for 1 h at room temperature, and then filtered. The filtrate was washed with three 50-ml portions of aqueous sodium hydrogen carbonate. The aqueous extracts were extracted with ethyl acetate (100 ml), and the organic phases were combined, dried (calcium sulfate), and evaporated. The residual syrup was dissolved in acetone (100 ml), whereupon a small amount of *N,N'*-dicyclohexylurea crystallized out. The mixture was filtered, the filtrate was evaporated, and the resultant syrup was treated twice more with acetone in the same way, to give the crude, syrupy aldehyde **2**, yield 8 g (80%). The syrup was distilled twice, and the fraction having b.p. 104–105°/0.5 torr was collected. This fraction was the pure, chromatographically homogeneous aldehyde **2**, yield 4.5 g (46%), $[\alpha]_D^{20}$ −131° (*c* 0.9, chloroform); R_F 0.21 (*A*), 0.69 (*B*), 0.39 (*C*); Schiff and Tollens positive; λ_{max}^{film} 3.70 (CHO), 5.72 (C=O), 7.24, 7.30 μm (CMe_2); n.m.r. data (see Fig. 1): τ 0.43 (1-proton singlet, $J_{5,6}$ ~0 Hz, H-6), τ 4.40 (1-proton doublet, $J_{1,2}$ 4.8 Hz, H-1), τ 5.40–5.83 (4-proton multiplet, H-2,3,4,5), τ 8.50, 8.56, 8.68 (3-, 3-, and 6-proton singlets, CMe_2).

Anal. Calc. for $C_{12}H_{18}O_6$: C, 55.80; H, 7.03. Found: C, 55.43; H, 6.76.

T.l.c. of the crude product showed the presence of five components, having R_F 0.28, 0.35, 0.39, 0.70, and 0.85 (developer *C*). The major component was that having R_F 0.39; it gave a positive reaction with the Schiff reagent, as did the side-product having R_F 0.35. The component having R_F 0.28 was chromatographically indistinguishable from *N,N'*-dicyclohexylurea. Attempts to purify the major component by chromatography on a column of silica gel were not successful.

Reduction of the aldehyde **2** *to the alcohol* **1**. — To a solution of the aldehyde **2** (200 mg) in water (30 ml) was added sodium borohydride (60 mg). The solution was kept for 4 h at room temperature, aqueous acetic acid was then added dropwise to bring the pH to ~5, and the solution was evaporated. T.l.c. of the residue revealed a single component, chromatographically indistinguishable from the alcohol **1**. The residue was dissolved in pyridine (5 ml), and *p*-toluenesulfonyl chloride (200 mg) was added. The product was isolated in the usual way[24] to give the *p*-toluenesulfonate **6**, indistinguishable from an authentic sample of **6**.

1,2:3,4-Di-O-*isopropylidene*-α-D-galacto-*hexodialdo-1,5-pyranose 6-oxime* (**3**). — To a solution of the 6-aldehyde **2** (0.5 g) in methanol (5 ml) was added a solution of hydroxylamine hydrochloride (140 mg) in a mixture of water (1 ml) and pyridine (1 ml). The solution was kept for 18 h at room temperature and then evaporated, and

the resultant syrup was dissolved in benzene (50 ml). The solution was successively washed with water, 10% sulfuric acid, 5% sodium hydrogen carbonate, and water, dried (calcium sulfate), and evaporated. The residue was crystallized from hexane to give **3** as colorless plates, yield 0.35 g (66%), m.p. 107–108°, $[\alpha]_D^{20}$ −116° (*c* 0.35, chloroform); R_F 0.20 (*A*), 0.82 (*B*), 0.54 (*C*); λ_{max}^{KBr} 2.91 (OH), 6.19 (C=N), 7.28, 7.30 μm (CMe_2); X-ray powder diffraction data: 10.77 m, 7.39 vs (1), 6.99 vw, 6.30 w, 5.82 w, 5.26 m, 4.95 s, 4.75 m, 4.51 m, 4.16 vw, 3.94 s, 3.60 vw, 3.31 m, 3.19 w, 3.09 w, 2.92 w.

Anal. Calc. for $C_{12}H_{19}NO_6$: C, 52.74; H, 7.01; N, 5.13. Found: C, 52.83; H, 7.22; N, 5.25.

1,2:3,4-Di-O-isopropylidene-α-D-galacto-hexodialdo-1,5-pyranose 6-(p-nitrophenyl)hydrazone (**7**). — A solution of (*p*-nitrophenyl)hydrazine hydrochloride (500 mg) in a mixture of water (5 ml) and pyridine (1 ml) was mixed with a solution of the 6-aldehyde **2** (500 mg) in methanol (5 ml). The solution was kept for 20 h at room temperature and then evaporated. The resultant syrup was dissolved in benzene (50 ml), and the solution was washed successively with water, 10% sulfuric acid, 5% sodium hydrogen carbonate solution, dried (calcium sulfate), and evaporated. The residue was crystallized from benzene–ethanol to give **7** as yellow needles, yield 600 mg (82%), m.p. 214–215°, $[\alpha]_D^{22}$ −83.5° (*c* 1.0, chloroform); R_F 0.55 (*A*), R_F 0.85 (*B*), R_F 0.76 (*C*); λ_{max}^{KBr} 3.07 (NH), 6.25 (C=N), 6.53, 7.26 μm (NO_2); X-ray powder diffraction data: 8.00 s (3,3), 7.51 m, 7.10 s, 6.71 m, 6.05 m, 5.80 s (2), 5.01 s, 4.56 vs (1), 4.75 m, 4.19 s, 4.00 s (3,3), 3.84 w, 3.65 w, 3.55 w.

Anal. Calc. for $C_{18}H_{23}N_3O_6$: C, 54.96; H, 5.89; N, 10.68. Found: C, 55.28; H, 6.08; N, 10.43.

1,2:3,4-Di-O-isopropylidene-α-D-galacto-hexodialdo-1,5-pyranose 6-aldehydrol (**8**). — A solution (~10%) of the aldehyde **2** was prepared in deuterium oxide. The n.m.r. spectrum (see Fig. 2) revealed the 6-aldehydrol **8** as the only detectable species in solution, n.m.r. data (internal standard, sodium 4,4-dimethyl-4-silapentane-1-sulfonate, τ = 10.00): τ 4.37 (1-proton doublet, $J_{1,2}$ 4.8 Hz, H-1), τ 4.95 (1-proton doublet, $J_{5,6}$ 7.1 Hz, H-6), τ 5.22 (1-proton quartet, $J_{2,3}$ 2.1 Hz, $J_{3,4}$ 7.6 Hz, H-3), τ 5.37–5.58 (multiplet, HOD, H-2,4), τ 6.33 (1-proton quartet, $J_{4,5}$ 1.7 Hz, H-5), τ 8.43, 8.52, 8.60 (3-, 3-, and 6-proton singlets, CMe_2).

Addition of 2 drops of deuterium oxide to 0.4 ml of a 10% solution of the aldehyde **2** in chloroform-*d* caused the signal at τ 0.43 for the aldehydic proton (H-6) to be diminished to about 30% of its original intensity, and a signal of ~20% of the intensity of one proton appeared at τ 4.95 (H-6 of the aldehydrol).

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REFERENCES

1 D. HORTON, J. B. HUGHES, AND J. M. J. TRONCHET, *Chem. Commun.*, (1965) 481.
2 D. HORTON, *Abstracts Papers Am. Chem. Soc. Meeting*, 150 (1965) 21D; *Abstracts Papers 4th Intern. Symp. Carbohyd. Chem., Kingston, Ontario*, (1967) 22.
3 J. L. GODMAN AND D. HORTON, *Carbohyd. Res.*, 6 (1968) 229.
4 D. HORTON AND J. M. J. TRONCHET, *Carbohyd. Res.*, 2 (1966) 315.
5 J. L. GODMAN, D. HORTON, AND J. M. J. TRONCHET, *Carbohyd. Res.*, 4 (1967) 392.
6 D. HORTON, J. B. HUGHES, AND J. K. THOMSON, *J. Org. Chem.*, 33 (1968) 728.
7 A. K. CHATTERJEE, A. E. EL ASHMAWY, D. HORTON, J. S. JEWELL, L. G. MAGBANUA, W. E. MAST, K. D. PHILIPS, AND J. D. WANDER, *Abstracts Papers Am. Chem. Soc. Meeting*, 154 (1967) 19D.
8 D. HORTON AND J. S. JEWELL, *Carbohyd. Res.*, 2 (1966) 251; 5 (1967) 149.
9 K. E. PFITZNER AND J. G. MOFFATT, *J. Am. Chem. Soc.*, 85 (1963) 3027; 87 (1965) 5661.
10 J. R. DYER, W. E. MCGONIGAL, AND K. C. RICE, *J. Am. Chem. Soc.*, 87 (1965) 654.
11 O. T. SCHMIDT, *Methods Carbohyd. Chem.*, 2 (1963) 324.
12 D. C. DEJONGH AND K. BIEMANN, *J. Am. Chem. Soc.*, 86 (1964) 67.
13 A. B. FOSTER, W. G. OVEREND, M. STACEY, AND L. F. WIGGINS, *J. Chem. Soc.*, (1949) 2542.
14 K. FREUDENBERG AND K. RASCHIG, *Ber.*, 60 (1927) 1633.
15 H. OHLE AND H. THIEL, *Ber.*, 66 (1933) 525.
16 D. HORTON, M. NAKADATE, AND J. M. J. TRONCHET, to be published.
17 A. E. EL ASHMAWY AND D. HORTON, *Carbohyd. Res.*, 1 (1965) 164.
18 D. HORTON AND FRANCES L. OLSEN, unpublished data.
19 D. HORTON AND J. D. WANDER, unpublished data.
20 E. L. ELIEL, N. L. ALLINGER, S. J. ANGYAL, AND G. A. MORRISON, *Conformational Analysis*, Wiley-Interscience, Inc., New York, N. Y. 1965, p. 19.
21 H. EL KHADEM, D. HORTON, AND T. F. PAGE, JR., *J. Org. Chem.*, 33 (1968) 734.
22 C. V. HOLLAND, D. HORTON, M. J. MILLER, AND N. S. BHACCA, *J. Org. Chem.*, 32 (1967) 3077.
23 C. CONE AND L. HOUGH, *Carbohyd. Res.*, 1 (1965) 1.
24 R. S. TIPSON, *Methods Carbohyd. Chem.*, 2 (1963) 248.
25 K. FREUDENBERG AND R. M. HIXON, *Ber.*, 56 (1923) 2119.

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THE OXIDATION OF D-GALACTOPYRANOSYL RESIDUES OF POLYSACCHARIDES TO D-GALACTOPYRANOSYLURONIC ACID RESIDUES

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ABSTRACT

Optimized conditions have been devised for the oxidation of polysaccharides containing terminal D-galactopyranosyl residues to new polysaccharides containing uronic acid, by a known two-stage sequence employing D-galactose oxidase and halogen. The isolation of D-galacturonic acid (as galactaric acid) from the hydrolyzates of an oxidized galactomannan confirms the conversion of D-galactopyranosyl residues into D-galactopyranosyluronic acid residues in that polymer. An aldobiouronic acid thought to be 6-*O*-(α-D-galactopyranosyluronic acid)-D-mannose was detected in the partial, acid hydrolyzate of this galacturonogalactomannan derived from guaran. Acidic fragments containing D-galacturonic acid were isolated from the partial, acid hydrolyzates of oxidized galactoglucomannan of spruce and oxidized arabinogalactan of larch; this indicates that the two-stage sequence will oxidize D-galactopyranosyl residues attached by α-D or β-D glycosidic bonds to polysaccharides of structural complexity greater than that of the galactomannan of guaran. The decrease in molecular weight of the larch arabinogalactan, together with the nonquantitative relationship between the disappearance of D-galactopyranosyl residues and the appearance of uronic acid, as a result of the oxidations, suggests that the reactions of this polymer do not follow a simple scheme. The development of a refined technique for the elucidation of the nature of the D-galactopyranosyl residues of polysaccharides will have to depend upon more certain knowledge of the action and specificity of the enzyme than is at present available.

INTRODUCTION

Recently, Avigad, Asensio, Amaral, and Horecker[1] investigated the action on various D-galactosides of a D-galactose oxidase isolated from the fungus *Polyporus circinatus*, and found that it converted the primary alcohol group of the D-galactose residue into an aldehyde group. This group, in turn, could be oxidized to a carboxylic acid group by conventional techniques. They also found that a galactomannan prepared from guar gum was readily oxidized by the enzyme, and that subsequent oxidation of this strongly reducing polysaccharide with bromine yielded an acidic polysaccharide. The uronic acid content of the acidic polymer (as determined by the

carbazole assay) indicated that a proportion of acid equal to 30% of the terminal D-galactose residues was formed by this two-stage oxidation sequence.

The purpose of the present investigation was to make sure that the two-stage oxidation described by Avigad *et al.*[1], consisting of the reaction of guaran with the enzyme D-galactose oxidase followed by conversion of the aldehyde groups into carboxylic acid groups by oxidation with halogen, results in the conversion of terminal D-galactopyranosyl residues of galactomannans into D-galactopyranosyluronic acid residues. It was also our purpose to determine whether other polysaccharides containing D-galactopyranosyl residues in different molecular environments, such as in the arabinogalactan and the galactoglucomannan of conifers, will undergo a similar oxidation, to yield polysaccharides containing D-galactopyranosyluronic acid residues.

RESULTS AND DISCUSSION

The galactomannan employed in this investigation was purified from a crude guaran sample supplied by Stein Hall, Inc. This polymer contains terminal D-galactopyranosyl residues linked by α-D-(1→6) glycosidic bonds to the polymer backbone, which consists of β-D-(1→4)-linked D-mannopyranosyl residues[2].

The arabinogalactan of western larch was chosen for investigation, because it has been shown by Bouveng[3] to be free from uronic acid and to be composed of a backbone of β-D-(1→3)-linked D-galactopyranosyl residues. Many of the C-6 positions of the backbone carry substituents, each side-chain containing an average of two sugar residues composed of β-L-(1→3)-linked L-arabinofuranosyl residues or of β-D-(1→6)-linked D-galactopyranosyl residues. Methylation evidence indicates that some non-terminal, as well as terminal, D-galactopyranosyl residues are present that have primary alcohol groups available for oxidation by the enzyme.

The galactoglucomannan of Engelmann spruce was used because it has been shown by Mills and Timell[4] to be a water-soluble polysaccharide composed chiefly of β-D-(1→4)-linked D-glucopyranosyl and D-mannopyranosyl residues. This polymer contains some terminal D-galactopyranosyl residues, linked by α-D-(1→6) glycosidic bonds to certain of the D-mannopyranosyl residues. Additional branches thought to be present include those linked to the C-6 position of D-glucopyranosyl residues and, in the case of galactoglucomannans from certain spruces, linkages of an unknown nature to lignin or to lignin debris[5,6]. It was concluded from analytical studies that the polymer employed in this investigation had 4–8% of glucuronoarabinogalactan impurity and 14% of lignin debris that could not be removed from the polymer by fractionation.

Relatively large batches of the three polymers were oxidized by a two-stage oxidation sequence employing D-galactose oxidase followed by oxidation of the aldehyde groups with iodine to carboxylic acid groups. The reaction time was diminished to 20% of that used by Avigad *et al.*[1] by increasing the concentration of the enzyme catalase (which destroys the by-product, hydrogen peroxide). The analyses of

the oxidized polysaccharides prepared in this investigation are summarized in Table I. The oxidation conditions employed here resulted in the destruction of 46% of the terminal D-galactopyranosyl residues of the galactomannan, and caused the appearance of a functional group which yields carbon dioxide under the conditions of a quantitative test for uronic acid[7]. Avigad *et al.*[1] achieved a 30% conversion of the D-galactopyranosyl residues of guaran into uronic acid.

TABLE I

CHARACTERIZATION OF OXIDIZED AND UNOXIDIZED GALACTOSE-CONTAINING POLYMERS

	Galactomannan		*Galactoglucomannan*			*Arabinogalactan*		
Number of oxidations	0	1	0	1	3	0	Oxidation control[c]	1
Yield[a], %		95		96	65		80	69
Molecular weight (osmotic pressure)	567,000	[b]	9,700	9,700	11,500	27,900	25,900	17,000
Galactose, %	41	25	23	16	11	81	81	80
Glucose, %	0	0	16	14	14.5	0	0	0
Mannose, %	59	53	58	60	62	0	0	0
Arabinose, %	0	0	1.7	1.7	1.5	19	15	13
Xylose, %	0	0	1.1	1.3	1.0	0	0	0
Uronic acid, %	0	21	0	7	10	0	3[d]	7
$[\alpha]_D^{30}$ (*c* 1, 0.1M NaOH)	+60	+43.3	+2		+10			

[a]All percentages are calculated on an ash-, moisture-, and lignin-free basis. [b]This polysaccharide would not dissolve completely in solvents necessary for molecular-weight determinations. [c]Oxidized only by the sodium hypoiodite stage, without preliminary oxidation with D-galactose oxidase. [d]No galacturonic acid, arabinuronic acid, or oligosaccharides containing these acids could be detected by paper chromatography.

Only 30% of the D-galactose residues of the galactoglucomannan could be oxidized in one sequence. Two additional oxidation sequences increased the conversion to 50%, and additional oxidations might have increased it even more. Once again, the close correspondence between the loss of D-galactopyranosyl residues, consumption of sodium hypoiodite, and uronic acid analysis, suggests that few side-reactions had occurred. It may be speculated that the aldehyde groups that are produced by the action of D-galactose oxidase react intra- or inter-molecularly to form hemiacetal or acetal linkages, and that these inhibit the action of the enzyme on these two polymers. Conversion of these groups into carboxylic acid groups removes the acetal linkages and allows further interaction of the polymer with the enzyme. It is possible that the lignin contaminant of the galactoglucomannan may also inhibit enzymic oxidation of that polysaccharide.

Based on methylation data[3], 40–42% of the D-galactopyranosyl residues of larch arabinogalactan have primary alcohol groups potentially available for reaction with D-galactose oxidase. The proportion of uronic acid introduced into the polymer corresponds to the conversion of 10% of the available D-galactopyranosyl residues,

on the assumption that only those residues, and not the L-arabinofuranosyl residues, are oxidized by these treatments.

The small loss of galactomannan and galactoglucomannan shown in Table I was thought to arise from the procedure used for separating the oxidized polymer from the enzyme. The greater losses of arabinogalactan arose from unknown reactions of the polymer with sodium hypoiodite and also with the enzyme. Little evidence for a decrease in molecular weight of the galactomannan and galactoglucomannan was observed, whereas the great loss in molecular weight of the arabinogalactan originated with the enzymic oxidation, and not with the sodium hypoiodite oxidation stage by itself. The lack of agreement between the appearance of uronic acid and the disappearance of D-galactopyranosyl residues suggests that the oxidation of arabinogalactan by this oxidation system does not follow the same path as the oxidation of the other two polymers. The known lability of L-arabinofuranosyl residues to acidic conditions makes it difficult to assign the loss of some of these residues to the action of D-galactose oxidase. The oxidation of the glucuronoarabinogalactan of black spruce by this same two-stage oxidation sequence results also in decrease of the molecular weight by half and in the introduction of "uronic acid" into the molecule, independent of the disappearance of the D-galactopyranosyl and L-arabinofuranosyl residues.

Paper-chromatographic examination* of the complete, acid hydrolyzates of all three oxidized polymers indicated that no uronic acid (or sugar) other than a galacturonic acid was produced as a result of the two-stage oxidation. Chromatographic tests with D-glucuronic acid (and D-glucurono-1,4-lactone), D-mannuronic acid (and D-mannurono-1,4-lactone), and D-galacturonic acid as controls showed that a multiple-development technique with pyridine-containing and acetic acid-containing solvents will separate these acids from one another, and, as a result, it was possible to demonstrate the presence of glucuronic acid as well as galacturonic acid in oxidized galactoglucomannans. The former acid was derived from the 4–8% of glucuronoarabinogalactan contaminant in this preparation of Engelmann-spruce galactoglucomannan and did not arise from the action of the oxidants.

Partial, acid hydrolysis of the oxidized galactomannan, the oxidized galactoglucomannan, and the oxidized arabinogalactan yielded a variety of hydrolysis products, some of which were shown by subsequent acid hydrolysis to be composed of galacturonic acid and various sugars. By developing chromatograms for two weeks in a pyridine-containing solvent and then for at least one week in an acidic solvent, it was possible to separate acidic fragments from the smaller proportion of nonacidic oligosaccharides. The chromatographic mobilities of these acids, together with some of their analytical properties, are given in Table II.

Quantitative, paper-chromatographic analysis of the hydrolyzed, acidic fragments from the oxidized galactoglucomannan showed galacturonic acid to occur with mannose, glucose, and galactose, and, in two instances, with both glucose and

*Sugars of the D or L series are not differentiated, unless further characterized by measurement of specific rotation.

TABLE II

ACIDIC FRAGMENTS FROM THE PARTIAL, ACID HYDROLYZATES OF CERTAIN OXIDIZED GALACTOSE-CONTAINING POLYMERS

Component	*Polymer source*	R_X[a]	*Yield, %*	*Molar ratio of sugars after hydrolysis*			
				Galacturonic acid	*Galactose*	*Glucose*	*Mannose*
A	galactomannan	0.17	2.4	1	0	0	1
A-1	galactoglucomannan	0.17	1.5	1	0	0	1
A-2[b]	galactoglucomannan	0.13	0.9	1	0	1	0
A-3	galactoglucomannan	0.11	0.3	1	1	0	0
B	arabinogalactan	0.11		1	1	0	0
A-4	galactoglucomannan	0.082	0.3	1	0	1	0.9
A-5	galactoglucomannan	0.064	0.2	1	0	0.9	1
A-6	galactoglucomannan	0.055	0.1	+	+	+	+

[a]Chromatographic mobility in 9:2:2 ethyl acetate–acetic acid–water, relative to the distance travelled by xylose. [b]Trace quantities of glucuronic acid and galactose were detected after hydrolysis. This acid migrates at the same rate as 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose, which is present in the unoxidized polysaccharide.

mannose residues; the characterization of these fragments is the subject of another communication[8]. This evidence shows that the D-galactopyranosyl residues of this extract, attached to glucose, mannose, and galactose residues, may be oxidized by the procedure employed here to D-galactopyranosyluronic acid residues. Similarly, the detection of fragments composed of D-galacturonic acid linked to D-galactose in the hydrolyzate of the oxidized arabinogalactan from larch shows that some D-galactopyranosyl residues linked to the polymer by β-D-glycosidic bonds are also susceptible to the oxidizing actions employed here. Because the D-galactopyranosyl residues of arabinogalactans are linked to the polymer both by β-D-(1→6) and β-D-(1→3) glycosidic bonds, the nature of the linkage cannot be determined from the evidence available. A study of the acids isolated from the partial, acid hydrolyzates of these two oxidized polysaccharides will be the subject of future communications.

The major, acidic fraction isolated from the partial, acid hydrolyzate of oxidized galactomannan (acid *A* in Table I) yielded equimolar quantities of galacturonic acid and mannose after hydrolysis, as determined by quantitative, paper chromatography. Treatment with sodium borohydride reduced only the mannose residues of acid *A*, and, as the equivalent weight is of the correct magnitude, this acid is an aldobiouronic acid in which a mannose residue forms the terminal, reducing group. Galacturonic acid was separated by preparative, paper chromatography from the acid hydrolyzate of the aldobiouronic acid as a syrup, which has not yet been crystallized. Oxidation of the syrup with bromine water resulted in the formation of a crystalline derivative that had an X-ray diagram identical to that of authentic galactaric acid.

These results show that the terminal D-galactopyranose residues of galactomannan are oxidized to D-galacturonic acid residues by the two-stage oxidation process employed here. As the galactomannan of guaran has been shown[2,9] to contain D-galactopyranosyl residues linked only by α-D-(1→6) glycosidic bonds to D-manno-

pyranosyl residues, it is concluded that acid *A* is 6-*O*-(α-D-galactopyranosyluronic acid)-D-mannose.

The introduction of uronic acid residues into polysaccharides stabilizes certain glycosidic linkages to acid hydrolysis, and facilitates study of the structural details of the polymer. Aspinall and co-workers[10] have developed a platinum-catalyzed oxidation of the primary alcohol groups of the sugar residues of polysaccharides by oxygen, and, although the method has been used to oxidize arabinogalactans[11], it is less specific than the enzymically catalyzed oxidation. D-Galactose oxidase oxidizes the hydroxymethyl groups of D-galactopyranosyl residues, but the model compounds employed by Avigad *et al.*[1] did not include compounds containing nonterminal galactopyranosyl residues having primary alcohol groups available for oxidation. The results of an investigation in this laboratory of the oxidation of agar suggest that an appreciable proportion of nonterminal D-galactopyranosyl residues were oxidized in that polysaccharide, and, hence, these residues may also have been oxidized in the larch arabinogalactan. This observation, together with the diminution of molecular weight of the arabinogalactan of larch and spruce on enzymic oxidation, the large losses of L-arabinofuranosyl residues, and the small losses of D-galactopyranosyl residues relative to production of uronic acid, suggests that the oxidation described by Avigad *et al.*[1] for simple carbohydrates, and in this work for linear polysaccharides having terminal branches, does not describe the oxidation of more-complex polymers containing nonterminal D-galactopyranosyl residues. The future development of more-refined techniques for the elucidation of the nature of D-galactopyranosyl residues in polysaccharides will have to depend on more-certain knowledge of the action and the specificity of the enzyme.

EXPERIMENTAL

Methods. — Polysaccharides and their degradation products were hydrolyzed by dissolving them in 72% sulfuric acid (1 ml per 50 mg), diluting the solutions after 5 min to a 4% concentration of sulfuric acid, and heating the solutions for 1 h at 120° in a household pressure-cooker. Partial, acid hydrolysis was achieved by heating 10 mg of a sample in 10 ml of M sulfuric acid for 3 h at 90°. Sulfate ions were removed from the hydrolyzate as barium sulfate, and the sugars were isolated, after filtration and de-ionization of the solutions with Amberlite IR-120 (H^+) resin, by evaporation in a rotary evaporator.

Quantitative, sugar analyses were made by the technique of Saeman *et al.*[12]. The spot-area technique of Fisher *et al.*[13] and others[14] was employed for the analysis of small quantities of material. Control experiments with D-galactose, D-mannose, D-glucose, D-glucuronic acid, D-galacturonic acid, their corresponding alditols, and 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose were made, in order to permit compensation for the losses of sugar that occurred during hydrolysis. The chromatographic reagents used were *p*-anisidine hydrochloride for reducing sugars, and alkaline silver nitrate for alditols. Chromatographic separations were effected with Whatman No. 1 or 3 MM paper, and the solvents were: *A*, 9:2:2 (v/v) ethyl acetate–acetic

acid–water; *B*, 8:2:1 (v/v) ethyl acetate–pyridine–water; and *C*, 18:3:1:4 (v/v) ethyl acetate–acetic acid–formic acid–water. Separations of alditols, sugars, and uronic acids from one another were achieved by first chromatographing mixtures in solvent *B* (for two weeks or less), followed by chromatographing for a suitable period of time (usually 1 week or less) with solvents *A* or *C*. Although consistent R_F values could not be obtained between identical chromatograms run on different occasions by this two-stage process, consistent separations were nevertheless possible, and the location of compounds was ascertained by including the appropriate, known sample as a control.

Other analyses made on the samples included lignin determinations[15,16], ash and moisture determinations, and uronic acid analysis by evolution of carbon dioxide[7]. The number-average molecular weight and the degree of polymerization (D. P.) were determined by the technique of Genung[17] by using a Mechrolab High-Speed Membrane Osmometer with Schleicher and Schuell 08 membranes.

Preparation of samples. — A solution of the galactomannan of guaran (obtained as a yellow, granular powder from Stein Hall, Inc.) in a 100-fold excess of water was heated to 90°, centrifuged in a Sharples steam supercentrifuge, and ethanol (2.5 vol.) was added to precipitate the product, which was washed with ether, and dried[18]. A solution (0.5%) of this preparation in 0.1M sodium hydroxide was prepared, and the resultant, deacetylated polymer was dialyzed free from inorganic salts, and isolated by freeze-drying. Found: D.P. 3600; ratio of D-galactose to D-mannose, 2:3; ash 0.2%; and nitrogen 0.2%.

The galactoglucomannan was isolated from the chlorite-liquor extract of a holocellulose preparation of a sample of Engelmann spruce (*Picea engelmanni*)[3] (obtained by room-temperature extraction with sodium chlorite), by a procedure involving the use of calcium ion to separate pectic substances, and of barium hydroxide in the presence of excess sodium hydroxide to separate soluble (4-*O*-methylglucurono)-arabinoxylans from the components having insoluble barium salts. The pH of the insoluble residue separated by centrifugation was adjusted to 7 in order to dissolve the galactoglucomannan but leave an insoluble barium salt of a lignin–galactan complex[6]. Repeated precipitation with Fehling solution was used to complete the purification of the galactoglucomannan. Analysis showed ratios of D-galactose:D-glucose:D-mannose of 12:8:3; $[\alpha]_D$ +2° (*c* 1, 0.1M sodium hydroxide); D.P. 60; ash, negligible; lignin, 14%.

Ultracentrifugation of a 1% solution of the galactoglucomannan for 1.5 h in a Spinco Model E analytical ultracentrifuge equipped with a schlieren optical system gave a bell-shaped distribution of molecular weights. Free-boundary electrophoresis of a 2% solution in borate buffer (pH 9.2) at 13 mA for 3.5 h at 4.5°, followed by quantitative analysis of the ascending and descending fractions, showed no significant change in the lignin content, but indicated that the ascending sample was richer in galactose residues. Careful examination of the hydrolyzates of this polymer showed the presence of a trace of an acid migrating at the same rate as 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose. Complete hydrolysis of this trace fragment

with acid yielded a glucuronic acid and a galactose, and this evidence, coupled with consideration of the amounts of compounds separated by electrophoresis suggested that 4–8% of a glucuronoarabinogalactan was present in this extract of Engelmann spruce.

Oxidation reactions. — Commercial D-galactose oxidase and peroxidase (used to decompose the hydrogen peroxide by-product that inhibits the activity of D-galactose oxidase) were obtained from Worthington Biochemical Corporation, Freehold, New Jersey. One unit of D-galactose oxidase activity is defined as the weight of enzyme that forms an amount of reaction product corresponding to a change in optical absorbance (by reaction with *O*-dianisidine) of one unit at 400 nm, after exactly 5 min of reaction with D-galactose under the conditions described by Avigad *et al.*[19]. One unit of peroxidase activity is defined as the quantity of enzyme that forms an amount of colored reaction-product corresponding to an optical absorbance of 0.050 at 470 nm, in 10 sec, according to the procedure of Chance and Maehly[20].

Samples, and controls containing D-galactose, D-glucose, D-mannose, galactomannan, or galactoglucomannan, were oxidized in duplicate with these enzymes in the presence of suitable buffers under a variety of conditions designed to optimize the general scheme described by Avigad *et al.*[1]. The reactions were terminated by heating the solutions rapidly to 90°. The change in reducing power, and hence, the extent of oxidation, was followed by the iodimetric method of Macleod and Robison[21]. In one experiment, for example, the reducing power of a solution of D-galactose was doubled to a maximum value in less than 2 h by the action of the enzyme, whereas the reducing value of solutions of D-glucose and D-mannose was unchanged even after prolonged treatment. The extent of oxidation of polysaccharides was found to depend on the proportion of peroxidase, as well as of D-galactose oxidase, in the reaction mixture. Under optimized conditions, control experiments showed that the extent of oxidation of galactomannan and galactoglucomannan reached a maximum after 4 h of reaction with D-galactose oxidase, and that the loss of D-galactopyranose residues, as estimated by iodine titration, agreed (within 6%) with the loss as measured by quantitative, sugar analysis. Uronic acid analyses of these products by the method of evolution of carbon dioxide yielded results that were lower (by 6–15%) than those determined by the other two techniques. In other experiments, chromatographic examination of the hydrolyzates of the three types of oxidized polysaccharide investigated here, with solvents *A*, *B*, and *C*, showed that no uronic acid other than galacturonic acid, no saccharinic acid derivative, and no sugar or uronic acid other than those originally present in the polymers, could be detected after this oxidation cycle.

Batches (1 g) of the three polysaccharides were oxidized for 4 h at 30° in a solution containing 0.5 mg of D-galactose residues per ml of solution, 2.2 units of D-galactose oxidase per mg of D-galactose residues, 1200 units of peroxidase per mg of D-galactose residues, and 0.6 ml of phosphate buffer of pH 7 (supplied by Matheson, Coleman, and Bell) per mg of D-galactose residues. Upon completion of the reaction, sufficient 20 mN iodine and solid sodium carbonate were added to bring the solution to 10 mg of iodine and 13 mg of sodium carbonate per mg of D-galactose residues.

After 4 h, the solution was heated quickly to 90°, and cooled to room temperature, and inorganic ions were removed with Amberlite MB-3 mixed-bed resin. The oxidized polymers were isolated in 85–95% yield after freeze-drying the solution. The effects of this oxidation cycle on the polysaccharides investigated here are given in Table I. As only 30% of the D-galactopyranosyl residues of the galactoglucomannan were oxidized by one multistage, oxidation process, two additional oxidation sequences were performed on it, and the extent of oxidation was observed to increase, from 30% to 52%.

Partial hydrolysis with acid. — In a typical experiment, the oxidized polysaccharide (1 g) was dissolved in 525 ml of distilled water at 90°. A similar volume of 2M sulfuric acid, also at 90°, was added to the hot solution of the hemicellulose, and, after 4 h at that temperature, the solution was cooled and the hydrolyzate was isolated by the procedure described earlier. The hydrolyzates were streaked on sheets of Whatman 3 MM paper (about 3 mg/cm) and eluted for two weeks with solvent *B*. The sheets were then air-dried, and chromatographed for one week in solvent *A*. Guide strips were cut from the chromatograms, and the migrating compounds were detected with *p*-anisidine or silver oxide. The areas of the chromatograms corresponding to the separated fragments were cut out, and eluted with water. After filtration, and treatment with charcoal, the eluates were evaporated to dryness in a rotary evaporator, and weighed. Small samples (1–2 mg) of the syrups were hydrolyzed, and the hydrolyzates were analyzed for sugar content by the method of Fisher *et al.*[13]. The results, together with the chromatographic mobilities of the fragments in a single solvent, are given in Table I.

Characterization of acid A from oxidized galactomannan. — The major fragment isolated from the partial, acid hydrolyzate of oxidized galactomannan (acid *A*) was a colorless syrup that could not be induced to crystallize. Quantitative, paper-chromatographic analysis after complete hydrolysis with acid indicated that acid *A* was composed of equal parts of D-galacturonic acid and D-mannose, and that the hydrolysis products from acid *A*, reduced with sodium borohydride by a standard technique[22], contained only a mannitol and D-galacturonic acid. The infrared spectrum of acid *A* was identical with that of acid *A*-1 from oxidized galactoglucomannan. Found: $[\alpha]_D$ +111° (*c* 1, water); equivalent weight, 391. Required for an aldobiouronic acid, equivalent weight, 356.

Paper partition-chromatography of the hydrolyzate of this acid permitted the separation and isolation of an acidic, colorless syrup that was chromatographically identical with D-galacturonic acid in solvents *A*, *B*, and *C*. Although these solvents did not reveal any impurity, the galacturonic acid has thus far failed to crystallize. Oxidation of the acid (5 mg) with 10 ml of saturated, aqueous bromine water for 2 h yielded crystals that were washed with ethanol and dried on a porous plate. The X-ray powder pattern of these crystals was identical with that of galactaric acid[23].

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REFERENCES

1 G. AVIGAD, D. AMARAL, C. ASENSIO, AND B. L. HORECKER, *J. Biol. Chem.*, 237 (1962) 2736.
2 F. SMITH AND R. MONTGOMERY, *The Chemistry of Plant Gums and Mucilages*, Reinhold Publishing Corp., New York, 1959.
3 H. O. BOUVENG, *Svensk Kem. Tidskr.*, 73 (1961) 3.
4 A. R. MILLS AND T. E. TIMELL, *Can. J. Chem.*, 41 (1963) 1389.
5 I. CROON, H. MEIER, AND B. LINDBERG, *Acta Chem. Scand.*, 13 (1959) 1299.
6 N. S. THOMPSON AND O. A. KAUSTINEN, *Tappi*, 49 (1966) 83.
7 R. L. WHISTLER AND A. R. JEANES, *Ind. Eng. Chem., Anal. Ed.*, 15 (1943) 317.
8 J. K. ROGERS AND N. S. THOMPSON, in press (1967).
9 R. L. WHISTLER AND D. F. DURSO, *J. Am. Chem. Soc.*, 73 (1951) 4189.
10 G. O. ASPINALL, I. M. CAIRNCROSS, AND A. NICHOLEM, *Proc. Chem. Soc.*, (1959) 270.
11 G. A. ADAMS, *Pulp Paper Mag. Can.*, 65 (1964) T13.
12 J. F. SAEMAN, W. E. MOORE, R. L. MITCHELL, AND M. A. MILLETT, *Tappi*, 37 (1954) 336.
13 R. B. FISHER, D. S. PARSONS, AND G. A. MORRISON, *Nature*, 161 (1948) 764.
14 H. D. FOWLER, *Nature*, 168 (1951) 1123.
15 TAPPI, T 222 m-54.
16 J. E. STONE AND M. J. BLUNDELL, *Anal. Chem.*, 23 (1951) 771.
17 L. B. GENUNG AND R. C. MALLETT, *Ind. Eng. Chem., Anal. Ed.*, 13 (1941) 369.
18 H. S. DUGAL AND J. W. SWANSON, unpublished research, The Institute of Paper Chemistry (1966).
19 G. AVIGAD, D. AMARAL, C. ASENSIO, AND B. L. HORECKER, *Biochem. Biophys. Res. Commun.*, 4 (1961) 474.
20 B. CHANCE AND A. G. MAEHLY, *Methods Enzymol.*, 2 (1955) 784.
21 M. MACLEOD AND R. ROBISON, *Biochem. J.*, 23 (1929) 517.
22 H. MEIER, *Acta Chem. Scand.*, 14 (1960) 1749.
23 *Methods of Analysis*, Association of Official Agricultural Chemists, Washington, D.C., 4th ed., 1935, p. 345.

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Notes

Separation of sugars on an ion-exchange resin*

INTRODUCTION

Several methods utilizing ion-exchange resins have been developed for the separation of mixtures of neutral carbohydrates. In 1952, Roseman, Abeles, and Dorfman[1] reported that the basic resin Amberlite IRA-400**, in the hydroxide form, retained only the reducing sugars when a mixture of reducing and nonreducing sugars was applied. In 1960, Hough, Priddle, and Theobald[2] resolved a mixture of raffinose, sucrose, and D-glucose on the basic resin De-Acidite FF. Mixtures of carbohydrates have also been resolved by chromatography on strongly basic anion-exchange resins in the borate form[3,4] and the sulfate form[5–7]. Austin, Hardy, Buchanan, and Baddiley[8] effected separations of isomeric glycosides on the hydroxyl form of Dowex-1 resin. Jonsson and Samuelson[9], using the cation-exchange resin Amberlite IR-120 in the lithium, sodium, and potassium forms, separated small amounts of monosaccharides. The cation-exchange resin Dowex-50 in the lithium form[10] resolved mixtures of oligosaccharides or methylated monosaccharides, and in the barium form[11] resolved mixtures of monosaccharides. In this laboratory, Dowex-50 in the potassium form has been used to resolve the sugars produced in an invertase–sucrose reaction[12], and the sugars in wheat bran[13]. The use of Dowex-50 (K^+) has now been extended to the separation of other sugars, some of which had not been studied previously. For the same separations, Dowex-50 (K^+) has been found superior to Dowex-50 (Li^+ or Ba^{+2}). Illustrated are separations of oligosaccharides, hexoses, pentoses, deoxy sugars, isopropylidene acetals of sugars, and methyl glycosides.

EXPERIMENTAL

A glass column (167 × 4.5 cm) having a coarse-fritted disc as a support was used. The resin (120 × 4.5 cm) was 200–400 mesh Dowex-50W X4 (K^+ form), and it was used in all separations without regeneration between experiments. Sugars were applied in about 5 ml of water and were chromatographed with water, only, as eluant. Fractions of 5 ml were collected with an automatic fraction-collector at a flow rate of 0.7–0.8 ml per min. The carbohydrate content of alternate tubes was determined by the phenol–sulfuric acid method[14]. Contents of tubes were pooled by peaks after analysis, and

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**Reference to a company or product name does not imply approval or recommendation of the product by the U. S. Department of Agriculture to the exclusion of others that may be suitable.

the sugars were identified by paper chromatography and, in some cases, by crystallization and m.p. Paper chromatograms were made on Whatman No. 1 paper in the descending way, with 6:4:3 (v/v) butyl alcohol–pyridine–water as eluant, and sugars were detected with alkaline silver nitrate.

RESULTS

The void volume was at fraction number 100. Fig. 1 shows the separation, from each other, of stachyose, raffinose, sucrose, D-glucose, D-fructose, and D-xylose (150 mg of each). Fig. 2 shows the separation of D-glucose, 1,2-*O*-isopropylidene-α-D-

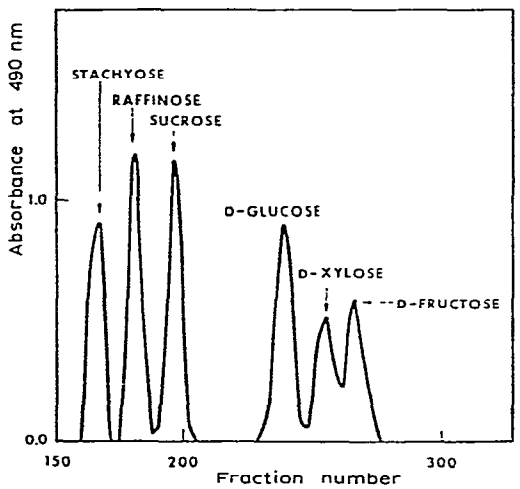

Fig. 1. Chromatography, on Dowex-50 (K^+) ion-exchange resin, of a mixture of stachyose, raffinose, sucrose, D-glucose, D-fructose, and D-xylose.

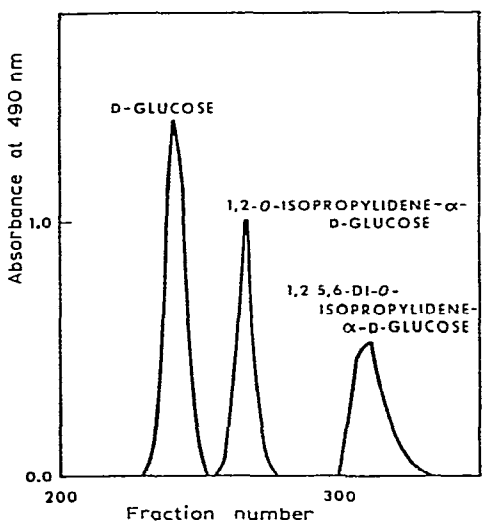

Fig. 2. Chromatography on Dowex-50 (K^+) ion-exchange resin of a mixture of D-glucose, 1,2-*O*-isopropylidene-α-D-glucofuranose, and 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose.

glucofuranose, and 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose (100 mg of each). Table I lists the sugars, together with their respective distribution coefficients[15] (K_D), which, in various synthetic mixtures, have been separated by this procedure. With the weight of sugar employed here (25–150 mg of each), it was found that, if the difference in distribution coefficient was less than 0.05, some overlapping occurred. For example, it was observed that overlapping occurred between D-fructose and D-xylose ($\Delta K_D = 0.04$), and between methyl α- and β-D-glucopyranoside ($\Delta K_D = 0.03$). The K_D values were reproducible. In all experiments, the yield of a recovered sugar was not less than 95%.

TABLE I

THE SUGARS SEPARATED ON DOWEX-50 (K^+) ION-EXCHANGE RESIN, AND THEIR DISTRIBUTION COEFFICIENTS

Sugar	*Distribution coefficient* (K_D)
Stachyose	0.27
Raffinose	0.33
Sucrose	0.39
D-Glucose	0.58
D-Galactose	0.66
D-Mannose	0.68
D-Fructose	0.69
L-Rhamnose	0.55
L-Arabinose	0.78
D-Lyxose	0.71
D-Ribose	0.85
D-Xylose	0.67
2-Deoxy-D-*erythro*-pentose	0.68
Methyl α-D-glucoside	0.51
Methyl β-D-glucoside	0.48
Methyl α-D-mannoside	0.67
1,2-*O*-Isopropylidene-α-D-glucofuranose	0.66
1,2:5,6-Di-*O*-isopropylidene-α-D-glucofuranose	0.85
2,3:4,5-Di-*O*-isopropylidene-β-D-fructopyranose	0.89

Fractionation of oligosaccharides according to molecular size, and separation of synthetic mixtures of monosaccharides on a preparative scale, have been achieved by this technique. Impurities are not introduced. The ion-exchange procedure for fractionating oligosaccharides is analogous to gel filtration (for example, with Sephadex), but possesses the advantage that the monosaccharides are concurrently resolved in a manner analogous to partition chromatography. Previously, resolution of mixtures of monosaccharides has been achieved by using variations on the charcoal–Celite columns of Whistler and Durso[16] or by partition chromatography on cellulose columns[17]. These procedures are usually very efficient, but the separations may require several weeks. Glycosides have previously been resolved on Dowex-1 resin, but free sugars were not recoverable[8]. Preparative, column-chromatographic fractionation of

cyclic acetals has been accomplished by using such stationary phases as alumina, silica gel, and cellulose[18], but none of these methods appear to be as simple as that described here. Moreover, in the method now reported, the parent sugar is recoverable.

Although the mixtures thus far separated have been relatively simple, the respective K_D values suggest that more-complex mixtures should be separable. The procedure is rapid and economical, and provides a useful extension to other methods of separating such mixtures.

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REFERENCES

1 S. ROSEMAN, R. H. ABELES, AND A. DORFMAN, *Arch. Biochem. Biophys.*, 36 (1952) 232.
2 L. HOUGH, J. E. PRIDDLE, AND R. S. THEOBALD, *Chem. Ind.* (London), (1960) 900.
3 L. P. ZILL, J. X. KHYM, AND G. M. CHENIAE, *J. Am. Chem. Soc.*, 75 (1953) 1339.
4 J. I. OHMS, J. ZEC, J. V. BENSON, AND J. A. PATTERSON, *Anal. Biochem.*, 20 (1967) 51.
5 O. SAMUELSON AND B. SWENSON, *Acta Chem. Scand.*, 16 (1962) 2056.
6 J. DAHLBERG AND O. SAMUELSON, *Acta Chem. Scand.*, 17 (1963) 2136.
7 O. SAMUELSON AND H. STRÖMBERG, *Carbohyd. Res.*, 3 (1966) 89.
8 P. W. AUSTIN, F. E. HARDY, J. G. BUCHANAN, AND J. BADDILEY, *J. Chem. Soc.*, (1963) 5350.
9 P. JONSSON AND O. SAMUELSON, *Anal. Chem.*, 39 (1967) 1156.
10 J. K. N. JONES, R. A. WALL, AND A. O. PITTET, *Can. J. Chem.*, 38 (1960) 2285.
11 J. K. N. JONES AND R. A. WALL, *Can. J. Chem.*, 38 (1960) 2290.
12 R. M. MCCREADY AND J. C. GOODWIN, *J. Chromatog.*, 22 (1966) 195.
13 R. M. SAUNDERS AND H. G. WALKER, Joint Meeting of American Association of Cereal Chemists and American Oil Chemists' Society, Washington, D. C., March 31–April 4, 1968.
14 M. DUBOIS, K. A. GILLES, J. K. HAMILTON, P. A. REBERS, AND F. SMITH, *Anal. Chem.*, 28 (1956) 350.
15 P. FLODIN, *J. Chromatog.*, 5 (1961) 103.
16 R. F. WHISTLER AND D. F. DURSO, *J. Am. Chem. Soc.*, 72 (1950) 677.
17 L. HOUGH, J. K. N. JONES, AND W. H. WADMAN, *J. Chem. Soc.*, (1949) 2511.
18 A. N. DE BELDER, *Advan. Carbohyd. Chem.*, 20 (1965) 219.

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Partial toluene-*p*-sulphonylation studies of methyl 6-*O*-trityl-β-D-galactofuranoside
Part II. Synthesis of 2,5,6- and 3,5,6-trimethyl ethers of D-galactose*

Few methyl ethers of D-galactofuranose are known. Of these, the 2,3,5-tri-*O*-methyl and 2,3,5,6-tetra-*O*-methyl derivatives have been known for some time[1,2], and syntheses of the 2,5- and 3,5-di-*O*-methyl derivatives have recently been reported.[3] In the present note, we report on syntheses of 2,5,6- and 3,5,6-tri-*O*-methyl-D-galactose.

The starting materials were methyl 2,5-di-*O*-methyl-3-*O*-tosyl**-6-*O*-trityl- and the corresponding 2-*O*-tosyl-β-D-galactofuranosides[3]. The former compound, on successive detritylation, methylation, detosylation, and hydrolysis yielded 2,5,6-tri-*O*-methyl-D-galactose having $[\alpha]_D^{27}$ −15.9° (water) and R_F 0.63 (solvent *A*); these values are consistent with its furanoid character. The M_G value (0.00) of the tri-*O*-methyl-D-galactose on electrophoresis in borate buffer supports the fact that position 2 is substituted, and oxidation with bromine water afforded a syrupy γ-lactone having C=O absorption[4] at 1785 cm^{-1}. The sugar was characterized as the crystalline amide.

Methyl 3,5-di-*O*-methyl-2-*O*-tosyl-6-*O*-trityl-β-D-galactofuranoside on similar detritylation, methylation, detosylation, and hydrolysis yielded 3,5,6-tri-*O*-methyl-D-galactose having $[\alpha]_D^{27}$ −40.5° (water), R_F 0.66, and M_G 0.60. The syrupy lactone derived from this sugar also had a strong C=O absorption at 1785 cm^{-1}. This sugar was characterized as the crystalline 3,5,6-tri-*O*-methyl-D-galactonamide.

Detritylation of methyl 2,3-di-*O*-methyl-5-*O*-tosyl-6-*O*-trityl-β-D-galactofuranoside[3] afforded crystalline methyl 2,3-di-*O*-methyl-5-*O*-tosyl-β-D-galactofuranoside, but scarcity of material precluded further work on this fraction.

EXPERIMENTAL

The general experimental methods have been described[3] in Part I.

*Methyl 2,5,6-tri-*O*-methyl-3-*O*-tosyl-β-D-galactofuranoside.* — A solution of methyl 2,5-di-*O*-methyl-3-*O*-tosyl-6-*O*-trityl-β-D-galactofuranoside (0.480 g) in dry chloroform containing 4% of hydrogen chloride was kept for 10 min at 0°. Neutralization (silver carbonate), filtration, and evaporation yielded a syrup that was purified by chromatography on a column of silica gel with ethyl acetate–light petroleum (b.p. 60–80°) (20:80) as eluant. The resulting homogeneous syrup (0.260 g) was methylated with methyl iodide (10 ml) and silver oxide (1 g). Filtration and evaporation gave a syrup which was decolourized (in ethereal solution) with charcoal to give a chromatographically homogeneous syrup (0.183 g, 60%), $[\alpha]_D^{28}$ −76.8° (*c* 2.72, chloroform) (Found: OCH_3, 31.45. calc.: 31.79%).

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**Tosyl = toluene-*p*-sulphonyl.

Methyl 3,5,6-tri-O-methyl-2-O-tosyl-β-D-galactofuranoside. — Methyl 3,5-di-*O*-methyl-2-*O*-tosyl-6-*O*-trityl-β-D-galactofuranoside (0.740 g), on similar detritylation and methylation, yielded the title compound as a syrup (0.450 g, 85%), $[\alpha]_D^{28}$ −56.2° (*c* 3.28, chloroform) (Found: OCH_3, 31.78%).

Methyl 2,5,6-tri-O-methyl-β-D-galactofuranoside. — A solution of methyl 2,5,6-tri-*O*-methyl-3-*O*-tosyl-β-D-galactofuranoside (0.137 g) in dry methanol (5 ml), after being mixed with sodium (0.200 g) in methanol (5 ml), was boiled under reflux. After 8 h, some of the starting material still remained (t.l.c., chloroform–acetone, 10:1), and a further quantity of sodium (0.150 g) in methanol (5 ml) was added and the solution refluxed for an additional 6 h. The mixture was cooled to room temperature, organic solvents were removed by aeration, water (5 ml) was added, and the solution was extracted continuously with chloroform for 12 h. The chloroform extract was dried (Na_2SO_4) and evaporated to a syrup (0.067 g, 81%), $[\alpha]_D^{27}$ −82.7° (*c* 1.8, chloroform) (Found: OCH_3, 52.29. Tri-*O*-methylhexoside calc.: 52.5%).

Methyl 3,5,6-tri-O-methyl-β-D-galactofuranoside. — Methyl 3,5,6-tri-*O*-methyl-2-*O*-tosyl-β-D-galactofuranoside (0.306 g) was similarly detosylated to yield a syrupy product (0.148 g, 94%), $[\alpha]_D^{27}$ −131.5° (*c* 3.18, chloroform) (Found: OCH_3, 52.30%).

2,5,6-Tri-O-methyl-D-galactose. — Methyl 2,5,6-tri-*O*-methyl-β-D-galactofuranoside (0.030 g) was hydrolyzed with 0.25N sulphuric acid (0.5 ml) for 5 h at 100°. Neutralization (barium carbonate), filtration, and evaporation gave a syrup (0.028 g, 98%), $[\alpha]_D^{27}$ −15.9° (*c* 2.8, water). Paper chromatography (solvent *A*) and paper electrophoresis showed a single component having R_F, R_G, and M_G values of 0.63, 0.78, and 0.00, respectively.

3,5,6-Tri-O-methyl-D-galactose. — Methyl 3,5,6-tri-*O*-methyl-β-D-galactofuranoside (0.149 g) was similarly hydrolyzed with 0.25N sulphuric acid (1.5 ml) to yield a syrup (0.121 g, 86%), $[\alpha]_D^{27}$ −40.5° (*c* 3.9, water). Paper chromatography (solvent *A*) and paper electrophoresis revealed a single spot, R_F 0.66, R_G 0.81, and M_G 0.60.

2,5,6-Tri-O-methyl-D-galactonamide. — A solution of 2,5,6-tri-*O*-methyl-D-galactose (0.028 g) in water (1.5 ml) was oxidized with bromine (5 drops) in the presence of barium carbonate (0.040 g) for 72 h in the dark. Bromine was removed by aeration, and the aqueous solution, after acidification with hydrochloric acid, was extracted continuously with chloroform for 24 h. The extract was dried (Na_2SO_4), filtered, and concentrated to a syrup (0.018 g) which was homogeneous (t.l.c., chloroform–acetone, 1:1). Distillation (bath temperature 135–140°/0.01 mm) gave the syrupy lactone, $\nu_{max}^{CHCl_3}$ 1785 cm^{-1}, which was dissolved in methanol saturated with ammonia. After 48 h at 0°, the solvent was removed by slow evaporation in a vacuum desiccator. The residue was crystallized twice from ethanol–ether to give 2,5,6-tri-*O*-methyl-D-galactonamide, m.p. 136°, $[\alpha]_D^{23}$ +38.2° (*c* 0.6, water).

Anal. Calc. for $C_9H_{19}NO_6$: C, 45.50; H, 8.02; N, 5.91; OCH_3, 39.2. Found: C, 45.50; H, 7.68; N, 6.10; OCH_3, 39.0.

3,5,6-Tri-O-methyl-D-galactonamide. — 3,5,6-Tri-*O*-methyl-D-galactose (0.078 g) in water (3 ml) was similarly oxidized with bromine (10 drops) in the presence of barium carbonate (0.150 g). The syrupy oxidation product (0.072 g), recovered as

above, was homogeneous by t.l.c. (chloroform–acetone 1:1), and was distilled (bath temperature 130–135°/0.01 mm) to give the syrupy lactone (0.055 g) $\nu_{max}^{CHCl_3}$ 1785 cm^{-1}. Treatment with methanolic ammonia and isolation as above gave 3,5,6-tri-*O*-methyl-D-galactonamide, m.p. 133–134°, $[\alpha]_D^{23}$ +5.4° (*c* 1.0, water).

Anal. Calc. for $C_9H_{19}NO_6$: C, 45.50; H, 8.02; N, 5.91; OCH_3, 39.2. Found: C, 45.46; H, 7.90; N, 5.95; OCH_3, 39.15.

Methyl 2,3-di-O-methyl-5-O-tosyl-β-D-galactofuranoside. — Methyl 2,3-di-*O*-methyl-5-*O*-tosyl-6-*O*-trityl-β-D-galactofuranoside (0.259 g) was dissolved in dry chloroform (10 ml), and detritylated with dry hydrogen chloride[3]. The dried material was washed with light petroleum (b.p. 65–110°) to remove chlorotriphenylmethane, yielding a white powder (0.158 g, 100%). Crystallization from isopropyl ether gave methyl 2,3-di-*O*-methyl-5-*O*-tosyl-β-D-galactofuranoside, m.p. 123–124.5°, $[\alpha]_D^{25}$ −55.5° (*c* 2.57, chloroform).

Anal. Calc. for $C_{16}H_{24}O_8S$: C, 51.05; H, 6.43; S, 8.52; OCH_3, 24.73. Found: C, 50.65; H, 6.29; S, 8.55; OCH_3, 25.08.

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REFERENCES

1 S. P. LUCKETT AND F. SMITH, *J. Chem. Soc.*, (1940) 1114.
2 W. N. HAWORTH, D. A. RUELL, AND G. C. WESTGARTH, *J. Chem. Soc.*, 125 (1924) 2468.
3 I. R. SIDDIQUI AND B. URBAS, *Carbohyd. Res.*, 5 (1967) 210.
4 S. A. BARKER, E. J. BOURNE, R. M. PINKARD, AND D. H. WHIFFEN, *Chem. Ind.* (London), (1958) 653

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Preparation of 2,3,2′,3′,6′-penta-*O*-acetyl-1,6-anhydrocellobiose

In the course of preparing 2,3,2′,3′,4′-penta-*O*-acetyl-1,6-anhydrocellobiose[1] (**1**) according to the method described by Lindberg and Selleby[2], a case of acetyl migration has been encountered. When a 10% solution of 2,3,2′,3′,4′-penta-*O*-acetyl-1,6-anhydro-6′-*O*-tritylcellobiose (**2**) in glacial acetic acid was detritylated with *one* equivalent of hydrogen bromide, followed by recovery in the usual way[1,2], compound **1** was formed[1] in a yield of 80%. However, when compound **2** was treated with *four* equivalents of hydrogen bromide under similar conditions, a new compound (**3**) was obtained in 40% yield; crystallized from chloroform–ether, it had m.p. 142°, $[\alpha]_D^{25}$ −80°.

above, was homogeneous by t.l.c. (chloroform–acetone 1:1), and was distilled (bath temperature 130–135°/0.01 mm) to give the syrupy lactone (0.055 g) $\nu_{max}^{CHCl_3}$ 1785 cm^{-1}. Treatment with methanolic ammonia and isolation as above gave 3,5,6-tri-*O*-methyl-D-galactonamide, m.p. 133–134°, $[\alpha]_D^{23}$ +5.4° (*c* 1.0, water).

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REFERENCES

1 S. P. LUCKETT AND F. SMITH, *J. Chem. Soc.*, (1940) 1114.
2 W. N. HAWORTH, D. A. RUELL, AND G. C. WESTGARTH, *J. Chem. Soc.*, 125 (1924) 2468.
3 I. R. SIDDIQUI AND B. URBAS, *Carbohyd. Res.*, 5 (1967) 210.
4 S. A. BARKER, E. J. BOURNE, R. M. PINKARD, AND D. H. WHIFFEN, *Chem. Ind.* (London), (1958) 653

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Compound **3** has the same elementary composition as **1**. Both **1** and **3** show absorption for hydroxyl group in their infrared spectra. Compound **1** can be converted into **2**, whereas **3** does not give a trityl ether. On acetylation with acetic anhydride and pyridine, compounds **1** and **3** afford the same hexaacetate **4**. Methylation of **3** with diazomethane[3] gives a monomethyl ether which, on saponification and acid hydrolysis, affords 4-*O*-methyl-D-glucose and D-glucose (identified by g.l.c.). All of these reactions indicate that **3** is 2,3,2′,3′,6′-penta-*O*-acetyl-1,6-anhydrocellobiose. Its formation clearly involves the migration[4,5] of an acetyl group from O-4′ to O-6′.

The n.m.r. spectra of **1** and **3** show a singlet for H-1 at τ 5.4 which is similar to that found in the spectra of 1,6-anhydro-β-D-glucopyranose triacetate and 1,6-anhydrocellobiose hexaacetate (**4**). The spectra of **1**, **3**, and **4** have a similar AB part of an ABX system in the region τ 5.9 to 6.3 corresponding to the two H-6 protons. The signals at τ 5.6 to 5.9, due, in the spectra of **3** and **4**, to the two H-6′ protons, are absent from the spectrum of **1**; the larger shielding effect of hydrogen, as compared to acetyl, has shifted them to higher field. Consequently, the signals in the region τ 6.3 to 6.6 represent four protons in the spectrum of **1** (probably H-4, H-5′, and two H-6′), three protons in the spectrum of **3** (probably H-4, H-4′, and H-5′), and two protons in the spectrum of **4** (probably H-4 and H-5′).

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REFERENCES

1 N. ROY AND T. E. TIMELL, *Carbohyd. Res.*, 6 (1968) 475.
2 B. LINDBERG AND L. SELLEBY, *Acta Chem. Scand.*, 14 (1960) 1051.
3 I. O. MASTRONARDI, S. M. FLEMATTI, AND E. G. GROSS, *Carbohyd. Res.*, 3 (1966) 177.
4 P. J. GAREGG, *Svensk Kem. Tidskr.*, 77 (1965) 1.
5 W. A. BONNER, *J. Org. Chem.*, 24 (1959) 1388.

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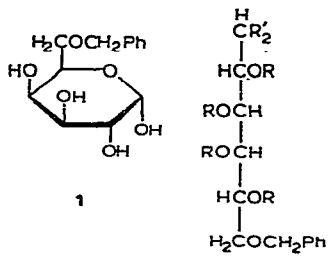
6-*O*-Benzyl-D-galactose and its dimethyl acetal

6-*O*-Benzyl-α-D-galactose (**1**) is a useful starting-material in synthetic work in which there is required a D-galactose derivative having the 6-hydroxyl group protected by a group that can subsequently be removed under mild conditions, such as hydrogenolysis. The dimethyl acetal (**5**) of the *aldehydo* form of **1** provides a convenient starting-point for the preparation of derivatives of D-galactoseptanose. The preparation of compounds **1** and **5** is now described.

The readily available 6-*O*-benzyl-1,2:3,4-di-*O*-isopropylidene-α-D-galactopyranose[1] was easily hydrolyzed, and a crystalline product was isolated; this was formulated as 6-*O*-benzyl-α-D-galactopyranose (**1**), because of its slow, negative mutarotation. The free sugar **1** was converted, in four steps, into the dimethyl acetal (**5**) in 50% overall yield; the steps were mercaptalation of **1** to give **2**, acetylation of **2** to give **3**, demercaptalation of **3** to give **4**, and deacetylation of **4** to give **5**. The five new compounds **1** to **5** crystallized well, in contrast to the corresponding D-*gluco* compounds[2]. The excellent yield obtained in the demercaptalation of **3** to give **4** was unexpected.



1

	R	R'
2	H	SEt
3	Ac	SEt
4	Ac	OMe
5	H	OMe

EXPERIMENTAL

General. — The microanalyses were performed by the Australian Microanalytical Service, Melbourne. All evaporations and concentrations were conducted in a rotary evaporator under diminished pressure at ≯ 40° (bath temp.).

6-O-Benzyl-α-D-galactopyranose (**1**). — Hot M hydrochloric acid (100 ml) was added to a solution of 6-*O*-benzyl-1,2:3,4-di-*O*-isopropylidene-α-D-galactopyranose (100 g) in *p*-dioxane (400 ml) at 100°, and the mixture was kept for 30 min at 100°, and cooled. The resulting crystals of compound **1** were filtered off, and the mother liquor was rendered acidic (pH 4), and evaporated to dryness. The residue, treated with *p*-dioxane, yielded more of **1**; total yield, over 90% (when pure starting material was used). The recrystallized compound had m.p. 96–98°, $[\alpha]_D^{25}$ +75.3° (5 min), +41.3° (2 days), and +32.7° (8 days) (*c* 1.0, methanol).

Anal. Calc. for $C_{13}H_{18}O_6$: C, 57.8; H, 6.7. Found: C, 57.7; H, 6.8.

6-O-Benzyl-D-galactose diethyl dithioacetal (**2**). — A mixture of finely powdered **1** (250 g), hydrochloric acid (d. 1.19, 750 ml) and ethanethiol (250 ml),

all precooled to 0°, was vigorously shaken for 5 min at 0°. The dithioacetal **2** crystallized, and was filtered off, washed with ice-cold water, and recrystallized from methanol as quickly as possible. The yield of pure **2** was 80–85%, m.p. 120°, $[\alpha]_D^{25}$ +3.4° (*c* 1.0, methanol).

Anal. Calc. for $C_{17}H_{28}O_5S_2$: C, 54.2; H, 7.5; S, 17.0. Found: C, 54.1; H, 7.5; S, 17.0.

2,3,4,5-Tetra-O-acetyl-6-O-benzyl-D-galactose diethyl dithioacetal (**3**). — The dithioacetal **2** (112 g), pyridine (360 ml), and acetic anhydride (720 ml) were mixed at 0°; the mixture was kept overnight at room temperature, and poured, with stirring, into ice–water (5 l). The acetate that crystallized was filtered off, and recrystallized from methanol (85% yield), m.p. 85°, $[\alpha]_D^{25}$ −12.6° (*c* 1.0, methanol).

Anal. Calc. for $C_{25}H_{36}O_9S_2$: C, 55.1; H, 6.7; S, 11.8. Found: C, 54.9; H, 6.8; S, 11.7.

2,3,4,5-Tetra-O-acetyl-6-O-benzyl-D-galactose dimethyl acetal (**4**). — The diethyl dithioacetal (**3**) (40 g) in dry methanol (400 ml) was vigorously stirred for 90 min with cadmium carbonate (48 g) and mercuric chloride (130 g) while being heated under reflux in an all-glass apparatus. The mixture was cooled, and filtered, and the filtrate was shaken with water (600 ml) and chloroform (600 ml). The chloroform layer was washed with water until free from chloride ions, (dried sodium sulfate,) and evaporated to a syrup which crystallized from ether at 0° (80% yield). Recrystallization gave **4** that had m.p. 58–60°, $[\alpha]_D^{25}$ −4.6° (*c* 1.0, methanol).

Anal. Calc. for $C_{23}H_{32}O_{11}$: C, 57.0; H, 6.7. Found: C, 56.9; H, 6.8.

*6-O-Benzyl-*aldehydo*-D-galactose dimethyl acetal* (**5**). — For the methanolysis, a solution of compound **4** (100 g) in 0.1% methanolic sodium methoxide (1 liter) was kept for 24 h at room temperature, and the solution was then concentrated. The product crystallized during the concentration, and was filtered off at intervals. Recrystallization from methanol gave **5** in 82% yield, m.p. 126–127°, $[\alpha]_D^{25}$ +13.2° (*c* 1.0, methanol).

Anal. Calc. for $C_{15}H_{24}O_7$: C, 56.9; H, 7.6. Found: C, 57.0; H, 7.6.

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REFERENCES

1 C. E. BALLOU AND H. O. L. FISCHER, *J. Amer. Chem. Soc.*, 76 (1954) 3188.
2 E. F. L. J. ANET, unpublished results.

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Equilibration of D-fructose with its 1,2-enediol[a]

Sugar 1,2-enediols have been suggested as intermediates in numerous carbohydrate reactions, including the Lobry de Bruyn–Alberda van Ekenstein transformation[1], the formation of saccharinic acids[1], the formation of furan derivatives[2], and sugar interconversions catalyzed by the hexose phosphate isomerases. Recently, the mechanism by which D-glucose, D-mannose, and D-fructose are interconverted in alkaline solution[3], and the mechanism of action of the D-glucose and D-mannose 6-phosphate isomerases[4,5] have been studied by means of isotope-exchange experiments, and are consistent with a 1,2-enediol intermediate.

The present work reports some experiments concerning the equilibrium between D-fructose and its hypothetical 1,2-enediol, by using the acquisition of carbon-bound deuterium as a measure of the extent of reaction. Experiments were conducted in deuterium oxide solutions containing (*a*) calcium hydroxide-d_2, the base most often used to effect alkaline isomerizations and degradations, (*b*) sodium hydroxide-*d*, with and without added calcium ion, and (*c*) sulfuric acid-d_2. The amount of deuterium incorporated was estimated by determining the position and extent of diminution of n.m.r. signals. Because of complicating side-reactions in alkaline solution, particularly the production of acid, D-fructose was isolated as its crystalline 2,3:4,5-di-isopropylidene acetal[6] prior to each determination of deuterium. The n.m.r. spectrum of the acetal, in chloroform-*d*, showed signals of the isopropylidene methyl groups as four singlets at τ 8.65, 8.60, 8.56, and 8.47. The 1-OH proton was coupled with the C-1 methylene group, and gave rise to a triplet centered at τ 7.32 (*J* 4.5 Hz), and the H-1 protons gave a doublet centered at τ 6.35 (*J* 5 Hz). Two prominent multiplets were also observed at τ 6.20–6.13, and at τ 5.74–5.63, each arising from two protons. That the doublet at τ 7.35 was due to the H-2 protons was evident when, on exchange with deuterium oxide, the triplet at τ 7.32 disappeared and the doublet collapsed to a singlet. Examination of n.m.r. spectra of 2,3:4,5-di-*O*-isopropylidene-D-fructose, prepared from D-fructose that had been equilibrated in alkaline deuterium oxide solution, indicated that measurable incorporation of deuterium occurred only at C-1. For measurement of the amount of incorporation, reproducible and comparable figures could be obtained by comparing the intensity of the signal arising from the H-1 protons, either with the signals of the isopropylidene methyl groups, or with the two downfield multiplets. In both cases, the results were reproducible with a precision of ± 0.1 proton per signal, as determined by experiments with both deuterated and nondeuterated 2,3:4,5-di-*O*-isopropylidene-D-fructose. The results of a number of experiments are shown in Table I.

Paper chromatography of the residue obtained after D-fructose had reacted for 3 h in aqueous calcium hydroxide, under conditions comparable to those used

[a]Contribution from the Missouri Agricultural Experiment Station. Journal Series Number 5287.

TABLE I

ACQUISITION OF CARBON-BOUND DEUTERIUM[a] BY D-FRUCTOSE AT C-1

Catalyst	*Reaction time (h)*	*Deuterium atoms incorporated*[b]
$Ca(OD)_2$ (note[c])	2	0.4
$Ca(OD)_2$ (note[c])	3	0.6
$Ca(OD)_2$ (note[c])	4	0.9
$Ca(OD)_2$ (note[c])	6	1.3
NaOD (0.03M)	3	0.7
NaOD (0.03M), 0.03M in $CaCl_2$	3	0.7
NaOD (0.03M), 0.10M in $CaCl_2$	3	0.6
D_2SO_4[b] (M)	7	0.0
D_2SO_4[b] (M)	10	0.0

[a]All reactions performed at 45°. [b]Calculated by comparison of the H-1 signal intensity with the intensities of downfield multiplets at τ 6.20–6.13 and at τ 5.74–5.63. [c]250 mg of $Ca(OH)_2$ suspended in 6.0 ml of deuterium oxide.

in the deuterium-exchange experiments, indicated that a fructose was the major component in the residue, although a glucose was detectable. Wolfrom and Lewis[7] have estimated that, at "equilibrium" in saturated lime-water (6 days, 35°) approximately 63.5% of a glucose and 31.0% of a ketose is present. Thus, it seems probable that the measurements described in the present work were made on compounds that had been isolated considerably before "equilibrium" was attained. It is evident, therefore, that no appreciable proportion of D-fructose-*1-d* arose *via* D-glucose in a Lobry de Bruyn–Alberda van Ekenstein type of transformation; it was formed by a reversible enolization of the parent sugar in deuterium oxide.

The experiments involving the use of sodium hydroxide-*d* solutions, with and without added calcium ions, indicate the absence of significant catalysis by calcium ions. Calcium and certain other divalent metal ions have been observed to accelerate the rate of alkaline degradation of 3-*O*-methyl-D-glucose, a reaction thought to proceed *via* a 1,2-enediol intermediate[8].

The absence of any measurable incorporation of deuterium by D-fructose in acid, and the relatively rapid incorporation in base, parallel the reactivity of this sugar in acidic and basic solutions. D-Fructose undergoes alkaline degradation[9] in dilute alkali at 25°, whereas in relative terms it is much more stable toward dehydration reactions in acid solutions[10].

EXPERIMENTAL

Materials and methods. — Descending paper-chromatography was performed with 10:4:3 ethyl acetate–pyridine–water as the irrigant, and aniline hydrogen phthalate as the spray reagent. T.l.c. was performed with silica gel as the support and 3:1:1 butanone–acetic acid–water as the irrigant. Spots were visualized by spraying with 10% ethanolic sulfuric acid followed by charring at 100°. N.m.r. spectra were

determined at 60 MHz with a Varian A-60 spectrometer. Samples, at a concentration of 200 g/l, were prepared in chloroform-*d* with tetramethylsilane (τ = 10.00) as the internal standard. 2,3:4,5-Di-*O*-isopropylidene-D-fructose was prepared by the method of Pacsu, Wilson, and Graf[6]; m.p. 96–7°. This product was homogeneous by t.l.c.

Treatment of D-*fructose with alkaline deuterium oxide.* — In a typical experiment, 1.0 g of D-fructose and 250 mg of calcium hydroxide were added to magnetically stirred deuterium oxide (6.0 ml) at 45°. After being stirred for 3 h, the suspension was applied to a column containing 10 ml of Dowex-50 (H^+). The effluent was evaporated to dryness, and the syrupy residue was treated with 20 ml of anhydrous acetone and 0.8 ml of concentrated sulfuric acid. This suspension was stirred for 0.5 h at 0° and then for 3 h at 25°. The resulting, yellow solution was rendered neutral with concentrated ammonium hydroxide, the suspension was filtered, and the filtrate was evaporated to a syrup. 2,3:4,5-Di-*O*-isopropylidene-D-fructose crystallized spontaneously after several h, and was recrystallized from ethanol–water; yield, 500 mg; m.p. and mixed m.p. 95–96°.

The same procedure was used in isolating D-fructose from the sodium hydroxide-*d* solutions, in which case 10.0 ml was utilized. Solutions containing calcium chloride were treated first with Dowex-50 (H^+) and then with Dowex-1 (CO_3^{2-}) prior to conversion into the isopropylidene acetal.

Treatment of D-*fructose with acidified deuterium oxide.* — To a flask containing 6.0 ml of M sulfuric acid in deuterium oxide, at 45°, was added 1.0 g of D-fructose. The solution was stirred for 10 h, and was then neutralized with Dowex-1 (CO_3^{2-}), evaporated to dryness, and converted into 2,3:4,5-di-*O*-isopropylidene-D-fructose as described above. Other experiments involving acidic solutions were handled in the same way. No correction was made for hydrogen ions introduced by the sulfuric acid.

Treatment of D-*fructose with aqueous calcium hydroxide.* — To a solution of 5.0 g of D-fructose in 30 ml of water was added 1.25 g of calcium hydroxide. The suspension was stirred for 15 min at 45°, and filtered, and a 5.0-ml aliquot of the filtrate was titrated with standard acid. The remainder of the filtrate was kept in a bath at 45° for an additional 3 h. At this time, titration indicated that 0.35 equivalent of acid per mole of D-fructose had been produced. The remainder of the alkaline solution was treated with Dowex-50 (H^+), and evaporated, and the residue used in paper-chromatographic experiments.

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REFERENCES

1 J. C. SPECK, Jr., *Advan. Carbohyd. Chem.*, 13 (1958) 63, and references therein.
2 E. F. L. J. ANET, *Advan. Carbohyd. Chem.*, 19 (1964) 181.
3 J. C. SOWDEN AND R. SCHAFFER, *J. Amer. Chem. Soc.*, 74 (1952) 505.
4 Y. J. TOPPER, *J. Biol. Chem.*, 225 (1957) 419.
5 I. A. ROSE AND E. L. O'CONNELL, *J. Biol. Chem.*, 236 (1961) 3086.

6 E. PACSU, E. J. WILSON, JR., AND L. GRAF, *J. Amer. Chem. Soc.*, 61 (1939) 2675.
7 M. L. WOLFROM AND W. L. LEWIS, *J. Amer. Chem. Soc.*, 50 (1928) 837.
8 J. KENNER AND G. N. RICHARDS, *J. Chem. Soc.*, (1957) 3019.
9 J. KENNER AND G. N. RICHARDS, *J. Chem. Soc.*, (1954) 1784.
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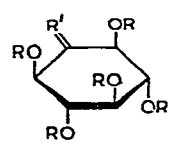
Carbohyd. Res., 7 (1968) 86–89

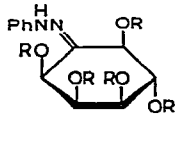
Formation of arylazocyclohexene derivatives on acylation of certain inosose phenylhydrazones*

Recent communications by Wolfrom and coworkers[1] describing the preparation of arylazoethylene derivatives from aldose phenylhydrazones prompt this report on related findings for inosose derivatives. The products resulting from acylation of *myo*-inosose-2 phenylhydrazone[2] (**1a**) and DL-*epi*-inosose-2 phenylhydrazone[2] (**2a**) in the presence of pyridine have now been examined. On treating a solution of penta-*O*-acetyl-*myo*-inosose-2 (**1d**)[3] in warm acetic acid with phenylhydrazine, Angyal and Matheson[4] obtained a pale-yellow, crystalline compound (m.p. 112–113°) to which they assigned the phenylhydrazone structure **1b**. However, it is now found that, when this reaction is performed, compound **1d** actually loses the elements of acetic acid, and, instead of giving phenylhydrazone **1b**, it yields the elimination product 6-phenylazo-5-cyclohexene-DL-*ido*-1,2,3,4-tetrol tetraacetate (**3a**). Moreover, compound **3a** can conveniently be prepared directly from **1a**.



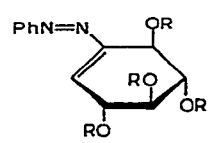
1

a: R = H, R′ = NNHPh
b: R = Ac, R′ = NNHPh
c: R = OCEt, R′ = NNHPh
d: R = Ac, R′ = O



2

a: R = H
b: R = Ac
c: R = OCEt



3

a: R = Ac
b: R = OCEt

EXPERIMENTAL

N.m.r. spectra were obtained on a Varian A-60 spectrometer, with tetramethylsilane (τ = 10.00) as an internal standard. I.r. spectra were recorded with a Perkin–Elmer grating Model 257, and u.v. spectra with a Beckman DK-2 spectrophotometer**. Analyses were made by Rolf A. Paulson of the Microchemical Analysis Section of this Division.

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6 E. PACSU, E. J. WILSON, JR., AND L. GRAF, *J. Amer. Chem. Soc.*, 61 (1939) 2675.
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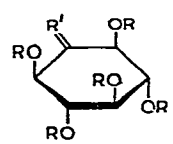

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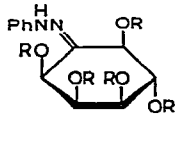
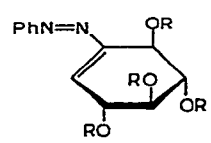
d: R = Ac, R′ = O



2

a: R = H
b: R = Ac
c: R = OCEt



3

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b: R = OCEt

EXPERIMENTAL

N.m.r. spectra were obtained on a Varian A-60 spectrometer, with tetramethylsilane (τ = 10.00) as an internal standard. I.r. spectra were recorded with a Perkin–Elmer grating Model 257, and u.v. spectra with a Beckman DK-2 spectrophotometer**. Analyses were made by Rolf A. Paulson of the Microchemical Analysis Section of this Division.

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6-Phenylazo-5-cyclohexene-DL-ido-*1,2,3,4-tetrol tetraacetate* (**3a**). — A mixture of the phenylhydrazone **1a** (3 g) with pyridine (50 ml) and acetic anhydride (20 ml) was stirred for 1 h at 10–15°, and for 1 h at 25°, and poured into ice–water; rapid recrystallization* of the precipitated product, from ethanol or aqueous methanol, afforded pale-yellow needles of **3a**, yield 3.5 g (75%), m.p. 112–114° (no depression of m.p. on admixture with the compound prepared as described earlier[4]). A sample of **3a** was purified by column chromatography [silica gel, 100–200 mesh; with 3:1 (v/v) heptane–ethyl acetate as eluant], and dried to constant weight at 25° and 10 torr.

Anal. Calc. for $C_{20}H_{22}N_2O_8$: C, 57.40; H, 5.30; N, 6.70. Found: C, 57.28; H, 5.48; N, 6.70.

Previously, only a nitrogen analysis had been reported[4]. Slow crystallization of **3a** from 95% ethanol at room temperature afforded orange crystals (m.p. 135–136°) having some properties identical with those of the product having m.p. 112–114°. The two products are probably dimorphs. Dimorphism has been reported for some inosose esters[4], but this observation has been questioned[5]. The compound showed a yellow spot having R_F 0.55 ±0.02 [t.l.c. on silica gel G, 3:1:1 (v/v) heptane–ethyl acetate–acetic acid, 60 min]. Spraying of the chromatogram with 1.5M sulfuric acid in methanol at room temperature caused the yellow spot of **3a** to disappear; however, it reappeared as a purple-brown spot on heating the plate for 2 min at 120°. Aromatic azo derivatives show R_F values higher than that of **3a**, and can thus be readily distinguished therefrom. Compound **3a** showed ultraviolet and visible spectra characteristic of the R−CH = CH−N = N−Ph chromophore[6] [λ_{max}^{MeOH} 222.5 (sh) (ε ~12,300), 228 (ε ~13,700), 233 (ε ~12,800), 302 (ε ~23,000), and 436 nm (ε ~400)] (Type A) that differ from those of either *myo*-inosose-2 phenylhydrazone (**1a**) [278 (ε ~23,500); 302 (sh) nm (ε ~10,200)] (Type B), or aromatic azo compounds, for example, azobenzene [λ_{max}^{MeOH} 229 (ε ~11,500), 318 (ε ~21,500), 442 nm (ε ~550)] (Type C). The u.v. and visible spectra of **3a** are similar to those reported for the β-elimination product from D-mannose phenylhydrazone[1], indicating a structural similarity between the compounds.

The i.r. spectrum[7] of **3a** shows $\nu_{max}^{Fluorolube}$ 1658 (C=C), 1584 (N=N), 1430 cm^{-1} (N=N) (see also Table I). In the n.m.r. spectrum ($CDCl_3$) of **3a**, the olefinic proton (H-5) appeared as a quartet centered at τ 3.05, due to vicinal coupling with H-4 ($J_{4,5}$ ~2.5 Hz) and long-range coupling with H-1 ($J_{1,5}$ ~1.0 Hz). These values of the coupling constants are comparable with the values $J_{1,2}$ ~3.1 Hz and $J_{2,4}$ ~1,2 Hz reported for methyl 3,4-dichloro-4-deoxy-α-D-*glycero*-pent-2-enopyranoside by Coxon and coworkers[8]; other pertinent signals occur at τ 7.82, 7.84, 7.86, and 8.00 (acetate methyl groups[9]).

To eliminate the possibility that rearrangement of compound **3a** had occurred during the chromatography on silica gel, crude **3a** was also purified by column chromatography on microcrystalline cellulose (Avicel) with 1:1 (v/v) ethyl acetate–

*Repeated recrystallization of **3a** from warm ethanol, or prolonged drying thereof under vacuum, results in further elimination, and consequent variable melting points.

ether or 2:1 (v/v) ethyl acetate–benzene as eluant. The yield of yellow-orange product **3a** isolated amounted to 72–77%. Some trailing material (10–15%) was difficult to separate from a yellowish impurity; the i.r. spectrum of this mixture, however, showed a band at 3300 cm^{-1}, possibly indicating the presence of a small proportion of phenylhydrazone **1b**. Colorless **1b** was not, however, isolated, even when acetylation of **1a** was conducted at 0–5° and the reaction mixture was carefully processed.

The u.v. and visible spectra of the orange compound **3a** (m.p. 135–136°) were identical with those of compound **3a** having m.p. 112–114°; λ_{max}^{MeOH} 222 (sh) ($\varepsilon \sim 11{,}800$), 228 ($\varepsilon \sim 13{,}000$), 232.5 ($\varepsilon \sim 12{,}200$), 304 ($\varepsilon \sim 22{,}500$), and 437 nm ($\varepsilon \sim 350$). The i.r. spectra of the two forms of **3a** were almost identical (see Table I); a minor difference observed for compound **3a** (m.p. 135–136°) in the region of 1105–1077 cm^{-1} may possibly arise from geometrical isomerism of the phenylazo group. However, no bathochromic shifts were observed in the u.v. and visible spectra such as would be expected for *cis*- and *trans*-azo compounds[10].

*Penta-O-propionyl-*myo*-inosose-2 phenylhydrazone* (**1c**). — In contrast to the reaction with acetic anhydride, treatment of **1a** with propionic anhydride and pyridine at 0–5° gave the pentapropionate **1c** (yield 40%) as colorless prisms; m.p. 90–91°.

Anal. Calc. for $C_{27}H_{36}N_2O_{10}$: C, 59.11; H, 6.61; N, 5.11. Found: C, 59.28; H, 6.50; N, 5.17.

Compound **1c** had a u.v. spectrum of Type B, and $\nu_{max}^{Fluorolube}$ 3300 (N–H), 1600 (C=N), 1532 cm^{-1} (N–H bending)[7]; n.m.r. ($CHCl_3$) τ 0.75 (N–H group), multiplet centered at τ 7.65 (CH_2), multiplet centered at τ 8.85 (CH_3).

The mother liquor gave a small crop of pale-yellow crystals having a u.v. spectrum of Type A; presumably, this compound has structure **3b**.

Compound **3b** was obtained on recrystallization of **1c** from aqueous ethanol at 50°. On storage for about two months at room temperature or for about four months at 5°, **1c** was converted into **3b**, as evidenced by its change in color from colorless (pure inosose-2 phenylhydrazone is always colorless) to yellow-orange (azo form). A sample of **1c** recrystallized from aqueous ethanol gave yellow-orange needles of **3b**, m.p. 72–74°; λ_{max}^{MeOH} 222 (sh) ($\varepsilon \sim 12{,}500$), 227.5 ($\varepsilon \sim 13{,}100$), 233 ($\varepsilon \sim 13{,}200$) 303 ($\varepsilon \sim 22{,}600$), and 439 nm ($\varepsilon \sim 300$). The major i.r. bands are listed in Table I.

Anal. Calc. for $C_{24}H_{30}N_2O_8$: C, 60.74; H, 6.37; N, 5.90. Found: C, 60.45; H, 6.45; N, 5.80.

Acylation of DL-epi-*inosose-2 phenylhydrazone* (**2a**). — Similarly, acetylation of **2a** at 0–5° gave pentaacetate **2b** (yield 65%), m.p. 134–135°; u.v. spectrum of Type B.

Anal. Calc. for $C_{22}H_{26}N_2O_{10}$: C, 55.22; H, 5.48; N, 5.85. Found: C, 55.20; H, 5.35; N, 5.80.

Propionylation of **2a** (0–5°) gave the pentapropionate **2c** (yield 50%), m.p. 112–113°; u.v. spectrum of Type B.

Anal. Calc. for $C_{27}H_{36}N_2O_{10}$: C, 59.11; H, 6.61; N, 5.11. Found: C, 59.22; H, 6.70; N, 5.20.

If the temperature of acylation of **1a** or **2a** was raised to 70–90°, further reactions

TABLE I

INFRARED ABSORPTION BANDS (CM^{-1}) FOR ACYL DERIVATIVES OF SOME INOSOSE PHENYLHYDRAZONES[a]

Assignment	*Compound number*					
	3a[b]	**3a**[c]	**3b**	**1c**	**2a**	**2c**
OH, NH				3297 m	3395 m	3390 m
C=O	1755 s	1745 s	1752 s	1760 w	1770 w	1762 s
				1747 s	1752 s	1750 s
				1720 m	1738 w	1730 s
C=C	1657 w	1650 w	1644 w			
C=N				1600 m	1630 m	1635 m
Phenyl ring	1590 w	1580 w	1590 w	1588 s	1600 s	1600 s
	1480 w	1482 w	1472 w	1495 s	1490 m	1490 m
N=N (azo)	1592 w	1575 w	1582 w			
N–H (bending)				1531 m	1510 s	1509 s
Fingerprint region						1490 m
			1420 m	1415 m		1420 sh
	1340 w	1340 w				1350 s
		1320 w	1330 m			
	1300 m	1300 w			1310 m	1315 m
	1280 w	1285 sh	1278 s		1285 sh	1280 s
	1270 w					
	1260 m	1260 sh		1265 sh	1265 m	
		1250 w		1255 s	1250 w	1255 s
	1240 w				1230 s	
	1220 s	1220 s			1215 s	
			1200 s		1200 sh	1210 m
				1190 s	1185 sh	1190 m
	1165 m		1165 w		1168 m	1165 s
	1160 w	1160 m		1160 s		
	1140 w	1150 w	1155 m		1140 sh	
		1130 w			1120 sh	1135 m

occurred, with the formation of aromatic azo compounds having u.v. spectra of Type C. Unless the reaction conditions were controlled, the acylation of inosose phenylhydrazones in the presence of pyridine (or other base) produced mixtures containing components having u.v. spectra of Types A, B, and C.

DISCUSSION

The major factors influencing the course of acylation of inosose phenylhydrazones in the presence of a basic catalyst are: (*1*) the thermodynamic (steric) stability of the inosose or its phenylhydrazone (as exemplified in acylation of **2a** to **2b**, compared with **1a** to **3a**), (*2*) the nature of the acyl group (acetyl, propionyl, benzoyl, etc.), and (*3*) the temperature at which the reaction is conducted.

Formation of **3a** from **1a** is probably a two-step process consisting of (*a*) ab-

TABLE I (cont.)

Assignment	*Compound number*					
	3a[b]	3a[c]	3b	1c	2a	2e
Fingerprint region (contd.)	1105 m	1100 w	1110 w		1100 m	1118 w
			1085 m	1085 m	1090 sh	
	1077 m		1068 m	1070 sh	1075 w	1070 s
		1060 s		1060 sh		1060 sh
	1050 s		1050 w		1055 s	
		1045 m	1038 w	1040 w		1040 m
	1020 s	1020 s	1025 sh	1020 w	1030 s	1020 m
			1015 m	1000 s		
			995 m		995 sh	995 w
	970 m	972 m			980 sh	970 sh
	960 s	950 m	962 m		950 m	960 sh
			948 w	940 m		940 w
	930 w		928 w		935 m	
	920 s	920 s		922 m	920 m	
	910 w	908 w			905 sh	910 w
			895 m		900 m	
	890 m	890 m	888 m	890 s	890 sh	894 m
					880 sh	882 m
		870 w	862 m	875 sh	865 sh	860 w
	850 s	855 s	847 w	840 w	850 sh	840 sh
					830 w	830 sh
			818 m	805 m		810 s
	780 s	782 s	775 sh			
			770 m		760 s	760 sh
	750 m	750 w	745 m	752 s	750 m	750 s
	695 s	690 s	690 s	690 m	700 s	695 s
	660 w	655 w				

[a]All spectra were recorded for Nujol mulls. [b]Compound 3a having m.p. 112–114°. [c]Compound 3a having m.p. 135–136°.

straction of an acidic imino proton by a nucleophile (base), followed by (*b*) γ-elimination of the acetoxy group; the mechanism is similar to that described for aldose phenylhydrazones[1].

A study of the chemistry of compound **3a** may be of interest in connection with the formation of phenylosazones from inosose phenylhydrazones. In the steroid[11] and other[12] fields, several compounds, similar in structure to **3a**, have recently been postulated as being precursors in formation of phenylosazones.

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REFERENCES

1 M. L. WOLFROM, A. THOMPSON, AND D. R. LINEBACK, *J. Org. Chem.*, 27 (1962) 2563; M. L. WOLFROM, G. FRAENKEL, D. R. LINEBACK, AND F. KOMITSKY, JR., *ibid.*, 29 (1964) 457; H. EL KHADEM, M. L. WOLFROM, Z. M. EL SHAFEI, AND S. H. EL ASHRY, *Carbohyd. Res.*, 4 (1967) 225.
2 T. POSTERNAK, *Biochem. Prepn.*, 2 (1952) 57.
3 H. E. CARTER, C. BELINSKEY, R. K. CLARK, JR., E. H. FLYNN, B. LYTLE, G. E. MCCASLAND, AND M. ROBBINS, *J. Biol. Chem.*, 174 (1948) 415.
4 S. J. ANGYAL AND N. K. MATHESON, *J. Chem. Soc.*, (1950) 3349.
5 T. POSTERNAK, *Cyclitols*, Holden-Day, Inc., San Francisco, Calif., 1965, p. 159.
6 A. J. FATIADI AND H. S. ISBELL, *Carbohyd. Res.*, 5 (1967) 302.
7 A. J. FATIADI, *J. Res. Natl. Bur. Std.*, 71A (1967) 277.
8 B. COXON, H. J. JENNINGS, AND K. A. MCLAUCHLAN, *Tetrahedron*, 23 (1967) 2395.
9 F. W. LICHTENTHALER, H. LEINERT, AND R. SNAUI, *Chem. Ber.*, 100 (1967) 2383.
10 J. BUCKINGHAM AND R. D. GUTHRIE, *Chem. Commun.*, (1967) 1241.
11 J. BUCKINGHAM AND R. D. GUTHRIE, *Chem. Commun.*, (1966) 781; *J. Chem. Soc. (C)*, (1967) 1700; A. HASSNER AND R. CATSOULACOS, *Chem. Commun.*, (1967) 121; *Tetrahedron Lett.*, (1967) 489.
12 L. CAGLIOTI, G. ROSINI, AND F. ROSSI, *J. Am. Chem. Soc.*, 86 (1967) 3865.

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Carbohyd. Res., 7 (1968) 89–94

A modified procedure for the colorimetric, ultramicro determination of reducing sugars with the alkaline ferricyanide reagent

Oxidation in an alkaline ferricyanide solution[1] is the basis for a convenient method commonly employed for the quantitative assay of reducing sugars and of end groups in polysaccharides. A variety of procedures is available for measuring the extent of reduction of ferricyanide (see refs. 2–12 for selected procedures and applications). The sensitive, colorimetric method for the determination of ferrocyanide by formation of the Prussian Blue complex is often used in biochemical research[8–12]. This method, however, suffers from some disadvantages inherent in the colloidal nature of the colored complex; its stability in solution is highly dependent on ionic strength, pH, and the presence of other hydrocolloids[8–10,12].

An alternative method for the colorimetric determination of ferrocyanide formed as a result of the oxidation of a sugar is based on an indirect assay involving formation of ferrous ions and their subsequent interaction with a reagent for iron[13,14]. Following this approach, conditions have now been defined for a sensitive assay of reducing sugars that uses 2,4,6-tripyridyl-*s*-triazine as the reagent for iron[15,16].

EXPERIMENTAL

Reagents. — *1. Potassium ferricyanide.* This reagent (0.7 mM) is made with 10 mM sodium hydroxide that contains 2% of sodium carbonate; kept in a brown bottle, it is stable for at least two weeks at room temperature.

REFERENCES

1 M. L. WOLFROM, A. THOMPSON, AND D. R. LINEBACK, *J. Org. Chem.*, 27 (1962) 2563; M. L. WOLFROM, G. FRAENKEL, D. R. LINEBACK, AND F. KOMITSKY, JR., *ibid.*, 29 (1964) 457; H. EL KHADEM, M. L. WOLFROM, Z. M. EL SHAFEI, AND S. H. EL ASHRY, *Carbohyd. Res.*, 4 (1967) 225.
2 T. POSTERNAK, *Biochem. Prepn.*, 2 (1952) 57.
3 H. E. CARTER, C. BELINSKEY, R. K. CLARK, JR., E. H. FLYNN, B. LYTLE, G. E. MCCASLAND, AND M. ROBBINS, *J. Biol. Chem.*, 174 (1948) 415.
4 S. J. ANGYAL AND N. K. MATHESON, *J. Chem. Soc.*, (1950) 3349.
5 T. POSTERNAK, *Cyclitols*, Holden-Day, Inc., San Francisco, Calif., 1965, p. 159.
6 A. J. FATIADI AND H. S. ISBELL, *Carbohyd. Res.*, 5 (1967) 302.
7 A. J. FATIADI, *J. Res. Natl. Bur. Std.*, 71A (1967) 277.
8 B. COXON, H. J. JENNINGS, AND K. A. MCLAUCHLAN, *Tetrahedron*, 23 (1967) 2395.
9 F. W. LICHTENTHALER, H. LEINERT, AND R. SNAUI, *Chem. Ber.*, 100 (1967) 2383.
10 J. BUCKINGHAM AND R. D. GUTHRIE, *Chem. Commun.*, (1967) 1241.
11 J. BUCKINGHAM AND R. D. GUTHRIE, *Chem. Commun.*, (1966) 781; *J. Chem. Soc. (C)*, (1967) 1700; A. HASSNER AND R. CATSOULACOS, *Chem. Commun.*, (1967) 121; *Tetrahedron Lett.*, (1967) 489.
12 L. CAGLIOTI, G. ROSINI, AND F. ROSSI, *J. Am. Chem. Soc.*, 86 (1967) 3865.

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EXPERIMENTAL

Reagents. — *1. Potassium ferricyanide.* This reagent (0.7 mM) is made with 10 mM sodium hydroxide that contains 2% of sodium carbonate; kept in a brown bottle, it is stable for at least two weeks at room temperature.

2. Ferric chloride. This reagent (2 mM) is made with a solution containing 0.2M citric acid and 2M sodium acetate in 5M acetic acid. It is advisable to dissolve the ferric salt in a small volume of the acid citrate–acetate solution, so as to provide amounts of reagents sufficient for only a day or two of use. Blank values tend to increase somewhat with older solutions.

3. 2,4,6-Tripyridyl-s-triazine (TPTZ). This compound was obtained from G. F. Smith Chemical Co., P.O. Box 5906 Station D, Columbus, Ohio 43222. The reagent employed is 2.5 mM (0.78 mg per ml) in 3M acetic acid; it is stable for at least a week at room temperature.

Procedure. — Reactions should be shielded from direct light. Aliquots (1.0 ml) of solutions containing 2 to 100 nmoles of reducing sugar are placed in test tubes (1.5 × 12.5 cm). Reagent 1 (1.0 ml) is added, and the tubes are kept for 10 min in a boiling-water bath. Reagent 2 (1.0 ml) and reagent 3 (2.0 ml) are then added immediately, without cooling. The solutions are stirred vigorously with a Vibromixer and, after 5 min in the dark, the absorbance at 595 nm (A_{595}) of the $Fe(TPTZ)_2^{2+}$ is read against a reagent blank.

COMMENTS

The color produced is stable for at least 6 h when the mixture is kept in the dark, and also when it is diluted with several volumes of water. Exposure to light may cause a slow increase in the blank values, due to photoreduction of ferricyanide.

A standard curve for the determination of D-glucose, and comparative values for other sugars, are presented in Fig. 1. The molar extinction coefficient for D-glucose obtained in the present procedure was 75,000 ±3%, corresponding to 3.3 mole equivalents of the violet $Fe(TPTZ)_2^{2+}$ (see Refs. 15 and 16). The reagents alone had A_{595} 0.15 to 0.25 for different batches of reagents and solutions; this value is probably attributable to presence of ferrous ions and other reducing contaminants in the reagents. If desired, most of these contaminations can be removed by prior reaction with TPTZ and extraction of the complex with nitrobenzene[15,16]. Samples taken from biological solutions after deproteinization with trichloroacetic acid or perchloric acid can be assayed without difficulty. Mercaptans, Cu^{2+}, Ag^{+}, CN^{-}, NO^{-}, or high concentrations of (ethylenediamine)tetraacetic acid may interfere with formation of color, but other ions commonly found in biological solutions do not affect the reaction[15,16]. The violet $Fe(TPTZ)_2^{2+}$ complex can be extracted into organic solvents, a property which can be advantageous in the determination of reducing sugars and of end groups of polysaccharides in turbid solutions, in suspensions, or in solutions in nonpolar solvents.

In general, the alkaline ferricyanide method for the determination of reducing sugars lacks specificity, and is susceptible to interference if other reducing components are present in the solution. The present procedure for the assay of the resulting ferrocyanide affords a stable, soluble color; this property has obvious advantages over measurements based on the unstable, Prussian Blue colloid. The method also pro-

vides one of the most sensitive assays available for the determination of reducing sugars. By further lessening the volumes of reagents used, amounts even smaller

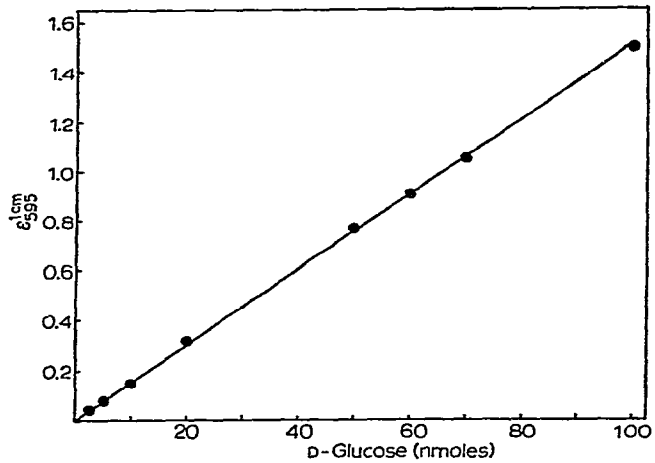

Fig. 1. Standard curve for D-glucose. In comparison with D-glucose (100%), the relative molar yields of color with representative sugars were: D-fructose, 104; 2-deoxy-D-*arabino*-hexose, 80; D-xylose, 95; D-glucuronic acid, 100; 2-amino-2-deoxy-D-glucose, 135; maltose, 140; melibiose, 110; D-glucitol < 0.1; methyl α-D-glucoside, < 0.2; and sucrose, $< 0.2\%$. Development of the violet $Fe(TPTZ)_2^{2+}$ resulting from the oxidation of D-glucose, was conducted as described in the text, in a final volume of 5.0 ml. Measurements were made with a Gilford model 300 and a Cary 14 spectrophotometers, with cuvettes of 1.0-cm light-path.

than 2 nmoles of sugar can be assayed. However, at these low levels, control of the reproducibility and accuracy of color formation is much more difficult, owing to the relatively larger contribution of trace contaminants in the reagents. The use of reagent solutions prepurified[15,16] by treatment with TPTZ is, under these circumstances, obligatory.

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REFERENCES

1 J. G. GENTELE, *Dinglers Polytech. J.*, 152 (1860) 68; *Chem. Zentr.*, 30 (1859) 504.
2 H. C. HAGEDORN AND B. N. JENSEN, *Biochem Z.*, 135 (1923) 46.

*On leave from the Hebrew University, Jerusalem, Israel.

3 W. Z. HASSID, *Ind. Eng. Chem. Anal. Ed.*, 9 (1937) 228.
4 R. L. WHISTLER AND J. N. BEMILLER, *Methods Carbohyd. Chem.*, 1 (1962) 395.
5 T. J. SCHOCH, *Methods Carbohyd. Chem.*, 4 (1964) 64.
6 T. E. FRIEDMAN, C. W. WEBER, AND N. F. WITT, *Anal. Biochem.*, 4 (1962) 358.
7 R. J. HENRY, *Clinical Chemistry, Principles and Technics*, Harper and Row, New York, 1964, p. 625.
8 J. T. PARK AND M. J. JOHNSON, *J. Biol. Chem.*, 181 (1949) 149.
9 G. T. ASHWELL, *Methods Enzymol.*, 3 (1957) 86.
10 R. I. MATELES, *Nature*, 187 (1960) 241.
11 R. G. SPIRO, *Methods Enzymol.*, 8 (1966) 3.
12 J. M. GHUYSEN, D. J. TIPPER, AND J. L. STROMINGER, *Methods Enzymol.*, 8 (1966) 685.
13 D. W. KROGMAN AND A. T. JAGENDORF, *Plant Physiol.*, 32 (1957) 373.
14 M. AVRON AND N. SHAVIT, *Anal. Biochem.*, 6 (1963) 549.
15 D. F. COLLINS, H. DIEHL, AND E. F. SMITH, *Anal. Chem.*, 31 (1959) 1862.
16 H. DIEHL, E. F. SMITH, L. MCBRIDE, AND R. CRYBERG, *The Iron Reagents*, G. F. Smith Chemical Co., Columbus, Ohio, 1965, p. 41.

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Carbohyd. Res., 7 (1968) 94–97

Laminaran of *Ishige okamurai**

Previously, Handa and Nisizawa[1] reported that the soluble laminaran isolated from *Eisenia bicyclis* Setchell was composed of β-(1→3)- and β-(1→6)-linked D-glucose residues in an approximate ratio of 2:1. This finding was mainly based on the isolation of a series of gentio- and laminari-oligosaccharides from a partial acid hydrolyzate of laminaran. Moreover, the β-D-(1→6)-linked residues were assumed to be located near the center of the molecule on the basis of alkali degradation. The structure of *Eisenia*-laminaran was thus considerably different from the structures of laminarans of the genus *Laminaria*[2], in spite of their belonging to the same family. In addition, no "insoluble" laminaran was found in *Eisenia*. From the determination of the amount of 2,3,4-tri-*O*-methyl-D-glucose obtained after hydrolysis of fully methylated laminaran and of the formaldehyde produced by the periodate oxidation following the Smith degradation procedure, it was found[3] that the β-D-linked units form a straight-chain structure which constitute about one-quart of the whole molecule. The absence of 2,4-di-*O*-methyl-D-glucose in the methylation products was also evidence for a nonbranched structure of this laminaran.

In the present work, the laminaran of *Ishige okamurai* Yendo, which belongs to the Chondariales, was investigated in order to compare its structure with that of other laminarans; differences may be expected, since this alga belongs to a family far remote from the Laminariales. *Ishige* grows at an intertidal zone and is distributed from the middle to the southern part of Japan.

Finely crushed dry fronds of *Ishige* (100 g) were extracted with about a 10-fold volume of 0.09M hydrochloric acid for 2 h at room temperature by the countercurrent

*Contribution No. 170 from the Shimoda Marine Biological Station, Tokyo Kyoiku University, Tokyo, Japan.

3 W. Z. HASSID, *Ind. Eng. Chem. Anal. Ed.*, 9 (1937) 228.
4 R. L. WHISTLER AND J. N. BEMILLER, *Methods Carbohyd. Chem.*, 1 (1962) 395.
5 T. J. SCHOCH, *Methods Carbohyd. Chem.*, 4 (1964) 64.
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10 R. I. MATELES, *Nature*, 187 (1960) 241.
11 R. G. SPIRO, *Methods Enzymol.*, 8 (1966) 3.
12 J. M. GHUYSEN, D. J. TIPPER, AND J. L. STROMINGER, *Methods Enzymol.*, 8 (1966) 685.
13 D. W. KROGMAN AND A. T. JAGENDORF, *Plant Physiol.*, 32 (1957) 373.
14 M. AVRON AND N. SHAVIT, *Anal. Biochem.*, 6 (1963) 549.
15 D. F. COLLINS, H. DIEHL, AND E. F. SMITH, *Anal. Chem.*, 31 (1959) 1862.
16 H. DIEHL, E. F. SMITH, L. MCBRIDE, AND R. CRYBERG, *The Iron Reagents*, G. F. Smith Chemical Co., Columbus, Ohio, 1965, p. 41.

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extraction procedure of Black *et al.*[4]. This acidic extraction was chosen in order to remove the cations combined with the cell-wall polysaccharides. The process was repeated 4 times to extract a total of 400 g of fronds. After removal of the acidic polysaccharides in the combined extract with cetyltrimethylammonium bromide, the supernatant obtained by centrifugation was treated with charcoal to remove colored impurities, and then dialyzed against tap water. The laminaran was precipitated by addition of ethanol and further purified by repeated precipitation with ethanol. The product was a white powder, easily soluble in water. No mannitol was detected, by paper chromatography, in the hydrolyzate of the laminaran. Eluates (2 ml each) obtained by gel-filtration on a Sephadex G-50 (super fine) column (1.2 × 76 cm) of a laminaran solution showed a single symmetrical peak which covered the fractions from No. 58 to No. 71.

Anal. Calc. for $C_6H_{10}O_5$: C, 44.44; H, 6.22. Found: C,43.85; H, 6.32.

The laminaran was free of ash and nitrogen. The optical rotation, $[\alpha]_D^{20} - 37.5°$ (*c* 1.34, water), and the infrared spectrum showing a type 2b absorption[5] at 890 cm^{-1}, suggest the presence of β-D-glucosidic linkages. The degree of polymerization estimated by hypoiodite oxidation was equivalent to about 19 D-glucose residues.

Paper chromatography of a partial acid hydrolyzate (0.05M oxalic acid, 100°, 30 min) demonstrated the presence of gentiobiose and laminaribiose; this indicates that the laminaran contains both β-(1→3)- and β-(1→6)-linked D-glucose residues.

Ishige laminaran was oxidized with 0.03M sodium metaperiodate in aqueous solution, in the dark, at 10°. The reaction mixture was assumed to attain maximum oxidation at 120 h on the basis of the oxidation of cellobiose used as control. The laminaran consumed 0.58 mole of periodate, and released 0.29 mole of formic acid and 0.057 mole of formaldehyde per anhydroglucose residue. The degree of polymerisation, calculated from the formaldehyde released, corresponds to about 18 glucose residues, and is in close agreement with the result of the hypoiodite oxidation.

An oxidized laminaran, obtained by periodate oxidation, was reduced with sodium borohydride, 0.05M sulfuric acid was added, and the reaction mixture was kept for 20 h at room temperature in order to cleave the acetal but not the glucosidic linkages. After this partial acid hydrolysis, the resulting product was subjected to an additional periodate oxidation, and the formaldehyde produced was determined by the chromotropic acid reagent, and found to be 0.16 mole per anhydroglucose residue. Therefore, the ratio of (1→6) linkages to the residual ones must be approximately 1 to 6.

Laminaran methylated by Kuhn's method[6] showed no OH absorption in the infrared spectrum. Its methoxyl content was also determined, and was found to be close to the 44.5% theoretical value for a tri-*O*-methylanhydroglucose. Paper chromatography of the hydrolyzate of the methylated laminaran demonstrated the presence of four *O*-methyl-D-glucoses, the spots of which corresponded to 2,3,4,6-tetra-*O*-, 2,3,4-tri-*O*-, 2,4,6-tri-*O*-, and 2,4-di-*O*-methyl-D-glucose; the spots were detected with the *p*-anisidine–hydrochloric acid reagent. Each methylglucose was extracted from the filter paper (Whatman 3MM), and determined with the phenol–sulfuric acid reagent. The molar ratios of the methylglucoses were found to be

approximately 1:1.66:6.23:0.42, when 2,3,4,6-tetra-*O*-methyl-D-glucose was taken as unity.

Thus it appears that the chemical structure of *Ishige* laminaran is composed of (1→3)- and (1→6)-linked D-glucose residues, and thus it is similar to that of *Eisenia* laminaran. The linkages are, however, in a different ratio, namely, in the former polysaccharide 4:1, in the latter one 3:1. Moreover, *Ishige* laminaran differs in having a single branch, but its position is not known. These results show that the structure of laminaran seems to depend on the source. In addition, it is probable that the variations in solubility of laminaran may be due not only to the degree of branching, as pointed out by Annan *et al.*[7], but also to the content of (1→6)-linkage.

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REFERENCES

1 N. HANDA AND K. NISIZAWA, *Nature*, 192 (1961) 1078.
2 F. B. ANDERSON, E. L. HIRST, D. J. MANNERS, AND A. G. ROSS, *J. Chem. Soc.*, (1958) 3233.
3 M. MAEDA AND K. NISIZAWA, *J. Biochem.* (Tokyo), 63 (1968) 199.
4 W. A. P. BLACK, W. J. CORNHILL, E. T. DEWAR, AND F. N. WOODWARD, *J. Appl. Chem.*, 1 (1951) 505.
5 S. A. BARKER, E. J. BOURNE, M. STACEY, AND D. H. WHIFFEN, *J. Chem. Soc.*, (1954) 171, 3468.
6 R. KUHN, H. TRISCHMANN, AND I. LÖW, *Angew. Chem.*, 67 (1955) 32.
7 W. D. ANNAN, E. L. HIRST, AND D. J. MANNERS, *J. Chem. Soc.*, (1965) 885.

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Bis(1,2:5,6-di-*O*-isopropylidene-3-*O*-thiocarbonyl-α-D-glucofuranose) disulfide

A rapid method is reported for the quantitative preparation of bis(1,2:5,6-di-*O*-isopropylidene-3-*O*-thiocarbonyl-α-D-glucofuranose) disulfide (**2**) from 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose (**1**). Yields of **2** approaching 100% are obtained within 1 h. By other methods[1], maximum yields were only 66% in 16 h.

Compound **2** is a useful intermediate for the following syntheses: preparation of 1,2-*O*-isopropylidene-α-D-glucofuranose 5,6-thionocarbonate[1] (by removal of the 5,6-isopropylidene group from **2**, followed by treatment with pyridine); preparation of *O*-(alkoxythiocarbonyl) derivatives[2] of **2** (by treating **2** with alcohols under specific conditions); and the indirect preparation of some *S*-alkylxanthates[3].

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3 M. MAEDA AND K. NISIZAWA, *J. Biochem.* (Tokyo), 63 (1968) 199.
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5 S. A. BARKER, E. J. BOURNE, M. STACEY, AND D. H. WHIFFEN, *J. Chem. Soc.*, (1954) 171, 3468.
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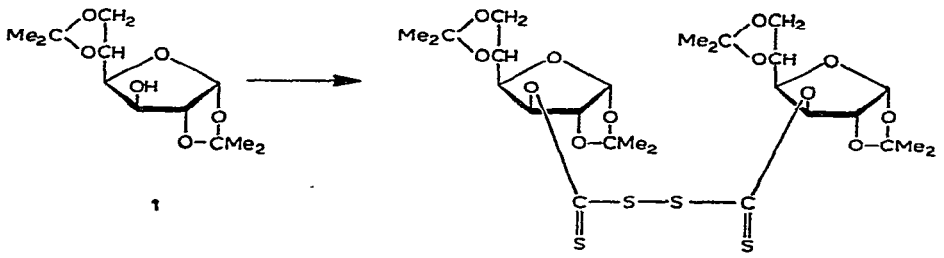
EXPERIMENTAL

Compound **1** (15 g) was dissolved in methyl sulfoxide (15 ml), and aqueous sodium hydroxide solution (5M, 15 ml) was added, along with carbon disulfide (10 ml).



1 2

The resultant yellow solution, which turned red almost immediately, was stirred for 7 min at 25°. The solution was cooled to 5° and acetic acid (5M) was added dropwise until the mixture became yellow (pH about 5). The xanthate present in the mixture was oxidatively crosslinked immediately with a cold, aqueous solution of iodine. The iodine was added as fast as was convenient, to minimize decomposition of the xanthate to other products. A dark-brown syrup resulted, which was separated and dissolved in ether. The extract was washed, first with aqueous sodium thiosulfate until the color was a bright yellow, and then with water. The ether layer was dried (anhydrous magnesium sulfate), and evaporated to yield **2** (19.6 g, 100%) as a heavy syrup which crystallized as needles on standing. T.l.c. (ethyl acetate–carbon disulfide, 9:1 v/v, as solvent, and Silica Gel G* as adsorbent) revealed a single component that had an R_F value the same as that of authentic **2**. After the product had been recrystallized from alcohol–water, its m.p. was undepressed in admixture with an authentic sample of **2**. Although yields for several preparations of **2** were almost 100% by this procedure, yields of only 60–70% of **2** were obtained when cooling to 5° was omitted.

This facile procedure should be applicable for preparation of xanthates and of oxidatively coupled xanthates of various hydroxy compounds.

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REFERENCES

1. W. M. DOANE, B. S. SHASHA, C. R. RUSSELL, AND C. E. RIST, *J. Org. Chem.*, 30 (1965) 162.
2. B. S. SHASHA, W. M. DOANE, C. R. RUSSELL, AND C. E. RIST, *Carbohyd. Res.*, 6 (1968) 34.
3. Unpublished results from this laboratory.

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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.

A synthesis of 6-amino-6-deoxy-D-mannose*

The 6-amino-6-deoxyhexoses are of interest in view of the occurrence[1] of 6-amino-6-deoxy-D-glucose in kanamycin A. As part of a program in this laboratory on the hydrolytic susceptibility of various substituted glycosides, the methyl α-D-glycoside of 6-amino-6-deoxy-D-mannopyranose was required. This report describes the synthesis of methyl 6-amino-6-deoxy-α-D-mannopyranoside hydrochloride (**4**) from D-mannose in a sequence of high-yielding steps.

The 4,6-benzylidene acetal[2] of methyl α-D-mannopyranoside[3] was acetylated to give the 2,3-diacetate[4] (**1**). Treatment of **1** with *N*-bromosuccinimide by the procedure of Hanessian[5] gave crystalline methyl 2,3-di-*O*-acetyl-4-*O*-benzoyl-6-bromo-6-deoxy-α-D-mannopyranoside (**2**). Treatment of the bromo derivative **2** with sodium azide in *N*,*N*-dimethylformamide gave the corresponding 6-azido derivative **3**. Removal of the ester groups from **3** by catalytic transesterification, followed by reduction of the azido group and conversion of the resultant amine into the hydrochloride salt gave the crystalline glycoside **4**.

The sequence affords an excellent preparative procedure for the amino glycoside **4** because all steps are amenable to large-scale work and no chromatographic purifications are required. The glycoside **4** can be obtained in over 60% net yield from the starting material **1** if the intermediates **2** and **3** are not recrystallized to high purity. The useful new reaction described by Hanessian[5], as used in the conversion of **1** into **2**, is particularly advantageous because it obviates the necessity of generating a good leaving group at C-6 by a less effective procedure; the conventional method involving selective sulfonylation of an unsubstituted glycoside at C-6 leads to mixtures of esters that may be troublesome to separate, and yields of the desired 6-ester are frequently low.

The glycoside **4** was further characterized as its crystalline *N*-acetylated derivative (**5**). Hydrolysis of the glycoside **4** gave 6-amino-6-deoxy-D-mannose hydrochloride (**6**) as a syrup. Details of characterization of the intermediates and products **1**–**6**, including n.m.r. spectral data, are given in the experimental section.

A synthesis of the syrupy amino sugar **6**, by way of methyl 2,3,4-tri-*O*-acetyl-6-*O*-*p*-tolylsulfonyl-α-D-mannopyranoside, was reported recently in the patent literature[6]. Crystalline methyl 6-acetamido-6-deoxy-α-D-mannopyranoside (**6**), having constants in good agreement with those found in the present work, was obtained from methyl α-D-mannopyranoside in ~15% net yield by a 6-step sequence.

EXPERIMENTAL

General methods. — Melting points were determined with a Thomas–Hoover

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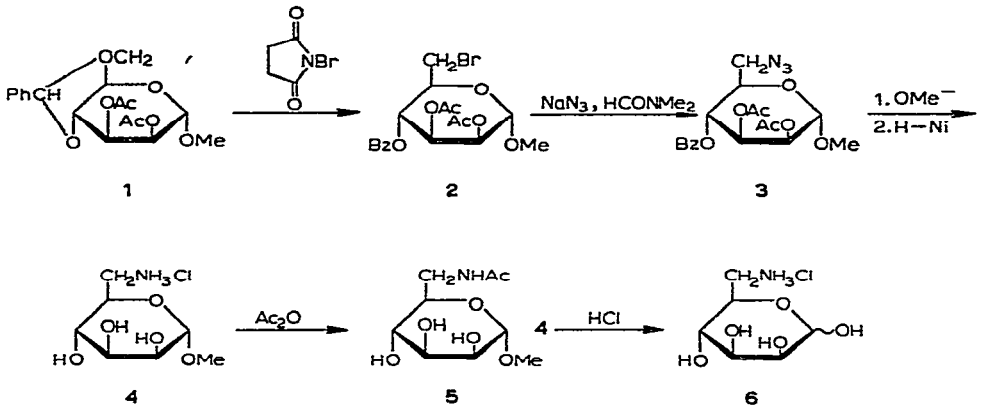
apparatus and are uncorrected. Specific rotations were determined in a 2-dm polarimeter tube. I.r. spectra were measured with a Perkin–Elmer Model 137 "Infracord" i.r. spectrophotometer. N.m.r. spectra were measured with Varian A-60, A-60A, or HA-100 spectrometers. Unless otherwise noted, spectra were measured at 35–40° with solutions (5–15%) in chloroform-*d* with an internal standard of tetramethylsilane



1 2 3

4 5 6

(τ = 10.00), or in deuterium oxide with sodium 4,4-dimethyl-4-silapentane-1-sulfonate (τ = 10.00) as the internal standard. Elemental analyses were performed by W. N. Rond. X-Ray powder diffraction data give interplanar spacings, Å, for CuKα radiation. The camera diameter was 114.54 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. Unless otherwise noted, R_F values refer to t.l.c. on Silica Gel G (E. Merck, Darmstadt, Germany), activated at 110°, with the developer indicated. Zones were detected with sulfuric acid or with ninhydrin. Paper chromatography was performed by downward irrigation on Whatman No. 1 paper, with detection by ninhydrin and by alkaline silver nitrate.

Methyl 2,3-di-O-acetyl-4,6-O-benzylidene-α-D-mannopyranoside (**1**). — A solution of methyl 4,6-*O*-benzylidene-α-D-mannopyranoside[2] (4.0 g) in a mixture of acetic anhydride (4 ml) and pyridine (200 ml) was heated for 3.5 h on a steam bath and was then kept for 4 h at room temperature. The solution was poured into ice–water (175 ml), whereupon an oil separated. The water layer was decanted and evaporated, and the residue was triturated with ether (100 ml). The ether solution was added to the oil, and the mixture was evaporated to give a pale-yellow syrup. The latter was dissolved in ethanol (200 ml), and the solution was passed through a bed of decolorizing carbon. The solution was evaporated to a syrup, which was converted into a solid mass by evaporating several small portions of methanol from it. The solid product, yield 4.4 g (87%), was sufficiently pure for subsequent conversions. It was chromatographically homogeneous and had m.p. 59–63°, $[\alpha]_D^{20}$ +21.4 ±0.6° (*c* 2, chloroform); R_F 0.35 [1:1 petroleum ether (b.p. 65–100°)–ether];

λ_{max}^{KBr} 5.75 (OAc), 14.35 μm (aryl); n.m.r. data (60 MHz, chloroform-*d*): τ 2.67 (5-proton multiplet, phenyl), τ 4.45 (1-proton singlet, benzylic H), τ 4.69 (2-proton multiplet, H-2,3), τ 5.35 (1-proton narrow multiplet, H-1), τ 5.61–6.26 (4-proton multiplet, H-4,5,6,6'), τ 6.62 (3-proton singlet, OMe), τ 7.85, 8.00 (3-proton singlets, OAc).

Anal. Calc. for $C_{18}H_{22}O_8$: C, 59.01; H, 6.05. Found: C, 58.89; H, 5.87.

This product was obtained by Coxon[3] as a syrup, $[\alpha]_D^{25}$ +23.1 (chloroform), in unspecified yield, after purification by column chromatography. The n.m.r. spectral data reported for **1** by Coxon[3] are in agreement with those measured in the present work.

*Methyl 2,3-di-*O*-acetyl-4-*O*-benzoyl-6-bromo-6-deoxy-*α*-*D*-mannopyranoside* (**2**). — To a suspension of *N*-bromosuccinimide (6.45 g, 0.0363 mole) in dry carbon tetrachloride (250 ml) was added the benzylidene acetal **1** (12.0 g, 0.0333 mole) followed by barium carbonate (10 g). The mixture was refluxed for 1.5 h, cooled to 0°, and filtered. The filtrate was evaporated, and the resultant syrup was crystallized from ethanol to give **2** as white needles, yield 10.4 g (71%), m.p. 119–120°, $[\alpha]_D^{20}$ +21.1 ±0.1° (*c* 2.3, chloroform); R_F 0.47 (1:1 petroleum ether (b.p. 65-110°)–ether); λ_{max}^{KBr} 5.78, 5.85 (OAc, OBz), 6.31, 6.40, 6.80 (aryl C=C), 14.1 μm (aryl); n.m.r. data (60 MHz, chloroform-*d*): τ 2.1, 2.6 (2- and 3-proton multiplets, phenyl), τ 4.60–4.90 (multiplets, 3 protons, H-2,3,4). τ 5.26 (1-proton, narrow multiplet, H-1), τ 5.92 (1-proton multiplet, H-5), τ 6.53 (5-proton multiplet, H-6,6', OMe), τ 7.85, 8.12 (3-proton singlets, OAc).

Anal. Calc. for $C_{18}H_{21}BrO_8$: C, 48.55; H, 4.75; Br, 17.95. Found: C, 48.15; H, 4.65; Br, 17.76.

*Methyl 2,3-di-*O*-acetyl-6-azido-4-*O*-benzoyl-6-deoxy-*α*-*D*-mannopyranoside* (**3**). — A solution of the 6-bromo derivative **2** (14 g) in *N*,*N*-dimethylformamide (120 ml) containing sodium azide (20 g) was stirred for 18 h at 51° and then poured into ice–water (500 ml), whereupon the product **3** crystallized, yield almost quantitative, m.p. 65°. Recrystallization from ethanol gave pure **3**; yield 10.5 g (78%), m.p. 71–72°, $[\alpha]_D^{20}$ +15.3 ±0.6° (*c* 2.3, chloroform); R_F 0.47 (1:1 petroleum ether (b.p. 65–110°)–ether); λ_{max}^{KBr} 4.80 (azide), 5.73, 5.83 (OAc, OBz), 6.30, 6.38, 6.79, 6.95 (aryl C=C), 14.05 μm (aryl); n.m.r. data (60 MHz, chloroform-*d*): τ 2.1, 2.55 (2- and 3-proton multiplets, phenyl), τ 4.40–4.80 (multiplets, 3 protons, H-2,3,4), τ 5.25 (1-proton, narrow multiplet, H-1), τ 5.93 (1-proton multiplet, H-5), τ 6.55 (multiplet, 5 protons, H-6,6', OMe), τ 7.85, 8.14, (3-proton singlets, OAc).

Anal. Calc. for $C_{18}H_{21}N_3O_8$: C, 53.07; H, 5.20; N, 10.31. Found: C, 52.87; H, 5.33; N, 10.06.

Methyl 6-amino-6-deoxy-α-D-mannopyranoside hydrochloride (**4**). — To a solution of the azido derivative **3** (1.67 g) in anhydrous methanol (20 ml) was added a small piece of sodium and the solution was kept for 18 h at room temperature. Solid carbon dioxide was added to neutralize the base and the solution was evaporated to a syrup. The latter was dissolved in water (20 ml) and washed with two 10-ml portions of chloroform. The combined chloroform solution was extracted with water (20 ml). The combined aqueous solutions were evaporated to 35 ml and then stirred

for 5 h at 50° with Raney nickel* (8 g). Filtration of the suspension and evaporation of the filtrate gave the free base of the title compound **4** as a syrup.

The syrup was dissolved in methanol (15 ml), 12M hydrochloric acid (1.1 ml) was added, and the solution was evaporated. Methanol (four 15-ml portions) was evaporated from the residue to remove excess hydrogen chloride, and the residue was crystallized from hot ethanol (15 ml); yield 2.0 g (71%), m.p. 161–162° (dec.), $[\alpha]_D^{20}$ +65.7 ±0.4° (*c* 2.2, water); R_F 0.67 (5:5:3:1 pyridine–ethyl acetate–water–acetic acid); $R_{2\text{-amino-2-deoxyglucose}}$ 1.07 (papergram, 5:5:3:1 pyridine–ethyl acetate–water–acetic acid); λ_{max}^{KBr} 3.1 (broad, NH_3^+), 5.28, 6.22, 6.42, 6.73 μm (NH_3^+); azide, carbonyl, and aryl ring-absorptions absent; n.m.r. data (100 MHz, 70°, deuterium oxide): τ 5.19 (1-proton doublet, $J_{1,2}$ 1.7 Hz, H-1), τ 5.7 (HOD, observed at τ 5.5 at 25°), τ 6.04 (1-proton quartet, $J_{2,3}$ 3.0 Hz, H-2), τ 6.09-6.60 (4-proton multiplet, H-3,4,5,6), τ 6.58 (3-proton singlet, OMe), τ 6.82 (1-proton quartet, $J_{5,6'}$ 8.0 Hz, $J_{6,6'}$ 13.2 Hz, H-6'); X-ray powder diffraction data: 8.25 w, 7.04 vw, 6.03 m, 5.47 s (3,3), 5.18 w, 4.67 s (3,3), 4.41 w, 4.14 w, 3.86 w, 3.69 vs (2), 3.51 vs (1), 3.22 m, 3.09 w, 3.00 vw, 2.94 m, 2.82 w, 2.76 w, 2.70 m.

Anal. Calc. for $C_7H_{16}ClNO_5$: C, 36.60; H, 7.02; Cl, 15.44; N, 6.10. Found: C, 36.54; H, 7.07; Cl, 15.23; N, 6.27.

Methyl 6-acetamido-6-deoxy-α-D-mannopyranoside (**5**). — To a solution of the hydrochloride salt **4** (0.50 g) in methanol (25 ml) was added 10 ml of Amberlite IRA-400 (OH^-) ion-exchange resin, together with acetic anhydride (1 ml), and the mixture was kept for 18 h at room temperature. The resin was filtered off, the filtrate was evaporated to low volume, and ether was added. Refrigeration of the solution gave the product **5** as leaflets, yield 0.22 g (43%), m.p. 171°, $[\alpha]_D^{20}$ +63.5 ±1.5° (*c* 2, water) [lit.[6] m.p. 171–172°, $[\alpha]_D^{30.5}$ +70° (*c* 2, methanol)]; R_F 0.67 (5:5:3:1 pyridine–ethyl acetate–water–acetic acid); λ_{max}^{KBr} 2.95 (OH) 6.10, 6.43 μm (NHAc); n.m.r. data (60 MHz, 70°, deuterium oxide): τ 5.25 (1-proton narrow doublet, H-1), τ 6.61 (3-proton singlet, OMe), τ 7.98 (3-proton singlet, NAc).

Anal. Calc. for $C_9H_{17}NO_6$: C, 45.95; H, 7.28; N, 5.95. Found: C, 45.83; H, 7.24; N, 5.94.

6-Amino-6-deoxy-D-mannose hydrochloride (**6**). — The glycoside **4** (0.5 g) in M hydrochloric acid (10 ml) was refluxed for 4 h, and the solution was evaporated to a syrup. Water (three 10-ml portions) was evaporated from the syrup to remove hydrochloric acid, the product was redissolved in water and decolorized with activated carbon. The solution was evaporated to give the amino sugar **6** as a colorless, ninhydrin-positive syrup that was essentially homogeneous by t.l.c. on cellulose (Eastman Chromagram Sheet No. 6065 with fluorescent indicator, 4:1:1 butyl alcohol–acetic acid–water), R_F 0.15, and by paper chromatography (5:5:3:1 ethyl acetate–pyridine–water–acetic acid), $R_{2\text{-amino-2-deoxyglucose}}$ 0.63.

The n.m.r. spectrum of **6** at 60 MHz in deuterium oxide showed the H-1 signals

*Raney nickel catalyst No. 28; the Raney Catalyst Division of the W. R. Grace Co., Chattanooga, Tennessee.

of the two pyranose anomers as narrow signals ($J_{1,2} \leqslant 1$ Hz) of approximately equal intensity, at τ 4.78 and 5.04.

The 2-hydroxy-1-naphthylmethylene Schiff-base derivative of **6**, prepared by the method of Jolles and Morgan[7], was obtained from methanol–acetone as an amorphous yellow powder that softened at 128° and decomposed at 140–150°.

Anal. Calc. for $C_{17}H_{19}O_6N$: N, 4.20. Found: N, 4.23.

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REFERENCES

1 J. D. DUTCHER, *Advan. Carbohyd. Chem.*, 18 (1963) 259; D. HORTON in R. W. JEANLOZ AND E. A. BALASZ, ed., *The Amino Sugars*, Academic Press, Inc., New York, Vol. IA, *in press.*
2 J. G. BUCHANAN AND J. C. P. SCHWARZ, *J. Chem. Soc.*, (1962) 4770.
3 J. E. CADOTTE, F. SMITH, AND D. SPRIESTERSBACH, *J. Amer. Chem. Soc.*, 74 (1952) 1501.
4 B. COXON, *Tetrahedron*, 21 (1965) 3481.
5 S. HANESSIAN, *Carbohyd. Res.*, 2 (1966) 86.
6 Y. ITO AND T. OKUDA, Japanese Patent 8003 ('67); *Chem. Abstr.*, 67 (1967) 117217.
7 Z. E. JOLLES AND W. T. J. MORGAN, *Biochem. J.*, 34 (1940) 1183.

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Preliminary communication

The carbohydrate–polypeptide linkages in a fungal glucoamylase

The glucoamylase [α-D-(1→4)-D-glucan glucohydrolase, E.C. 3.2.1.3] of *Aspergillus niger* has been reported[1] to consist of two isoenzymes[2], each of which is a glycoprotein containing D-mannose as the preponderant carbohydrate (8–15%), with lesser proportions of D-glucose (2–3%) and D-galactose (0.2%). No evidence has to our knowledge been reported concerning the nature of the linkage between the carbohydrate moieties and the polypeptide chain in these isoenzymes. In this laboratory, we have now obtained evidence that both of the isoenzymes of the glucoamylase of *A. niger* contain carbohydrate residues that are glycosidically linked through D-mannose to residues of serine and threonine in the protein.

The isoenzymes were purified by chromatography on DEAE-cellulose as reported by Pazur and Ando[2], and were designated glucoamylase I and II according to their electrophoretic mobility[3].

Purified glucoamylase II was treated with 0.1M sodium hydroxide–0.3M sodium borohydride for 12 days at 4°, and then the base was neutralized with glacial acetic acid until evolution of hydrogen ceased. Amino acid analysis* of an acid hydrolyzate of this material, compared with a similar analysis of the purified glucoamylase II, revealed a decrease in the proportion of serine and threonine, an increase in the proportion of alanine, and the appearance of 2-aminobutyric acid. Chromatography of the modified enzyme on Sephadex G-25 resulted in the isolation of two carbohydrate-containing components, one of which was retained by the column. Both components were hydrolyzed with M hydrochloric acid. Analysis* by g.l.c. of the acetylated hydrolyzate of the component retained by the column revealed the presence of mannitol hexaacetate plus peaks corresponding in retention time to those for

*Amino acid analyses were performed with a Beckman Model 120C Amino Acid Analyzer under conditions for the analysis of acid hydrolyzates. Analyses by g.l.c. were made on a F & M Model 5750 Gas Chromatograph equipped with a flame-ionization detector. A stainless-steel column (6 ft×0.125 in) packed with a stationary phase of 3% ECNSS-M on Gas Chrom Q (100–120 mesh) was used for the acetates, which were prepared by warming the sample in pyridine–acetic anhydride. The column was operated at a temperature of 190°, with nitrogen as the carrier gas (14 ml/min). A stainless-steel column (9 ft×0.125 in) packed with a stationary phase of 3% SE-52 on Anakrom SD (90–100 mesh) was used for the per(trimethylsilyl) ethers, which were prepared by shaking the sample with an excess of a mixture of chlorotrimethylsilane and hexamethyldisilazane in pyridine (TRI-SIL, Pierce Chemical Company, Rockford, Illinois). Nitrogen was used as the carrier gas at a flow rate of 10 ml/min. The chromatograph was programmed to maintain the temperature at 120° for 3 min after injection of the sample, and then to increase at 6°/min to 185°, followed by maintenance of the temperature at 185° until the components had emerged from the column (33–39 min).

acetylated hexoses. Investigation of the per(trimethylsilyl) ethers of components in the hydrolyzate showed peaks corresponding to mannose, glucose, galactose, and a hexitol. The hexitol per(trimethylsilyl) ethers were not resolved on the column used for the analysis of the per(trimethylsilyl) ethers. The carbohydrate component not retained by the gel-filtration column gave the same results on g.l.c. investigation of the acetate and per(trimethylsilyl) ether derivatives of the acid hydrolyzate, except that no hexitol component was observed.

Purified glucoamylase I was treated with 0.1M sodium hydroxide–0.3M sodium borohydride under the conditions already described for glucoamylase II. Amino acid analysis of an acid hydrolyzate of the resulting product yielded the same results as those described for glucoamylase II. Two carbohydrate-containing components, showing the same behavior as the two obtained from glucoamylase II, were obtained by chromatography of the product on Sephadex G-25. Analysis by g.l.c. of the acetates and per(trimethylsilyl) ethers of products in an acid hydrolyzate of these carbohydrate components gave results the same as those observed for glucoamylase II.

Treatment of glycoproteins with alkaline borohydride is known to effect β-elimination of carbohydrate moieties glycosidically linked to the hydroxyl group of serine or threonine residues, with concomitant reduction of the reducing end of the eliminated carbohydrate to the corresponding alditol[4–7]. The decrease in the proportion of serine and threonine observed in the two isoenzymes after treatment with alkaline borohydride, and the simultaneous increase in the proportion of alanine and appearance of 2-aminobutyric acid, indicate that serine and threonine are involved through their hydroxyl groups in glycosidic linkages. The alanine and 2-aminobutyric acid observed are formed, presumably, under the reducing conditions, from the unsaturated amino acid residues produced by β-elimination of the glycosidically linked carbohydrate substituent.

The presence of D-mannitol hexaacetate in the acetylated hydrolyzates of the carbohydrate fragments obtained from the alkaline borohydride treatment of the two isoenzymes indicates that D-mannose is involved in the linkage between the carbohydrate and the polypeptide. The presence of galactitol hexaacetate in these acetylated hydrolyzates cannot be ruled out completely, because of the very small proportion of D-galactose present in the enzyme and the similarity in retention time between galactitol hexaacetate and acetylated α-D-galactose or α-D-glucose. However, it is evident that D-mannitol hexaacetate is present in a much greater concentration than galactitol hexaacetate, and there was no evidence for the presence of D-glucitol hexaacetate.

The ratio of the amount of D-mannitol hexaacetate to acetylated hexoses in the acid hydrolyzates was approximately unity, as determined by g.l.c. This would indicate that the carbohydrate components are present in the isoenzymes as side chains having relatively low degree of polymerization. The ratio of *ca.* unity indicates that the side chains could be disaccharide residues, but this point must be verified further, as this ratio could also be the result of averaging of a distribution of sizes of side chain. The other carbohydrate component, which was not retained by the gel-filtration

column, is believed to be glycoprotein from which the carbohydrate had not been eliminated.

From the evidence presented, it may be concluded that glucoamylase I and II from *A. niger* contain carbohydrate moieties that are glycosidically linked through D-mannose to the hydroxyl groups of serine and threonine in the polypeptide chain of the enzyme; these carbohydrate residues are present as small, oligomeric side-chains. This conclusion is in contrast to the suggestion by Pazur, Kleppe, and Ball[1] that the carbohydrates are esterified to the carboxyl groups of aspartic acid and glutamic acid in these isoenzymes. Further work is in progress to obtain quantitative data for the carbohydrates and the amino acids involved in the linkages.

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REFERENCES

1 J. H. PAZUR, K. KLEPPE, AND E. M. BALL, *Arch. Biochem. Biophys.*, 103 (1963) 515.
2 J. H. PAZUR AND T. ANDO, *J. Biol. Chem.*, 234 (1959) 1966.
3 E. C. WEBB, *Nature*, 203 (1964) 821.
4 H. C. ROBINSON, A. TELSER, AND A. DORFMAN, *Proc. Natl. Acad. Sci. U.S.*, 56 (1966) 1859.
5 G. DAWSON AND J. R. CLAMP, *Biochem. J.*, 103 (1967) 5P.
6 E. E. GRABNER, C. W. HALL, AND E. F. NEUFELD, *Arch. Biochem. Biophys.*, 116 (1966) 391.
7 K. TANEKA AND W. PIGMAN, *J. Biol. Chem.*, 240 (1965) PC 1487.

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Carbohyd. Res., 7 (1968) 106–108

Corrigenda

Carbohydrate Research, 6 (1968)

Page 171 line 1 of Abstract, for amylase read amylose.

Page 466 line 13 and 14 up should read λ_{max}^{EtOH} 256, 306, 390 nm (log ε 5.00, 4.74, 5.03), λ_{min}^{EtOH} 279, 327 nm (log ε 4.53, 4.70).

Page 467 line 11 down should read λ_{max}^{EtOH} 242, 282, 384 nm (log ε 4.00, 3.89, 4.12), λ_{min}^{EtOH} 265, 323 nm (log ε 3.84, 3.30).

Page 471
The ultraviolet absorption spectra readings on the right hand side of Table I should read as follows:

	λ_{max}^{EtOH}	log ε	λ_{min}^{EtOH}	log ε
Line 2 from above should read	392, 311, 259	4.4, 4.1, 4.3	333, 285	4.0, 3.9
Line 5 from below should read	406, 335, 265	3.4, 3.0, 3.5	351, 322	2.9, 3.0
Line 4 from below should read	394, 316, 264	5.0, 4.8, 4.9	343, 287	4.7, 4.5
Line 3 from below should read	400, 330, 256	4.3, 3.9, 4.3	346, 280	3.8, 3.7
Line 2 from below should read	406, 317, 259	4.4, 4.0, 4.4	356, 284	3.9, 3.6
Line 1 from below should read	400, 330, 256	3.3, 2.9, 3.3	347, 284	2.9, 2.8

Line 1 below Table I number 4 should read D-arabinose and number 5 should read L-arabinose.

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STUDIES ON URONIC ACID MATERIALS

PART XXVIII*. SOME STRUCTURAL FEATURES OF *Acacia drepanolobium* GUM

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(Received November 10th, 1967; in revised form, December 12th, 1967)

ABSTRACT

Linkage analysis of Fraction *A* of *A. drepanolobium* gum yielded 3-*O*-β-L-arabinopyranosyl-L-arabinose, 3-*O*-β-L-arabinofuranosyl-L-arabinose, 3-*O*-β-D-galactopyranosyl-D-galactose, 6-*O*-β-D-galactopyranosyl-D-galactose, and the β-D-(1→3)- and β-D-(1→6)-linked trisaccharides. The *O*-methyl derivative of degraded gum *A* was analysed, after methanolysis, by gas–liquid partition chromatography. After paper-chromatographic separation of a hydrolysate of methylated, degraded gum *A*, the resulting *O*-methyl sugars were characterised. Degraded gum *A* was subjected to a Smith degradation, and the product was examined by linkage and methylation analysis. Degraded gum *A* was subjected to a controlled Smith degradation, and the product was shown to contain arabinitol. This shows that at least some of the reducing galactose residues of degraded gum *A* are substituted at both C-3 and C-6. The structural evidence obtained shows that, although *A. drepanolobium* gum Fraction *A* and *A. arabica* gum are very similar, some differences do exist between these two gums.

INTRODUCTION

The gum polysaccharide from *Acacia drepanolobium* Harms ex Sjösted is not completely water-soluble. In a previous paper[2], the gum was subjected to a graded extraction procedure whereby three fractions were obtained: (*A*) water-soluble, 80%; (*B*) salt-soluble, 8%; (*C*) a gel soluble in dilute alkali, 12%. Analytical data for these fractions have been given[2], and the identity of the four aldobiouronic acids present in each fraction has been established[2]. This Paper presents the results of a structural study of the water-soluble fraction of *Acacia drepanolobium* gum.

EXPERIMENTAL

The origin of the gum[2], the preparation[2] of the freeze-dried, electrodialysed sample of the cold water-soluble fraction of the gum, and the standard analytical

*For Part XXVII, see Ref. 1.

methods[3] have been described. Paper chromatography was carried out on Whatman No. 1 and 3MM papers with the following solvent system (v/v): (*a*) benzene–butyl alcohol–pyridine–water (1:5:3:3, upper layer); (*b*) ethyl acetate–acetic acid–formic acid–water (18:3:1:4); (*c*) ethyl acetate–pyridine–water (10:4:3); (*d*) butyl alcohol–ethanol–water (4:1:5, upper layer); (*e*) butanone–water–ammonia (sp. gr. 0.88) (200:17:1); (*f*) butanone–acetic acid–water (9:1:1, saturated with boric acid). R_{Gal} and R_G values refer to distances moved relative to D-galactose and 2,3,4,6-tetra-*O*-methyl-D-glucose, respectively.

Gas–liquid partition chromatography (g.l.c.) of mixtures of methyl glycosides was carried out on a Chromatograph Type S3A (Gas Chromatography Ltd.), fitted with flame-ionisation detectors, at nitrogen flow-rates of *ca.* 100 ml/min on columns of (*i*) 15% by weight of poly(butane-1,4-diol succinate) on Celite (120 × 0.5 cm) at 175°; (*ii*) 15% by weight of poly(ethylene glycol adipate) on Celite (75 × 0.5 cm) at 160°. Retention times (*T*) are given relative to that of methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucopyranoside.

Degraded gums *A* and *B* were methylated successively with methyl sulphate and sodium hydroxide, and with methyl iodide and silver oxide. The reduced (lithium aluminium hydride), methylated, degraded gum *A* was methylated by Kuhn's method[4]. Oligosaccharides were methylated by the method of Perila and Bishop[5]. Methanolyses were carried out under reflux for 7 h with dry 5% methanolic hydrogen chloride.

RESULTS

Determination of conditions for autohydrolysis. — The time required for complete autohydrolysis of an *Acacia* gum differs from species to species, since it is dependent on such features as the proportion of uronic acid groups present; at 98°, *A. senegal* gum requires 50 h, whereas *A. nubica* gum requires[1] 120 h. In a trial experiment, a solution (4%) of soluble *A. drepanolobium* gum was heated on a boiling water-bath for 120 h. Aliquots (2 ml) were withdrawn at intervals, filtered, and diluted to 10 ml. The changes in the specific rotation and in free reducing power (alkaline ferricyanide method[6]) are shown in Table I. In a parallel experiment, a 4% solution of the soluble gum was also heated on a boiling water-bath for 120 h. At intervals, aliquots (6 ml) were withdrawn and filtered, and portions (5 ml) were then added to 2M sodium chloride (5 ml). The resulting gum solution in M sodium chloride was used to obtain the values for the limiting-viscosity number $[\eta]$ shown in Table I. From the kinetic data (Table I), it was deduced that a period of 84 h was required for complete autohydrolysis of a 4% solution of the gum.

Autohydrolysis of water-soluble A. drepanolobium *gum to give degraded gum* A. — A solution of the gum (50 g in 1.25 l of water) was heated on a boiling water-bath for 84 h. The solution was then cooled, filtered, and dialysed against distilled water (4 l), the distilled water being changed daily over a period of 4 days. Freeze-drying gave degraded gum *A* (11 g, 22%), $[\alpha]_D$ +31° (*c* 1.0, water) (Found: uronic acid, 17;

TABLE I

THE CHANGES OCCURRING DURING AUTOHYDROLYSIS OF *A. drepanolobium* GUM

Time (*h*)	$[\alpha]_D$ (*degrees*)	*Reducing power*[a]	$[\eta]$ (*ml/g*)
0	+82	1.56	25.1
2	+84	5.20	—
5	—	—	18.4
9	+89	12.7	—
10	—	—	13.2
24	+91	22.2	7.5
48	+92	29.7	5.2
72	+92	32.6	3.6
96	+92	32.8	3.5
120	+90	33.0	—

[a]Expressed as mg of arabinose per ml of autohydrolysate.

galactose, 80; arabinose, 3; methoxyl, 0.73%). Hydrolysis (N sulphuric acid, 7 h) of degraded gum *A* indicated the presence of the four aldobiouronic acids identified previously[2], namely 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose, 4-*O*-(α-D-glucopyranosyluronic acid)-D-galactose, 6-*O*-(4-*O*-methyl-β-D-glucopyranosyluronic acid)-D-galactose, and 4-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-D-galactose, having, respectively, R_{Gal} 0.22, 0.27, 0.59, and 0.65 in solvent (*b*).

The dialysates (4 × 4 l) from the autohydrolysate were concentrated to a syrup (30 g). Chromatographic examination showed the presence of galactose, arabinose, rhamnose (trace), and two neutral disaccharides having the mobilities of (*1*) 3-*O*-β-L-arabinopyranosyl-L-arabinose [R_{Gal} 0.77, solvent (*a*); 0.71, solvent (*b*)] and (*2*) 3-*O*-β-L-arabinofuranosyl-L-arabinose [R_{Gal} 1.25, solvent (*b*); 1.47, solvent (*e*)]. Disaccharide (*1*) was isolated by fractionation of a portion of the dialysate on 3MM papers in solvent (*b*). The product (50 mg) had $[\alpha]_D$ +195° (*c* 1.0), gave only arabinose on acid hydrolysis, and had the same paper-chromatographic mobility [R_{Gal} 0.77, solvent (*a*); 0.71, solvent (*b*)] as an authentic specimen of 3-*O*-β-L-arabinopyranosyl-L-arabinose. A portion (15 mg) was methylated: g.l.c. examination of the methanolysis products revealed the methyl glycosides of 2,3,4-tri-*O*-methyl-L-arabinose [(*i*) *T* 1.01; (*ii*) *T* 0.82], 2,5-di-[(*i*) *T* 1.81, 3.25; (*ii*) *T* 1.26, 2.18] and 2,4-di-*O*-methyl-L-arabinose [(*i*) *T* 2.08, 2.18; (*ii*) *T* 1.48, 1.56]. Hydrolysis of the methyl glycosides, followed by paper chromatography in solvent (*d*), confirmed the presence of 2,3,4-tri-*O*-methyl-L-arabinose (R_G 0.79) and 2,5- and 2,4-di-*O*-methyl-L-arabinose (R_G 0.84 and 0.65, respectively). The phenylosazone of the disaccharide had m.p. 233–234° (decomp.) (from water).

Disaccharide (*2*) was isolated by fractionation of a portion of the dialysate on 3MM papers in solvent (*e*). The product (32 mg) had $[\alpha]_D$ +89.7° (*c* 0.64), gave only arabinose on acid hydrolysis, and had the same paper-chromatographic mobility [R_{Gal} 1.25, solvent (*b*); 1.47, solvent (*e*)] as an authentic specimen of 3-*O*-β-L-arabinofuranosyl-L-arabinose. A portion (10 mg) was methylated: g.l.c. examination of the

methanolysis products revealed the methyl glycosides of 2,3,5-tri-[(*i*) *T* 0.56, 0.73; (*ii*) *T* 0.47, 0.63] and 2,5-di-[(*i*) *T* 1.82, 3.24; (*ii*) *T* 1.27, 2.20] and 2,4-di-*O*-methyl-L-arabinose [(*i*) *T*, 2.07, 2.17; (*ii*) *T* 1.46, 1.57]. Hydrolysis of the methyl glycosides, followed by paper chromatography in solvent (*d*), confirmed the presence of 2,3,5-tri-(R_G 0.98), 2,5-di-(R_G 0.84), and 2,4-di-*O*-methyl-L-arabinose (R_G 0.65). The phenylosazone of the disaccharide had m.p. 196–198° (from water).

Partial hydrolysis of degraded gum A. — Degraded gum A was subjected to partial, acid hydrolysis with 0.5N sulphuric acid (1 h, boiling water-bath). After neutralisation (barium carbonate), filtration, deionisation [Amberlite IR-120 (H^+) resin], and concentration, the resulting syrup (4.8 g) was fractionated on Whatman 3MM papers to give four fractions.

Fraction 1 (62 mg) had $[\alpha]_D$ +60° (*c* 0.2), gave only galactose on acid hydrolysis, and had the same paper-chromatographic mobility as an authentic specimen of 3-*O*-β-D-galactopyranosyl-D-galactose [R_{Gal} 0.48, solvent (*a*); 0.53, solvent (*c*)]. A portion (15 mg) was methylated; g.l.c. showed the methanolysis products to be the methyl glycosides of 2,3,4,6-tetra- [(*i*) *T* 1.68; (*ii*) *T* 1.66], 2,4,6-tri- [(*i*) *T* 3.76, 4.23; (*ii*) *T* 3.00, 3.45] and 2,5,6-tri-*O*-methyl-D-galactose [(*i*) *T* 3.98; (*ii*) *T* 3.26]. Hydrolysis of the methyl glycosides, followed by paper chromatography [solvent (*d*)], showed the presence of 2,3,4,6-tetra-, 2,4,6-tri- and 2,5,6-tri-*O*-methyl-D-galactose (R_G 0.90, 0.72, and 0.88, respectively). The disaccharide, recrystallised from aqueous acetone, gave needles of 3-*O*-β-D-galactopyranosyl-D-galactose monohydrate, m.p. and mixed m.p. 156–159°.

Fraction 2 (94 mg) had $[\alpha]_D$ +29° (*c* 0.94), gave only galactose on acid hydrolysis, and was identical with an authentic specimen of 6-*O*-β-D-galactopyranosyl-D-galactose [R_{Gal} 0.29, solvent (*a*); 0.37, solvent (*c*)]. A portion (20 mg) was methylated; g.l.c. showed the methanolysis products to be the methyl glycosides of 2,3,4,6-tetra- [(*i*) *T* 1.68; (*ii*) *T* 1.66], 2,3,5-tri-[(*i*) *T* 4.05, 5.39; (*ii*) *T* 3.28, 4.37], and 2,3,4-tri-*O*-methyl-D-galactose [(*i*) *T* 6.45; (*ii*) *T* 5.30]. Hydrolysis of the methyl glycosides, followed by paper chromatography [solvent (*d*)], showed the presence of 2,3,4,6-tetra-, 2,3,5-tri- (R_G 0.86) and 2,3,4-tri-*O*-methyl-D-galactose (R_G 0.71). After recrystallisation from water, the phenylosazone of 6-*O*-β-D-galactopyranosyl-D-galactose had m.p. and mixed m.p. 200–203°.

Fraction 3 (9 mg) had $[\alpha]_D$ +46° (*c* 0.18), gave D-galactose and 3-*O*-β-D-galactopyranosyl-D-galactose on partial hydrolysis with acid, and had[7] D.P. = 2.90. On paper chromatography, the trisaccharide was identical [R_G 0.22, solvent (*a*); 0.23, solvent (*c*)] with an authentic specimen of the β-(1→3)-linked D-galactosetrisaccharide. A portion (4 mg) was methylated; g.l.c. showed the presence of the methyl glycosides of 2,3,4,6-tetra-, 2,4,6-tri- and 2,5,6-tri-*O*-methyl-D-galactose (retention times as for Fraction 1).

Fraction 4 (19 mg) had $[\alpha]_D$ +20° (*c*, 0.38), gave galactose and 6-*O*-β-D-galactopyranosyl-D-galactose on partial hydrolysis with acid, and had[17] D.P. = 2.95. On paper chromatography, the trisaccharide was identical [R_G 0.15, solvent (*a*); 0.18, solvent (*b*)] with an authentic specimen of the β-(1→6)-linked D-galactose-

trisaccharide. A portion (8 mg) was methylated; g.l.c. showed the presence of the methyl glycosides of 2,3,4,6-tetra-, 2,3,5-tri-, and 2,3,4-tri-*O*-methyl-D-galactose (*T*-values as for Fraction 2). Paper chromatography of the hydrolysate showed the presence of the same three methylated sugars (R_G values as for Fraction 2).

Borohydride reduction of degraded gum A. — Degraded gum *A* (500 mg) was dissolved in water (100 ml), and sodium borohydride (400 mg) was added. After 24 h, further sodium borohydride (100 mg) was added. After 6 h, the solution was dialysed for 2 days. The freeze-dried product was hydrolysed to yield galactose, arabinose, and the same four aldobiouronic acids that were obtained from unreduced, degraded gum *A*. In addition, paper chromatography [solvent (*f*)] showed the presence of galactitol; arabinitol was absent.

Degraded gum *A* gave no formaldehyde on periodate oxidation. The yield of formaldehyde from reduced, degraded gum *A* (40.4 mg) was 180 μg (0.25 h), 230 μg (0.5 h), 235 μg (1 h), 245 μg (2 h), 245 μg (6 h), 250 μg (24 h); calculation gives a value of 4,800 for the $\overline{M}\eta$ of degraded gum *A*. Molecular-sieve chromatography[3,8], with a calibrated Bio-Gel P300 column, indicated a value of 5,200 for the $\overline{M}\eta$ of degraded gum *A*.

Methylation of degraded gum A. — Degraded gum *A* (1.34 g) was methylated to give a product (974 mg), $[\alpha]_D$ +18° (*c* 1.00, chloroform) (Found: methoxyl, 40.1%; not raised on further attempted methylation). Methanolysis of a portion of this product, followed by g.l.c., gave the results shown in Table II. The methyl glycosides

TABLE II

THE METHANOLYSIS AND HYDROLYSIS PRODUCTS FROM METHYLATED, DEGRADED GUM *A*

Relative retention time (T) *of methyl glycosides*[a]		R_G *in solvent* (d)	*Relative molar propns.*	O-*Methyl sugars identified*
column (i)	*column* (ii)			
0.56, 0.73	0.49, 0.63	0.98	tr	2,3,5-tri-*O*-methyl-L-arabinose
1.01	0.82	0.79	tr	2,3,4-tri-*O*-methyl-L-arabinose
1.68	1.66	0.90	++	2,3,4,6-tetra-*O*-methyl-D-galactose
(3.00), (3.76), (4.25)	2.45, (3.04), (3.50)	0.72	$\frac{+}{2}$	2,3,6-tri-*O*-methyl-D-galactose
(3.76), (4.25)	(3.04), (3.50)	0.72	++	2,4,6-tri-*O*-methyl-D-galactose
6.45	5.30	0.72	+++	2,3,4-tri-*O*-methyl-D-galactose
14.5, 16.3	9.9, 11.3	0.53	+++	2,4-di-*O*-methyl-D-galactose
2.38, (3.00)	2.20, 2.75	—	++	2,3,4-tri-*O*-methyl-D-glucuronic acid[b]
—	—	0.59	+	2,6-di-*O*-methyl-D-galactose
—	—	0.34	+	2-*O*-methyl-D-galactose

[a]Figures in parentheses indicate incompletely resolved components. [b]As methyl ester methyl glycoside.

were hydrolysed, and paper chromatography in solvents (*d*) and (*e*) indicated the presence of 2,6-di- and 2-*O*-methyl-D-galactose, in addition to the *O*-methyl sugars shown in Table II.

Methanolysis of methylated, degraded gum A. — Methylated, degraded gum *A* (790 mg) was heated under reflux for 8 h with 5% methanolic hydrogen chloride (80 ml). The resulting mixture of methyl glycosides was hydrolysed with sulphuric acid (N, 100 ml) on a boiling water-bath for 4 h. After being cooled, the solution was neutralised (barium carbonate), filtered, deionised [Amberlite resin IR-120 (H^+)], and concentrated to a syrup (750 mg). The mixture of *O*-methyl sugars was fractionated on Whatman 3MM papers by double development in solvent (*e*) to give seven fractions.

Fraction 1 (58 mg) had $[\alpha]_D$ +117° (*c* 0.58), and was chromatographically identical with 2,3,4,6-tetra-*O*-methyl-D-galactose [R_G 0.89, solvent (*d*); 0.72, solvent (*e*)]. The derived 2,3,4,6-tetra-*O*-methyl-*N*-phenyl-D-galactosylamine had m.p. 192–195° (lit.[9], 192-197°), after recrystallisation from ethyl acetate.

Fraction 2 (39 mg) had $[\alpha]_D$ +100° (*c* 0.39); demethylation gave galactose and arabinose. A portion (5 mg) was heated under reflux for 7 h with 5% methanolic hydrogen chloride, and g.l.c. indicated the presence of 2,3,5-tri- (+) [(*i*) *T* 0.56, 0.73; (*ii*) *T* 0.48, 0.63], 2,3,4-tri-*O*-methyl-L-arabinose (++) [(*i*) *T* 1.00, (*ii*) *T* 0.82], and 2,3,4,6-tetra-*O*-methyl-D-galactose (++++) [(*i*) *T* 1.68; (*ii*) *T* 1.66].

Fraction 3 contained three components when examined in solvent (*c*). The fraction was therefore refractionated on 3MM papers by double development in solvent (*e*) to give Fractions 3(*a*), (*b*), and (*c*).

Fraction 3(a) (26 mg) had $[\alpha]_D$ +98° (*c* 0.26), and paper chromatography indicated the presence of 2,3,6-tri- (++) and 2,4,6-tri-*O*-methyl-D-galactose (+). After reflux with methanolic hydrogen chloride, g.l.c. indicated the presence of the methyl glycosides of these two *O*-methyl sugars.

Fraction 3(b) (54 mg) had $[\alpha]_D$ +95° (*c* 0.54). A portion (5 mg) was refluxed with methanolic hydrogen chloride, and g.l.c. indicated the presence of 2,4,6-tri- with a trace of 2,3,4-tri-*O*-methyl-D-galactose. The derived 2,4,6-tri-*O*-methyl-*N*-phenyl-D-galactosylamine, after recrystallisation from ethyl acetate (twice) and from acetone–ether–light petroleum (1:1:1), had m.p. 163–165° (lit.[9], 170°).

Fraction 3(c) (31 mg) had $[\alpha]_D$ +110° (*c* 0.31) and was identical (R_{Gal} 0.71) with 2,3,4-tri-*O*-methyl-D-galactose in solvent (*d*). This was confirmed by g.l.c. examination of the methyl glycoside. 2,3,4-Tri-*O*-methyl-*N*-phenyl-D-galactosylamine was prepared, and, after recrystallisation from ethyl acetate, had m.p. 164–167° (lit.[9], 164–169°).

Fraction 4 (22 mg) had $[\alpha]_D$ +80° (*c* 0.44), and was identical [R_G 0.54, solvent (*d*); 0.19, solvent (*e*)] with 2,6-di-*O*-methyl-D-galactose. After periodate oxidation, paper chromatography in solvent (*d*) revealed a bright-yellow spot (*p*-anisidine hydrochloride spray) having the same mobility (R_G 0.20) as 2-methoxymalonaldehyde.

Fraction 5 (42 mg) had $[\alpha]_D$ +88° (*c* 0.42) and behaved identically [R_G 0.49, solvent (*d*); 0.12, solvent (*e*)] with 2,4-di-*O*-methyl-D-galactose. Recrystallisation

from acetone containing 1% of water gave 2,4-di-*O*-methyl-D-galactose monohydrate, m.p. 96–97°. The X-ray diffractogram was identical with that of an authentic specimen. The derived 2,4-di-*O*-methyl-*N*-phenyl-D-galactosylamine had, after recrystallisation from acetone, m.p. 216–219° (lit.[9], 214–228°).

Fraction 6 (28 mg) had $[\alpha]_D$ +81° (*c* 0.56) and behaved identically in solvent (*d*) with an authentic specimen of 2-*O*-methyl-D-galactose (R_G 0.31). Recrystallisation from glacial acetic acid gave crystals, m.p. 151–154° (lit.[9], 145–148°).

Fraction 7 (76 mg) had $[\alpha]_D$ +53° (*c* 0.76). After reflux with methanolic hydrogen chloride, g.l.c. indicated the presence of the methyl ester methyl glycoside of 2,3,4-tri-*O*-methyl-D-glucuronic acid. This was reduced (sodium borohydride, 200 mg) for 12 h; excess of borohydride was destroyed by the addition of Amberlite resin IR-120 (H^+), and borate was removed by distillation as methyl borate. Thick-paper chromatography yielded 2,3,4-tri-*O*-methyl-D-glucose (26 mg). A portion (5 mg) was refluxed with methanolic hydrogen chloride for 7 h; g.l.c. indicated the presence of the methyl glycoside of 2,3,4-tri-*O*-methyl-D-glucose.

Reduction of methylated, degraded gum A. — Lithium aluminium hydride (50 mg) was added to methylated, degraded gum *A* (50 mg) in tetrahydrofuran (10 ml), and the mixture was refluxed for 3 h. After cooling, the excess hydride was destroyed by the addition of ethyl acetate and water; the mixture was taken to dryness and extracted with chloroform. This extract was concentrated to a syrup which was methylated with methyl iodide (1 ml) and silver oxide (1 g) in *N*,*N*-dimethylformamide (1 ml) to give a product (36 mg), $[\alpha]_D$ +19° (*c* 0.72, chloroform). After conversion into the methyl glycosides, g.l.c. gave the results shown in Table III. Examination of the hydrolysate of the methyl glycosides by paper chromatography [solvents (*d*) and (*e*)] indicated the presence of 2,6-di- and 2-*O*-methyl-D-galactose, in addition to the *O*-methyl sugars shown in Table III.

Partial methanolysis of methylated, degraded gum A. — Methylated, degraded gum *A* (100 mg) was refluxed with 2% methanolic hydrogen chloride (15 ml) for 2 h. The methanolysate was neutralised (silver carbonate), filtered, and evaporated to a syrup that was hydrolysed with sulphuric acid (0.5N, 10 ml) for 5 h on a boiling water-bath. After being cooled, the solution was neutralised (barium carbonate), filtered, deionised [Amberlite IR-120 (H^+)], and then fractionated on a column (3 × 33 cm) of Duolite A-4 resin in the formate form. Neutral *O*-methyl sugars were eluted with water (3 l); this fraction was not investigated further. Acidic *O*-methyl sugars were then eluted with 5% aqueous formic acid (500 ml); after removal of the formic acid and water, paper chromatography of the resulting syrup in solvent (*d*) indicated that there were no neutral *O*-methyl sugars present. This syrup was treated with 5% methanolic hydrogen chloride for 7 h; the product was examined by g.l.c., and this indicated that the methyl glycosides of 2,3,4-tri-*O*-methyl-D-glucuronic acid, 2,3,6-tri-, 2,3,4-tri-, 2,6-di-, and 2,4-di-*O*-methyl-D-galactose were present. Examination of the hydrolysate of these methyl glycosides by paper chromatography in solvents (*d*) and (*e*) confirmed these results.

Smith degradation[10] *of degraded gum* A. — Degraded gum *A* (1.0 g) was dissolved

TABLE III

THE METHANOLYSIS AND HYDROLYSIS PRODUCTS FROM METHYLATED, REDUCED, DEGRADED GUM *A*

Relative retention time (T) *of methyl glycosides*[a]		R_G *in solvent* (d)	*Relative molar propns.*	O-*Methyl sugars identified*
column (i)	*column* (ii)			
0.57, 0.73	0.49, 0.63	0.98	tr	2,3,5-tri-*O*-methyl-L-arabinose
(1.00)	0.82	0.79	tr	2,3,4-tri-*O*-methyl-L-arabinose
(1.00), 1.40	1.00, 1.39	1.00	+ +	2,3,4,6-tetra-*O*-methyl-D-glucose
1.68	1.66	0.90	+ +	2,3,4,6-tetra-*O*-methyl-D-galactose
2.96, (3.75), (4.25)	2.45, (3.04), (3.50)	0.72	+/2	2,3,6-tri-*O*-methyl-D-galactose
(3.75), (4.25)	(3.04), (3.50)	0.72	+ +	2,4,6-tri-*O*-methyl-D-galactose
6.45	5.30	0.72	+ + + +	2,3,4-tri-*O*-methyl-D-galactose
14.6, 16.4	9.9, 11.3	0.53	+ + +	2,4-di-*O*-methyl-D-galactose
—	—	0.59	+	2,6-di-*O*-methyl-D-galactose
—	—	0.34	+	2-*O*-methyl-D-galactose

[a] As for Table II.

in water (25 ml) and 0.5M sodium metaperiodate solution (25 ml) was added; 6.8 mmoles of periodate was reduced, and 4.0 mmoles of formic acid was released after 96 h, when the reaction was stopped by the addition of ethylene glycol (2 ml). The solution was dialysed for 48 h, sodium borohydride (0.5 g) was then added, and the mixture was kept at room temperature for 30 h. After dialysis for 48 h, the polyalcohol was hydrolysed (N sulphuric acid, room temperature, 48 h). The solution was neutralised (barium carbonate), filtered, deionised [Amberlite IR-120 (H^+)], and dialysed. The syrup (390 mg) obtained from the dialysate was shown by paper chromatography in solvents (*a*), (*b*), and (*c*) to contain glycerol and glycolaldehyde as the main components, together with small proportions of slower-moving, non-reducing glycosides. A portion of the dialysate was hydrolysed (N sulphuric acid, 7 h), and paper chromatography [solvents (*a*) and (*b*)] showed a trace of galactose to be present. After further dialysis (48 h), degraded gum *B* [93 mg, yield 9%], $[\alpha]_D$ +9° (*c* 0.93), was isolated as the freeze-dried product.

Mild hydrolysis (0.5N sulphuric acid, 1 h) of degraded gum *B* (10 mg) followed by paper chromatography [solvent (*a*)] showed the presence of galactose and two neutral disaccharides having the chromatographic mobilities of 3-*O*-β-D-galactopyranosyl-D-galactose (R_{Gal} 0.48) and 6-*O*-β-D-galactopyranosyl-D-galactose (R_{Gal} 0.29).

Degraded gum *B* was exhaustively methylated. Methanolysis, followed by g.l.c. examination of the mixture of methyl glycosides, gave the results shown in

Table IV. Paper-chromatographic examination of a hydrolysate of the methyl glycosides [solvents (*d*) and (*e*)] indicated the presence of 2,6-di- and 2-*O*-methyl-D-galactose, in addition to the *O*-methyl sugars identified as methyl glycosides by g.l.c.

TABLE IV

THE METHANOLYSIS AND HYDROLYSIS PRODUCTS FROM METHYLATED, DEGRADED GUM *B*

Relative retention time (T) *of methyl glycosides*[a]		R_G *in solvent* (d)	*Relative molar propns.*	*O-Methyl sugars identified*
column (i)	*column* (ii)			
1.68	1.66	0.90	+ +	2,3,4,6-tetra-*O*-methyl-D-galactose
3.76, 4.25	3.00, 3.45	0.72	+ + + + +	2,4,6-tri-*O*-methyl-D-galactose
6.45	5.29	0.72	+ +	2,3,4-tri-*O*-methyl-D-galactose
14.6, 16.4	9.9, 11.3	0.53	+ +	2,4-di-*O*-methyl-D-galactose
2.38, 3.00	2.20, 2.75	—	tr	2,3,4-tri-*O*-methyl-D-glucuronic acid[b]
—	—	0.59	+	2,6-di-*O*-methyl-D-galactose
—	—	0.34	+	2-*O*-methyl-D-galactose

[a]Figures in parentheses indicate incompletely resolved components; [b]As methyl ester methyl glycoside.

Controlled Smith degradation[10] *of degraded gum* A. — Degraded gum *A* (1.0 g) was treated in the same way as described for the Smith degradation, with the exception that the periodate oxidation was carried out at 2°, giving degraded gum *C* (105 mg, 10%) which was isolated by freeze-drying. A portion of this product (20 mg) was hydrolysed, and paper chromatography in solvent (*f*) indicated the presence of galactose and arabinitol; galactitol was absent.

DISCUSSION

Autohydrolysis of Fraction *A* of *A. drepanolobium* gum effected the release of all of the rhamnose, most of the arabinose, and some galactose. Degraded gum *A*, isolated after dialysis, contained D-galactose (80%), L-arabinose (3%), and uronic acid (17%). Molecular-sieve chromatography[3,8] gave estimated values of 5,200 and over 200,000 for the number-average molecular-weights of degraded gum *A* and *A. drepanolobium* gum Fraction *A*, respectively. Degradation on autohydrolysis is much greater than can result from the simple removal of arabinose and rhamnose from the periphery. It does not appear to have been caused by the presence of any internal, acid-labile arabinofuranose residues, since acid hydrolysis of borohydride-reduced, degraded gum *A* yielded only galactitol. There is no evidence of any galactofuranose residues in the polysaccharide, although, under the experimental circumstances, traces of di- and tri-*O*-methyl-D-galactofuranoses would be difficult to detect by g.l.c. and paper chromatography. The possibility that some galactopyranosidic bonds may be unusually sensitive to mild conditions of autohydrolysis has already been proposed for other plant gums[3,12,13].

An examination of methylated, degraded gum *A* showed the presence of 2,3,4,6-tetra-(+ +), 2,4,6-(+ +), 2,3,6-($\pm$), and 2,3,4-tri-(+ + +), 2,4-(+ + +) and 2,6-di-(+), and 2-*O*-methyl-D-galactose (+), 2,3,4-tri-*O*-methyl-D-glucuronic acid (+ +), and traces of 2,3,5- and 2,3,4-tri-*O*-methyl-L-arabinose. The presence of 2-*O*-methyl-D-galactose is probably due to undermethylation. This shows that the units **1** and **2** are structural fragments of degraded gum *A*. Methylation has shown traces of terminal, non-reducing L-arabinofuranose residues to be present in the

L-Ara*f*-(1→ L-Ara*p*-(1→

1 **2**

autohydrolysed gum of *A. senegal*[11], and terminal, non-reducing L-arabinopyranose residues to be present in degraded gum *A* of *A. arabica*[12]. Lithium aluminium hydride reduction of methylated, degraded gum *A*, followed by a Kuhn methylation[4], yielded 2,3,4,6-tetra-*O*-methyl-D-glucose after methanolysis. This confirms that D-glucuronic acid, and its 4-*O*-methyl analogue, are terminal, non-reducing end-groups in degraded gum *A*. Partial methanolysis was carried out on methylated, degraded gum *A*; after hydrolysis of the methyl glycosides, the methylated aldobiouronic acids were separated from the neutral *O*-methyl sugars. Methanolysis of the methylated aldobiouronic acids, followed by g.l.c. examination of the mixture of methyl glycosides, showed the presence of 2,3,4-tri-*O*-methyl-D-glucuronic acid, and 2,3,6- and 2,3,4-tri-, and 2,4- and 2,6-di-*O*-methyl-D-galactose. This indicates that units **3–6** are structural fragments of degraded gum *A*.

α-D-G*p*A-(1→4)-D-Gal*p*-(1→
or
4-*O*-Me-α-D-G*p*A-(1→4)-D-Gal*p*-(1→

3

β-D-G*p*A-(1→6)-D-Gal*p*-(1→
or
4-*O*-Me-β-D-G*p*A-(1→6)-D-Gal*p*-(1→

4

α-D-G*p*A-(1→4)-D-Gal*p*-(1→
3
↑
or
4-*O*-Me-α-D-G*p*A-(1→4)-D-Gal*p*-(1→
3
↑

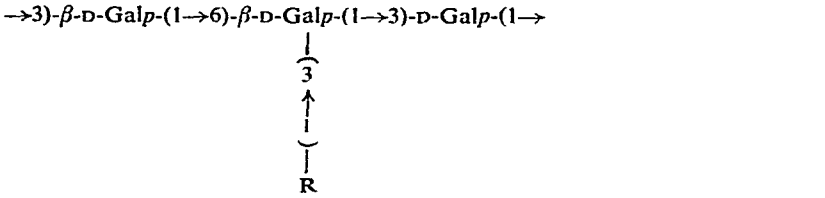
5

β-D-G*p*A-(1→6)-D-Gal*p*-(1→
3
↑
or
4-*O*-Me-β-D-G*p*A-(1→6)-D-Gal*p*-(1→
3
↑

6

Smith degradation of degraded gum *A* yielded degraded gum *B*. Partial, acid hydrolysis of degraded gum *B* gave 3-*O*-β-D-galactopyranosyl-D-galactose and 6-*O*-β-D-galactopyranosyl-D-galactose. An examination of methylated, degraded gum *B* indicated the presence of 2,3,4,6-tetra-(+ +), 2,4,6-(+ + + + + +) and 2,3,4-tri-(+ +), 2,6-(+) and 2,4-di-(+ +), and 2-*O*-methyl-D-galactose (+), with a trace of 2,3,4-tri-*O*-methyl-D-glucuronic acid. The presence of a trace of glucuronic acid in degraded gum *B* may be explained by incomplete oxidation of degraded gum *A*, and the presence of 2,6-di- and 2-*O*-methyl-D-galactose may arise from undermethyl-

ation. The proportion of the *O*-methylgalactoses obtained from methylated, degraded gum *B* is evidence that degraded gum *B* is a galactan composed predominantly of β-D-(1→3) glycosidic linkages, with a few β-D-(1→6) branch points. The identification of some 2,3,4-tri-*O*-methyl-D-galactose from methylated, degraded gum *B* indicates that unit 7 is a possible structural feature of degraded gum *A*.



where R = L-Araf-; L-Arap-; D-Galp-; →4)-D-Galp-; or →6)-D-Galp-

7

A controlled Smith degradation of degraded gum *A* yielded degraded gum *C*. Periodate oxidation of degraded gum *A* did not yield formaldehyde, so it may be concluded that the reducing galactose residue is substituted at C-6. The fact that degraded gum *C* contains arabinitol is evidence that at least some of the reducing galactose residues of degraded gum *A* are substituted[14] at C-3 (see Fig. 1).

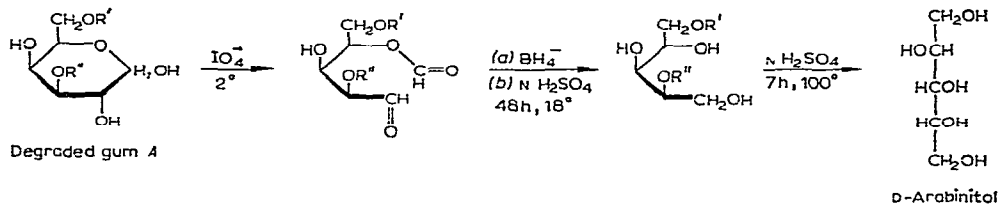


Fig. 1

Mild, acid hydrolysis of degraded gum *A* afforded 3-*O*-β-D-galactopyranosyl-D-galactose, 6-*O*-β-D-galactopyranosyl-D-galactose, and two trisaccharides, which were shown by methylation analysis and polarimetry to be the β-D-(1→3)- and β-D-(1→6) linked galactose trisaccharides. Characterisation of these oligosaccharides shows that units **8** and **9** are structural fragments of degraded gum *A*.

-β-D-Galp-(1→6)-β-D-Galp-(1→6)-D-Galp-(1→ -β-D-Galp-(1→3)-β-D-Galp-(1→3)-D-Galp-(1→

8 **9**

Two disaccharides were obtained from the diffusate of the autohydrolysate of Fraction *A*. Methylation and preparation of crystalline phenylosazones showed these to be 3-*O*-β-L-arabinofuranosyl-L-arabinose and 3-*O*-β-L-arabinopyranosyl-L-arabinose. The fact that both disaccharides are released under autohydrolysis conditions suggests that, in each case, the reducing L-arabinose residue was initially present in the furanose form. Thus units **10** and **11** are structural fragments of Fraction *A* of *A. drepanolobium* gum.

On the basis of the available evidence, Fraction *A* of *A. drepanolobium* gum is similar to *A. arabica* gum[12]. Both gums have high positive specific rotations, and the

-β-L-Araf-(1→3)-L-Araf-(1→ . -β-L-Araf-(1→3)-L-Araf-(1→

10 **11**

yields and properties of the degraded gums obtained from them are strikingly similar. There are, however, two important differences; degraded gum *A* from Fraction *A* of *A. drepanolobium* gum contains traces of terminal, non-reducing L-arabinopyranosyl and L-arabinofuranosyl residues; degraded gum *A* from *A. arabica* gum contains only terminal, non-reducing L-arabinopyranosyl residues[12]. Partial methanolysis of methylated, degraded gum *A* from *A. drepanolobium* gum has shown that units **3–6** are structural fragments of degraded gum *A*. Partial methanolysis of methylated, degraded gum *A* of *A. arabica* gum[12] has shown that only units **3**, **4**, and **6** are structural fragments of degraded *A. arabica* gum.

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REFERENCES

1 Part XXVII: D. M. W. ANDERSON AND G. M. CREE, *Carbohyd. Res.*, submitted.
2 D. M. W. ANDERSON AND I. C. M. DEA, *Carbohyd. Res.*, 7 (1967) 461.
3 D. M. W. ANDERSON AND J. F. STODDART, *Carbohyd. Res.*, 2 (1966) 104.
4 R. KUHN, H. TRISCHMANN, AND I. LÖW, *Angew. Chem.*, 67 (1955) 32.
5 O. PERILA AND C. T. BISHOP, *Can. J. Chem.*, 39 (1951) 815.
6 W. Z. HASSID, *Anal. Chem.*, 8 (1936) 138.
7 T. E. TIMELL, *Svensk Papperstidn.*, 19 (1960) 668.
8 D. M. W. ANDERSON, I. C. M. DEA, S. RAHMAN, AND J. F. STODDART, *Chem. Commun.*, (1965) 145.
9 R. L. WHISTLER, *Methods Carbohyd. Chem.*, 5 (1964) 298.
10 I. J. GOLDSTEIN, G. W. HAY, B. A. LEWIS, AND F. SMITH, *Abstracts Papers Am. Chem. Soc. Meeting*, 135 (1959) 3D.
11 D. M. W. ANDERSON, SIR EDMUND HIRST, AND J. F. STODDART, *J. Chem. Soc.* (*C*), (1966) 1959.
12 D. M. W. ANDERSON, SIR EDMUND HIRST, AND J. F. STODDART, *J. Chem. Soc.* (*C*), (1967) 1476.
13 A. M. STEPHEN, *South African J. Lab. Clin. Med.*, (1962) 76.
14 F. SMITH AND R. MONTGOMERY, *The Chemistry of Plant Gums and Mucilages*, Reinhold, New York, 1959.

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N.M.R.-STUDIEN AN ZUCKERN UND CYCLANOLEN

V.* ZUSAMMENHANG ZWISCHEN STERISCHER ORIENTIERUNG VON *O*- UND *N*-ACETYL-GRUPPEN UND DER SIGNALLAGE IHRER METHYL-RESONANZEN BEI CYCLIT- UND AMINOCYCLIT-POLYACETATEN

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ABSTRACT

Absorption ranges are deduced for axial and equatorial *O*- and *N*-acetyl-resonances of cyclitol and aminocyclitol polyacetates on the basis of 41 ($CDCl_3$) and 22 (Me_2SO-d_6) compounds, allowing configurational and conformational assignments. Scope and limitations are discussed; in particular, application to compounds having substituents other than acetoxy and acetamido groups.

ZUSAMMENFASSUNG

Anhand der N.M.R.-Daten von 41 ($CDCl_3$) bzw. 22 (Me_2SO-d_6) Cyclit- und Aminocyclit-polyacetaten werden Absorptionsbereiche axialer und äquatorialer *O*- und *N*-Acetyl-Resonanzen abgeleitet, die eine eindeutige Konfigurations- und Konformationsermittlung gestatten. Geltungsbereich und Einschränkungen, insbesondere bei Verbindungen mit anderen Substituenten als Acetoxy- bzw. Acetamino-Gruppen, werden diskutiert.

EINFÜHRUNG

Unter den physikalischen Methoden zur Konfigurations- und Konformationsermittlung carbocyclischer Sechsring-Verbindungen ist die eingehende N.M.R-Analyse der Ringprotonen eine der leistungsfähigsten[2]. Ihre Anwendbarkeit wird jedoch bei gleichartig polysubstituierten Cyclohexan-Derivaten eingeschränkt durch die Komplexität der Ring-CH-Multiplette, die meist nur mit hochauflösenden Geräten (100 bzw. 220 MHz) und unter Anwendung der Spinentkopplungstechnik analysiert werden können[3]. Bei Cycliten und Aminocycliten kann diese Schwierigkeit dadurch umgangen werden, daß die scharfen, nicht durch Spin–Spin-Kopplung beeinflußten Substituenten-Resonanzen herangezogen werden, wie z. B. — im Falle der entsprechenden Polyacetate — die Methyl-Resonanzen von Acetoxy- bzw. Acetamino-Gruppen, deren Signallage von der axialen bzw. äquatorialen Orientierung abhängt.

Die ersten diesbezüglichen Beobachtungen gehen auf die grundlegenden Arbeiten von *Lemieux* und Mitarbb.[4] zurück, die an einer Reihe von Aldopyranose-

*IV. Mitteilung, Ref. 1.

und Inositpolyacetaten zeigten, daß die Methylsignale axialer Acetoxy-Gruppen bei tieferem Feld erhalten werden als die äquatorialer. Diese Ergebnisse wurden in der Folge bestätigt[5–11] und konnten insbesondere auf Acetamino-Gruppen ausgedehnt werden, deren Methyl-Resonanzen eine analoge Abhängigkeit der Signallage von der sterischen Orientierung aufweisen und auf Grund einer Verschiebung nach höherem Feld sich nur bei axialer Anordnung denjenigen äquatorialer Acetoxy-Gruppen überlagern[6,7,10].

Mit der zunehmenden Anwendung dieser rein empirischen Befunde zur Konfigurations- und Konformations-bestimmung wurden verschiedentlich Absorptionsbereiche für axiale und äquatoriale Acetoxy- und Acetamino-Gruppen vorgeschlagen, die jedoch keine universelle Gültigkeit besitzen konnten, da sie aus den N.M.R.-Daten einer beschränkten Anzahl von Verbindungen eines bestimmten Typs (z.B. Anhydrozucker[8], Cyclite und Aminocyclite[10]), abgeleitet wurden. Die insbesondere bei Verbindungen mit anderen Substituenten als Acetoxy- und Acetamino-Gruppen beobachteten, z. T. beträchtlichen Abweichungen der Acetyl-Signallagen von diesen Bereichen[12], werden hieraus verständlich, obwohl dies verschiedentlich Zweifel an der Zuverlässigkeit der Methode aufwarf[13].

Im Hinblick auf die Einfachheit der Konfigurations- und Konformationsermittlung von Cyclit- und Aminocyclit-polyacetaten aus der Signallage der Acetyl-Resonanzen sowie zur Reevaluation der bisher hiermit vorgenommenen konfigurativen Zuordnungen erschien uns eine Klärung von Geltungsbereich und Einschränkungen dieser Methode erforderlich, die im folgenden für die Lösungsmittel Chloroform-*d*, Deuteriumoxid, und Methylsulfoxid-d_6 gegeben wird.

DISKUSSION

1. *Lage der Acetyl-Resonanzen in Deuteriochloroform*

In Tabelle 1 sind die Acetyl-Resonanzen von 41 vollacetylierten Inositen, Aminoinositen sowie deren Monodesoxy-Derivaten zusammengestellt. Dabei sind Polyacetate, die andere Substituenten als Acetoxy- und Acetamino-Gruppen enthalten (OH, C=O, OMs oder aromatische Reste) oder deren Konformation nicht eindeutig fixiert ist, nicht berücksichtigt. Aus den vorgenommenen Zuordnungen lassen sich

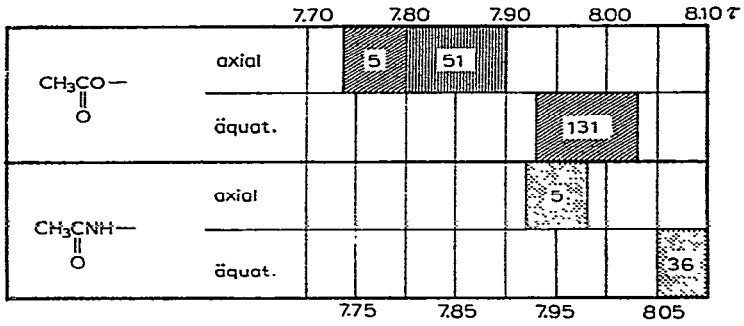


Abb. 1. Lage der Acetyl-Resonanzen von Inosit- und Inosamin-polyacetaten in $CDCl_3$. (Die Zahlen geben die Anzahl der in diesem Bereich liegenden Signale wieder).

die in Abb. 1 schematisch dargestellten Bereiche für die Lage axialer und äquatorialer Acetoxy- bzw. Acetamino-Gruppen ableiten.

Wie sich aus Abbild. 1 ergibt, sind die Resonanzbereiche für axiale und äquatoriale Acetoxy-Gruppen ebenso wie die für äquatoriale Acetamino-Gruppen klar voneinander getrennt, und zwar im Mittel um 0.15 p.p.m.

Hierdurch kann bei Verbindungen dieses Typs die Konformation und damit die Konfiguration des zugrundeliegenden Cyclits bzw. Aminocyclits in einfacher Weise ermittelt werden.

TABELLE I

LAGE DER ACETYL-RESONANZEN VON INOSIT- UND INOSAMIN-POLYACETATEN IN $CDCl_3$

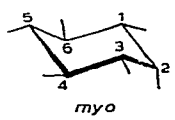
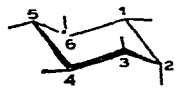
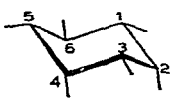
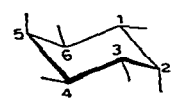
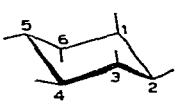
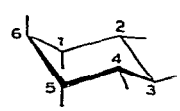
Konformation	*Hexa- bzw. Penta-acetat von*	*OAc*		*NHAc*		*Lit.*
		axial	*äquat.*	*axial*	*äquat.*	
	1 *scyllo*-Inosit	—	8.02 (6)			
	2 1-Desoxy-1-nitro-*scyllo*-inosit	—	7.99 (5)			
	3 *scyllo*-Inosamin	—	7.94 (2) 7.97 (3)		8.08	
	4 *scyllo*-Inosadiamin-(1,2)	—	7.95 (2) 8.03 (2)		8.09 (2)	21
scyllo	**5** *scyllo*-Inosadiamin-(1,3) (Streptamin)	—	7.97 (2) 8.02 (2)		8.09 (2)	
	6 2-Desoxy-*scyllo*-inosadiamin-(1,3) (2-Desoxy-streptamin)	—	7.96 (2) 8.02		8.07 (2)	
	7 *myo*-Inosit	7.80	8.00 (5)			
	8 (—)-1-Desoxy-*myo*-inosit (Viburnit)	7.85	7.98 (2) 8.00 8.01			15
	9 1-Desoxy-1-nitro-*myo*-inosit	7.87	8.02 (4)			
	10 *myo*-Inosamin-(1)	7.78	7.95 7.98 (2) 8.03		8.08	
	**11** *myo*-Inosamin-(2)	—	7.97 (2) 7.98 (3)	7.92		
myo	**12** *myo*-Inosamin-(4)	7.80	7.97 (2) 8.00 8.03		8.10	16
	13 3-Desoxy-*myo*-inosamin-(1)	7.83	7.98 (3)		8.08	14
	14 3-Desoxy-*myo*-inosamin-(4)	7.82	7.94 7.98 8.01		8.07	17
	15 *myo*-Inosadiamin-(1,3)	7.74	7.94 (2) 7.97		8.10 (2)	18
	16 *myo*-Inosadiamin-(4,6)	7.81	7.94 8.00 (2)		8.10 (2)	19
	17 3-Desoxy-*myo*-inosadiamin-(4,5)	7.85	7.93 8.01		8.06 (2)	20

TABELLE I

LAGE DER ACETYL-RESONANZEN VON INOSIT- UND INOSAMIN-POLYACETATEN IN $CDCl_3$

Konformation		*Hexa- bzw. Penta-acetat von*	*OAc*		*NHAc*		*Lit.*
			axial	*äquat.*	*axial*	*äquat.*	
	18	(—)-*chiro*-Inosit	7.81 (2)	7.96 (2) 8.00 (2)			
	19	(—)-1-*O*-Methyl-*chiro*-inosit (Quebrachit)	7.86 (2)	7.97 8.00 8.02			
	**20**	*chiro*-Inosamin-(1)	7.79 7.81	7.94 7.97 8.01		8.06	18
chiro *	**21**	*chiro*-Inosamin-(5)	7.81 7.86	7.97 8.00 8.02		8.10	
	22	*chiro*-Inosadiamin-(5,6)	7.82 (2)	7.98 (2)		8.06 (2)	21
	23	*epi*-Inosit	7.85 (2)	7.98 8.02 (3)			15
	24	3-Desoxy-*epi*-inosit	7.90 (2)	7.95 7.97 (2)			17
	25	*epi*-Inosamin-(1)	7.86 (2)	7.95 8.00 8.03		8.09	19
	**26**	*epi*-Inosamin-(2)	7.78	7.96 8.00 8.02	7.96		
epi	**27**	3-Desoxy-*epi*-inosamin-(2)	7.83	7.99 (3)	7.94		17
	28	*epi*-Inosamin-(6)	7.84 (2)	8.00 (3)		8.09	19
	29	2-Desoxy-*epi*-inosadiamin-(1,6)	7.82	7.97 8.00		8.05 (2)	20
	30	*neo*-Inosit	7.84 (2)	8.01 (4)			
	**31**	1,4-Didesoxy-1,4-dinitro-*neo*-inosit	7.88 (2)	7.98 (2)			
	32	*neo*-Inosamin-(1)	7.83 (2)	7.98 8.01 8.03		8.10	
neo	**33**	*neo*-Inosamin-(2)	7.82	7.96 (4)	7.94		
	34	*neo*-Inosadiamin-(1,4)	7.82 (2)	8.00 (2)		8.08 (2)	
	35	*allo*-Inosamin-(5)	7.83 7.85	7.95 8.00		8.05 (2)	14
	**36**	1-Desoxy-*allo*-inosamin-(5)	7.84 7.87	7.98 8.01		8.08	14
allo	**37**	*allo*-Inosadiamin-(1,5)	7.86 7.89	7.96 (2)	7.96	8.05	21
	38	3-Desoxy-3-nitro-*muco*-inosit	7.75 7.83 (2)	7.96 (2)			
	**39**	*muco*-Inosamin-(3)	7.87 (3)	7.96 (2)		8.07	
	40	1-Desoxy-*muco*-inosamin-(4)	7.85 (2)	7.96 7.98		8.09	22
muco	**41**	1-Desoxy-*muco*-inosamin-(2,3)	7.86 7.88	7.97		8.06 (2)	

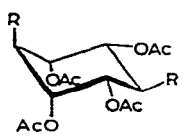
*Die Konfigurationsbezeichnung der vom optisch aktiven Inosit abgeleiteten Aminoinosite durch den Präfix *rac* (z.B. *rac*-Inosamin-4) ist irreführend, da auch Inosamine, die sich von *myo*-, *neo*-, *muco*- und *allo*-Inosit ableiten, „racemisch" sind. Dem Vorschlag eines Gutachters folgend, benutzen wir den Präfix *chiro* anstelle *rac*.

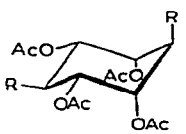
Die Methyl-Resonanzen axialer Acetamino-Gruppen — von den äquatorialen ebenfalls im Mittel um 0.15 p.p.m. getrennt — fallen dagegen in den Bereich äquatorialer Acetoxy-Gruppen, sodaß eine absolute Zuordnung der in diesem Bereich auftretenden Signale nur durch Vermessung der entsprechenden *N*-Trideuterioacetyl-*O*-acetyl-Derivate möglich ist.

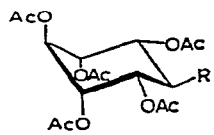
Zur Ermittlung der Konfiguration jedoch ist dies gewöhnlich nicht erforderlich, da diese sich zwanglos aus dem Fehlen einer äquatorialen Acetamino-Resonanz (z. B. Verbindungen **11**, **26**, **27** und **33** in Tab. 1) ableiten läßt.

Naturgemäß sind die in Abb. 1 angegebenen Bereiche für axiale und äquatoriale Acetoxy- und Acetamino-Gruppen nur so lange gültig, als die betreffenden Verbindungen in ihrer Konformation — gewöhnlich der Sessel-Konformation mit der geringsten Anzahl axialer Substituenten — eindeutig fixiert sind. Bei Verbindungen dagegen, die drei axiale und drei äquatoriale Acetoxy-Gruppen besitzen, wie z.B. die Hexaacetate von *cis*-, *allo*- und *muco*-Inosit, sind die beiden möglichen Sesselkonformationen sterisch und energetisch äquivalent, so daß eine leichte Sessel–Sessel-Inversion erfolgen kann, die je nach Inversionsgeschwindigkeit (d. h. der Temperatur, bei der die Probe vermessen wird) zu einem mehr oder weniger ausgeprägten Zusammenfallen der Acetoxy-Resonanzen führt.

So werden z. B. für Hexaacetyl-*muco*-inosit (**42**) und *N*,*N*-Diacetyl-*muco*-inosadiamin-(3,6)-tetraacetat (**45**), ebenso wie für die entsprechenden 3,6-Di-*O*-methylsulfonyl- (**44**) und 3,6-Diazido- (**43**) Derivate[23] Acetyl-Resonanzen erhalten, die auf eine schnelle Sessel–Sessel-Inversion schließen lassen. Wird jedoch in **42** eine Acetoxy-Gruppe durch eine Acetamino-Gruppe ersetzt, so tritt eine beachtliche Verlagerung des Inversions-Gleichgewichtes der beiden Sessel-Konformationen ein, dahingehend, daß die Konformation mit äquatorialer Acetamino-Gruppe bevorzugt ist. Dies geht eindeutig hervor aus der Lage und der Art der Aufspaltung der Acetyl-Resonanzen im *N*-Acetyl-*muco*-inosamin-(3)-pentaacetat (**39**, vgl. Tab. I). Einen entsprechenden, konformationsstabilisierenden Effekt besitzt die Nitrogruppe im *muco*-Derivat **38**.



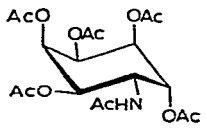




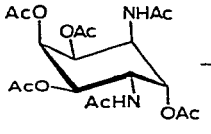
42	R = OAc	[OAc:7.92 (2), 7.95 (4)]	**38** R = NO_2
43	R = N_3	[OAc:7.88 (4)]	**39** R = NHAc
44	R = OMs	[OAc:7.88 (4)]	
45	R = NHAc	[OAc:7.92 (4); NHAc:8.03 (2)]	

Ähnliche Verhältnisse liegen bei Hexa-*O*-acetyl-*allo*-inosit vor, der nur *ein* 1H-Signal im τ 8-Gebiet liefert[24], während im entsprechenden Amino-Derivat **35** auf Grund der Aufspaltung der Acetyl-Resonanzen in die in Abb. 1 gegebenen Bereiche (vgl. Tab. I) die Konformation mit äquatorialer Orientierung der Acetamino-Gruppe eindeutig fixiert ist. Überraschend erscheint jedoch, daß auch in *N*,*N*-

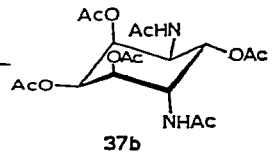
Diacetyl-*allo*-inosadiamin-(1,5)-tetraacetat trotz gleicher Anzahl axialer und äquatorialer Substituenten die Acetyl-Resonanzen in die in Abb. 1 festgelegten Bereiche aufgespalten sind. Es muß somit eine der beiden möglichen Sesselkonformationen (**37a** bzw. **37b**) fixiert sein — wahrscheinlich **37b**, da die 1,3-diaxiale Wechselwirkung zwischen einer Acetoxy- und Acetamino-Gruppe (**37a**) größer sein dürfte als die zwischen zwei axialen Acetoxy-Gruppen.



35

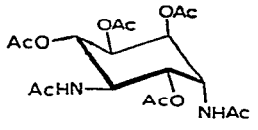


37a

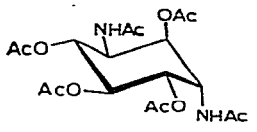


37b

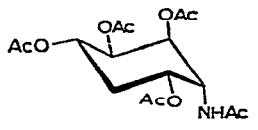
Abweichungen von der Sesselkonformation mit geringster Zahl axialer Substituenten treten offenbar auch bei Verbindungen mit *chiro*-Konfiguration ein, wenn sie eine Acetoxy- und Acetamino-Gruppe in 1,2-diaxialer Anordnung besitzen. So zeigen z. B. die drei bisher bekannten Verbindungen dieser Art (**46–48**) Acetyl-Resonanzen[14,21], die nicht mehr im Sinne der in Abb. 1 gegebenen Bereiche interpretierbar sind und das Vorliegen einer *Skew*-Konformation oder eine schnelle Sessel–Sessel-Inversion wahrscheinlich machen. Interessanterweise tritt bei Verbindungen mit zwei 1,2-diaxialen Acetoxy-Gruppen ein derartiger Effekt nicht auf, wie aus der Lage der Acetyl-Resonanzen von **18–22**: (vgl. Tab. I) hervorgeht.



46



47



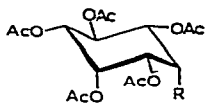
48

Acetyl-Resonanzen[14,21]

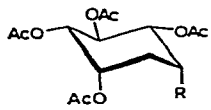
46	7.84, 7.96 (3), 8.02 (2)
47	7.82, 7.99 (3), 8.02 (2)
48	7.86, 7.90, 7.92 (2), 8.03

1. *Anwendbarkeit der Bereiche von Abb. 1 auf Cyclit-polyacetate mit anderen Substituenten als Acetoxy- und Acetamino-Gruppen.* — Wird in einem Inosit-hexaacetat eine Acetoxy-Gruppe durch eine Nitro–Gruppe ersetzt (Verbb. **2**, **9**, und **31** im Vergleich zu **1**, **7**, und **30**) oder durch eine Methoxy-Gruppe wie im Quebrachit-pentaacetat (**19**), so treten nur geringfügige, im Rahmen der Bereiche in Abb. 1 bleibende Abweichungen auf. Entsprechendes gilt für die Eliminierung einer Acetoxygruppe, wie z. B. der Vergleich der *epi*-Verbindungen **23** und **26** mit ihren 3-Desoxy-*epi*-Analogen **24** und **27** zeigt. Auf Grund dieser Befunde muß der früher beschriebenen[14], als *N*-Acetyl-3-desoxy-*epi*-inosamin-(1)-tetraacetat (**49**) angesehenen Verbindung (Acetyl-Resonanzen bei τ 7.88, 7.93 (2), 7.99 und 8.05) die nach dem Synthese-

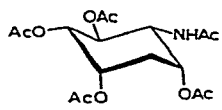
weg mögliche Konfiguration eines *N*-Acetyl-6-desoxy-*myo*-inosamin-(5)-tetraacetat (**50**) zugeordnet werden.



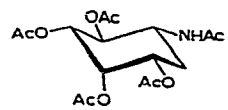
25 R = OAc
26 R = NHAc



24 R = OAc
27 R = NHAc



49



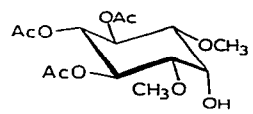
50

Dagegen lassen sich die in Abb. 1 gegebenen Bereiche nicht ohne Einschränkung auf Verbindungen übertragen, die neben Acetoxy-, Acetamino-, Nitro- und Methoxy-Gruppen noch andere Substituenten besitzen. Bereits bei 1,2-*O*-Isopropyliden-*myo*-inosit-tetraacetat (Acetoxy-Resonanzen bei τ 7.92, 7.97 und 8.01 (2) sind im Vergleich zu *myo*-Inosit-hexaacetat (**7**, Tab. I) die Resonanzen der dem Dioxolan-Ring benachbarten Acetoxy-Gruppen nach niedrigerem Feld verschoben. Ähnliche paramagnetische Verschiebungen treten bei Verbindungen auf, die nicht voll acetyliert sind (OH als Nachbarsubstituent) oder Methylsulfonyloxy-Gruppen enthalten, während bei aromatischen Substituenten diamagnetische Verschiebungen der benachbarten Acetoxy-Resonanzen beobachtet werden.

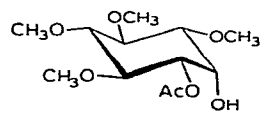
Teilweise acetylierte Inosite und Inosamine. — Die für voll acetylierte Inosite und Inosamine abgeleiteten Bereiche (Abb. 1) lassen sich nicht auf nur teilweise acetylierte Verbindungen übertragen. Zwar zeigt 4,5,6-Tri-*O*-acetyl-1,3-di-*O*-methyl-*myo*-inosit (**51**) noch die in Analogie zu *trans*-Cyclohexantriol-triacetat (s. unten) erwarteten Resonanzen[25] bei τ 7.97 (2) und 8.01, dagegen sind die Acetoxy-Signale der vier Penta-*O*-acetyl-*myo*-inosite (Tab. II) ohne Ausnahme nicht mehr im Sinne der in Abb. 1 gegebenen Werte interpretierbar. Offenbar bedingt eine zur Acetoxy-Gruppe vicinale Hydroxyl-Funktion eine Verschiebung zu niedrigeren τ-Werten.

So geben z. B. auch die Strukturisomere **52** und **53** trotz eindeutig verschiedener sterischer Orientierung der Acetoxy-Gruppen jeweils ein 3H-Signal[25] bei τ 7.84.

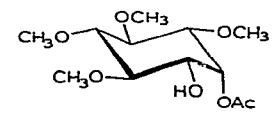
Entsprechendes gilt für *N*-Acetyl-inosamine, deren Acetamino-Resonanzen bei wesentlich tieferen τ-Werten (um τ 7.95) erhalten werden und keine eindeutigen Rückschlüsse auf die sterische Orientierung mehr zulassen.



51



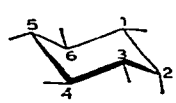
52



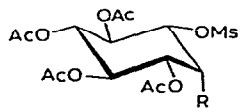
53

TABELLE II

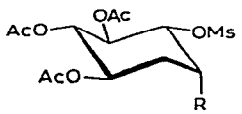
ACETOXY RESONANZEN VON PENTA-*O*-ACETYL-*myo*-INOSITEN[25]

	Pentaacetat	*Acetoxy-Resonanzen*
	1, 2, 3, 4, 5	7.83, 7.90, 7.93, 7.97, 8.01
	1, 2, 3, 4, 6	7.79 (2), 7.89, 7.99 (2)
	1, 2, 4, 5, 6	7.78, 7.92, 7.98 (2), 8.01
	1, 3, 4, 5, 6	7.89 (2), 7.98 (3)

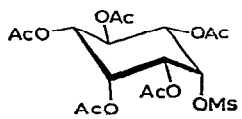
O-Methylsulfonyl-inosit-polyacetate. — Beim Vergleich der Lage der Acetyl-Resonanzen in den Methansulfonaten **54–58** (Tab. III) mit den für die entsprechenden vollacetylierten Verbindungen **7**, **8** und **23** erhaltenen (vgl. Tab. I) ergibt sich, daß die einer Methylsulfonyloxy-Gruppe benachbarten äquatorialen Acetoxy-Resonanzen im Mittel um 0.07 p.p.m. nach niederem Feld verschoben sind. Hierdurch kann der in Abb. 1 für äquatoriale Acetoxy-Gruppen angegebene Bereich unterschritten werden (z. B. Signale bei τ 7.91 und 7.92 bei **54**, **55** und **57**). Inwieweit ein ähnlicher Effekt auch bei axialen Acetoxygruppen auftritt — Hexa-*O*-acetyl-*myo*-inosit zeigt die axiale Acetoxy-Resonanz bei τ 7.80, **54** bei τ 7.78 — läßt sich erst abschätzen, wenn weitere Verbindungen dieses Typs bekannt sind.



54 R = OAc
55 R = OMs



56 R = OAc
57 R = OMs



58

TABELLE III

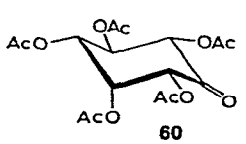
METHYL-RESONANZEN VON *O*-(METHYLSULFONYL)-INOSIT-POLYACETATEN[16,22,26]

Verbindung	*OMs*		*OAc*	
	axial	*äquat.*	*axial*	*äquat.*
54	—	7.00	7.78	7.92 8.00 (3)
55	6.80	6.89	—	7.91 (2) 7.98 (2)
56	—	6.99	7.83	7.93 7.97 (2)
57	6.82	6.89	—	7.92 7.97 (2)
58	6.92	—	7.85	7.94 (2) 8.00 8.02

Keto-Verbindungen. — Die in Abb. 1 angegebenen Bereiche sind bei Acetoxy-Gruppen, die einer Carbonyl-Funktion benachbart sind, nicht anwendbar. So zeigen z. B. die Pentaacetate der *scyllo*- bzw. *epi*-(2)-Inosose (**59** bzw. **60**) zwei Acetoxy-Resonanzen, die aus dem äquatorialen Bereich paramagnetisch verschoben sind (τ 7.88 bzw. 7.89), was wahrscheinlich auf den Entschirmungs(„deshielding")-Effekt der Carbonylgruppe zurückzuführen ist.

OAc (τ)
7.88 (2)
7.96 (2)
7.98

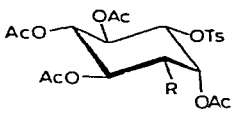
59



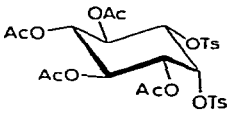
OAc (τ)
7.86
7.89 (2)
7.95
7.98

60

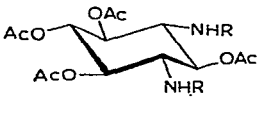
Cyclit-polyacetate mit aromatischen Substituenten. — Eine starke Verschiebung der Acetoxy-Resonanzen nach höheren τ-Werten tritt erwartungsgemäß dann ein, wenn sich die Methylprotonen im Bereich diamagnetischer Abschirmung[27] eines aromatischen Substituenten befinden. So sind z. B. bei Penta-*O*-acetyl-1-*O*-*p*-Tolylsulfonyl-*myo*-inosit (**61**) bei Vergleich mit den für Hexa-*O*-acetyl-*myo*-inosit erhaltenen Werten beide der *p*-Tolylsulfonyl-Gruppe benachbarten Acetoxy-Resonanzen nach höheren Feld verschoben, und zwar um 0.08 und 0.10 p.p.m. Bei zwei benachbarten *p*-Tolylsulfonyl-Gruppen ist dieser Effekt additiv, wie das τ 7.96 Signal der axialen Acetoxy-Gruppe in **62** zeigt (vgl. Tab. IV).



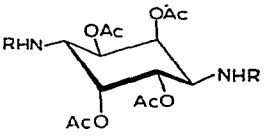
61 R = OAc
62 R = OTs



63



64
R = CO_2CH_2Ph



65
R = CO_2CH_2Ph

TABELLE IV

INOSIT-POLYACETATE MIT AROMATISCHEN SUBSTITUENTEN

Verbindung	*axiale OAc*	*äquat. OAc*	*Lit.*
61	7.88	8.00 (2), 8.02, 8.10	52
62	7.96	7.96, 8.01, 8.10	52
63	—	7.95, 8.00, 8.04, 8.18	52
64	—	8.10, 8.17 (2), 8.23	5
65	7.82 (2)	8.05 (2)	5

Eine entsprechende Verschiebung von Acetoxy-Resonanzen wird bei Benzyloxycarboxylamino-Substituenten erhalten, wobei die Abschirmungseffekte offenbar differenzierter sind. Das Streptamin-Derivat **64** zeigt im Vergleich zum Hexaacetat (**5**, Tab. I) beträchtliche Verschiebungen (0.1–0.2 p.p.m.). Im Tetra-*O*-acetyl-di-*N*-benzyloxycarbonyl-*neo*-inosadiamin-(1,4) (**65**) dagegen befinden sich die beiden axialen Acetoxy-Gruppen offenbar außerhalb des Bereichs diamagnetischer Abschirmung des aromatischen Kerns, wie aus der Signallage von τ 7.82 (*N*,*N*-Diacetyltetraacetat **34**: τ 7.82, vgl. Tab. I) hervorgeht, während die Resonanzen der beiden äquatorialen Acetoxy-Gruppen nur eine Verschiebung von 0.05 p.p.m. aufweisen. Die konformative Einstellung der Benzyloxycarbonyl-Gruppe in Relation zu den Acetoxy-Gruppen ist somit in **65** eine völlig andere als in **64**.

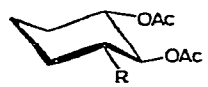
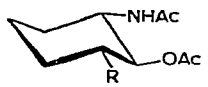
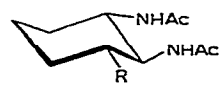
Analoge diamagnetische Verschiebungen von Acetoxy-Resonanzen werden durch die beiden aromatischen Kerne eines Dibenzylphosphoryloxy-Restes ausgelöst, wie anhand von sechs Penta-*O*-acetyl-inosit-dibenzylphosphaten gezeigt werden konnte[28]. Im Hinblick auf die bei einer Reihe von aromatisch substituierten Zucker- und Aminozucker-polyacetaten erhaltenen Werte[12] sind auch für Cyclit-polyacetate, die Benzoyl-, Benzyl-, Trityl- oder ähnliche Substituenten enthalten, Abweichungen

von den in Abb. 1 angegebenen Bereichen zu erwarten, so daß nur unter Berücksichtigung dieser Effekte eine Konfigurationsermittlung möglich ist.

2. *Polyacetate von Cyclanolen und Aminocyclanolen.* — Die Acetyl-Resonanzen von *O*-Acetyl-cyclohexanol (τ 7.98) und *N*-Acetyl-cyclohexylamin (τ 8.09) liegen in dem für äquatoriale Acetoxy- bzw. Acetamino-Gruppen abgeleiteten Bereichen (vgl. Abb. 1). Entsprechendes ist für vollacetylierte Di-, Tri- und Tetrahydroxycyclohexane bzw. deren Amino-Analoga zu erwarten, sofern all-äquatoriale Orientierung der Substituenten vorliegt. Wie aus den in Tab. V zusammengestellten Daten hervorgeht, ist dies bei allen bisher bekannten Verbindungen dieses Typs der Fall.

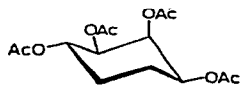
TABELLE V

LAGE DER ACETYL-RESONANZEN ALL-AQUATORIALER CYCLANOL- UND AMINOCYCLANOLACETATE

Verbindung		*äquat. OAc*	*äquat. NHAc*	*Lit.*
O-Acetyl-cyclohexanol		7.98		
N-Acetyl-cyclohexylamin		—	8.09	
	R = H	8.00 (2)		
	R = OAc	7.96 (2)		
		7.99		
	R = NHAc	7.95	8.07	10, 11
		7.99		
	R = H	7.99	8.09	
	R = OMe	7.93	8.09	11
	R = OEt	7.94	8.07	10
	R = OAc	8.01	8.11 (2)	29
	R = NHAc		8.10 (3)	30
	R = H	7.95 (2)	8.07	31
	R = e-Ph	7.98 (2)	8.08	32
	R = a-OEt	7.98 (2)	8.10	1

Bei Verbindungen dagegen, die axiale Acetoxy- bzw. Acetamino-Gruppen enthalten, können Abweichungen von den in Abb. 1 gegebenen Bereichen auftreten. So liegen zwar die Resonanzen axialer Acetoxy-Gruppen, sofern sie, wie in **66** und **76**, von zwei äquatorialen flankiert sind, noch im üblichen axialen Bereich (vgl. Tab. VI), jedoch sind solche mit nur einer vicinalen Acetoxy-Gruppe (**67**, **68**, **72–75**) nach Tab. VI an die obere Grenze dieses Bereiches verschoben. Im Falle der Verbindung **69** wird er sogar überschritten. Allgemein ergibt sich aus den Daten der Tab. VI, daß die Methyl-Resonanzen axialer Acetoxy-Gruppen umso mehr in den Bereich äquatorialer fallen, je weniger Nachbarsubstituenten (OAc bzw. NHAc) vorhanden sind. Diese Folgerung wird bestätigt durch die Acetyl-Signallagen von Steroiden, die

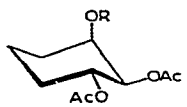
im Ring A Acetoxy-Gruppen besitzen. Wie sich aus den von Tori und Komori[33] tabellierten Werten ableiten läßt, tritt hierbei eine beträchtliche Überlappung der Bereiche für axiale (τ 7.91–7.97, 15 Beispiele) und äquatoriale Acetoxy-Gruppen (τ 7.95–8.02, 23 Beispiele) ein.



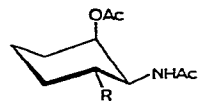
66

67 R = OAc
68 R = NHAc

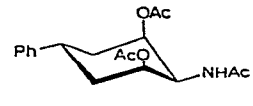
69



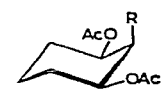
70 R = CH_3
71 R = Et
72 R = Ac



73 R = OAc
74 R = NHAc



75



76 R = OAc
77 R = NHAc

TABELLE VI

LAGE DER ACETYL-RESONANZEN VON CYCLANOL- UND ACETAMINOCYCLANOL-ACETATEN MIT AXIALEN GRUPPEN

Verbindung	*OAc*		*NHAc*		*Lit.*
	axial	*äquat.*	*axial*	*äquat.*	
66	7.86	7.97			
		8.00 (2)			
67	7.89	7.96			15
		7.97			
		8.00			
68	7.90	7.96		8.08	
		8.02			
69	7.92 (2)	7.98		8.06	1
70		7.90		8.08	11
71		7.90		8.07	11
72	7.90	7.97		8.04	11
73	7.90	7.97		8.08	31
74	7.89			8.07 (2)	34
75	7.88 (2)			8.05	32
76	7.88	7.96 (2)			
77		7.95 (2)	7.99		35

II. *Lage der Acetyl-Resonanzen in D_2O.*

Auf Grund der Unlöslichkeit einiger Cyclit-polyacetate in Deuteriochloroform, z. B. Tri-*N*-acetyl-*cis*-inosatriamin-(1,3,5)-triacetat[36] ist verschiedentlich D_2O als Lösungsmittel herangezogen worden. Wie sich aus den in Tab. VII zusammengestellten Werten ergibt, weisen die Acetyl-Signallagen zwar keine beträchtlichen Unterschiede gegenüber den $CDCl_3$-Daten auf, jedoch ergibt sich aus der geringen (0.02–0.04 p.p.m.) Verschiebung der Acetoxy-Resonanzen nach niederem Feld,

daß die $CDCl_3$-Bereiche (Abb. 1) nicht uneingeschränkt auf D_2O übertragen werden können. Entsprechende Ergebnisse wurden bei einigen Zucker-polyacetaten erhalten[37].

TABELLE VII

SIGNALLAGEN VON ACETYL-RESONANZEN IN D_2O

Verbindung	*OAc*		*NHAc*	
	axial	*äquat.*	*axial*	*äquat.*
N,N-Diacetyl-*scyllo*-inosadiamin-(1,3)-tetraacetat		7.94 (4)		8.08 (2)
N,N-Diacetyl-*myo*-inosadiamin-(2,4)-tetraacetat[38]		7.95 (2) 7.99 (2)	7.90	8.07
Tri-*N*-acetyl-*cis*-inosatriamin-(1,3,5)-triacetat[36]	7.74 (3)			8.05 (3)
Di-*O*-acetyl-2*t*-acetaminocyclohexandiol-(1*r*, 3*c*)		7.96 (2)		8.07
Di-*O*-acetyl-2*c*-acetaminocyclohexandiol-(1*r*, 3*t*) (73)	7.87	7.95		8.06
Acetaminocyclohexan				8.05

III. *Lage der Acetyl-Resonanzen im Methylsulfoxid-*d_6.

In jeder Hinsicht günstiger zur Konformations- und Konfigurationsermittlung auf Grund der chemischen Verschiebungen der Acetyl-Resonanzen erweist sich Methylsulfoxid-d_6. Abgesehen von den im Vergleich zu Chloroform und D_2O besseren Lösungseigenschaften sind die Absorptionsbereiche axialer und äquatorialer *O*- und *N*-Acetyl-Gruppen klarer voneinander getrennt, wodurch die Zuordnungen erleichtert werden.

In Tab. VIII sind die Acetyl-Resonanz-Signallagen von 22 Cyclit- und Aminocyclit-polyacetaten zusammengestellt. Aus den vorgenommenen Zuordnungen ergibt sich die in Abb. 2 schematisch dargestellten Korrelation zwischen chemischer Verschiebung des Acetyl-Signals und der sterischen Orientierung der Acetyl-Gruppe.

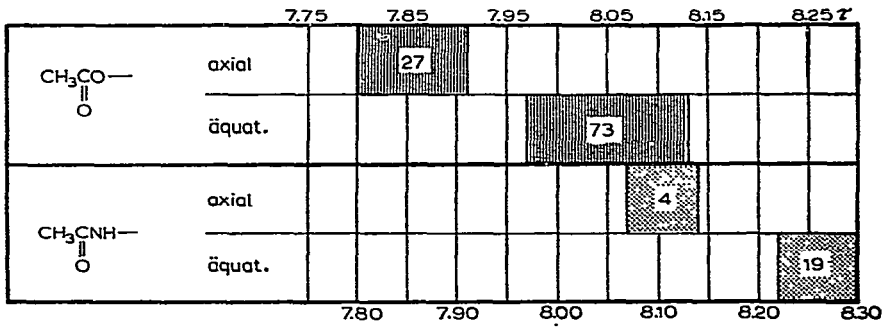
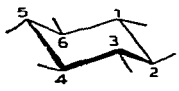
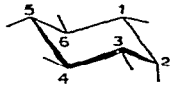
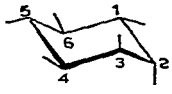
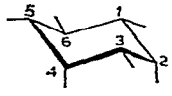
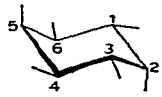
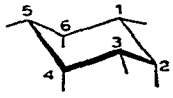


Abb. 2. Lage der Acetyl-Resonanzen von Inosit- und Inosaminpolyacetaten in Methylsulfoxid-d_6.

TABELLE VIII

LAGE DER ACETYL-RESONANZEN VON INOSIT- UND INOSAMIN-HEXAACETATEN IN Me_2SO-d_6

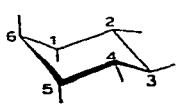
Konformation	*Polyacetat von*	*OAc*		*NHAc*	
		axial	*äquat.*	*axial*	*äquat.*
 *scyllo*	*scyllo*-Inosadiamin-(1,3) (Streptamin)		8.10 (4)		8.30 (2)
	1-Desoxy-*scyllo*-inosamin-(2)		8.02 (2) 8.06 (2)		8.22
	1-Desoxy-1-nitro-*scyllo*-inosit		8.06 (5)		
	myo-Inosit	7.85	8.04 (3) 8.06 (2)		
	1-Desoxy-1-nitro-*myo*-inosit	7.87	8.03 (3) 8.05 (2)		
 *myo*	*myo*-Inosamin-(1)	7.84	8.03 8.07 (3)		8.23
	myo-Inosamin-(2)		8.05 (3) 8.10 (2)	8.10	
	myo-Inosadiamin-(1,2)[26]		8.05 8.08 (3)	8.14	8.23
	myo-Inosadiamin-(1,3)	7.86	8.08 (2) 8.11		8.26 (2)
	Di-*N*-formyl-*myo*-inosadiamin-(1,3)[39]	7.86	8.05 (2) 8.09		
	myo-Inosadiamin-(4,6)[19]	7.86	8.09 (3)		8.25 (2)
	(—)-*chiro*-Inosit	7.82 (2)	8.01 (2) 8.04 (2)		
 *chiro*	(—)-1-*O*-Methyl-*chiro*-inosit (Quebrachit)	7.85 (2)	8.00 8.04 (2)		
	chiro-Inosamin-(5)	7.85 (2)	8.07 (2) 8.10 (1)		8.27
	6-Desoxy-*chiro*-inosadiamin-(1,3)[20]	7.90	8.02 8.13	8.13	8.23
 *epi*	*epi*-Inosamin-(1)[19]	7.88 7.90	8.05 8.08 8.13		8.22
	epi-Inosamin-(2)	7.80	7.97 8.02 8.05 (2)	8.07	
	epi-Inosamin-(6)[23]	7.89 (2)	8.10 (3)		8.23
 *neo*	*neo*-Inosamin-(1)	7.87 (2)	8.05 (3) 8.07 (3)		8.24
	neo-Inosadiamin-(1,4)	7.89 (2)	8.11 (2)		8.25 (2)
	Di-*N*-formyl-*neo*-inosadiamin-(1,4)	7.85 (2)	8.08 (2)		
 *cis*	*cis*-Inosatriamin-(1,3,5)[36]	7.91 (3)			8.25 (3)

Im Vergleich zu den für Chloroform erhaltenen Absorptionsbereichen wird in Methylsulfoxid-d_6 eine generelle diamagnetische Verschiebung der Acetyl-Resonanzfrequenzen beobachtet, die interessanterweise für Acetoxy-Gruppen verhältnismäßig klein (0.05 p.p.m.), für Acetamino-Gruppen dagegen sehr beträchtlich ist (0.25 p.p.m.), ein Effekt, der zweifellos mit der völlig andersartigen NH-Solvatation zusammenhängt. Durch diesen Lösungsmittel-Effekt wird der Gesamtabsorptionsbereich um 0.14 p.p.m. breiter, was eine schärfere Trennung der individuellen Bereiche und somit eine exaktere Differenzierung zwischen OAc- und NHAc-Resonanzen gestattet.

Bei Verbindungen mit drei axialen und drei äquatorialen Substituenten und der dadurch leicht erfolgenden Sessel–Sessel-Inversion beobachtet man, analog zu den bei $CDCl_3$ erhaltenen Ergebnissen, ein mehr oder weniger ausgeprägtes Zusammenfallen der Acetylsignale. Dies ist z. B. der Fall bei *muco*-Inosamin-hexaacetaten (vgl. Tab. IX), deren Acetyl–Resonanzen mit Ausnahme der *muco*-2,4-Verbindung Mittelwerte darstellen, bedingt durch den bereits bei normaler Meßtemperatur (34–36°) relativ schnellen Übergang der beiden Alternativ-Konformationen. Infolge der Temperaturabhängigkeit dieser Sessel-Sessel-Inversion dürfte jedoch durch Tieftemperaturmessungen eine Aufspaltung in die in Abb. 2 gegebenen Bereiche zu erreichen sein.

TABELLE IX

LAGE DER ACETYL-RESONANZEN POLYACETYLIERTER *muco*-INOSAMINE

muco-*Hexaacetate*	*Acetyl-Resonanzen* (τ)
	
Inosamin-(3) (**39**)	7.98 (5), 8.16
Inosadiamin-(2,3)[38]	7.94 (2), 7.98, 8.09, 8.23 (2)
Inosadiamin-(2,4)[26]	7.87, 7.90 (2), 8.08, 8.21 (2)
Inosadiamin-(1,4) (**45**)[23]	7.91 (4), 8.10 (2)
Inosatetramin-(1,2,4,5)[23]	7.91 (2), 8.09 (4)

Im Falle von *N*-Acetyl-*muco*-inosamin-(3)-pentaacetat (**39**) läßt sich hierbei eine Abhängigkeit der Konformation vom Lösungsmittel feststellen. In $CDCl_3$ ist auf Grund der deutlich aufgespaltenen Acetylresonanzen (vgl. Tab. I) die Konformation mit äquatorialer Acetamino-Gruppe (**39**) eindeutig bevorzugt, d. h. letztere besitzt ein größeres „sterisches Geswicht" als eine Acetoxy-Gruppe. In Methylsulfoxid dagegen ist die sterische Präferenz der Acetamino-Gruppe nicht vorhanden; die Acetyl-Resonanzen (Tab. IX) indizieren vollständige Aquilibrierung der beiden Alternativ-Konformationen.

Hinsichtlich der Anwendbarkeit der Me_2SO-Absorptionsbereiche (Abb. 2) auf Cyclit-polyacetate mit anderen Substituenten als Acetoxy- und Acetamino–Gruppen ergibt sich aus den bisher vorliegenden Daten ein zu $CDCl_3$ analoger Gültigkeitsbereich. Substitution einer Acetoxy-Gruppe durch OMe, NO_2, NHCHO oder H bedingt nur geringfügige, im Rahmen der Bereiche von Abb. 2 bleibende

Abweichungen. Aromatische Gruppen dagegen zeigen die erwartete diamagnetische Verschiebung benachbarter Acetoxy-Gruppen, wie z. B. aus den für Tetra-*O*-acetyl-di-*N*-benzyloxycarbonyl-streptamin (**64**) erhaltenen Acetoxy-Resonanzen [τ 8.12, 8.17 (2), 8.23] hervorgeht.

Auch für Cyclanol- und Acetaminocyclanol-acetate werden zu $CDCl_3$ analoge Verhältnisse erhalten, wie sich aus den in Tab. X zusammengestellten Daten ergibt: bei all-äquatorialer Orientierung der Gruppen liegen die Acetyl-Resonanzen innerhalb der normalen Bereiche (Abb. 2), während bei Vorliegen isolierter axialer Acetoxy-Gruppen diese überschritten werden.

TABELLE X

LAGE DER ACETYL-RESONANZEN VON CYCLANOL- UND AMINOCYCLANOL-ACETATEN

Verbindung	*OAc*		*NHAc*
	axial	*äquat.*	*äquat.*
O-Acetyl-cyclohexanol		8.04	
Acetamino-cyclohexan			8.24
Di-*O*-acetyl-*trans*-cyclohexandiol-(1,2)		8.05 (2)	
O-Acetyl-*trans*-2-acetamino-cyclohexanol-(1)		8.07	8.24
Di-*O*-acetyl-2*t*-acetamino-cyclohexandiol-(1*r*,3*c*)		8.07 (2)	8.26
Di-*O*-acetyl-3*c*-acetamino-cyclohexandiol-(1*r*,2*t*)		8.06 8.09	8.27
1*r*,2*t*,3*t*-Triacetamino-cyclohexan			8.26 (3)
Di-*O*-acetyl-2*c*-acetamino-cyclohexandiol-(1*r*,3*t*) (73)	7.96	8.05	8.25
4*c*-Acetamino-cyclohexantriol-(1*r*,2*c*,3*t*) (68)	7.94	8.10 (2)	8.27

IV. *Abschließende Betrachtungen*

Obwohl die Unterschiede in der chemischen Verschiebung zwischen axialer und äquatorialer *O*- bzw. *N*-Acetyl-Gruppen durch eine Vielzahl von Beispielen belegt sind, steht eine befriedigende theoretische Deutung dieses Effekts noch aus. Bezeichnend ist aber, daß in $CDCl_3$ die Resonanzen primärer[40] und acyclischer sekundärer[43] Acetoxy-Gruppen Signallagen aufweisen, die mit denen für äquatorial orientierte Ring-OAc (vgl. Abb. 1) übereinstimmen. Hieraus läßt sich folgern, daß die Abschirmung der Methylprotonen einer Acetoxy-Gruppe sich bei äquatorialer Anordnung an einem Sechsring nicht ändert, dagegen bei axialer einer paramagnetischen Abschirmung („deshielding") unterliegt, wie sie in ähnlicher Größe auch bei Enol- und Phenol-acetaten[44] beobachtet wird. Da diese paramagnetische Verschiebung umso kleiner ist, je „isolierter" die axiale Acetoxy-Gruppe steht (vgl. S. 13), dürfte dieser Effekt nicht ausschließlich auf die Anisotropie der C–C-Bindungen zurückzuführen sein, wie sie z. B. zur Deutung der umgekehrt liegenden Unterschiede äquatorialer und axialer Ring-CH herangezogen wurde[45]. Vielmehr

ist anzunehmen, daß die Konformation der Acetyl-Gruppe als solche — bei äquatorialer Orientierung ähnlich wie bei CH_2OAc-Gruppen konformativ ungehindert, bei axialer Anordnung durch 1,3-diaxiale Wechselwirkungen mit den Ring-CH sowie durch benachbarte äquatoriale Substituenten konformativ eingeschränkt — der wesentliche Faktor dieser paramagnetischen Verschiebung ist.

EXPERIMENTELLER TEIL

Synthesen und Eigenschaften der von uns vermessenen Verbindungen sind mit Ausnahme von **1**, **7**[46], **21**[47], **26**[48], **30**[49], **32**[50] und **68**[51] in *l.c.* 5, 6 und 10 beschrieben. Die Spektren wurden mit dem Varian A-60 Spektrometer mit Konzentrationen von 0.1–0.2M in Deuteriochloroform aufgenommen. Als innerer Standard diente Tetramethylsilan; die so ermittelten τ-Werte sind mit einem Fehler von höchstens ± 0.02 behaftet. Die Lage der Acetyl-Resonanzen von 0.1–0.2M–Lösungen sind praktisch unabhängig von der Konzentration; so zeigen z. B. die fünf Acetyl-Signale von *N*-Acetyl-*myo*-inosamin-(1)-pentaacetat (**10**, Tab. I) bei Konzentrationen zwischen 100 und 10 mg/ml (0.23–0.02M-Lösungen) nur Abweichungen von $< \pm 0.01$ p.p.m.

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LITERATUR

1 IV. Mitteil.: F. W. LICHTENTHALER UND H. K. YAHYA, *Ber.*, 101 (1968) 908
2 E. L. ELIEL, N. L. ALLINGER, S. J. ANGYAL, UND G. A. MORRISON, *Conformational Analysis*, Interscience Publishers, Inc., New York, 1965, S. 152 und 398 ff.; G. E. MCCASLAND, *Advan. Carbohyd. Chem.*, 20 (1965) 47.
3 G. E. MCCASLAND, S. FURUTA, L. F. JOHNSON, UND J. N. SHOOLERY, *J. Org. Chem.*, 29 (1964) 2354; G. E. MCCASLAND, M. O. NAUMANN, UND L. J. DURHAN, *ibid.*, 31 (1967) 3079.
4 R. U. LEMIEUX, R. K. KULLNIG, H. J. BERNSTEIN, UND W. G. SCHNEIDER, *J. Am. Chem. Soc.*, 80 (1958) 6098.
5 F. W. LICHTENTHALER UND H. O. L. FISCHER, *J. Am. Chem. Soc.*, 83 (1961) 2005.
6 F. W. LICHTENTHALER, *Ber.*, 94 (1961) 3071.
7 A. C. RICHARDSON UND K. A. MCLAUCHLAN, *J. Chem. Soc.*, (1962) 2499.
8 L. D. HALL, L. HOUGH, K. A. MCLAUCHLAN, UND K. G. R. PACHLER, *Chem. Ind.* (London), (1962) 1465.
9 J. C. SOWDEN, C. H. BOWERS, L. HOUGH, UND S. SHUTE, *Chem. Ind.* (London), (1962) 1827.
10 F. W. LICHTENTHALER, *Ber.*, 96 (1963) 2047.
11 F. A. L. ANET, R. A. B. BANNARD, UND L. D. HALL, *Can. J. Chem.*, 41 (1963) 2331.
12 Vgl., z. B.: D. HORTON, J. B. HUGHES, J. S. JEWELL, K. D. PHILIPS, UND W. M. TURNER, *J. Org. Chem.*, 32 (1967) 1073.
13 R. U. LEMIEUX UND J. D. STEVENS, *Can. J. Chem.*, 43 (1965) 2070; T. D. INCH, J. R. PLIMMER, UND H. G. FLETCHER, JR., *J. Org. Chem.*, 31 (1966) 1827.
14 M. NAKAJIMA, A. HASEGAWA, UND F. W. LICHTENTHALER, *Ann.*, 680 (1964) 21.

15 H. Z. SABLE, Western Reserve University, Cleveland, Ohio, persönliche Mitteilung.
16 T. SUAMI UND K. YABE, *Bull. Chem. Soc. Japan*, 38 (1965) 855.
17 M. NAKAJIMA, A. HASEGAWA, UND T. KUROKAWA, *Ann.*, 689 (1965) 235.
18 M. NAKAJIMA, N. KURIHARA, A. HASEGAWA, UND T. KUROKAWA, *Ann.*, 689 (1965) 243.
19 T. SUAMI UND S. OGAWA, *Bull. Chem. Soc. Japan*, 40 (1967) 1925
20 M. NAKAJIMA, A. HASEGAWA, UND T. KUROKAWA, *Ann.*, 689 (1965) 229.
21 M. NAKAJIMA, A. HASEGAWA, UND F. W. LICHTENTHALER, *Ann.*, 669 (1963) 75.
22 T. SUAMI UND K. YABE, *Bull. Chem. Soc. Japan*, 39 (1966) 1931.
23 T. SUAMI, F. W. LICHTENTHALER, UND S. OGAWA, *Bull. Chem. Soc. Japan*, 40 (1967) 1488.
24 S. BROWNSTEIN, *Can. J. Chem.*, 40 (1962) 870.
25 S. J. ANGYAL UND G. J. H. MELROSE, *J. Chem. Soc.*, (1965) 6494.
26 T. SUAMI, F. W. LICHTENTHALER, UND S. OGAWA, *Bull. Chem. Soc. Japan*, 39 (1966) 170.
27 H. SUHR, *Anwendungen der Kernmagnetischen Resonanz in der Organischen Chemie*, Springer Verlag, Berlin, 1965, S. 33 ff.
28 N. KURIHARA, H. SHIBATA, H. SAKEI, UND M. NAKAJIMA, *Ann.*, 701 (1967) 225.
29 T. SUAMI UND S. OGAWA, *Bull. Chem. Soc. Japan*, 38 (1965) 758.
30 F. W. LICHTENTHALER, T. NAKAGAWA, UND A. EL-SCHERBINEY, *Angew. Chem.*, 79 (1967) 530; *Angew. Chem. Intern. Ed. Engl.*, 6 (1967) 568; *Chem. Ber.*, 101 (1968) im Druck.
31 F. W. LICHTENTHALER, *Ber.*, 96 (1963) 845.
32 F. W. LICHTENTHALER, H. LEINERT, UND U. SCHEIDEGGER, *Ber.*, 101 (1968) im Druck.
33 K. TORI UND T. KOMENO, *Tetrahedron*, 21 (1965) 309.
34 T. SUAMI UND S. OGAWA, *Bull. Chem. Soc. Japan*, 37 (1964) 733.
35 T. SUAMI UND S. OGAWA, *Bull. Chem. Soc. Japan*, 37 (1964) 194.
36 F. W. LICHTENTHALER UND H. LEINERT, *Ber.*, 99 (1966) 903.
37 F. W. LICHTENTHALER UND H. P. ALBRECHT, *Ber.*, 99 (1966) 575.
38 T. SUAMI, S. OGAWA, Y. NAKASHIMA, UND H. SANO, *Bull. Chem. Soc. Japan*, 40 (1967) 2958.
39 F. W. LICHTENTHALER, H. LEINERT, UND T. SUAMI, *Ber.*, 100 (1967) 2383.
40 Die Acetoxy-Resonanzen der Verbb.[41] Nr. 79, 140, 167 und 261 sowie[42] Nr. 217, 281 und 682 liegen im Bereich von τ 7.93–8.01.
41 NMR-Spectra Catalog, Varian Associates, Palo Alto, California, 1962/63.
42 Sadtler Standard NMR-Spectra, Vol. 1 und 2, Sadtler Research Laboratories Inc., Philadelphia, Pa., 1965.
43 Isopropylacetat und 3-Acetoxybuttersäure-methylester zeigen Acetoxy-Resonanzen bei τ 7.97.
44 Vinyl- und 2-Propenyl-acetat zeigen Acetoxy-Signale bei τ 7.88, die Phenolacetate[42] Nr. 130, 252, 699 und 1122 zwischen τ 7.70 und 7.82.
45 L. M. JACKMAN, *Applications of Nuclear Magnetic Resonance Spectroscopy*, Pergamon Press, London, 1959, S. 117.
46 *Beilsteins Handbuch der Organischen Chemie*, Bd. VI (2. Erg.-Werk), S. 1157.
47 M. NAKAJIMA, N. KURIHARA, UND A. HASEGAWA, *Ber.*, 95 (1962) 141.
48 E. L. MAY UND E. MOSETTIG, *J. Org. Chem.*, 14 (1949) 1137.
49 S. J. ANGYAL UND N. K. MATHESON, *J. Am. Chem. Soc.*, 77 (1955) 4343.
50 G. R. ALLEN, JR., *J. Am. Chem. Soc.*, 79 (1957) 1167.
51 M. NAKAJIMA UND N. KURIHARA, unpubliziert.
52 S.J. ANGYAL P.F. GILHAM UND G.F.H. MELROSE, *J.Chem. Soc.*, (1965) 5252.

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NITROGEN-CONTAINING CARBOHYDRATE DERIVATIVES

PART V*. SYNTHESIS AND CONFIGURATIONAL DETERMINATION OF CARBOHYDRATE *C*-NITRO ALCOHOLS BY OPTICAL ROTATORY DISPERSION AND CIRCULAR DICHROISM

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ABSTRACT

An epimeric pair 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, was synthesized from D-mannose. The absolute configuration at C-2 was based on the rules relating the signs of the Cotton effect of the o.r.d. curves and the c.d. maxima to the configuration at C-2 of the carbohydrate *C*-nitro alcohols. This assignment was confirmed by chemical degradation of 2-acetamido-1,2-dideoxy-1-nitro-D-*glycero*-D-*talo*-heptitol to D-serine. Another epimeric pair, 2-acetamido-1,2-dideoxy-1-nitro-D-*glycero*-L-*manno*-heptitol and 2-acetamido-1,2-dideoxy-1-nitro-D-*glycero*-L-*gluco*-heptitol, was synthesized, and the configurations at C-2, determined by optical methods, agreed with those established by chemical methods.

INTRODUCTION

In a previous publication[1] from this laboratory, we described an improved method for the synthesis of 2-acetamido-2-deoxy-D-mannose from D-arabinose by way of the nitromethane synthesis. The original method, which was reported by Sowden[2] and O'Neill[3], consists of (*i*) preparation of 3,4,5,6-tetra-*O*-acetyl-1-nitro-D-*arabino*-1-hexene by heating 2,3,4,5,6-penta-*O*-acetyl-1-deoxy-1-nitro-D-mannitol in benzene solution in the presence of sodium hydrogen carbonate, (*ii*) treatment of 3,4,5,6-tetra-*O*-acetyl-1-nitro-D-*arabino*-1-hexene with methanolic ammonia to produce 2-acetamido-1,2-dideoxy-1-nitro-D-hexitols, and (*iii*) conversion of the nitromethyl group into an aldehydic group by application of the Nef reaction. In the improved method, 2,3,4,5,6-penta-*O*-acetyl-1-deoxy-1-nitro-D-mannitol was directly transformed, by the action of ammonia, to an epimeric mixture of 2-acetamido-1,2-dideoxy-1-nitro-D-mannitol (70% yield) and 2-acetamido-1,2-dideoxy-1-nitro-D-glucitol (8% yield) without isolating the intermediate, 3,4,5,6-tetra-*O*-acetyl-1-nitro-D-*arabino*-1-hexene. The same reaction has now been applied to the synthesis of 2-acetamido-1,2-dideoxy-1-nitro-D-*manno*-heptitol and 2-acetamido-1,2-dideoxy-1-

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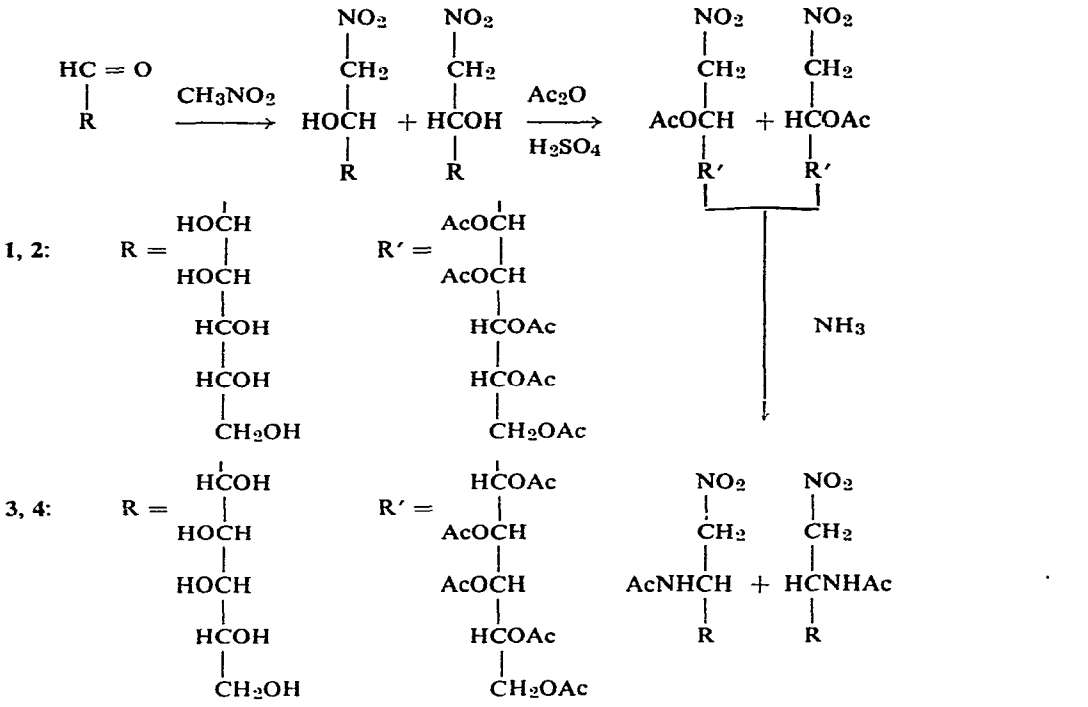
nitro-D-*galacto*-heptitol. The sequence for the synthesis of 2-acetamido-1,2-dideoxy-1-nitro-D-heptitols is illustrated in the following scheme.



The absolute configuration at C-2 of the 2-acetamido-1,2-dideoxy-1-nitro-D-heptitols (**1, 2**) was first predicted from the o.r.d.[4] and the c.d. curves[5] and then confirmed chemically (a preliminary communication[6] has already been published). This paper reports further studies and detailed procedures for the synthesis and the configurational determination of 2-acetamido-1,2-dideoxy-1-nitro-D-heptitols.

DISCUSSION

Almost pure 2,3,4,5,6,7-hexa-*O*-acetyl-1-deoxy-1-nitro-D-*glycero*-D-*galacto*-heptitol was synthesized from D-mannose according to Sowden and Schaffer[7]. The acetylated deoxynitroheptitol was treated with anhydrous ammonia in methanol. Upon concentration the reaction mixture gave a crystalline product **1**. After extraction of acetamide with chloroform, a crystalline product **2**, the epimer of **1**, was obtained from the mother liquor.

The absolute configuration at C-2 of the epimeric pair of these compounds, **1** and **2**, was determined by examination of the o.r.d. and c.d. curves. Compound **1** exhibited a negative Cotton effect and negative c.d. maxima at 276 nm and at about 308 nm, and compound **2** a positive Cotton effect and positive c.d. maxima at 266 nm and 313 nm.

TABLE I

COTTON EFFECT AND THE CIRCULAR DICHROISM OF CARBOHYDRATE *C*-NITRO ALCOHOLS IN WATER

Compound	*Configuration of the 2-acetamido group in the conventional Fischer projection formula*	*First extremum of the Cotton effect*		*Maxima of circular dichroism*	
2-Acetamido-1,2-dideoxy-1-nitro-D-*glycero*-D-*talo*, heptitol (**1**)	left	$[\varphi]^{a}_{346-343}$	−236°	$[\theta]^{b}_{309-336}$ $[\theta]_{276}$	−1185 −1304
2-Acetamido-1,2-dideoxy-1-nitro-D-*glycero*-D-*galacto*-heptitol (**2**)	right	$[\varphi]_{347}$	−171°	$[\theta]_{313}$ $[\theta]_{266}$	1130 801
2-Acetamido-1,2-dideoxy-1-nitro-D-*glycero*-L-*manno*-heptitol (**3**)	right	$[\varphi]_{332-328}$	607°	$[\theta]^{c}_{310}$ $[\theta]_{275}$	942 2067
2-Acetamido-1,2-dideoxy-1-nitro-D-*glycero*-L-*gluco*-heptitol (**4**)	left	$[\varphi]_{344}$	−80°	$[\theta]_{316-310}$ $[\theta]_{270}$	−409 −394

[a]$[\varphi]$, molecular rotation. [b]$[\theta]$, molecular ellipticity. [c]Molecular ellipticity at 310 nm (shoulder)

The structure, 2-acetamido-1,2-dideoxy-1-nitro-D-*glycero*-D-*talo*-heptitol (the acetamido group on C-2 is on the left in the conventional Fischer projection formula), was assigned to compound **1** and 2-acetamido-1,2-dideoxy-1-nitro-D-*glycero*-D-*galacto*-heptitol (the acetamido group is on the right) to compound **2**, on the basis of the rules relating the signs of the c.d. maxima and the Cotton effect of the o.r.d. curves to the configuration at C-2 of the carbohydrate *C*-nitro alcohols [4–6].

(*a*) Carbohydrate *C*-nitro alcohols that have a hydroxyl group on the right side of the C-2 adjacent to the nitromethyl group in the conventional Fischer projection formula, exhibit positive c.d. maxima (or shoulders) at about 280 nm and 310 nm, and positive Cotton effects associated with the 310 nm-band, whereas those having the hydroxyl group on the left side of the C-2 show negative c.d. maxima (or shoulders) and negative Cotton effects.

(*b*) Neither the sign of the Cotton effect nor the ellipticity is affected by the replacement of the C-2 hydroxyl group by an acetamido group.

(*c*) The absolute configurations of other asymmetric centers do not contribute significantly to either the sign of the Cotton effect or the ellipticity.

The configuration at C-2 of **1** was determined as previously described[6], D-serine being the final product of the degradation of **1**.

Compound **1** was converted into syrupy 2-acetamido-2-deoxy-D-heptose by decomposition of the nitro group by the method of Nef[1–3], and then reduced to 2-acetamido-2-deoxy-D-heptitol with sodium borohydride in aqueous solution. After decomposition of excess sodium borohydride and deionization with cation-

exchange resins, the borate ion was removed by many additions of methanol followed by evaporation. Crystalline 2-acetamido-2-deoxy-D-heptitol was obtained from **1** in a yield of about 68%. One mole of the heptitol was oxidized with four moles of sodium periodate in water, and the product was directly oxidized with bromine. Hydrolysis of the acetamido linkage with sulfuric acid and purification on a column of Dowex-50 (H^+) gave a crystalline product, which was recrystallized from ethanol–water; colorless needles, dec. 225°, $[\alpha]_D^{23}$ −15.6° (*c* 5.2, M HCl). The elemental analysis, melting point, optical rotation, paper-chromatographic properties [R_F 0.28, (4:1:2) butyl alcohol–acetic acid–water], and the infrared spectrum of this product were in accord with those of D-serine[8]. The overall yield of D-serine from 2-acetamido-2-deoxy-D-heptitol was 55%. The isolation of D-serine proves that compound **1** is 2-acetamido-1,2-dideoxy-1-nitro-D-*glycero*-D-*talo*-heptitol (the acetamido group is on the left in the Fischer projection) and, accordingly, compound **2** is 2-acetamido-1,2-dideoxy-1-nitro-D-*glycero*-D-*galacto*-heptitol (the acetamido group is on the right), in agreement with the aforementioned rules.

As a model experiment, 2-acetamido-1,2-dideoxy-1-nitro-D-mannitol, which showed negative c.d. maxima and a negative Cotton effect, was subjected to the same sequential reactions. The absolute configuration of 2-acetamido-1,2-dideoxy-1-nitro-D-mannitol at C-2 (the acetamido group is on the left) was established by the isolation of 2-acetamido-2-deoxy-D-mannose when the 2-acetamido-1,2-dideoxy-1-nitro-D-mannitol was subjected to the Nef reaction[1-3]. The failure to isolate L-serine, after the reaction sequence, coupled with the isolation of D-serine suggests that inversion did not take place during the sequence of transformation. Similarly, another epimeric pair of 2-acetamido-1,2-dideoxy-1-nitro-D-heptitols, **3** and **4**, was synthesized from D-galactose, and the absolute configuration at C-2 was determined by both optical and chemical methods. The structure 2-acetamido-1,2-dideoxy-1-nitro-D-*glycero*-L-*manno*-heptitol (the acetamido group is on the right) was predicted for the main product, **3**, which was obtained by treatment of the acetylated nitro alcohol[9] with ammonia, since it exhibited positive c.d. maxima and a positive Cotton effect. The final product resulting from the degradation of **3** was shown to be L-serine, thus confirming structure **3**, and, accordingly, **4** is 2-acetamido-1,2-dideoxy-1-nitro-D-*glycero*-L-*gluco*-heptitol. The o.r.d. and c.d. data are shown in Table I. Thus, the validity of the rules was further demonstrated.

The production of 2-acetamido-1,2-dideoxy-1-nitro-D-heptitols from the nitro-alcohol acetates is stereoselective. The ratio of the yield of compound **1** to that of compound **2** was approximately 5:2, and the ratio of the yield of compound **3** to that of compound **4** was about 7:2. The same result was obtained whether the starting material was a mixture of acetylated epimeric nitro alcohols or one of the epimers. The yields of 2-acetamido-1,2-dideoxy-1-nitro-D-mannitol and 2-acetamido-1,2-dideoxy-1-nitro-D-glucitol from the acetylated nitro hexitol were 70% and 8%, respectively[1]. In the major product, the acetamido group at C-2 and the hydroxyl group at C-3 are in a *cis* relationship. Since 2-acetamido-1,2-dideoxy-1-nitro-D-mannitol and its epimer were also synthesized[2] by the addition of ammonia to 3,4,5,6-

tetra-*O*-acetyl-1-nitro-D-*arabino*-1-hexene, it is reasonable to assume that during the treatment of the acetylated nitro alcohols with ammonia, three steps of reaction, namely formation of an olefinic bond, addition of ammonia to the olefinic bond, and migration of the acetyl group, occurr. O'Neill[3] rationalized the stereoselective addition of ammonia to the olefinic bond on the basis of rotational conformation, and the same explanation can be applied to the stereoselective production of 2-acetamido-1,2-dideoxy-1-nitro-D-heptitols.

EXPERIMENTAL

General. — Melting points are not corrected. Ascending paper chromatography was performed on Toyo Roshi No. 53 filter paper in the following solvents: A, butyl alcohol–pyridine–water (6:4:3); B, butyl alcohol–acetic acid–water (4:1:2). O.r.d. and c.d. were measured on water solutions with a JASCO Model ORD/UV-5 automatically recording spectropolarimeter, Nippon Bunko.

2-Acetamido-1,2-dideoxy-1-nitro-D-glycero-D-talo-*heptitol* (**1**) *and 2-acetamido-1,2-dideoxy-1-nitro*-D-glycero-D-galacto-*heptitol* (**2**). — Anhydrous ammonia was passed into a cooled (0°) suspension of 2,3,4,5,6,7-hexa-*O*-acetyl-1-deoxy-1-nitro-D-*glycero*-D-*galacto*-heptitol[7] (10.8 g) in methanol (200 ml). The crystals dissolved gradually to give a yellow solution and, after 10 to 15 min, a vigorous uptake of ammonia began and continued for 2 h. After approximate saturation, the solution was kept with a drying tube for 16 h at room temperature, and then concentrated *in vacuo* to a crystalline mass. Filtration and washing with ethanol gave crude **1** (2.8 g, 46%). Two recrystallizations from methanol gave a product having m.p. 180–181° (dec.), $[\alpha]_D^{23}$ +21.4° (*c* 5.3, water).

Anal. Calc. for $C_9H_{18}N_2O_8$: C, 38.29; H, 6.38; N, 9.92. Found: C, 38.87; H, 6.13; N, 10.02.

The mother liquor and washings were concentrated to a syrup, which was then extracted three times with chloroform to remove acetamide. After evaporation of the chloroform, crystals appeared in the residue. Filtration and washing with ethanol gave **2** (1.1 g, 18%). After being recrystallized three times from ethanol, the product had m.p. 159.5–160.5° (dec.), $[\alpha]_D^{23}$ −26.4° (*c* 5.0, water).

Anal. Calc. for $C_{19}H_{18}N_2O_8$: C, 38.29; H, 6.38; N, 9.92. Found: C, 38.62; H, 6.17; N, 9.94.

Determination of the absolute configuration at C-2 of (**1**). *(i) 2-Acetamido-2-deoxy*-D-glycero-D-talo-*heptose* (**5**). — In a barium hydroxide solution (16 ml) prepared from crystalline barium hydroxide octahydrate (80 g) and water (1000 ml) was dissolved **1** (1 g). This solution was added dropwise into cold, conc. sulfuric acid (1.2 g) with vigorous stirring, and the mixture was kept overnight in the cold. After neutralization with the barium hydroxide solution, the precipitated barium sulfate was centrifuged off, and the combined supernatant and washings were concentrated to a pale-yellow syrup. Paper chromatography of this syrup showed a spot of 2-acetamido-2-deoxy-D-*glycero*-D-*talo*-heptose (**5**) (R_F 0.53, solvent A) and a trace of **1** (R_F 0.82, solvent A).

(*ii*) *2-Acetamido-2-deoxy*-D-glycero-D-talo-*heptitol* (**6**). — To a cooled solution of syrupy **5** in water (20 ml) was added a solution of sodium borohydride (200 mg) in cold water (20 ml) with stirring. After 2 h. of stirring, Dowex-50 (H^+) was added. The mixture was stirred for 1 h, and then the resin was filtered off. The filtrate was concentrated to a syrup, and the boric acid was removed by repeated addition of methanol to the syrup and subsequent evaporation *in vacuo*. On being kept in the refrigerator, the syrup crystallized. The crystals were filtered off and washed with ethanol (611 mg, yield from **1** 68%). Three recrystallizations from ethanol gave a product having m.p. 118–119° (dec.), $[\alpha]_D^{25}$ +41.3° (*c* 4.1, water).

Anal. Calc. for $C_9H_{19}NO_7$: C, 42.68; H, 7.56; N, 5.53. Found: C, 41.55; H, 7.67; N, 5.47.

(*iii*) *Isolation of* D-*serine* (**7**). — A cold 0.3M sodium periodate solution (30 ml, 9 mmoles of sodium periodate) was added to an ice-cooled solution of **6** (511 mg, 2.02 mmoles) in water (50 ml), under mechanical stirring in the dark. After 3 h, the optical density of the remaining sodium periodate was measured at 233 nm[10]. Instead of the 0.92 mmole expected to be unchanged, 1.8 mmoles of sodium periodate were found to be unchanged (0.22 mmole of **6** had not been oxidized). After an additional hour of stirring at 20°, the pH of the reaction mixture was adjusted to approximately 5 by addition of barium hydroxide solution, and the precipitate was discarded. Bromine (2.4 g) and barium carbonate (1.2 g) were added. to the combined filtrate and washings The mixture was shaken until the bromine dissolved, and the solution was kept 48 h in the dark at room temperature. The excess bromine was removed by aeration, and the solution was mixed with a slurry of silver carbonate. After 20 h of stirring, the insoluble salts were filtered off, and the bromine-free filtrate was treated with hydrogen sulfide. After separation of the precipitate, the decolorized solution was concentrated under reduced pressure to a thin syrup. This syrup was dissolved in M sulfuric acid (15 ml) and heated for 2 h on a boiling water bath. After cooling, the pH of the solution was adjusted to 4 with barium hydroxide solution, and the barium sulfate was centrifuged off. The supernatant was placed on a column of Dowex-50 (H^+) resin, and the column was washed with water, and then eluted with 5% ammonium hydroxide, at a flow rate of about 1 ml per minute. Concentration of the fractions, that contained ninhydrin-positive materials, gave a yellow crystalline product. After treatment of the solution in water with charcoal, the product was recrystallized by dissolution in a small volume of water and addition of ethanol. The resulting fine needles were filtered off and washed with ethanol; yield 118 mg (55%), m.p. 225° (dec.), $[\alpha]_D^{23}$ −15.6° (*c* 5.2, M hydrochloric acid). The infrared spectrum and paper-chromatographic properties (R_F 0.28, solvent B) of this product were identical with those of D-serine.

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.

2-Acetamido-1,2-dideoxy-1-nitro-D-glycero-L-manno-*heptitol* (**3**) *and 2-acetamido-1,2-dideoxy-1-nitro*-D-glycero-L-gluco-*heptitol* (**4**). — A cooled (0°) suspension of 2,3,4,5,6,7-hexa-*O*-acetyl-1-deoxy-1-nitro-D-*glycero*-L-*manno*-heptitol[9] (17.1 g) in

methanol (340 ml) was saturated with anhydrous ammonia. The crystals dissolved during this process. The reaction mixture, protected from moisture, was kept overnight at room temperature. The solution was concentrated to a crystalline mass. After filtration, crystalline **3** was washed with ethanol; yield 6.0 g (61%), m.p. 181.0–183.0°; three recrystallizations from methanol gave a product having m.p. 189.0–190.0° (dec.), $[\alpha]_D^{23}$ +12.9° (*c* 4.8, water).

Anal. Calc. for $C_9H_{18}N_2O_8$: C, 38.29; H, 6.38; N, 9.92. Found: C, 38.57; H, 6.33; N, 9.63.

The filtrate was decolorized with carbon and concentrated to give crystalline **4**; yield 1.7 g (17%). After two recrystallizations from ethanol, the product melted at 144.5–145.5° (dec.), $[\alpha]_D^{23}$ +10.7° (*c* 5.2, water).

Anal. Calc. for $C_9H_{18}N_2O_8$: C, 38.29; H, 6.38; N, 9.92. Found: C, 37.85; H, 6.16; N, 9.77.

Determination of the absolute configuration at C-2 of **3**. (*i*) *2-Acetamido-2-deoxy*-D-glycero-L-manno *heptitol* (**9**). — Compound **3** (1 g) was degraded to syrupy 2-acetamido-2-deoxy-D-*glycero*-L-*manno*-heptose (**8**) by the procedure described above for compound **1**. When the resulting syrup was reduced with sodium borohydride (200 mg) as described for 2-acetamido-2-deoxy-D-*glycero*-D-*talo*-heptose (**5**), 562 mg of crystalline 2-acetamido-2-deoxy-D-*glycero*-L-*manno*-heptitol (**9**) was obtained. The overall yield from **3** was 63%. After recrystallization from methanol, the product had m.p. 163.0–163.5° (dec.), $[\alpha]_D^{25}$ +4.6° (*c* 4.9, water).

Anal. Calc. for $C_9H_{19}NO_7$: C, 42.68; H, 7.56; N, 5.53. Found: C, 42.12; H, 7.25; N, 5.37.

(*ii*) *Isolation of* L-*serine* (**10**). — 2-Acetamido-2-deoxy-D-*glycero*-L-*manno*-heptitol (**9**) (520 mg, 2.06 mmoles) in water (50 ml) was oxidized with a 0.3M sodium periodate solution (30 ml, 9 mmoles) at 0° in the dark. After 3 h, the remaining sodium periodate was determined spectrophotometrically[10]. As the result suggested that about 10% of 2-acetamido-2-deoxy-D-*glycero*-L-*manno*-heptitol had not been oxidized, the periodate oxidation was continued for 2 h. The reaction mixture was then neutralized to pH 6.0 with barium hydroxide solution, and the precipitate was filtered off and discarded. Oxidation with bromine (2.4 g) in the presence of barium carbonate (1.2 g), hydrolysis of the acetamido linkage with M sulfuric acid, and purification on a column of Dowex-50 (H^+) resin were performed as described for the degradation of **1**. The final product was recrystallized twice from water–ethanol; yield 140 mg (overall yield from **9** was 60%), m.p. 227° (dec.), $[\alpha]_D^{23}$ +15.2° (*c* 5.2, M hydrochloric acid).

The infrared spectrum and paper-chromatographic properties (R_F 0.28, solvent B) of this product were indistinguishable from those of L-serine.

Anal. Calc. for $C_3H_7NO_3$: C, 34.28; H, 6.71; N, 13.33. Found: C, 34.53; H, 6.76; N, 13.27.

Degradation of 2-acetamido-1,2-dideoxy-1-nitro-D-*mannitol* (**11**) *to* D-*serine*. (*i*) *2-Acetamido-1,2-dideoxy-1-nitro*-D-*mannitol* (**11**). — This compound was synthesized from D-arabinose and converted into 2-acetamido-2-deoxy-D-mannose (**12**) according to the method previously reported[1].

(*ii*) *2-Acetamido-2-deoxy-*D*-mannitol* (**13**). — A solution of sodium borohydride (160 mg) in cold water (20 ml) was added to a cooled (0°) solution of 2-acetamido-2-deoxy-D-mannose (1 g, as monohydrate) in water (20 ml) under mechanical stirring. After stirring for 1 h at room temperature, Dowex-50 (H^+) resin was added and stirred for 1 h. Filtration and concentration gave a syrup, from which the boric acid was removed by evaporation with methanol, as described above. The crystals were filtered off and washed with ethanol; yield 818 mg (88%). Recrystallization from methanol–ether gave a product having m.p. 136–137° (dec.), $[\alpha]_D^{22}$ −8.6° (*c* 5.2, water).

Anal. Calc. for $C_8H_{17}NO_6$: C, 43.06; H, 7.68; N, 6.28. Found: C, 43.26; H, 7.45; N, 6.35.

(*iii*) *Isolation of* D*-serine.* — 2-Acetamido-2-deoxy-D-mannitol (**13**) (667 mg) was degraded to D-serine by the procedure described for **1**; the yield of D-serine was 200 mg (64%), m.p. 223° (dec.), $[\alpha]_D^{23}$ −14.9° (*c* 4.8, M hydrochloric acid). The infrared spectrum and the paper-chromatographic properties (R_F 0.28, solvent B) of this product were indistinguishable from those of D-serine.

Anal. Calc. for $C_3H_7NO_3$: C, 34.28; H, 6.17; N, 13.33. Found: C, 34.60; H, 6.33; N, 13.32.

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REFERENCES

1 C. SATOH, AND A. KIYOMOTO, *Chem. Pharm. Bull.* (Tokyo), 12 (1964) 615.
2 J. C. SOWDEN AND M. L. OFTEDAHL, *J. Am. Chem. Soc.*, 82 (1960) 2303.
3 A. N. O'NEILL, *Can. J. Chem.*, 37 (1959) 1747.
4 C. SATOH, A. KIYOMOTO, AND T. OKUDA, *Chem. Pharm. Bull.* (Tokyo), 12 (1964) 518.
5 C. SATOH, A. KIYOMOTO, AND T. OKUDA, *Carbohyd. Res.*, 5 (1967) 140.
6 C. SATOH AND A. KIYOMOTO, *Carbohyd. Res.*, 3 (1966) 248.
7 J. C. SOWDEN AND R. SCHAFFER, *J. Am. Chem. Soc.*, 73 (1951) 4662.
8 E. FISCHER AND W. A. JACOBS, *Ber.*, 39 (1906) 2945.
9 J. C. SOWDEN AND D. R. STROBACH, *J. Am. Chem. Soc.*, 82 (1960) 954.
10 G. O. ASPINALL AND R. J. FERRIER, *Chem. Ind.* (London), (1957) 1216.

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ÉTUDE CHIMIQUE DE LA PECTINE DE RACINES D'ŒILLET (*Dianthus caryophyllus* L.)

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ABSTRACT

Pectic substances having D-galacturonic acid, D-galactose, and L-arabinose as main components, in the weight ratios 55:24:16 were isolated from carnation roots. Alcoholic precipitation, DEAE-cellulose chromatography, and paper electrophoresis gave fractions having the highest content of neutral or acidic polysaccharides, but not in a pure state. It was not possible to isolate a galacturonan devoid of neutral sugars. Partial hydrolysis and methylation of the acid polysaccharide showed a backbone of α-(1→4)-linked D-galacturonic acids. Isolation of *O*-(D-galactosyluronic acid)-(1→2)-rhamnose, *O*-(D-galactosyluronic acid)-D-galactose, and *O*-(D-galactosyluronic acid)-L-arabinose proves that neutral sugars are combined in the socalled "pectic acid" molecule through glycosidic linkages. So far, the aldobiouronic acid, *O*-(D-galactosyluronic acid)-L-arabinose seem not to have been found in pectic substance hydrolyzates.

RÉSUMÉ

Les substances pectiques isolées des racines d'œillet sont constituées principalement d'acide D-galacturonique, D-galactose et L-arabinose dans le rapport 55:24:16, en poids. Le fractionnement par précipitation alcoolique, chromatographie sur DEAE-cellulose et électrophorèse a conduit à des fractions enrichies en polysaccharide neutre ou en polysaccharide acide, mais il ne nous fut pas possible d'isoler une galacturonane dépourvue d'oses neutres. L'étude de la fraction acide par hydrolyse partielle et méthylation montre que le squelette de la chaîne est constitué par des restes d'acide α-D-galactosiduronique reliés en (1→4). L'obtention d'acides *O*-galactosiduronique-(1→2)-rhamnose, galactosiduronique-galactose et galactosiduronique arabinose indique que des oses neutres sont inclus au sein de la molécule d' «acide pectique» par des liaisons osidiques. L'acide galactosiduronique-arabinose est un acide aldobiouronique qui ne semble pas avoir été signalé jusqu'à maintenant dans les hydrolysats de substances pectiques.

INTRODUCTION

Les substances pectiques, polysaccharides complexes contenant une forte proportion de résidus d'acide D-galactosiduronique, sont présentes chez tous les

végétaux. Cependant, leur proportion est variable suivant les tissus considérés. Elles sont localisées dans les parois cellulaires de toutes les plantes supérieures.

Les travaux de Hirst et Jones[1] sur la structure des pectines suggéraient la présence de trois polysaccharides homogènes, une galactane, une arabane et une galacturonane, reliés simplement entre eux par des forces secondaires. Cependant, les essais de purification de ces trois polysaccharides furent le plus souvent infructueux et l'étude de l'hydrolysat partiel de pectines de diverses origines: luzerne[2], sisal[3], pomme[4], écorce de sapin[5] et bois de pin maritime[6] a montré que la galacturonane pouvait contenir dans sa molécule des oses neutres tels que le rhamnose et le xylose. Si bien que la structure des pectines reste encore discutée.

Au cours d'un travail sur les oligosaccharides des racines d'œillet (*Dianthus caryophyllus* L.) effectué dans ce laboratoire[7], on avait constaté la présence, chez cette espèce, de quantités appréciables de substances pectiques. Les racines de Caryophyllacées ont une composition caractéristique en oligosaccharides qui n'ont été trouvés que dans cette famille, ce qui reflète un métabolisme glucidique particulier, et peut faire considérer les Caryophyllacées comme le terme ultime d'une série évolutive[8]. Il était alors intéressant d'étudier les substances pectiques de l'œillet afin de préciser, si elles aussi, possédaient une structure particulière.

RÉSULTATS ET DISCUSSION

L'étude critique de différentes méthodes d'extraction de la pectine des racines d'œillet (extraction des racines fraîches par l'eau à 100° après stabilisation alcoolique, extraction sans stabilisation alcoolique, extraction des racines sèches, extraction en milieu acide dilué) a montré qu'elles n'entraînaient pas de différences notables dans la composition du produit obtenu.

La pectine étudiée a été extraite à partir des racines fraîches, par l'eau à 100°. La solubilisation des acides organiques par l'eau chaude a entraîné une acidification du milieu (pH 3,5) suffisante pour solubiliser la pectine. Telle quelle la pectine obtenue donne encore un résidu de calcination simple de 8%. Nous avons constaté la présence dans la solution de pectine d'ions potassium correspondant à la salification d'une partie des carboxyles. Pour déterminer le pourcentage d'estérification, il était nécessaire d'opérer sur un polysaccharide dont les carboxyles avaient été libérés. Nous avons traité la pectine par une solution de gaz chlorhydrique dans le 2-propanol dans les conditions décrites par Bender[9]. Après ce traitement, le résidu de calcination est tombé à 4%.

Cette pectine est assez peu méthylée (5,65%) comparativement à celles de pomme ou de citron dans lesquelles la teneur en groupes «méthoxy » est d'environ 7%. La teneur en acide D-galacturonique est plus faible que celle trouvée pour les autres pectines généralement étudiées. Ceci se traduit d'ailleurs par une viscosité plus faible des solutions de pectine d'œillet. La viscosité relative par rapport à l'eau des solutions à 1% est de 1,35.

Pour confirmer l'influence de la teneur en acide D-galacturonique sur la viscosité

TABLEAU I

COMPARAISON ENTRE LA PECTINE D'ŒILLET ET LES PRINCIPALES PECTINES ÉTUDIÉES ANTÉRIEUREMENT

Origine de la pectine	*Acide* D-*galacturonique* %	OCH_3 %	CH_3CO %
Œillet	50	5,65	0,83
Pomme	70–80	6–9	0
Citron	62–85	7–10	0
Betterave	60–65	5–7	5–10

des pectines, nous avons étudié les variations de la viscosité[10], en fonction de la température, de solutions tamponnées à pH 6,8. Les mêmes mesures ont été effectuées avec les pectines de pomme et de citron comme termes de comparaison (Fig. 1).

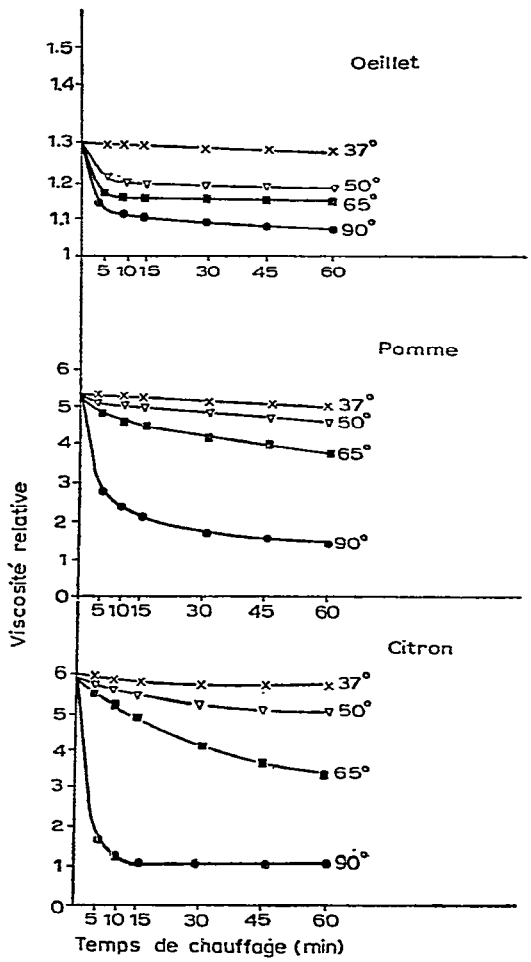

Fig. 1. Variations de la viscosité relative des pectines d'œillet, pomme et citron, à pH 6,8, en fonction de la température(+37°, +50°, +65° et +90°).

La chute de viscosité est approximativement proportionnelle au pourcentage d'estérification des pectines. Cependant, il semble qu'un autre facteur intervienne. Bien que la pectine d'œillet ait un pourcentage d'estérification (58%) supérieur à celui de la pectine de pomme utilisée (35,7%), la diminution de sa viscosité est proportionnellement moins importante. Ceci est probablement dû au fait que la pectine de pomme est beaucoup plus riche en acide D-galacturonique que celle d'œillet. Leur taux respectif en acide D-galacturonique est de 74% et 55%.

Les essais de précipitation fractionnée de la pectine d'œillet par l'éthanol conduisent à des fractions de composition différente, enrichies soit en oses neutres (fractions précipitant avec 70–80 et 80–90% d'alcool), soit en acide D-galacturonique (fractions précipitant avec 50 et 50–70% d'alcool). Mais il ne nous a pas été possible d'isoler une fraction neutre pure, ni un polysaccharide composé uniquement d'acide D-galacturonique. Par ailleurs, les essais de fractionnement par le bromure de cétyltriméthylammonium(Cetavlon) se sont montrés infructueux pour isoler le polysaccharide acide.

TABLEAU II

FRACTIONNEMENT DE LA PECTINE PAR L'ALCOOL ÉTHYLIQUE

Résidus	*Pectine native*	*Fractions précipitant pour des concentrations alcooliques de*			
		0–50%	50–70%	70–80%	80–90%
Acide D-galacturonique %	55	60	55	35	25
D-Galactose %	24	20	25	37	41
L-Arabinose %	16	15	17	25	30

Le fractionnement sur cellulose DEAE selon le procédé de Neukom[11] confirme l'hétérogénéité de la pectine. Si la majeure partie du complexe arabinogalactane est éluée dans les premières fractions avec un tampon phosphate 0,005M, une partie des oses neutres reste fixée sur la colonne et n'est éluée progressivement que par l'hydroxyde de sodium en même temps que la fraction acide, elle-même hétérogène.

Par électrophorèse sur papier, il nous a été possible de séparer la pectine d'œillet en deux composants: a) une arabinogalactane, neutre, migrant vers la cathode et ne contenant plus d'acide D-galacturonique; b) un polysaccharide acide, migrant vers l'anode, constitué pour sa plus grande part d'acide galacturonique (92,5%), mais contenant encore quelques traces d'oses neutres (arabinose, galactose, xylose et rhamnose). Nous n'avons pu malheureusement isoler le complexe polysaccharidique neutre en quantité suffisante pour étudier sa structure.

L'impossibilité, au cours de ces divers essais, d'obtenir un polysaccharide acide complètement dépourvu d'oses neutres tend à prouver que des résidus de sucres neutres sont combinés par des liaisons glycosidiques à des molécules d'acide D-galacturonique de la polygalacturonane. C'est ce qu'a confirmé l'analyse des produits

de l'hydrolyse partielle de la pectine: à côté d'oses neutres et des acides oligogalacturoniques, nous avons pu isoler plusieurs acides aldobiouroniques. La chromatographie d'un hydrolysat partiel sur Amberlite CG 400 a permis en effet de distinguer cinq fractions.

Dans la première fraction, éluée par l'eau, nous avons identifié les oses neutres suivants,: D-galactose, L-arabinose, xylose, rhamnose, fucose, 2-*O*-méthylxylose 2-*O*-méthylfucose et 2-*O*-méthylrhamnose. Les quatre premiers sont retrouvés de façon constante dans toutes les pectines. Par contre, les sucres *O*-méthylés, en particulier le 2-*O*-méthylfucose et le 2-*O*-méthylxylose, ont été plus rarement signalés[2]. Il est possible qu'ils proviennent d'une contamination par des polysaccharides tels que les xylanes, présents en petites quantités.

L'emploi d'un gradient exponentiel d'acide formique a conduit ensuite à l'élution des fractions II à V, renfermant respectivement l'acide D-galacturonique et ses oligomères (acides di-, tri- et tétragalacturoniques). Ceux-ci furent isolés par précipitation de leurs sels de calcium, et une purification finale fut réalisée, si nécessaire, par chromatographie préparative sur papier.

La méthylation des acides oligogalacturoniques, suivie de la réduction des groupes carboxyles par le borohydrure de potassium et de l'hydrolyse des produits méthylés, conduit au 2,3-di-*O*-méthylgalactose. Ceci démontre que les résidus galacturoniques de l'acide pectique d'œillet étaient reliés par des liaisons glycosidiques (1→4), comme dans les autres pectines. De plus, le pouvoir rotatoire dextrogyre élevé du di- et du trigalacturonate de calcium conduit à affecter à ces liaisons la configuration α-D.

A côté des oligosaccharides composés d'acide D-galacturonique, nous avons isolé par chromatographie sur papier six acides aldobiouroniques dont la composition fut déterminée par hydrolyse à l'acide nitrique. Cinq de ces acides accompagnent l'acide D-galacturonique dans la fraction II: *O*-D-galactosyluronique (1→2)-rhamnose, (D-galactosyluronique)-fucose, (D-galactosyluronique)-2-*O*-méthylxylose, (D-galactosyluronique)-2-*O*-méthylfucose et (D-galactosyluronique)-arabinose. Ce dernier possède vraisemblablement[12] une liaison osidique (1→4). Le sixième acide aldobiouronique est un galactosyluronique-galactose, isolé de la fraction III où il accompagne l'acide digalacturonique.

L'acide *O*-(D-galactosyluronique)-(1→2)-rhamnose semble être un des constituants caractéristiques des hydrolysats pectiques[1,2,4,6], et on a envisagé que certains résidus de rhamnose pourraient servir de points de liaison entre deux chaînes polygalacturoniques[2]. L'acide galactosyluronique-galactose a déjà été isolé à partir des pectines de luzerne[2] et de pomme[4]. Par contre, à notre connaissance, l'acide galactosyluronique-arabinose n'a pas encore été signalé comme constituant des acides pectiques, ce qui montre que, tout comme le galactose, l'arabinose n'est pas seulement présent dans le polysaccharide neutre.

L'ensemble de ces résultats nous permet de penser que la pectine d'œillet présente une structure comparable à celle des autres pectines. Le métabolisme particulier des oligosaccharides chez les Caryophyllacées ne semble donc pas influencer

la composition des pectines, polysaccharides des parois cellulaires et des espaces intercellulaires.

PARTIE EXPÉRIMENTALE

Techniques générales. — La chromatographie sur papier est effectuée sur papier Whatman n° 1 et 3MM dans les solvants suivants: a) alcool butylique–pyridine–eau (9:5:4, volumes) pour les oses neutres; b) acétate d'éthyle–acide acétique–acide formique–eau (18:3:1:4, volumes) pour les acides uroniques. Le révélateur utilisé est celui à la β-naphtylamine et au thymol[13]. Les migrations chromatographiques des oses neutres sont indiquées par rapport à celle du galactose (R_{Gal}), et celles des dérivés uroniques par rapport à celle de l'acide D-galacturonique (R_{GalA}).

L'analyse qualitative de la pectine totale et de ses produits de fractionnement a été effectuée après hydrolyse en tubes scellés pendant 5 h à 100° dans H_2SO_4 0,5M (10 mg de produit par 1 ml). L'hydrolysat fut neutralisé par le carbonate de baryum, puis désionisé avec de l'Amberlite IR-120 (H^+).

Extraction de la pectine d'œillet. — Les racines nettoyées le plus rapidement possible après leur récolte sont concassées, puis traitées deux fois pendant 5 h par cinq parties d'eau au bain-marie bouillant. Après filtration suivie de centrifugation, le centrifugat limpide est concentré au 1/5 de son volume par évaporation sous pression réduite (température du bain extérieur: 50° maximum). L'extrait aqueux est additionné de trois fois son volume d'alcool éthylique à 95% et fournit un précipité gélatineux. Celui-ci est dissous par l'eau et à nouveau précipité par l'alcool, et ceci deux fois. Le produit obtenu est solubilisé dans l'eau et la solution est dialysée pendant 24 h contre de l'eau distillée afin d'éliminer sels minéraux et sucres. La fraction non-dialysable est alors précipitée par trois volumes d'alcool à 95%.

La purification de l'extrait est suivie par mesure, après chaque précipitation, de la viscosité des solutions. Pour limiter au maximum la dégradation des extraits, les filtrations, précipitations alcooliques, dialyses et centrifugations sont effectuées au voisinage de 0°.

Détermination des caractères physico-chimiques de la pectine. — La viscosité des solutions à 1% de pectine est mesurée à l'aide du viscosimètre de Baume, en enceinte thermorégulée à 25° (Fig. 1). Le pouvoir rotatoire est $[\alpha]_D^{20} + 117°$ (*c* 0,8, eau).

Le pourcentage d'estérification a été déterminé après saponification de la pectine (0,5 g) par l'hydroxyde de sodium 0,5M (20 ml) pendant 30 min à 25°. Les carboxyles libérés par saponification sont dosés en retour par l'hydroxyde de sodium 0,1M.

Pour la détermination de la teneur en groupes « acétyles », la pectine (0,5 g) est laissée en contact avec l'hydroxyde de sodium 0,125M (25 ml) pendant 1 h à 15°. L'acide acétique libéré est déplacé par l'acide sulfurique et entraîné par distillation. Il est dosé par l'hydroxyde de sodium 0,05M.

Dosage des constituants de la pectine. — Le dosage du galactose, de l'arabinose et de l'acide galacturonique a été effectué après hydrolyse chlorhydrique (HCl M,

2 h, 100°). L'hydrolysat est désionisé par passage sur Duolite A 102-d. Dans l'éluat aqueux, nous avons dosé le galactose (24%) par la technique à la cystéine–acide sulfurique de Dische[14] et l'arabinose (16%) par la technique à l'orcinol–acide chlorhydrique de Bial[15]. L'acide galacturonique (55%), fixé par la résine, a été élué par de l'acide formique à 5% et dosé par la technique au carbazole–acide sulfurique de Dische[16].

Essais de fractionnement de la pectine

a) Précipitation fractionnée par l'éthanol. — Une solution de pectine à 1% fut soumise au fractionnement par l'alcool éthylique aux concentrations suivantes: 0–50%, 50–70%, 70–80% et 80–90%. Les constituants des fractions ont été dosés par les techniques indiquées ci-dessus (voir tableau II).

b) Précipitation par le bromure de cétyl triméthylammonium(Cétavlon). — Un volume égal de solution à 7% de bromure de cétyltriméthylammonium est ajouté à une solution de pectine à 2%. Le précipité, séparé par centrifugation, est lavé par l'éthanol pour éliminer l'excès de réactif. Le polysaccharide est ensuite regénéré par décomposition du complexe par l'acide acétique 5M et précipitation par l'éthanol. La solution aqueuse du polysaccharide est désionisée avec de l'Amberlite IR-120 (H^+) et IR-4B (OH^-), puis concentrée. La chromatographie de l'hydrolysat acide du précipité révèle la présence d'oses neutres en quantité importante à côté de l'acide D-galacturonique.

c) Chromatographie sur DEAE cellulose. — Une solution de pectine (200 mg) dans 10 ml de tampon phosphate 0,005M de pH 6,1 est adsorbée sur une colonne de DEAE cellulose (20 g, diamètre 2 cm, forme phosphate). L'élution est effectuée avec 300 ml de chacun des tampons phosphate suivants, ajustés à pH 6,1 (0,005M, 0,025M, 0,05M, 0,1M, 0,25M), puis avec 300 ml de chacune des solutions d'hydroxyde de sodium suivantes (0,1M, 0,2M et 0,3M). Le débit de la colonne est de 1 ml/min. Les fractions (20 ml) sont testées par la réaction à l'anthrone[17] pour les oses neutres et la réaction au carbazole–acide sulfurique[16] pour les acides uroniques. Après désionisation à l'aide de résines échangeuses d'ions, la composition des fractions correspondant à chacun des pics d'élution est étudiée après hydrolyse sulfurique.

d) Electrophorèse. — Elle a été effectuée sur papier Whatman n° 3MM, en tampon pyridine–acide acétique de pH 6,5 (pyridine 100 ml, acide acétique 3 ml, eau distillée q.s.p. 1000 ml). La migration a lieu sous 500 volts, soit environ 11 volts/cm. Les bandes sont révélées à l'aide du réactif à la β-naphtylamine–thymol : la fraction neutre se révèle en brun, la fraction acide en rouge.

Après élution des bandes correspondant à chacune des deux fractions, la composition des polysaccharides ainsi obtenus est déterminée après hydrolyse chlorhydrique.

Hydrolyse partielle de la pectine. — La pectine (5 g) est chauffée à reflux au bain-marie bouillant avec de l'acide sulfurique 0,5M (500 ml) pendant 3 h. L'hydrolysat est neutralisé par le carbonate de baryum, puis désionisé par passage sur une colonne d'Amberlite IR-120 (H^+). La colonne est lavée à l'eau jusqu'à ce que l'éluat

ne donne plus de coloration verte avec le réactif à l'anthrone. L'éluat est concentré sous pression réduite, puis séché sous vide.

L'hydrolysat ainsi obtenu (3,5 g) est chromatographié sur une colonne de 40 ml d'Amberlite CG-400 sous forme formiate. La colonne est d'abord éluée à l'eau (500 ml), puis avec un gradient exponentiel d'acide formique 0–M. Ce gradient est réalisé grâce à un flacon mélangeur à volume constant, contenant 500 ml d'eau, et alimenté par un flacon de réserve contenant 2 litres d'acide formique M. Enfin, la colonne est lavée avec de l'acide formique 2M. L'éluat est recueilli par fractions de 10 ml. Par enregistrement polarimétrique, nous localisons 5 pics d'élution.

Fraction I (0,950 g). — Elle est composée de 8 oses neutres qui furent isolés, après hydrolyse, par chromatographie préparative sur papier (solvant a) et identifiés au moyen d'un de leurs dérivés caractéristiques ou par chromatographie : galactose (méthylphénylhydrazone, p.f. 186°), arabinose (diphénylhydrazone, p.f. 207–208°), xylose (R_{Gal} 1,50), fucose (méthylphénylhydrazone, p.f. 172°), rhamnose (3,4-dibromophénylhydrazone, p.f. 125°), 2-*O*-méthylxylose (anilide, p.f. 125°), 2-*O*-méthylfucose (p.f. 148–151°) et 2-*O*-méthylrhamnose (R_{Gal} 2,15).

Fraction II (447 mg). — Elle contient 6 composés séparés par chromatographie préparative dans le solvant b:

Acide galacturonique (400 mg). – Il a été caractérisé par la 2,5-dichlorophénylhydrazone, p.f. 180–181°.

Composé II_A (22,5 mg). – $R_{Gal\,A}$ 0,80 (solvant b). Par hydrolyse prolongée (HNO_3 à 3% contenant 0,05% d'urée pendant 15 h à 100°), il libère de l'acide galacturonique et du rhamnose. Il n'est pas révélé par le chlorure de triphényltétrazolium[18], ce qui suggère une liaison osidique (1→2).

Composé II_B (10 mg). – R_{GalA} 0,50 (solvant b). Par hydrolyse nitrique prolongée, il libère de l'acide galacturonique et du fucose. Il donne une coloration rouge avec le chlorure de triphényltétrazolium, et par conséquent ne possède pas de liaison (1→2).

Composé II_C (8 mg). – R_{GalA} 0,40 (solvant b). Par hydrolyse nitrique prolongée, il libère de l'acide galacturonique et de l'arabinose. La coloration orangée obtenue lors de la révélation par la diphénylamine–urée suggère[19] une liaison osidique (1→4).

Composé II_D (traces).– R_{GalA} 1,30 (solvant b). Par hydrolyse nitrique, il libère de l'acide galacturonique et du 2-*O*-méthylxylose.

Composé II_E (traces). – R_{GalA} 1,80 (solvant b). Par hydrolyse nitrique, il libère de l'acide galacturonique et du 2-*O*-méthylfucose.

Fraction III (150 mg). — R_{GalA} 0,20 (solvant b); $[\alpha]_D^{20}$ +116° (*c* 1.2, acide chlorhydrique M). Elle a été isolée à l'état de sel de calcium. Les dosages de l'acide galacturonique par la technique au carbazole–acide sulfurique et du nombre de groupes réducteurs par celle de Nelson-Somogyi donnent un rapport égal à 1,95 (en théorie 2 pour le digalacturonate de calcium).

A côté de l'acide digalacturonique, un composé (12 mg) dont le R_{GalA} est de 0,18 a été isolé à partir de cette fraction. Il libère par hydrolyse nitrique de l'acide galacturonique et du galactose.

Fraction IV (80 mg). — R_{GalA} 0,05 (solvant b); $[\alpha]_D^{20}$ +149° (*c* 0.35, acide chlor-

hydrique M). Le rapport acide galacturonique sur groupes réducteurs est de 2,87 (en théorie 3 pour le trigalacturonate de calcium).

Fraction V (367 mg). — Elle est constituée par un mélange d'acide tétragalacturonique et d'homologues supérieurs de l'acide galacturonique. L'acide tétragalacturonique a été isolé à l'état de sel de calcium. Le rapport acide galacturonique sur groupes réducteurs est égal à 4,4 (en théorie 4 pour le tétragalacturonate de calcium pur).

Afin de confirmer l'existence d'une liaison osidique (1→4) entre les unités galacturoniques des oligomères de l'acide galacturonique, nous avons effectué une méthylation suivie de la réduction des groupes carboxyles de l'acide galacturonique et de l'hydrolyse des produits méthylés. Ils conduisirent tous à du 2,3-di-*O*-méthyl-D-galactose.

Méthylation. — L'acide oligogalacturonique (20 mg) est méthylé: (*a*) — Dans un premier stade avec de l'hydroxyde de sodium 10 M (20 ml) et du sulfate de méthyle (15 ml) ajouté goutte à goutte. Le liquide entre en ébullition puis la température diminue .L'agitation est maintenue pendant une nuit à la température du laboratoire. Après une nouvelle addition de sulfate de méthyle (3 ml) et d'hydroxyde de sodium (2 g), l'agitation est encore maintenue pendant 24 h. Après dilution avec de l'eau distillée (250 ml), les produits méthylés sont extraits par du chloroforme (250 ml) utilisé par fractions de 30 ml. Les solutions chloroformiques sont lavées à l'eau distillée jusqu'à neutralité, puis deshydratées à l'aide du sulfate de sodium anhydre. (*b*) — Dans un second stade, la méthylation est effectuée avec de l'iodure de méthyle (3 ml) et de l'oxyde d'argent (1 g) en présence de *N*,*N*-diméthylformamide (6 ml). L'agitation est maintenue, à l'obscurité, sous réfrigérant à reflux, pendant une nuit. Après filtration, le produit méthylé est extrait par le chloroforme (100 ml). La solution est lavée avec une solution de cyanure de potassium à 0,05%, puis avec de l'eau distillée, deshydratée et évaporée. Cette méthylation est recommencée deux fois.

Réduction des carboxyles par le borohydrure de potassium. — Le produit méthylé (10 mg) est estérifié par chauffage pendant 7 h à 100° en présence de gaz chlorhydrique à 2% dans le méthanol (2 ml). La solution neutralisée par le carbonate d'argent est évaporée. La réduction est effectuée directement sur le résidu par addition d'eau distillée (0,5 ml) et d'une solution de borohydrure de potassium (0,3 ml, 6,7%). Après décomposition de l'excès de borohydrure par de l'acide acétique, désionisation et évaporation de la solution, le résidu est hydrolysé par l'acide sulfurique 0,25M (5 ml) pendant 7 h à 100°.

Identification du 2,3-di-O-méthylgalactose. — Les dérivés méthylés du galactose obtenus sont séparés par chromatographie dans le mélange alcool butylique–alcool éthylique–eau (5:1:4, volumes). La chromatographie révèle la présence de deux taches dont l'une en plus grande quantité. Ce dernier corps a été isolé par chromatographie préparative et caractérisé par comparaison avec un témoin de 2,3-di-*O*-méthylgalactose. Leurs mobilités chromatographiques sont identiques. L'oxydation periodique des deux composés (dosage par spectrophotométrie[19]) se déroule selon

une cinétique comparable et la consommation d'oxydant est identique (0,95 mole par mole de dérivé).

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Nous adressons nos vifs remerciements à Monsieur le Professeur J. E. Courtois pour l'intérêt qu'il a porté à ce travail.

BIBLIOGRAPHIE

1 E. L. HIRST ET J. K. N. JONES, *J. Chem. Soc.*, (1939) 454.
2 G. O. ASPINALL ET J. R. S. FANSHAWE, *J. Chem. Soc.*, (1961) 4215.
3 G. O. ASPINALL ET A. CANAS-RODRIGUEZ, *J. Chem. Soc.*, (1958) 4020.
4 A. J. BARRETT ET D. H. NORTHCOTE, *Biochem. J.*, 94 (1965) 617.
5 S. S. BHATTACHARJEE ET T. E. TIMELL, *Can. J. Chem.*, 43 (1965) 758.
6 A. ROUDIER ET J. GALZIN, *Bull. Soc. Chim. France*, (1966) 2481.
7 U. ARIYOSHI, Thèse Doct. Univ. Pharm. Paris, 1961.
8 J. E. COURTOIS ET F. PERCHERON, *Bull. Soc. Botan. France*, (1965) 29.
9 W. A. BENDER, DANS R. L. WHISTLER (Ed.), *Industrial Gums*, Academic Press, New York, 1959, p. 421.
10 P. ALBERSHEIM, H. NEUKOM ET H. DEUEL, *Arch. Biochem. Biophys.*, 90 (1960) 46.
11 H. NEUKOM, H. DEUEL, W. J. HERI ET W. KUNDDIG, *Helv. Chim. Acta*, 43 (1960) 64.
12 R. W. BAILEY, *J. Chromatog.*, 8 (1962) 175.
13 F. PETEK, Thèse Doct. Univ. Paris, 1962.
14 Z. DISCHE ET L. B. SHETTLES, *J. Biol. Chem.*, 175 (1948) 595.
15 H. G. ALBAUM ET W. W. UMBREIT, *J. Biol. Chem.*, 167 (1947) 369.
16 Z. DISCHE, *Biochem. Z.*, 189 (1927) 77.
17 R. DREYWOOD, *Ind. Eng. Chem.*, 11 (1962) 175.
18 K. WALLENFELS, *Naturwissenchaften*, 37 (1950) 491.
19 G. O. ASPINALL ET R. J. FERRIER, *Chem. Ind.* (London), 80 (1961) 57.

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QUANTUM CHEMISTRY OF CARBOHYDRATES

PART I. THE ELECTRONIC STRUCTURE OF SOME PENTOSES

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ABSTRACT

The simple LCAO-MO method of inductive parameters, in the form derived by Del Re, has been applied to the calculation of the electronic structure of some pentoses. The calculated data have been used for the interpretation of some chemical properties of pentoses.

INTRODUCTION

When the reactivity of carbohydrates is being studied and interpreted, attention is usually paid mainly to stereochemical factors[1]. Electronic factors are rarely invoked, since the data based on qualitative theories are not reliable and rather descriptive. Moreover, the complex molecular structure of carbohydrates limits the application of quantitative methods.

In this paper, we attempt the approximate quantum-mechanical calculation of the electronic distribution in carbohydrate molecules and the correlation of the resulting data with typical reactions of carbohydrates. Pentoses have been chosen for study, since the calculations are somewhat easier than for hexoses.

THE METHOD AND THE RESULTS OF CALCULATION

The strictest method of calculation of the electronic and spatial structure of complex, saturated molecules involves extensions of the Hückel method[2,3]. However, the exact geometry of a molecule must be known for such calculations; in the case of pentoses, the structure is not so well known as for other carbohydrates, because of the indefinite position of the hydrogen atoms in the hydroxyl groups. In this connection, only certain methods[4-6] of calculation of σ-electronic structure can be used. We have applied to carbohydrates the method developed by Del Re[4] to calculate the molecular electronic structure of saturated compounds, *e.g.*, amino acids[7,8] and unsaturated molecules[1-11].

The principle of calculation is based on an adaptation of the simple molecular

orbital (MO) method. Each MO is localized at one of the bonds of the molecule $\mu\nu$ and is constructed on the base AO φ_μ, φ_ν:

$$\psi_{\mu\nu} = c_\mu\varphi_\mu + c_\nu\varphi_\nu. \tag{1}$$

The matrix elements of the Hamiltonian operator for μ and ν atoms are determined as follows:

$$H_{\mu\mu} = \alpha + \delta_\mu\beta$$
$$H_{\mu\nu} = \varepsilon_{\mu\nu}\beta \tag{2}$$

where α and β are empirical values of standard coulomb and resonance integrals, and $\varepsilon_{\mu\nu}$ is a dimensionless parameter characterizing the bond type and is independent of its environment. The values δ_μ are determined by equation (3):

$$\delta_\mu = \delta_\mu^0 + \sum_{\nu \text{ adj. to } \mu} \gamma_{\mu(\nu)}\delta_\nu. \tag{3}$$

Here, δ_ν^0 and $\gamma_{\mu(\nu)}$ are parameters depending only on the nature of an atom and bond[4,10]. Table I shows the values of parameters used in this work.

TABLE I

PARAMETERS USED FOR CALCULATION

Atom, bond	$\varepsilon_{\mu\nu}$	$\gamma_{\mu(\nu)}$	$\gamma_{\nu(\mu)}$	δ_μ^0	$\delta^0{}_\nu$
C_{sp^3} (H)	1.0	0.3	0.4	0.07	0
C_{sp^3} (H)				0.12	
C_{sp^3} (C)	1.0	0.1	0.1	0.07	
C_{sp^3} (C)				0.12	
C_{sp^2} (O_{sp^2})	0.95	0.1	0.1	0.07	0.40
C_{sp^2} (O_{sp^2})	0.7			0.12	0.28
O (H)	0.45	0.3	0.4	0.40	0

Equation (3) forms a system of linear, heterogeneous equations, the number of which equals the number of atoms in a molecule. The equation systems were solved[12] with the help of an electronic computer M-20.

The data obtained may be used to calculate bond charges[13]

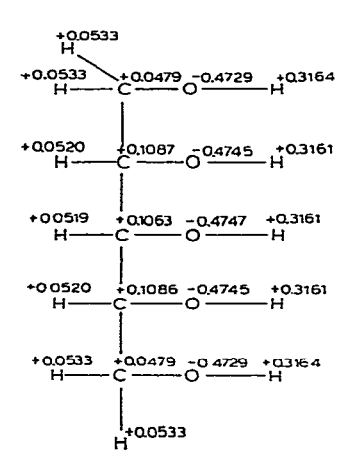
$$Q_{\mu\nu} = \frac{\delta_\nu - \delta_\mu}{2\varepsilon_{\mu\nu}}\left[1 + \left(\frac{\delta_\mu - \delta_\nu}{2\varepsilon_{\mu\nu}}\right)^2\right]^{-\frac{1}{2}} \tag{4}$$

and effective charges on atoms

$$q_\mu = \sum_\nu Q_{\mu\nu} \tag{5}$$

The results of the calculations for some pentoses are given as molecular diagrams **1-8**. The electronic charges on the atoms having π-orbitals in molecules **2-3**

were found in σ,π-approximation, *i.e.*, by summing σ- and π-charges. The π-charges were calculated for the carbonyl group by using the HMO-method with Streitwieser parameters[14].



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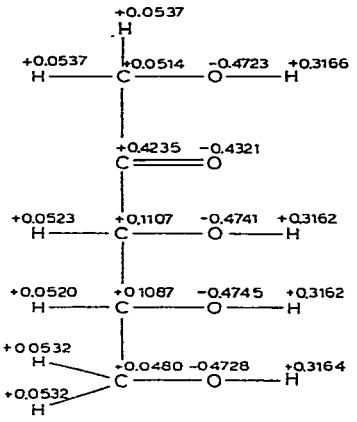

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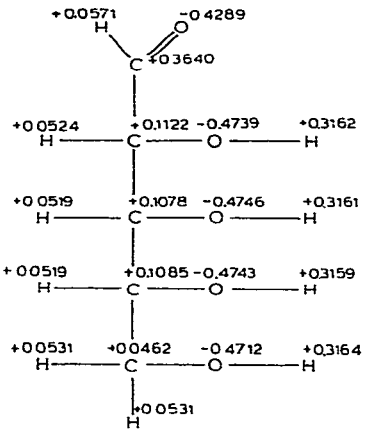

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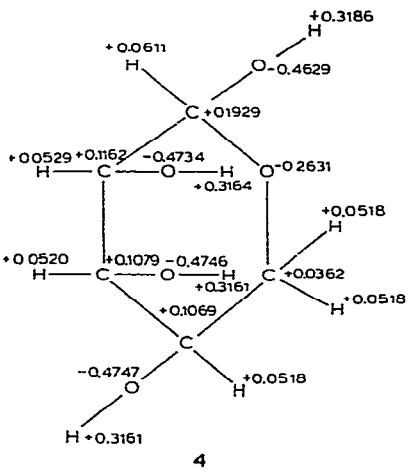

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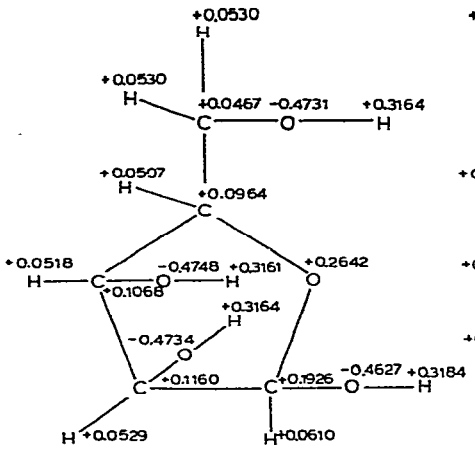
DISCUSSION

The calculation makes possible some quantitative conclusions concerning the electronic properties of carbohydrates; these are usually based only on qualitative and intuitive considerations. First of all, the presence is shown of positive charges on all of the carbon atoms of various types of carbohydrate molecule (alditol, carbonyl form, hydrated *aldehydo*-form, pyranoid and furanoid rings, and enediol).

The fact explains the susceptibility of carbohydrate carbon atoms to nucleophilic attack. This property may be associated with the general instability of the carbon



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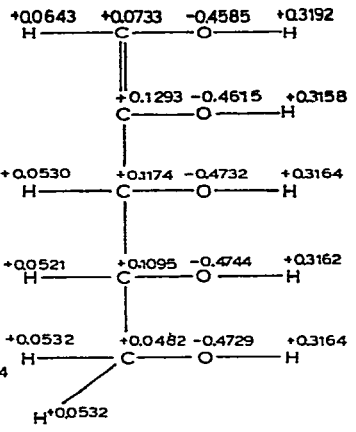

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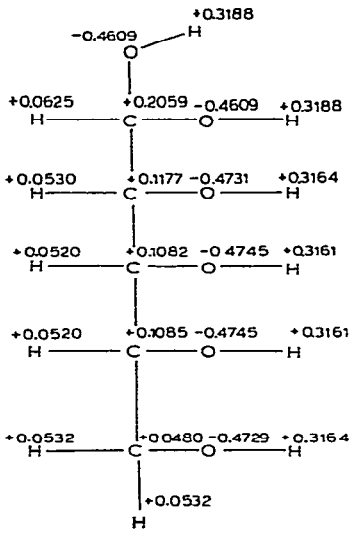

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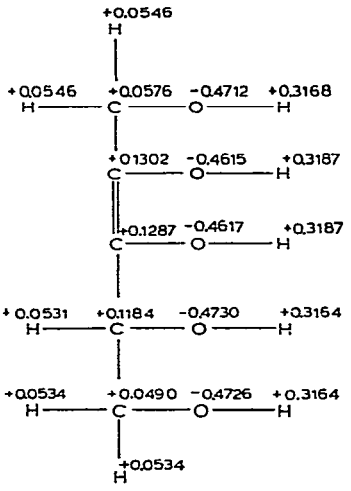

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skeleton of carbohydrates, which are inclined to fragmentation and isomerization in alkaline media[15,16].

From the point of view of distribution of electronic density, the hydroxyl groups of secondary carbon atoms do not differ significantly in reactivity[17]. As would be expected, the carbon atom of the terminal primary alcohol group carries a positive charge that is considerably less than that of the secondary atoms.

In the cyclic forms (**4** and **5**) of the pentoses, the electronic density on C-1 has a higher value than that for the carbonyl (**2**) and hydrated open-chain structures (**6**). This corresponds to a weakening of the aldehyde character of the acetal carbon atom, as observed experimentally. The positive charge on the carbon atom adjacent to the

carbonyl group or centre is somewhat higher than that for the other carbon atoms in the carbonyl, cyclic, or hydrated forms. The acidic nature of the hemiacetal hydroxyl group becomes evident from the molecular diagrams **4** and **5**, which show a decrease in negative charge on the hemiacetal oxygen atom (in comparison with the other hydroxyl groups) and consequent labilisation of the proton. It should be noted also that the electronic density of the ring oxygen atom is rather low, which makes it less susceptible to electrophilic attack. Thus, it is more probable that, in acid media, the proton attacks the glycosidic oxygen atom in preference to the oxygen atom of the pyranoid or furanoid ring.

The probable involvement of enediolic forms in the Lobry de Bruyn–Alberda van Ekenstein[18] and saccharinic acid rearrangements[16] of carbohydrates stimulated the calculation of structures **7** and **8**. The molecular diagrams show considerable positive charges on the double bonds. Structure **7** may be considered to be similar to a 2-hydroxyglycal, which is characterised by proton attack at C-2 in addition reactions with water or hydrogen chloride. It is possible that the cloud of π-electrons, first of all interacting with H^+, is shifted towards C-2 which carries the larger positive charge.

REFERENCES

1 E. L. ELIEL, N. L. ALLINGER, S. J. ANGYAL, AND G. A. MORRISON, *Conformational Analysis*, Interscience, New York, 1965.
2 M. WOLFSBERG AND L. HELMHOLTZ, *J. Chem. Phys.*, 20 (1952) 837.
3 H. HOFFMAN, *J. Chem. Phys.*, 39 (1963) 1937.
4 G. DEL RE, *J. Chem. Soc.*, (1958) 4031.
5 C. SANDORFY, *Can. J. Chem.*, 33 (1955) 1337.
6 K. FUKUI, *Bull. Chem. Soc. Japan*, 33 (1960) 1197.
7 G. DEL RE, B. PULLMAN, AND T. YONEZAWA, *Biochim. Biophys. Acta*, 75 (1963) 153.
8 T. YONEZAWA, G. DEL RE, AND B. PULLMAN, *Bull. Chem. Soc. Japan*, 37 (1964) 985.
9 L. B. KIER, *Tetrahedron Letters*, (1965) 3273.
10 H. BERTHOD AND A. PULLMAN, *J. Chem. Phys.*, 62 (1965) 942.
11 V. I. MINKIN, YU. A. ZHDANOV, E. A. MEDIANTZEVA, AND YU. A. OSTROUMOV, *Tetrahedron*, 23 (1967) 3651.
12 YU. A. OSTROUMOV, *Computer programme for MO calculations on M-20*, Rostov-on-Don, 1965.
13 R. S. MULLIKEN, *J. Chem. Phys.*, 46 (1949) 539.
14 A. STREITWIESER, JR., *Molecular Orbital Theory for Organic Chemists*, Wiley, New York, 1961.
15 N. S. MAJAT AND O. P. GOLOVA, *Usp. Khim.*, 28 (1959) 1114.
16 YU. A. ZHDANOV AND G. N. DOROFEENKO, *Chemical Transformations of the Carbon Skeleton of Carbohydrates*, Moscow, 1962, p. 27.
17 N. K. KOCHETKOV, I. V. TORGOV, AND M. M. BOTVINIK, *Chemistry of the Natural Compounds*, Moscow, 1963.
18 H. S. ISBELL, *J. Res. Natl. Bur. Std.*, 32 (1944) 45.

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THE INTRODUCTION OF UNSATURATED LINKAGES INTO SOME CARBOHYDRATES BY PYROLYSIS OF CYCLIC, ORTHOFORMIC ESTERS

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ABSTRACT

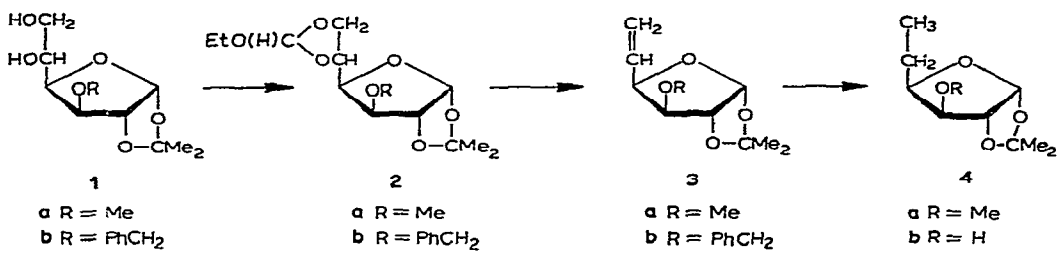
The conversion of the 5,6-diol system in two D-glucofuranose derivatives into carbon–carbon double bonds by pyrolysis of the cyclic ethyl orthoformic esters has been shown to proceed in high yield. 5,6-*O*-(Ethoxymethylene)-1,2-*O*-isopropylidene-3-*O*-methyl-α-D-glucofuranose and 3-*O*-benzyl-5,6-*O*-(ethoxymethylene)-1,2-*O*-isopropylidene-α-D-glucofuranose were prepared from the corresponding 5,6-diols by treatment with ethyl orthoformate. When these compounds were heated in the presence of a carboxylic acid, they were converted into 5,6-dideoxy-1,2-*O*-isopropylidene-3-*O*-methyl-α-D-*xylo*-hex-5-enofuranose and 3-*O*-benzyl-5,6-dideoxy-1,2-*O*-isopropylidene-α-D-*xylo*-hex-5-enofuranose, respectively. On hydrogenation, the latter compounds yielded 5,6-dideoxy-1,2-*O*-isopropylidene-3-*O*-methyl-α-D-*xylo*-hexofuranose and 5,6-dideoxy-1,2-*O*-isopropylidene-α-D-*xylo*-hexofuranose, respectively.

INTRODUCTION AND DISCUSSION

The number of methods available for the formation of unsaturated carbohydrates has increased in recent years, and this development has occasioned interest in such compounds for use as synthetic intermediates[1,2]. We have been concerned with devising simple procedures for converting *cis*-1,2-diols into the corresponding olefins by way of 2-alkoxy-1,3-dioxolane derivatives[3]. We are examining the carboxylic acid-catalysed, thermal breakdown of cyclic orthoformates of carbohydrate diols, to ascertain what limitations the kind of protective group, used elsewhere on the carbohydrate molecule, places on the conditions for successful elimination. This paper describes the introduction of terminal unsaturation into two D-glucofuranose derivatives.

The 2-ethoxy-1,3-dioxolanes required as intermediates are readily prepared under mild conditions by exchange between 1,2-diols and ethyl orthoformate in the presence of a carboxylic acid as the catalyst. When 1,2-*O*-isopropylidene-3-*O*-methyl-α-D-glucofuranose (**1a**) was heated with ethyl orthoformate in the presence of a trace of acetic acid, there was formed a liquid product that gave analyses corresponding to those calculated for the orthoformate expected and that showed no hydroxyl

absorption in its i.r. spectrum. In the n.m.r. spectrum, the resonances of H-1 and the orthoformyl proton gave an overlapping, 2-proton triplet. One of the two peaks attributed to the resonance of the orthoformyl proton had the same chemical shift as the high-field branch of the H-1 doublet. It had been found that, where there is the possibility of stereoisomerism in 2-ethoxy-1,3-dioxolanes, both isomers are formed, except under extreme conditions of steric interaction, when a single stereoisomer may result[4]. Since there is little such interaction in the present example, the product is considered to be a mixture of diastereoisomers of 5,6-*O*-(ethoxymethylene)-1,2-*O*-isopropylidene-3-*O*-methyl-α-D-glucofuranose (**2a**). On heating this mixture in the presence of benzoic acid, ethanol and carbon dioxide were evolved, and the unsaturated compound 5,6-dideoxy-1,2-*O*-isopropylidene-3-*O*-methyl-α-D-*xylo*-hex-5-enofuranose (**3a**) was formed in 80% yield. The n.m.r. spectrum of this product showed the presence of vinylic protons in the terminal double bond[5], and the structure of the product was confirmed by catalytic hydrogenation to form 5,6-dideoxy-1,2-*O*-isopropylidene-3-*O*-methyl-α-D-*xylo*-hexofuranose (**4a**), which was identical with the compound obtained by methylation of the known[6,7] 5,6-dideoxy-1,2-*O*-isopropylidene-α-D-*xylo*-hexofuranose (**4b**).



The exchange reaction between ethyl orthoformate and 3-*O*-benzyl-1,2-*O*-isopropylidene-α-D-glucofuranose (**1b**) gave a liquid product that gave analyses corresponding to those calculated for the orthoformate, and that showed no hydroxyl absorption in its i.r. spectrum. In the n.m.r. spectrum, the H-1 doublet and the signal of the orthoformyl proton were resolved. The latter consisted of two bands, at τ 4.29 and τ 4.32, the integrated areas of which were in the approximate ratio 2:3, and the total integral of which corresponded to one proton. The product was, therefore, considered to be a mixture of the diastereoisomers of 3-*O*-benzyl-5,6-*O*-(ethoxymethylene)-1,2-*O*-isopropylidene-α-D-glucofuranose (**2b**). Pyrolysis of the mixture of diastereoisomers in the presence of triphenylacetic acid (used because it is less volatile than benzoic acid) gave an 86% yield of 3-*O*-benzyl-5,6-dideoxy-1,2-*O*-isopropylidene-α-D-*xylo*-hex-5-enofuranose (**3b**). The presence of the vinyl group in this product was confirmed by its n.m.r. spectrum, and its physical properties corresponded with those described by English and Levy[8] for the compound obtained by treatment of 5,6-anhydro-3-*O*-benzyl-1,2-*O*-isopropylidene-α-D-glucofuranose with methyllithium. The product obtained by English and Levy[8], after catalytic hydrogena-

tion of the unsaturated compound, has been shown by Hall, Hough, and Pritchard[7] to be identical with 5,6-dideoxy-1,2-*O*-isopropylidene-α-D-*xylo*-hexofuranose (**4b**) prepared by a number of other methods. Hydrogenation of our unsaturated compound **3b** gave the same product (**4b**), the identity of which was confirmed by a separate synthesis[6,7].

EXPERIMENTAL

General methods. — Melting points were determined on a Kofler micro hot-stage and are uncorrected. Refractive indexes were determined with a Bellingham and Stanley Abbé "60" refractometer. I.r. spectra of Nujol mulls and of liquid films were recorded with either a Unicam SP-200 or a Perkin–Elmer Model 275 i.r. spectrometer, and the principal bands are reported. The n.m.r. spectra were recorded at 100 MHz with a Varian HR-100 spectrometer, in carbon tetrachloride solution with tetramethylsilane ($\tau = 10.00$) as the internal standard. Chemical shifts were determined from a side band by assuming a linear scale. Chemical-shift values are given on the τ-scale, and correspond to the mid-point of each singlet or symmetrical multiplet. For narrow, unsymmetrical multiplets, the weighted mean value is given, and, for complex multiplets, the upper and lower limits of the chemical shifts are given. Microanalyses were performed by the Australian Microanalytical Service, Melbourne.

*Preparation of the mixture of diastereoisomers of 5,6-*O*-(ethoxymethylene)-1,2-*O*-isopropylidene-3-*O*-methyl-α-D-glucofuranose* (**2a**). — Compound **1a** (2.0 g, 8.5 mmole), ethyl orthoformate (1.3 g, 8.8 mmole), and one drop of glacial acetic acid were heated for 2 h at 115–125°, during which time ethanol distilled from the mixture. On distillation from metallic sodium, the resulting liquid, gave the mixture of diastereoisomeric orthoformic esters as a colourless oil, yield 2.23 g (90%), b.p. 120° at 0.2 torr, n_D^{25} 1.4527, $[\alpha]_D^{21}$ −8.4° (*c* 9.6, ethanol); ν_{max} 1459, 1379, 1212, 1083, 1018, 853 cm^{-1}; n.m.r. data: τ 4.3 (2-proton apparent triplet, orthoformyl-proton diastereoisomers, and doublet, $J_{1,2}$ 3.5 Hz, H-1), τ 5.56 (1-proton doublet, $J_{2,3} < 0.5$ Hz, H-2), τ 5.70–6.58 (7-proton multiplet, H-3,4,5,6,6′, and ethoxy CH_2), τ 6.60 (3-proton singlet, OMe), τ 8.58, 8.74 (3-proton singlets, CMe_2), τ 8.83 (3-proton triplet, *J* 7 Hz, Me of ethoxyl group).

Anal. Calc. for $C_{13}H_{22}O_7$: C, 53.8; H, 7.6. Found: C, 53.9; H, 7.7.

*5,6-Dideoxy-1,2-*O*-isopropylidene-3-*O*-methyl-α-D-*xylo*-hex-5-enofuranose* (**3a**). — The mixture of diastereoisomers **2a** (2.0 g) and benzoic acid (0.02 g) was heated from 150 to 170° over a period of 5 h, during which time ethanol and carbon dioxide (80% yield, by titration with barium hydroxide) were evolved. The residue was dissolved in ether, anhydrous potassium carbonate was added to neutralize the benzoic acid, the suspension was filtered, and the filtrate was evaporated. Distillation of the residue gave the product as a colourless liquid, yield 1.1 g (80%), b.p. 80° at 2 torr, n_D^{25} 1.4557, $[\alpha]_D^{21}$ −43.1° (*c* 1.5, ethanol); ν_{max} 1360, 1200, 1145, 1065, 1015, 918, 857 cm^{-1}; n.m.r. data: τ 3.92–4.30 (2-proton multiplet, H-5 multiplet and H-1 doublet $J_{1,2}$ 4.5 Hz), τ 4.55–4.9 (2-proton multiplet, H-6,6′), τ 5.48 (2-proton multi-

plet, H-2,4), τ 6.38 (1-proton doublet, $J_{3,4}$ 3.5 Hz, H-3), τ 6.60 (3-proton singlets, OMe), τ 8.57, 8.74 (3-proton singlets, CMe_2).

Anal. Calc. for $C_{10}H_{16}O_4$: C, 60.0; H, 8.1. Found: C, 59.9; H, 8.0.

*5,6-Dideoxy-1,2-O-isopropylidene-3-O-methyl-α-*D*-xylo-hexofuranose* (**4a**). — On hydrogenation in ethanol solution over Adams' platinum oxide catalyst, compound **3a** (1.0 g) absorbed one molar equivalent of hydrogen. Distillation of the residue obtained on removal of the catalyst and the solvent gave the product as a colourless liquid, yield 0.96 g (95%), b.p. 60° at 0.1 torr, n_D^{25} 1.4314, $[\alpha]_D^{21}$ −53.1° (*c* 2.1, ethanol); ν_{max} 1470, 1387, 1217, 1168, 1131, 1097, 1028, 896, 866 cm^{-1}; n.m.r. data: τ 4.30 (1-proton doublet, $J_{1,2}$ 3.9 Hz, H-1), τ 5.60 (1-proton doublet, $J_{2,3}$ 0.5 Hz, H-2), τ 6.11 (1-proton sextet, H-4), τ 6.57 (1-proton doublet, $J_{3,4}$ 3.2 Hz, H-3), τ 6.66 (3-proton singlet, OMe), τ 8.45 (2-proton quartet, *J* 7 Hz, showing evidence of a further splitting of 1.0 Hz, H-5,5'), τ 8.61, 8.77 (3-proton singlets, CMe_2), τ 9.1 (3-proton triplet, *J* 7 Hz, H-6,6',6'').

Anal. Calc. for $C_{10}H_{18}O_4$: C, 59.4; H, 9.0. Found: C, 59.5; H, 9.2.

Compound[7] **4a** (0.188 g, 1.0 mmole) was added to a suspension of sodium hydride (0.06 g) in ether (5 ml), under nitrogen at one atmosphere pressure. The mixture was warmed until the evolution of hydrogen had ceased (0.5 h) and then cooled in ice, and a solution of methyl sulphate (0.1 ml) in ether (3 ml) was added dropwise. When addition had been completed, the mixture was boiled under reflux for 4 h. A further quantity of sodium hydride (0.05 g) was added, and the procedure was repeated. After the mixture had been heated under reflux and then kept overnight, it was treated with concentrated ammonia solution and kept for 2 h. The ether layer was separated, washed with water, dried ($CaSO_4$), and evaporated to dryness. Distillation of the residue gave the product as a colourless oil, yield 0.13 g (64%), b.p. 60° at 0.1 torr, n_D^{25} 1.4345, $[\alpha]_D^{23}$ −50.0° (*c* 1.6, ethanol), $[\alpha]_D^{20}$ −74° (*c* 5.0, chloroform). The i.r. and n.m.r. spectra were identical with those already given.

*Preparation of the mixture of diastereoisomers of 3-O-benzyl-5,6-O-(ethoxymethylene)-1,2-O-isopropylidene-α-*D*-glucofuranose* (**2b**). — Compound **1b** (0.52 g, 1.7 mmole), ethyl orthoformate (0.31 g, 2.1 mmole), and one drop of glacial acetic acid were heated from 110 to 120° over a period of 3 h, during which time ethanol distilled from the mixture. Distillation of the residue from metallic sodium gave the mixture of diastereoisomeric orthoformic esters as a colourless liquid, yield 0.57 g (93%), b.p. 150–155° at 0.1 torr, n_D^{25} 1.5321, $[\alpha]_D^{21}$ −15.3° (*c* 2.0, ethanol); ν_{max} 1460, 1380, 1215, 1118, 1080, 1015, 950, 862, 722, 700 cm^{-1}; n.m.r. data: τ 2.74 (5-proton singlet, phenyl protons), τ 4.25 (1-proton doublet, $J_{1,2}$ 3.9 Hz, H-1), τ 4.29, 4.32 (1-proton, giving rise to two resonance maxima, the approximate ratio of the integrated peaks being 2:3; orthoformyl C-H in diastereoisomers), τ 5.43 (2-proton singlet, benzylic CH_2), τ 5.58 (1-proton doublet, $J_{2,3} < 0.5$ Hz, H-2), τ 5.60–6.12 (5-proton multiplet, H-3,4,5,6,6'), τ 6.54 (2-proton quartet, *J* 7 Hz, ethoxy CH_2), τ 8.63, 8.82 (3-proton singlets, CMe_2), τ 8.84 (3-proton triplet, Me of ethoxyl group).

Anal. Calc. for $C_{19}H_{26}O_7$: C, 62.3; H, 7.2. Found: C, 62.3; H, 7.3.

*3-O-Benzyl-5,6-dideoxy-1,2-O-isopropylidene-α-*D*-xylo-hex-5-enofuranose* (**3b**).

— The mixture of diastereoisomers **2b** (0.57 g) and triphenylacetic acid (0.02 g) was heated from 150 to 170° over a period of 5 h, during which time ethanol and carbon dioxide were evolved. The residue was dissolved in ether, solid potassium carbonate was added, and, after being kept, the suspension was filtered, and the filtrate was evaporated to dryness. Distillation of the residue gave the product as a colourless liquid, yield 0.37 g (86%), b.p. 124–128° at 0.2 torr, n_D^{25} 1.5378, $[\alpha]_D^{21}$ −55.5° (*c* 3.0, ethanol) [lit.[8] b.p. 124–129° at 0.2 torr, $[\alpha]_D^{28}$ −56.4° (*c* 3.22, chloroform)]; ν_{max} 1458, 1379, 1207, 1182, 1079, 1018, 921, 862, 720, 700 cm^{-1}; n.m.r. data: τ 2.80 (5-proton singlet, phenyl protons), τ 3.85–4.25 (2-proton multiplet, H-5 multiplet and H-1 doublet, $J_{1,2}$ 3.8 Hz, H-1,5), τ 4.61, 4.77, 4.87 (2-proton multiplet, H-6,6'), τ 5.4–6.0 (4-proton multiplet, H-2,4, and benzylic CH_2), τ 6.27 (1-proton doublet, $J_{3,4}$ 3.0 Hz, H-3), τ 8.60, 8.79 (3-proton singlets, CMe_2).

Anal. Calc. for $C_{16}H_{20}O_4$: C, 69.5; H, 7.3. Found: C, 69.8; H, 7.4.

5,6-Dideoxy-1,2-O-isopropylidene-α-D-xylo-hexofuranose (**4b**). — Hydrogenation of **3b** in ethanol over palladium–carbon by the method of English and Levy[8] gave a crystalline product which, after sublimation at 70° at 0.5 torr followed by crystallisation from light petroleum (b.p. 60–80°), consisted of colourless plates that, on slow heating, partially melted from 58° and were transformed into needles that had m.p. 77–78°, $[\alpha]_D^{21}$ −21.2° (*c* 3.0, ethanol), $[\alpha]_D^{22}$ −23.6° (*c* 1.39, chloroform); ν_{max} 1262, 1228, 1174, 1102, 1078, 1027, 940, 897, 870, 863, 798 cm^{-1}; n.m.r. data: τ 4.29 (1-proton doublet, $J_{1,2}$ 3.5 Hz, H-1), τ 5.67 (1-proton doublet, $J_{2,3} < 0.5$ Hz, H-2), τ 6.13 (2-proton multiplet, H-3,4), τ 7.3 (1-proton, broad singlet which disappeared on deuterium-exchange, OH), τ 8.4 (2-proton multiplet, H-5, 5'), τ 8.6, 8.78 (3-proton singlets CMe_2), τ 9.04 (3-proton triplet, *J* 7 Hz, H-6,6',6''). The compound was hydrolysed with dilute acid, and the osazone was prepared at room temperature by the method of Jones and Thompson[6]. Repeated recrystallisation from ethanol–water gave dark-yellow needles, m.p. 167–169° which, after a final recrystallisation from methanol–water, had m.p. 169–171°, $[\alpha]_D^{22}$ +31→+16.2° (24 h) [*c* 0.82, 3:2 (v/v) pyridine–ethanol]. 5,6-Dideoxy-D-*threo*-hexulose phenylosazone has been described as having m.p. 167–168°, $[\alpha]_D^{20}$ +47→20° (24 h) [*c* 0.60, 3:2 (v/v) pyridine–ethanol][9].

A sample of **4b** was prepared from 1,2-*O*-isopropylidene-5,6-di-*O*-*p*-tolylsulphonyl-α-D-glucofuranose (m.p. 154–158° from methanol) by treatment with sodium iodide in butanone followed by catalytic hydrogenation of the unsaturated intermediate[6,7]. After sublimation at 70° at 0.1 torr and recrystallisation from light petroleum (b.p. 60–80°), the compound was obtained as colourless plates which, on being heated, behaved in the way already described and had m.p. 76–78°. A mixed m.p. again showed the same behaviour, and no depression was observed. The i.r. and n.m.r. spectra of the respective compounds were also identical.

REFERENCES

1 R. J. FERRIER, *Advan. Carbohyd. Chem.*, 20 (1965) 67.
2 E. ALBANO, D. HORTON, AND T. TSUCHIYA, *Carbohyd. Res.*, 2 (1966) 349.

3 G. CRANK AND F. W. EASTWOOD, *Aust. J. Chem.*, 17 (1964) 1392.
4 J. S. JOSAN AND F. W. EASTWOOD, unpublished results.
5 R. H. BIBLE, JR., *Interpretation of N.M.R. Spectra*, PLENUM Press, New York, 1965.
6 J. K. N. JONES AND J. L. THOMPSON, *Can. J. Chem.*, 35 (1957) 955.
7 L. D. HALL, L. HOUGH, AND R. A. PRITCHARD, *J. Chem. Soc.*, (1961) 1537; *cf.*, H. OHLE AND E. DICKHÄUSER, *Ber.*, 58 (1925) 2593.
8 J. ENGLISH AND M. F. LEVY, *J. Amer. Chem. Soc.*, 78 (1956) 2846.
9 P. A. J. GORIN, L. HOUGH, AND J. K. N. JONES, *J. Chem. Soc.*, (1955) 2699.

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GLUCURONIC ESTERS

PART V*. SYNTHESIS AND PROPERTIES OF BENZYL 2,3,4-TRI-*O*-BENZYL-1-CHLORO-1-DEOXY-β-D-GLUCOPYRANURONATE

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ABSTRACT

By the action of thionyl chloride on benzyl 2,3,4-tri-*O*-benzyl-D-glucopyranuronate at 0°, a crystalline benzyl 2,3,4-tri-*O*-benzyl-1-chloro-1-deoxy-D-glucopyranuronate was obtained. On the basis of its chemical behavior, the β-D configuration has been established for this compound. The n.m.r. spectra of the glucuronic acid halides are described and discussed.

INTRODUCTION

In Part III[1] of this series, benzyl 2,3,4-tri-*O*-benzyl-1-bromo-1-deoxy-α-D-glucopyranuronate (**1a**) and benzyl 2,3,4-tri-*O*-benzyl-1-chloro-1-deoxy-α-D-glucopyranuronate (**1b**) were described as intermediates in the synthetic pathway to the fully benzylated derivative of D-glucuronic acid having the C-1 hydroxyl group unblocked. Both halides, prepared from the corresponding 1-*O*-acetyl derivative, were obtained as oils showing positive rotation in chloroform; the α-D configuration of compound **1a** was established by its conversion into benzyl 1-*O*-acyl-2,3,4-tri-*O*-benzyl-β-D-glucopyranuronates[2]. Because of the rather low yields of compound **1b**, other methods leading to 1-chloro compounds were also investigated. In this paper, the synthesis and characteristics of crystalline benzyl 2,3,4-tri-*O*-benzyl-1-chloro-1-deoxy-β-D-glucopyranuronate (**2**) are presented.

RESULTS AND DISCUSSION

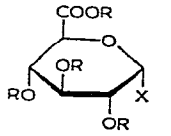
Glaudemans and Fletcher[3] obtained 2,3,5-tri-*O*-benzyl-D-arabinofuranosyl chloride of nearly pure α-configuration by the action of hydrogen chloride in dichloromethane on an anomeric mixture of the corresponding 1-*O*-*p*-nitrobenzoyl derivative. The same treatment applied to benzyl 2,3,4-tri-*O*-benzyl-1-*O*-*p*-nitrobenzoyl-α-D-glucopyranuronate[2] failed; even after a six-day reaction period neither *p*-nitrobenzoic acid nor compound **1b** could be detected, and, from the reaction mixture, 67% of the starting ester was recovered. When the same ester was treated with

*Part IV: D. Keglević, N. Pravdić, and J. Tomašić, *J. Chem. Soc.* (*C*), (1968) 511.

hydrogen chloride in acetic acid, a rapid formation of *p*-nitrobenzoic acid, followed by slow appearance of the chloro derivative, was observed. It was found that, in this case, the reaction proceeded through transesterification, affording benzyl 1-*O*-acetyl-2,3,4-tri-*O*-benzyl-D-glucopyranuronate[1], which was then subsequently converted into the chloro compound **1b**.

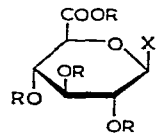
When benzyl 2,3,4-tri-*O*-benzyl-D-glucopyranuronate[1] was treated with thionyl chloride at 0°, following essentially the procedure of Baddiley and his collaborators[4], a crystalline product of high purity was obtained. It showed negative rotation in chloroform, and the elemental analysis corresponded to the fully benzylated compound, benzyl 2,3,4-tri-*O*-benzyl-1-chloro-1-deoxy-β-D-glucopyranuronate (**2**). Apparently, this is the first crystalline example of this class of compound.

The chloride **2** proved to be surprisingly stable; its physical constants have not changed after storage in a stoppered vial at room temperature for one year. Treatment of compound **2** with benzyl alcohol gave benzyl tetra-*O*-benzyl-α-D-glucopyranuronate (**3**) having $[\alpha]_D$ +35°, whereas the same reaction performed with the bromide **1a** resulted in the formation of the β-D anomer **4** showing $[\alpha]_D$ −33°.



1a X = Br
1b X = Cl
3 X = OCH_2Ph
5 X = $OCOC_6H_4$–OMe(*p*)

R = CH_2Ph



2 X = Cl
4 X = OCH_2Ph

At room temperature, a suspension of compound **2** and the silver salt of *p*-methoxybenzoic acid in benzene gave, after five days, 38% of benzyl 2,3,4-tri-*O*-benzyl-1-*O*-*p*-methoxybenzoyl-α-D-glucopyranuronate[2] (**5**); under identical conditions, the dextrorotatory chloride **1b** remained unchanged. On the other hand, at reflux temperature with silver *p*-methoxybenzoate, compound **2** yielded an almost equimolar mixture of anomeric *p*-methoxybenzoyl derivatives, whereas compound **1b** gave a mixture enriched with the β-D anomer. As expected, in the presence of silver perchlorate, the configuration of the 1-*O*-*p*-methoxybenzoyl derivative did not greatly depend on the initial configuration of the chloride; with each halide, an anomeric mixture, enriched in the α-D anomer **5**, was obtained.

Hence, in comparison with compound **1b**, the crystalline chloride **2** proved to be more reactive, thus representing a convenient intermediate in the synthesis of 1-*O*-acyl-α-D-glucopyranuronates.

The n.m.r. spectra of the benzylated halides **2**, **1a**, and **1b** could not be analyzed readily, due to the presence of benzyl groups. We then turned our attention to the acetylated halides and compared the spectra of the anomeric pair of 1-chloro derivatives [**6** (Ref. 5) and **7**]. Heyns *et al.*[6] recorded the n.m.r. spectra of methyl 2,3,4-tri-*O*-acetyl-1-bromo-1-deoxy-α-D-glucopyranuronate and its β-D anomer, and established the *C1* conformation for the α-D anomer. The spectrum of methyl 2,3,4-tri-*O*-acetyl-

1-chloro-1-deoxy-α-D-glucopyranuronate (**6**) proved to be considerably simpler than the spectra of the corresponding aldopyranosyl halides[7]; due to the lack of C-6 protons, the signal for H-5 appears as a doublet. The splitting patterns and magnitudes of the coupling constants[8,9] in the spectrum fully support the *trans*-diaxial arrangement of the ring protons and are consistent with the formulation of the α-D anomer **6** in the *C1* conformation. In the spectrum of the β-D anomer **7***, the ring protons are observed as four-proton and one-proton multiplets with narrow spacings. If this compound had been in the *C1* conformation, the H-1 signal would have been a wide doublet (1,2-diaxial coupling) and the other part of the spectrum would have been similar to that of the α-D anomer. It seems plausible that, in this case, conformational inversion has occurred, and that compound **7** in chloroform-*d* adopts the *1C* conformation. This conformation is supported by the fact that the signals for the methoxy and all acetoxy groups are observed at lower field than in the spectrum of the α-D anomer **6**, indicative[8] of axial orientation. On the other hand, steric repulsion of axial groups may cause some flattening of the ring, and the existence of this compound in a flexible form cannot be excluded. Moreover, the large coupling of the one-proton multiplet at τ 5.72–5.88, which probably arises from H-5 ($J_{4,5}$ 9.6 Hz), makes the possibility of a conformation in the flexible cycle even more probable[7].

Although the spectra of the fully benzylated halides **1a**, **1b**, and **2** are not very informative, the small coupling ($J_{1,2}$ 3.9 and 3.6 Hz) of the doublets at τ 3.81 and 3.99 in the spectrum of the α-D anomers **1a** and **1b**, respectively, denote the equatorial–axial orientation, thus providing an indication of the α-D configuration[13]. On the other hand, in the spectrum of the β-D-chloro derivative **2**, no signal has been found in the region from τ 3.0–4.4. Since, in the spectra of all of the α-D anomers described, the signal for H-1 appears as a narrow doublet in the region 3.35–3.99, the nonexistence of such a signal in the spectrum of compounds **2** and **7**, as well as in that of methyl 2,3,4-tri-*O*-acetyl-1-bromo-1-deoxy-β-D-glucopyranuronate[6], suggests the β-D configuration for compound **2**.

There is not enough evidence to assign the *1C* conformation to compound **2**, although this assumption would be consistent with the published n.m.r. data for various aldopyranosyl halides[12,14,15]. Horton *et al.*[12] have pointed out that the favored conformation of the acetylated aldopyranosyl halides in chloroform solution is that chair form in which the halogen atom is axial, *i.e.*, the *1C* conformation for the β-D anomers.

On the basis of the findings presented in this paper, the β-D configuration has been established for the chloro derivative **2**. Baddiley and his collaborators first used thionyl chloride in the synthesis of 1-chloro derivatives bearing nonparticipating groups in the molecule; from tetra-*O*-benzyl-α-D-glucose[4] or -galactose[16], they obtained the α-D anomer of the corresponding chloride. Since, for benzyl 2,3,4-tri-*O*-benzyl-D-glucopyranuronate, the β-D configuration, or at least a strong predominance

*This compound, prepared already by Heyns *et al.*[6], was obtained also by the action of thionyl chloride on methyl 2,3,4-tri-*O*-acetyl-D-glucopyranuronate[10], as well as from methyl tetra-*O*-acetyl-β-D-glucopyranuronate[11] by treatment with aluminum chloride[12].

of this configuration was proposed[1], it would follow that the reaction with thionyl chloride proceeds without isomerization at the anomeric carbon atom.

EXPERIMENTAL

Melting points are uncorrected. Specific rotations were measured in chloroform (*ca.* 1% at 20–23°). Thin-layer chromatography was conducted on Silica Gel G (Merck), components being detected by spraying with 10% sulfuric acid and heating at 100°. Column chromatography was conducted on silica gel (0.2–0.5 mm, Merck). The i.r. spectra were recorded for Nujol mulls on a Perkin–Elmer Model 137 instrument. The n.m.r. spectra were obtained for chloroform-*d* solutions with a Varian A-60A spectrometer, and tetramethylsilane as an internal standard.

*Treatment of benzyl 2,3,4-tri-*O*-benzyl-1-*O*-p-nitrobenzoyl-α-*D*-glucopyranuronate with acetic acid–dry hydrogen chloride.* — The title ester[2] (250 mg) was added to an acid solution (5 ml, 2.6 g of dry hydrogen chloride per 100 ml of acetic acid); the reaction was monitored by t.l.c. in ether–light petroleum (1:2). After storage for 6 days at room temperature, chloroform was added, and the reaction mixture was processed in the standard way. The remaining oil was chromatographed on a silica-gel column (15 g) with ether–light petroleum (1:2). The solvent displaced 25% of the chloride **1b**, showing $[\alpha]_D$ +28°, followed by benzyl 1-*O*-acetyl-2,3,4-tri-*O*-benzyl-D-glucopyranuronate[1] (35%), $[\alpha]_D$ +22°. Further elution with chloroform and ethanol gave benzyl 2,3,4-tri-*O*-benzyl-D-glucopyranuronate[1] (10%) and *p*-nitrobenzoic acid (60%).

*Benzyl 2,3,4-tri-*O*-benzyl-1-chloro-1-deoxy-β-D-glucopyranuronate* (**2**). — Benzyl 2,3,4-tri-*O*-benzyl-D-glucopyranuronate[1] (554 mg) was dissolved in freshly distilled thionyl chloride (5 ml), and the solution was kept for 48 h at 0° and then concentrated *in vacuo*. Residual traces of the reagent were removed by codistillation with toluene, and the crude oily residue, on storage in a vacuum desiccator over sodium hydroxide, started to crystallize. Recrystallization from light petroleum–ether gave compound **2** as fine needles (380 mg, 66%), m.p. 85–86°, $[\alpha]_D$ −6°.

Anal. Calc. for $C_{34}H_{33}ClO_6$: C, 71.26; H, 5.80; Cl, 6.19. Found: C, 71.04; H, 5.79; Cl, 5.98.

*Benzyl tetra-*O*-benzyl-α-D-glucopyranuronate* (**3**). — A solution of compound **2** (120 mg) in absolute benzene (5 ml) and benzyl alcohol (1 ml) was stirred with freshly prepared silver carbonate (120 mg) for 6 h at room temperature in the dark. T.l.c. (ether–light petroleum, 1:2) of the reaction mixture then showed the absence of compound **2**. The precipitate was filtered off and washed with benzene. The filtrate and washings were combined, the solvent was evaporated, and benzyl alcohol was distilled off *in vacuo*. The residual oil was chromatographed on a column of silica gel (25 g) with ether–light petroleum (1:2). The product was isolated as a colorless oil (88 mg, 66%), $[\alpha]_D$ +35°.

Anal. Calc. for $C_{41}H_{40}O_7$: C, 76.38; H, 6.25. Found: C, 76.11; H, 6.31.

*Benzyl tetra-*O*-benzyl-β-D-glucopyranuronate* (**4**). — Benzyl 2,3,4-tri-*O*-benzyl-1-bromo-1-deoxy-α-D-glucopyranuronate[1] (**1a**, 230 mg) was treated in the same way

as described for the preparation of compound **3**, except that the reaction mixture was shaken overnight. After the removal of the silver salts, the solvent and benzyl alcohol were distilled off, and the residual oil was crystallized from methanol. Recrystallization from the same solvent gave 118 mg (49%) of compound **4**, m.p. 93–94°, $[\alpha]_D$ −33°.

Anal. Calc. for $C_{41}H_{40}O_7$: C, 76.38; H. 6.25. Found: C, 76.65; H, 6.47.

T.l.c. of compound **4** in several solvent systems showed a slightly higher mobility than that for compound **3**.

Reactions of compound **2** *with silver* p-*methoxybenzoate.* — (*a*) *Without heating.* A suspension of compound **2** (100 mg) and silver *p*-methoxybenzoate (50 mg) in absolute benzene (8 ml) was stirred in the dark for 5 days at room temperature. Examination of the reaction mixture (t.l.c., benzene–ether, 15:1) revealed a trace of product after one day; after 5 days, compound **2** was still present. The insoluble salts were filtered off, and washed with benzene, and the filtrates were concentrated *in vacuo* leaving a crystalline residue. Recrystallization from ethanol gave benzyl 2,3,4-tri-*O*-benzyl-1-*O*-*p*-methoxybenzoyl-α-D-glucopyranuronate[2] (**5**) as needles (45 mg, 38%), m.p. and mixed m.p. 110–111°, $[\alpha]_D$ +29°. The i.r. spectrum was superimposable on that of the compound prepared earlier[2].

(*b*) *Under reflux.* The mixture described above was heated under reflux for 30 h. The product (58%) was obtained as the mixture of anomers, m.p. 89–93°, $[\alpha]_D$ +3°.

In a similar preparation, the α-chloro derivative **1b** was refluxed for 10 h; the resulting mixture of anomers (28%) showed m.p. 88–98°, $[\alpha]_D$ −11°.

(*c*) *With silver perchlorate.* To a solution of compound **2** (285 mg) in chloroform (10 ml), silver *p*-methoxybenzoate (140 mg) and silver perchlorate (30 mg) were added, and the mixture was refluxed for 5 h. Benzyl 2,3,4-tri-*O*-benzyl-1-*O*-*p*-methoxybenzoyl-D-glucopyranuronate (164 mg, 48%) showed m.p. 93–109°, $[\alpha]_D$ +22°.

In a similar preparation with compound **1b**, the product had m.p. 92–105°, $[\alpha]_D$ +15°.

*Methyl 2,3,4-tri-*O*-acetyl-1-chloro-1-deoxy-β-*D*-glucopyranuronate* (**7**). — (*a*) Methyl 2,3,4-tri-*O*-acetyl-D-glucopyranuronate[10] (0.8 g) was treated with freshly distilled thionyl chloride (10 ml) as described in the preparation of compound **2**. The crystalline residue, after removal of the reagent, was triturated with absolute ether. The product (520 mg, 62%) showed m.p. 142–144°, $[\alpha]_D$ −20°; Heyns *et al.*[6] gave m.p. 151–152°, $[\alpha]_D$ −15.8°.

Anal. Calc. for $C_{13}H_{17}ClO_9$: C, 44.27; H, 4.86; Cl, 10.05. Found: C, 44.01; H, 4.88; Cl, 10.32.

(*b*) A solution of methyl tetra-*O*-acetyl-β-D-glucopyranuronate[11] (2.0 g) in absolute chloroform (20 ml) was shaken with crushed aluminium chloride[12] (5 g) for 18 h at room temperature. The mixture was poured onto ice–water and worked up by the standard procedure; the colorless syrup spontaneously crystallized (1.4 g, 75%). The optical rotation, m.p., and i.r. spectrum were identical with those of compound 7 prepared as in (*a*).

N.m.r. data. (*a*) *Methyl 2,3,4-tri-*O*-acetyl-1-chloro-1-deoxy-α-*D*-glucopyranuronate* (**6**). — Signals at τ 3.70 (doublet, $J_{1,2}$ 4.0 Hz, H-1), 4.43 (triplet, $J_{2,3}=J_{3,4}$ 9.5 Hz, H-3), 4.82 (quartet, $J_{3,4}$ 9.5, $J_{4,5}$ 9.8 Hz, H-4), 5.02 (quartet, $J_{1,2}$ 4.0, $J_{2,3}$ 9.5 Hz, H-2), 5.46 (doublet, $J_{4,5}$ 9.8 Hz, H-5), 6.32 (OCH_3, 3 protons), 7.97 and 8.00 (OAc, 9 protons).

(*b*) *Methyl 2,3,4-tri-*O*-acetyl-1-chloro-1-deoxy-β-*D*-glucopyranuronate* (**7**). — Signals in the regions τ 4.48–4.94 (4-proton multiplet), 5.72–5.88 (1-proton multiplet), singlet at τ 6.22 (OCH_3, 3 protons), 7.91 and 7.96 (OAc, 9 protons).

(*c*) *Benzyl 2,3,4-tri-*O*-benzyl-1-bromo-1-deoxy-α-*D*-glucopyranuronate* (**1a**). — Signals at τ 2.81–2.96 (aromatic protons), 3.81 (doublet, $J_{1,2}$ 3.9 Hz, H-1), and 5.0–6.8 (methylene and ring protons).

(*d*) *Benzyl 2,3,4-tri-*O*-benzyl-1-chloro-1-deoxy-α-*D*-glucopyranuronate* (**1b**). — Signals at τ 2.70–2.85 (aromatic protons), 3.99 (doublet, $J_{1,2}$ 3.6 Hz, H-1), and in the region 4.9–6.3 (methylene and ring protons).

(*e*) *Benzyl 2,3,4-tri-*O*-benzyl-1-chloro-1-deoxy-β-*D*-glucopyranuronate* (**2**). — Signals in the region τ 2.7–2.9 (aromatic protons), and 4.7–6.6 (methylene and ring protons).

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REFERENCES

1 N. PRAVDIĆ AND D. KEGLEVIĆ, *Tetrahedron*, 21 (1965) 1897.
2 D. KEGLEVIĆ, N. PRAVDIĆ, AND J. TOMAŠIĆ, *J. Chem. Soc., (C)*, (1968) 511.
3 C. P. J. GLAUDEMANS AND H. G. FLETCHER, JR., *J. Org. Chem.*, 28 (1963) 3004.
4 P. W. AUSTIN, F. E. HARDY, J. G. BUCHANAN, AND J. BADDILEY, *J. Chem. Soc.*, (1964) 2128.
5 W. F. GOEBEL AND F. H. BABERS, *J. Biol. Chem.*, 111 (1935) 347.
6 K. HEYNS, W. P. TRAUTWEIN, F. G. ESPINOSA, AND H. PAULSEN, *Ber.*, 99 (1966) 1183.
7 D. HORTON AND W. N. TURNER, *J. Org. Chem.*, 30 (1965) 3387.
8 L. D. HALL, *Advan. Carbohydrate Chem.*, 19 (1964) 51.
9 R. U. LEMIEUX, J. D. STEVENS, AND R. R. FRASER, *Can. J. Chem.*, 40 (1962) 1955.
10 N. PRAVDIĆ AND D. KEGLEVIĆ, *J. Chem. Soc.*, (1964) 4633.
11 G. N. BOLLENBACK, J. W. LONG, D. G. BENJAMIN, AND J. A. LINDQUIST, *J. Am. Chem. Soc.*, 77 (1955) 3310.
12 C. V. HOLLAND, D. HORTON, AND J. S. JEWELL, *J. Org. Chem.*, 32 (1967) 1818.
13 R. U. LEMIEUX, R. K. KULLNIG, H. J. BERNSTEIN, AND W. G. SCHNEIDER, *J. Am. Chem. Soc.*, 80 (1958) 6098.
14 B. COXON, *Tetrahedron*, 22 (1966) 2281.
15 L. D. HALL AND J. F. MANVILLE, *Carbohyd. Res.*, 4 (1967) 512.
16 P. W. AUSTIN, F. E. HARDY, J. G. BUCHANAN, AND J. BADDILEY, *J. Chem. Soc.*, (1965) 1419.

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SYNTHESIS OF 1,6-ANHYDRO DERIVATIVES OF 2-AMINO-2-DEOXY-β-D-TALOPYRANOSE AND 4-AMINO-4-DEOXY-β-D-TALOPYRANOSE*†

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ABSTRACT

The oxime (**2**) of 1,6-anhydro-3,4-*O*-isopropylidene-β-D-*lyxo*-hexopyranos-2-ulose (**1**) in aqueous hydrochloric acid is reduced stereospecifically by hydrogen and a platinum catalyst, with concomitant hydrolysis of the *O*-isopropylidene group, to give 2-amino-1,6-anhydro-2-deoxy-β-D-talopyranose hydrochloride (**3**) in high yield. Hydrolysis of the anhydro sugar **3** under more vigorous conditions gave crystalline 2-amino-2-deoxy-α-D-talopyranose hydrochloride (**4**). In aqueous solution, the amino sugar **4** exists as a 1:2 mixture of the α and β-D-pyranose anomers. Reduction of 1,6-anhydro-2,3-*O*-isopropylidene-β-D-*lyxo*-hexopyranos-4-ulose oxime (**5**) in aqueous hydrochloric acid, with hydrogen over platinum, also occurred stereospecifically, to give a crystalline product formulated as 4-amino-1,6-anhydro-4-deoxy-β-D-talopyranose hydrochloride (**6**), which was further characterized as the peracetylated derivative **7**. Attempted hydrolysis of **6** to give 4-amino-4-deoxy-D-talose hydrochloride (**8**) gave only unchanged **6** and tarry products, presumably because the free 4-amino sugar derivative **8** undergoes rapid destruction in the acid solution by way of a pyrrole derivative.

INTRODUCTION

A series of investigations in this laboratory has been concerned with amino sugars related to antibiotic substances, and with the behavior of 4-amino-4-deoxy sugars under conditions of acid hydrolysis[3]. New amino sugars of defined structure can be obtained by modification of readily available amino sugars. This route, which has been used for the preparation of some 4-amino-4-deoxy sugars[3], is limited by the availability of suitable amino sugars as precursors. Amino sugars of defined structure can also be obtained by sequences involving displacement of "isolated" sulfonyloxy groups by such nitrogen nucleophiles as hydrazine[4] or azide ion[5,6]. Such

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displacements, at secondary positions[5], are subject to steric limitations[7], especially in bridged ring-systems[8].

The procedure introduced by Lindberg and Theander[9] for synthesis of amino sugars, by reduction of aldosulose oximes, is not stereochemically definitive because the reaction can, and frequently does, give rise to an epimeric mixture of amino sugars. The stereoselectivity of the reduction can, in many cases, be influenced by the procedure used for reduction. The advent of high-yielding methods[10,11] for preparation of aldosulose derivatives makes the oxime route attractive as a preparative method for amino sugars, especially if high stereoselectivity is attainable in the reduction step. A further potential advantage is the fact that the aldosulose precursors can be deuterated or tritiated at positions *alpha* to the carbonyl group; if suitably bridged derivatives are used, the labeling can be made stereospecific and also position-specific[1,12]. Such labeling methods can be used for preparing specifically deuterated or tritiated amino sugars for biochemical studies.

The present report describes the reduction of the oxime (**2**) of 1,6-anhydro-3,4-*O*-isopropylidene-β-D-*lyxo*-hexopyranos-2-ulose[12] (**1**) and the oxime[11] (**5**) of 1,6-anhydro-2,3-*O*-isopropylidene-β-D-*lyxo*-hexopyranos-4-ulose, with hydrogen over platinum. The reductions are highly stereoselective, and provide high-yielding, preparative routes to the 1,6-anhydrides of 2-amino-2-deoxy-D-talopyranose and 4-amino-4-deoxy-D-talopyranose.

DISCUSSION

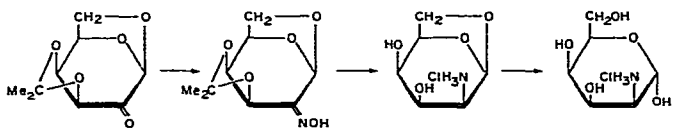
1,6-Anhydro-3,4-*O*-isopropylidene-β-D-*lyxo*-hexopyranos-2-ulose[12] (**1**) was converted in high yield into a crystalline oxime (**2**) that was chromatographically homogeneous and that appeared to be a single, geometrical isomer; in its n.m.r. spectrum, it showed the low-field (τ 1.17) signal anticipated[11,13] for the hydroxyl proton of the oxime group. Reduction of the oxime with hydrogen over platinum, in acidified aqueous ethanol, led to reduction to the amine, together with hydrolysis of the *O*-isopropylidene group, to give syrupy 2-amino-1,6-anhydro-2-deoxy-β-D-talopyranose hydrochloride (**3**) in almost quantitative yield. Evidence that the reduction had proceeded stereospecifically to **3** was provided by paper chromatography with the Fischer–Nebel[14] solvent system. In this system, the product **3** was homogeneous, and its mobility (R_{GN} relative to 2-amino-2-deoxy-D-glucose) was 1.93. A sample of 2-amino-1,6-anhydro-2-deoxy-β-D-galactopyranose[15,16] had R_{GN} 1.72. The latter compound was cleanly separated from **3**, and was absent from the product obtained by reduction of the oxime **2**.

The anhydride **3** was characterized by hydrolysis to 2-amino-2-deoxy-D-talose hydrochloride, isolated as the crystalline α-D-pyranose anomer **4**, identical by its X-ray diffractogram with an authentic sample[17]. The crude hydrolyzate contained a major component, R_{GN} 1.08, corresponding to **4**, and a minor componen., R_{GN} 1.93, corresponding to the anhydride **3**, but 2-amino-2-deoxy-D-galactose hydrochloride (R_{GN} 0.88) was absent.

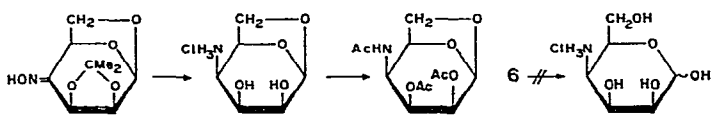
The amino sugar **4** exhibits downward mutarotation[17]. At equilibrium in deuterium oxide, the n.m.r. spectrum of the solution shows two narrow multiplets at τ 4.62 and 4.82, in the ratio of approximately 1:2, that are assigned to H-1 of the α-D-pyranose and β-D-pyranose anomers, respectively. The observed positions and multiplicities of these H-1 signals coincide almost exactly with the corresponding signals of 2-amino-2-deoxy-α- and -β-D-mannopyranose hydrochloride[18] (axial $-NH_3Cl$ group), and are quite different from those of the H-1 signals of 2-amino-2-deoxy-α- and -β-D-galactopyranose (and -D-glucopyranose) hydrochloride[19]; the latter sugars have an equatorially attached $-NH_3Cl$ group.

Reduction of 1,6-anhydro-2,3-*O*-isopropylidene-β-D-*lyxo*-hexopyranos-4-ulose oxime[11] (**5**) by the procedure used for the 2-oxime **2** gave a 77% yield of a crystalline product, m.p. 170–185° (dec.), $[\alpha]_D$ −66° (in water), formulated as 4-amino-1,6-anhydro-4-deoxy-β-D-talopyranose hydrochloride (**6**). There was no indication that a significant proportion of any isomeric product was formed. It is evident, therefore, that reduction of the oxime **5** (a mixture of *syn* and *anti* isomers[11]) proceeds with high stereoselectivity.

Formulation of the reduction product as the D-*talo* isomer **6** is based on a number of lines of evidence. By analogy with the behavior on reduction of the oxime **2**, the 2-ketone[12] **1**, and 1,6-anhydro-2,3-*O*-isopropylidene-β-D-*lyxo*-hexopyranos-4-ulose[12], it was anticipated that attack of hydrogen on **5** would be from the unhindered face of the molecule, to give the D-*talo* derivative **6**. Furthermore, 4-amino-1,6-anhydro-4-deoxy-D-mannose hydrochloride (the 4-epimer of **6**) has been reported[15] to have m.p. 175–180°, $[\alpha]_D$ −100.4° (in water); this specific rotation is substantially different from that observed for **6**.



1 **2** **3** **4**



5 **6** **7** **8**

Acetylation of **6** with acetic anhydride–sodium acetate gave crystalline 4-acetamido-2,3-di-*O*-acetyl-1,6-anhydro-4-deoxy-β-D-talopyranose (**7**). The n.m.r. spectrum of **7** was very similar to that of 1,6-anhydro-β-D-talopyranose triacetate[12], except that a signal of an NH proton was present and the H-4 signal was at higher field in **7** than in the 4-acetoxy analog. It is known[20] that a methine proton having a geminal acetamido group gives a signal at higher field than one in a similar environment that has a geminal acetoxy group.

Attempts to hydrolyze the anhydro sugar **6** to the free amino sugar, namely, 4-amino-4-deoxy-D-talose hydrochloride (**8**), were unsuccessful, as had been anticipated[3]. The anhydro sugar **6** was quite resistant under conditions of acid hydrolysis, as evidenced by the recovery of 70% of the starting material after 3 h at 100° in M hydrochloric acid. The formation of tar in the reaction, especially under more vigorous hydrolytic conditions, and the absence from the reaction product of ninhydrin-positive substances other than the starting material, indicated that the amino sugar **8** was being destroyed as fast as it was being liberated. Such destruction of 4-amino-4-deoxy sugars under acid hydrolytic conditions is well known[21,22], and is presumed to proceed by dehydration processes through the furanoid form, leading to pyrrole derivatives which subsequently polymerize.

EXPERIMENTAL

General methods. — Solutions were evaporated below 50° under diminished pressure. Melting points were determined with a Thomas–Hoover melting-point apparatus (Arthur H. Thomas Co., Philadelphia, Pennsylvania), and are uncorrected. Specific rotations were measured in a 2-dm tube. I.r. spectra were measured with a Perkin–Elmer Model 137 "Infracord" i.r. spectrophotometer. N.m.r. spectra were measured at 60 MHz with a Varian A-60 n.m.r. spectrometer. Chemical shifts are given on the τ-scale, and, unless otherwise stated, spectra were measured at ~40° with solutions (~10%) in chloroform-*d*, with tetramethylsilane ($\tau = 10.00$) as the internal standard. Spectral analyses are first-order, and the *J* values recorded refer to the observed splittings. Microanalyses were determined 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: m, moderate; s, strong; w, weak; v, very. The strongest lines are numbered (1, strongest), and double numbers indicate approximately equal intensities. T.l.c. was performed with Silica Gel G (E. Merck, Darmstadt, Germany), activated at 110°, as the adsorbent. Unless otherwise stated, the adsorbent layer was 250 μm thick, the developing solvent was 3:1 chloroform–ether, and indication was effected with sulfuric acid. Paper chromatography was performed on Whatman No. 1 paper by the descending method, with 5:5:3:1 pyridine–ethyl acetate–water–acetic acid according to the procedure of Fischer and Nebel[14], and indication was effected with ninhydrin and with alkaline silver nitrate. R_{GN} values refer to mobility relative to that of 2-amino-2-deoxy-D-glucose hydrochloride.

*1,6-Anhydro-3,4-O-isopropylidene-β-*D-lyxo-*hexopyranos-2-ulose oxime* (**2**). — To a solution of hydroxylamine hydrochloride (1.00 g) in methanol (50 ml) were added water (0.1 ml), potassium hydrogen carbonate (1.0 g), and 1,6-anhydro-3,4-*O*-isopropylidene-*β*-D-*lyxo*-hexopyranos-2-ulose[12] (**1**, 526 mg). The mixture was boiled under reflux for 30 min on a steam bath, and evaporated to dryness, and the residue was extracted with two 75-ml portions of chloroform. The extract was dried (magnesium sulfate) and evaporated to dryness. The resultant syrup crystallized

from chloroform–petroleum ether (b.p. 30–60°) to give **2** as a white powder, yield 414 mg (75%), m.p. 139–140°, $[\alpha]_D^{20}$ −29 ±2° (*c* 1, chloroform); R_F 0.85 (t.l.c.); λ_{max}^{KBr} 2.95 (OH), and 7.25 μm (CMe_2); n.m.r. data: τ 1.17 (1-proton broadened singlet, disappears on deuteration, OH of oxime), τ 4.33 (1-proton singlet, H-1), τ 4.78 (1-proton doublet of narrow multiplets), 5.29–5.52 (2-proton multiplet) (H-3,4,5), τ 5.78 (1-proton doublet of narrow multiplets, $J_{6,6'}$ 8 Hz, $J_{5,6'}$ ~0.5 Hz, *endo* proton, H-6'), τ 6.12–6.38 (1-proton multiplet, *exo* proton, H-6), τ 8.41, 8.53 (3-proton singlets, CMe_2).

Anal. Calc. for $C_9H_{13}NO_5$: C, 50.23; H, 6.09; N, 6.51. Found: C, 50.40; H, 6.24; N, 6.70.

*2-Amino-1,6-anhydro-2-deoxy-β-*D*-talopyranose hydrochloride* (**3**). — To a mixture of 95% ethanol (25 ml) and conc. hydrochloric acid (0.3 ml) were added platinum oxide (0.10 g) and the 2-oxime[12] **2** (510 mg). The mixture was shaken under hydrogen at 50 lb.in^{-2} pressure for 16 h at room temperature. The catalyst was removed by filtration, and the solution was evaporated to give **3**, as a colorless syrup, in essentially quantitative yield; λ_{max}^{film} 2.9–3.0 (OH), 3.20, 6.30, and 6.68 μm (NH_3^+). The product was chromatographically homogeneous, R_{GN} 1.93 (papergram), and gave positive reactions with ninhydrin and with alkaline silver nitrate. The product was clearly distinguishable from a reference sample of 2-amino-1,6-anhydro-2-deoxy-β-D-galactopyranose*; the latter had R_{GN} 1.72 (papergram).

*Hydrolysis of 2-amino-1,6-anhydro-2-deoxy-β-*D*-talopyranose hydrochloride* (**3**). — The syrupy product from the preceding experiment in M hydrochloric acid was heated for 2 h on a steam bath. The solution was evaporated (coevaporation with propyl alcohol and toluene) to give a white, crystalline residue, yield 155 mg, m.p. 170–180° (dec.). Paper-chromatographic examination of this product revealed a major, ninhydrin-positive component, R_{GN} 1.07, chromatographically indistinguishable from 2-amino-2-deoxy-D-talose hydrochloride[17] (**4**), and a minor product, R_{GN} 1.93, indistinguishable from the anhydro sugar **3**, the intensity of which was ~15% of that of the main product. No additional components were detected by alkaline silver nitrate, and no zone corresponding to 2-amino-2-deoxy-D-galactose hydrochloride (R_{GN} 0.88, papergram) was present. Recrystallization of the product from ethanol gave 2-amino-2-deoxy-α-D-talopyranose hydrochloride (**4**), X-ray powder diffraction data: 7.26 m, 6.39 vw, 5.42 vw, 5.18 s (1,1), 4.92 w, 4.67 s (1,1), 4.18 vw, 4.07 s (2,2), 3.66 m, 3.37 s (2,2), 3.12 m. The X-ray diffractogram was indistinguishable from that of an authentic sample[17].

The n.m.r. spectrum of a solution of **4** at mutarotational equilibrium in deuterium oxide (sodium 4,4-dimethyl-4-silapentane-1-sulfonate as internal standard) showed the H-1*e* signal[18,19] at τ 4.62 as a narrow, unresolved multiplet, $J_{1e,2} < 1$ Hz, and the H-1*a* signal[18,19] at τ 4.82 as a narrow doublet, $J_{1a,2}$ ~1.5 Hz; the intensities of the two signals were in the approximate ratio of 1:2.

*4-Amino-1,6-anhydro-4-deoxy-β-*D*-talopyranose hydrochloride* (**6**). — To a

*The authors thank Dr. R. W. Jeanloz for a reference sample of this compound [15,16].

mixture of 95% ethanol (25 ml), M hydrochloric acid (0.25 ml), and platinum oxide (50 mg) was added 1,6-anhydro-2,3-*O*-isopropylidene-β-D-*lyxo*-hexopyranos-4-ulose oxime[11] (**5**, 217 mg), and the mixture was shaken under hydrogen at 50 lb.in^{-2} pressure for 6 h at room temperature. The catalyst was removed by filtration, hydrochloric acid (20 ml) was added to the filtrate, and after 3h at 25° the filtrate was evaporated to a colorless syrup; this was dissolved in hot abs. ethanol, toluene was added, and the ethanol was allowed to evaporate slowly. The product **6** crystallized as flakes when the solution was cooled; yield 152 mg (77%), m.p. 170–185° (dec.); $[\alpha]_D^{20}$ −66 ±1° (*c* 1, water); R_F 0.75 (papergram, 54:27:19 butyl alcohol–ethanol–water, ninhydrin spray); λ_{max}^{KBr} 2.9–3.1 (OH), 6.30 and 6.70 μm (NH_3^+); X-ray powder diffraction data: 7.85 w, 6.39 m, 5.47 w, 4.93 vs (2,2), 4.60 vs (2,2), 4.37 vw, 4.15 s (3), 3.92 m, 3.74 vs (2,2), 3.34 m, 3.12 m, 2.97 vs (1), 2.83 w, 2.76 w, 2.66 m.

Anal. Calc. for $C_6H_{12}ClNO_4$: C, 36.46; H, 6.12; Cl, 17.94; N, 7.09. Found: C, 36.41; H, 6.16; Cl, 18.18; N, 7.33.

*4-Acetamido-2,3-di-*O*-acetyl-1,6-anhydro-4-deoxy-β-*D*-talopyranose* (**7**). — A mixture of acetic anhydride (8 ml), anhydrous sodium acetate (50 mg), and 4-amino-1,6-anhydro-4-deoxy-β-D-talopyranose hydrochloride (**6**, 105 mg) was heated for 30 min on a steam bath, and then boiled for 1 min. The reaction mixture was poured into ice–water (25 ml), and the stirred solution was allowed to warm to room temperature. The solution was extracted with four 75-ml portions of chloroform, and the chloroform extracts were combined, dried (magnesium sulfate), and evaporated to a pale-yellow syrup. A solution of the syrup in ether crystallized on slow evaporation, to give **7** as long needles, yield 75 mg (60%), m.p. 140–140.5°, $[\alpha]_D^{20}$ −85 ±2° (*c* 2.0, chloroform); R_F 0.15 (t.l.c.); λ_{max}^{KBr} 3.10 (NH), 5.75 (OAc), 6.0–6.1, and 6.50 μm (NHAc); n.m.r. data: τ 4.0–4.2 (1-proton broad doublet, disappears on deuteration, NH), τ 4.56 (1-proton multiplet, H-3), τ 4.68 (1-proton, narrow doublet overlapping H-3 signal, H-1), τ 5.10 (1-proton, sharp quartet, $J_{1,2}$ 1.7 Hz, $J_{2,3}$ 4.6 Hz, H-2), τ 5.32–5.62 (2-proton multiplet, appearance changes on deuteration, H-4,5), τ 5.75 [1-proton doublet, $J_{6,6'}$ 7.6 Hz, $J_{5,6'} \leqslant 0.5$ Hz, H-6′ (*endo* proton at C-6)], τ 6.15-6.40 [1-proton multiplet, H-6 (*exo* proton at C-6)], τ 7.82 (3-proton singlet, 3-OAc), τ 7.98, 8.00 (3-proton singlets, 2-OAc, NAc).

Anal. Calc. for $C_{12}H_{17}NO_7$: N, 4.88. Found: N, 5.02.

*Attempted hydrolysis of 4-amino-1,6-anhydro-4-deoxy-β-*D*-talopyranose hydrochloride* (**6**). — A solution of **6** (40 mg) in M hydrochloric acid (20 ml) was boiled under reflux for 3 h and then evaporated (codistillation with propyl alcohol). Addition of toluene to the resultant brown syrup caused separation of a crystalline product, yield 28 mg (70%), shown to be the pure starting material **6** by t.l.c. on microcrystalline cellulose[23] (5:5:3:1 pyridine–ethyl acetate–water–acetic acid, detection with ninhydrin) and by a comparative i.r. spectrum. The uncrystallized portion of the reaction product was found to contain a component having the chromatographic characteristics of **6**, and a number of other products that were detected by alkaline silver nitrate, but there was no other ninhydrin-positive component.

The experiment was repeated, but with 4M hydrochloric acid and a heating period of 2 h. The solution became very dark and gave a black syrup after evaporation. No ninhydrin-positive component could be detected in this product by t.l.c.

REFERENCES

1 D. HORTON AND J. S. JEWELL, *Carbohyd. Res.*, 3 (1966) 255.
2 A. K. CHATTERJEE, A. E. EL ASHMAWY, D. HORTON, J. S. JEWELL, LIGAYA G. MAGBANUA, W. E. MAST, KERSTIN D. PHILIPS, AND J. D. WANDER, *Abstracts Papers Am. Chem. Soc. Meeting*, 154 (1967) 19D.
3 A. E. EL ASHMAWY AND D. HORTON, *Carbohyd. Res.*, 1 (1965) 164; 3 (1967) 191.
4 K. FREUDENBERG, O. BURKHART, AND E. BRAUN, *Ber.*, 59 (1926) 714; M. L. WOLFROM, F. SHAFIZADEH, R. K. ARMSTRONG, AND T. M. SHEN HAN, *J. Amer. Chem. Soc.*, 80 (1959) 3716; D. HORTON, M. L. WOLFROM, AND A. THOMPSON, *J. Org. Chem.*, 26 (1961) 5069.
5 M. L. WOLFROM, J. BERNSMANN, AND D. HORTON, *J. Org. Chem.*, 27 (1962) 4505; E. J. REIST, R. R. SPENCER, B. R. BAKER, AND L. GOODMAN, *Chem. Ind.* (London), (1962) 1794.
6 F. D. CRAMER, *Methods Carbohyd. Chem.*, 1 (1962) 242; D. HORTON AND A. E. LUETZOW, *Carbohyd. Res.*, 7 (1968) 101.
7 D. HORTON, J. S. JEWELL, AND H. S. PRIHAR, *Can. J. Chem.*, 46 (1968) in press.
8 A. K. CHATTERJEE, D. HORTON, AND J. S. JEWELL, *Carbohyd. Res.*, 6 (1968) 212.
9 B. LINDBERG AND O. THEANDER, *Acta Chem. Scand.*, 13 (1959) 1226; *cf.* D. HORTON, in R. W. JEANLOZ and E. A. Balazs, (Eds.), *The Amino Sugars*, Vol. IA, Academic Press Inc., New York, 1968 *in press.*
10 J. D. ALBRIGHT AND L. GOLDMAN, *J. Amer. Chem. Soc.*, 87 (1965) 4214; B. LINDBERG AND K. N. SLESSOR, *Carbohyd. Res.*, 1 (1966) 492; W. SOWA AND G. H. S. THOMAS, *Can. J. Chem.*, 44 (1966) 836.
11 D. HORTON AND J. S. JEWELL, *Carbohyd. Res.*, 2 (1966) 251.
12 D. HORTON AND J. S. JEWELL, *Carbohyd. Res.*, 5 (1967) 149.
13 D. HORTON, M. NAKADATE, AND J. M. J. TRONCHET, *Carbohyd. Res.*, 7 (1968) 56.
14 F. G. FISCHER AND H. J. NEBEL, *Z. Physiol. Chem.*, 302 (1955) 10.
15 S. P. JAMES, F. SMITH, M. STACEY, AND L. F. WIGGINS, *J. Chem. Soc.*, (1946) 625.
16 R. W. JEANLOZ, D. M. SCHMID, AND P. J. STOFFYN, *J. Amer. Chem. Soc.*, 79 (1957) 2586.
17 R. KUHN AND H. FISCHER, *Ann.*, 612 (1958) 65.
18 D. HORTON, J. S. JEWELL, AND KERSTIN D. PHILIPS, *J. Org. Chem.*, 31 (1966) 3843.
19 D. HORTON, J. S. JEWELL, AND KERSTIN D. PHILIPS, *J. Org. Chem.*, 31 (1966) 4022.
20 D. HORTON, J. B. HUGHES, J. S. JEWELL, KERSTIN D. PHILIPS, AND W. N. TURNER, *J. Org. Chem.*, 32 (1967) 1073.
21 E. J. REIST, D. E. GUEFFROY, AND L. GOODMAN, *J. Amer. Chem. Soc.*, 87 (1965) 677.
22 S. W. GUNNER, W. G. OVEREND, AND N. R. WILLIAMS, *Carbohyd. Res.*, 4 (1967) 498.
23 M. L. WOLFROM, D. L. PATIN, AND ROSA M. DE LEDERKREMER, *J. Chromatog.*, 17 (1965) 488.

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SYNTHESIS OF *o*-NITROPHENYL β-D-GALACTOSIDE 6-PHOSPHATE, A SUBSTRATE OF THE STAPHYLOCOCCAL β-D-GALACTOSIDASE

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ABSTRACT

o-Nitrophenyl β-D-galactoside 6-phosphate has been synthesized. It is a substrate for the staphylococcal β-D-galactosidase, but is not a substrate for the crystalline β-D-galactosidase of *E. coli*.

INTRODUCTION AND DISCUSSION

Recently, the staphylococcal β-D-galactosidase was characterized as an enzyme that cleaves the glycosidic bond in phosphorylated β-D-galactosides[1,2], but the position of the phosphate on the D-galactose moiety has not yet been clarified. Also, a substrate was not available for use in a simple colorimetric test for this new enzyme.

Assuming that the phosphate is bound at the 6-position of the D-galactose glycone in a substrate for the staphylococcal β-D-galactosidase, we synthesized the hitherto unknown *o*-nitrophenyl β-D-galactoside 6-phosphate (ONPG-6-P). The ONPG-6-P is split by the staphylococcal β-D-galactosidase, but not by the crystalline β-D-galactosidase of *Escherichia coli*.

The first step in the synthesis was to prepare an *o*-nitrophenyl β-D-galactoside (ONPG) having protected hydroxyl groups, except at the primary position. Applying the method of Hurst and McInnes[3], methanolysis (employing a slightly basic or acidic catalyst) of fully trimethylsilylated ONPG gave chromatographically homogeneous, crystalline *o*-nitrophenyl 2,3,4-tri-*O*-(trimethylsilyl)-β-D-galactoside (**1**) in good yield. Evidence that compound **1** contained a free primary hydroxyl group was obtained by acetylation, followed by removal of the trimethylsilyl groups, which gave known[4] *o*-nitrophenyl 6-*O*-acetyl-β-D-galactoside.

The hydroxyl group at position 6 in compound **1** was phosphorylated with cyanoethylphosphoric acid (2-cyanoethyl dihydrogen phosphate) and dicyclohexylcarbodiimide[5]. The trimethylsilyl groups were removed by treatment in a slightly acidic methanol–water mixture at 25° to give 71% of alkali-labile ONPG and its derivatives, 87% of which was cyanoethylphosphorylated. Ammonia at 55° splits the cyanoethylphosphoric acid ester bond of the diester. The alkaline conditions applied resulted in very little splitting of the glycosidic bond. The removal of the cyanoethyl group from the sugar-bound phosphoric acid was monitored by thin-layer

chromatography, and the crude product resulting from this reaction sequence was pure enough to be used as a testing substrate for staphylococcal β-D-galactosidase. The above synthetic method should be applicable to 6-phosphates of other hexosides.

EXPERIMENTAL

Thin-layer chromatography (t.l.c.) was performed on cellulose MN300 (Macherey and Nagel) and Silicagel G and F (Merck).

Paper chromatography was performed on Whatman 3MM paper. Crystalline β-D-galactosidase was a gift of Prof. Dr. K. Wallenfels. Staphylococcal β-D-galactosidase was a crude enzyme-preparation made by sonicating cells of a β-D-galactosidase constitutive strain in the presence of glass beads. The extract had no activity toward *o*-nitrophenyl β-D-galactoside.

Analytical procedures. — ONPG was determined quantitatively with the crystalline β-D-galactosidase of *E. coli* and a 0.1M tris buffer, pH 7.6, containing 0.05M NaCl and 0.05M $MgCl_2$. After incubation for 10 h at 25°, *o*-nitrophenol (ONP) was measured[6] at 405 nm in a Beckman DB spectrophotometer.

o-Nitrophenyl β-D-galactoside 6-phosphate (ONPG-6-P) was determined with crude staphylococcal β-D-galactosidase or by a combination of alkaline phosphatase and *E. coli*-β-D-galactosidase in the same buffer used for the ONPG assay.

The total amount of alkali-labile ONPG and its derivatives was estimated by saponification with 10N NaOH overnight at 25°. The reaction mixture was then neutralized with conc. HCl and diluted to a suitable absorbance with 0.33M Na_2CO_3. The extinction coefficient[7] of ONP in sodium carbonate is 4.8×10^6 cm^2 · mole^{-1} at 415 nm.

The phosphate determination was carried out according to Marinetti[8].

Chromatography. — T.l.c. of ONPG and its phosphorylated derivatives was performed with cellulose powder and butyl alcohol–pyridine–water (6:4:3). Trimethylsilylated ONPG and its derivative were separated on Silica Gel G or F with ether–hexane (2:1). Paper chromatography of ONPG and its phosphorylated derivatives was made on Whatman 3MM paper with the butyl alcohol–pyridine–water solvent. The spots were detected under ultraviolet light, cut out, and eluted with water.

o-Nitrophenyl 2,3,4-tri-O-(trimethylsilyl)-β-D-galactopyranoside. — ONPG (2 g) was treated[9] with 12 ml of hexamethyldisilazane and 8 ml of chlorotrimethylsilane in 50 ml of pyridine overnight at 25°. The solution was evaporated under diminished pressure, the residue was mixied with in dry hexane, and pyridine hydrochloride and NH_4Cl were removed by filtration. After evaporation, the colorless syrup (t.l.c., R_F 0.7, ether–hexane), presumably *o*-nitrophenyl 2,3,4,6-tetra-*O*-(trimethylsilyl)-β-D-galactopyranoside, was used without further purification.

The foregoing syrup was dissolved in 140 ml of dry methanol. The cooled (ice-bath) solution was treated with 17 ml of 0.8mM K_2CO_3 in 25 ml of methanol and left for 45 min at 0°. T.l.c. showed one spot, R_F 0.3 (ether–hexane). The solution was carefully neutralized with acetic acid, and crystallisation occurred during the evapor-

ation of the methanol. After recrystallisation from a small amount of methanol, the product (2.3 g, 67%) had m.p. 87°, $[\alpha]_D^{22}$ −72.3° (*c* 1.22, chloroform).

Anal. Calc. for $C_{21}H_{39}NO_8Si_3$: C, 48.71; H, 7.59; N, 2.71. Found: C, 48.20; H, 7.64; N, 2.80.

o-*Nitrophenyl β-*D*-galactopyranoside 6-cyanoethylphosphate.* To 8 mmoles of 2-cyanoethyl dihydrogen phosphate[5] (cyanoethylphosphoric acid) in 20 ml of pyridine was added 1 g (1.93 mmoles) of *o*-nitrophenyl 2,3,4-tri-*O*-(trimethylsilyl)-*β*-D-galactopyranoside and 3.4 g of dicyclohexylcarbodiimide. The mixture was left for two days at 25° in a well-stoppered flask. Water (10 ml) was then added, and the mixture was left for 30 min. After evaporation, 300 ml of methanol, 50 ml of water, and 2 ml of glacial acetic acid were added, and the solution was stirred overnight at 25°. After evaporation to a small volume, *N,N'*-dicyclohexylurea was removed by filtration. T.l.c. on cellulose showed essentially one spot (R_F 0.4) and a trace of ONPG (R_F 0.8). Total ONPG and cyanoethylphosphorylated ONPG was 1.35 mmoles; 87% of the alkali-labile products had been cyanoethylphosphorylated.

o-*Nitrophenyl-β-*D*-galactopyranoside 6-phosphate.* Syrupy *o*-nitrophenyl *β*-D-galactopyranoside 6-cyanoethylphosphate was dissolved in ammonium hydroxide containing 20% of NH_3 and heated for 2 h at 55°. During the reaction, a yellow color developed due to some splitting of the glycosidic bond. The ammonia was removed by evaporation under diminished pressure. T.l.c. showed essentially one spot (R_F 0.24), some unconverted ONPG, very little cyanoethylphosphorylated product, and a small amount of an unidentified substance having R_F 0.06.

Analysis for total ONPG and derivatives, 1.25 mmoles; analysis for ONPG-6-P, 0.93 mmole (74%), equivalent to a 48% yield. Further purification of the product was done by paper chromatography to yield a homogeneous preparation. A sample was analyzed for ONPG-6-P and phosphate. Both methods for assaying ONPG-6-P gave identical values. A phosphorus determination gave 1.1 mole per mole of ONPG-6-P. The product had $[\alpha]_D^{22}$ −57.1° (*c* 1.4, water), calculated for the free acid.

o-*Nitrophenyl 6-*O*-acetyl-β-*D*-galactopyranoside.* — A sample of 2,3,4-tri-*O*-(trimethylsilyl)-*β*-D-galactopyranoside was heated in a boiling water-bath with 10% excess of acetic anhydride in pyridine for 1 h. The solvents were evaporated, and the trimethylsilyl groups were removed by boiling for 4 h in a methanol–water mixture[3]. The product (> 90%) crystallized readily from water and had m.p. 155–156°, $[\alpha]_D^{22}$ −24.7° (*c* 0.43, water); lit.[4], m.p. 155°, $[\alpha]_D^{22}$ −26.2° (*c* 1.5, water).

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REFERENCES

1 W. HENGSTENBERG, J. B. EGAN, AND M. L. MORSE, *Proc. Natl. Acad. Sci. U.S.*, 58 (1967) 274.
2 E. P. KENNEDY AND G. A. SCARBOROUGH, *Proc. Natl. Acad. Sci. U.S.*, 58 (1967) 225.
3 D. T. HURST AND A. G. MCINNES, *Can. J. Chem.*, 43 (1965) 2004; A. G. MCINNES, *ibid.*, 43 (1965) 1998.
4 Y. Z. FROHWEIN, *Nature*, 196 (1962) 775.
5 G. M. TENER, *J. Am. Chem. Soc.*, 83 (1961) 159.
6 K. WALLENFELS, O. P. MALHOTRA, AND D. DABICH, *Biochem. Z.*, 333 (1960) 377.
7 L. DOUB AND T. M. VANDENBELT, *J. Am. Chem. Soc.*, 71 (1949) 2414.
8 G. V. MARINETTI, *J. Lipid Res.*, 3 (1962) 9.
9 C. C. SWEELEY, R. BENTLEY, M. MAKITA, AND W. W. WELLS, *J. Am. Chem. Soc.*, 85 (1963) 2497.

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SOME 7-(β-D-GLUCOPYRANOSYLOXY)COUMARINS FOR USE AS FLUOROGENIC SUBSTRATES

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ABSTRACT

Several 7-(β-D-glucopyranosyloxy) derivatives of 3-substituted coumarins were synthesized as potential substrates for the enzyme β-D-glucosidase. Two of these were prepared by Knoevenagel condensation of 4-(β-D-glucopyranosyloxy)-2-hydroxybenzaldehyde with ethyl acetoacetate and ethyl cyanoacetate, respectively. One of these, 3-acetyl-7-(β-D-glucopyranosyloxy)coumarin, was hydrolyzed by β-D-glucosidase at a rate 1.9 times that of 7-(β-D-glucopyranosyloxy)-4-methylcoumarin. This behavior provides a method potentially more sensitive than established procedures for the measurement of β-D-glucosidase activity.

INTRODUCTION

Synthetic substrates that release a fluorescent moiety in enzymic reactions have been used for the study of many hydrolytic enzymes. Among such enzymes are β-D-glucuronidase[1], arylsulfatase[2], and β-D-glucosidase[3]. A highly sensitive procedure for the measurement of β-D-glucosidase has been described by Robinson[3], who prepared 7-(β-D-glucopyranosyloxy)-4-methylcoumarin and showed that it is cleaved to 7-hydroxy-4-methylcoumarin by the enzyme. While trying to measure β-D-galactosidase and β-D-glucuronidase activities in single nerve-cells by using the D-galactoside and D-glucuronoside of 7-hydroxy-4-methylcoumarin[4] as substrates, it was found that the β-D-glucosidase activity, measured by using Robinson's substrate, was too low to allow accurate determination. We then decided to investigate ways by which the sensitivity of this method could be increased. In a parallel study[5], we have prepared a series of 3-substituted 7-hydroxycoumarins that have fluorescence intensities up to 3.6 times that of 7-hydroxy-4-methylcoumarin. We intended to utilize this increased fluorescence by preparing the β-D-glucopyranosides of the 3-substituted coumarins, thus obtaining a more sensitive method for the measurement of β-D-glucosidase. However, the excitation and fluorescence wavelengths of the 3-substituted 7-hydroxycoumarins are such that they evoke excessive light-scattering in the solvent, increasing the instrumental blank by an amount equal to the increase in fluorescence intensity that these substances display. Despite this behavior, the β-D-glucopyranosides of the 3-substituted 7-hydroxycoumarins are useful, in that at least one of them is

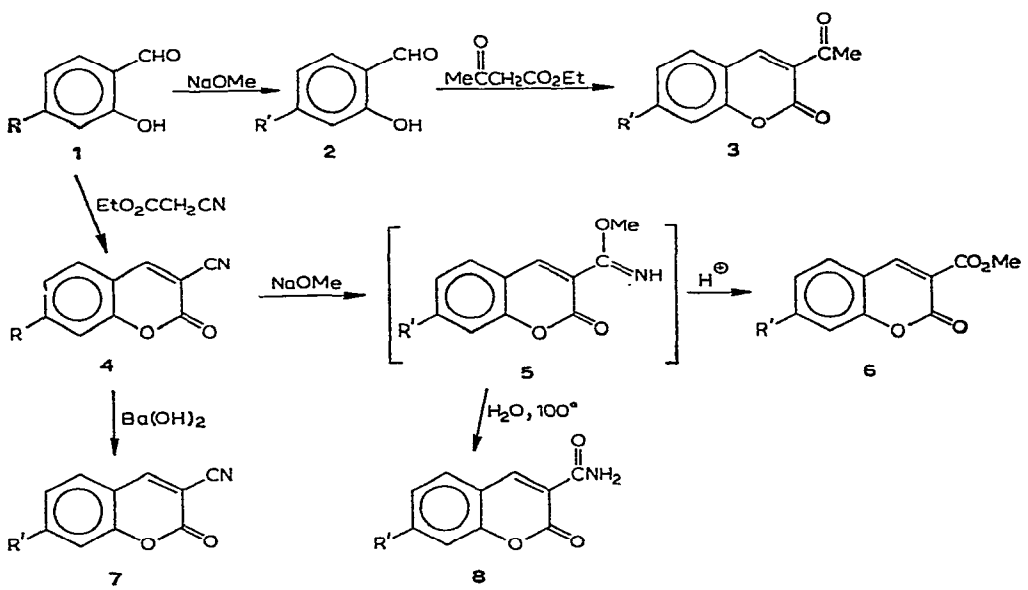
acted on by β-D-glucosidase at a rate 1.9 times that observed with 7-(β-D-glucopyranosyloxy)-4-methylcoumarin.

In this paper, we describe the preparation and fluorescence-spectral properties of five new coumarin D-glucopyranosides, and the enzymic rates of hydrolysis of two of them.

RESULTS AND DISCUSSION

We first tried to prepare the β-D-glucopyranosides of 3-substituted 7-hydroxycoumarins by direct condensation of tetra-*O*-acetyl-α-D-glucopyranosyl bromide with the 3-cyano-, 3-acetyl-, and 3-(ethoxycarbonyl) derivatives of 7-hydroxycoumarin. This method, which Robinson[3] used to prepare 7-(β-D-glucopyranosyloxy)-4-methylcoumarin, was found to be useful only for the preparation of 3-(ethoxycarbonyl)-7-(tetra-*O*-acetyl-β-D-glucopyranosyloxy)coumarin. The yield in this reaction was only 10%, apparently because of low reactivity of the hydroxycoumarin, as half of the starting coumarin was recovered. 3-Acetyl- and 3-cyano-7-hydroxycoumarin gave no product in this reaction.

3-(Ethoxycarbonyl)-7-(tetra-*O*-acetyl-β-D-glucopyranosyloxy)coumarin was transesterified and deacetylated by the action of sodium methoxide in methanol to give a 60% yield of 7-(β-D-glucopyranosyloxy)-3-(methoxycarbonyl)coumarin (**6**). Treatment of the D-glucoside tetraacetate with barium hydroxide deacetylated the tetra-*O*-acetyl-D-glucose residue and also saponified the 3-(ethoxycarbonyl) group, to give 3-carboxy-7-(β-D-glucopyranosyloxy)coumarin in 20% yield.



R = tetra-*O*-acetyl-β-D-glucopyranosyloxy
R′ = β-D-glucopyranosyloxy

In order to prepare 3-acetyl- and 3-cyano-7-(β-D-glucopyranosyloxy)coumarins, another route was taken. It was found that ethyl acetoacetate undergoes the Knoevenagel reaction with 4-(β-D-glucopyranosyloxy)-2-hydroxybenzaldehyde (**2**) to give 3-acetyl-7-(β-D-glucopyranosyloxy)coumarin (**3**). When this reaction was tried with ethyl cyanoacetate, mixtures were obtained which appeared, on t.l.c., to contain the product, but which did not yield the crystalline D-glucoside. It was found that 3-cyano-7-(tetra-*O*-acetyl-β-D-glucopyranosyloxy)coumarin (**4**) could be obtained in 50% yield by the cyclization of ethyl cyanoacetate with 2-hydroxy-4-(tetra-*O*-acetyl-β-D-glucopyranosyloxy)benzaldehyde (**1**). Treatment of **4** with sodium methoxide in methanol gave an intermediate which, although not isolated, was believed to be the iminoether **5**. When **5** was warmed with dilute acetic acid, the ester **6** was formed in 22% yield. When **5** was boiled with water, 3-carboxamido-7-(β-D-glucopyranosyloxy)-coumarin (**8**) was obtained in 39% yield. 3-Cyano-7-(β-D-glucopyranosyloxy)-coumarin (**7**) was finally obtained by deacetylation of **4** with barium hydroxide.

The structures of the D-glucopyranosides were confirmed in each case by comparison of the infrared spectrum of the D-glucoside with that of the corresponding 7-methoxycoumarin. In every instance (see Experimental), the spectra of the pair showed close correspondence. Elemental analyses were satisfactory for all but the 3-carboxycoumarin D-glucopyranoside, which repeatedly gave erratic results.

TABLE I

EXCITATION, FLUORESCENCE, AND ULTRAVIOLET ABSORPTION MAXIMA OF 7-HYDROXYCOUMARINS COMPARED WITH THOSE OF THEIR β-D-GLUCOPYRANOSIDES

7-Hydroxycoumarin	*7-(β-D-glucopyranosyloxy)coumarin*	*Excitation* (λ_{max}, *nm*)	*Fluorescence* (λ_{max}, *nm*)	*Absorption* (λ_{max}, *nm*)
4-Methyl-		367	449	359
	4-methyl-[a]	331	380	314
3-Carboxy-[b]		396	450	385
	3-carboxy-	359	402	329
3-(Carboxamido)-[c]		398	445	400
	3-(carboxamido)-	355	398	340
3-Acetyl-[d]		419	458	413
	3-acetyl-	377	421	350
3-(Ethoxycarbonyl)-[b]		398	445	402
	3-(ethoxycarbonyl)-	361	404	342
3-Cyano-[d]		408	450	407
	3-cyano-	360	407	265, 344

[a]See ref 3. [b]See ref. 7. [c]See ref. 5. [d]See ref. 8.

The fluorescence and absorption properties of the coumarin D-glucosides are compared with those of their aglycons in Table I. The values show that each of the D-glucosides has excitation and fluorescence maxima that occur at wavelengths shorter than those for the corresponding maxima of the aglycon. Thus, the excitation and fluorescence of the aglycon can be resolved satisfactorily in each case from that of

the D-glucoside by use of a fluorescence spectrometer. It is also clear from the Table that the absorption maxima of the D-glucosides are at a sufficiently short wavelength that they do not absorb light of the excitation wavelength for the aglycon. Since the D-glucoside may be used as a substrate at mM concentration, such a filter effect might, in an adverse situation, be great.

The D-glucosides of 3-substituted coumarins have limited stability in aqueous solution, even at pH 7.4. Solutions, initially free from aglycon, kept overnight and then examined by a fluorescence spectrometer are observed to contain the aglycon. This hydrolytic formation of the hydroxycoumarin is accelerated at high pH values. The instability of D-glucosides of phenol toward base is known[6], and the D-glucosides of 3-substituted coumarin appear to have this property. Because of this instability, there is a limitation on the amount of time that should be used in incubation with an enzyme. However, the instability of D-glucosidases is actually a more limiting factor than that of the substrates.

In order to obtain the highest sensitivity in enzyme assays, it is necessary to lessen to a minimum the contamination of coumarin D-glucosides by the aglycon. We have found that 7-(β-D-glucopyranosyloxy)-4-methylcoumarin can be obtained in very pure form by recrystallization from pyridine–heptane. We have obtained this D-glucoside exhibiting a fluorescence, on an equimolar basis, 5.5×10^{-5} that of 7-hydroxy-4-methylcoumarin, when both are examined with the excitation and fluorescence wavelengths of the aglycon. Crystallization of the 3-substituted coumarin D-glucosides from water, accompanied by treatment with charcoal, results in a high degree of purification. We have obtained the following fluorescence ratios of D-glucoside to aglycon by this means: 3-acetyl, 4.0×10^{-5}; 3-methoxycarbonyl, 4.3×10^{-5}; and 3-carboxy, 9.1×10^{-5}. The last value is high, because of the difficulty of separating the aglycon from the D-glucoside by crystallization. Another purification procedure which we have found useful is chromatography on Florisil columns, with a methanol gradient in chloroform as eluant. This technique effects complete separation of the aglycon from the D-glucoside.

We have found that the 3-acetyl and 3-(methoxycarbonyl) derivatives of 7-hydroxycoumarin are released from the corresponding D-glucosides by the β-D-glucosidase present in rat brain. By using a homogenate diluted 100-fold, and incubating at 37° and pH 4.7, the aglycon was formed at the following rates from the D-glucosides stated: 4-methyl-, 1.3 mmole.kg^{-1} h^{-1}; 3-(methoxycarbonyl)-, 1.1 mmole.kg^{-1} h^{-1}; 3-acetyl-, 2.5 mmole kg^{-1} h^{-1} (all weights on a wet basis). The enzyme activity with all of the substrates has a pH optimum of 4.7. It thus seems that the rates of hydrolysis of certain of the 7-(β-D-glucopyranosyloxy)-3-substituted coumarins may provide an analytical advantage over the 4-methyl compound. A complete study of the rates of hydrolysis of these substrates will be presented in the future.

EXPERIMENTAL

Melting points were obtained on a Fisher–Johns block and are uncorrected.

I.r. spectra were recorded for Nujol mulls, or for solutions (in 0.1-mm sodium chloride cells), with a Perkin–Elmer 237-B spectrophotometer. Thin-layer chromatograms were read by u.v. light. Microanalyses were performed by Micro-Tech Laboratories, Skokie, Illinois.

*3-(Ethoxycarbonyl)-7-(tetra-*O*-acetyl-β-*D*-glucopyranosyloxy)coumarin.* — To a solution of 3-(ethoxycarbonyl)-7-hydroxycoumarin[7] (23.4 g, 0.10 mole) in acetone (500 ml) was added tetra-*O*-acetyl-α-D-glucopyranosyl bromide (12.3 g, 0.15 mole) followed by M sodium hydroxide (300 ml). The solution was stirred overnight at room temperature, and most of the solvent was then removed under diminished pressure with a rotary evaporator. The resulting precipitate was collected, and washed twice with M sodium hydroxide (70 ml) and once with water. The residue that remained was crystallized from ethanol, to give 5.4 g (9.6%) of crude product, m.p. 173–177°. If desired up to 53% of the starting coumarin could be recovered by acidification of the filtrate obtained from the extraction with base.

For analysis, the product was crystallized from ethanol to give fine white needles, m.p. 179°. T.l.c. in 3:7 ethyl acetate–benzene gave one spot, R_F 0.2; in 4:1:5 butyl alcohol–ethanol–water, a spot at R_F 0.7.

Anal. Calc. for $C_{26}H_{28}O_{14}$: C, 55.3; H, 5.00. Found: C, 55.2; H, 5.05.

7-β-D-Glucopyranosyloxy-3-(methoxycarbonyl)coumarin (**6**). — (*a*) To a suspension of 3-(ethoxycarbonyl)-7-(tetra-*O*-acetyl-β-D-glucopyranosyloxy)coumarin (3.4g, 6 mmoles) in anhydrous methanol (300 ml) was added 0.1M methanolic sodium methoxide (90 ml) with stirring at room temperature. After the suspended D-glucoside had dissolved, the solution was refrigerated overnight. The solution was then neutralized with 0.1M hydrochloric acid (90 ml), and the methanol was removed under diminished pressure. The material that separated was crystallized from water, to give 1.4 g (60%) of **6**, m.p. 151–156°, which was recrystallized from 85% methanol to a constant m.p. of 153–157°. T.l.c. on silica gel G in 4:1:5 butyl alcohol–ethanol–water gave a trace of material at R_F 0.07, and a main spot at R_F 0.6. For analysis, the sample was dried at 60–70° *in vacuo*. The material obtained in this way gave the correct analysis for a hemihydrate.

Anal. Calc. for $C_{17}H_{18}O_{10}\cdot 0.5\ H_2O$: C, 52.3; H, 4.85. Found: C, 52.0; H, 4.82.

When dried at 160°, the sample lost 2.3% of its weight, and gave analytical values correct for the anhydrous compound.

Anal. Calc. for $C_{17}H_{18}O_{10}$: C, 53.4; H, 4.75. Found: C, 53.5; H, 4.90.

The i.r. spectrum of the hemihydrate in methyl sulfoxide solution bore a close resemblance to that of 7-methoxy-3-(methoxycarbonyl)coumarin[9], providing additional support for the structures proposed. Strong and moderate bands were given in the region below 2000 cm^{-1}, where the solvent is transparent: D-glucoside **6**, 1765, 1750, 1610, 1220, and 995 cm^{-1}; 7-methoxy-3-(methoxycarbonyl)coumarin[7]; 1760, 1745, 1605, and 1215 cm^{-1}.

(*b*) *From 3-cyano-7-(tetra-*O*-acetyl-β-*D*-glucopyranosyloxy)coumarin* (**4**). — The reaction was performed as in the preparation of 3-(carboxamido)-7-(β-D-glucopyranosyloxy)coumarin (**8**) through the step of neutralization with ion-exchange

resin. Following this step, the solvents were removed *in vacuo*, and the residue was dissolved in 30 ml of M acetic acid. The solution was briefly warmed on a steam-bath, and then kept overnight at 4°; the solvents were removed, and the residue was crystallized from ethanol–water. The precipitate that formed was recrystallized from water, to give the ester in 22% yield. The i.r. spectrum of the product was identical to that of authentic compound **6**.

3-Carboxy-7-(β-D-glucopyranosyloxy)coumarin. — A suspension of 3-(ethoxycarbonyl)-7-(tetra-*O*-acetyl-β-D-glucopyranosyloxy)coumarin (1.0 g, 1.8 mmole) in 0.3M barium hydroxide (200 ml) was shaken for 1 h. Carbon dioxide was passed through the resulting solution until no further precipitation occurred. The barium carbonate was filtered off, and extracted with hot water, the suspension was filtered, and the combined filtrates were passed through a column of Rexyn AG-50 (H^+) resin (Fisher Scientific Co.). Crystallization from 17:3 methanol–water gave 0.14 g (20%) of the product, m.p. 210° (dec.). T.l.c. on silica gel G in 4:1:5 butyl alcohol–acetic acid–water showed a strongly fluorescing spot at R_F 0.2 (product), which was contaminated by 3-carboxy-7-hydroxycoumarin (R_F 0.6) and, prior to crystallization, the starting D-glucoside tetraacetate (R_F 0.8). Further recrystallization did not entirely remove the free coumarin acid.

The i.r. spectra, in methyl sulfoxide solution, of 3-carboxy-7-methoxycoumarin[9] and 3-carboxy-7-(β-D-glucopyranosyloxy)coumarin show good agreement. Strong and moderate bands were observed in the region below 2000 cm^{-1}, where the solvent is transparent: D-glucoside, 1750, 1610, 1560, 1495, and 1215 cm^{-1}; 3-carboxy-7-methoxycoumarin, 1750, 1610, 1560, 1505, and 1215 cm^{-1}.

3-Acetyl-7-(β-D-glucopyranosyloxy)coumarin (**3**). — To a solution of 4-(β-D-glucopyranosyloxy)2-hydroxybenzaldehyde[10] (1.68 g, 13 mmole) in 18 ml of *N*,*N*-dimethylformamide was added ethyl acetoacetate (3.0 g, 10 mmole), absolute ethanol (120 ml), piperidine (0.6 ml), and glacial acetic acid (0.06 ml). The mixture was kept overnight at room temperature, and the yellow product that crystallized was washed with ethanol; yield 3.3 g (91%), m.p. 246–251° (dec.). Recrystallization from boiling water gave light-yellow needles, m.p. 249° (dec.). T.l.c. of recrystallized samples on silica gel G, with 4:1:5 butyl alcohol–ethanol–water, occasionally revealed contamination of the D-glucoside (R_F 0.5) by 3-acetyl-7-hydroxycoumarin (R_F 0.7). Separation of the free coumarin from the D-glucoside could be achieved by recrystallization or by column chromatography as described below, with 9:11 methanol–chloroform as the limit eluant.

Anal. Calc. for $C_{17}H_{18}O_9$: C, 55.7; H, 4.95. Found: C, 56.3; 56.2; H, 5.21, 5.16.

In order to establish the structure better, a portion of the D-glucoside (30 mg) was acetylated with acetic anhydride (0.15 ml) in pyridine (0.2 ml) for 48 h at room temperature; the product was precipitated with ice–water, and recrystallized twice from ethanol; colorless needles, m.p. 159–160°.

Anal. Calc. for $C_{25}H_{26}O_{13}$: C, 56.2; H, 4.90. Found: C, 56.5; H, 5.01.

Additional support for the structure was provided by the close similarity of the infrared spectra of the D-glucoside **3** and 3-acetyl-7-methoxycoumarin[11]. I.r. spectra

were obtained in Nujol mulls; strong and moderate bands are given for the region below 2000 cm^{-1}: D-glucoside **3**, 1725, 1670, 1615, 1590, 1550, 1500, 1275, 1215, 1160, 1135, 1110, 1075, 1040, 995, 895, 880, 820, and 770 cm^{-1}; 3-acetyl-7-methoxycoumarin, 1730, 1670, 1615, 1590, 1545, 1500, 1295, 1245, 1225, 1205, 1125, 1015, 985, 835, 765, and 725 cm^{-1}.

3-Cyano-7-(tetra-O-acetyl-β-D-glucopyranosyloxy)coumarin (**4**). — Absolute ethanol was added with warming to 2-hydroxy-4-(tetra-*O*-acetyl-β-D-glucopyranosyloxy)benzaldehyde[12] (4.6 g, 10 mmole) and ethyl cyanoacetate (1.13 g, 10 mmole). When dissolution occurred, piperidine (0.4 ml) and glacial acetic acid (0.04 ml) were added, and the swirled mixture was cooled immediately in ice. After the initial exothermic reaction, the solution was kept overnight at room temperature. If a precipitate formed, it was collected; otherwise, the oil that occasionally resulted was triturated with ether until it solidified. The resulting material, yield 3.2 g (62%), had m.p. ~238° (dec.). Recrystallization from glacial acetic acid gave material having m.p. 238–239° (dec.). T.l.c. in 3:7 ethyl acetate–benzene gave a single spot, R_F 0.3. The product had $\nu_{max}^{CHCl_3}$ 2230 cm^{-1} (nitriile).

Anal. Calc. for $C_{24}H_{23}NO_{12}$: C, 55.7; H, 4.48; N, 2.71. Found: C, 55.6; H, 4.37; N, 2.84.

3-Cyano-7-(β-D-glucopyranosyloxy)coumarin (**7**). — A suspension of compound **4** (2.0 g, 3.9 mmole) in 0.3M barium hydroxide (400 ml) was shaken for 1 h. The resulting solution was treated with carbon dioxide until it was neutral to litmus, and the barium carbonate was filtered off, and extracted once with hot water. The combined filtrates were passed through a column of Rexyn AG-50 (H^+) resin (Fisher Scientific Co.), and the eluate was evaporated under diminished pressure. The residue was crystallized from water, to give 0.58 g (43%) of light-yellow platelets, which gave a single spot, R_F 0.6, by t.l.c. in 4:1:5 butyl alcohol–ethanol–water. This material had a poorly defined m.p. until it had been heated *in vacuo* for 2 h at 160°; it then melted at 213–214°. Analysis indicated that this product was the hemihydrate. Further heating did not provide the anhydrous D-glucoside.

Anal. Calc. for $C_{16}H_{15}NO_8 \cdot 0.5\,H_2O$: C, 53.6, H, 4.50; N, 3.91. Found: C, 53.4; H, 4.27; N, 3.77.

Additional support for the structure was given by the i.r. spectrum of the D-glucoside (**7**) in methyl sulfoxide solution, which bore a close resemblance to that of 3-cyano-7-methoxycoumarin[13]. Bands of strong and moderate intensity were observed in the region below 2000 cm^{-1}, where the solvent is transparent: D-glucoside **7**, 1730, 1605, 1555, 1500, 1260, 1245, 1165, and 765 cm^{-1}; 3-cyano-7-methoxycoumarin, 1735, 1615, 1600, 1555, 1505, 1270, 1250, 1160, and 765 cm^{-1}. Both compounds had $\nu_{max}^{Me_2SO}$ 2220 cm^{-1} (nitrile).

3-Cyano-7-hydroxycoumarin could be separated from 3-cyano-7-(β-D-glucopyranosyloxy)coumarin by column chromatography, with an elution gradient of 0→74% methanol in chloroform.

3-(Carboxamido)-7-(β-D-glucopyranosyloxy)coumarin (**8**). — To a cold solution of 3-cyano-7-(tetra-*O*-acetyl-β-D-glucopyranosyloxy)coumarin (2.07 g, 4 mmole) in

200 ml of anhydrous methanol was added 0.1M methanolic sodium methoxide (60 ml). The mixture was stirred overnight at 4°, and then neutralized, either with Rexyn AG-50 resin or with 0.1M hydrochloric acid. The solution was concentrated *in vacuo* to 15 ml, and the resulting precipitate was collected after the pH had been adjusted to 6. The product was heated briefly in boiling water, treated with charcoal, and cooled, to give yellow crystals, yield 0.58 g (39%), m.p. 255–256° (dec.). T.l.c. on silica gel G, in 4:1:5 butyl alcohol–ethanol–water, showed a single spot, R_F 0.6. A chromatogram of the crude material in 4:1:5 butyl alcohol–acetic acid–water showed the presence of trace amounts of 3-carboxy-7-(β-D-glucopyranosyloxy)coumarin.

Anal. Calc. for $C_{16}H_{17}NO_9$: C, 52.3; H, 4.67; N, 3.81. Found: C, 52.5; H, 4.77; N, 3.85.

3-(Carboxamido)-7-methoxycoumarin, for i.r. spectral comparison with **8**, was prepared as follows. 3-Carboxy-7-methoxycoumarin[9] (2.38 g, 10.8 mmole) in 15 ml of chloroform and 5 ml of thionyl chloride was heated under reflux until evolution of hydrogen chloride ceased. The product was precipitated with petroleum ether, and recrystallized twice from chloroform–petroleum ether; yield, 0.93 g (36%), m.p. 144–145°. Ammonia was passed through a solution of the acid chloride (0.25 g, 1.1 mmole) in *p*-dioxane. The precipitated product was filtered off, and crystallized from aqueous ethanol; yield 0.14 g (60%), m.p. 240–241° (preheated stage).

Anal. Calc. for $C_{11}H_9NO_4$: C, 60.3; H, 4.14; N, 6.39. Found: C, 60.2; H, 4.09; N, 6.52.

An i.r. spectral comparison of the D-glucoside **8** with 3-(carboxamido)-7 methoxycoumarin in methyl sulfoxide solution showed close similarity. Bands of strong and moderate intensity were observed in areas of solvent transparency below 2000 cm^{-1}: D-glucoside **8**, 1710, 1670, 1610, 1585, 1560, 1500, 1230, and 800 cm^{-1}; 3-(carboxamido)-7-methoxycoumarin, 1710, 1675, 1620, 1585, 1560, 1510, and 1235 cm^{-1}.

Column chromatography of coumarin D-*glucosides.* — Florisil was washed with 2:1 (v/v) redistilled chloroform–methanol and finally with chloroform. An all-glass column (20 × 1.4 cm), having a ball-joint at the top and a glass-frit septum and Teflon stopcock at the bottom, was packed with Florisil under chloroform. A solution of the D-glucoside (5 mg) in methanol was evaporated to dryness in the presence of about 6 ml of Florisil. The D-glucoside, adsorbed to the Florisil, was placed on the top of the column, and the column was eluted with a continuous gradient of methanol in chloroform to the methanol limit required for the particular D-glucoside. A typical gradient, which was close to linear, was obtained in the following way. A 1-liter Büchner filter-flask was filled with 1 liter of the limit eluant (37:13 methanol–chloroform, in the case of 3-cyano-7-hydroxycoumarin and its D-glucoside). This flask was connected to a 10 × 15 cm, 1-liter, cylindrical, glass reagent-bottle by Teflon tubing reaching to the bottom of each vessel. The cylindrical bottle contained 653 ml of distilled chloroform (an amount equal in weight to the 1 liter of 37:13 methanol–chloroform) and was stirred by a bar magnet. This mixing bottle was connected to the column by Teflon tubing which reached to the bottom of the mixing bottle and

was sealed into the ball-joint top of the column by rubber tubing. The top of the column was placed 2 feet below the bottom of the mixing flask, to provide a siphoning pressure sufficient to maintain a constant flow. When the bridge between the conical and cylindrical flask was filled and the siphon started, the levels in the two containers fell together to give the gradient. Elution was conducted at 2 ml/min, and 10-ml fractions were collected automatically. When a different limit-eluant was used, the volume of chloroform was adjusted to match the density of the limit. In the two cases where Florisil chromatography has been performed (the 3-acetyl and 3-cyano derivatives), the aglycon was eluted before, and was completely resolved, from the D-glucoside.

Spectral measurements. — Fluorescence measurements of the 7-hydroxycoumarins were obtained on an Aminco–Bowman fluorescence spectrometer (American Instrument Co., Silver Spring, Md., U. S. A.). All data are corrected to lines in the spectrum of mercury. The fluorescence of the β-D-glucopyranosides was measured on a Baird SF-1 fluorescence spectrometer (Baird-Atomic, Inc., Cambridge, Mass., U. S. A.) and are corrected. Both instruments used an Osram XB165 xenon arc and a 1P28 photomultiplier tube. Samples were measured in pH 8.5 buffer, except for 7-hydroxy-4-methylcoumarin and its D-glucoside, which were measured at pH 10. Concentrations: hydroxycoumarins, 0.1 μM; coumarin glucosides, 0.1 mM. The absorption data were obtained with a Bausch and Lomb Spectronic 505 u.v. spectrophotometer. 7-Hydroxy-4-methylcoumarin was obtained from the Eastman Organic Chemicals Co., Rochester, N.Y., U. S. A.

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REFERENCES

1 J. A. R. MEAD, J. N. SMITH, AND R. T. WILLIAMS, *Biochem. J.*, 61 (1955) 569.
2 W. R. SHERMAN AND E. F. STANFIELD, *Biochem. J.*, 102 (1967) 905.
3 D. ROBINSON, *Biochem. J.*, 63 (1956) 39.
4 E. ROBINS, *J. Histochem. Cytochem.*, 8 (1960) 431.
5 W. R. SHERMAN AND E. ROBINS, *Anal. Chem.*, 40 (1968) 803.
6 C. E. BALLOU, *Advan. Carbohyd. Chem.*, 9 (1954) 59.
7 H. VON PECHMANN AND E. GRAEZER, *Ber.*, 34 (1901) 378.
8 V. BALAIAH, T. R. SESHADRI, AND V. VENKATESWARLU, *Proc. Indian Acad. Sci.*, 16A (1942) 68.
9 W. BAKER AND C. B. COLLIS, *J. Chem. Soc.*, (1949) Suppl. 1, 512.
10 V. L. REICHEL AND R. SCHICKLE, *Ann.*, 553 (1942) 101.
11 F. M. DEAN, A. ROBERTSON, AND W. B. WHALLEY, *J. Chem. Soc.*, (1950) 895.
12 A. ROBERTS AND R. ROBINSON, *J. Chem. Soc.*, (1927) 245.
13 W. BAKER AND C. S. HOWES, *J. Chem. Soc.*, (1953) 119.

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SUBSTRATE SPECIFICITY OF D-GALACTOSE OXIDASE*

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ABSTRACT

The rate of oxidation of methyl ethers of D-galactose and 2-amino-2-deoxy-D-galactose, and of oligosaccharides and polysaccharides containing D-galactosyl residues having a free hydroxyl group at C-6, has been followed by various procedures that depend either upon the hydrogen peroxide or the aldehyde groups produced, or upon the unoxidized D-galactose residues remaining in the reaction mixture. Derivatives of D-galactose having substituents on the hydroxyl group at C-4 are not oxidized. 2-Amino-2-deoxy-D-galactose residues having glycosyl substituents at C-3 are not oxidized by the enzyme, and therefore, neither are chondroitin 4-sulfate nor dermatan sulfate. No completely satisfactory procedure was found for following the oxidation reaction to termination, which, in none of the cases studied, was 100% complete.

INTRODUCTION AND DISCUSSION

D-Galactose oxidase (D-galactose:oxygen oxidoreductase EC 1.1.3.9) has been stated to oxidize those residues of D-galactopyranose in which the primary hydroxyl group at C-6 is unsubstituted[1,2]. The reaction has been applied widely to modify D-galactopyranosyl residues in carbohydrates[3,4], to introduce a tritium label into such compounds [5–7], and to analyze for these residues[2,8–15]. Several peculiarities of the reaction have gone unanswered, however. For example, the reaction does not go to completion[2,5], unusual kinetics are sometimes observed[2–7], and analytical data are not consistent[2]. As a result, the value of application of this enzyme to structural studies is dubious. The present paper considers the influence of substituents on the rate of oxidation of the D-galactopyranosyl residues, in an attempt to clarify some of the above questions.

The effect of chloride, sulfate, phosphate, cyanide, formate, hydrazine, and

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hydroxylamine was first studied. Of these, the last four completely inhibit the enzyme, although the influence of hydroxylamine[16] on the coupled peroxidase system complicates its evaluation as an inhibitor. Phosphate of concentrations up to 0.1M at pH 7.0 was not inhibiting, but, at pH 5.6, a diminution in the rate of oxidation was noted. Sodium sulfate was not inhibiting at concentrations up to 0.4M, whereas both sodium chloride and potassium chloride inhibited the reaction noticeably at 0.16M and to the extent of 45% at 0.4M. The inhibition by chloride may well explain the variability noted by some workers in determining the D-galactose in carbohydrate polymers not oxidized by D-galactose oxidase[17,18]. The oxidized polymer was hydrolyzed with hydrochloric acid, and the residual D-galactose was determined, after neutralization, by using D-galactose oxidase assay. Such difficulties would appear to be avoided if sulfuric acid is used for the hydrolysis.

The extent of oxidation of available D-galactose residues in a carbohydrate can be estimated by several methods, all of which have certain disadvantages. Perhaps the simplest is that in which the hydrogen peroxide produced by the oxidation reaction is coupled, through a peroxidase, to a chromogen[10]. Such a system serves well in estimation of the initial course of the oxidation, but suffers from several disadvantages when the reaction is followed to completion. The color produced is diminished in the presence of other electron acceptors[12], and is affected by the precipitation of chromogen from solution[13]. Such side reactions decrease the absorbance value for the final oxidation, although, for *o*-nitrophenyl α-D-galactopyranoside, 1,5-anhydro-D-galactitol, and raffinose, the results so obtained, calculated from a standard curve determined with hydrogen peroxide, are of the same order of magnitude as that calculated by a determination of D-galactose residues that were unoxidized (see Table I).

As has been noted by Avigad *et al.*[2] and several other workers, the initial velocities of the oxidation reaction differ for various substrates. In certain cases, this factor in itself presents the problem of determining the percentage of D-galactose residues having free hydroxyl groups at C-6 in an unknown carbohydrate. The problem is complicated, however, by the fact that some of the D-galactosyl residues in a carbohydrate having such a free hydroxyl group at C-6 are not oxidized. As may be noted in Table I, a D-galactose residue substituted at O-4 by a methyl group is not a substrate for D-galactose oxidase. This is true for 4-*O*-methyl-D-galactose and 2,4-di-*O*-methyl-D-galactose, but not for D-galactose substituted by methyl groups at O-2 or O-3. It may also be noted that 2-amino-2-deoxy-D-galactose residues having the hydroxyl group at C-3 substituted by a methyl group are oxidized a little more slowly than 2-amino-2-deoxy-D-galactose, which itself is oxidized more slowly than D-galactose. If, however, the hydroxyl group at C-3 of 2-amino-2-deoxy-D-galactose is substituted by a D-glucopyranosyl or a D-glucopyranosyluronic acid residue, the oxidation is completely checked. This inhibition may be the result of steric effects, as can be seen for the slow oxidation of 2′-*O*-(α-L-fucopyranosyl)lactose and the resistance of uridine 5′-(α-D-galactopyranosyl pyrophosphate) to oxidation[2]. From these results, it is therefore not surprising that chondroitin 4-sulfate and dermatan

TABLE I

SUBSTRATE SPECIFICITY OF D-GALACTOSE OXIDASE

Substrate[a]	*Relative rate of oxidation*	*% oxidized, as determined by*		
		Formation of H_2O_2	*Park–Johnson procedure (24 h)*	*Residual D-galactose*
D-Galactose	100	86		
o-Nitrophenyl β-D-galactopyranoside	57	84	78	86
Methyl α-D-galactopyranoside	118	84		
1-*O*-(α-D-Galactopyranosyl)-*myo*-inositol[b]	37	60		
2-*O*-Methyl-D-galactose[b]	50	68		
3-*O*-Methyl-D-galactose[c]	172			
4-*O*-Methyl-D-galactose[b]	0	0		
2,4-Di-*O*-methyl-D-galactose[b,c]	0	0		
2,3-Di-*O*-methyl-D-galactose[b]	76	75		
1,2:3,4-Di-*O*-isopropylidene-α-D-galactose	0			
1,5-Anhydro-D-galactitol	134	96	20	95
O-α-D-Galactopyranosyl-(1→3)-D-galactose[c]	48			
Raffinose	145	96	46	98
2′-*O*-(α-D-Fucopyranosyl)lactose[d]	4			
2-Amino-2-deoxy-D-galactose	71			
Methyl 2-acetamido-2-deoxy-3-*O*-methyl-α-D-galactopyranoside[e]	54			
Chondrosine[e]	0	0		
O-β-D-Glucopyranosyl-(1→3)-2-acetamido-2-deoxy-D-galactose[e]	0	0		
O-β-D-Galactopyranosyl-(1→3)-2-acetamido-2-deoxy-D-galactose[c]	39			
Guaran	150	76	3	95
Ovomucoid	87	86		
Chondroitin 4-sulfate[f]	0			
Dermatan sulfate[f]	0			

[a]Reaction solution contained 100 mg of available D-galactose/l. [b]Provided by Dr. B. A. Lewis of the University of Minnesota. [c]Provided by Dr. G. F. Springer of Northwestern University. [d]Provided by Dr. V. Ginsberg of the National Institutes of Health. [e]Provided by Dr. R. W. Jeanloz of Harvard University Medical School. [f]Provided by Dr. J. A. Cifonelli, LaRabida Institute, Chicago.

sulfate, both of which have a free hydroxyl group at C-6 of their 2-amino-2-deoxy-D-galactose residues, but have substituents at O-3 and O-4, are not oxidized by D-galactose oxidase. Several carbohydrate-containing biopolymers are oxidizable to some extent by D-galactose oxidase, but serious questions must now be raised concerning any structural deductions that can be made from such information. If oxidation can be noted by any of the methods mentioned above, it would seem clear that some of the D-galactopyranosyl residues have free hydroxyl groups at C-6. However, a negative result in no way excludes the possibility of occurrence, within the molecule,

of D-galactopyranosyl residues that might have been expected to be oxidized. Further work is continuing on this subject.

For a while, it was thought that the incomplete oxidation of the substrates for D-galactose oxidase was due to inhibition by the product. It would seem, however, that this is not the case. A reaction that has come to a halt cannot be significantly reactivated by the addition of fresh D-galactose oxidase, and the extent of oxidation is not significantly different if the initial concentrations of substrate are varied. In the latter instance, it would have been expected that the concentration of product in the reaction systems would have varied; and, with it, the extent of the product inhibition. This behavior was, however, not noted for the oxidation of *o*-nitrophenyl β-D-galactopyranoside in concentrations that were varied several-fold. This also is an area for further study.

EXPERIMENTAL

Materials. — D-Galactose oxidase was obtained from Worthington Biochemical Company and the Miles Laboratories, Inc. Catalase (beef liver, 185,000 units per ml) and peroxidase (horseradish, 400 units per mg) from the Worthington Biochemical Company were used without further purification. β-D-Galactosidase was obtained from Calbiochem. The various derivatives of D-galactose were either purchased from Pfanstiehl Chemical Company and used without purification, or were donated as indicated by the footnotes in Table I.

DEAE-Cellulose was successively washed with 0.5M hydrochloric acid and 0.5M sodium hydroxide followed, each time, by a thorough washing with water. After regeneration with alkali, the DEAE-cellulose was equilibrated with 0.02M phosphate buffer, pH 7.0.

Purification of D-galactose oxidase. — Crude D-galactose oxidase (41 mg) from Miles Laboratories was dissolved in approximately 25 ml of 0.02M phosphate buffer, pH 7.0, and the dark solution was passed through a column (7.0 × 0.9 cm) of DEAE-cellulose equilibrated with the same buffer. The column was then washed with buffer solution (twice the initial volume). A dark pigment remained at the top of the column. The eluate was collected immediately after addition of the sample, and was divided into two fractions to separate the more-concentrated peak from the tailing fractions. In a typical experiment, D-galactose oxidase (17,000 units) was added to a column; the first two-thirds of the eluate contained 13,000 units (250 units per ml) and the remaining one-third of the eluate contained 1,500 units (60 units per ml).

Assay of D-galactose oxidase activity. — To one ml of enzyme solution was added 0.4 ml of *o*-tolidine (recrystallized from acetone–water, 100 mg/l of water), 0.4 ml of peroxidase (100 mg/l of water), and 0.2 ml of 0.25M D-galactose solution. All of the solutions were preheated to 30° and kept at this temperature during the assay, which was continuously recorded at 420 nm with a Gilford spectrophotometer, Model 2000, with a 1-cm light-path. One unit of enzymic activity corresponds to

a net absorbancy of 1.0 at 420 nm after reaction for 10 min at 30°. This value, when converted into moles of hydrogen peroxide produced (and, therefore, D-galactose oxidized) can be redefined as 30 nmoles of D-galactose oxidized per min at 30°.

Inhibitor studies. — The inhibitor studies were conducted with a Galactostat kit (Worthington Biochemical Company), from which the enzyme, dissolved in 25 ml of distilled water, and the chromogen solution were kept separated. According to the above assay, the enzyme solution was equivalent to 11.3 units per ml. Studies on the effect of potassium chloride, sodium chloride, sodium sulfate, and sodium formate were made with D-galactose (1.39×10^{-4}M) as the substrate. Corresponding studies with sodium cyanide, sodium bisulfite, hydrazine sulfate, and hydroxylamine hydrochloride were made with methyl α-D-galactopyranoside (1.39×10^{-4}M) as the substrate. In each instance, 0.4 ml of chromogen solution, 0.4 ml of enzyme solution, and appropriate volumes of the inhibitor solution and the substrate solution (to make a final volume of 1.6 ml) were mixed and allowed to react at 30° or 37°. As in the enzyme assay, the absorbance at 420 nm was measured periodically during 1 h.

Relative rates of oxidation. — The rates of oxidation of various substrates (see Table I) were determined in relation to that of D-galactose. In each case, 0.5 ml of D-galactose oxidase (70 units, purified on DEAE-cellulose), 0.5 ml of peroxidase (100 mg/l), 0.5 ml of *o*-tolidine (200 mg/l), and 0.5 ml of the substrate solution (the concentration of the reaction solution was 1.39×10^{-4}M) were mixed at 30°. The rates of oxidation were followed spectrophotometrically for 10 min at 30°, at 420 nm in a 1-cm cell.

Extent of oxidation. — Attempts were made to measure the extent of the final oxidation of various substrates by (*a*) using the color produced by the chromogen, as read against a standard curve determined with hydrogen peroxide, (*b*) measuring the aldehyde formed, with use of the Park–Johnson reagents[19], or (*c*) measuring the D-galactose remaining after oxidation had apparently ceased. For *o*-nitrophenyl β-D-galactopyranoside, the residual substrate was determined by hydrolysis with β-D-galactosidase according to the procedure of Avigad[5]. These various methods will be illustrated below (*A–D*) by considering the particular substrate, *o*-nitrophenyl β-D-galactopyranoside.

A. The reaction solution was prepared exactly as already described for the determination of relative rates of oxidation. The color produced by the *o*-tolidine chromogen was determined continuously in the Gilford spectrophotometer at 420 nm until such time as the color produced was at the maximum. The equivalence of this color to the hydrogen peroxide produced by the reaction was derived from a standard curve. The latter was determined by mixing 0.5 ml of *o*-tolidine solution (200 mg/l), 0.5 ml of peroxidase solution (100 mg/l), and 1.0 ml of solutions containing various amounts (0–230 nmoles) of hydrogen peroxide in water; the color so produced was read immediately against an appropriate blank. The maximum color, equivalent to 84% of complete oxidation, was obtained in approximately 1 h with *o*-nitrophenyl β-D-galactopyranoside.

B. By the procedure of Avigad[5], a mixture containing 0.5 ml of D-galactose

oxidase (125 units per ml, purified by DEAE-cellulose chromatography), 0.5 ml of catalase (0.1 ml of commercial suspension diluted to 25 ml with water), 0.5 ml of *o*-nitrophenyl β-D-galactopyranoside (5.56×10^{-4}M), and 0.5 ml of water was allowed to react for 24 h at 30°. A blank determination was made by omitting the glycoside. To 1 ml of the final reaction solution was added an equal volume of β-D-galactosidase (240 mg/l) in 0.01M phosphate buffer, pH 7.0, which was mM in magnesium chloride. The amount of *o*-nitrophenol released was determined continuously at 405 nm, and read after 30–45 min when the absorbance was at the maximum. The correspondence of this color to the original concentration of unoxidized *o*-nitrophenyl β-D-galactopyranoside was determined from a standard curve. Eight separate determinations gave results that varied from 71–77% of complete oxidation; the average value was 75%.

C. The extent of oxidation, followed by the formation of aldehyde groups, was measured by an oxidation system similar to that already described for Avigad's procedure[5], except that samples were taken periodically from the reaction mixture and analyzed by the Park–Johnson procedure[19]. The resulting color, read at 690 nm in a Coleman Jr. colorimeter, was converted into equivalents of D-galactose from a preconstructed, standard curve determined with D-galactose. The extent of oxidation, as determined by this procedure, was 75–85%; average value 78%.

D. The alternative procedure of determining the amount of residual D-galactose, after oxidation and hydrolysis with sulfuric acid, used an oxidation system similar to that already described for Avigad's procedure[5]. When maximal oxidation had been achieved, as judged by the results by that procedure, 1 ml of the solution was mixed with 1 ml of 1.5M sulfuric acid, and the mixture was heated for 6 h at 100°. The solution was then de-ionized by passing it through a bed of mixed resin containing Amberlite G-45 (OH^-) and Rexyn-101 (H^+). The eluate was evaporated to dryness *in vacuo*, the residue was dissolved in 0.6 ml of distilled water, and the content of D-galactose was determined by means of D-galactose oxidase.

The results obtained with other substrates are summarized in Table I.

ACKNOWLEDGMENT

The authors are indebted to the colleagues noted in Table I for various derivatives of D-galactose.

REFERENCES

1 G. AVIGAD, C. ASENSIO, D. AMARAL, AND B. L. HORECKER, *Biochem. Biophys. Res. Commun.*, 4 (1961) 474.
2 G. AVIGAD, D. AMARAL, C. ASENSIO, AND B. L. HORECKER, *J. Biol. Chem.*, 237 (1962) 2736.
3 J. C. ROBINSON AND J. E. PIERCE, *Arch. Biochem. Biophys.*, 106 (1964) 348.
4 G. F. SPRINGER, Y. NAGAI, AND H. TEGTMEYER, *Biochemistry*, 5 (1966) 3254.
5 G. AVIGAD, *Carbohyd. Res.*, 3 (1967) 430.
6 J. E. G. BARNETT, *Carbohyd. Res.*, 4 (1967) 267.
7 A. G. MORELL, C. J. A. VAN DEN HAMER, I. H. SCHEINBERG, AND G. ASHWELL, *J. Biol. Chem.*, 241 (1966) 3745.

8 S. A. BARKER, G. PARDOE, AND M. STACEY, *Nature*, 197 (1963) 231.
9 R. M. BRADLEY AND J. N. KANFER, *Biochim. Biophys. Acta*, 84 (1964) 210.
10 C. H. DEVERDIER AND M. HJELM, *Clin. Chim. Acta*, 7 (1962) 742.
11 W. FISCHER AND J. ZAPF, *Z. Physiol. Chem.*, 337 (1964) 186.
12 W. FISCHER AND J. ZAPF, *Z. Physiol. Chem.*, 339 (1964) 54.
13 M. HJELM, *Clin. Chim. Acta*, 15 (1967) 87.
14 E. REREM AND C. LEWIS, *Anal. Biochem.*, 3 (1962) 230.
15 J. SEMPERE, C. GANCEDO, AND C. ASENSIO, *Anal. Biochem.*, 12 (1965) 590.
16 D. AMARAL, L. BERNSTEIN, D. MORSE, AND B. L. HORECKER, *J. Biol. Chem.*, 238 (1963) 2281.
17 W. BUTLER AND L. CUNNINGHAM, *J. Biol. Chem.*, 241 (1966) 3882.
18 U. LINDAHL, *Biochim. Biophys. Acta*, 130 (1966) 361.
19 J. T. PARK AND M. J. JOHNSON, *J. Biol. Chem.*, 181 (1949) 149.

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THE CHEMICAL STRUCTURE OF KEFIRAN, THE WATER-SOLUBLE POLYSACCHARIDE OF THE KEFIR GRAIN

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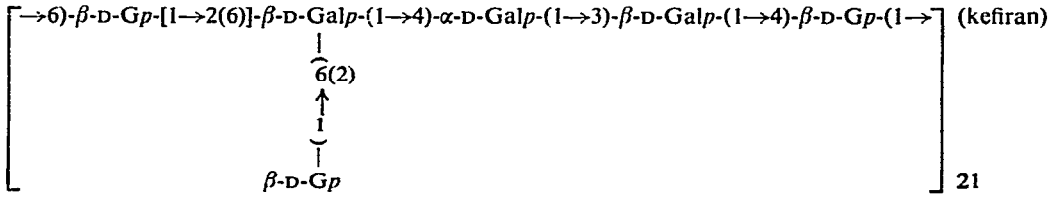
ABSTRACT

From kefir grains, a water-soluble polysaccharide, kefiran, was isolated, consisting of approximately equal proportions of galactose and glucose residues. A β-D-(1→6) glucanase from *Trichoderma viride* was able to fragment the polysaccharide, the main products being equimolar amounts of glucose and a pentasaccharide, kefirose. The results of methylation studies on both kefiran and kefirose, of periodate-oxidation analysis of kefiran, and of a study on the products of partial hydrolysis of kefirose with acid provided substantial evidence for assigning the following structures to kefirose and kefiran, respectively.

β-D-G*p*-(1→2)-β-D-Gal*p*-(1→4)-α-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-D-G (kefirose)



(kefiran)

The variety of linkage types may account for the rather poor accessibility of kefiran to enzymic attack. The latter property might be important in the ecology of the kefir grain.

INTRODUCTION

The kefir grain is the inoculating material used for the conversion of milk into the Russian beverage kefir. It consists of yeasts and bacteria, preponderantly *Saccharomyces delbrückii* and *Lactobacillus brevis*. At least 24% of the dry material of the kefir grain consists of the capsular polysaccharide of *Lactobacillus brevis*[1]. The polysaccharide is a water-soluble glucogalactan containing approximately equal amounts of D-glucose and D-galactose residues[1].

The present study is concerned with the chemical structure of this polysaccharide.

RESULTS AND DISCUSSION

A crude preparation of kefiran was obtained by disintegrating kefir grains with a homogenizer and precipitating the polysaccharide from the viscous filtrate with

ethanol. Purification was achieved by freezing an aqueous solution of the crude material, followed by thawing, when the polysaccharide was obtained as a spongy gel releasing most of the liquid by syneresis. The purified kefiran had $[\alpha]_D$ +68° (*c* 1, water) and contained approximately 50% of galactose and 50% of glucose residues. Neither repeated freezing and thawing, nor fractionation with ethanol, indicated inhomogeneity of the polysaccharide. A molecular weight of 20,600 (corresponding to an average of 127 hexose units) was estimated by using the hypoiodite method.

On periodate oxidation, the polysaccharide consumed 1.13 moles of periodate per mole of hexosyl residue, yielding 0.30 mole of formic acid. Reduction of the resulting polyaldehyde and hydrolysis of the polyalcohol yielded a mixture of galactose, tetritol, and glycerol in the proportions of 1.00:1.55:3.08. From the tetritol fraction, D-threitol and erythritol were isolated.

Kefiran was methylated by the Haworth procedure and subsequently by the method of Wallenfels and Bechtler[15]. The methylated polysaccharide had $[\alpha]_D$ +18° (chloroform). After hydrolysis, four products were detected on paper chromatograms. Separation by preparative paper-chromatography yielded 2,3,4,6-tetra-*O*-methyl-D-glucose (13%), tri-*O*-methylglucoses (33%), tri-*O*-methylgalactoses (38%), and 3,4-di-*O*-methyl-D-galactose (16%). The tri-*O*-methylglucoses were separated on a carbon–Celite column by using a gradient of butanone–water, to afford approximately equal amounts of 2,3,4- and 2,3,6-tri-*O*-methyl-D-glucose. The tri-*O*-methylgalactoses were partly separated on carbon–Celite, with a gradient of ethanol–water, to give approximately equal amounts of 2,3,6- and 2,4,6-tri-*O*-methyl-D-galactose.

The results of periodate oxidation and methylation indicate that kefiran has a main chain with branch points at O-2 and O-6 of some of the D-galactose residues; the branches are terminated by D-glucose residues. The remaining D-galactose residues are linked through positions 4 and 3, and the remaining D-glucose residues are linked through positions 4 and 6. All of these linkage types are present in approximately equal amounts.

Several, crude enzyme-preparations from various sources failed to hydrolyze kefiran; only a crude cellulase from *Trichoderma viride* was able to degrade the polysaccharide, during prolonged incubation, to give mainly glucose and a pentasaccharide, in approximately equimolar quantities. The pentasaccharide, kefirose, had $[\alpha]_D$ +90° (water) and contained three galactose and two glucose residues. After reduction with sodium borohydride and hydrolysis, the ratio of galactose to glucose was 3:1, indicating that the reducing end of kefirose is a glucose residue.

Methylation of kefirose gave a product having $[\alpha]_D$ +35° (chloroform), which, after hydrolysis, gave six products (paper chromatography). Fractionation by preparative paper-chromatography yielded 2,3,4,6-tetra-*O*-methyl-D-glucose, 2,3,6-tri-*O*-methyl-D-glucose, a mixture of 2,3,6- and 2,4,6-tri-*O*-methyl-D-galactose, 3,4,6-tri-*O*-methyl-D-galactose, 2,6-di-*O*-methyl-D-galactose, and 4,6-di-*O*-methyl-D-galactose, in molar proportions of 1.0:1.2:1.9:1.0:0.6:0.1.

The presence of di-*O*-methylgalactoses in the mixture is attributable to incom-

plete methylation. Apart from the di-*O*-methylgalactoses, the results demonstrate that kefirose has the same linkage types as kefiran, except for the 6-linked D-glucose residue and the branching point at one of the D-galactose residues, which are not present in kefirose. One non-reducing end-group (a D-glucose residue as in kefiran) is present in kefirose, and the 2,6-disubstituted D-galactose residue in kefiran corresponds with a 2-substituted D-galactose residue in kefirose. Thus, kefirose is a straight-chain pentasaccharide terminated by D-glucose residues, and having three interior D-galactose residues.

In order to obtain information on the sequence of the linkage types, kefirose was fragmented by mild hydrolysis with acid, and the oligosaccharides produced were separated by paper chromatography. The results are recorded in Table I. Fraction I was 2-*O*-β-D-glucopyranosyl-D-galactose originating from the non-reducing end of kefirose. Fraction II contained, *inter alia*, 4-*O*-β-D-galactopyranosyl-D-glucose (lactose), which could only have originated from the reducing end of kefirose. The non-crystalline part of Fraction II ($[\alpha]_D$ +76°) yielded galactose and a small proportion of glucose on hydrolysis; the main component was therefore a galactosylgalactose or a mixture of galactosylgalactoses, and the optical rotation was indicative of at least some α-D links. From this material, a small quantity of 4-*O*-β-D-galactopyranosyl-D-galactose was isolated.

TABLE I

OLIGOSACCHARIDES OBTAINED BY PARTIAL, ACID HYDROLYSIS OF KEFIROSE (1 G)

Fraction	*Yield (mg)*	*Component sugars*	*Mol. weight*	$[\alpha]_D$ *(water) (degrees)*
I	63	Gal + G	363	+ 32
II	180	Gal + less G	—	—
III	105	Gal + G	526	+ 42
IV	42	Gal	521	+139
V	61	Gal + less G	702	+ 95
VI	32	Gal + less G	678	+108
VII	49	Gal + G	825	+ 85

Fraction III was a trisaccharide, probably a glucosylgalactosylgalactose, and thus represented three sugar units from the non-reducing end of kefirose; the specific optical rotation was suggestive of β-D links.

Fraction IV was a trisaccharide consisting of galactose residues, corresponding to the interior of the kefirose molecule remaining after loss of the glucose end-groups. According to the methylation analysis, the following structures were possible:

(*a*) D-Gal*p*-(1→4)-D-Gal*p*-(1→3)-D-Gal*p* and (*b*) D-Gal*p*-(1→3)-D-Gal*p*-(1→4)-D-Gal*p*. Structure (*a*) should consume 6 moles and (*b*) 5 moles of periodate; moreover, (*a*) would leave no intact D-galactose and (*b*) would leave one mole of D-galactose. The observed consumption of periodate was at least 5.5 moles per mole of hexosyl residue; only a trace of intact galactose was found in the hydrolyzate of the boro-

hydride-reduced product. Fraction IV therefore had structure (*a*). The rather high optical rotation was indicative of α-D linkages. Since Fraction III, containing three residues from the non-reducing part of the kefirose molecule, probably has exclusively β-D linkages, it is likely that the (1→3)-bond is of the α-D type, all other bonds of the kefirose molecule being of the β-D type.

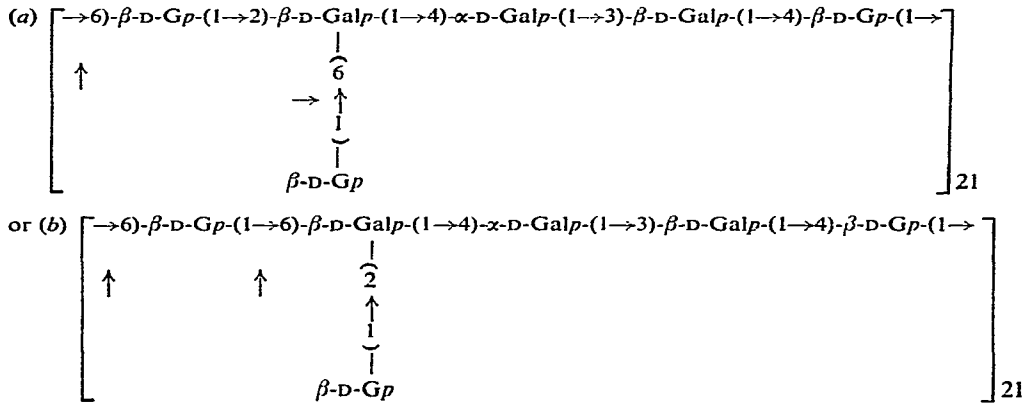
Fraction V was a glucosylgalactotriose, while Fraction VI was a galactotriosylglucose. Both Fractions V and VI had rather high optical rotations, indicating the presence of an α-D linkage in addition to the β-D linkages. This was in harmony with the structures of the other oligosaccharides.

Fraction VII was unchanged kefirose. From the yields of the oligosaccharides, it may be concluded that, during acid hydrolysis, the weakest bond in the kefirose molecule is the α-D-(1→3) linkage.

The evidence obtained permits the assignment of the following structure for kefirose:

β-D-G*p*-(1→2)-β-D-Gal*p*-(1→4)-α-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-D-G

The release of kefirose and glucose from kefiran by the *Trichoderma* enzyme implies that kefiran contains hexasaccharide repeating-units, consisting of kefirose to which a D-glucosyl residue is linked at position 6 of the 2-linked D-galactosyl residues. The difference in specific optical rotation between kefiran and kefirose indicates that the linkages split by the enzyme are of the β-D type. It follows that there are two possibilities for the structure of kefiran:



Each of these structures would consume 1.17 moles of periodate per mole of hexosyl residue (found 1.13) and produce 0.33 mole of formic acid (found 0.30). After periodate oxidation, reduction of the polyaldehyde, and hydrolysis, galactose, erythritol, D-threitol, and glycerol would be released in ratios of 1:1:1:3; the observed ratios of galactose, tetritols, and glycerol were 1.00:1.55:3.08.

The linkages which would be split by the *Trichoderma* enzyme in either (*a*) or (*b*) are indicated by arrows. Obviously, the enzyme responsible for the hydrolysis is a β-(1→6)-glucanase [β-(1→6)-glucan 6-glucanohydrolase), which is known to

be present, although in small proportions, in *Trichoderma* species, including *T. viride*[2]. Considerably greater amounts of this enzyme have been found in many *Penicillium* species, in several *Aspergillus* species, and in other fungi[2]. However, kefiran was not attacked by several crude fungal and bacterial enzyme-preparations; one of these was derived from *Aspergillus niger*, of which species at least one strain produces a β-D-(1→6)-glucanase[2]. Attempts to find micro-organisms which were able to hydrolyze the polysaccharide led to the isolation of bacteria identified as belonging to the genus *Arthrobacter*; they attacked the polysaccharide slowly[3].

The resistance of kefiran to enzymic attack is possibly due to the succession of different linkage types and the presence of some uncommon linkage types, and explains the capacity of kefir grain to survive microbial contamination.

EXPERIMENTAL

Melting points are corrected. Specific optical rotations were equilibrium rotations unless otherwise specified. Evaporations were done below 30°. Paper chromatograms were made by the descending method on either Whatman No. 1 or 3 MM papers, with the following solvents (v/v): (*A*) butyl alcohol–pyridine–water (6:4:3); (*B*) butyl alcohol–ethanol–water (40:11:19); (*C*) butanone–water (2:1); (*D*) butyl alcohol saturated with water. For detection of sugar spots, anisidine phosphate[4] was used; sugar alcohols were detected with a silver nitrate reagent[5]. In addition to the former reagent, triphenyltetrazolium chloride[6], aniline–diphenylamine[7], and periodate–benzidine[8] were also used.

Preparation of kefiran and determination of the sugar composition. — The polysaccharide was isolated and purified by repeated precipitation with ethanol, followed by two freezing and thawing treatments as described earlier[1]. During the two latter treatments, *ca.* 15% of the material was lost. Third and fourth freezing and thawing treatments gave losses of 0.5 and 0.1%, respectively. The resulting preparation was dissolved in water to form a 1% solution. Fractional precipitation with ethanol yielded four fractions (Table II). All subsequent experiments were carried out with the preparation obtained after two freezing and thawing treatments.

TABLE II

FRACTIONATION OF KEFIRAN WITH ETHANOL

Precipitate from	*Yield* (%)	*Glucose in hydrolysate* (%)	$[\alpha]_D$ (*degrees*)
20% Ethanol	25	49.7	+65.8
30% Ethanol	57	50.5	+66.6
40% Ethanol	15	50.8	+65.9
Dissolved residue	3	—	+72.6

The polysaccharide was hydrolyzed, and the sugar composition of the hydrolyzate was determined by a modified hypoiodite method[9], before and after removal of glucose with glucose oxidase (D-glucose aerodehydrogenase, Boehringer).

Determination of molecular weight. — The hypoiodite method was used[9].

Periodate oxidation. — Periodate consumption and formic acid production were determined by the method of Chanda *et al.*[10]. In Table III, the results are given in moles per mole of hexosyl residue. The data were extrapolated to zero time to obtain net values for periodate consumption and formic acid production. Kefiran (100 mg) was oxidized with 0.02N periodate (100 ml) for 8 days at room temperature. Barium chloride hydrate (490 mg) was added to precipitate periodate and iodate, and, after several days, the salts were removed by filtration. Potassium borohydride (300 mg) was added to reduce the polyaldehyde, and, after a day, the solution was acidified with acetic acid. The solution was passed through a mixed bed of ion-exchange resins Amberlite IR-120 and IR-45, and the eluate was concentrated to dryness. Boric acid was removed by repeated evaporation of methanol from the residue. The residue (88 mg) was hydrolyzed in 0.1N sulphuric acid (10 ml) for 4 h at 90°, and neutralized with barium carbonate, and the filtered solution was concentrated to a small volume. The mixture was fractionated by paper chromatography with solvent (*A*). The following components were eluted from the paper strips.

TABLE III

PERIODATE CONSUMPTION AND FORMIC ACID PRODUCTION DURING OXIDATION OF KEFIRAN (MOLES PER MOLE OF HEXOSYL RESIDUE)

Time (days)	*1*	*3*	*6*	*10*	*14*	*17*	*21*
Periodate consumption	1.13	1.17	1.27	1.22	1.27	1.32	1.36
Formic acid production	0.277	0.310	0.323	0.349	0.360	0.376	0.408

D-Galactose, m.p. 163–165°, $[\alpha]_D$ +77.9° (water); lit.[10,11], m.p. 167°, $[\alpha]_D$ +81.1°.

Erythritol (5 mg) crystallized spontaneously when the tetritol fraction (12 mg) was dissolved in ethanol–ethyl ether. After recrystallization, the m.p. and mixed m.p. was 118° (lit.[12] 118.5°). The mother liquor, on treatment with benzaldehyde in the presence of hydrochloric acid[13], gave di-*O*-benzylidene-D-threitol, m.p. and mixed m.p. 221°.

Glycerol was identified as its tris(*p*-nitrobenzoate)[14], m.p. and mixed m.p. 192–194°.

Methylation of kefiran. — Kefiran (10 g) was methylated in the usual way by the Haworth procedure. After extraction of the partly methylated product with chloroform from the neutralized solution, the solvent was removed, and the product was dissolved in methyl sulphoxide (50 ml). Methylation was completed by two consecutive treatments with methyl iodide and barium oxide[15]. The methylated polysaccharide was extracted with chloroform from the semi-solid mixture, and the extract was shaken with sodium thiosulphate solution to remove iodine. The chloroform solution was concentrated, the syrupy residue was dissolved in benzene (25 ml), and an equal volume of light petroleum was added. A small amount of impurity was removed by centrifugation, and light petroleum (200 ml) was added to precipitate the bulk of the methylated product. The material was washed with 10% chloroform in hexane and redissolved in chloroform–benzene (2:3, 25 ml). The solution was poured

into light petroleum (200 ml) with stirring. The precipitate was washed with chloroform–hexane and pentane, and finally dried, The product (10.4 g) did not show hydroxyl absorption in the infrared. (Found: OMe, 41.8%).

Hydrolysis of methylated kefiran. — Methylated kefiran (2 g) was dissolved in 70% sulphuric acid (10 ml) and then diluted to 100 ml. The solution was heated for 4 h at 100°, neutralized with barium carbonate, filtered, and concentrated. The methyl sugars were separated by paper chromatography in solvent (*B*); the following fractions were obtained.

Fraction *1* (175 mg) was 2,3,4,6-tetra-*O*-methyl-D-glucose. After recrystallization from pentane, it had m.p. 95–96°, $[\alpha]_D$ +81.5° (*c* 1.6, water); lit.[16], m.p. 96°, $[\alpha]_D$ +84°. The "anilide" had m.p. 136–137°; lit[16], m.p. 137–138°.

Fraction *2*, on demethylation[17], yielded glucose with a trace of galactose. On paper chromatograms in solvents (*B*) and (*C*), this fraction had the same mobility as 2,3,6-tri-*O*-methyl-D-glucose, although the colour developed with anisidine reagent was not the same. The results of periodate oxidation of kefiran indicated the presence of both (1→4) and (1→6) linkages; therefore, both 2,3,4- and 2,3,6-tri-*O*-methylhexoses could be expected in the hydrolyzate of the methylated polysaccharide. Fraction *2* was therefore expected to contain 2,3,4- as well as 2,3,6-tri-*O*-methyl-D-glucose. The product (600 mg) was subjected to fractionation on a carbon–Celite column with a gradient of butanone–water[18]. The subfractions obtained were tested by applying drops on filter paper and developing with anisidine reagent; subfractions yielding similar colours were combined to give four fractions. The first of these (157 mg) contained 2,3,6-tri-*O*-methyl-D-glucose contaminated with tri-*O*-methylgalactoses. The second preparation (107 mg) contained exclusively 2,3,6-tri-*O*-methyl-D-glucose, m.p. 111–112°, $[\alpha]_D$ +70° (*c* 1.6, water); lit.[19], m.p. 121–123°, $[\alpha]_D$ +70.5°; its bis(*p*-nitrobenzoate) had m.p. 191–192° (lit.[20], m.p. 189–190°). The third preparation (96 mg) was a mixture, and the fourth one (196 mg) was 2,3,4-tri-*O*-methyl-D-glucose, $[\alpha]_D$ +72° (*c* 3.4, acetone); lit.[16], +70.5°. The "anilide" had m.p. 147–149°; lit.[21,22], m.p. 145–146° and 150°.

Fraction *3* yielded exclusively galactose on demethylation. In solvents (*B*) and (*C*), the material behaved as a single compound, although the results of periodate oxidation and the identity of fraction *4* indicated the presence of 2,3,6- and 2,4,6-tri-*O*-methyl-D-galactoses. Attempts at separating the tri-*O*-methylgalactoses (800 mg) on a carbon–Celite column with a gradient of ethanol[18] resulted in a partial separation. The first fraction (107 mg) contained material eluted before the peak attained its maximum, and was 2,4,6-tri-*O*-methyl-D-galactose, m.p. 102–104°, $[\alpha]_D$ +86° (*c* 1.4, water); lit.[23], m.p. 102–105°, $[\alpha]_D$ +89° (water). It gave an "anilide", m.p. 171–172° (lit.[24], m.p. 170–179°). The corresponding amide, prepared in the usual way, had m.p. 166° (lit.[25], 2,4,6-tri-*O*-methyl-D-galactonamide, m.p. 167°). The second fraction (117 mg) was the eluate around the maximum of the peak; part of it (47 mg) crystallized and was 2,4,6-tri-*O*-methyl-D-galactose. The third fraction contained the material (318 mg) trailing behind the maximum of the peak; it was oxidized with bromine water in the presence of sodium carbonate to give 2,3,6-tri-*O*-

methyl-D-galactono-1,5-lactone, m.p. 99° (from ether–pentane), $[\alpha]_D$ −38° (*c* 3.1, water); lit.[26,27], m.p. 100°, $[\alpha]_D$ −37° (water).

Fraction *4* was a methyl ether of galactose as indicated by demethylation, and its mobility on paper chromatograms suggested that it was a di-*O*-methylgalactose. The sugar crystallized from ethanol–acetone–ethyl acetate and had m.p. 109–116°. Recrystallization from 95% ethanol–ether yielded a modification having m.p. 169–171°, $[\alpha]_D$ +92.6 (10 min)→+115.6° (24 h) (*c* 3.6, water); 3,4-di-*O*-methyl-D-galactose[28] has m.p. 164–166°, $[\alpha]_D$ +95→117° (water). There was no depression of m.p. on admixture with authentic material.

Attempted estimation of the amounts of tri-O-methyl sugars. — Samples of Fractions *2* (2,3,4- and 2,3,6-tri-*O*-methyl-D-glucose) and *3* (2,3,6- and 2,4,6-tri-*O*-methyl-D-galactose) (50 mg) were dissolved in water (1 ml) and reduced with potassium borohydride (75 mg in 2 ml of water) overnight. The solutions were then acidified with acetic acid, deionized with Amberlite IR-120 (H^+), and concentrated to dryness. Methanol was evaporated several times from the residue to remove boric acid. Reducing end-group estimation by the hypoiodite method showed that in both preparations 96% of the reducing groups had been reduced by the borohydride. The tri-*O*-methylalditol mixtures were dissolved in water (20 ml), and to a sample (0.8 ml) was added 0.1M sodium metaperiodate (0.4 ml). After 30 min at room temperature, periodate consumption[10] and formaldehyde production[29] were determined.

The tri-*O*-methylgalactitol mixture consumed 0.49 mole of periodate per mole, with the production of a trace of formaldehyde. This means that half of the quantity of tri-*O*-methylgalactitol was resistant to periodate, the other half consuming one mole per mole. Since 2,4,6-tri-*O*-methyl-D-galactitol is expected to be resistant towards periodate, whereas the 2,3,6-isomer should consume one mole, it is concluded that the two tri-*O*-methylgalactoses were present in equimolar amounts.

The tri-*O*-methylglucitol mixture consumed 0.71 mole of periodate per mole while 0.35 mole of formaldehyde was produced. On complete oxidation, a periodate consumption of 1.00 mole was expected. Pure 2,3,6-tri-*O*-methyl-D-glucitol consumed 0.81 mole of periodate per mole. When the consumption by this compound in the mixture (0.71 − 0.35 = 0.36 mole) is corrected for incomplete oxidation, a value of 0.44 is obtained; this means that 44% of the mixture was 2,3,6-tri-*O*-methyl-D-glucitol. Pure 2,3,4-tri-*O*-methyl-D-glucitol consumed 0.93 mole of periodate per mole and gave rise to 0.89 mole of formaldehyde. Correction of the formaldehyde produced from the tri-*O*-methylglucitol mixture indicated that 39% of the mixture consisted of 2,3,4-tri-*O*-methyl-D-glucitol.

Enzymic hydrolysis of kefiran. — The following, crude enzyme-preparations were tested for their ability to hydrolyze the polysaccharide: *Myrothecium* cellulase[30]; Pektolyt (De Betuwe, Tiel), a crude commercial preparation derived from *Aspergillus niger*; Luizym (Luitpoldwerke, Munich), derived from *Aspergillus oryzae*; Hydralase D (Jacques Wolf, Passaic, N.Y.), from *Aspergillus oryzae*; Rapidase, an α-amylase from *Bacillus subtilis*; Rhozyme, an α-amylase probably from *Bacillus stearothermophilus*; Takadiastase (Parke, Davis and Co., London), from *Aspergillus oryzae*;

Hemicellulase (Mann Research Laboratories, New York) of fungal origin; Hydrolase (Calbiochem, Los Angeles) from *Aspergillus oryzae*; Helisol (Swiss Ferment Comp., Basle, Switzerland); *Cryptochiton stelleri* enzyme, lyophilized juice from the sugar gland[31,32]; *Trichoderma viride* cellulase (Meiji Seika Kaisha Ltd., Tokyo), a crude preparation.

Solutions (1 ml, 3%) of kefiran were incubated with the enzymes (10 mg; in the case of *Myrothecium* cellulase, 0.1 ml) and incubated for several weeks at 37°; a drop of toluene was added to prevent microbial contamination. The viscous solution was liquefied by the *Trichoderma* enzyme, whereas none of the other enzymes showed any activity. On inspection by paper chromatography of the hydrolyzate of kefiran with *Trichoderma* cellulase, glucose, a trace of a disaccharide, and a slow-moving oligosaccharide were present. A blank experiment (cellulase incubated without substrate) demonstrated that no sugars were produced from the enzyme preparation.

Preparation of kefirose. — *Trichoderma* cellulase (1 g) was dissolved in water (10 ml) and dialyzed for several hours at 0°. The solution was brought to 20% saturation with ammonium sulphate, and the precipitated material was removed. The filtrate was adjusted to 60% saturation, and the precipitate was dissolved in water (5 ml) and added to a solution of kefiran (5 g) in water (100 ml). The solution was incubated at 35° (with addition of toluene), and samples were taken periodically for determination of reducing end-groups by the hypoiodite method. After 190 days, the increase in reducing power was negligible; by that time, the percentage of hydrolysis was 42%, whereas determination (hypoiodite method in conjunction with glucose oxidase) of the amount of glucose released was 19.1%.

The hydrolyzate was fractionated on a carbon–Celite column by elution with ethanol–water mixtures[33]. Kefirose (3.4 g) was eluted with 20–40% ethanol. End-group determination with hypoiodite yielded a value for the molecular weight of 814 (calc. for a pentasaccharide, 828). The sugar composition was determined in the hydrolyzate, before and after reduction with borohydride, as described for kefiran.

Methylation of kefirose and hydrolysis of methylated kefirose. — The oligosaccharide was methylated by the Haworth procedure followed by the Purdie method. The product contained *ca.* 47% of methoxyl group (calc., 49.5%), as estimated from the hydroxyl absorption in the infrared by comparison with 2,3,4,6-tetra-*O*-methyl-D-glucose. Repeated methylation by the Purdie method did not result in a decrease of the hydroxyl peak near 3500 cm^{-1}. After hydrolysis of the product (100 mg) in N sulphuric acid (5 ml) for 4 h at 100°, paper-chromatographic inspection of the neutralized ($BaCO_3$) hydrolyzate in solvents (*B*) and (*C*) showed the presence of six sugar spots. The components were separated on sheets of filter paper with solvent (*B*), the following fractions being obtained.

Fraction (*a*) (17 mg) was 2,3,4,6-tetra-*O*-methyl-D-glucose, m.p. 85–86° (from pentane); lit.[16] 96°. The "anilide" had m.p. 137–138°; lit.[16] 137–138°.

Fraction (*b*) (19 mg) crystallized from ethanol–ethyl ether–light petroleum and had m.p. 110–111°; lit.[19] for 2,3,6-tri-*O*-methyl-D-glucose, 121–123°. The bis(*p*-nitrobenzoate) had m.p. 190–191°; lit.[20] 189–190°.

Fraction (*c*) (31 mg) was a mixture of 2,3,6- and 2,4,6-tri-*O*-methyl-D-galactose. It was partly resolved into its components, as described for the mixture of tri-*O*-methylgalactoses from kefiran. The first part of the eluate contained 2,4,6-tri-*O*-methyl-D-galactose, m.p. 102–104° (lit.[23] 102–105°), which yielded an "anilide", m.p. 170° (lit.[24] 170–179°). From the last part of the eluate, the corresponding lactone was prepared, m.p. 99° (lit.[26] for 2,3,6-tri-*O*-methyl-D-galactono-1,5-lactone, 100°).

Fraction (*d*) (16 mg), on demethylation, yielded galactose, di-*O*-methylgalactose, and mono-*O*-methylgalactoses, as disclosed by paper chromatography in solvent (*B*). The methyl sugar gave a bright spot on paper chromatograms sprayed with triphenyltetrazolium chloride, indicating that the C-2 hydroxyl group was unsubstituted. It crystallized from ethanol–ethyl acetate–pentane and, after recrystallization, had m.p. 87–89°, $[\alpha]_D$ +144 (10 min)→ +110° (*c* 1.3, water) (lit.[34] for 3,4,6-tri-*O*-methyl-D-galactose, m.p. 88–89°, $[\alpha]_D$ +154→ +110°).

Fraction (*e*) (9 mg), on demethylation, yielded galactose. The substance crystallized from ethanol–ethyl acetate–pentane and, after recrystallization, had m.p. 126°. It reacted weakly with triphenyltetrazolium chloride, which indicated that the C-2 hydroxyl group was substituted. On paper chromatograms (solvent *A*), the sugar migrated at the same rate as authentic 2,6-di-*O*-methyl-D-galactose, and the colours developed with the anisidine reagent were similar. On paper electrophoresis in 0.05M sodium borate, both substances had M_G 0.29. The sugar was thus identified as 2,6-di-*O*-methyl-D-galactose (lit.[35], m.p. 128–130°).

Fraction (*f*) (2 mg) was also a di-*O*-methylgalactose, as indicated by demethylation and by its mobility on paper chromatograms. The colour developed on the chromatogram with triphenyltetrazolium chloride indicated that the C-2 hydroxyl group was unsubstituted. The methyl sugar crystallized and had m.p. 137–138°. On paper chromatograms (solvent *A*), it moved at the same rate as authentic 4,6-di-*O*-methyl-D-galactose, and the colours developed with the anisidine reagent were similar. On paper electrophoresis in 0.05M sodium borate, both substances had M_G 0.18. The sugar was thus identified as 4,6-di-*O*-methyl-D-galactose (lit.[36], m.p. 131–133°).

Partial, acid hydrolysis of kefirose. — Kefirose (1 g) was dissolved in 1% nitric acid (80 ml) and heated for 1.5 h at 100°. After neutralization, the solution was concentrated and applied to sheets of filter paper. The sugars were separated by chromatography in solvent (*D*) for 4 days, and the eluates from the paper strips containing the oligosaccharides were concentrated to dryness. Samples of the oligosaccharides (2 mg) in water (0.2 ml) were reduced with potassium borohydride (6 mg in 0.2 ml of water) overnight; the solutions were acidified with acetic acid, and ions were removed with Amberlite IR-120 and IR-45. After concentration of the solutions to dryness, boric acid was removed by repeated distillation of methanol *in vacuo*. Samples of the oligosaccharides and the reduced oligosaccharides were hydrolyzed in N sulphuric acid for 4 h at 100°. The neutralized ($BaCO_3$) and filtered solutions were used for paper-chromatographic examination in solvent (*A*).

Fraction I was a disaccharide of galactose and glucose. After reduction with

borohydride and hydrolysis, only glucose was present. The glucosylgalactose had m.p. 171–172°, $[\alpha]_D$ +32° (*c* 1.7, water); lit.[37] for 2-*O*-β-D-glucopyranosyl-D-galactose, m.p. 171–172°, $[\alpha]_D$ +42.6°. The sugar did not react with the triphenyltetrazolium chloride reagent, and its reducing power towards Somogyi reagent was 2.5% as compared to glucose; this behaviour was expected for a (1→2)-linked disaccharide[38,39].

Fraction II was a mixture of disaccharides, part of which (90 mg) crystallized. The crystalline material yielded glucose and galactose on hydrolysis, and galactose after reduction and hydrolysis. The recrystallized galactosylglucose had m.p. 220–221°, $[\alpha]_D$ +53° (*c* 2.0, water); lit.[40] for 4-*O*-β-D-galactopyranosyl-D-glucose, m.p. 223°, $[\alpha]_D$ +55.3°. The mother liquor contained 90 mg of disaccharides which had $[\alpha]_D$ +76° (*c* 2, water). Part of this material (23 mg) crystallized and yielded exclusively galactose on hydrolysis. After recrystallization, the sugar had m.p. 209–210°, $[\alpha]_D$ +82 (10 min)→ +63° (*c* 2.3, water); lit.[41] 4-*O*-β-D-galactopyranosyl-D-galactose, m.p. 210–212°, $[\alpha]_D$ +67°.

Fraction III was a trisaccharide containing glucose and (more) galactose residues. After borohydride reduction and hydrolysis, both sugars were present in approximately equal amounts, as judged from the spots on paper chromatograms. Probably, the sugar was a glucosylgalactosylgalactose and not a galactosylglucosylgalactose, since such a trisaccharide could not be formed from kefirose.

The oligosaccharide of fraction IV was a galactose trisaccharide. The sugar (10 mg) was dissolved in water (2 ml) and reduced with potassium borohydride (30 mg) overnight. By paper-chromatographic examination along with known quantities of the oligosaccharide, it could be deduced that not more than 2% of the reducing groups were present after reduction. After recovery of the reduced compound, it was dissolved in water (5 ml) and 0.1M sodium metaperiodate (3 ml) was added. Periodate consumption was 5.6 moles per mole of trisaccharide after 1.5 h; 6.4 moles after 4 h, and 6.9 moles after 6.5 h. Extrapolation of these values to zero time yielded a minimum value of 5.5 moles of periodate per mole of trisaccharide. The remainder of the solution was reduced with borohydride (30 mg) overnight; the solution was treated as usual, and the deionized product was hydrolyzed. The neutralized hydrolyzate was examined by means of paper chromatography (solvent *A*).

Fractions V and VI were tetrasaccharides of glucose and (more) galactose. The hydrolyzate of reduced V still contained more galactose than glucose, whereas the hydrolyzate of VI contained exclusively galactose.

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REFERENCES

1 J. W. M. LA RIVIÈRE, P. KOOIMAN, AND K. SCHMIDT, *Arch. Mikrobiol.*, 59 (1967) 269.
2 E. T. REESE, F. W. PARRISH, AND M. MANDELS, *Can. J. Microbiol.*, 8 (1962) 327.
3 J. W. M. LA RIVIÈRE, in preparation.
4 S. MUKHERJEE AND H. C. SRIVASTAVA, *Nature*, 169 (1952) 330.
5 W. E. TREVELYAN, D. P. PROCTER, AND J. S. HARRISON, *Nature*, 166 (1950) 444.
6 K. WALLENFELS, *Naturwissenschaften*, 37 (1950) 491.
7 J. L. BUCHAN AND R. I. SAVAGE, *Analyst*, 77 (1952) 401.
8 J. A. CIFONELLI AND F. SMITH, *Anal. Chem.*, 26 (1954) 1132.
9 R. WILLSTÄTTER AND G. SCHÜDEL, *Ber.*, 51 (1918) 780.
10 S. K. CHANDA, J. K. N. JONES, AND E. G. V. PERCIVAL, *J. Chem. Soc.*, (1950) 1289.
11 C. N. RIIBER, *Ber.*, 56 (1923) 2185.
12 A. S. PERLIN AND C. DRICE, *Can. J. Chem.*, 33 (1955) 1216.
13 R. C. HOCKETT, *J. Am. Chem. Soc.*, 57 (1935) 2260.
14 A. M. UNRAU, *Can. J. Chem.*, 42 (1964) 916.
15 K. WALLENFELS AND G. BECHTLER, *Angew. Chem.*, 75 (1963) 1014.
16 J. C. IRVINE AND J. W. H. OLDHAM, *J. Chem. Soc.*, 119 (1921) 1744.
17 L. HOUGH, J. K. N. JONES, AND W. H. WADMAN, *J. Chem. Soc.*, (1950) 1702.
18 B. LINDBERG AND B. WICKBERG, *Acta Chem. Scand.*, 8 (1954) 569.
19 J. C. IRVINE AND E. L. HIRST, *J. Chem. Soc.*, 121 (1922) 1213.
20 P. A. REBERS AND F. SMITH, *J. Am. Chem. Soc.*, 76 (1954) 6097.
21 S. PEAT, E. SCHLÜCHTERER, AND M. STACEY, *J. Chem. Soc.*, (1939) 581.
22 J. D. GEERDES, B. A. LEWIS, AND F. SMITH, *J. Am. Chem. Soc.*, 79 (1957) 4209.
23 D. J. BELL AND S. WILLIAMSON, *J. Chem. Soc.*, (1938) 1196.
24 F. BROWN, E. L. HIRST, AND J. K. N. JONES, *J. Chem. Soc.*, (1949) 1757.
25 E. G. V. PERCIVAL AND J. C. SOMERVILLE, *J. Chem. Soc.*, (1937) 1615.
26 L. HOUGH AND D. B. POWELL, *J. Chem. Soc.*, (1960) 16.
27 J. K. N. JONES AND M. B. PERRY, *J. Am. Chem. Soc.*, 79 (1957) 2787.
28 J. S. D. BACON AND D. J. BELL, *J. Chem. Soc.*, (1939) 1869.
29 M. LAMBERT AND A. C. NEISH, *Can. J. Research*, 28B (1950) 83,
30 P. KOOIMAN, P. A. ROELOFSEN, AND S. SWEERIS, *Enzymologia*, 16 (1953) 237.
31 B. J. D. MEEUSE AND W. FLUEGEL, *Nature*, 181 (1958) 699.
32 B. J. D. MEEUSE AND W. FLUEGEL, *Arch. Neerl. Zool.*, 13 (1958) Suppl. 1, 301.
33 R. L. WHISTLER AND D. F. DURSO, *J. Am. Chem. Soc.*, 72 (1950) 677.
34 R. KUHN AND H. H. BAER, *Ber.*, 88 (1955) 1537.
35 J. W. H. OLDHAM AND D. J. BELL, *J. Am. Chem. Soc.*, 60 (1938) 323.
36 J. S. D. BACON, D. J. BELL, AND J. LORBER, *J. Chem. Soc.*, (1940) 1147.
37 A. M. GAKHOKIDZE AND N. D. KUTIDZE, *J. Gen. Chem. USSR (Engl. Transl.)*, 22 (1952) 167.
38 S. HAQ AND W. J. WHELAN, *Nature*, 178 (1956) 1222.
39 D. J. BELL, in *Modern Methods of Plant Analysis*, K. PEACH AND M. V. TRACEY (Eds.), Vol. 2, Springer, Berlin, 1955, p. 9.
40 J. R. CLAMP, L. HOUGH, J. L. HICKSON, AND R. L. WHISTLER, *Advan. Carbohyd. Chem.*, 16 (1961) 159.
41 H. O. BOUVENG AND H. MEIER, *Acta Chem. Scand.*, 13 (1959) 1884.

Notes

Resistance of secondary *p*-tolylsulfonyloxy groups in bridged ring-systems to displacement by nucleophiles*

Sodium azide in a dipolar, aprotic solvent such as *N,N*-dimethylformamide is a useful reagent for effecting nucleophilic displacement by azide ion in systems where forcing conditions are required for reaction to take place[1]. The reagent was used[2,3] in 1962 for displacement of an "isolated" secondary *p*-tolylsulfonyloxy group[4] in a sugar derivative; reduction of the resultant azido derivative provides a convenient, high-yielding procedure for synthesis of amino sugars. Since 1963, the sodium azide–*N,N*-dimethylformamide reagent, or variants thereof, has been employed extensively for synthesis of amino sugars from sulfonic esters of suitably protected, secondary alcohols[5,6].

"Isolated" secondary *p*-tolylsulfonyloxy groups[4] are normally displaced by azide with concomitant inversion of configuration, presumably through a conventional SN2 process. In situations where the "isolated" sulfonyloxy group can be oriented antiparallel to the ring oxygen-atom in a pyranoid system, a more complex pathway involving ring contraction may be operative[7–9].

The relative ease with which "isolated" secondary *p*-tolylsulfonyloxy groups undergo direct SN2 displacement by azide ion varies widely from one type of system to another. Thus, with 1,2:5,6-di-*O*-isopropylidene-3-*O*-*p*-tolylsulfonyl-α-D-glucofuranose, the reaction fails completely, even under vigorous forcing conditions[2]. In contrast, the D-allofuranose analog reacts readily with strong nucleophiles[10], including azide[11]. This difference in behavior may be explained[2] in terms of hindrance to approach of the charged nucleophile in the D-glucose derivative, where the entering group must attack from the *endo* side of the "envelope" of the bicyclo[3.3.0] fused-ring system[12].

In the present report, it is shown that the mono-*p*-toluenesulfonates (**1–4**) of four stereoisomeric 1,6-anhydro-*O*-isopropylidene-β-D-hexopyranoses, each having a bicyclo[3.2.1] ring system with an additional, fused, 5-membered ring, are all completely inert to sodium azide–*N,N*-dimethylformamide and related reagents, under conditions considerably more severe than those that usually lead to successful displacements in other systems.

The 4-*p*-toluenesulfonate[13] (**3**) of 1,6-anhydro-2,3-*O*-isopropylidene-β-D-mannopyranose and the 2-*p*-toluenesulfonate[14] (**4**) of 1,6-anhydro-3,4-*O*-isopropyl-

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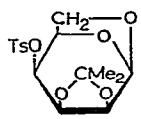
idene-β-D-galactopyranose were prepared by procedures described in the literature. *p*-Toluenesulfonylation of 1,6-anhydro-2,3-*O*-isopropylidene-β-D-talopyranose[15] gave the 4-*p*-toluenesulfonate (**1**) and, similarly, 1,6-anhydro-3,4-*O*-isopropylidene-β-D-talopyranose[15] was converted into its 2-*p*-toluenesulfonate (**2**). Substances **1** and **3** are 4-epimers, and **2** and **4** are 2-epimers.



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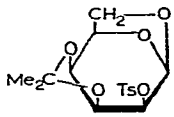

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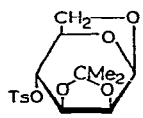

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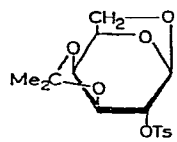


4

Each of the four substances **1**–**4** was refluxed with an excess of sodium azide or lithium azide in *N*,*N*-dimethylformamide or methyl sulfoxide for periods of 2–7 days. In each case, the starting material was recovered in high yield, and i.r. spectroscopic examination of the mother liquors failed to reveal any trace of organic azide in the product (Table I). The addition of water and urea to the reaction mixture[2] did not have any observed effect. Treatment of substance **3** with anhydrous hydrazine at reflux for 7 days gave back the starting material in high yield.

TABLE I

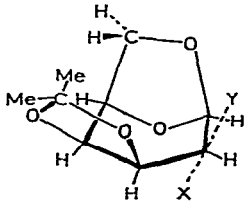
ATTEMPTED DISPLACEMENT OF THE *p*-TOLYLSULFONYLOXY GROUP BY AZIDE IN 1,6-ANHYDRO-*O*-ISOPROPYLIDENE-*O*-*p*-TOLYLSULFONYLHEXOPYRANOSES

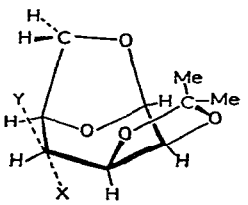
Starting material	*Solvent*	*Experimental conditions*	*Recovery (%) of crystalline starting material*[a]
1	$HCONMe_2$	2 days at reflux	72
2	$HCONMe_2$	7 days at reflux	86
2	$HCONMe_2$[b]	3 days at reflux	89
3[c]	$HCONMe_2$[d]	32 h at 110° and 2 days at reflux	50
3[c]	Me_2SO[e]	42 h at 150°	70
4[f]	$HCONMe_2$	7 days at reflux	87

[a]Identical to authentic material by mixed m.p. and comparative i.r. spectra. In each experiment the noncrystallized portion of the product, shown by t.l.c. to be mainly starting material, was examined by i.r. spectroscopy and was found to exhibit no detectable absorption for the azide group in the region 4.0–5.0 μm. [b]Water (1 ml) and urea (50 mg) added to the mixture, to simulate the conditions used by Wolfrom, Bernsmann, and Horton[2]. [c]See Ref. 13. [d]Dichloromethane was used to extract the product. [e]Chloroform was used to extract the product. [f]See Ref. 14.

The observed results suggest that, in each of the four systems, there is severe steric hindrance to the achievement of a transition state (**1a**–**4a**) having five groups around one carbon atom. This hindrance probably involves steric crowding of the potential leaving group as well as hindrance to attack by the nucleophile.

A similar lack of reactivity has been noted with 2- and 4-sulfonic esters of methyl 3,6-anhydro-α-D-glucopyranoside and related derivatives[16], and chlorosulfates of 1,6-anhydrohexopyranoses have been found[17] to be unreactive with nucleophiles. It may be that extreme lack of reactivity is a general characteristic of *p*-tolylsulfonyloxy groups, in bicyclo[3.2.1] systems formed by fusion of a tetrahydropyran and a tetrahydrofuran ring, whenever neighboring groups that could provide anchimeric assistance are absent.

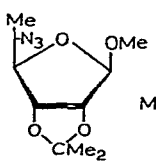




2a X = N_3^-, Y = OTs **1a** X = N_3^-, Y = OTs

4a X = OTs, Y = N_3^- **3a** X = OTs, Y = N_3^-

It is noteworthy that no reaction leading to an azido sugar with concomitant ring-contraction is observed, despite the fact that two of the derivatives (**1** and **2**) have the *p*-tolylsulfonyloxy group antiparallel to the ring oxygen-atom. This behavior is in contrast to that of methyl 6-deoxy-2,3-*O*-isopropylidene-4-*O*-(methylsulfonyl)-α-L-mannopyranoside (**6**), which reacts with azide ion in *N*,*N*-dimethylformamide to give[7–9], by ring contraction, methyl 5-azido-5,6-dideoxy-2,3-*O*-isopropylidene-α-L-talopyranoside (**5**). The conversion of **6** into **5** appears to involve an intermediate



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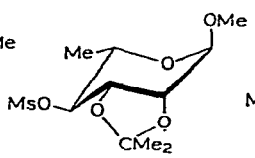

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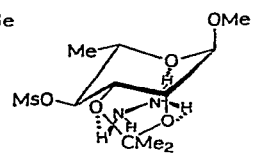

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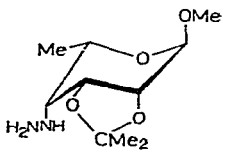
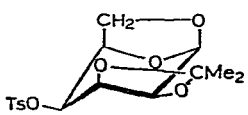


8

or transition state in which O-5 is partially bonded to C-4 before the O-5–C-5 bond is completely broken, because complete retention of configuration at C-1 is observed. It can be seen from the conformational representation (**1b**) of the anhydro derivative **1** that formation of an oxonium bridge by O-5 between C-5 and C-4 would lead to an extremely strained system.



1b

Jarý and coworkers[9] observed that, although sodium azide–*N*,*N*-dimethylformamide converted substance **6** into the ring-contracted product **5**, treatment of **6** with hydrazine led to a simple SN2 displacement at C-4 to give methyl 4,6-dideoxy-4-hydrazino-2,3-*O*-isopropylidene-α-L-talopyranoside (**8**). A reason for this difference in reactivity was not advanced. Following an earlier suggestion from these laboratories[2], it may be noted that the stereochemistry of substance **6** is such that a molecule of hydrazine could be accommodated, by hydrogen bonding with O-2, O-3, and O-5, in a situation (**7**) ideal for direct, rearside attack on C-4 by an electron pair on a nitrogen atom. An azide ion approaching this position would, on the other hand, encounter considerable electronic interference because of the nonbonding electron clouds of O-2, O-3, and O-5,

The results of this work indicate that the azide displacement reaction cannot be used with the sulfonic esters **1**–**4** as a preparative route to amino sugars, and the observations suggest that related displacement reactions in bicyclo[3.2.1] fused-ring systems may suffer from similar limitations*.

The reduction of aldosulose oximes provides a satisfactory preparative route to 4-amino-1,6-anhydro-4-deoxy-2,3-*O*-isopropylidene-β-D-talopyranose[19,20] and 2-amino-1,6-anhydro-2-deoxy-3,4-*O*-isopropylidene-β-D-talopyranose[20,21].

EXPERIMENTAL

General methods. — Solutions were evaporated under diminished pressure. Melting points were determined with a Thomas–Hoover melting-point apparatus (Arthur H. Thomas Co., Philadelphia, Pennsylvania) and are uncorrected. Infrared spectra were measured with a Perkin–Elmer Model 137 "Infracord" instrument. Microanalyses were determined by W. N. Rond. T.l.c. was performed with Silica Gel G (E. Merck, Darmstadt, Germany), activated at 110°, as the adsorbent, and 3:1 chloroform–ether as the developer, and indication was effected with sulfuric acid.

*1,6-Anhydro-2,3-*O*-isopropylidene-4-*O*-p-tolylsulfonyl-β-D-talopyranose* (**1**). — To a solution of 1,6-anhydro-2,3-*O*-isopropylidene-β-D-talopyranose[15] (160 mg) in dry pyridine (15 ml) at 0° was added a solution of purified *p*-toluenesulfonyl chloride (150 mg) in cold, dry pyridine (5 ml). The reaction mixture was kept for 18 h at room temperature and was then poured into ice–water (25 ml), whereupon white crystals of compound **1** separated. The product was filtered, washed with water, and dried; yield 173 mg (69%). After recrystallization from ethanol, the product had m.p. 140–140.5°, $[\alpha]_D^{20}$ −56 ±2° (*c* 1, chloroform); R_F 0.74; λ_{max}^{KBr} 6.25 (aryl C=C), 7.30 μm (CMe_2).

Anal. Calc. for $C_{16}H_{20}O_7S$: C, 53.92; H, 5.66; S, 9.00. Found: C, 53.87; H, 5.81; S, 9.13.

*1,6-Anhydro-3,4-*O*-isopropylidene-2-*O*-p-tolylsulfonyl-β-D-talopyranose* (**2**). — This product was prepared from 1,6-anhydro-3,4-*O*-isopropylidene-β-D-talopyranose[15]

*In a protic system, the 2-*O*-methylsulfonyl analog of 4 undergoes methanolysis to give methyl 2,6-anhydro-3,4-*O*-isopropylidene-α and β-D-talopyranoside[18].

(236 mg) and *p*-toluenesulfonyl chloride (230 mg) by the procedure used in the preceding experiment, to give compound **2** as fine, powdery crystals, yield 320 mg (77%), m.p. 177.5–178°, R_F 0.72; λ_{max}^{KBr} 6.23 (aryl C=C), 7.28 μm (CMe_2).

Anal. Calc. for $C_{16}H_{20}O_7S$: C, 53.92; H, 5.66; S, 9.00. Found: C, 53.77; H, 5.66; S, 9.02.

Attempted displacement of the p-*tolylsulfonyloxy group by azide in 1,6-anhydro-*O-*isopropylidene-*O-p-*tolylsulfonylhexopyranoses.* — The results of these experiments are summarized in Table I. To a solution of ~150 mg of the appropriate 1,6-anhydro-*O*-isopropylidene-*O*-*p*-tolylsulfonylhexopyranose (**1–4**), in the solvent noted (25 ml), was added the azide salt (~75 mg), and the mixture was heated for the period specified. The solution was evaporated, and the solid residue was extracted with three 100-ml portions of carbon tetrachloride. The extract was concentrated and then refrigerated, whereupon unreacted starting material crystallized. The remainder of the material from the reaction was isolated by evaporation of the mother liquors.

*Treatment of 1,6-anhydro-2,3-*O-*isopropylidene-4-*O-p-*tolylsulfonyl-β-*D-*mannopyranose* (**3**) *with hydrazine.* — Substance **3** (1.0 g) was refluxed with anhydrous hydrazine (10 ml; technical, anhydrous 95+%, Olin Mathieson Chemical Corp., New York) for 48 h under nitrogen. When the solution was cooled, crystalline starting material was obtained, yield 0.5 g.

T.l.c. of the filtrate revealed that compound **3** was the principal component of the mother liquor. The experiment was repeated, and the period of reflux was extended to 6 days. Evaporation of the solution and crystallization of the product from methanol gave compound **3**, yield 0.75 g (75%). The remaining mother liquor gave a positive test with ninhydrin, but no crystalline product could be isolated.

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REFERENCES

1 D. HORTON AND D. H. HUTSON, *Advan. Carbohyd. Chem.*, 18 (1963) 163–165.
2 M. L. WOLFROM, J. BERNSMANN, AND D. HORTON, *J. Org. Chem.*, 27 (1962) 4505.
3 E. J. REIST, R. R. SPENCER, B. R. BAKER, AND L. GOODMAN, *Chem. Ind.* (London), (1962) 1794.
4 R. S. TIPSON, *Advan. Carbohyd. Chem.*, 8 (1953) 107.
5 A. C. RICHARDSON, *Ann. Repts. Progr. Chem.*, 62 (1965) 371.
6 A. C. RICHARDSON, *Ann. Repts. Progr. Chem.*, 63 (1966) 489.
7 C. L. STEVENS, R. P. GLINSKI, K. G. TAYLOR, P. BLUMBERGS, AND F. SIROKMAN, *J. Am. Chem. Soc.*, 88 (1966) 2073.
8 S. HANESSIAN, *Chem. Commun.*, 21 (1966) 796.
9 J. JARÝ, P. NOVÁK, Z. KSANDR, AND Z. SAMEK, *Chem. Ind.* (London), (1967) 1490.
10 K. W. BUCK, A. B. FOSTER, R. HEMS, AND J. M. WEBBER, *Carbohyd. Res.*, 3 (1966) 137.
11 D. T. WILLIAMS AND J. K. N. JONES, *Can. J. Chem.*, 45 (1967) 7.
12 A. C. COPE AND T. Y. SHEN, *J. Am. Chem. Soc.*, 78 (1956) 3177.
13 A. E. KNAUF, R. M. HANN, AND C. S. HUDSON, *J. Am. Chem. Soc.*, 63 (1941) 1447.

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14 R. M. HANN AND C. S. HUDSON, *J. Am. Chem. Soc.*, 64 (1942) 2435.
15 D. HORTON AND J. S. JEWELL, *Carbohyd. Res.*, 5 (1967) 149.
16 M. L. WOLFROM, Y.-L. HUNG, P. CHAKRAVARTY, G. U. YUEN, AND D. HORTON, *J. Org. Chem.*, 31 (1966) 2227.
17 A. G. COTTRELL, E. BUNCEL, AND J. K. N. JONES, *Can. J. Chem.*, 44 (1966) 1483.
18 N. A. HUGHES, *Chem. Commun.*, (1967) 1072.
19 D. HORTON AND J. S. JEWELL, *Carbohyd. Res.*, 3 (1966) 255.
20 A. K. CHATTERJEE, D. HORTON, J. S. JEWELL, AND K. D. PHILIPS, *Carbahyd. Res.*, 7 (1968) 173.
21 A. K. CHATTERJEE, A. E. EL ASHMAWY, D. HORTON, J. S. JEWELL, L. G. MAGBANUA, W. E. MAST, K. D. PHILIPS, AND J. D. WANDER, *Abstracts Papers Am. Chem. Soc. Meeting*, 154 (1967) 19D.

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Carbohyd. Res., 7 (1968) 212–217

On the transglycosylase activity of lysozyme*

Sharon and Seifter[1] were the first to show that the incubation of a tetrasaccharide, obtained from bacterial cell-wall with egg-white lysozyme, produced a disaccharide in addition to compounds having a low R_F value. On the basis of this observation, they concluded that lysozyme catalyzes transglycosylation as well as hydrolyzes the bacterial cell-wall oligosaccharides. Maksimov *et al.*[2] also observed the formation of an insoluble chitin-like product during the incubation of chitin oligosaccharides with lysozyme. In the preceding papers[3,4] of this series, it was reported that *p*-nitrophenyl β-glycosides of chitin oligosaccharides were hydrolyzed by lysozyme to liberate *p*-nitrophenol which can be assayed colorimetrically, whereas *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside is not susceptible to lysozyme. In this paper, a clear demonstration of the transglycosylase activity of lysozyme was attempted by incubating *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside[5] (**1**), as the glycosyl acceptor, with lysozyme in the presence of a chitin oligosaccharide, as the glycosyl donor. Fig. 1 shows the liberation of *p*-nitrophenol during a typical experiment. In the case of tetra-*N*-acetylchitotetraose as the glycosyl donor, a smooth liberation of *p*-nitrophenol was observed. Paper chromatography of the reaction mixture after catalytic hydrogenation, which enables to detect the resulting *p*-aminophenyl glycosides with the diazotized *p*-aminobenzenesulfonic acid reagent[6], revealed an intense spot ($R_{compound\ 1}$ 0.55) at the area expected for glycosides of chitin oligosaccharides. The spot was also detected with the benzidine–periodic acid reagent[7], and it was, therefore, assumed to be one of the *p*-aminophenyl glycosides of chitin oligosaccharides produced by transglycosylase activity of lysozyme and subsequent catalytic hydrogenation. Tri-*N*-acetylchitotriose induced the liberation of *p*-nitrophenol only after a short induction period, and at a rate much slower than that of

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the tetraose. Since tri-*N*-acetylchitotriose reacts slowly, the transglycosylation was probably accelerated by the formation of small amounts of higher oligosaccharides during the induction period. In contrast to the cases of the trimer and tetramer, the incubation of di-*N*-acetylchitobiose and **1** with lysozyme did not cause the liberation of any detectable amounts of *p*-nitrophenol, even after 100 h at 40°. This result clearly indicates that lysozyme can catalyze neither hydrolysis nor polymerization of di-*N*-acetylchitobiose under the present experimental conditions.

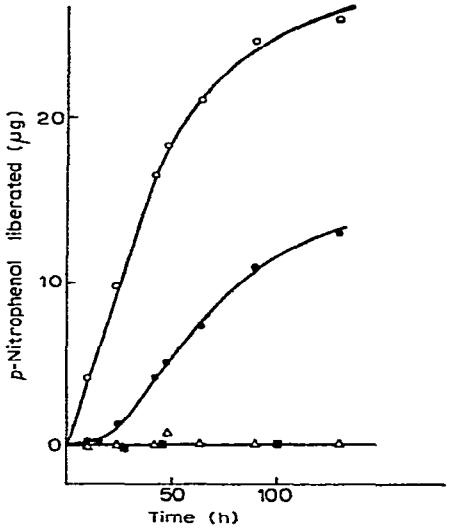
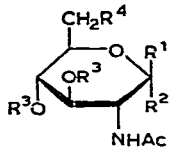


Fig. 1. The lysozyme-catalyzed liberation of *p*-nitrophenol from **1** in the presence of tetra-*N*-acetylchitotetraose (○), tri-*N*-acetylchitotriose (●), and di-*N*-acetylchitobiose (△), and from **4** in the presence of tetra-*N*-acetylchitotetraose (■). Experimental details are given in the text.

In order to elucidate the structural specificity requirements of the sugar residue whose glycosidic bond undergoes attack by lysozyme (residue D in the schema proposed by Blake *et al.*[9]), the transglycosylation reaction was applied to *p*-nitrophenyl 2-acetamido-2,6-dideoxy-β-D-glucopyranoside (**4**). Compound **4** was synthesized by



	R^1	R^2	R^3	R^4
(1)	$OC_6H_4NO_2$-*p*	H	H	OH
(2)	H	Cl	Ac	H
(3)	$OC_6H_4NO_2$-*p*	H	Ac	H
(4)	$OC_6H_4NO_2$-*p*	H	H	H

formation of the glycosyl chloride from 2-acetamido-1,3,4-tri-*O*-acetyl-2,6-dideoxy-β-D-glucopyranose[8], followed by condensation with *p*-nitrophenol, and subsequent deacetylation. Although incubation of **4** with lysozyme in the presence of tetra-*N*-acetylchitotetraose did not liberate any detectable amounts of *p*-nitrophenol, as shown in Fig. 1, examination of the reaction product by paper chromatography, after catalytic hydrogenation, revealed the presence of *p*-aminophenyl glycosides of chitin

oligosaccharides ($R_{compound\ 4}$ 0.33, 0.49). This result suggests that the hydroxyl group at C-5 of "the residue D" may be essential for the attack by lysozyme of the glycosidic bond of the residue. This assumption is in agreement with the three-dimensional lysozyme–substrate model[9] which suggests interaction between lysozyme and the hydroxyl group at C-5 of the distorted "sugar residue D". Final evidence will have to await the isolation of transfer product derived from **4** or the synthesis of the *p*-nitrophenyl β-glycoside of chitin oligosaccharides whose hydroxyl group at C-6 of "the residue D" is reduced. Work is now in progress along these lines.

EXPERIMENTAL

*General** — Descending paper chromatography was performed on Toyo Roshi No. 53 paper in butyl alcohol–pyridine–water (6:4:3). Di-*N*-acetylchitobiose, m.p. 260–262° (dec.), $[\alpha]_D^{26}$ +17° (*c* 0.42, water, after 4 h); tri-*N*-acetylchitotriose, m.p. 304–306°, $[\alpha]_D^{28}$ +2° (*c* 0.94, water, after 2 h); and tetra-*N*-acetylchitotetraose, $[\alpha]_D^{26}$ −3° (*c* 0.52, water, after 2 h) were prepared according to the procedure of Rupley[10]. Egg-white lysozyme (crystallized 4 times, lot No. 4103) was kindly provided by Seikagaku Kogyo Co.

*2-Acetamido-3,4-di-*O*-acetyl-2,6-dideoxy-α-*D*-glucopyranosyl chloride* (**2**). — A solution of 2-acetamido-1,3,4-tri-*O*-acetyl-2,6-dideoxy-β-D-glucopyranose[8] (1.50 g), in glacial acetic acid (20 ml), saturated at 0° with hydrochloric acid, was kept for 48 h at room temperature. After dilution with chloroform (50 ml), the solution was washed once with water, twice with an ice-cold saturated solution of sodium hydrogen carbonate, and finally with water. The solution was dried with sodium sulfate, and the crystalline residue, obtained after evaporation of the solvent, was recrystallized from ethyl acetate–hexane to give white needles (1.00 g, 71%), m.p. 145° (dec.), $[\alpha]_D^{25}$ +153° (*c* 0.88, chloroform).

Anal. Calc. for $C_{12}H_{18}NO_6Cl$: C, 46.83; H, 5.89; N, 4.56. Found: C, 46.74; H, 5.92; N, 4.55.

p-*Nitrophenyl 2-acetamido-3,4-di-*O*-acetyl-2,6-dideoxy-β-*D*-glucopyranoside* (**3**). — To a solution of **2** (830 mg) and *p*-nitrophenol (535 mg) in acetone (12 ml) was added 1.0M sodium hydroxide (3.88 ml). The mixture was kept for 16 h at 5°. After evaporation of the acetone, the crystals formed were collected, washed with ice-cold water until the washings were colorless, and dried, white needles (370 mg, 34%), m.p. 239–240° (dec.). Recrystallization from methanol–chloroform raised the m.p. to 245° (dec.), $[\alpha]_D^{25}$ −8° (*c* 0.61, chloroform).

Anal. Calc. for $C_{18}H_{22}N_2O_9$: C, 52.68; H, 5.40; N, 6.83. Found: C, 52.53; H, 5.47; N, 6.76.

p-*Nitrophenyl 2-acetamido-2,6-dideoxy-β-*D*-glucopyranoside* (**4**). — A suspension of **3** (273 mg) in dry methanol (6.5 ml) was warmed to 40°, 1.0M sodium methoxide (0.26 ml) was added, and the mixture was shaken until complete dissolution. After

*For conditions, see reference 3.

a further heating for 10 min at 40°, the mixture was kept overnight at 5°. The solution was evaporated, and the residue was recrystallized from water–methanol to give white needles (140 mg, 62%), m.p. 216–217° (dec.), $[\alpha]_D^{25}$ −11° (*c* 0.54, water).

Anal. Calc. for $C_{14}H_{18}N_2O_8$: C, 51.53; H, 5.56; N, 8.59. Found: C, 51.64; H, 5.73; N, 8.24.

Transglycosylation reaction. — All enzymic experiments shown in Fig. 1 were performed as follows: a 2.5×10^{-3}M solution of glycoside (**1** or **4**) in a 0.04M sodium citrate buffer (pH 5.1), containing 5 mg lysozyme per ml and a drop of toluene to inhibit bacterial growth, was incubated at 40° in the presence of an equimolar concentration of chitin oligosaccharide. A blank solution was prepared in the same way but without chitin oligosaccharide. At various time intervals, aliquots (1 ml) were withdrawn, and the reaction was interrupted by addition of 0.2M borate buffer (pH 9.8, 2.0 ml) according to Woollen *et al.*[11]. The color was measured at 400 nm, and the values obtained were corrected for the blank. The amount of *p*-nitrophenol liberated by the enzyme was determined from the molar extinction coefficient(1.8×10^{-2}) of the chromophore at this wavelength. At the end of the incubation period, the reaction mixture was transferred into a Visking cellophane tube and dialyzed thoroughly at 5° against several changes of distilled water. The combined dialyzates were concentrated, and the residue was dissolved in aqueous methanol. The solution was then hydrogenated at room temperature and normal pressure in the presence of 10% palladium on charcoal. After the rapid absorption of hydrogen had ceased, the mixture was filtered, the filtrate was evaporated, and the residue was dissolved in distilled water. A sample of the solution was chromatographed (descending) on a sheet of Toyo Roshi No. 53 paper for 12 h at 20°. Aminophenyl glycosides were detected with benzidine–periodic acid[7] and diazotized *p*-aminobenzenesulfonic acid[6].

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REFERENCES

1 N. SHARON AND S. SEIFTER, *J. Biol. Chem.*, 239 (1964) PC2398.
2 V. I. MAKSIMOV, E. D. KAVERZNEVA, AND N. A. KRAVCHENKO, *Biokhimiya*, 30 (1965) 1007.
3 T. OSAWA, *Carbohyd. Res.*, 1 (1966) 435.
4 T. OSAWA AND Y. NAKAZAWA, *Biochim. Biophys. Acta*, 130 (1966) 56.
5 D. H. LEABACK AND P. G. WALKER, *J. Chem. Soc.*, (1957) 4754.
6 B. N. AMES AND H. K. MITCHELL, *J. Amer. Chem. Soc.*, 74 (1952) 252.
7 J. A. CIFONELLI AND F. SMITH, *Anal. Chem.*, 26 (1954) 1132.
8 C. J. MOREL, *Helv. Chim. Acta*, 41 (1958) 1501.
9 C. C. F. BLAKE, L. N. JOHNSON, G. A. MAIR, A. C. T. NORTH, D. C. PHILLIPS, AND V. R. SARMA, *Proc. Roy. Soc.* (London), *Ser. B.*, 167 (1967) 378.
10 J. A. RUPLEY, *Biochim. Biophys. Acta*, 83 (1964) 245.
11 J. W. WOOLLEN, R. HEYWORTH, AND P. G. WALKER, *Biochem. J.*, 78 (1961) 111.

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SULPHATES OF MONOSACCHARIDES AND DERIVATIVES

PART VI[1] D-GLUCOSE 2-SULPHATE, D-GALACTOSE 2- AND 3-SULPHATE, AND D-GALACTOSE 2,3-DISULPHATE

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ABSTRACT

D-Glucose 2-sulphate, D-galactose 2- and 3-sulphates, and D-galactose 2,3-disulphate have been synthesized by definitive routes and isolated as amorphous barium salts From periodate-oxidation studies on these compounds, it is concluded that, where a sulphate group is located at the 2-position of glucose or galactose, oxidation proceeds *via* the open-chain form

The infrared spectra of the synthetic sugar-sulphates and of some derivatives have been studied It is shown that the spectra of sugar sulphates, particularly those having a sulphate group at position 2, cannot be used to predict the position of the sulphate group

INTRODUCTION

Of the isomeric D-galactose monosulphates, both the 4- and 6-isomers have been synthesized by definitive routes[2,3], but the 2- and 3-sulphates have been prepared only in small yields or by routes which leave some doubt as to the position of the sulphate group[1,4] The definitive syntheses of D-galactose 2- and 3-sulphates are now described For purposes of comparison, the 2-sulphate of D-glucose and the 2,3-disulphate of D-galactose have also been synthesized

DISCUSSION

D-Galactose 2-(barium sulphate) was prepared by sulphation, with the pyridine–sulphur trioxide reagent[5], of 1,3,4,6-tetra-*O*-acetyl-α-D-galactopyranose[6], followed by catalytic deacetylation of the monosulphate D-Glucose 2-(barium sulphate) was prepared in the same manner from 1,3,4,6-tetra-*O*-acetyl-α-D-glucopyranose D-Galactose 3-(barium sulphate) was prepared by sulphation of 4,6-*O*-ethylidene-1,2-*O*-isopropylidene-α-D-galactose[7], followed by hydrolytic removal of the blocking groups* D-Galactose 2,3-di-(barium sulphate) was prepared from benzyl 4,6-*O*-benzylidene-β-D-galactopyranoside[2], the blocking groups being removed by catalytic hydrogenation In every case, the barium salt of the sugar sulphate was purified by chromatography on a cellulose column

*An identical synthesis has been reported by A Stoffyn and P Stoffyn, *J Org Chem*,, 32 (1967) 4001

In view of the importance of strictly defining the positions to be sulphated the possibility of migration of the blocking groups during the sulphation process must be considered Acetyl groups are known to migrate, particularly under alkaline conditions, but that no migration of the acetyl groups in 1,3,4,6-tetra-*O*-acetyl-α-D-galactopyranose could have occurred during the sulphation was shown in the following manner The expected product, D-galactose 2-sulphate, was readily distinguishable from D-galactose 3- and 6-sulphate by its chromatographic and electrophoretic mobilities[8,9] The only other possibility, D-galactose 4-sulphate, is readily distinguished from the 2-sulphate by the characteristic colour given on thin-layer chromatograms sprayed with the diphenylamine–aniline reagent[8,10], the 2-sulphate gives a brown colour and that of the 4-sulphate is blue–grey In the synthesis of D-galactose 3-sulphate, the intermediate 4,6-*O*-ethylidene-1,2-*O*-isopropylidene-α-D-galactose was readily characterised as the crystalline 3-*O*-*p*-tolylsulphonyl derivative[7]

The chromatographic and electrophoretic properties of these sulphates have already been reported[8,9] The barium salt of the synthetic D-galactose 3-sulphate was identical in chromatographic and electrophoretic properties with a monosulphate fraction, isolated from the direct sulphation products of D-galactose, which had been provisionally identified as the 3-sulphate[1]

In previous reports on the oxidation of sugar sulphates with dilute solutions of sodium metaperiodate, it was concluded that the patterns of oxidation could best be explained by assuming that the sugar sulphates are being oxidised in the pyranoid-ring forms[2,11] This pattern of oxidation holds for D-glucose 3- and 6-sulphates, and for D-galactose 4- and 6-sulphates In contrast, Suzuki and Strominger[12] reported that the oxidation patterns for the 4- and 6-sulphates of 2-acetamido-2-deoxy-D-

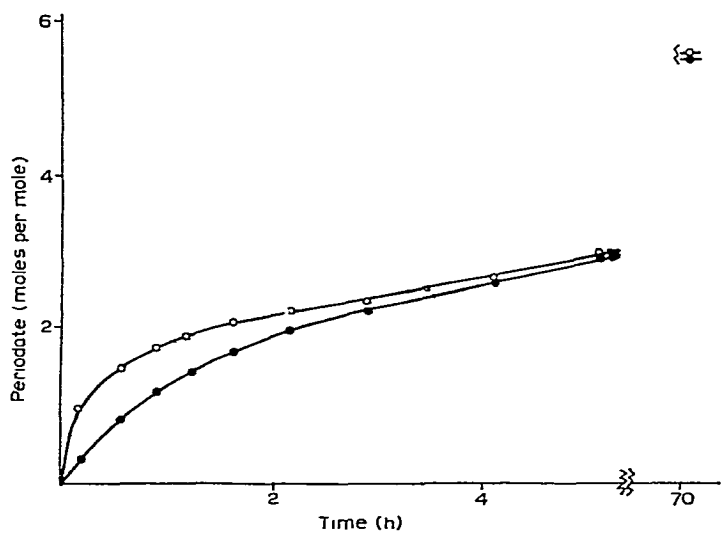
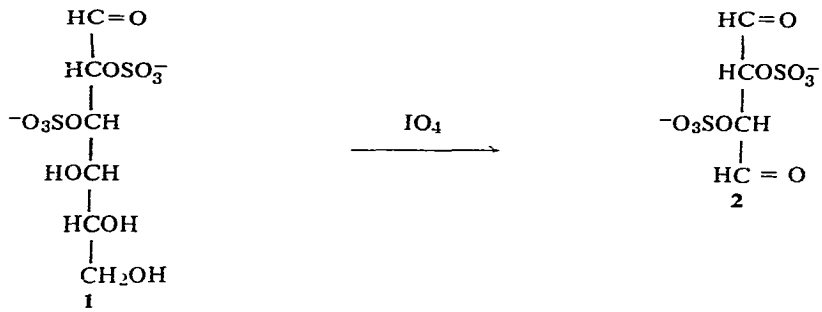


Fig 1 Periodate oxidation of D-galactose 2-(sodium sulphate) (○) and D-glucose 2-(sodium sulphate) (●)

galactose suggested that these compounds were oxidised in the open-chain forms We found that when D-galactose 3-(sodium sulphate) was oxidised with unbuffered sodium metaperiodate, the reaction followed the pattern for oxidation of the sugar in the pyranose form, one mole of periodate per mole of sugar sulphate being consumed rapidly (14 min), the oxidation then slowing down until, even at 100 h, only 3 9 moles per mole had been consumed This is very similar to the pattern of oxidation of D-glucose 3-sulphate[11]

The reductions of periodate by D-galactose 2-(sodium sulphate) and D-glucose 2-(sodium sulphate) are shown in Fig 1 D-Galactose 2-sulphate rapidly consumed 1 mole of oxidant per mole The oxidation then proceeded more slowly (but nevertheless more rapidly than the oxidation of galactose 3-sulphate), the theoretical 6 moles per mole being approached at 70 h The oxidation of D-glucose 2-sulphate was slower in the initial stages, but, after 4 h, the rate of oxidation became identical with that of D-galactose 2-sulphate It is suggested that oxidation of these 2-sulphates proceeds *via* the acyclic form of the sugar sulphate The faster initial rate of oxidation for the galactose isomer compared with that of the glucose isomer may reflect a greater equilibrium concentration of open-chain form in the galactose series than in the glucose series Whatever the reason, the relatively fast and complete oxidation of both 2-sulphates can be used as a diagnostic test

D-Galactose 2,3-di-(sodium sulphate), which cannot be oxidised in a pyranoid-ring form, is nevertheless oxidised smoothly by periodate, 2 moles of oxidant per mole of sugar sulphate being consumed in 4 h, after which the reaction terminates Oxidation of the acyclic form **1**, should lead to the consumption of 2 moles of oxidant per mole, with production of L-*threo*-tetrodialdose 2,3-disulphate (**2**)



1 **2**

After prolonged oxidation of the disulphate (for 168 h) in neutral solution the products were examined by paper electrophoresis in a neutral buffer A component migrating at a rate slightly faster than that of the parent hexose disulphate, was the major product detected From the known rates of migration of various sugar sulphates[3 9], this component was judged to be a disulphate with molecular weight less than that of hexose disulphates, in agreement with a structure such as **2**

Infrared spectroscopy has often been used for locating the sulphate group in a carbohydrate sulphate[13] This method, which is based on a survey of carbohydrate

sulphates, assumes that, for the sulphates of D-hexopyranoses in the *C1* conformation[14], the position of the absorption band at 800–850 cm^{-1} (due to C-O-S bond vibration) varies with the location of the sulphate group An equatorial sulphate (*e g*, D-glucose 3-sulphate) gives peak absorption at 830 cm^{-1}, whereas an axial sulphate (*e g*, D-galactose 4-sulphate) gives a maximum at 850 cm^{-1} In contrast, acyclic alkyl sulphates[15] absorb in the region 760 to 810 cm^{-1} We have recently commented on this method for assigning sulphate groups and have suggested that it may not always be reliable[16]

TABLE I

ABSORPTION MAXIMA FOR SYNTHETIC SUGAR SULPHATES IN THE 800–860 cm^{-1} REGION

Sugar sulphate (barium salt)	*Absorption maxima (cm^{-1})*	
	Film	*KBr disc*
D-Galactose 2-sulphate	850	844
D-Galactose 3-sulphate	828	835
4,6-*O*-Ethylidene-1 2-*O*-isopropylidene-α-D-galactose 3-sulphate	856	—
D-Galactose 2,3-disulphate	858[a]	858[b]
Benzyl 4,6-*O*-benzylidene-β-D-galactoside 2,3- disulphate	832	—
D-Glucose 2-sulphate	839	832

[a]Also a broad shoulder at 810–820 cm^{-1}, [b]Very broad peak

In Table I are given the absorption maxima in the region 800–900 cm^{-1} for the synthetic sugar sulphates and for some of their derivatives Since it has been shown[16] that the physical state of the specimen may affect the wavelength of maximum absorption, values are given for (*a*) thin films evaporated from aqueous (or aqueous ethanolic) solution on to silver chloride plates, and (*b*) potassium bromide discs

For D-galactose 3-sulphate, the absorption maximum at about 830 cm^{-1} agrees with that for a sulphate group having an equatorial orientation, suggesting that this sugar sulphate exists in the normal chair conformation of the pyranose ring, whether as a syrupy film or in the crystalline matrix of a potassium bromide disc. In contrast, the intermediate, 4,6-*O*-ethylidene-1,2-*O*-isopropylidene-α-D-galactose 3-sulphate, has a maximum at 856 cm^{-1} This shift in the absorption maximum may be attributed to a twisting of the ring, due to the fusion of the three ring systems, although it seems doubtful if this is the only explanation, since a complete change in the orientation of this sulphate group from equatorial to axial seems unlikely In benzyl 4,6-*O*-benzylidene-β-D-galactopyranoside 2,3-disulphate, where the acetal ring probably locks the sugar ring in the *C1* conformation, the absorption maximum is at the expected 832 cm^{-1} Removal of the acetal group and scission of the glycosidic bond gives D-galactose 2,3-disulphate, in which the major peak appears at 858 cm^{-1}. It seems likely that the equilibrium mixture present in the amorphous disulphate

salt contains ring forms other than the pyranose, or even the open-chain form. Mutual repulsion of the adjacent $-SO_4^-$ groups might be expected to give a conformation in which these groups are orientated axially and *trans* to one another, with a consequent shift in absorption maximum towards 850 cm^{-1} Indeed, a shoulder at about 820 cm^{-1} indicates the presence of at least one other form, in addition to that giving the major peak

The 2-sulphates of D-glucose and D-galactose both show absorption at higher frequencies than expected, particularly when in the form of syrups, and this effect is more pronounced with the galactose isomer Clearly, these sugars either do not have the expected pyranoid-ring conformations or, alternatively, factors other than the conformation affect the position of maximum absorption This is in agreement with the findings of Anderson *et al*[17], who showed that 2-sulphates of derivatives of 3,6-anhydro-D-galactose also display anomalous absorption

EXPERIMENTAL

General methods — The methods of paper electrophoresis in pyridine–acetic acid buffer and in borate buffer have been described[2,3]. Paper chromatography employed the solvent systems previously described[1], and the technique of thin-layer chromatography of sugar sulphates has been reported[8] For the purification of sugar sulphates by cellulose-column chromatography, the method of Peat *et al.*[3] was used with the modification that 75% (v/v)-aqueous ethanol was the eluant for disulphates, after monosulphates had been eluted from the column in the usual manner

Infrared spectra were measured on either a Perkin–Elmer Model 225 or a Unicam SP 200G spectrometer For thin films, the sugar sulphate in concentrated aqueous (or aqueous alcoholic) solution was layered on a silver chloride plate and evaporated to dryness in a vacuum desiccator over phosphoric oxide

D-*Galactose 2-(barium sulphate)* — 1,3,4,6-Tetra-*O*-acetyl-α-D-galactopyranose[6] had m p 144–146°, $[\alpha]_D^{18}$ +141° (*c* 0 5, chloroform). To this compound (12 g) in dry *N*,*N*-dimethylformamide (150 ml) was added pyridine–sulphur trioxide complex (12 0 g) The mixture was left at room temperature for 36 h under anhydrous conditions, and the sulphated product was then recovered as described previously[3]. The syrupy product was dried throughly and then deacetylated with barium methoxide in methanol[18] The recovered sugar sulphate in water (5 ml) was poured into a mixture of ethanol (90 ml) and ether (30 ml) The precipitate was recovered on the centrifuge, washed with ether, and dried (yield 8 1 g) Paper electrophoresis indicated that the preponderant product was a galactose monosulphate, with traces of galactose The product was purified by chromatography on a cellulose column (120 × 10 cm) to give the pure D-galactose 2-(barium sulphate) as a white, amorphous powder (5 3 g), $[\alpha]_D^{18}$ +49.6° (*c* 1 0, water) [Found S, 9.7 $(C_6H_{11}O_9S)_2Ba$ calc · S, 9 8%]

D-*Glucose 2-(barium sulphate)* — 1,3,4,6-Tetra-*O*-acetyl-α-D-glucopyranose[6] had m p 108–110°, $[\alpha]_D^{18}$ +141° (*c* 1 2, chloroform) The tetra-*O*-acetyl compound (10 5 g) in dry *N*,*N*-dimethylformamide (150 ml) was sulphated as described above,

and then deacetylated The D-glucose 2-(barium sulphate) was purified on the cellulose column to yield a white, hygroscopic powder (4 8 g), $[\alpha]_D^{18}$ +40 5° (*c* 1 0, water) [Found S, 9 9. $(C_6H_{11}O_9S)_2Ba$ calc . S, 9 8%].

D-*Galactose 3-(barium sulphate)* — 4,6-*O*-Ethylidene-α-D-galactose[7], m p 183–184°, $[\alpha]_D^{18}$ +92°, was converted into 4,6-*O*-ethylidene-1,2-*O*-isopropylidene-α-D-galactose[7], care being taken to keep the reaction mixture anhydrous in order to prevent migration of the ethylidene group The syrupy product (8 0 g) migrated as a single zone on paper chromatography The *p*-tolylsulphonyl derivative had m p 115–116°, $[\alpha]_D^{18}$ +121°, lit[7] m p 115–118°, $[\alpha]_D$ +119°

To 4,6-*O*-ethylidene-1,2-*O*-isopropylidene-α-D-galactose (2 5 g) in dry pyridine (8 ml) was added pyridine–sulphur trioxide (2 5 g) in dry pyridine (17 ml) The mixture was stirred under anhydrous conditions for 7 h at 25° and then for 12 h at 15° The sulphation product was recovered as described previously[3] The syrupy 4,6-*O*-ethylidene-1,2-*O*-isopropylidene-α-D-galactose 3-(barium sulphate) (3 5 g) had $[\alpha]_D^{18}$ +72° (*c* 0 86, water) Examination of this product by paper electrophoresis in neutral buffer revealed that sulphation was almost complete A portion (1 5 g) of the syrup was refluxed for 5 5 h in a mixture (120 ml) of acetone, water, and conc sulphuric acid (96 48 2 4, v/v) The cooled mixture was neutralised (barium hydroxide), the barium sulphate removed on the centrifuge, and the solution evaporated to dryness (yield, 1 0 g) The product contained D-galactose 3-sulphate, D-galactose, and traces of 4,6-*O*-ethylidene-D-galactose 3-sulphate Chromatography on a cellulose column (70 × 6 cm) gave pure D-galactose 3-(barium sulphate) as a white powder (120 mg), $[\alpha]_D^{18}$ +45° (*c* 0 32, water) [Found C, 20 6; H, 3 4, S, 9 9 $(C_6H_{11}O_9S)_2Ba$ calc · C, 22 0, H, 3 4, S, 9 8%]

D-*Galactose 2,3-di-(barium sulphate)* — Benzyl 4,6-*O*-benzylidene-β-D-galactopyranoside[2] (4 5 g) in dry pyridine (10 ml) was stirred with pyridine–sulphur trioxide (9 9 g) under anhydrous conditions The temperature was slowly raised to 60° and maintained for 6 h at 60–65°, and the mixture was then left at room temperature overnight The sulphation product was isolated from the mixture in the usual way to yield a buff-coloured powder (6 9 g) The product (4 0 g) in 80% (v/v) aqueous ethanol (100 ml) was reduced for 2 days with hydrogen at 5 atmos over 5% palladium-on-carbon[19] (4 g) The catalyst was filtered off, and washed with 80% aqueous ethanol, and the combined filtrates were evaporated to dryness The solid was again reduced as described above for 24 h, and the product (1 86 g) was recovered Examination by paper electrophoresis revealed that the major product migrated as a disulphate, with smaller quantities of monosulphates and free galactose The D-galactose 2,3-di-(barium sulphate) was purified by chromatography on a cellulose column, and precipitated from aqueous solution with ethanol (10 vol) as a white powder (0 58 g), $[\alpha]_D^{18}$ +72° (*c* 0 36, water) (Found S, 13 2 $C_6H_{10}O_{12}S_2Ba$ calc S, 13 5%) The disulphate had R_F 0 16 in the solvent butyl alcohol–acetic acid–water (50 12 25), compared with 0 45 for galactose 2-(barium sulphate) On paper electrophoresis in 0 1M borate buffer (pH 10 0), it had M_G 2 9 (*cf*, galactose 2-sulphate, 1 02, galactose 3-sulphate, 1 33)

Periodate oxidations — To the barium salt of the sugar sulphate (15 mg) in water (5 ml) was added a slight excess of sodium sulphate in water (1 ml) Precipitated barium sulphate was removed on the centrifuge, and portions (1 ml) of the supernatant solution were incubated in the dark at 35° with 0 018M sodium metaperiodate (5 ml) At suitable intervals, the periodate remaining was determined[20] The oxidant consumed in moles per mole (time in h) for D-galactose 3-sulphate was 0 97 (0 25), 1 61 (0 80), 2 05 (2 0), 2 15 (3 0); 2 6 (5 0), 3 9 (100) For D-galactose 2,3-disulphate, the corresponding results were: 0 45 (0 30), 0 90 (0 75), 1 45 (1 4), 1 82 (2 5), 1 95 (4 0), 1 98 (50) After 168 h, the reaction mixture from the disulphate was adjusted to pH 10 with aqueous barium hydroxide, and the precipitated barium salts were removed on the centrifuge Excess of barium was precipitated with carbon dioxide and the filtrate was evaporated to dryness Sugar fragments were extracted from the residue with hot 75% aqueous ethanol, and the extracts were evaporated to dryness The product was then examined by paper electrophoresis in pyridine–acetic acid buffer The major product detected on the paper migrated at a rate slightly faster than that of the parent disulphate Traces only of non-migrating material were detected

D-Galactose 2-sulphate and D-glucose 2-sulphate were separately oxidised, and the results are given in Fig 1

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REFERENCES

1 Part V *J Chem Soc*, (1963) 2242
2 J R Turvey and T P Williams, *J Chem Soc*, (1962) 2119
3 S Peat, J R Turvey, M J Clancy, and T P Williams, *J Chem Soc*, (1960) 4761
4 D Grant and A Holt, *J Chem Soc*, (1960) 5026
5 R B Duff, *J Chem Soc*, (1948) 1597
6 B Helferich and J Zirner, *Ber*, 95 (1962) 2604
7 D H Ball and J K N Jones, *J Chem Soc* (1958) 905
8 D M Bowker and J R Turvey, *J Chromatog*, 22 (1966) 486
9 J R Turvey, *Advan Carbohydrate Chem*, 20 (1965) 183
10 R W Bailey and E J Bourne, *J Chromatog*, 4 (1960) 206
11 J R Turvey, M J Clancy, and T P Williams, *J Chem Soc*, (1961) 1692
12 S Suzuki and J L Strominger, *J Biol Chem*, 235 (1960) 2768
13 S F D Orr, *Biochim Biophys Acta*, 14 (1954) 173 A G Lloyd and K S Dodgson, *Biochim Biophys Acta*, 46 (1961) 116
14 R E Reeves, *Advan Carbohydrate Chem*, 6 (1951) 107
15 A G Lloyd, N Tudball, and K S Dodgson, *Biochim Biophys Acta*, 52 (1961) 413
16 J R Turvey, D M Bowker, and M J Harris, *Chem Ind* (London), (1967) 2081
17 N S Anderson, T C S Dolan, D A Rees, G P Mueller, D J Stancioff, and N F Stanley, *J Chem Soc*, C, (1968) 602
18 H S Isbell, *Bur Standards J Research*, 5 (1930) 1179
19 R Mozingo, *Org Syn*, 26 (1946) 78
20 G O Aspinall and R J Ferrier, *Chem Ind* (London), (1957) 1216

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REACTION OF SOME SULFONIC ESTERS OF D-MANNITOL WITH METHOXIDE, SYNTHESIS OF 2,3 4,5-DIANHYDRO-D-IDITOL*

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ABSTRACT

3,4-Di-*O*-(methylsulfonyl)- and 3,4-di-*O*-*p*-tolylsulfonyl-D-mannitol and their 1,2,5,6-tetraacetates have been synthesized and crystallized By the action of an excess of methanolic barium methoxide during 2 h at 25°, each of these compounds is converted into 2,3 4,5-dianhydro-D-iditol, the structure of which is assigned on the basis of chemical and physical properties, including n m r spectroscopic evidence

INTRODUCTION

In 1947, Wiggins[1] reported the preparation of 1,4 3,6-dianhydro-D-iditol (**6**) by the following series of reactions D-Mannitol (**1**) was condensed with acetone to give 1,2 5,6-di-*O*-isopropylidene-D-mannitol (**2**), and **2** was *p*-toluenesulfonylated to afford the 3,4-di-*p*-toluenesulfonate (**3b**) of **2** Compound **3b** was then treated with 70% acetic acid during 3 h at 70°, giving an unstated yield of an uncharacterized syrup that was formulated as 3,4-di-*O*-*p*-tolylsulfonyl-D-mannitol (**4b**) This crude syrup was acetylated with boiling acetic anhydride and sodium acetate to give a 65% yield of a syrup which, it was supposed, was the 1,2,5,6-tetraacetate (**5b**) of **4b** Then, by the action of an excess of sodium methoxide on the crude, syrupy **5b**, Wiggins[1] obtained a syrup, presumed to be 1,4 3,6-dianhydro-D-iditol (**6**) (yield, 54%, calc on **5b**)

However, the syrupy dianhydride had $[\alpha]_D$ −16 7° (water)[1], and, although it crystallized, Wiggins could not recrystallize it In contrast, the enantiomorph (**6-e**) of **6** is readily crystallized and recrystallized, and has[2] m p 64° and $[\alpha]_D$ +20 8° (water), and its hemihydrate has[1] m p 43–45° and $[\alpha]_D$ + 18 4° (water) Moreover, on benzoylation of the syrupy dianhydride (**6**), the yield of the 2,5-dibenzoate of **6** was only 48 5% of the theoretical, instead of the almost 100% to be expected Similarly, methanesulfonylation of the syrup gave the 2,5-dimethanesulfonate of **6**, but in only 40 3% yield Thus, it seems reasonable to conclude that the actual content of compound **6** in Wiggins' syrupy **6** was probably less than 50% (which means that the actual yield of **6** was less than 27%, calc on **5b**)

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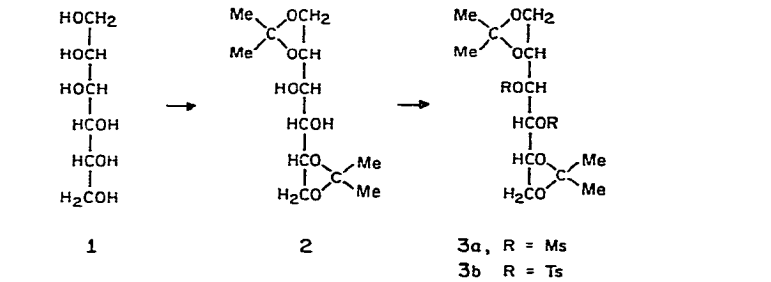
From this evidence alone, it would seem that, not only must Wiggins' "dianhydro-D-iditol" have been grossly impure, but the syrupy tetraacetate (**5b**) and, therefore, the syrupy di-*p*-toluenesulfonate (**4b**) from which it was prepared were probably impure also Consequently, we decided to re-investigate some of these reactions



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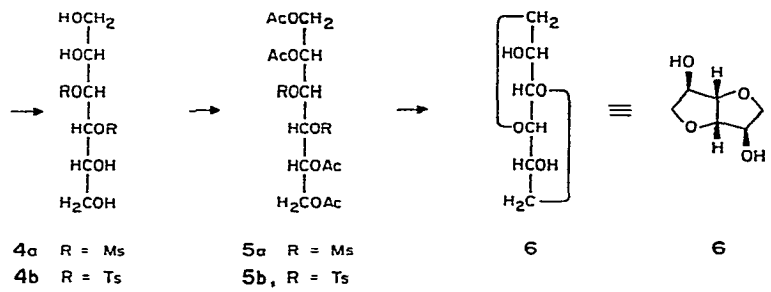
3b R = Ts



4a R = Ms 5a R = Ms 6 6
4b R = Ts 5b, R = Ts

RESULTS AND DISCUSSION

In the present study, the preparation of each compound in the sequence **1** to **5b** has been improved, and compound **4b** and its tetraacetate **5b** (obtained as syrups by Wiggins' have been crystallized and characterized In addition, compound **4a** and its tetraacetate (**5a**) have been synthesized from **3a** (already known), and have also been crystallized and characterized

A polarimetric study (see Fig 1) of the formation of **4b** from **3b** revealed why Wiggins' syrupy di-*p*-toluenesulfonate **4b** was impure On treating **3b** with 4 1 (v/v) acetic acid–water at 65°, we found that, at first, the optical rotation rises, reaching a maximum after the elapse of 45 min At this stage, hydrolysis of the isopropylidene acetal groups was essentially complete, because, on arresting the reaction, and removing the solvent, a 93% yield of crystalline **4b** was obtained (Crystalline **4a** was obtained in a yield of 83% by similar treatment of **3a**) However, if the reaction in aqueous acetic acid at 65° is permitted to proceed beyond 45 min, the optical rotation slowly falls, suggesting slow liberation of free *p*-toluenesulfonic acid, possibly with associated dehydrations Consequently, as Wiggins had conducted the hydrolysis with 70% acetic acid for 4 times as long (3 h) at a higher temperature (70°), his syrupy **4b** must

have contained artifacts, and these would have led to contamination of his tetraacetate **5b**

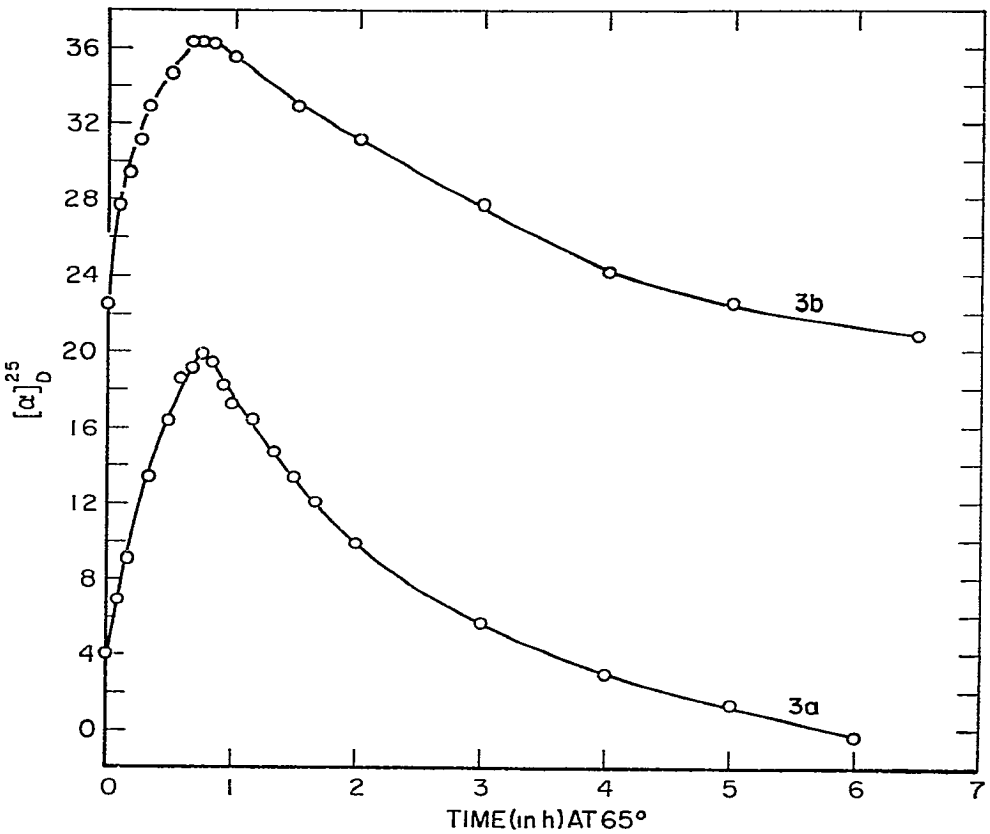


Fig 1 Rate of hydrolysis of 1,2 5,6-di-*O*-isopropylidene-3,4-di-*O*-(methylsulfonyl)-D-mannitol (**3a**) and of 1,2 5,6-di-*O*-isopropylidene-3,4-di-*O*-*p*-tolylsulfonyl-D-mannitol (**3b**) in 4 1 (v/v) acetic acid–water at 65°

Acetylation of our crystalline **4a** and **4b** afforded the crystalline tetraacetates **5a** and **5b**, respectively Because the properties of our **4b** and **5b** were quite different from those reported by Wiggins[1] for these two compounds, we decided to saponify the crystalline tetra-*O*-acetyl-di-*O*-*p*-tolylsulfonyl derivative **5b** under his conditions, in the expectation of obtaining a high yield of pure **6**, this product was not, however, obtained Treatment of our pure, crystalline **5b** in chloroform with an excess of sodium methoxide in methanol at 0°, followed by 4 h at 25° (with isolation as described by Wiggins[1], except that carbon dioxide was used instead of 0 5M sulfuric acid to neutralize the base), gave a practically quantitative yield (calculated as dianhydrohexitol) of a product consisting of colorless crystals and a colorless syrup The crystalline product had an elementary analysis corresponding to that calculated for a dianhydrohexitol hemihydrate, but (*1*) its melting point was much higher than that reported[1,2]

for 1,4 3,6-dianhydro-L-iditol (**6-e**) or its hemihydrate, (*2*) its specific rotation (in water) was much higher than that expected for **6** on the basis of that of **6-e**, and (*3*) its infrared spectrum differed significantly from that of authentic* **6-e** Thin-layer chromatograms of the syrup on Silica Gel G†‡, with ethyl acetate as the developer, showed the presence of three compounds, the fastest-moving of which had the same R_F value as authentic **6-e**

In an attempt to increase the yield of the new, crystalline dianhydro-D-iditol, the reaction time was increased (16 h at 25°), but t l c of the reaction mixture with 99 1 (v/v) ethyl acetate–methanol showed two main components and three trace components After one development, the fastest-moving component had the same R_F value as that of **6-e**, but a second development with the same solvent mixture showed that they differed Similar treatment of pure, crystalline **5a** in chloroform with methanolic sodium methoxide followed by 2 days in a refrigerator and then 4 days at 25°, caused almost complete disappearance of the component having the same R_F value (1 development) as **6-e** and the appearance of several new components, suggesting the formation of methyl ethers

As the conversion of the tetraacetates **5a** or **5b** into a dianhydrohexitol involves deacetylation, it seemed possible that the acetylation step might be unnecessary This was verified, treatment of pure, crystalline **4a** with an excess of methanolic sodium methoxide only (for 24 h at 25°) gave a mixture similar to that from the tetraacetate **5a** (16 h at 25°)

It was then decided to determine the effect of conducting the reaction for 2 h (instead of 4 h, as described by Wiggins[1]) at 25°, and to employ methanolic barium methoxide (without chloroform) instead of sodium methoxide Under these conditions, only one product was formed (t l c), and compounds **4a** and **4b** (and **5a** and **5b**) each gave an excellent yield of a crystalline compound (*A*) having an elementary analysis agreeing with that calculated for a dianhydrohexitol Its specific rotation was much higher than that expected for compound **6** on the basis of the specific rotation of the enantiomorph[2] (**6-e**), and its melting point and i r spectrum differed from those of **6-e**

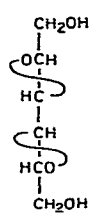
Compound *A* was found to have two free hydroxyl groups, because, on acetylation with acetic anhydride–pyridine (160 min at 25°), it afforded a crystalline compound having an elementary composition agreeing with that calculated for a di-*O*-acetyldianhydrohexitol, yield 97% A crystalline dibenzoate was also prepared, its properties differed from those expected for the 2,5-dibenzoate of **6** on the basis of those

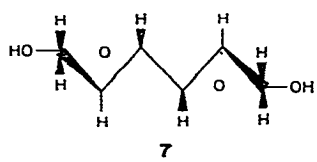
*Samples of this compound and its 2,5-dibenzoate were kindly supplied by Dr H G Fletcher, Jr , of the National Institutes of Health, Bethesda, Maryland 20014

†From E Merck A-G, Darmstadt, Germany pretreated with water (2 ml per g), air-dried overnight, and activated for 1 h at 110°

‡Certain commercial products and instruments are identified in this paper in order to specify adequately the experimental procedure In no case does such identification imply recommendation or endorsement by the National Bureau of Standards, nor does it imply that the product or equipment identified is necessarily the best available for the purpose

of the dibenzoate[2] of **6-e** The n m r spectrum of compound *A*, measured in methyl sulfoxide-d_6, showed signals of the hydroxyl protons as a 2-proton, apparent triplet, and hence the two hydroxyl groups are both primary[3] This conclusion was confirmed by the observation that compound *A*, found to be stable in aqueous solution at 25°, is unaffected by sodium metaperiodate in water during 24 h at 25°, showing that the two hydroxyl groups of compound *A* are not vicinal As a further confirmation, the n m r spectra of the diacetate and dibenzoate, measured in chloroform-*d*, showed a downfield shift of the signals of four protons Consequently, the two anhydro rings of compound *A* must involve carbon atoms 2,3,4, and 5 Unless rearrangement occurred after the initial reaction, a 2,5 3,4-dianhydride is ruled out because of the method of preparation, suggesting that compound *A* is the 2,3 4,5- or 2,4 3,5-dianhydride The n m r spectra of compound *A* and its 1,6-diacetate and 1,6-dibenzoate were consistent with either of these structures (and the respective esters thereof) The n m r spectrum of compound *A* showed only four main groups of signals, and, for some compounds, this situation could be attributed to chemical-shift averaging caused by completely free rotation at the C-1–C-2, C-3–C-4, and C-5–C-6 bonds However, inspection of molecular models of the 2,3 4,5- and 2,4 3,5-dianhydrides of D-iditol reveals that, with suitable orientation of the hydroxymethyl groups, each molecule can have a simple, two-fold axis of symmetry vertical to the C-3–C-4 bond As compound *A* and its two diesters are optically active (and therefore lack a plane of symmetry), the optical asymmetry at C-2 differs from that at C-5, and that at C-3 differs from that at C-4 Consequently, it seems reasonable to attribute the observed chemical-shift equivalence of H-1 with H-6 or H-6′, of H-1′ with H-6′ or H-6, of H-2 with H-5, and of H-3 with H-4, to the presence of the two-fold axis of symmetry Such symmetry, which does not make the molecule optically inactive, is nevertheless sufficient to give chemical-shift equivalence of protons located similarly on each half of the chain of carbon atoms Because the 2,4 3,5-dianhydride structure has two oxetan rings, seldom encountered in sugar chemistry, we have assigned the 2,3 4,5-dianhydride structure (**7**) to compound *A*.





7

Incidentally, occasion was taken to prepare the hexakis(methanesulfonate) and hexakis-(*p*-toluenesulfonate) of **1**

EXPERIMENTAL

General methods — All melting points are corrected I r spectra were recorded with a Perkin–Elmer Model 137 "Infracord" or (for spectra of higher resolution)

with a Model 257 grating i r spectrophotometer N m r spectra were recorded with a Varian A-60 n m r spectrometer, with tetramethylsilane (τ 10 00) as internal standard

1,2·5,6-Di-O-isopropylidene-D-mannitol (**2**) — The method of Baer[4] was simplified Anhydrous zinc chloride (70 4 g) was placed in a 500-ml Erlenmeyer flask, 352 ml of acetone (reagent grade) was added, the flask was quickly stoppered, and the suspension was swirled until the salt had dissolved The suspension was filtered (fluted, soft paper) into a 500-ml, round-bottomed flask containing 36 4 g (0 2 mole) of **1** and an egg-shaped stirring-bar, and the suspension was magnetically stirred (under a reflux condenser) in a bath of cool water until the alditol had just dissolved (2 h) The solution was poured immediately, with stirring, into a beaker containing a solution of 88 g of potassium carbonate in 88 ml of water (If necessary, the resulting mixture could then safely be kept at room temperature for several days, with no deleterious effects) The suspension was now filtered with suction The precipitate was stirred three times with chloroform, and the suspension was filtered each time, the combined extracts were dried (anhydrous sodium sulfate), and evaporated to dryness, giving 25 4 g of colorless crystals The aqueous layer was extracted three times with chloroform, and the combined extracts were similarly treated, to give 16 8 g of colorless crystals

For recrystallization, the total product was dissolved in chloroform (1 ml per g), heptane (10 ml per g) was added, the mixture was boiled under reflux, the hot solution was filtered (fluted, soft paper), and the filtrate was diluted with heptane (10 ml per g) and kept overnight in a refrigerator, to give 27.7 g (53%) of colorless crystals of **2** (pure enough for the next step), m p 120–121°, ν_{max}^{KCl} 1387 (CMe_2), 1376 (CMe_2), 1160, 1126, and[5] 1044 cm^{-1} (1,3-dioxolane ring)

1,2 5,6-Di-O-isopropylidene-3,4-di-O-(methylsulfonyl)-D-mannitol (**3a**) — The method of Bladon and Owen[6] was improved In a 250-ml, three-necked, round-bottomed flask (equipped with a stirrer, pentane thermometer, and pressure-equalizing dropping funnel) was placed 13 2 g (0 05 mole) of **2**, 30 ml of dry pyridine was added, and the solution was cooled in ice–salt to $-10°$ A solution of 9 5 ml of methanesulfonyl chloride in 20 ml of dry pyridine was now added dropwise, with stirring, at such a rate that the temperature remained below $-5°$ (12 min) The mixture was then stirred for 1 h at $-10°$, the bath was removed, and stirring was continued for a further 5 h The mixture was recooled to $-10°$, and water was added in portions (1+1+1+2+5+10 ml) at 5-min intervals, with stirring, so that the temperature remained below $-5°$ Water (100 ml) was added, the suspension was filtered, and the colorless crystals were washed thoroughly with water, and dried at 0 1 torr over phosphorus pentaoxide, wt , 19 8 g, m p 138–141° The filtrate and washings were combined and extracted with chloroform, the extracts were combined and washed successively with excess potassium hydrogen sulfate solution water, sodium hydrogen carbonate solution, and water, dried (anhydrous sodium sulfate), and evaporated to dryness, to give 1 0 g of crystalline product, total yield 20 8 g (99%) The compound (**3a**) was recrystallized from boiling absolute methanol (6 ml per g), m p 143–144°

(lit [6] m p 143–144°), $[\alpha]_D^{25}$ +4 1° (*c* 4 2, 4 1 acetic acid– water), ν_{max}^{Nujol} no OH bands, 1359 (S = O), 1175 (S = O, 1,3-dioxolane), 1145, and 1040 cm^{-1} (1,3-dioxolane) The compound should be stored in a refrigerator

1,2 5,6-Di-O-isopropylidene-3,4-di-O-p-tolylsulfonyl-D-mannitol (**3b**) — To minimize concomitant formation of the mono-*p*-toluenesulfonate[7], the procedure of Brigl and Gruner[8] was modified[9] To 52 5 g (0 2 mole) of **2** in a 1-liter Erlenmeyer flask was added 336 ml of dry pyridine, then 84 g of *p*-toluenesulfonyl chloride was added in one portion, the flask was stoppered, and the mixture was swirled at room temperature until the crystals had dissolved Crystallization of pyridinium chloride began after about 4 h The mixture was kept for 7 days at 25°, and then cooled in ice–salt to below −1° It was processed as for **3a**, but with double the proportions of water, followed by an equal volume of water, and the resulting, colorless crystals were removed by filtration, washed with water, pressed, and dissolved in chloroform The chloroform solution was washed as for **3a**, dried, and evaporated to dryness, affording a practically quantitative yield of colorless crystals Recrystallization from boiling absolute methanol (5 ml per g) gave 91 6 g (80%) of **3b**, m p 119–120°, $[\alpha]_D^{26}$ +8 6° (*c* 4 0, chloroform), $[\alpha]_D^{27}$ +22 5° (*c* 1 0, 4 1 acetic acid–water) [lit [8] m p 120–121°, $[\alpha]_D$ +9 3° (chloroform)]; ν_{max}^{Nujol} no OH bands, 1605 (phenyl C = C), 1355 and 1183 (S = O), 820 cm^{-1} (*p*-substituted phenyl)

After shorter reaction times, the yield and m p of once-recrystallized product were 3 days, 60%, 113–115°, 5 days, 70%, 119–120°

Rate of hydrolysis of **3a** *by aqueous acetic acid* — Finely powdered **3a** (20 000 g) was weighed into a 50-ml volumetric flask, 4 1 (v/v) acetic acid–water was added to the mark, the suspension was shaken at room temperature until all of the crystals had dissolved (15 min), and the solution was cooled in ice Portions (6 ml) of this solution were placed in Pyrex test tubes, which were then cooled in Dry Ice–chloroform, sealed, and stored in the refrigerator until used For a determination, a tube was warmed to room temperature and was then heated, in a bath maintained at 65°, for a chosen period of time The tube was removed from the bath, rapidly cooled in Dry Ice–chloroform, opened, warmed to room temperature, and the specific rotation of the solution determined (2-dm tube) The results are given in Fig 1, from which it may be seen that, after the elapse of 45 min at 65°, hydrolysis of the isopropylidene groups was complete, thereafter, slow hydrolysis of the sulfonic ester groups continued After 45 min, the solution had $[\alpha]_D^{27}$ +19 9° (calc on the original wt of **3a**); $[\alpha]_D^{27}$ +24 6° (calc as **4a**)

Rate of hydrolysis of **3b** *by aqueous acetic acid* — Finely powdered **3b** (10 000 g) was weighed into a 100-ml volumetric flask, and 4 1 (v/v) acetic acid–water was added to the mark The determinations were then performed as described for the hydrolysis of **3a** The results are given in Fig 1, from which it may be seen that the hydrolysis is similar to that of **3a** After 45 min, the solution had $[\alpha]_D^{27}$ +36 3° (calc on the original wt of **3b**), $[\alpha]_D^{27}$ +42 2° (calc as **4b**)

3,4-Di-O-(methylsulfonyl)-D-mannitol (**4a**). — In a 500-ml, round-bottomed flask were placed 200 ml of acetic acid, 50 ml of water, and an egg-shaped, magnetic

stirring-bar, the flask was closed with a loose-fitting stopper bearing a thermometer, and the solution was heated (in a bath at 67°), with stirring, until its temperature reached 65° (30 min) Compound **3a** (10 5 g, 0 025 mole) was now added, the flask was closed, and the suspension was stirred at 65° until all of the crystals had dissolved (2 min) The resulting solution was heated for 45 min at 65°, cooled in ice, and evaporated under diminished pressure (bath temp 35°) to a colorless syrup which was (3 times) dissolved in 50 ml of water and the solution was re-evaporated to a syrup The syrup was dissolved in 50 ml of absolute ethanol, and the solution was evaporated to a syrup which crystallized, the mass was stirred with absolute ethanol (5 ml per g) and filtered, to give colorless crystals, wt 6 1 g (72%), m p 111–114° The mother liquor afforded a second crop, 0 9 g, m p 104–108° The total yield was 7 0 g (83%) It was recrystallized from absolute ethanol (5 ml per g), m p 113–115°, $[\alpha]_D^{26}$ +30 6° (*c* 1 0, water), ν_{max}^{Nujol} 3546 (OH), 1359 (S = O), 1170 cm^{-1} (S = O)

Anal Calc for $C_8H_{18}O_{10}S_2$ C, 28 4, H, 5 4, S, 19 0. Found C, 28 5, H, 5 2, S, 18 9

On recrystallization from cold absolute methanol (30 ml per g) by the addition of dry ether (50 ml per g), it crystallized with 1 molecular proportion of methanol; m p 102–103° (softens at 77°), $[\alpha]_D^{27}$ +28 4° (*c* 1 0, water)

Anal Calc for $C_8H_{18}O_{10}S_2$ CH_3OH. C, 29 2, H, 6 0, S, 17 3 Found C, 29 0, H, 5 8, S, 17 5

3,4-Di-O-p-*tolylsulfonyl*-D-*mannitol* (**4b**). — The conditions of Brigl and Gruner[8] were modified as follows the concentration of **3b** in the initial solution was almost halved, a temperature of 65° (instead of 60°) was used, and the reaction time was diminished from 4 h to 45 min Compound **3b** (11 4 g, 0 02 mole) was treated as described for the preparation of **4a** from **3a**, after removal of the aqueous acetic acid at the conclusion of the hydrolysis, the resulting colorless syrup was dissolved in chloroform, and the solution was washed successively with aqueous sodium hydrogen carbonate solution (until free from acid) and water, dried (anhydrous sodium sulfate), and evaporated to dryness under diminished pressure, giving a colorless, flaky glass which was dried at 0 2 torr, wt 9 7 g (99%) This was dissolved in chloroform (10 ml per g) and the solution was refrigerated, to give (in two crops) 9 1 g (93%) of colorless crystals, m p 128–129° Alternatively, the flaky glass was dissolved in absolute methanol (1 ml per g), water was added dropwise to faint opalescence, and the solution was refrigerated, to give colorless crystals, wt 8 7 g (89%), m p 130–131° It was recrystallized from cold absolute methanol (12 ml per g) by gradual addition of pentane (24 ml per g), m p 133–134°, $[\alpha]_D^{25}$ +50 4° (*c* 1 0, absolute methanol), +40 1° (*c* 1.0, 4 1 acetic acid–water), +47 1° (*c* 1 0, dry pyridine), ν_{max}^{Nujol} 3448 (OH), 1600 (phenyl C = C), 1351, 1190, and 1176 (S = O), 820 cm^{-1} (*p*-substituted phenyl)

Anal Calc for $C_{20}H_{26}O_{10}S_2$· C, 49 0, H, 5 3; S, 13 1 Found C, 49 1; H, 5 5; S, 12 9

1,2,5,6-Tetra-O-*acetyl-3,4-di*-O-(*methylsulfonyl*)-D-*mannitol* (**5a**) — To a solution of 10 ml of acetic anhydride in 10 ml of dry pyridine at 0° was added 3 4 g (0 01 mole) of **4a**, and the suspension was swirled at 0° until the crystals had dissolved

The solution was kept overnight at 25°, and poured, with stirring, onto 100 ml of chopped ice, to give 3 5 g of colorless crystals The aqueous solution was extracted with chloroform and the extract was processed as usual, to give a further 1 5 g of product, total yield, quantitative The compound was recrystallized from absolute methanol (8 ml per g) by adding pentane (5 ml per g), m p 81–83°, $[\alpha]_D^{29}$ +30 2° (*c* 1 0, absolute methanol), ν_{max}^{Nujol} no OH bands, 1745 (ester C = O), 1351, 1176 cm^{-1} (S = O)

Anal Calc for $C_{16}H_{26}O_{14}S_2$· C, 37 9, H, 5 2, S, 12 7 Found C, 38 1; H, 5 1, S, 12 6

*1,2,5,6-Tetra-*O*-acetyl-3,4-di-*O*-p-tolylsulfonyl-*D*-mannitol* (**5b**) — The following procedure gave a crystalline product To 9 8 g (0 02 mole) of **4b** was added 30 ml of dry pyridine, the flask was quickly stoppered, and the suspension was swirled until the crystals had dissolved The colorless solution was cooled for 5 min in ice–salt, 30 ml of acetic anhydride was added, and the solution was swirled and then kept in the bath for 5 min The solution was now kept for 3 h at 25°, and poured onto chopped ice, with stirring The mixture was extracted with chloroform, and the extract was washed successively with potassium hydrogen sulfate solution, water, and sodium hydrogen carbonate solution, dried (anhydrous sodium sulfate), and evaporated to dryness under diminished pressure, giving a pale-yellow syrup (12 9 g) which was dissolved in 66 ml of absolute ethanol at room temperature After being kept overnight at room temperature, an almost solid mass of colorless crystals was obtained, this was refrigerated overnight, and filtered, to give 11 3 g (86%) of crystals These were recrystallized from absolute ethanol (5 ml per g), m p 83–84°, $[\alpha]_D^{27}$ +28 5° (*c* 1 0, chloroform), ν_{max}^{Nujol} no OH bands, 1754 (ester C = O), 1600 (phenyl C = C), 1176 (S = O), 813 cm^{-1} (*p*-substituted phenyl)

Anal Calc for $C_{28}H_{34}O_{14}S_2$ C, 51 1, H, 5 2, S, 9 7 Found C, 50 8, H, 5 2, S, 9 6

*2,3.4,5-Dianhydro-*D*-iditol* (**7**) — Methanolic barium methoxide was prepared by the method of Weltzien and Singer[10], as this gives a more concentrated solution than the method of Isbell[11] To a solution of 3,4-di-*O*-*p*-tolylsulfonyl-D-mannitol (**4b**, 19 7 g, 0 04 mole) in 525 ml of absolute methanol was added 146 ml (0 08 mole) of 0 56M barium methoxide in methanol, causing immediate formation of a colorless precipitate The suspension (protected from carbon dioxide) was stirred magnetically for 2 h at 25°, and then carbon dioxide was passed in for 10 min Water (200 ml) was added, giving a colorless solution, and carbon dioxide was passed in until the solution was neutral (Alkacid test paper) The resulting suspension was gently heated until boiling and then cooled somewhat, decolorizing carbon (1 g) was added, and the suspension was boiled briefly, cooled, and filtered with suction The insoluble matter was washed with three 10-ml portions of water, and the filtrate and washings were combined, and evaporated to dryness under diminished pressure The resulting, colorless, crystalline mass was dried at 30°/0 05 torr, and extracted with three 250-ml portions of boiling 2-butanone under reflux The extracts were combined, and evaporated to dryness under diminished pressure, giving colorless crystals which

were dried at 30°/0 05 torr over phosphorus pentaoxide, wt 5 8 g (99%) On recrystallization from ethanol–pentane (yield 5 3 g, 91%) or ethyl acetate (15 ml/g), it had m p 100–101°, $[\alpha]_D^{25}$ +82 1° (*c* 1 0, water), unchanged after 48 h at 25°, $[\alpha]_D^{25}$ +84 5° (*c* 1 0, abs methanol), ν_{max}^{Nujol} 3290 (OH), 3010, 1242, 886, 763 cm^{-1} (epoxide), n m r data (methyl sulfoxide-d_6, 60 MHz) τ 5 16 (2-proton triplet, $J_{OH,1} = J_{OH,1'} = J_{OH,6} = J_{OH\ 6}$ 5 4 Hz, two OH groups), τ 6 17–6 56 (4-proton complex multiplet, H-1, H-1′, H-6, H-6′), τ 6 95 (2-proton, complex multiplet, H-2 and H-5), τ 7 24 (2-proton, apparent singlet, H-3 and H-4) On addition of a trace of trifluoroacetic acid, the triplet at τ 5 16 and a water peak at τ 6 64 disappeared and were replaced by a sharp singlet at τ 5 77 (exchangeable OH), and the complex multiplet at τ 6 17–6 56 became a pair of quartets forming the AB part of an ABX subsystem composed of H-1, H-1′, and H-2, or H-6, H-6′, and H-5 The signal at τ 7 24 now appeared as a narrow, apparent triplet On addition of a further quantity of trifluoroacetic acid, the singlet at τ 5 77 moved downfield to τ 4 80, thus confirming its assignment as an OH resonance The AB quartets showed $J_{1,2} = J_{5\ 6} \sim 3$ 5 Hz, $J_{1\ 2} = J_{5\ 6} \sim 4$ 5 Hz, and $J_{1\ 1'} = J_{6\ 6'}$ 12 3 Hz

Anal Calc for $C_6H_{10}O_4$ C, 49 3, H, 6 9 Found C, 49 1, H, 7 1

1 6-Di-O-acetyl-2,3 4,5-dianhydro-D-iditol —To a solution of 0 292 g (2 mmoles) of **7** in 14 ml of dry pyridine at 0° was added 10 ml of acetic anhydride, with stirring and exclusion of moisture The mixture was stirred for 5 min, the ice bath was removed, and the solution was kept for 160 min and then poured, with stirring, onto chopped ice The mixture was extracted with chloroform, and the extract was processed in the usual way, dried (anhydrous sodium sulfate), and evaporated to dryness The resulting syrup crystallized from ethanol–pentane, yield of crystals, 0 440 g (96 6%) It was recrystallized from hot isopropyl alcohol, colorless crystals, m p 48–49 5°, $[\alpha]_D^{22}$ +87 0° (*c* 2 1, chloroform), ν_{max}^{Nujol} no OH bands, 1739 (ester C = O), 1258 (ester C–O), 1227 (ester C–O), 889 (epoxide), 763 cm^{-1} (epoxide), n m r data ($CDCl_3$, 60 MHz) τ 5 58 (2-proton quartet, $J_{1,2} = J_{5,6} \sim 3$ 3 Hz, $J_{1\ 1'} = J_{6\ 6}$ 12 5 Hz, H-1 and H-6), τ 6 02 (2-proton quartet, $J_{1'\ 2} = J_{5\ 6'} \sim 5$ 5 Hz, $J_{1\ 1'} = J_{6\ 6'}$ 12 5 Hz, H-1′ and H-6′), τ 6 79 (2-proton, complex multiplet, H-2 and H-5), τ 7 02 (2-proton, narrow triplet, H-3 and H-4), τ 7 92 (6-proton singlet, two acetyl methyls)

Anal Calc for $C_{10}H_{14}O_6$ C, 52 2; H, 6 1 Found C, 52 1, H, 6 2.

2,3 4,5-Dianhydro-1,6-di-O-benzoyl-D-iditol —To a solution of 0 73 g (5 mmoles) of 7 in 4 ml of dry pyridine, cooled in ice–salt, was added 1 5 ml of benzoyl chloride with stirring The crystalline mass was kept for 1 h at 10°, and then recooled in ice–salt, moist pyridine (5 ml containing 1 drop of water) was added, the suspension was stirred, and cooling was continued for 30 min Saturated, aqueous sodium hydrogen carbonate solution (2 ml) was added, with stirring, slight effervescence occurred, the crystals dissolved, and then new crystals formed The mixture was kept for 30 min at 0°, a further 2 ml of the hydrogen carbonate solution was added (giving more crystals), and the suspension was stirred and filtered The crystals were washed successively with water, sodium hydrogen carbonate solution, and water, and dissolved in chloroform The solution was washed successively with sodium hydrogen carbonate

and water, dried (anhydrous sodium sulfate), and evaporated to dryness to give a mass of colorless crystals that was dried at 25°/0 3 torr, wt 1 77 g (100%) It was recrystallized from absolute ethanol (20 ml per g), m p 95–96°, $[\alpha]_D^{21}$ +63 8° (*c* 1 0, chloroform), ν_{max}^{Nujol} no OH bands, 1721 (benzoic ester), 1595 (C = C), 1311, 1282 (benzoic ester), 1245 (epoxide), 1176 (ester C–O), 1120 (benzoic ester C–O), 895, 886 (epoxide), 768 (epoxide), 707 cm^{-1} (phenyl), n m r data ($CDCl_3$, 60 MHz) τ 1 86–2 05 (4-proton, complex multiplet, aromatic *ortho*-protons), τ 2 40–2 74 (6-proton, complex multiplet, aromatic *meta*- and *para*-protons), τ 5 29 (2-proton quartet, $J_{1,2} = J_{5\,6} \sim 3\,3$ Hz, $J_{1\,1'} = J_{6\,6'}$ 12 4 Hz, H-1 and H-6), τ 5 73 (2-proton quartet, $J_{1',2} = J_{5\,6'} \sim 5\,3$ Hz, $J_{1,1} = J_{6,6}$ 12 3 Hz, H–1′ and H–6′), τ 6 62 (2-proton, complex multiplet, H-2 and H-5), τ 6 88 (2-proton, narrow triplet, H-3 and H–4)

Anal Calc for $C_{20}H_{18}O_6$ C, 67 79; H, 5 12 Found C, 67 73, H, 5 15

1,2,3,4,5,6-Hexa-O-(*methylsulfonyl*)-D-*mannitol* — A suspension of **1** (9.1 g, 0 05 mole) in 90 ml of dry pyridine was stirred for 5 min, and then cooled to −10° With stirring and cooling, 28 5 ml of methanesulfonyl chloride was rapidly added, and, after 30 min, the cooling bath was removed The mixture was stirred for 5 h at room temperature, recooled to −5°, and processed as for the preparation of **3a**, giving faintly pink crystals, wt 32 1 g (99%) It was recrystallized from *N,N*-dimethylformamide (5 ml per g) by treatment with decolorizing carbon, filtration, and addition of absolute methanol (20 ml per g), colorless crystals, m p 183–184° (softening at 178°, bath preheated to 168° and heated at 4°/min), $[\alpha]_D^{26}$ +32 9° (*c* 2 08, *N,N*-dimethylformamide), ν_{max}^{Nujol} no OH bands, 1361, 1351, 1337, 1182, 1167 (S = O), 901, 862 cm^{-1} (C–O–S)

Anal Calc for $C_{12}H_{26}O_{18}S_6$ C, 22 2, H, 4 0, S, 29 6 Found C, 22 0, H, 3 8, S, 29 3

1,2,3,4,5,6-Hexa-O-p-*tolylsulfonyl*-D-*mannitol* — A suspension of **1** (1 82 g, 0 01 mole) in 18 ml of dry pyridine was stirred, and cooled to −5° *p*-Toluenesulfonyl chloride (14 3 g) was added, and the mixture was stirred for 30 min The cooling bath was then removed, and the mixture was kept for 2 days at room temperature, recooled to −5°, and treated with water as for the preparation of **3b** The resulting mixture was extracted with chloroform, and the extract was washed successively with aqueous potassium hydrogen sulfate solution, water, aqueous sodium hydrogen carbonate solution, and water, it was then dried (anhydrous sodium sulfate) and evaporated under diminished pressure to a syrup The syrup was dissolved in acetone, the solution was treated with decolorizing carbon, the mixture was filtered, and the filtrate was evaporated to dryness The product was crystallized from acetone (1 ml per g) by addition of absolute ethanol (5 ml per g) to give crop 1, 7 23 g, m p 124–126°; crop 2, 0 1 g, m p 120–126°, total yield 7 33 g (66%) Recrystallized from acetone–absolute ethanol, the product had m p 127–128°, $[\alpha]_D^{27}$ +29 3° (*c* 2 0, acetone), ν_{max}^{Nujol} no OH bands, 1592 and 1488 (phenyl C = C), 1348 (S = O), 1206 and 1119 (*p*-substituted phenyl), 1190 and 1172 (S = O), 870 (C–O–S), and 815 cm^{-1} (*p*-substituted phenyl)

Anal Calc for $C_{48}H_{50}O_{18}S_6$ C, 52 06; H, 4 6, S, 17 4 Found C, 52 02, H, 4 5, S, 17 4

After other reaction times, the yields of crude, crystalline product were 1 day, 39%, 5 days, 68%

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REFERENCES

1 L F WIGGINS, *J Chem Soc*, (1947) 1403
2 H G FLETCHER, JR, AND R M GOEPP, JR, *J Am Chem Soc*, 67 (1945) 1042, 68 (1946) 939
3 O L CHAPMAN AND R W KING, *J Am Chem Soc*, 86 (1964) 1256
4 E BAER, *J Am Chem Soc*, 67 (1945) 338
5 R S TIPSON, H S ISBELL, AND J E STEWART, *J Res Natl Bur Std*, 62 (1959) 257
6 P BLADON AND L N OWEN, *J Chem Soc*, (1950) 598
7 P BLADON AND L N OWEN, *J Chem Soc*, (1950) 604
8 P BRIGL AND H GRUNER, *Ber*, 67 (1934) 1969
9 See R S TIPSON, *J Org Chem*, 9 (1944) 235
10 W WELTZIEN AND R SINGER, *Ann*, 443 (1925) 104
11 H S ISBELL, *Bur Std J Res*, 5 (1930) 1179

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Araucaria bidwillii GUM

PART II [1]. FURTHER STUDIES ON THE POLYSACCHARIDE COMPONENTS

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ABSTRACT

Fractionation of the polysaccharide exudate from *Araucaria bidwillii* yields an arabinogalactan, and, as the major component, a highly branched acidic polysaccharide of similar structural type, but of different quantitative composition to that examined in Part I Acetolysis of the carboxyl-reduced polysaccharide furnished the following oligosaccharides, 4-*O*-L-rhamnopyranosyl-D-glucose, *O*-L-rhamnopyranosyl-(1→4)-*O*-D-glucopyranosyl-(1→6)-D-galactose, 6-*O*-D-glucopyranosyl-D-galactose, 6-*O*-(4-*O*-methyl-D-glucopyranosyl)-D-galactose, 3-*O*-β-D-galactopyranosyl-D-galactose and the polymer-homologous tri- and tetra-saccharides, 6-*O*-D-galactopyranosyl-D-galactose and the polymer-homologous trisaccharide, and *O*-D-galactopyranosyl-(1→6)-*O*-D-galactopyranosyl-(1→3)-D-galactose The structural significance of these oligosaccharides is assessed, and evidence for the acid-catalysed anomerisation of certain glycosidic linkages during acetolysis is discussed Some aspects of the structure of the arabinogalactan have been examined

INTRODUCTION

In Part I[1], we described the results of an examination of an acidic polysaccharide from the resinous exudate of bunya pine (*Araucaria bidwillii*), which had been discovered by Professor A J Birch, F R S , in the course of an investigation of the terpenoid components of the resin The polysaccharide was shown to possess a highly branched structure with a core of D-galactose residues mutually joined by (1→3) and (1→6)-linkages and with outer chains terminated by end groups of L-arabinofuranose, L-rhamnopyranose, D-galactopyranose, D-glucuronic acid, and 4-*O*-methyl-D-glucuronic acid In addition, the sites of attachment of the end groups of D-glucuronic acid (and its 4-methyl ether) were established by the characterisation, as products of partial acid hydrolysis, of the two aldobiouronic acids, 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose (**1**) and 6-*O*-(4-*O*-methyl-β-D-glucopyranosyluronic acid)-D-galactose (**2**) Through the kindness of Dr C M Stewart, Division of Forest Products C S I R O , Australia, we obtained a supply of the native exudate gum from which

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a fresh sample of the carbohydrate portion was isolated A further investigation of the carbohydrate gum was undertaken to assess the homogeneity of the constituent polysaccharide and to ascertain the site of attachment of the L-rhamnopyranose end-groups

RESULTS AND DISCUSSION

Examination of the water-soluble portion of the gum by chromatography on diethylaminoethylcellulose and diethylaminoethyl-Sephadex showed that the acidic polysaccharide was the main component, but that small proportions of a neutral polysaccharide (probably an arabinogalactan) and a mixture of chromatographically mobile sugars were also present The acidic polysaccharide contained, as constituent sugars, glucuronic acid (in part as the 4-methyl ether), galactose, arabinose, and rhamnose, but these sugars were present in significantly different proportions (approximate molar percentages, 18, 56, 17, and 9) from those in the previously examined[1] gum sample (10, 69, 16, and 5) Examination of the cleavage products from the methylated acidic polysaccharide indicated that, with one significant exception, the sugar residues were present in the same types of linkage The detection of 2,3-di-*O*-methylglucuronic acid showed that a proportion of the glucuronic acid residues carried glycosidic substituents at C-4 This observation suggested that L-rhamnopyranose residues might be attached to this position as in gum arabic[2] This type of structural feature, if present in the previous polysaccharide sample, would have been quantitatively less significant in view of the lower proportions of both glucuronic acid and rhamnose residues and may have escaped detection

The acidic polysaccharide was converted into the carboxyl-reduced derivative by treatment of the acetylated 2-hydroxyethyl ester with lithium borohydride[3] Methylation of a sample of the modified derivative, followed by gas–liquid chromatographic examination of the cleavage products, showed that it was fully representative of the parent polysaccharide Acetolysis of the carboxyl-reduced polysaccharide, followed by de-*O*-acetylation of the products, gave a mixture of sugars from which the following oligosaccharides were isolated by adsorption and partition chromatography 4-*O*-L-rhamnopyranosyl-D-glucose (**3**), *O*-L-rhamnopyranosyl-(1→4)-*O*-D-glucopyranosyl-(1→6)-D-galactose (**4**), 6-*O*-D-glucopyranosyl-D-galactose (**5**), 6-*O*-(4-*O*-methyl-D-glucopyranosyl)-D-galactose (**6**), 3-*O*-β-D-galactopyranosyl-D-galactose (**7**), the polymer-homologous tri- (**8**) and tetra-saccharides (**9**), 6-*O*-D-galactopyranosyl-D-galactose (**10**), the polymer-homologous trisaccharide (**11**), and *O*-D-galactopyranosyl-(1→6)-*O*-D-galactopyranosyl-(1→3)-D-galactose (**12**) Oligosaccharide **7** was also isolated as the crystalline sugar The structures of these oligosaccharides were assigned on the basis of paper chromatography of the products of partial hydrolysis of the sugars and the derived glycitols, and by gas–liquid chromatography of the cleavage products of the methylated oligosaccharides and the methylated oligosaccharide glycitols

β-D-G*p*A-(1→6)-D-Gal (**1**) 4-*O*-Me-β-D-G*p*A-(1→6)-D-Gal (**2**)

L-Rha*p*-(1→4)-D-G (**3**) L-Rha*p*-(1→4)-D-G*p*-(1→6)-D-Gal (**4**)
D-G*p*-(1→6)-D-Gal (**5**) 4-*O*-Me-D-G*p*-(1→6)-D-Gal (**6**)
β-D-Gal*p*-(1→3)-D-Gal (**7**) *β*-D-Gal*p*-(1→3)-*β*-D-Gal*p*-(1→3)-D-Gal (**8**)
β-D-Gal*p*-(1→3)-*β*-D-Gal*p*-(1→3)-*β*-D-Gal*p*-(1→3)-D-Gal (**9**)
D-Gal*p*-(1→6)-D-Gal (**10**) D-Gal*p*-(1→6)-D-Gal*p*-(1→6)-D-Gal (**11**)
D-Gal*p*-(1→6)-D-Gal*p*-(1→3)-D-Gal (**12**)

The formation, from the carboxyl-reduced polysaccharide, of the oligosaccharides (**7**–**12**) containing galactose only is fully consistent with the presence in this polysaccharide sample of a branched framework of galactose residues in which (1→3)-linkages predominate in the inner chains and (1→6)-linkages are located in the outer chains The oligosaccharides **5** and **6** clearly have their origin in units of the two aldobiouronic acids (**1** and **2**) present in the parent polysaccharide The characterisation of the two rhamnose containing oligosaccharides (**3** and **4**) provides clear evidence that some, if not all, of the L-rhamnopyranose end-groups are attached by (1→4)-linkages to residues of D-glucuronic acid In this respect, therefore, the *Araucaria bidwillii* polysaccharide resembles gum arabic[2]

Despite quantitative differences in the proportions of the constituent units, our results show that the nature of the sugar residues and of the linkages between them in the present sample of acidic polysaccharide from *Araucaria bidwillii* gum are the same as those in the previously examined sample Evidence concerning the configuration of glycosidic linkages in the polysaccharide is not yet complete In both series of investigations, the low specific rotations of the polysaccharides and their methylated derivatives have suggested that the D-glucuronic acid and D-galactose residues have the *β*-configuration Furthermore, the two aldobiouronic acids (**1** and **2**) formed on partial hydrolysis of the earlier sample[1] were assigned *β*-D-glucopyranosyluronic acid linkages on the basis of specific rotations However, examination of the oligosaccharides formed on acetolysis of the carboxyl-reduced polysaccharide showed that all those containing (1→6)-linkages (**4**–**6**, **10**–**12**) had more-positive specific rotations than would be expected for compounds having *β*-D-glucopyranosyl or *β*-D-galactopyranosyl units In the case of the disaccharide, 6-*O*-D-glucopyranosyl-D-galactose (**5**), comparison of the n m r signals assigned to anomeric protons[4] (τ 5 02–5 03, J 2 8–3 0 Hz) in the derived glycitol and in melibiitol, and of changes in specific rotation of the two disaccharide glycitols during periodate oxidation[5] pointed to the presence of an *α*-D-glycosidic linkage Since the disaccharide **5** clearly originated from the same structural unit in the parent polysaccharide as the aldobiouronic acid **1**, it is probable that acid-catalysed anomerisation took place during the acetolysis of the carboxyl-reduced polysaccharide and that a high proportion of other (1→6)-linkages were similarly affected It is well known that Lewis acids catalyse the anomerisation of glycosidic linkages, and amongst disaccharides those containing (1→6)-linkages undergo the reaction most readily[6] This type of anomerisation accompanying the fragmentation of polysaccharides has been encountered previously during the acetolysis of ivory-nut mannans[7], larch glucomannan[8], and soybean galactomannan[9], when only a small proportion of the (1→4)-linked *β*-D-mannopyranosyl residues

were altered It is noteworthy that, in the present investigation, no evidence for the anomerisation of (1→3)-linked β-D-galactopyranosyl residues was obtained

Only a preliminary investigation of the neutral arabinogalactan has been so far undertaken The results obtained, however, clearly show that any residual traces of the arabinogalactan which were present in the acidic polysaccharide preparation were insufficient to have caused serious contamination The arabinogalactan, which was isolated in homogeneous form by chromatography on diethylaminoethyl-Sephadex, gave, on hydrolysis, arabinose and galactose in the molar ratio of 4 1, and partial hydrolysis yielded traces of oligosaccharides with the chromatographic mobilities of 3- and 6-*O*-β-D-galactopyranosyl-D-galactose, and 3-*O*-L-arabinofuranosyl-L-arabinose A sample of the polysaccharide was converted into its methylated derivative, but all attempts to raise the methoxyl content to its theoretical value failed However, infrared and n m r. spectroscopy indicated the presence of only trace amounts of non-etherified hydroxyl groups, furthermore, no unsuspected structural features were detected Gas–liquid chromatography of the methanolysis products from the methylated polysaccharide showed the presence of methyl glycosides of 2,3,5-tri-, 2,5- and 3,5-di-*O*-methylarabinose and, in trace amount, of 2,3,4,6-tetra-*O*-methylgalactose, no other galactose derivatives were detected A further sample of the arabinogalactan was degraded by Smith's procedure[10] of sequential periodate oxidation, borohydride reduction, and graded acid hydrolysis, and furnished an arabinan devoid of galactose residues Methanolysis of the methylated arabinan gave methyl glycosides of 3,5-di-*O*-methylarabinose, and, in smaller amount, of 2,3,5-tri- and 2,5-di-*O*-methylarabinose These results show that the arabinogalactan contains a highly ramified structure, although the nature of the branching points and the structural role of galactose residues remain to be established

EXPERIMENTAL

Paper chromatography was carried out on Whatman No 1 and 3MM papers with the following solvent systems (v/v) (*A*) ethyl acetate–pyridine–water (10 4 3), (*B*) butyl alcohol–pyridine–water (3 2 2), (*C*) ethyl acetate–acetic acid–formic acid–water (18 3 1 4), (*D*) butyl alcohol–ethanol–water (4 1 5, upper layer), R_G-Values of methylated sugars refer to rates of movement relative to 2,3,4,6-tetra-*O*-methyl-D-glucose in solvent *D* Gas–liquid chromatography was carried out on columns of (*a*) 5% byw eight of poly(neopentyl glycol adipate) at 150°, (*b*) 10% by weight of polyphenyl ether [*m*-di-(*m*-phenoxyphenoxy)benzene] at 200°, (*c*) 15% by weight of poly(ethylene glycol adipate) at 175° on Celite Retention times (*T*) are quoted relative to that of methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucopyranoside Unless otherwise stated, optical rotations were observed for water solutions at *ca* 18° Small-scale methylations of oligosaccharides were performed by the procedure of Kuhn *et al*[11]

Fractionation of the gum and isolation of polysaccharide components — The crude-gum nodules were crushed and dispersed in water, and the resulting suspension

was extracted continuously with light petroleum (b p 40–60°) for 4–5 days to remove terpenoid resin The aqueous solution was filtered through glass-fibre paper and freeze-dried to give the carbohydrate gum Examination of the crude gum by ultracentrifugation in water and by boundary electrophoresis in pyridine–acetic acid buffer showed, in each case, a single, slightly unsymmetrical peak

A portion (90 mg) of the gum was chromatographed on diethylaminoethylcellulose (phosphate form)[12], and the column (30 × 4 cm, 30 g) was eluted successively with 0 025M (500 ml), 0 05M (500 ml), 0 1M (500 ml), 0 25M, and 0 5M sodium dihydrogen phosphate buffer (500 ml) at pH 6 Analysis of the fractions with the phenol-sulphuric acid reagent[13] showed that a small amount of carbohydrate was eluted with 0 025M buffer, but that the major portion of the polysaccharide was eluted with 0 5M buffer

The gum (4 g) was chromatographed on a column (60 × 4 5 cm) of diethylaminoethyl-Sephadex A-50 (formate form), and elution with water (2000 ml), and water containing 1% (3000 ml), 5% (4000 ml), and 10% (3000 ml) of formic acid gave Fractions *1–4* Fraction *1* was concentrated and poured into ethanol (4 vol) to give Fraction *1a* (70 mg), and the supernatant liquid was concentrated to give Fraction *1b* (60 mg) Fractions 2–4 were each adjusted to pH 5 by the addition of potassium acetate, dialysed, concentrated, and poured into ethanol (6 vol) to give the polysaccharide contents (0 17, 0 55, and 2 03 g) The polysaccharide from Fraction *1a* had $[\alpha]_D$ +30° and gave arabinose and galactose on hydrolysis Fraction *1b* consisted of a syrupy mixture of sugars shown by chromatography to include galactose, arabinose, rhamnose, and a series of galactose-containing oligosaccharides The polysaccharides from Fractions 2–4 had, respectively, $[\alpha]_D$ −8 3°, −10 0°, and −9 5°, and contained 10, 14, and 20% of uronic acid residues (carbazole–sulphuric acid reagent[14]) Each of these polysaccharide fractions gave, on hydrolysis, qualitatively similar mixtures of sugars, including galactose, arabinose, rhamnose, and acidic sugars

The arabinogalactan (Fraction *1a*) was isolated, on a preparative scale, by chromatography on diethylaminoethyl-Sephadex A-50 (formate form) as described above On the larger columns used, elution with water gave arabinogalactan (Fraction *1a*) and soluble sugars (Fraction *1b*) as discrete fractions Crude gum (160 g) furnished arabinogalactan (5 5 g), $[\alpha]_D$ +30°, which gave single, symmetrical peaks when examined by ultracentrifugation and by boundary electrophoresis in borate buffer Hydrolysis of the polysaccharide gave arabinose and galactose in the molar ratio of 4 1, and partial hydrolysis afforded traces of oligosaccharides having the chromatographic mobilities of 3- and 6-*O*-β-D-galactopyranosyl-D-galactose, and 3-*O*-L-arabinofuranosyl-L-arabinose

The acidic polysaccharide component of the gum was isolated on a preparative scale by precipitation from aqueous solution with ethanol It had $[\alpha]_D$ −9 5° (*c* 0 9) and uronic anhydride (by decarboxylation) 18%, and gave, on hydrolysis, galactose, arabinose, rhamnose, and two acidic disaccharides having the chromatographic mobilities of 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose and 6-*O*-(4-*O*-

methyl-β-D-glucopyranosyluronic acid)-D-galactose, R_{Gal} 0 20 and 0 61 in solvent *C* Hydrolysis of the polysaccharide with N sulphuric acid for 4 h at 100°, followed by chromatographic separation of the neutral sugars and estimation by Wilson's procedure[15], showed that galactose, arabinose, and rhamnose were formed in the molar ratio of 4 5 2 1 On the assumption that these conditions of hydrolysis liberated the acidic sugar residues mainly in the form of the two above-mentioned aldobiouronic acids, the polysaccharide had the following composition (approx molar percentages) glucuronic acid (and its 4-methyl ether), 18, galactose, 56, arabinose, 17, and rhamnose, 9 Examination of the acidic polysaccharide by chromatography on diethylaminoethylcellulose (phosphate form), as described above, showed that more than 97% of the polysaccharide was eluted in a single band with 0 5M phosphate buffer

Methylation of the acidic polysaccharide —The polysaccharide (2 g) was methylated successively with methyl sulphate and 30% aqueous sodium hydroxide, and methyl iodide and silver oxide, to give methylated gum (1 2 g), $[\alpha]_D$ −62 7° (*c* 1 08, chloroform) (Found OMe, 42 6%) A sample of the methylated gum was heated with methanolic hydrogen chloride, and examination of the cleavage products by g l c on columns (*a*) and (*c*) indicated the presence of methyl glycosides of the following sugars (approximate proportions in parenthesis) 2,3,4-tri-*O*-methylrhamnose (+ +), 2,3,5-tri (+ + +), 2,3,4-tri- or 3,5-di- (trace), and 2,5-di-*O*-methylarabinose (+), 2,3,4,6-tetra- (+ + +), 2,3,4- (+ + +) and 2,4,6-tri- (+), and 2,4-di-*O*-methylgalactose (+ + +), and 2,3,4-tri (+ +) and 2,3-di-*O*-methylglucuronic acid (+ +)

Preparation of carboxyl-reduced gum — The acidic polysaccharide (45 g) in water (2250 ml) was passed through a column of Amberlite resin IR-120 (H^+) to remove metal ions, ethylene oxide (600 ml) was added, and the solution was kept for 7 days, during which time the pH of the solution rose from 2 55 to 6 5 Dialysis of the solution, followed by concentration and addition of ethanol (5 vol), afforded the 2-hydroxyethyl ester (42 g) The polysaccharide ester was acetylated[16] to give polysaccharide glycol ester acetate (54 g). Lithium borohydride (40 g) in tetrahydrofuran (1000 ml) was added, slowly with stirring, to the glycol ester acetate (54 g) in tetrahydrofuran (1000 ml) Polysaccharide was immediately precipitated, and the reaction was continued by heating under reflux for 18 h Excess of borohydride was destroyed by the careful addition of water, further water (2000 ml) was added, and the solution was neutralised with dilute sulphuric acid, and concentrated to remove tetrahydrofuran The aqueous solution was dialysed, concentrated, and poured into ethanol (5 vol) to give carboxyl-reduced gum (37 5 g) The modified polysaccharide had $[\alpha]_D$ −5 7° (*c* 1 27) and uronic anhydride, < 1%, and was essentially homogeneous when examined by ultracentrifugation and boundary electrophoresis Hydrolysis gave rhamnose, arabinose, galactose, glucose, and 4-*O*-methylglucose

Successive treatments of the carboxyl-reduced gum (200 mg) with methyl sulphate and 30% aqueous sodium hydroxide, and methyl iodide and silver oxide, gave the methylated derivative (120 mg), $[\alpha]_D$ −52° (*c* 0 96, chloroform) (Found OMe, 42 2%) A sample of the methylated polysaccharide was heated with methanolic

hydrogen chloride, and examination of the cleavage products by g l c on columns (*b*) and (*c*) indicated the presence of methyl glycosides of the following sugars (approximate proportions in parenthesis) 2,3,4-tri-*O*-methylrhamnose (+ +), 2,3,5-tri-(+ + +), 2,3,4-tri- or 3,5-di- (trace), and 2,5-di-*O*-methylarabinose (+), 2,3,4,6-tetra- (+ + +), 2,3,4- (+ + +) and 2,4,6-tri- (+), and 2,4-di-*O*-methylgalactose (+ + +), and 2,3,4,6-tetra- (+ +) and 2,3,6-tri-*O*-methylglucose (+ +)

Acetolysis of carboxyl-reduced gum — The carboxyl-reduced gum (23 g) was converted[16] into the acetylated derivative (30 5 g) The polysaccharide acetate (30 g) was added with stirring to a mixture of acetic anhydride (324 ml) and conc sulphuric acid (9 7 ml) at 3° during 15 min, stirring was continued for a further 15 min, and the solution was set aside for 20 h at room temperature The resulting solution was poured into ice–water (750 ml) with stirring, and, after stirring for a further 15 min, the mixture was extracted with chloroform (4 × 250 ml) The chloroform extracts were washed with water and aqueous sodium hydrogen carbonate, dried, and concentrated to a syrup The syrupy acetates were treated with methanolic 5% barium methoxide at 0° for 48 h, and the resulting solution was poured into water (200 ml), neutralised with dilute sulphuric acid, filtered, treated with ion-exchangers, and concentrated to a syrup (20 g) The mixture of sugars was adsorbed on a column (52 × 5 5 cm) of charcoal–Celite (1 1), and the column was eluted successively with water (4 l), and water containing a gradient of 0→5% (4 l), 5% (4 l), a gradient of 5→10% (4 l), 10% (3 l), a gradient of 10→15% (4 l), and 15% of ethanol (4 l) Fractions containing monosaccharides were discarded, and those containing oligosaccharides were further separated, as necessary, by filter-sheet chromatography to give ten chromatographically pure oligosaccharides (**3–12**)

Examination of oligosaccharides — *Oligosaccharide* **3** The sugar (108 mg), R_{Gal} 0 79 in solvent *C*, $[\alpha]_D$ −9 5° (*c* 3 3), gave, on hydrolysis, rhamnose and glucose, and, on hydrolysis of the derived glycitol (borohydride reduction), rhamnose and glucitol The methanolysis products from the methylated disaccharide were examined by g l c on column (*a*) and had the retention times of methyl glycosides of 2,3,4-tri-*O*-methylrhamnose and 2,3,6-tri-*O*-methylglucose Methanolysis of the methylated glycitol furnished methyl 2,3,4-tri-*O*-methylrhamnopyranoside and 1,2,3,5,6-penta-*O*-methylglucitol Oligosaccharide **3** was methylated successively with methyl sulphate and sodium hydroxide, and methyl iodide and silver oxide in *N*,*N*-dimethylformamide, to give methylated disaccharide (43 mg), $[\alpha]_D$ −27° (*c* 1 10, chloroform). Hydrolysis of a sample of the methylated derivative, followed by chromatography of the hydrolysate in solvent *D*, showed two components and indicated that etherification was complete The major portion (40 mg) of the methylated disaccharide was hydrolysed in N sulphuric acid for 4 h at 100°, the solution was neutralised with barium carbonate, filtered, concentrated, and separated by filter-sheet chromatography in solvent *D* to give (*a*) 2,3,4-tri-*O*-methyl-L-rhamnose (10 mg), R_G 0 96, $[\alpha]_D$ +21° (*c* 1.03), which was converted into the aniline derivative, m p and mixed m p 111°, and (*b*) 2,3,6-tri-*O*-methyl-D-glucose (13 mg), R_G 0 80, $[\alpha]_D$ +69° (*c* 1 32), which was converted into the 1,4-di-*p*-nitrobenzoate, m p and mixed m p 191°

Oligosaccharide **4** The sugar (73 mg), R_{Gal} 0 42 and 0 16 in solvents *B* and *C*, $[\alpha]_D$ +56° (*c* 1 2), gave rhamnose, glucose, and galactose on hydrolysis, and rhamnose, glucose, and galactitol on hydrolysis of the derived glycitol Partial, acid hydrolysis gave rhamnose and 6-*O*-glucopyranosylgalactose (oligosaccharide **5**) The methanolysis products from the methylated trisaccharide were examined by g l c on column (*a*) and had the retention times of methyl glycosides of 2,3,4-tri-*O*-methylrhamnose, 2,3,6-tri-*O*-methylglucose, and 2,3,4- and 2,3,5-tri-*O*-methylgalactose Methanolysis of the methylated glycitol furnished methyl glycosides of 2,3,4-tri-*O*-methylrhamnose and 2,3,6-tri-*O*-methylglucose, and 1,2,3,4,5-penta-*O*-methylgalactitol

Oligosaccharide **5** The sugar (37 mg), R_{Gal} 0 51 and 0 28 in solvents *B* and *C*, $[\alpha]_D$ +75° (*c* 2 0), gave glucose and galactose on hydrolysis, and glucose and galactitol on hydrolysis of the derived glycitol The methanolysis products from the methylated disaccharide were examined by g l c on column (*c*) and had the retention times of methyl glycosides of 2,3,4,6-tetra-*O*-methylglucose, and 2,3,4- and 2,3,5-tri-*O*-methylgalactose Methanolysis of the methylated glycitol gave methyl glycosides of 2,3,4,6-tetra-*O*-methylglucose together with 1,2,3,4,5-penta-*O*-methylgalactitol The n m r spectrum of the glycitol in deuterium oxide showed a doublet attributable[4] to the anomeric proton (τ 5 03, J 3 0 Hz) In a parallel experiment, 6-*O*-α-D-galactopyranosyl-D-glucitol (melibiitol) showed a similar doublet (τ 5 02, J 2 8 Hz) Samples (10 mg) of the glycitol and of melibiitol were each oxidised in water (1 ml) containing sodium metaperiodate (50 mg), constant optical rotations were observed after 4 h, the products having $[\alpha]_D$ +76° and +78°, respectively

Oligosaccharide **6** The sugar (15 mg), R_{Gal} 0 88 and 0 61 in solvents *B* and *C*, gave 4-*O*-methylglucose and galactose on hydrolysis, and 4-*O*-methylglucose and galactitol on hydrolysis of the derived glycitol The methanolysis products from the methylated disaccharide were examined by g l c on column (*a*) and had retention times of methyl glycosides of 2,3,4,6-tetra-*O*-methylglucose, and 2,3,4- and 2,3,5-tri-*O*-methylgalactose Methanolysis of the methylated glycitol gave 1,2,3,4,5-penta-*O*-methylgalactitol and methyl glycosides of 2,3,4,6-tetra-*O*-methylglucose

Oligosaccharide **7** The sugar (105 mg), R_{Gal} 0 70 and 0 28 in solvents *B* and *C*, was recrystallised from ethanol–water and was characterised as 3-*O*-β-D-galactopyranosyl-D-galactose by m p and mixed m p 164–169°, $[\alpha]_D$ +87→+65° (*c* 0 92), and by X-ray powder photography Examination of the methanolysis products from the methylated disaccharide by g l c on columns (*a*) and (*c*) showed the presence of peaks attributable to methyl glycosides of 2,3,4,6-tetra-, 2,4,6- and, probably, 2,5,6-tri-*O*-methylgalactose Methanolysis of the methylated glycitol gave 1,2,3,5,6-(= 1,2,4,5,6-) penta-*O*-methylgalactitol and methyl glycosides of 2,3,4,6-tetra-*O*-methylgalactose

Oligosaccharide **8** The sugar (50 mg), R_{Gal} 0 38 in solvent *B*, $[\alpha]_D$ +58° (*c* 0 5), gave galactose only on hydrolysis, and 3-*O*-galactopyranosylgalactose (oligosaccharide 7) on partial hydrolysis Comparison of the colorimetric reactions of the sugar and the derived glycitol with the phenol–sulphuric acid reagent[17] indicated that it was a trisaccharide The methanolysis products from the methylated tri-

saccharide were examined by g l c on column (*a*) and had retention times of methyl glycosides of 2,3,4,6-tetra- and 2,4,6-tri-*O*-methylgalactose Methanolysis of the methylated glycitol gave methyl glycosides of the same two sugars and 1,2,3,5,6-penta-*O*-methylgalactitol in approximately equimolecular proportions

Oligosaccharide **9** The sugar (25 mg), R_{Gal} 0 31 in solvent *B*, $[\alpha]_D$ +51° (*c* 0 86), gave galactose only on hydrolysis, and oligosaccharides **7** and **8** on partial hydrolysis. Comparison of the colorimetric reactions of the sugar and the derived glycitol with the phenol–sulphuric acid reagent[17] indicated that it was a tetrasaccharide The methanolysis products from the methylated tetrasaccharide were examined by g l c on column (*a*) and had the retention times of methyl glycosides of 2,3,4,6-tetra- and 2,4,6-tri-*O*-methylgalactose (approximate proportions of 1 3) Methanolysis of the methylated glycitol gave methyl glycosides of the same two sugars and 1.2,3,5,6-penta-*O*-methylgalactitol in the approximate proportions of 1 2 1

Oligosaccharide **10** The sugar (21 mg), R_{Gal} 0 46 and 0 24 in solvents *B* and *C*, $[\alpha]_D$ +126° (*c* 0 9), gave galactose only on hydrolysis and was chromatographically similar to 6-*O*-β-D-galactopyranosyl-D-galactose The methanolysis products from the methylated disaccharide were examined by g l c on column (*c*) and had the retention times of methyl glycosides of 2,3,4,6-tetra-, and 2,3,4- and 2,3,5-tri-*O*-methylgalactose Methanolysis of the methylated glycitol gave 1,2,3,4,5-penta-*O*-methylgalactitol and methyl glycosides of 2,3,4,6-tetra-*O*-methylgalactose.

Oligosaccharide **11** The sugar (7 mg), R_{Gal} 0 22 and 0 07 in solvents *B* and *C*, gave galactose only on hydrolysis Comparison of the colorimetric reactions of the sugar and the derived glycitol with the phenol–sulphuric acid reagent[17] indicated that it was a trisaccharide The methanolysis products of the methylated trisaccharide were examined by g l c on column (*a*) and had retention times of methyl glycosides of 2,3,4,6-tetra-, and 2,3,4- and 2,3,5-tri-*O*-methylgalactose

Oligosaccharide **12** The sugar (95 mg), R_{Gal} 0 24 and 0 05 in solvents *B* and *C*, m p 160–168°, $[\alpha]_D$ +84° (*c* 1 5), gave galactose only on hydrolysis Comparison of the colorimetric reactions of the sugar and the derived glycitol with the phenol–sulphuric acid reagent[17] indicated that it was a trisaccharide Partial, acid hydrolysis of the trisaccharide gave 3- and 6-*O*-galactopyranosylgalactose (oligosaccharides **7** and **10**), whereas partial hydrolysis of the glycitol gave 6-*O*-galactopyranosylgalactose as the sole disaccharide Degradation of the trisaccharide in oxygen-free lime-water for 16 h at room temperature gave galactose and 6-*O*-galactopyranosylgalactose The methanolysis products from the methylated trisaccharide were examined by g l c on column (*a*) and had retention times of methyl glycosides of 2,3,4,6-tetra-, and 2,3,4- and 2,4,6-tri-*O*-methylgalactose Methanolysis of the methylated glycitol furnished 1,2,3,5,6-penta-*O*-methylgalactitol, and methyl glycosides of 2,3,4,6-tetra- and 2,3,4-tri-*O*-methylgalactose

Methylation of the arabinogalactan — Arabinogalactan (200 mg) was methylated successively with methyl sulphate and 30% aqueous sodium hydroxide, and methyl iodide and silver oxide, to give methylated arabinogalactan (110 mg), $[\alpha]_D$

+51° (*c* 0 3, chloroform) (Found. OMe, 32 3%; not raised on further methylation). A further sample of polysaccharide was methylated in the same way and remethylated with methyl iodide and silver oxide in *N*,*N*-dimethylformamide to give methylated arabinogalactan of the same methoxyl content, whose i r and n m r spectra indicated the absence of hydroxyl groups and of unsuspected functional groups. G l c of the methanolysis products from the methylated arabinogalactan on columns (*a*) and (*b*) showed the presence of methyl glycosides of 2,3,5-tri-, 2,5- and 3,5-di-*O*-methylarabinose, and, in small proportion, of 2,3,4,6-tetra-*O*-methylgalactose A sample of methylated arabinogalactan was hydrolysed, and paper chromatography of the hydrolysate showed the above-mentioned sugars together with small proportions of 2- and another mono-*O*-methylarabinose A further quantity of the hydrolysate was oxidised with bromine water, and g l.c of the products on column (*a*) showed the presence of 2,3,5-tri-, and 2,5- and 3,5-di-*O*-methylarabinonolactones

Degradation of periodate-oxidised arabinogalactan — Arabinogalactan (3 88 g) was oxidised with 0 1M sodium metaperiodate solution (390 ml) for 30 h (consumption of reagent, determined spectrophotometrically[18], was constant and corresponded to 0 48 mol per sugar residue) Ethylene glycol (5 ml) was added to destroy excess of periodate, and the solution was dialysed for 4 days, concentrated (to 200 ml), and treated with potassium borohydride (1.5 g) for 2 days The resulting solution was stirred with Amberlite resin IR-120 (H^+) for 15 min to destroy excess borohydride and to remove metal cations, and then repeatedly evaporated to dryness with methanol to remove boric acid The residue was treated with N sulphuric acid for 2 5 h at room temperature, neutralised with barium carbonate, filtered, concentrated, and poured into ethanol (5 vol) The sticky precipitate, which separated, was removed at the centrifuge, washed with ethanol, dissolved in water, and freeze-dried to give degraded arabinan (0 7 g), $[\alpha]_D$ +45° (*c* 0 82) The supernatant liquid and ethanol washings were combined and concentrated to a syrup (0 3 g), chromatography of which showed the presence of glycerol and a trace of arabinose Hydrolysis of the syrup gave galactose, arabinose, and glycerol

Degraded arabinan (100 mg) was methylated in methyl sulphoxide with methyl iodide and sodium hydride[19], and then in *N*,*N*-dimethylformamide with methyl iodide and silver oxide, to give methylated arabinan (52 mg), $[\alpha]_D$ +58° (*c* 0 6, chloroform) (Found OMe, 38 7%) G l c of the methanolysis products from the methylated polysaccharide on columns (*a*) and (*b*) showed the presence of methyl glycosides of 2,3,5-tri-, and 2,5- and 3,5-di-*O*-methylarabinose (major product) A further sample was hydrolysed, and chromatography of the hydrolysate showed the above-mentioned sugars and a mono-*O*-methylarabinose

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REFERENCES

1 The paper by G O ASPINALL AND R M FAIRWEATHER, *Carbohyd Res*, 1 (1965) 83, is to be regarded as Part I
2 G O ASPINALL, A J CHARLSON, E L HIRST, AND R YOUNG, *J Chem Soc*, (1963) 1696, G O ASPINALL AND R YOUNG, *ibid*, (1965) 3003
3 D A REES AND J W B SAMUEL, *Chem Ind* (London), (1965) 2008
4 J. M VAN DER VEEN, *J Org Chem*, 28 (1963) 564
5 P. A J GORIN AND A S PERLIN, *Can J Chem*, 37 (1959) 1930
6 *Cf.* B LINDBERG, *Acta Chem Scand*, 3 (1949) 1153
7 G O. ASPINALL, R B RASHBROOK, AND G KESSLER, *J Chem. Soc*, (1958) 215
8 G O ASPINALL, J E MCKAY, AND R BEGBIE, *J Chem Soc*, (1962) 214
9 G O ASPINALL AND J N C WHYTE, *J Chem Soc*, (1964) 5058
10 I J GOLDSTEIN, G W HAY, B A LEWIS, AND F. SMITH, *Methods Carbohyd Chem*, 5 (1965) 361
11 R KUHN, H TRISCHMANN, AND I LOW, *Angew Chem*, 62 (1955) 32
12 H NEUKOM, H DEUEL, W. J HERI, AND W KUNDIG, *Helv Chim Acta*, 43 (1960) 64
13 M DUBOIS, K A GILLES, J K HAMILTON, P A REBERS, AND F SMITH, *Anal Chem*, 28 (1956) 350
14 E A MCCOMB AND R M MCCREADY, *Anal Chem*, 24 (1952) 1630
15 C M WILSON, *Anal Chem*, 31 (1959) 1199
16 J F CARSON AND W D MACLAY, *J Am Chem Soc*, 68 (1946) 1015
17 T E TIMELL, *Svensk Papperstidn*, 63 (1960) 668
18 G O ASPINALL AND R J FERRIER, *Chem Ind* (London), (1957) 1216
19 P A SANDFORD AND H E CONRAD, *Biochemistry*, 5 (1966) 1508

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SOME FURTHER RING-OPENING REACTIONS OF METHYL 4,6-*O*-BENZYLIDENE-2,3-DIDEOXY-2,3-EPIMINO-α-D-ALLOPYRANOSIDE AND ITS DERIVATIVES

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(Received December 19th, 1967)

ABSTRACT

Modifications have been made to the preparation of methyl 4,6-*O*-benzylidene-2,3-dideoxy-2,3-epimino-α-D-allopyranoside from derivatives of 2-amino-2-deoxy-D-glucose, which permit it to be prepared in high yield and on a large scale Further, ring-opening reactions of the epimine and its *N*-substituted derivatives have been studied with halide ion under neutral and acidic conditions It has been found that anomalous, diequatorial ring-opening occurs when the free epimine is treated with ammonium halides (except the fluoride) in *N*,*N*-dimethylformamide, but, with its *N*-substituted derivatives, diaxial ring-opening predominates, although diequatorial ring-opening is significant in many cases Under acidic conditions, the free epimine undergoes hydrolysis of the benzylidene group, without rupture of the epimine ring, but its *N*-substituted derivatives undergo predominant, diaxial ring-opening before hydrolysis of the benzylidene group

Ring-opening reactions of the *N*-methanesulphonyl and *N*-acetyl derivatives with azide occur *trans*-diaxially and *trans*-diequatorially, respectively

INTRODUCTION

Ring-opening reactions of carbohydrate epoxides have been widely studied[1], whereas those of the recently accessible[2-10] nitrogen-analogues, the epimines, have not With azide as the nucleophile, the results obtained showed that ring-opening is not always analogous to that observed in the case of epoxides, which affords predominantly *trans*-diaxial[3] products For example, whereas the *allo*-epimine **7** and its *N*-tosyl and *N*-2,4-dinitrophenyl derivatives (**11**) undergo *trans*-diaxial ring-opening, the *N*-benzoyl derivative **9** gave the *trans*-diequatorial *gluco*-stereoisomer[11] The corresponding

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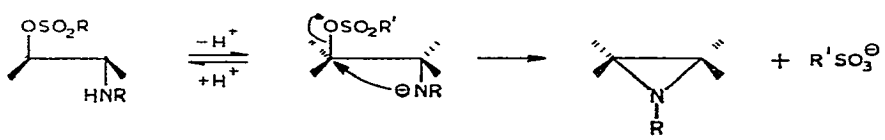
manno-epimine and its *N*-tosyl derivative underwent ring-opening to give *trans*-diaxial products, but the *N*-benzoyl derivative rearranged mainly to the oxazoline, although some of the *trans*-diaxial product was obtained Similar, anomalous ring-openings occur[12] with the *N*-benzoyl-*allo*-epimine in the β-D-glycoside series, in this case, *trans*-diequatorial ring-opening occurred with thiolacetate, azide, and even hydroxide (from basic alumina)

The above reactions were carried out under neutral or slightly basic conditions *N*-Acylepimines are extremely sensitive towards acid[13], and, with hydrogen halides, they underwent rapid opening to give *trans*-diaxial halo-amines in good yields The three-membered ring was so acid-sensitive that it was possible to effect ring opening without hydrolysis of the benzylidene substituent These studies have now been extended by a study of the *allo*-epimine **7**.

DISCUSSION

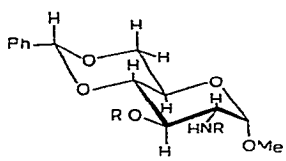
Various methods[2-4,6] available for the preparation of methyl 4,6-*O*-benzylidene-2,3-dideoxy-2,3-epimino-α-D-allopyranoside (**7**) are unsuitable for large-scale work One method[4] utilized the elimination of a sulphonyloxy group situated *trans* to a vicinal benzamido substituent; consequently, the readily available methyl 2-benzamido-4,6-*O*-benzylidene-2-deoxy-3-*O*-mesyl-α-D-glucopyranoside (**1**) should be a convenient precursor for the epimine However, previous results showed that the reduction of compound **1** with lithium aluminium hydride was unsatisfactory, and treatment with ethanolic sodium ethoxide, to give the epimine, was complicated by competing hydrolysis of the sulphonic ester to give alcohol **2** We reasoned that a larger sulphonate group at C-3 and a bulky alkoxide anion would hinder the approach of the base, and hence prevent competing hydrolysis. Accordingly, the 3-toluene-*p*-sulphonate **3** was converted almost quantitatively into the epimine **7** by sodium isopropoxide Lithium aluminium hydride was much less convenient and only gave a 60% yield of the epimine Furthermore, since higher temperatures might favour the chair-to-boat conformational change (**1**→**6**), a probable prerequisite of epimine formation[9], the action of sodium hydroxide on the compound **1** in the higher boiling solvent 2-methoxyethanol was examined and found to give the epimine **7** in 90–95% yield The latter reaction is the method of choice, and the epimine **7** can now be regarded as a useful and readily obtainable synthetic intermediate

Base-catalysed epimine formation from α,β-*trans*-acylamido-sulphonyloxy systems probably requires a *trans*-coplanar arrangement of participating substituents and the formation of the powerfully nucleophilic anion of the acylamido group

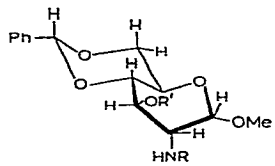


Thus, under mildly basic conditions, which would not favour anion formation, predominant attack by the carbonyl oxygen-atom occurs to give the oxazoline, and

thence the *cis*-acylamido alcohol[14]. Consequently, the rate of formation of the epimine must be related to the pK_a of the amide, and, since acylamide derivatives are considerably weaker acids than are sulphonamide derivatives[15], *N*-sulphonyl derivatives (*e g*, **5**) should be converted more readily into epimines than the corresponding *N*-acyl derivatives and, furthermore, competing oxazoline formation would be excluded This approach has been used successfully in the furanoside series[8] and has now been applied to the preparation of the *N*-mesyl-*allo*-epimine **10** from methyl



1 R = Bz R' = Ms
2 R = Bz R = H
3 R = Bz R = Ts
4 R = R = H
5 R = R = Ms



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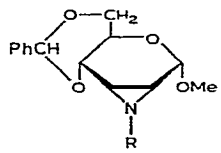
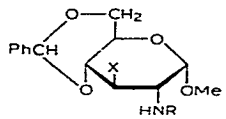
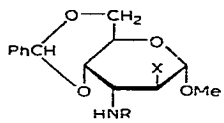


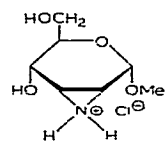
7 R = H
8 R = Ac
9 R = Bz
10 R = Ms
11 R = DNP



12 R = H X = Cl
13 R = Ac X = Cl
14 R = Bz X = Cl
15 R = DNP X = Cl
16 R = H, X = Br
17 R = Ac, X = Br
18 R = H X = I
19 R = Ac, X = I
20 R = Bz X = N_3
21 R = Ac, X = N_3



22 R = H X = Cl
23 R = Ac, X = Cl
24 R = Bz X = Cl
25 R = DNP, X = Cl
26 R = Ac X = I
27 R = H, X = N_3
28 R = Ms X = N_3



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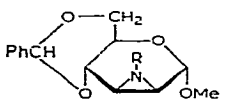
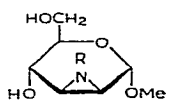
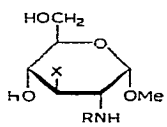


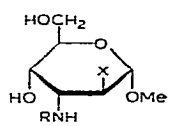
30 R = H
31 R = Ac



32 R = H
33 R = Ac



34 R = H, X = Cl
35 R = Ac X = Cl
36 R = Bz X = Cl
37 R = Ac, X = Br
38 R = Ac X = I



39 R = H, X = Cl
40 R = Ac X = I
41 R = Bz, X = Cl
42 R = Ac, X = Cl

2-amino-4,6-*O*-benzylidene-2-deoxy-α-D-glucopyranoside (**4**). The latter amine was obtained from the readily available *N*-benzoyl derivative **2** by hydrolysis with sodium hydroxide in 95% 2-methoxyethanol, *N*-benzoyl groups are not usually removed by aqueous sodium hydroxide[16], conditions which are effective for the removal of *N*-acetyl groups[16 17] The amino-alcohol **4** gave the di-*N*,*O*-mesyl derivative **5**, in high yield, which was converted into the *N*-mesyl-*allo*-epimine **10** by the action of sodium acetate in boiling 95% 2-methoxyethanol This is probably the most reliable route for epimine synthesis, but the removal of the alkali-stable *N*-mesyl group presents difficulties

The acetylepimino ring is very labile towards acid[13], and the two *N*-acetyl epimines **8** and **31** undergo ring-opening with hydrogen halides to give diaxial acetamido-halo derivatives (*e g*, **23**) before hydrolysis of the benzylidene substituent Consequently, selective removal of the benzylidene group from these derivatives was not possible However, the free epimines **7** and **30** are less prone to ring-opening than are their *N*-acyl derivatives, so that the benzylidene blocking groups can thereby be selectively removed. Thus, the *allo*-epimine **7**, upon treatment with hydrochloric acid in acetone, gave a 70% yield of methyl 2,3-dideoxy-2,3-epimino-α-D-allopyranoside hydrochloride (**29**) The *manno*-epimine **32** was similarly prepared from compound **30** Intermediates that crystallised from the reaction mixtures were shown to be the respective hydrochlorides of the starting epimines **7** and **30**, since, with alkali, they could be reconverted into the epimines Despite the fact that the epimines are very weak bases[18], these salts were stable over periods of several months The stability of the epimine salts to ring-opening was surprising, since the presence of a positive charge on nitrogen should weaken the C-N bond

The ring-opening reactions of the *allo*-epimine and its *N*-substituted derivatives, under essentially neutral conditions with ammonium halides in *N*,*N*-dimethylformamide, were then studied as an alternative route to halo-amino-pyranosides, under comparable conditions, the *allo*-epimine **7** is susceptible to *trans*-diaxial ring-opening by azide[11] With ammonium chloride, the *allo*-epimine **7** gave a 40% yield of a chloro-amine, which had not been encountered previously, although the ring-opening reactions with sodium azide have always been carried out in the presence of ammonium chloride, which acts as a buffer, presumably because azide is a much better nucleophile than chloride The chloroamine gave a crystalline *N*-acetyl derivative, which was different from the syrupy acetamido-chloro-altropyranoside[13] **23** Furthermore, when the *N*-acetyl-*allo*-epimine **8** was treated with hydrochloric acid in acetone[13], the syrupy altropyranoside **23** was the major product (80%), but a minor (3%), isomeric product was also isolated and was identical with the product obtained from the "ammonium chloride reaction" Provided that *trans*-opening of the epimine ring occurs, the new chloro-amine must be the 2-amino-3-chloro-glucoside **12** Acid hydrolysis of compound **12** gave methyl 2-amino-3-chloro-2,3-dideoxy-α-D-glucopyranoside (**34**), the molecular rotation of which (+32 6°) was similar to that (+30 8°) of methyl 2-acetamido-2-deoxy-α-D-glucopyranoside[19], but different from that (+21 6°) of the corresponding allopyranoside[20] Unequivocal support for the *gluco*-stereochemistry was provided by n m r spectroscopy (see below)

The *allo*-epimine **7** also reacted with ammonium bromide and iodide to give 52 and 54%, respectively, of the corresponding halo-derivatives The *N*-acetyl derivative of the iodo-amine was different from the syrupy 3-acetamido-2-iodo-altropyranoside **26**, obtained[13] by the action of hydriodic acid on the acetylepimine **8** Hence, the new iodo-derivative was the glucopyranoside **18** Removal of the benzylidene group from the *N*-acetyl derivative **19** gave methyl 2-acetamido-2,3-dideoxy-3-iodo-α-D-glucopyranoside (**38**), which again was different from the 3-acetamido-2-iodo-altropyranoside (**40**) previously described[13] The bromo derivative was also considered

to be the glucopyranoside **16**, since, after *N*-acetylation and removal of the benzylidene group, the molecular rotation of the product (+28 3°) was in close agreement with that (+28 6°) of the corresponding iodo-derivative and compared favourably with the value (+30 8°) reported[19] for methyl 2-acetamido-2-deoxy-α-D-glucopyranoside

The structures of the three halo-amines were proved beyond doubt by their n m r spectra The three products exhibited very similar n m r. spectra (Table I), suggesting that they were all configurationally identical In all three spectra, the H-1 resonances were low-field doublets at τ 5 3 (J 3 5 Hz), which suggested an *ax-eq* (*cis*)-arrangement of H-1 and H-2 An *eq-eq* (*trans*)-relationship, as found in α-D-mannopyranosides and α-D-altropyranosides, gives rise to a smaller (0 6–1 7 Hz)[21] coupling constant The highest field resonance due to a single proton was observed at τ *ca* 7 0 in all three spectra as a quartet (J 3 5 and 10 0 Hz), the splittings of which suggested it to be H-2 In the case of the chloro- and bromo-derivatives, **12** and **16**, this was confirmed by irradiation at the frequency of H-1, when the quartet collapsed to a doublet with a splitting of 10 Hz (Fig 1) The large coupling constant of H-2 and

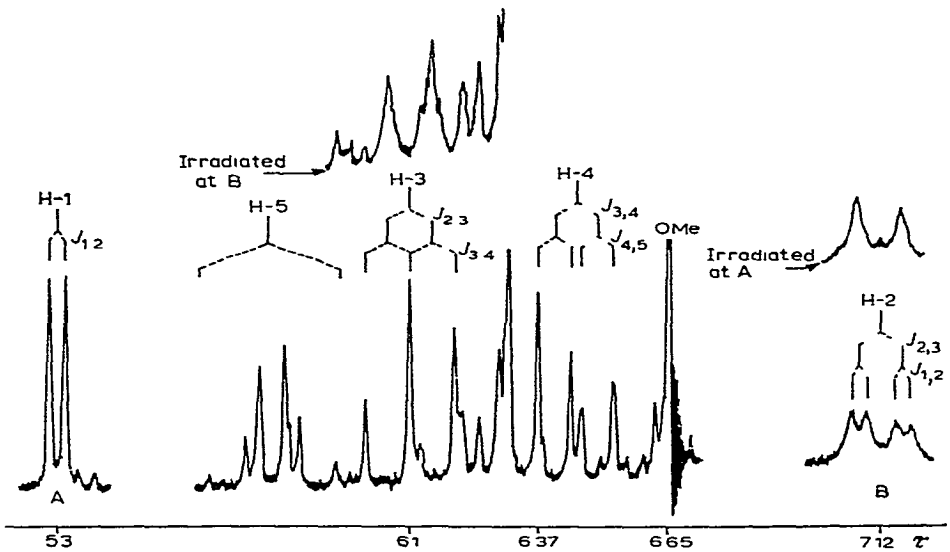


Fig 1 The low-field portion of the 100 MHz n m r spectrum of **12** showing the results of decoupling experiments

H-3 clearly indicates that these protons possess a *trans*-diaxial relationship[22], thereby establishing the *gluco*-stereochemistry of these products The assignment of all other ring-proton resonances was possible for the chloro derivative (Fig 1) The triplet at τ 6 1 (J 10 Hz) collapsed into a doublet when the H-2 quartet was irradiated, showing it to be due to H-3, and consequently a quartet (J 10 and 7 3 Hz) at τ 6 46 was assigned to H-4 The observed first-order coupling constants are clearly in accord with the *C1* conformation of the D-glucopyranose ring The H-5 resonance was observed at τ 5 8 as an octet with an increase in intensity of the limbs towards the centre of the multiplet The general structure of this multiplet was reminiscent of the

AB part of an ABX system, but integration showed it to be due to a single proton A similar resonance was observed in the bromo and iodo derivatives, but, in these cases, it was partly overlapped with other resonances

TABLE I

CHEMICAL SHIFTS (τ VALUES) AND FIRST-ORDER COUPLING CONSTANTS (Hz) AT 100 MHz

Compound	Chloro-amine (12)	Bromo-amine (16)	Iodo-amine (18)
H-1	5 3 d[a]	5 3 d	5 32 d
H-2	7 12 q	7 02 q	6 94 q
H-3	6 10 t	5 96 t	5 86 t
H-4	6 46 q	—	—
H-5	5 80 cm	5 77 cm	5 78 cm
H-6	—	—	—
OMe	6 65 s	6 64 s	6 62 s
NH_2	8 40 s	8 38 s	8 40 s
$J_{1,2}$	3 5	3 5	3 5
$J_{2,3}$	10 0	10 1	10 0
$J_{3,4}$	10 0	10 0	10 0
$J_{4,5}$	7 3	—	—

[a]s = singlet, d = doublet, t = triplet, q = quartet, cm = complex multiplet

Previous results[11,12] on the ring-opening of the α- and β-anomers of derivatives of the D-*allo*-epimine (**7** for the α-anomer) suggested that the *N*-benzoyl derivatives were alone in being anomalous by virtue of diequatorial ring-opening Our results obtained with the free epimine were therefore of some note, because they constitute the first examples of diequatorial ring-opening of the epimine **7**. We have therefore investigated the action of ammonium chloride on the *N*-substituted *allo*-epimines in order to assess whether the anomaly was a function of the reagent or of the substrate, and find that the *N*-benzoyl, *N*-acetyl, and, *N*-2,4-dinitrophenyl derivatives (**9**, **8**, and **11**) each gives mixtures of the *gluco*- and *altro*-isomers, in which the latter preponderated Thus, the *N*-benzoylepimine **9** gave a mixture of two chloro derivatives, the minor component (15%) of which was shown to be the *gluco*-isomer by comparison with the *N*-benzoyl derivative **14** derived from the amino-chloro-glucoside **12** The syrupy, major product (35%), isolated after chromatography, was identical with the chloro derivative formed by the action of hydrochloric acid on the *N*-benzoylepimine **9**. It was best characterised by acid hydrolysis to give crystalline methyl 3-benzamido-2-chloro-2,3-dideoxy-α-D-altropyranoside (**41**), which could be synthesised from the known[13] methyl 3-acetamido-2-chloro-2,3-dideoxy-α-D-altropyranoside (**42**) by acid hydrolysis followed by *N*-benzoylation

The action of ammonium chloride on the *N*-acetylepimine **8** gave mainly (42%) the known altropyranoside **23**, together with a small proportion of the glucopyranoside **13** Likewise, the *N*-2,4-dinitrophenyl derivative **11** afforded mainly the altropyranoside **25**, but the overall recovery of products was only 40%. Hence,

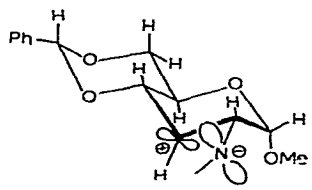
it appears that the pattern by which ammonium halides react with the *allo*-epimine and its derivatives is quite different from that for a mixture of sodium azide and ammonium chloride, where the azide anion is the predominant nucleophile

The above results suggest that the reported[11] failure to characterise a product when the *N*-acetylepimine **8** was heated under reflux with a mixture of sodium azide and ammonium chloride in *N*,*N*-dimethylformamide for 3 h may well have been due to ready de-*N*-acetylation under basic conditions We have now found that, with a shorter reaction time (5–20 min), the *N*-acetylepimine **8** undergoes *trans*-diequatorial ring-opening to give methyl 2-acetamido-3-azido-4,6-*O*-benzylidene-2,3-dideoxy-α-D-glucopyranoside **21**, but that extensive de-*N*-acetylation of the epimine also occurs Since the free epimine is less reactive towards azide than is the *N*-acetyl derivative, the epimine **7** was obtained as a side product. The *gluco*-configuration of the azido-pyranoside was shown by its synthesis[23] from methyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-3-*O*-mesyl-α-D-allopyranoside by replacement with sodium azide in *N*,*N*-dimethylformamide. The preparation of the 3-azide **21** from the *N*-acetylepimine **8** was not satisfactory, because the yields of 20–30% could not be maintained on a larger scale

The *N*-mesylepimine underwent reaction with sodium azide–ammonium chloride in *N*,*N*-dimethylformamide to give a crystalline azide identical with that obtained by mesylation of the known[11] methyl 3-amino-2-azido-4,6-*O*-benzylidene-2,3-dideoxy-α-D-altropyranoside (**27**), hence, normal *trans*-diaxial ring-opening had occurred Surprisingly, the same azide was obtained by the action of sodium azide on methyl 4,6-*O*-benzylidene-2-deoxy-3-*O*-mesyl-2-methanesulphonamido-α-D-glucopyranoside (**5**) This observation can be explained by initial ring closure of compound **5** to the *N*-mesylepimine **10**, followed by *trans*-diaxial ring-opening This further illustrates the ease with which the sulphonamido-sulphonate system undergoes cyclisation to the epimine

The pattern of ring-opening of the *allo*-epimine and its derivatives is confusing and unpredictable, and it is hard to account for the results An S_N2 process would favour the 2,3-*trans*-diaxial arrangement, whereas the products arising from an S_N1 mechanism would be governed by the relative stabilities of the two possible carbonium ions, and 2,3-*cis*, as well as 2,3-*trans*, products might be expected The tentatively suggested[11], diequatorial ring-opening of the *N*-benzoylepimine by prior isomerisation to the oxazoline [namely, methyl 4,6-*O*-benzylidene-2,3-dideoxy-3,2-(2-phenyl-1-oxa-3-azaprop-2-eno)-α-D-allopyranoside], followed by ring-opening by azide at C-3 to give the glucopyranoside, is not valid, since the oxazoline derivative, which can be easily prepared from compound **1** by the action of alkali, is quite stable towards azide in boiling *N*,*N*-dimethylformamide, Dr Guthrie has informed us that he has reached the same conclusion. An S_N1 reaction would involve initial heterolysis of a C–N bond to give a carbonium ion at either C-2 or C-3, whichever is the more favourable. There are two factors which would favour heterolysis of the C-3–N bond. Firstly, the electron-withdrawing effect of the anomeric acetal group would have a destabilising effect upon a positive charge situated at C-2, but not at C-3 Secondly,

cleavage of the C-3–N bond would give the C-3 carbonium ion **43** having the negatively charged nitrogen in an equatorial position Molecular models show that, if both charged centres were in a state of sp^2-hybridisation, the unoccupied *p*-orbital at C-3 and the occupied *p*-orbital on nitrogen could become parallel and that this could result in a certain amount of delocalisation by $p\pi$ overlap This overlap would be enhanced if deformation of the C-2–N bond occurred to allow C-3 and the nitrogen atom to become closer This transition state could be envisaged as an intramolecular



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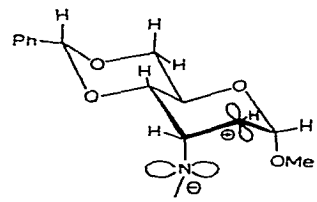

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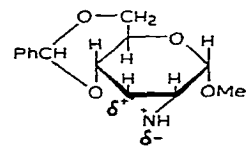

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ion-pair **45**, which would explain why the *trans* and not the *cis* product is obtained If cleavage of the C-2–N bond occurred to give the C-2 carbonium ion **44**, molecular models suggest that no $p\pi$ overlap could occur, although some $p\sigma$ overlap might be possible Stabilisation of carbonium ions by orbital overlap with a β-π system has been previously proposed[24] for the homoallylic system

The foregoing discussion merely explains how these *trans*-diequatorial products may arise, but does not explain why ring-opening is S_N2 in some cases and S_N1 in others Obviously, it would be dangerous to attempt an explanation of this duality of mechanism from our qualitative results

No "anomalous" diequatorial ring-openings have been observed for the corresponding *manno*-epimine **30** This is, perhaps, not surprising, since it would require the formation of the C-2 carbonium ion, which, as previously stated, would be destabilised by the adjacent, electron-withdrawing acetal group

EXPERIMENTAL

All evaporations were effected under diminished pressure, optical rotations were determined for chloroform solutions, unless otherwise stated, and melting points were determined on a Kofler micro-heating stage and are uncorrected Thin-layer chromatography was performed on either Silica Gel G (Merck) or Whatman Chromedia SG 41, and components were located as brown-black spots by spraying with 10% sulphuric acid in ethanol and heating above 120° Silica gel (Hopkins and Williams, or Davison) was utilised for column chromatography

Methyl 2-benzamido-4,6-O-benzylidene-2-deoxy-3-O-tosyl-α-D-glucopyranoside (**3**). — A solution of methyl 2-benzamido-4,6-*O*-benzylidene-2-deoxy-α-D-glucopyranoside[9] (**2**) (50 g) in pyridine (700 ml) was treated with toluene-*p*-sulphonyl chloride (75 g) and stored for 4 days at room temperature, when t l c (chloroform–ether, 1 1) indicated that reaction was almost complete The reaction mixture was

poured into ice–water, and the crystalline precipitate was recrystallised from ethanol containing a little chloroform to give the product (52 4 g, 75%), m p 179–184° (decomp) A further recrystallisation from ethanol gave material having m p. 193–195° (decomp), $[\alpha]_D$ +30° (Found C, 62 1; H, 5 4, N, 2 6 $C_{28}H_{29}NO_8S$ calc C, 62 3, H, 5 4, N, 2 6%)

Methyl 2-amino-4,6-O-benzylidene-2-deoxy-α-D-glucopyranoside (**4**). — A solution of the amide **2** (10 g) in 95% 2-methoxyethanol (100 ml) containing sodium hydroxide (8 g) was heated under reflux for 4 h, and was then cooled, diluted with water, and extracted three times with chloroform The combined extracts were washed with water, dried ($MgSO_4$), and evaporated Recrystallisation of the residue from ethanol gave needles (4 8 g, 62%), m p 175°, $[\alpha]_D$ +112 5° (*c* 1 0) (Found C, 60 0, H, 6 9, N, 5.1 $C_{14}H_{19}NO_5$ calc C, 59 8, H, 6 8, N, 5 0%)

Starting material was recovered when the preparation was repeated by using 2-methoxyethanol without added water

Methyl 4,6-O-benzylidene-2-deoxy-2-methanesulphonamido-3-O-methanesulphonyl-α-D-glucopyranoside (**5**) — Methanesulphonyl chloride (0 9 ml) was added dropwise to a solution of 0 5 g of the amine **4** in pyridine (4 ml), and the reaction mixture was kept overnight at 4° Addition of ice–water to the mixture gave a crystalline product which was recrystallised from ethanol to give compound **5** as needles (0 62 g, 68%), m p 207 5–208 5°, $[\alpha]_D$ +47° (*c* 1 0) (Found C, 44 1, H, 5 7, N, 3 2, S, 14 2 $C_{16}H_{23}NO_9S_2$ calc C, 43 9, H, 5 5, N, 3 2, S, 14 6%)

Methyl 4,6-O-benzylidene-2,3-dideoxy-2,3-epimino-α-D-allopyranoside (**7**) — (*a*) The sulphonate **3** (10 g) was added to a solution of sodium (1 5 g) in isopropyl alcohol (1 litre) at room temperature The solution was heated under reflux on a water bath for 1 h, during which time sodium toluene-*p*-sulphonate was precipitated After filtration, the solution was evaporated to dryness, and the residue was partitioned between water and chloroform The aqueous layer was extracted with a further portion of chloroform, and the combined chloroform solutions were washed twice with water, dried (Na_2SO_4), and evaporated to a chromatographically pure syrup which rapidly crystallised (crude yield, 4 4 g, 90%) Recrystallisation from ethyl acetate–light petroleum gave the epimine **7** as needles, m p 154–157° (after crystal transitions at 125 and 143°), $[\alpha]_D$ +148° (*c* 0 4) It was identical (mixed m p , i r spectrum, and chromatography) with authentic material[4]

(*b*) The sulphonate **3** (0 2 g) was dissolved in tetrahydrofuran (10 ml) and treated slowly with lithium aluminium hydride (0 2 g) The mixture was kept for 24 h at room temperature and processed, as described previously[9], to give epimine **7** (52 mg, 60%), m p 151–152°

(*c*) Methyl 2-benzamido-4,6-*O*-benzylidene-2-deoxy-3-*O*-mesyl-α-D-glucopyranoside[9] (**1**, 60 g) was dissolved in a solution of sodium hydroxide (32 g) in 95% aqueous 2-methoxyethanol (600 ml), and the solution was heated under reflux for 2 h It was then diluted with water and extracted with chloroform (3 × 200 ml) The chloroform extract, after drying ($MgSO_4$), was evaporated, and the residue was recrystallised from ethyl acetate–light petroleum to give the epimine **7** as needles

(31 g, 92%), m p. 141–145°, $[\alpha]_D$ +147° (*c* 1.0) The product was identical with the lower-melting form of the epimine obtained by Buss *et al*[4], who reported m p 143–145°, $[\alpha]_D$ +147°.

Methyl 4,6-O-benzylidene-2,3-dideoxy-2,3-(methanesulphonylepimino)-α-D-allopyranoside (**10**) — (*a*) To a solution of 0 1 g of the 3-*O*-mesyl-2-sulphonamido-glucoside **5** in 95% aqueous 2-methoxyethanol (2 ml) was added sodium acetate (0 1 g), and the mixture was heated under reflux for 1 h Addition of water then gave a crystalline product (65 mg, 80%) which was recrystallised from ethanol to give the methanesulphonylepimine **10**, m p 161–163°, $[\alpha]_D$ +121° (*c* 1 0) (Found C, 52 5, H, 5 9; N, 4 1, S, 9 2 $C_{15}H_{19}NO_6S$ calc C, 52 6, H, 5 6, N, 4 1, S, 9 4%)

(*b*) The epimine **7** (0 1 g) was methanesulphonylated, in the usual way, to give, after recrystallisation from ethanol, the *N*-mesyl derivative **10** (0 1 g, 90%), m p 161 5–162 5°, $[\alpha]_D$ +121° (*c* 1 0)

Ring-opening reactions of methyl 4,6-O-benzylidene-2,3-dideoxy-2,3-epimino-α-D-allopyranoside (**7**) *and its derivatives with ammonium halides* — (*a*) *The action of ammonium chloride on the epimine* To a solution of epimine **7** (1 g) in *N,N*-dimethylformamide (50 ml) was added ammonium chloride (0 4 g), and the mixture was heated under reflux for 1 h The solution was evaporated to dryness, and a solution of the residue in methanol was passed through a column of Amberlite IRA-400 (OH^- form) resin The eluate was evaporated to dryness, and the residue was recrystallised from isopropyl alcohol to yield methyl 2-amino-4,6-*O*-benzylidene-3-chloro-2,3-dideoxy-α-D-glucopyranoside (**12**), (0 5 g, 40%), m p 155–162°. Two recrystallisations from ethanol–light petroleum yielded a sample having m p 162° (very slow heating), $[\alpha]_D$ +42° (*c* 1 3) More-rapid heating caused partial melting at 155°, with a slow crystal transition to a form melting at 162° Extremely slow heating above 150° allowed the transition to occur without melting, to give a sharp m p of 162° (Found C, 55 9, H, 5 9, Cl, 12 0, N, 4 7 $C_{14}H_{18}ClNO_4$ calc C, 56 1, H, 6 0, Cl, 11.8, N, 4 7%)

N-Acetylation of the chloro-amine **12** (30 mg) in ethanol (0 4 ml) with acetic anhydride (0 04 ml) gave methyl 2-acetamido-4,6-*O*-benzylidene-3-chloro-2,3-dideoxy-α-D-glucopyranoside (**13**) (from ethanol) (80%), subl 293°, $[\alpha]_D$ +6° (*c* 0.4) (Found C, 56 3, H, 6 0, Cl, 10 6, N, 4 1 $C_{16}H_{20}ClNO_5$ calc C, 56 2, H, 5 85, Cl, 10 4, N, 4 1%)

Similarly the *N*-benzoylepimine **14** was prepared in 70% yield and had m p 273–274° (from ethanol), $[\alpha]_D$ +36° (*c* 0 5) (Found· C, 62 4, H 5 5, Cl, 8.7, N, 3 6 $C_{21}H_{22}ClNO_5$ calc C, 62 5, H, 5 45, Cl, 8 8, N, 3 5%)

The *N*-2,4-dinitrophenyl derivative **15**, prepared in the usual way[13], had m p 301–302° (from chloroform–ethanol), $[\alpha]_D$ −69° (*c* 0 4) (Found C, 51 4, H, 4 5, N, 9 0 $C_{20}H_{20}ClN_3O_8$ calc C, 51 6, H, 4 3, N, 9 0%)

(*b*) *The action of ammonium bromide on the epimine* A solution of 1 g of the epimine **7** in *N,N*-dimethylformamide (10 ml) was heated for 20 min at 130° with ammonium bromide The addition of water to the reaction mixture gave a crystalline solid (0 7 g, 52%), recrystallisation of which from ethanol–light petroleum gave

methyl 2-amino-4,6-*O*-benzylidene-3-bromo-2,3-dideoxy-α-D-glucopyranoside (**16**) as needles, m p. 150–150 5°, $[\alpha]_D$ +15° (*c* 0 74) (Found C, 49 0; H, 5.4; N, 4 1. $C_{14}H_{18}BrNO_4$ calc . C, 48.9, H, 5 2, N, 4 1%)

The *N*-acetyl derivative **17**, obtained as above, was recrystallised from *N*,*N*-dimethylformamide, and decomposed at *ca.* 275° without melting; $[\alpha]_D$ +31° (*c* 0 5, *N*,*N*-dimethylformamide) (Found C, 49 8, H, 5 3, N, 3.6 $C_{16}H_{20}BrNO_5$ calc · C, 49 9, H, 5 2, N, 3 6%)

(*c*) *The action of ammonium iodide on the epimine* The epimine **7** (1 g) was treated, as above, with ammonium iodide The product separated out as a gel that solidified when filtered Recrystallisation from ethanol gave methyl 2-amino-4,6-*O*-benzylidene-2,3-dideoxy-3-iodo-α-D-glucopyranoside (**18**) (0 8 g, 54%), m p 138–140°, $[\alpha]_D$ −20° (*c* 0 5) (Found C, 43 4, H, 4 4, N, 3 5 $C_{14}H_{18}INO_4$ calc C, 43 0, H, 4 6; N, 3 6%)

The *N*-acetyl derivative **19**, obtained in the usual way, had m p 271° (decomp) (from chloroform), $[\alpha]_D$ +4° (*c* 0 36, *N*,*N*-dimethylformamide) (Found C, 44 1, H, 4 7, N, 3 2 $C_{16}H_{20}INO_5$ calc · C, 44 4, H, 4 6, N, 3 2%)

(*d*) *The action of ammonium chloride on the* N-*acetylepimine* **8** To a solution of methyl 2,3-(acetylepimino)-4,6-*O*-benzylidene-2,3-dideoxy-α-D-allopyranoside (**8**) (0 5 g) in *N*,*N*-dimethylformamide (50 ml) was added ammonium chloride (0 2 g), and the mixture was heated under reflux for 2 h The solution was evaporated to dryness, and the syrupy residue was partitioned between water and chloroform The aqueous layer was extracted with a second portion of chloroform, and the combined organic layers were washed twice with water and dried (Na_2SO_4) T l c (chloroform–ether, 1 1) indicated the presence of one predominant, fast-moving component, a small proportion of a slightly slower moving component, and two further components near the starting line The mixture was chromatographed on a column of silica gel with chloroform–ether (1·1) as eluant, and the two fast-moving components were isolated

The major component, obtained as a syrup (0 236 g, 42%), was shown to be methyl 3-acetamido-4,6-*O*-benzylidene-2-chloro-2,3-dideoxy-α-D-altropyranoside (**23**) by comparison with an authentic sample[13], and was characterised as follows A solution of the syrup in acetone (15 ml) and conc hydrochloric acid (0 2 ml) was heated under reflux for 5 min Excess of solid sodium hydrogen carbonate was added, and the solution was filtered, and evaporated to an impure, yellow syrup which could not be crystallised The main component was obtained pure after chromatography on a column of silica gel, and was crystallised from ethyl acetate Recrystallisation from ethyl acetate yielded methyl 3-acetamido-2-chloro-2,3-dideoxy-α-D-altropyranoside (**42**) (18 mg), m p 135–137°, lit [13], m p 136–138°

The second component, isolated in small yield from the column, was recrystallised from isopropyl alcohol to give methyl 2-acetamido-4,6-*O*-benzylidene-3-chloro-2,3-dideoxy-α-D-glucopyranoside (**13**) identical (i r spectrum and sublimation temp) with the samples prepared from the chloro-amine **12** (see above), and by the action of hydrochloric acid in acetone on the *N*-acetylepimine **8** (see below)

(*e*) *Action of ammonium chloride on the* N-*benzoylepimine* **9** To a solution of the *N*-benzoylepimine (0 5 g) in *N*,*N*-dimethylformamide (50 ml) was added ammonium chloride (0 2 g), and the mixture was heated under reflux for 1 h and processed as described for the *N*-acetylepimine above T l c (chloroform) showed the presence of two fast-moving components, coincident with compounds **24** and **14**, respectively Evaporation of the chloroform solution, with recrystallisation of the residue from ethanol–ether, yielded methyl 2-benzamido-4,6-*O*-benzylidene-3-chloro-2,3-dideoxy-α-D-glucopyranoside (**14**) (85 mg), m p 273–274°, identical (i r. spectrum and mixed m p) with the product prepared above from compound **12**

The mother liquors were subjected to column chromatography to yield methyl 3-benzamido-4,6-*O*-benzylidene-2-chloro-2,3-dideoxy-α-D-altropyranoside (**24**) as a syrup (190 mg), $[\alpha]_D$ +68° (*c* 0 7) (Found C, 60 7, H, 5 5, N, 3 5 $C_{21}H_{22}ClNO_5$ calc C, 62 5, H, 5 5, N, 3 6%)

(*f*) *Action of ammonium chloride on the* (*2,4-dinitrophenyl*)*epimine* (**11**) To a solution of compound **11** (100 mg) in *N*,*N*-dimethylformamide (5 ml) was added ammonium chloride (50 mg), and the mixture was heated under reflux for 1 5 h T l c (ether–light petroleum, 1 1) indicated the presence of two products, one being on the starting line The solution was evaporated (to *ca* half volume) and poured into water After storage for 1 h at *ca* 4°, the precipitate was separated by centrifugation, washed with water, and then shaken with a 2 1 mixture of acetone–ethanol (10 ml) T l c indicated that the extract contained largely the fast-moving component, and the solid residue consisted largely of the compound which failed to move during t l c

The solid residue was recrystallised from chloroform–ethanol to yield methyl 4,6-*O*-benzylidene-3-chloro-2,3-dideoxy-2-(2,4-dinitrophenylamino)-α-D-glucopyranoside (**15**) (12 mg, 11%), m p 300–302°, $[\alpha]_D$ −69° (*c* 0 4), identical (i r spectrum and mixed m p) with the product derived from compound **12**

The extract was evaporated to dryness, and the residue was recrystallised from isopropyl alcohol to yield methyl 4,6-*O*-benzylidene-2-chloro-2,3-dideoxy-3-(2,4-dinitrophenylamino)-α-D-altropyranoside (**25**) (31 mg, 29%), slightly contaminated with the above isomer. A pure sample was obtained by extraction with ethanol, giving granular crystals m p 190–193°, $[\alpha]_D$ −35° (*c* 0 5) (Found C, 51 7, H, 4 5, N, 8 9 $C_{20}H_{20}ClN_3O_8$ calc C, 51 6, H, 4 3, N, 9 0%)

The action of hydrochloric acid on (*a*) *methyl 4,6-O-benzylidene-2,3-dideoxy-2,3-epimino-*α*-D-mannopyranoside* (**30**) — (*i*) A solution of the epimine (0 57 g) in acetone (20 ml) was cooled in an acetone–solid CO_2 bath, and 4N hydrochloric acid (1 ml) was added The mixture was then allowed to warm up to room temperature, when fine needles of methyl 4,6-*O*-benzylidene-2,3-dideoxy-2,3-epimino-α-D-mannopyranoside hydrochloride (0 4 g, 62%) separated, m p 153–165° (decomp) (Found C, 56 5, H, 6 3, Cl, 11 5; N, 4 6 $C_{14}H_{18}ClNO_4$ calc C, 56 1, H, 6 0, Cl, 11 8, N, 4 7%). The hydrochloride was very soluble in water, giving an acidic solution, but was not very soluble in ethanol from which it could, with care, be recrystallised It was indefinitely stable in the dry, crystalline state, but was unstable in solution Acetylation of

the hydrochloride, in the usual way, gave a quantitative yield of the *N*-acetylepimine **31**, and treatment with aqueous alkali gave the free epimine **30**

(*ii*) The epimine (14 g) was dissolved in acetone (250 ml), and conc hydrochloric acid (10 ml) was added The hydrochloride was precipitated initially, but, on being shaken vigorously for 15 min, this was transformed into an oil, and t l c (chloroform–methanol, 4 ·1) indicated the presence of a single product The reaction mixture was diluted with water to dissolve the oily product, sodium hydrogen carbonate (15 g) was then added cautiously in small portions, and the solution was extracted once with ether to remove benzaldehyde and evaporated to dryness The residue was extracted with hot ethyl acetate (3 × 200 ml), and the combined extracts were concentrated to *ca* 125 ml, when scratching induced crystallisation The product, methyl 2,3-dideoxy-2,3-epimino-α-D-mannopyranoside (**32**), was usually obtained, initially, as an unstable hydrate, m p 115–117°, ν_{max} *ca* 1650 cm^{-1} When the product was kept at room temperature for several days or dried *in vacuo* for 2 h at 100°, it gave the anhydrous product (6 4 g, 68%), m p 150–153°, $[\alpha]_D$ +107° (*c* 1, water) (Found· C, 47 9, H, 7 35, N, 7 95 $C_7H_{13}NO_4$ calc C, 47 9, H, 7 4, N, 8 0%)

The *N*-acetylepimine **33**, m p 124–125°, $[\alpha]_D$ +68° (*c* 1 2, water), was prepared, by the action of acetic anhydride in ethanol, in 94% yield (Found C, 49 6, H, 6 95, N, 6 35 $C_9H_{15}NO_5$ calc. C, 49 7, H, 6 9, N, 6 45%)

(*b*) *Methyl 4,6-O-benzylidene-2,3-dideoxy-2,3-epimino-α-D-allopyranoside* (**7**) — (*i*) The epimine (3 5 g) in acetone (60 ml) was treated with conc hydrochloric acid (2 5 ml), and the mixture was shaken for 30 min at room temperature The crystalline product was filtered off and washed with acetone, to give methyl 2,3-dideoxy-2,3-epimino-α-D-allopyranoside hydrochloride (**29**) (2 24 g, 80%), m p 118–122°, $[\alpha]_D$ +134° (*c* 2, water) Recrystallisation from methanol–ether gave the hydrochloride, m p 120–122° (Found C, 39 9, H, 6 7, N, 6 7 $C_7H_{14}ClNO_4$ calc C, 39 7, H, 6 6, N, 6 6%) The hydrochloride was stable in the crystalline state for several months, but underwent a slow transformation into a crisp, amorphous solid

(*ii*) When a cooled solution of the epimine (50 mg) in acetone (0 5 ml) was treated with one equivalent of hydrochloric acid in acetone (0 16 ml), methyl 4,6-*O*-benzylidene-2,3-dideoxy-2,3-epimino-α-D-allopyranoside hydrochloride crystallised immediately (46 mg, 81%), m p 108–112°, $[\alpha]_D$ +137° (*c* 1, water) (Found C, 55 9, H, 6 2, Cl, 11 8, N, 4 7 $C_{14}H_{18}ClNO_4$ calc C, 56 1, H, 6 1, Cl, 11 8, N, 4 7%)

(*c*) *Methyl 2,3-(acetylepimino)-4,6-O-benzylidene-2,3-dideoxy-α-D-allopyranoside*[13] (**8**) — To a solution of the *N*-acetylepimine **8** (5 g) in acetone (250 ml), conc hydrochloric acid (1 36 ml, 1 equiv) was added After 3 h at room temperature, the solution was evaporated to a syrup, which was co-concentrated with water and partitioned between water and chloroform T l c (chloroform–ether, 1 1) indicated the presence of a very small amount of a slower moving product, in addition to the expected chloro-amide **23** Elution from a column of silica gel, with chloroform–ether (1 1), yielded the syrupy, faster-moving product (4 5 g, 80%) shown by its i r spectrum to be methyl 3-acetamido-4,6-*O*-benzylidene-2-chloro-2,3-dideoxy-α-D-altropyranoside[13] (**23**) and by conversion[13] into the crystalline methyl 3-acetamido-

2-chloro-2,3-dideoxy-α-D-altropyranoside (**42**) (see above) Distillation afforded an analytical sample (Found C, 56 2, H, 5.8, N, 3.9. $C_{16}H_{20}ClNO_5$ calc.· C, 56 2, H, 5 85, N, 4 1%)

The minor product (0 156 g, 3%) was methyl 2-acetamido-4,6-*O*-benzylidene-3-chloro-2,3-dideoxy-α-D-glucopyranoside (**13**), which sublimed at 293°, and had $[\alpha]_D$ +6° (*c* 0 4) The infrared spectrum was identical with that of the *N*-acetyl derivative of the chloro-amine **12** described above

(*d*) *Methyl 2,3-(benzoylepimino)-4,6-O-benzylidene-2,3-dideoxy-α-D-allopyranoside* (**9**) — (*i*) Conc hydrochloric acid (0 454 ml) was dissolved in acetone (10 ml), and 1 ml (1 equiv) of this solution was added to a solution of the *N*-benzoylepimine **9** (200 mg) in acetone (9 ml) T.l c (chloroform–ether, 1 1) indicated that reaction had ceased after *ca* 2 h, the solution then contained a little starting material, together with one fast- and one slow-moving component After 3 h, the solution was evaporated, and co-concentrated with water The residue was fractionated on a column of silica gel, with chloroform–ether (1 1) as eluant, to give the faster-moving, syrupy chloroamide **24**, which was dissolved in acetone (1 5 ml) to which conc hydrochloric acid (0 1 ml) was then added After 30 min, the solution was neutralised (Ag_2CO_3), and evaporated to a syrup Benzaldehyde was removed by co-concentration with water, followed by co-concentration with ethanol The resulting syrup crystallised spontaneously to yield methyl 3-benzamido-2-chloro-2,3-dideoxy-α-D-altropyranoside (**41**) (7 mg), m p 154–157° Recrystallisation from ethanol–light petroleum gave a sample identical (mixed m p and i r spectrum) with (*i*) the slower moving component isolated from the column by elution with acetone, (*ii*) the product obtained below from the action of an excess of hydrochloric acid on the *N*-benzoylepimine **9** and (*iii*) the product obtained from the corresponding *N*-acetyl derivative **42** by deacetylation, followed by *N*-benzoylation

(*ii*) To a solution of the *N*-benzoylepimine **9** (1 g) in acetone (40 ml), an excess of conc hydrochloric acid (0 6 ml) was added, and the solution was heated under reflux for 10 min An excess of solid sodium hydrogen carbonate was added, and the solution was filtered and evaporated to a crystalline residue The solid was extracted with chloroform, and the extract was evaporated to a residue (0 47 g, 55%) that was recrystallised from ethanol–light petroleum to yield methyl 3-benzamido-2-chloro-2,3-dideoxy-α-D-altropyranoside as needles, m p 159–160°, $[\alpha]_D$ +22° (*c* 0 4) (Found C, 53 3, H, 5 75, Cl, 10 8, N, 4 3 $C_{14}H_{18}ClNO_5$ calc C, 53 2, H, 5 7, Cl, 11 25, N, 4 4%)

Methyl 3-amino-2-chloro-2,3-dideoxy-α-D-altropyranoside (**39**) *and its N-benzoyl derivative* (**41**) — Methyl 3-acetamido-2-chloro-2,3-dideoxy-α-D-altropyranoside[13] (**42**) (1 g) was heated for 1 h at 100° with N hydrochloric acid (2 ml) The solution was evaporated to a syrup which was co-concentrated twice with ethanol The resulting syrup crystallised on the addition of isopropyl alcohol, yielding compound **39** hydrochloride (0 58 g, 60%), m p 185–190° (decomp) Recrystallisation from isopropyl alcohol–light petroleum gave granular crystals, m p 192–194° (decomp.),

$[\alpha]_D$ +72° (*c* 0 8, water) (Found C, 34 1, H, 6 0, Cl, 28 4, N, 5 7 $C_7H_{15}Cl_2NO_4$ calc C,33 9, H, 6.0, Cl, 28 6; N, 5 6%)

A solution of the above hydrochloride (100 mg) in water was passed through a column of Amberlite IRA-400 (OH^- form) and evaporated Recrystallisation of the residue from isopropyl alcohol gave the free amine **39** (64 mg, 75%), m p 128–130°, $[\alpha]_D$ +87° (*c* 0 5, water) A further recrystallisation gave an analytical sample, m p 132–134° (Found C, 39 7; H, 6 9; N, 6 55 $C_7H_{14}ClNO_4$ calc C, 39.7; H, 6 6, N, 6 6%)

To a solution of the free amine (20 mg) in ethanol (1 ml), benzoic anhydride (25 mg) was added After 1 h at room temperature, the solution was evaporated to a syrup which was partitioned between water (5 ml) and ether (5 ml) The organic layer was extracted with a further portion of water, and the combined aqueous layers were washed with ether Evaporation of the aqueous solution, with recrystallisation of the residue from ethanol–light petroleum, yielded the chloro-amide **41** (14 mg, 47%), m p 158–160°, identical (i r spectrum and mixed m.p) with samples prepared above.

Acid hydrolysis of 4,6-O-benzylidene derivatives — (*a*) Compound **12** (500 mg) was dissolved in a mixture of acetone (10 ml) and chloroform (10 ml) Conc hydrochloric acid (1 ml) was added, and the mixture was shaken for 30 min , when the crystals that had formed were converted into an oil The mixture was evaporated to a syrup, and benzaldehyde was removed by co-concentration with water A solution of the resulting syrup in methanol was passed through a column of Amberlite IRA-400 (OH^- form) and evaporated Recrystallisation of the residue from ethyl acetate gave methyl 2-amino-3-chloro-2,3-dideoxy-α-D-glucopyranoside (**34**) (200 mg, 57%), m p 127–128°, $[\alpha]_D$ +156° (*c* 1, water) (Found C, 39 5, H, 6 6, N, 6 8 $C_7H_{14}ClNO_4$ calc C, 39 7, H, 6 7, N, 6 6%)

(*b*) Compound **14** (0 1 g) was dissolved in a 1 1 mixture of chloroform and acetone (15 ml), and conc hydrochloric acid (0 4 ml) was added The mixture was shaken for 2 h at room temperature (an oil was initially deposited, but later crystallised) and then cooled in the refrigerator to give methyl 2-benzamido-3-chloro-2,3-dideoxy-α-D-glucopyranoside (**36**) (39 mg) The mother liquors were evaporated to dryness, and co-concentrated with water to remove benzaldehyde The residue was recrystallised from ethanol–light petroleum, giving a second crop (16 mg, total yield, 55 mg, 70%), m p 245–246° (decomp), $[\alpha]_D$ +136° (*c* 0,6 methanol) (Found C, 53 2, H, 5 9, Cl, 11 1, N, 4 6 $C_{14}H_{18}ClNO_5$ calc C, 53 2, H, 5 7, Cl, 11 25; N, 4 4%)

(*c*) A suspension of compound **17** (0 6 g) in a 1% solution of hydrogen chloride in methanol (25 ml) was stirred until a clear solution was obtained (*ca* 1 h) The neutralised ($PbCO_3$) mixture was evaporated to give methyl 2-acetamido-3-bromo-2,3-dideoxy-α-D-glucopyranoside (**37**), which, after recrystallisation from ethanol–light petroleum, gave needles (0 35 g, 76%), m p 185–186°, $[\alpha]_D$ +95° (*c* 0 5, water) (Found C, 36 4, H, 5 5; N, 4 5 $C_9H_{16}BrNO_5$ calc C, 36 2, H, 5 4, N, 4 7%)

(*d*) Compound **19** (0 6 g) was hydrolysed as in (*c*) to give (from ethanol–light

petroleum) methyl 2-acetamido-2,3-dideoxy-3-iodo-α-D-glucopyranoside (**38**) (0 4 g, 84%), m p 189°, $[\alpha]_D$ +83° (*c* 0 55, water) (Found C, 31 0, H, 4 6, N, 3 9 $C_9H_{16}INO_5$ calc C, 31 3, H, 4.6; N, 4 1%)

Methyl 2-azido-4,6-O-benzylidene-2,3-dideoxy-3-methanesulphonamido-α-D-altropyranoside (**28**) — (*a*) A solution of the 3-methanesulphonate **5** (80 mg) in *N,N*-dimethylformamide (1 ml) was heated under reflux with sodium azide (80 mg) for 10 min. Addition of water to the hot reaction mixture gave a brown solid, recrystallisation of which from ethanol gave white needles (60 mg, 65%), m p 147–150°, $[\alpha]_D$ +54.3° (*c* 1 0) (Found· C, 47 0, H, 5 2, N, 14 7, S, 8 6 $C_{16}H_{20}N_4O_6S$ calc C, 46 9, H, 5 2, N, 14.6; S, 8.6%)

(*b*) The methanesulphonylepimine **10** (80 mg) was treated with sodium azide (80 mg) and ammonium chloride (5 mg) in *N,N*-dimethylformamide, as above The product (60 mg, 65%), m p 147–150°, was identical with the product from (*a*) (i r spectra and mixed m p)

(*c*) Methyl 3-amino-2-azido-4,6-*O*-benzylidene-2,3-dideoxy-α-D-altropyranoside[11] (**27**) (100 mg) was methanesulphonylated in the usual way to give compound **28** as needles (70 mg, 56%), m p 147–149°, identical with the product obtained in (*a*) and (*b*) above

The ring-opening of methyl 2,3-(acetylepimino)-4,6-O-benzylidene-2,3-dideoxy-α-D-allopyranoside (**8**) *with azide* — A solution of compound **8** (1 g) was heated under reflux with sodium azide (0 8 g) and ammonium chloride (0 4 g) in *N,N*-dimethylformamide (10 ml) for 5 min Addition of water to the hot solution caused the slow crystallisation of methyl 2-acetamido-3-azido-4,6-*O*-benzylidene-2,3-dideoxy-α-D-glucopyranoside (**21**) (0 35 g, 31%) Recrystallisation from ethanol gave needles, m p 255–256 5°, $[\alpha]_D$ +36° (*c* 1 0) (Found C, 54 85, H, 5 8, N, 16 25 $C_{16}H_{20}N_4O_5$ calc C, 55 2, H, 5 75, N, 16 1%) The identity of the azide was confirmed by comparison with a sample prepared by the action of sodium azide in *N,N*-dimethylformamide on methyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-3-*O*-mesyl-α-D-allopyranoside[23]

After removal of the azide, the mother liquors were extracted with chloroform (2 × 30 ml), and the dried ($MgSO_4$) extracts were evaporated to dryness to give a syrup which crystallised from ethanol to give the *allo*-epimine[4] (**7**) (0 1 g, 12%), identified by comparison with an authentic sample[4]

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REFERENCES

1 F H NEWTH, *Quart Rev* (London), 13 (1959) 30
2 J E CHRISTENSEN AND L GOODMAN, *J Amer Chem Soc* , 82 (1960) 4738

3 R D GUTHRIE AND D MURPHY, *J Chem Soc*, (1963) 5288
4 D H BUSS, L HOUGH, AND A C RICHARDSON, *J Chem Soc*, (1963) 5295
5 W MEYER ZU RECKENDORF, *Ber*, 97 (1964) 325
6 B R BAKER AND T NEILSON, *J Org Chem*, 29 (1964) 1047, 1051, 1057, 1063
7 B R BAKER AND T L HULLAR, *J Org Chem*, 30 (1965) 4038, 4045, 4049
8 B R BAKER AND T. L HULLAR, *J Org Chem*, 30 (1965) 4053
9 C F GIBBS, L HOUGH, AND A C RICHARDSON, *Carbohyd Res*, 1 (1965) 290
10 A D BARFORD AND A C RICHARDSON, *Carbohyd Res*, 4 (1967) 408
11 R D GUTHRIE AND D MURPHY, *J Chem Soc*, (1965) 3828
12 W MEYER ZU RECKENDORF, *Ber*, 97 (1964) 325
13 D H BUSS, L HOUGH, AND A C RICHARDSON, *J Chem Soc*, (1965) 2736
14 G E MCCASLAND, R K CARTER, AND H E CLARK, *J Amer Chem Soc*, 71 (1949) 637, S WINSTEIN, L GOODMAN, AND R BOSCHAN, *J Amer Chem Soc*, 72 (1950) 2311
15 G B BARLIN AND D D PERRIN, *Quart. Rev* (London), 20 (1966) 75, G E K BRANCH AND J O CLAYLON, *J Amer Chem Soc*, 50 (1928) 1680
16 R D GUTHRIE AND G P B MUTTER, *J Chem Soc*, (1964) 1614
17 A C RICHARDSON, *J Chem Soc*, (1964) 5364, *Carbohyd Res*, 4 (1967) 422
18 P E FANTA, in A WEISSBERGER (Ed), *Heterocyclic Compounds with Three and Four Membered Rings*, Part 1, Interscience, 1964, p 527
19 R KUHN, F ZILLIKEN, AND A GAUHE, *Ber*, 86 (1953) 466
20 R W. JEANLOZ, *J Amer Chem Soc*, 79 (1957) 2591
21 B COXON, *Tetrahedron*, 21 (1965) 3481
22 L D HALL, *Advan Carbohyd Chem*, 19 (1964) 51
23 Y ALI AND A C RICHARDSON, *Chem Commun*, (1967) 554
24 M SIMONETTA AND S WINSTEIN, *J Amer Chem Soc*, 76 (1954) 18

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GLUCURONOXYLOFUCAN, A CELL-WALL COMPONENT OF *Ascophyllum nodosum*[1] PART I

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ABSTRACT

A sulphated glucuronoxylofucan containing L-fucose (49%), D-xylose (10%), and D-glucuronic acid (11%) has been extracted from the cell-walls of *Ascophyllum nodosum*, after removal from the weed of laminaran, fucoidan, and the major part of the alginic acid Partial hydrolysis of the extract led to the characterisation of 3-*O*-(β-D-glucopyranosyluronic acid)-L-fucose as a major structural feature of the molecule, and to the separation of small quantities of 3-*O*-β-D-xylopyranosyl-L-fucose and 4-*O*-α-L-fucopyranosyl-D-xylose From the results of alkali treatment and mild methanolysis studies, deductions are made concerning the site of the sulphate groups Characterisation of the fragments found in the hydrolysates, after periodate oxidation, reduction, and hydrolysis of the initial polysaccharide, the degraded polysaccharide recovered after partial hydrolysis, the alkali-treated polysaccharide, and the degraded material recovered after methanolysis, indicates that at least some of the glucuronic acid residues are (1→4)-linked, that some of the fucose residues are vulnerable to periodate, and that the molecule is branched with end-group and (1→4)-linked xylose residues situated near the periphery of the molecule

INTRODUCTION

Fucoidan, the sulphated, fucose-containing polysaccharide of the brown seaweeds, has been extracted, with hot water and dilute acid, from *Fucus vesiculosus*, *F spiralis*, *Laminaria cloustoni*, *Himanthalia lorea*[2], and *Macrocystis pyrifera*[3], and subjected to a variety of purification techniques Even the most highly purified sample from *H lorea* still contained, in addition to ester sulphate and fucose, about 5% of galactose, 2% of xylose, and 3% of uronic acid Likewise, unpurified and purified *Macrocystis* fucoidan contained a proportion of galactose which corresponded to about 6 2% of the L-fucose content, whereas the proportion of xylose in these *Macrocystis* samples decreased from 2 5 to 0 5% on purification From results on the latter species, the author concluded that at least two heteropolymers were present (one contains L-fucose and D-galactose in the approximate ratio of 18 1, and the other contains a small proportion of D-xylose), and that the presence of a pure fucan as a major constituent of *Macrocystis* is unlikely Methylation and periodate-oxidation

studies[4] on the fucoidan from *F. vesiculosus* indicated a branched structure, with (1→2)-links predominating, and sulphate linked to C-4 and/or C-3, and this structure has been confirmed by acetolysis[5] and partial hydrolysis studies[6] Osmometric determinations of the molecular weight gave values of 133,000 ±20,000[5] (and sedimentation and diffusion measurements indicated a value of 7.8×10^4) In salt solution, the molecules are considered to exist as random coils [2]

Recently, another fucose-containing polysaccharide, ascophyllan, has been separated from the alginic acid extracted from *Ascophyllum nodosum* with dilute alkali[7] This material (6% of the dried weed) contained 25.2% of fucose, 26% of xylose, 19.2% of sodium hexuronate, about 12% of ester sulphate (as $NaSO_3$), and 12% of protein Partial hydrolysis studies with 0.5N oxalic acid at 100° indicated a glucuronic acid backbone with long side-chains of sulphated fucose and xylose 3-*O*-β-D-Xylopyranosyl-L-fucose has been identified as one of the hydrolytic fragments[8]

Examination of the weed residues (30% of the dried weed) of *Ascophyllum nodosum*, after removal of laminaran, fucoidan, and alginic acid with hot water and alkali, revealed the presence of additional sulphated polysaccharides containing fucose, xylose, and glucuronic, mannuronic, and guluronic acids, together with traces of glucose and galactose The present paper describes the separation and characterisation of a glucuronoxylofucan from these residues

DISCUSSION

Extractions of the residual weed with hydrochloric acid at pH 2, with 2N sulphuric acid, with M hydroxylamine at room temperature, and with ammonium oxalate–oxalic acid at 80° were investigated The last method was found to give the best yield (10% of the initial dried weed) of purest polysaccharide Preliminary treatment with formaldehyde polymerised phenolic materials and prevented their simultaneous extraction with the polysaccharides Without this treatment, a dark-brown instead of a cream polysaccharide resulted The oxalate extract was found to comprise the major sugars detected in these weed residues and to contain about 18% of ester sulphate The presence of mannuronic and guluronic acids suggested contamination with alginic acid, and it was possible to remove this by precipitation as the calcium salt from an aqueous solution (approximately 0.1% concentration with respect to the estimated content of alginic acid) of the extracted material The fucose-containing polysaccharide (7.6% of the initial weed) was recovered from the aqueous supernatant as a cream powder on freeze-drying It had $[\alpha]_D$ −225° (*c* 1.0, water) and contained approximately 49% of L-fucose, 10% of D-xylose, 12% of sodium uronate, 21% of sodium hydrogen sulphite, and 4% of protein The proportions of sugars were obtained from standard graphs of the peak heights of gas–liquid chromatograms of the TMS derivatives of the derived alcohols and reduced glucuronic acid, since this method gave more-repeatable results than those determined by the

method of Wilson[17]. The latter is more dependent on the exact conditions used, particularly the temperature for developing the paper chromatograms Furthermore, the g l.c results are in much better agreement with the proportion of glucuronic acid found by decarboxylation After separation from an acid hydrolysate, the L-fucose and D-xylose were characterised as crystalline materials and derivatives, and the uronic acid was identified as crystalline D-glucurono-6,3-lactone and by reduction to D-glucose, which was characterised with glucose-oxidase[9]

Attempted fractionation of this polysaccharide by precipitation with ethanol or copper acetate or on columns of DEAE-cellulose, Sephadex 200G, and Biogel was unsuccessful Furthermore, a considerable proportion of the polysaccharide appeared to be irreversibly bound on these columns It is therefore considered to be a heteropolysaccharide, and this was confirmed by partial hydrolysis studies

Hydrolysis with N oxalic acid led to the isolation from the hydrolysate of an aldobiouronic acid which was characterised as 3-*O*-(β-D-glucopyranosyluronic acid)-L-fucose Autohydrolysis of the free-acid polysaccharide in a dialysis sac gave a different pattern of oligosaccharides and led to the recovery from the dialysate of a number of neutral and sulphated fragments. A 3-*O*-β-D-xylopyranosyl-L-fucose and a 4-*O*-α-L-fucopyranosyl-D-xylose were separated from the neutral fraction and tentatively characterised Complete separation and characterisation of the acidic fragments proved impossible Nevertheless, repeated separation on Whatman No. 1 paper, followed by ionophoresis at pH 6, led to the isolation of what appeared to be four single entities (see Table I) They contained different proportions of ester sulphate, fucose, xylose, and glucuronic acid, had negative rotations, migrated to a greater or lesser degree on ionophoresis at pH 6, and the presence of the above aldobiouronic acid, 3-*O*-(β-D-glucopyranosyluronic acid)-L-fucose, could be detected chromatographically on partial hydrolysis of three of them

TABLE I

SULPHATED OLIGOSACCHARIDES SEPARATED FROM THE AUTOHYDROLYSATE

Fraction		$[\alpha]_D$ (*degrees*)	R_{Fuc} *Solvent*		*Sulphate* (%)	*Constituents*[b]
No	*Wt (mg)*		*1*	*2*		
6	87	−61	0 47	0 46	22 6	Fucose, glucuronic acid, xylose (3 1 1)
7	15	−90	0 32	0 26	—	Fucose, glucuronic acid, xylose (4 1 1)
8	54	−75	[a]	[a]	13 8	Fucose, glucuronic acid, xylose
9	92	−93	[a]	[a]	6 0	Fucose, glucuronic acid, xylose

[a]Very low mobility, separated by ionophoresis (pH 6) [b]Ratios determined from reduced and trimethylsilylated derivatives

In view of the fact that hydrolysis of ascophyllan with 0 5N oxalic acid for 1 h at 100° cleaved most of the fucose and xylose and left a glucuronic acid backbone[7], similar hydrolysis of the present glucuronoxylofucan was performed The degraded polymer (20% of the starting material) recovered from the hydrolysate was compared with the degraded polysaccharide (15% of the starting material) recovered after hydrolysis for 5 h with N oxalic acid, and analogous material (18% of the starting material) recovered from an autohydrolysis of the free-acid polysaccharide Chromatographic examination of hydrolysates of each of these degraded materials revealed fucose and glucuronic acid as the major constituents, with lesser amounts of xylose There appeared to be a higher proportion of fucose in the material recovered after the oxalic acid hydrolyses In addition to these degraded polysaccharides, insoluble material (*ca* 18, 3, and less than 2%, respectively) was recovered from each of the above hydrolysis experiments All of them had low carbohydrate[10] and high nitrogen contents, and hydrolysates showed the presence of fucose as the major constituent, with lesser amounts of glucuronic acid, xylose, and some oligouronic acids Amino acids were also detected.

After treatment of the glucuronoxylofucan with alkali[11], the residual polysaccharide, recovered in 70% yield, showed no diminution in sulphate content, although the reaction solution contained free sulphate ions The uronic acid content had increased from 12 to 15%, and chromatographic analysis of a hydrolysate of the recovered material showed only the sugars present in the initial material From this it may be concluded that the sulphate groups are not linked to units in which there are adjacent *trans*-hydroxyl-groups It follows that any (1→4)-linked or end-group xylose, glucuronic acid, and fucose must be devoid of ester sulphate (the end-group fucose could be sulphated on C-4), unless such (1→4)-linked units occurred at branch points or are disulphated

Although methanolic hydrogen chloride[12] cleaved a proportion of the sulphate groups and caused a certain amount of degradation, apparently no preferential cleavage of any one sugar occurred

Essentially all of the xylose units in the polysaccharide were vulnerable to periodate oxidation, and this confirmed their presence as (1→4)-linked or end-group, non-sulphated residues The small proportion of glycerol detected in the hydrolysate of the polyalcohol recovered after periodate oxidation and reduction of the glucuronoxylofucan indicates that, in the main, the xylose occurs as end group, although the tentative identification of a fucosyl-(1→4)-xylose in the partial acid hydrolysate confirms a proportion of (1→4)-linked units Further confirmation of these results was obtained by examination of the hydrolysates and the reduced hydrolysates of the corresponding polyalcohols derived from the initial polysaccharide (*P*), the alkali-treated polysaccharide (*A*), the methanolysed material (*M*), and the degraded polysaccharide (*D*) recovered after autohydrolysis (see Table II) The respective hydrolysates were reduced in order to detect the derived L-gulonic acid by g l c , since D-glucuronic acid was retained on the columns under the conditions of the experiments Erythronic acid could be detected by paper chromatography,

TABLE II

CONSTITUENTS OF THE HYDROLYSATES AND REDUCED HYDROLYSATES FROM THE RESPECTIVE POLYALCOHOLS

Polyalcohol	*Analysis*[a]	*Fucose*[b]	*Fucitol*[c]	D *Glucuronic acid*[b]	L-*Guloniс acid*[c]	*Erythronic acid*[d]	*Glyceric acid*[d]	*Propane-1,2-diol*[d]	*Glycerol*[d]
P	a	××××[e]	××××	××	××	××	×	—	×
	b	××××××	××××××	—	××	××	×?	×	×××
M	a	×××	××	××	××	?	?	—	×
	b	××××	××××	—	×××	××	?	×	××
A	a	×××××	×××××	××	××	×××	××	—	×
	b	×××××	×××××	—	×××	×××	×	×	××
D	a	×××	×××	××	××	×	××	—	×
	b	×××	×××	—	××	××	×××	×	××

[a] a = paper chromatography, b = gas–liquid chromatography [b] Hydrolysate [c] Reduced hydrolysate [d] Unaffected by reduction [e] ×××××= major component, ×× = minor component, × = trace.

and as its TMS derivative on g l c , in all four hydrolysates, indicating the presence of (1→4)-linked glucuronic acid units in the polysaccharide Small quantities of glyceric acid were also detected, and the proportion of this appeared to be greater in the hydrolysate of the polyalcohol from the autohydrolysed material This could only be derived from end-group or (1→2)-linked glucuronic acid, and it seems probable that autohydrolysis results in the removal of fucose and/or xylose residues, and the exposure of glucuronic acid at the ends of the chains The presence of propane-1,2-diol confirmed that some of the fucose is oxidised by the periodate

In conclusion, all of the results point to a highly branched molecule in which the xylose residues are at the periphery of the molecule No evidence has so far been obtained that the backbone consists of residues of a single sugar, but 3-*O*-(*β*-D-glucopyranosyluronic acid)-L-fucose is a major structural feature The majority of the ester sulphate is apparently linked to the fucose residues

EXPERIMENTAL

All solutions were evaporated under diminished pressure below 60° Specific rotations were measured in'water at 18° Paper chromatography was carried out on Whatman No 1 paper with the following solvent systems (v/v) (1) butyl alcohol–ethanol–water (40 11 19); (2) ethyl acetate–acetic acid–formic acid–water (18 3 1 4), (3) pyridine–ethyl acetate–acetic acid–water (5 5 1 2), as descending eluant equilibrated with pyridine–ethyl acetate–water (11 40 60), for detection of uronic acid Papers were sprayed with a saturated aqueous solution of aniline oxalate (1) or dipped in silver nitrate[13] (2), and acids were detected with Bromocresol Green (3) Ionophoresis of sulphated sugars was carried out on Whatman No 1 or 3 MM paper in pyridine–acetic acid buffer (0 05N, pH 6) for 40 min at 2000 volts R_{GalA}, R_{Fuc}, and R_{Xyl} are the rates of travel relative to galacturonic acid, fucose, and xylose, respectively Gas–liquid chromatography[14] was performed on columns of acid-washed Celite coated with 7.5% by weight of poly(butane-1,4-diol succinate) (column 1) at an operating temperature of 175°, and 10% of polyphenyl ether [*m*-bis(*m*-phenoxyphenoxy)benzene] (column 2) at an operating temperature of 175°, for methylated sugars Retention times are expressed relative to that of methyl 2,3,4,6-tetra-*O*-methyl-*β*-D-glucopyranoside For the trimethylsilylated (TMS) sugars and sugar alcohols, the liquid phase was column 2 at 155°, 3% of S E 30 (column 3) at 155°, and 15% of Apiezon K (column 4) at 155° and 175°, and the retention times are expressed relative to that of the TMS derivative of xylitol All reductions were carried out with potassium borohydride in aqueous solution The carbohydrate content of the polysaccharides was determined by the phenol–sulphuric acid method[10] and calculated from a graph constructed from a solution containing fucose glucuronic acid xylose in the ratio of 5 1 1 by weight Unless otherwise stated, hydrolyses were carried out with 90% formic acid in sealed tubes in an atmosphere of carbon dioxide for 6 h at 100°, followed by hydrolysis of formic esters by dilution (5 vol) and further heating for 2 h at 100°.

Extraction of the glucuronoxylofucan — Powdered, dried-weed residues (33% of the dried weed) of *Ascophyllum nodosum* (30 g), recovered after extraction of the dried weed with hot water and dilute alkali, were allowed to stand overnight with formaldehyde and then extracted with ammonium oxalate–oxalic acid solution (1 5 l, 0 25% w/v with respect to each, pH 2 8) for 6 h at 80° After removal of the residual solid (16 5 g), the solution was dialysed against distilled water until free from oxalate ions The solution from the dialysis sac was then concentrated and freeze-dried to a cream solid (10 g) Paper chromatography of a hydrolysate revealed the presence of fucose, xylose, glucuronic, mannuronic, and guluronic acids (and the respective lactones of these acids) (solvents 1, 2, and 3). To an aqueous solution of the extract (10 g in 1700 ml containing *ca* 1.7 g of alginic acid, determined from the proportions of mannuronic and glucuronic acids in the extract), a solution of calcium chloride (80 ml, 2% w/v) was added slowly with stirring, and the mixture was allowed to stand for 2 h The precipitated, gelatinous calcium alginate (1 75 g after freeze-drying) was removed by centrifugation, washed once with water, and freeze-dried as a slurry with water (Found SO_4^{2-}, nil) Chromatographic analysis of a hydrolysate showed only the presence of mannuronic and guluronic acids The combined supernatant and washings were dialysed against distilled water until free from Ca^{2+}, and then concentrated and freeze-dried to a cream solid (*P*) (7.64 g), $[\alpha]_D$ −225° (*c* 1 0) [Found carbohydrate content[10], 55, uronic acid, 10–11 (decarboxylation[15]), SO_4^{2-}, 20–22 8[16]; N_2, 0 69%] Chromatographic analysis of a hydrolysate revealed the presence of fucose, xylose, and glucuronic acid in the relative proportions[17] of 6 8 1 3 4 Reduction with borohydride to the respective alcohols, and estimation of the peak heights of the trimethylsilylated derivatives of fucitol, xylitol, and reduced glucuronic acid (gulonic acid) on g l c (column 4), against standard graphs of known weights of these compounds, gave a ratio of fucitol xylitol reduced glucuronic acid of 5·1 1

Partial hydrolysis and characterisation of the constituent sugars — (*i*) *Auto-hydrolysis of the free-acid polysaccharide* The polysaccharide (*P*) (5 g) was dissolved in water (50 ml), and stirred with excess of Amberlite IR-120 (H^+) resin, and the resulting solution was heated in a dialysis sac immersed in distilled water at 100° with stirring The dialysate was replaced by fresh, distilled water after 1, 3, and 5 5 h The three dialysates were combined and concentrated to about half volume, neutralised with ammonium hydroxide, and concentrated to a light-brown syrup The precipitated solid in the dialysis sac was washed with water, suspended in water, and freeze-dried (72 mg) [Found carbohydrate content, 15, N_2, 4 11%] The solution in the dialysis sac was neutralised with ammonia, concentrated, and freeze-dried to a cream solid (*D*) (940 mg), $[\alpha]_D$ −87° [Found carbohydrate content[10], 60, uronic anhydride (decarboxylation[15]), 22, SO_4^{2-}, 11[16], N_2, 0 85%] Hydrolysis of an aliquot gave[17] fucose xylose glucuronic acid = 3 5 1 2 5

The brown, syrupy dialysate, which contained glycerol from the dialysis sac, was fractionated on a cellulose column eluted with butyl alcohol two-thirds saturated with water After 8 litres had been collected, the eluant was changed to butyl alcohol

saturated with water, and the column was finally washed with water A number of mixed fractions were obtained, and either refractionated on paper or discarded

Fraction 1 (76 mg) was crystalline L-fucose, m p and mixed m p 139–140° after recrystallisation, $[\alpha]_D$ −88° The derived methylphenylhydrazone had m p 179–180°.

Fraction 2 (1 5 mg) was crystalline D-xylose, m p and mixed m p 147–149°, $[\alpha]_D$ +ve.

Fraction 3 (64 mg) was a neutral syrup, $[\alpha]_D$ −48°, which gave a pink spot (spray 1) with R_{Xyl} 0.71, R_{Fuc} 0 62 (solvent 2), R_{Xyl} 0 78, R_{Fuc} 0 63 (solvent 1) [*cf.* (1→3)-xylobiose, R_{Xyl} 0 81 (solvent 1) and 0 67 (solvent 2)] On hydrolysis, it gave fucose and xylose (paper chromatography), and had[18] DP of 2 The non-reduced aliquot corresponded to 46 μg of equivalent quantities of xylose (22 μg) and fucose (24 μg), and the reduced aliquot corresponded to 26 μg of xylose or 88 μg of fucose[19], indicating that xylose is the unreduced moiety and that the disaccharide is a xylosyl-fucose G l c of the derived glycosides, after methylation[20] and methanolysis, gave peaks having the retention times of 2,3,4-tri-*O*-methylxylose and 2,4-di-*O*-methyl-fucose [*T*, 1 68, 1 47 (column 1), and 1 0, 0 87 (column 2)], confirming the structure as xylosyl-(1→3)-fucose

Fraction 4 (11 3 mg) was a neutral syrup, $[\alpha]_D$ −60°, which gave a yellowish orange spot (spray 1) having R_{Fuc} 0 53, R_{Xyl} 0 63 (solvent 1), R_{Fuc} 0 65, R_{Xyl} 0 75 (solvent 2). An aliquot, on hydrolysis, showed equal quantities of fucose and xylose (paper chromatography) Measurement of the DP gave a value of 2, when calculated as a fucosylxylose[19] as for Fraction 3 G l c examination of the trimethylsilylated derivatives of the hydrolysate of the reduced material showed the presence of xylitol and absence of fucitol Peaks having the retention times of methyl 2,3,4-tri-*O*-methyl-fucosides and methyl 2,3-di-*O*-methylxylosides were obtained on g l c examination of the methanolysate after methylation[20]

Fraction 5 (10 mg) was an acidic syrup [R_{Fuc} 0 24 (solvent 1), R_{Fuc} 0 64 (solvent 3)], which partly crystallised, m p and mixed m p with D-glucurono-6,3-lactone 172° It was reduced with potassium borohydride, after conversion into the ester glycoside with methanolic hydrogen chloride Hydrolysis of this reduced material gave only glucose, which was identified by glucose oxidase[9]

The remaining four fractions were all separated as acidic syrups, and their properties are given in Table I

Partial hydrolysis of Fractions 7–9 gave spots having the mobilities of the free sugars, unchanged material, and the aldobiouronic acid, 3-*O*-(β-D-glucopyranosyl-uronic acid)-L-fucose, separated from an oxalic acid hydrolysate of the polysaccharide Fractions 8 and 9 appeared to be very similar, except for the higher sulphate and lower xylose content of the former

(*ii*) (*a*) *Hydrolysis with* N *oxalic acid* A 5% solution of the polysaccharide *P* (1 g) was heated for 2 h at 100° with N oxalic acid The degraded polysaccharide was precipitated from an aliquot (5 ml) with ethanol and returned to the oxalic acid solution, and the whole was reheated for a further 3 h An amorphous, brown pre-

cipitate (0.13 g) was then removed, and the 2-h and 5-h hydrolysates were neutralised separately with calcium carbonate, deionised with IR-120 (H^+) resin, and examined chromatographically Both gave spots having the mobility of fucose, xylose, and at least five slower oligosaccharide spots The proportion of the less-mobile oligosaccharides appeared to be less in the 5-h hydrolysate The combined hydrolysates were neutralised with ammonia and freeze-dried to give a cream solid (*H*) In a separate 5-h hydrolysis on a further portion of polysaccharide (1 g), the acidic supernatant was dialysed against distilled water until the dialysate was neutral The slightly acid solution from the dialysis sac was neutralised with calcium carbonate, filtered, concentrated, and freeze-dried to a cream solid (150 mg) Chromatographic analysis of a hydrolysate of this material revealed the presence of fucose and glucuronic acid (and its lactone) as major spots, with trace quantities of xylose G l c (columns 2 and 4) of the TMS derivatives of the reduced hydrolysate gave peaks having the retention times of fucitol and reduced glucurono-6,3-lactone in approximately equal quantities

Repeated separation on No 17 paper of the solid *H* derived from the 2-h and 5-h oxalic acid hydrolysate, and ionophoresis at pH 6 0, resulted in the isolation of a pure aldobiouronic acid (10 mg)

The aldobiouronic acid had $[\alpha]_D$ $-18°$ (*c* 0 16), R_{Fuc} 0 56 (solvent 1), R_{Fuc} 0 63, R_{GalA} 1 05 (solvent 2), M_{GalA} (pH 6 0) 1.1 [a yellow spot (spray 1) compared with the pink–orange spot of galacturonic acid] The acid and the derived glycitol (borohydride reduction) gave yellow colorations with the periodate–*p*-rosaniline spray[21], indicating that malonaldehyde derivatives were formed on periodate oxidation and pointing to the absence of a 4-*O*-substituted fucose derivative[22] A positive colour with triphenyltetrazolium hydroxide indicated the absence of a 2-*O*-substituent[23] After conversion into the free-acid form [IR-120 (H^+) resin], it was esterified with diazomethane and reduced with a 2% solution of potassium borohydride The recovered disaccharide in water (1 ml) was treated as follows an aliquot (0 25 ml) was hydrolysed, and chromatographic examination of the hydrolysate showed spots having the mobilities of glucose and fucose (solvents 1 and 2) The glucose was confirmed with glucose oxidase[9] Determination[18] of the DP gave 80 μg as an equivalent mixture of glucose and fucose, and the reduced material corresponded to 63 μg as fucose or 35 μg as glucose, indicating a glucosylfucose[19] The remainder of the disaccharide solution was concentrated to dryness and methylated[20], and the product was heated with 4% methanolic hydrogen chloride for 5 h The recovered glycosides gave peaks on the g l c having the retention times of methyl tetra-*O*-methylglucoside and methyl 2,4-di-*O*-methylfucoside [*T* 1 68, 1.47 (column 1), and 1 0, 0 87 (column 2)]

A trace quantity of another oligouronic acid, R_{Fuc} 0 36 (solvent 1) and R_{Fuc} 0.44 (solvent 2), was separated Complete hydrolysis, reduction, and conversion of the derived alcohols into their TMS derivatives gave peaks on g l c having retention times of fucitol and reduced glucurono-6,3-lactone [gulonic acid, *T* 2 5 (column 4), 1 9 (column 2)] compared with authentic D-glucurono-6,3-lactone reduced in a parallel experiment Reduction before hydrolysis gave a hydrolysate containing

fucose and glucuronic acid (paper chromatography) G l c of the derived TMS derivatives gave peaks having the retention times of fucose and fucitol (the glucuronic acid derivative is retained on the column under these conditions) Partial hydrolysis of this fraction gave unchanged material, glucuronosyl-(1→3)-fucose, and fucose (paper chromatography) This material is tentatively identified as a glucuronosylfucosylfucose

(*ii*) (*b*) *Hydrolysis with* 0 5N *oxalic acid* The polysaccharide *P* (1 2 g) was heated for 1 h at 100° in 0 5N oxalic acid The residual solid was removed by centrifugation, washed with water (2 × 5 ml), stored overnight in ethanol, filtered, and dried with ethanol and ether (217 mg) The combined acidic supernatant and first washings were dialysed against changes of distilled water for 24 h The slightly acid solution in the dialysis sac was neutralised with calcium carbonate, filtered, concentrated, and freeze-dried to a cream solid (240 mg) Chromatographic examination of hydrolysates of the residual solid and the non-dialysable material showed the presence of fucose as the major constituent, with equal quantities of xylose and glucuronic acid, and some partially hydrolysed fragments, which were present in greater amount in the non-dialysable hydrolysate Trace quantities of mannuronic acid were also present Amino acids were detected (ninhydrin) in the hydrolysate from the insoluble residue

Alkali treatment of glucuronoxylofucan — The polysaccharide *P* (1 6 g) was treated with sodium hydroxide according to the method of Rees[11] After dialysis against distilled water, degraded polysaccharide *A* (1 12 g) was recovered by freeze-drying [Found SO_4^{2-}, 19 2, uronic acid (decarboxylation), 15%] Chromatographic analysis of a hydrolysate revealed only the presence of fucose, xylose, and glucuronic acid, and this was confirmed by reduction of an aliquot of the hydrolysate with borohydride, and g l c of the TMS derivatives of the resulting alcohols

Methanolysis of the glucuronoxylofucan — The polysaccharide *P* (3 3 g) was shaken with 0 09M methanolic hydrogen chloride at room temperature After 2 h, 4 h, and 6 h, portions of the undissolved polysaccharide *M* (1 3 g, 0 45 g, 0 30 g, respectively) were removed, and, after 26 h, the residual, undissolved material (0 41 g) was recovered Each of the methanolysed materials was dissolved in water, and exhaustively dialysed against distilled water to remove absorbed sulphate The polysaccharides retained in the dialysis sacs were recovered by freeze-drying [Found SO_4^{2-} (after 2 h), 8 5, 4 h, 7 7, 6 h, 7 5, 26 h, 8 0% uronic anhydride (26 h), 22 05%] Chromatographic analysis of hydrolysates of each of the solids revealed fucose, glucuronic acid (major constituents), xylose, and faint spots having the mobility of glucurono- and mannurono-lactones

Periodate oxidation of the initial polysaccharide, methanolysed polysaccharide (26 h), alkali-treated polysaccharide, and the degraded polysaccharide from the autohydrolysis — The four materials (*P*, *M*, *A*, and *D*) (400 mg of each) were separately oxidised with 0 016M sodium periodate (250 ml) at room temperature in the dark Aliquots (1 ml) were withdrawn after 2, 4, 6 5, and 24 h, and the reduction of periodate was measured Oxidation was found to be complete after 6 5 h

Polysaccharide	P	M	A	D
Moles of periodate reduced per C_6 residue	0 33	0 83	0 40	1 0 (calc on the basis of 100% carbohydrate)

After 24 h, the excess of periodate in each solution was removed by the addition of ethylene glycol (1 ml), and the mixtures were set aside for 2 h The respective solutions were dialysed against several changes of distilled water and then each was concentrated to *ca.* 100 ml. Potassium borohydride (0.4 g, 1 g, 0.5 g, and 1 g, respectively) was added to each of the solutions which were kept overnight Excess borohydride was decomposed with acetic acid, and the solutions were treated with Amberlite IR-120 (H^+) resin, brought to neutrality with ammonium hydroxide, and concentrated Addition of ethanol (4 vol) precipitated the polyalcohols as white, amorphous solids The respective recoveries were from *P*, 355 mg, *M*, 272 mg, *A*, 235 mg, and *D*, 207 mg

Aliquots (15 mg) of these polyalcohols were hydrolysed Paper chromatography, and g l c of the derived TMS derivatives of the hydrolysates and reduced hydrolysates, revealed the products set out in Table II. Each of the substances was compared with authentic materials, which had the following retention times relative to that of xylitol Column 2 fucose, 1 37, 1 93, fucitol, 1 24, gulonic acid, 1 9, erythronic acid, 2 3, glyceric acid, 1.2, 1 3, glycerol, 0 31, propane-1,2-diol, 0 3, 0 63 Column 3 fucose, 0 78, 0 93, fucitol, 1 44, gulonic acid, 2 0; propane-1,2 diol, 1 0, 0 76 Column 4 fucose, 1 08, 1 47. fucitol, 1 47, gulonic acid, 2 5, erythronic acid, 0 75, glyceric acid, 0 54, propane-1,2-diol, 0 27, 0 53

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REFERENCES

1 A brief account of part of this work has already been published *Chem Ind* (London), (1967) 511
2 E G V Percival and A G Ross, *J Chem Soc*, (1950) 717, G Bernardi and G F Springer, *J Biol Chem*, 237 (1962) 75
3 R G Schweiger, *J Org Chem*, 27 (1962) 4267, 4270
4 J Conchie and E G V Percival, *J Chem Soc*, (1950) 827
5 A N O'Neill, *J Am Chem Soc*, 76 (1954) 5074
6 R H Côté, *J Chem Soc*, (1959) 2248
7 B Larsen, A Haug, and T L Painter, *Acta Chem Scand*, 20 (1966) 219
8 B Larsen, *Acta Chem Scand*, 21 (1967) 1395
9 M R Salton, *Nature*, 186 (1960) 966
10 M Dubois, K A Gilles, J K Hamilton, P A Rebers, and F Smith, *Anal Chem*, 28 (1956) 350
11 D A Rees, *J Chem Soc*, (1961) 5168
12 T G Kantor and M Schubert, *J Am Chem Soc*, 79 (1957) 152
13 D J Bell, in *Modern Methods of Plant Analysis*, K Paech and M V Tracey (Eds), Vol 2, Springer, Berlin, 1955, p 9
14 G O Aspinall, *J Chem Soc*, (1963) 1676
15 T P Nevell, *Methods Carbohyd Chem*, 3 (1963) 39
16 A S Jones and D S Letham, *Chem Ind* (London), (1954) 662

17 C M WILSON, *Anal Chem*, 31 (1959) 1199
18 T E TIMELL, *Svensk Papperstidn*, 63 (1960) 668
19 C W. FORD AND ELIZABETH PERCIVAL, *J Chem Soc*, (1965) 7042
20 O PERILA AND C T BISHOP, *Can J Chem*, 39 (1961) 815
21 F E HARDY AND J G BUCHANAN, *J Chem Soc*, (1963) 5881
22 G O ASPINALL, I W COTTRELL, SYLVIA V EGAN, I M MORRISON, AND J N C WHYTE, *J Chem Soc (C)*, (1967) 1071
23 R W BAILEY, S A BARKER, E J BOURNE, P M GRANT, AND M STACEY, *J Chem Soc*, (1958) 1895, D S FEINGOLD, G AVIGAD, AND S HESTRIN, *Biochem J*, 64 (1956) 351

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THE SYNTHESIS OF D-ARCANOSE*

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ABSTRACT

Oxidation of methyl 4,6-*O*-benzylidene-2-deoxy-α-D-*lyxo*-hexopyranoside (**3**) with ruthenium tetroxide gave the 3-ketone **4** in high yield Treatment of ketone **4** with methylmagnesium iodide gave predominantly methyl 4,6-*O*-benzylidene-2-deoxy-3-*C*-methyl-α-D-*xylo*-hexopyranoside (**5**), which was converted into the 3-*O*-methyl derivative (**6**) The reaction of compound **6** with *N*-bromosuccinimide afforded methyl 4-*O*-benzoyl-6-bromo-2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-D-*xylo*-hexopyranoside (**7**) in high yield Compound **7** was converted, by catalytic debenzoylation and reductive debromination, into methyl α-D-arcanoside (**9**). Acid-catalyzed hydrolysis of glycoside **9** gave D-arcanose (**10**) Configurational studies support the 2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-*xylo*-hexose structure for arcanose

INTRODUCTION

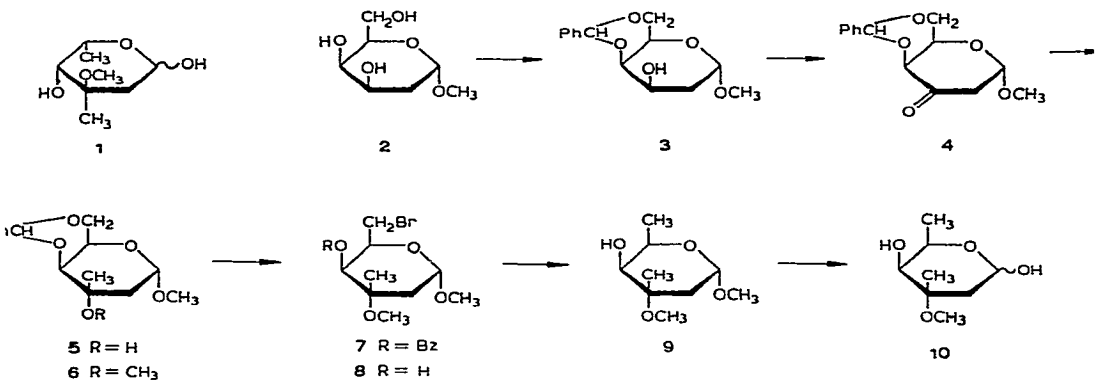
Lankamycin, a relatively new medium-spectrum antibiotic, active against gram-positive bacteria, is produced, together with another antibiotic, lankacidin, by *Streptomyces violaceoniger*[2], a micro-organism isolated from a sample of soil in Ceylon Acid hydrolysis of lankamycin, which belongs to the macrolide group[2], gives two sugar components, namely, lankavose (4,6-dideoxy-3-*O*-methyl-D-*xylo*-hexose), and the 4-*O*-acetyl derivative of a new branched-chain sugar, arcanose[3,4] By a combination of n m r and degradative studies, the 2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-L-*xylo*-hexose structure **1** was proposed for arcanose The structure was supported further by conversion[5] of L-arcanose into the diastereoisomer, L-cladinose, by a simple oxidation–reduction process at C-4

RESULTS AND DISCUSSION

In continuation of our syntheses of branched-chain sugars, we have now prepared D-arcanose by a route similar to that employed[6] in the synthesis of L-mycarose (2,6-dideoxy-3-*C*-methyl-L-*ribo*-hexose) and L-cladinose (2,6-dideoxy-3-*C*-methyl-3-

*For a preliminary communication, see Ref 1

O-methyl-L-*ribo*-hexose) Configurational investigations have led to the assignment of a D-*xylo* configuration to the hexose, in agreement with that assigned previously[5].



Methyl 2-deoxy-α-D-*lyxo*-hexopyranoside[7] (**2**) was converted into the 4,6-*O*-benzylidene derivative **3** by using copper sulfate as catalyst Oxidation of methyl 4,6-*O*-benzylidene-2-deoxy-α-D-*lyxo*-hexopyranoside (**3**) with a mixture of ruthenium dioxide and potassium metaperiodate in chloroform, in the presence of potassium carbonate[8], occurred readily to give methyl 4,6-*O*-benzylidene-2-deoxy-α-D-*threo*-hexopyranosid-3-ulose (**4**) in 89% yield The modified procedure for ruthenium tetroxide oxidation is a significant improvement over that published earlier[9], by which the hexopyranosid-3-ulose **4** was prepared from compound **3** in 47% yield Compound **4** in benzene was treated then with methylmagnesium iodide in ether at 0° The addition appeared to be highly stereoselective, giving predominantly one compound (**5**), which was separated by column chromatography from a trace of another product (presumably the alternative addition product) which migrated at a lower rate on a thin-layer chromatogram Grignard reagents are known to attack ketones preferentially from the least-hindered side of the molecule[10] A consideration of the conformation of compound **4** (Fig 1) suggests that attack by the Grignard reagent from the side resulting in the formation of the axial alcohol (route *a*) would be sterically more favorable than attack from the side resulting in the formation of the equatorial alcohol (route *b*) In route *b*, considerable steric interaction would be expected between the attacking species and the axial methoxyl group at C-1 Treatment of compound **5** with methyl sulfate and sodium hydroxide in tetrahydrofuran gave the 3-*O*-methyl derivative **6** in high yield.

Recently, Hanessian[11] reported a novel ring-opening of benzylidene acetals of sugars by *N*-bromosuccinimide to give, in the case of 4,6-*O*-benzylidene derivatives, the corresponding 6-bromo-4-benzoates Treatment of compound **6** with 1.1 equivalents of *N*-bromosuccinimide in boiling carbon tetrachloride, in the presence of an excess of barium carbonate, afforded an almost quantitative yield of methyl 4-*O*-benzoyl-6-bromo-2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-D-*xylo*-hexopyranoside (**7**) After 15 min, examination of the reaction mixture by thin-layer chromatography

showed that the starting material had all reacted Removal of the benzoyl group at C-4 by catalytic hydrolysis, followed by debromination with lithium aluminum hydride, gave crystalline methyl 2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-D-*xylo*-hexopyranoside (**9**) Acid-catalyzed hydrolysis [Rexyn-101 (H^+) ion-exchange resin] of the glycoside **9** afforded crystalline D-arcanose (**10**), whose physical constants were

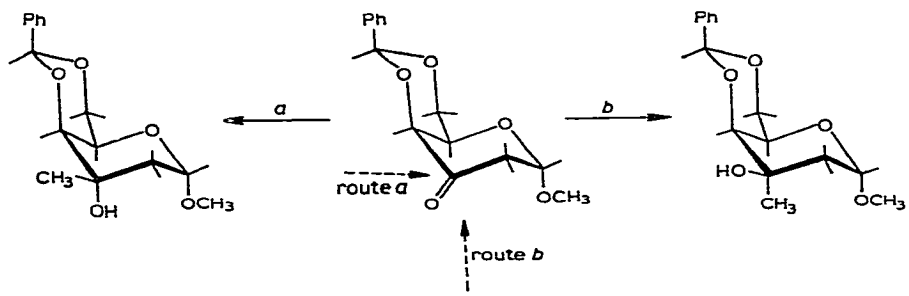


Fig 1 Addition of methylmagnesium iodide to methyl 4,6-*O*-benzylidene-2-deoxy-α-D-*threo*-hexopyranosid-3-ulose (**4**)

in good agreement with those reported for L-arcanose[3] The infrared and n m r spectra of **10** were identical with those of authentic L-arcanose, and the two compounds were indistinguishable by paper and thin-layer chromatography.

The n m r spectrum of glycoside **9** in chloroform-*d* showed two different *C*-methyl signals A singlet at τ 8 80 was assigned to the C-3 methyl group The C-5 methyl signal is observed as a doublet also centered at τ 8 80 (*J* 7 Hz) An equatorial C-5 methyl has been reported[12] previously to give a signal at τ 8 76–8 84 With acetyl chloride–dimethylaniline[13]*, compound **5** gave a syrupy acetate whose n m r spectrum showed an acetyl signal at τ 8 02 Tertiary acetoxy groups possessing the axial orientation in a number of cyclanols have been reported[14] recently to give n.m r signals in the region τ 7 93–8 04 With compound **5** in the *C1* (D) conformation, the n m r data support the previously assigned *xylo* configuration for arcanose[5] Further evidence for the axial orientation of the hydroxyl group at C-3 in compound **5**, and hence for the D-*xylo*-configuration for the compound, was obtained by application of the method of Overend *et al*[15] for the assignment of configuration to branched-chain sugars The infrared spectrum of compound **5** in carbon tetrachloride (c <0 005M) showed strong absorption at 3520 cm^{-1}, which was indicative of 1,3-diaxial, hydrogen-bonded interaction. Such interaction would arise if compound **5** had the *xylo* (but not the *lyxo*) configuration

EXPERIMENTAL

General methods — Solutions were concentrated below 50° under diminished pressure Melting points were determined on a Fisher–Johns melting-point apparatus and are uncorrected. Optical rotations were measured with a Bendix ETL-NPL

*We thank Professor F W Lichtenthaler for suggesting the acetylation procedure

automatic polarimeter, Type 143A, at 20 ±2° Infrared spectra were measured on a Beckman-IR5A spectrophotometer. N m r. spectra were determined at 60 MHz in chloroform-*d* with tetramethylsilane as internal standard Thin-layer chromatography (t l c) was performed, unless otherwise stated, with Silica Gel G as the adsorbent, and 2 3 ethyl acetate–petroleum ether (b p 60–80°) as the developing solvent The developed plates were air-dried, sprayed with 5% ethanolic sulfuric acid, and heated at about 150°. Paper chromatography was carried out by the descending method on Whatman No 1 filter paper in the following solvent systems butyl alcohol saturated with water (*A*), 6 4 3 butyl alcohol–pyridine–water (*B*); 3 1·1 butyl alcohol–ethanol–water (*C*) Sugars were detected[16] by spraying the chromatograms with a 1 1 mixture of vanillin (1% in ethanol) and perchloric acid (3% in water), and heating for 2 min

Methyl 4,6-O-benzylidene-2-deoxy-α-D-lyxo-hexopyranoside (**3**) — Methyl 2-deoxy-α-D-*lyxo*-hexopyranoside[7] (4 g) was stirred with freshly distilled benzaldehyde (15 ml) and anhydrous copper sulfate until t l c revealed no starting material (24 h) Addition of water and petroleum ether to the mixture gave a crystalline solid which was removed by filtration Recrystallization from methanol–water gave compound **3** as needles, yield 4 8 g (80%), m p 184–185°, $[\alpha]_D$ +104° (*c* 0 3, ethanol), lit[7], m p 178–179°, $[\alpha]_D^{20}$ +106° (*c* 0 62, chloroform)

Methyl 4,6-O-benzylidene-2-deoxy-α-D-threo-hexopyranosid-3-ulose (**4**) — Compound **3** (4 g) was stirred with a mixture of potassium metaperiodate (3 34 g, 2 equiv), ruthenium dioxide (0 10 g), and potassium carbonate in ethanol-free chloroform (20 ml) and water (20 ml) T l c did not show any starting material after 5 h Propyl alcohol (1 ml) was added, and the mixture was stirred for 15 min to destroy excess of ruthenium tetroxide The mixture was filtered through Celite, and the aqueous layer was extracted with chloroform (2 × 25 ml) The extracts were combined, dried ($MgSO_4$), and concentrated to give a crystalline product which was recrystallized from isopropyl alcohol, yield 3 5 g (89%), m p 134–135°, $[\alpha]_D$ +154° (*c* 0 7, chloroform), λ_{max}^{Nujol} 5 8 μm (C=O), lit[9], m p 132–133°, $[\alpha]_D$ +150° (chloroform)

Methyl 4,6-O-benzylidene-2-deoxy-3-C-methyl-α-D-xylo-hexopyranoside (**5**). — A solution of ketone **4** (3 g) in dry benzene (50 ml) was added dropwise to a stirred solution of methylmagnesium iodide [prepared from magnesium (4 3 g) and methyl iodide (14 6 ml)] in ether (200 ml) at 0° After being kept at room temperature overnight, the reaction mixture was poured into ice–water (500 ml), and the aqueous layer was extracted with chloroform Concentration of the dried extracts yielded a viscous syrup (3 1 g) T l c revealed the presence of a major component, R_F 0 43, and a trace of another component, R_F 0 14 Fractionation on silica gel (200 g), with 2 3 ethyl acetate–petroleum ether (b p 60–80°) as eluent, gave the major component **5** as a syrup, yield 2 8 g (91%), b p 135–140°/0 03 mm, $[\alpha]_D$ +159° (*c* 1 1, chloroform), λ_{max}^{film} 2 85 (OH), 14 4 μm (Ph), n m r data τ 2 32–2 8 (5-proton multiplet, Ph), τ 4 54 (1-proton singlet, benzylidene-methine H), τ 5 18 (1-proton multiplet, H-1), τ 6 75 (3-proton singlet, OMe), τ 7 8–8 2 (1-proton quartet, H-2 *eq*), τ 8 25–8.64 (1-proton quartet, H-2 *ax*), τ 8 8 (3-proton singlet, C-3 Me)

Anal. Calc. for $C_{15}H_{20}O_5$. C, 64 3, H, 7 2 Found C, 63 9, H, 7 6

Methyl 4,6-O-benzylidene-2-deoxy-3-C-methyl-3-O-methyl-α-D-xylo-hexopyranoside (**6**) — Methyl sulfate (25 ml) was added dropwise to a slurry of compound **5** (2 38 g) and finely powdered sodium hydroxide (5 g) in tetrahydrofuran (50 ml) After the mixture had been stirred for 24 h at room temperature, it contained the 3-*O*-methyl derivative **6** and a small amount of unchanged starting material Fractionation of the syrupy product on silica gel, with 1 4 ethyl acetate–petroleum ether (b p 60–80°) as eluent, gave compound **6** as a syrup, yield 1 96 g (82%), which crystallized on standing in a refrigerator, m p 38–39°, $[\alpha]_D$ +119° (*c* 0 8, chloroform), R_F 0 52 (t l c), λ_{max}^{KBr} 14 2 μm (Ph), no absorption attributable to OH, n m r data τ 2 32–2 8 (5-proton multiplet, Ph), τ 4 5 (1-proton singlet, benzylidene-methine H), τ 5 25 (1-proton multiplet, H-1), τ 6 72, 6 78 (3-proton singlets, C-1 OMe and C-3 OMe), τ 8 84 (3-proton singlet, C-3 Me)

Anal Calc for $C_{16}H_{22}O_5$ C, 65 3, H, 7 5 Found C, 65 4, H, 7 6

Methyl 4-O-benzoyl-6-bromo-2,6-dideoxy-3-C-methyl-3-O-methyl-α-D-xylo-hexopyranoside (**7**) — A solution of compound **6** (964 mg) and *N*-bromosuccinimide (643 mg) in dry carbon tetrachloride (34 ml) containing barium carbonate (3 34 g) was heated for 15 min at reflux temperature T l c showed that the starting material had all reacted The reaction mixture was filtered, and the filtrate was washed with sodium hydrogen carbonate solution and water, and concentrated to yield a syrup, which was chromatographed on silica gel, with 1 4 ethyl acetate–petroleum ether (b p 60–80°) as eluent Compound 7 was obtained as a syrup, yield 1 12 g (91%), $[\alpha]_D$ +134° (*c* 0 9, chloroform), R_F 0 75 (t l c), λ_{max}^{film} 5 8 (OBz), 6 21, 14 0 μm (Ph), n m r data τ 1 8–2 0, 2 3–2 55 (multiplets, 5 protons, Ph), τ 5 1 (1-proton multiplet, H-1), τ 6 53, 6 68 (3-proton singlets, C-1 OMe and C-3 OMe), τ 8 86 (3-proton singlet, C-3 Me)

Anal Calc for $C_{16}H_{21}BrO_5$ C, 51 5; H, 5 6, Br, 21 4 Found C, 51 5, H, 5 6, Br, 21 5

Methyl 6-bromo-2,6-dideoxy-3-C-methyl-3-O-methyl-α-D-xylo-hexopyranoside (**8**) — A solution of compound 7 (1 02 g) in dry methanol (15 ml) in which sodium (~ 0 1 g) had been dissolved was left for 4 h at room temperature The debenzoylated compound **8** was isolated in the usual manner [yield 0 61 g (86%)], and recrystallized from pentane to give needles, m p 62–63°, $[\alpha]_D$ +134° (*c* 0 7, chloroform), R_F 0 49 (t l c); λ_{max}^{KBr} 2 9 μm (OH), no absorption attributable to OBz, n m r data, τ 6 58, 6 75 (3-proton singlets, C-1 OMe and C-3 OMe), τ 8 8 (3-proton singlet, C-3 Me)

Anal Calc for $C_9H_{17}O_4Br$ C, 40 2, H, 6 3, Br, 29 8 Found C, 40 4, H, 6 5, Br, 30 0

Methyl 2,6-dideoxy-3-C-methyl-3-O-methyl-α-D-xylo-hexopyranoside (*methyl α-D-arcanoside*) (**9**) — Compound **8** (540 mg) and lithium aluminum hydride (500 mg) in dry ether were heated for 3 h at reflux temperature The reduced product was isolated in the usual manner, and recrystallized from pentane to give compound **9** as needles, yield 237 mg (62%), m p 93–94°, $[\alpha]_D$ +167° (*c* 0 7, chloroform), R_F 0 21 (t l c), λ_{max}^{KBr} 2 9 μm (OH), n m r data τ 5 35 (1-proton multiplet, H-1), τ 6 69,

6 79 (3-proton singlets, C-1 OMe and C-3 OMe), τ 8 8 (3-proton singlet, C-3 Me), τ 8 8 (3-proton doublet, J 6 5 Hz, C-5 Me)

Anal Calc for $C_9H_{18}O_4$ C, 56 8, H, 9 5 Found C, 56 6, H, 9 3

*2,6-Dideoxy-3-C-methyl-3-O-methyl-*D-xylo-*hexose* (D-*arcanose*) (**10**) — Methyl α-D-arcanoside (**9**) (177 mg) in water (2 5 ml) was stirred with Rexyn-101 resin (H^+) for 4 h The filtered solution was passed through a small column of Duolite A-4 resin (OH^-) to remove any trace of acid Concentration of the solution gave a colorless syrup which crystallized on trituration with ether Recrystallization from ethyl acetate–petroleum ether (b p 60–80°) gave D-arcanose (**10**) as prisms, yield 55 mg (31%), m p 101–102°, $[\alpha]_D$ +19 8° (*c* 1 0, ethanol), λ_{max}^{KBr} 2 93 μm (OH), n m r data τ 6 81 (3-proton singlet, C-3 OMe) τ 8 78 (3-proton singlet, C-3 Me), τ 8 78 (3-proton doublet, J 6 5 Hz, C-5 Me), lit [3] for L-arcanose, m p 96–98°, $[\alpha]_D$ −19 2° (*c* 4 6, ethanol) The infrared and n m r spectra of the synthetic product were identical with those of authentic L-arcanose The two compounds were indistinguishable by paper chromatography and had R_{Rha} 3 1 (solvent *A*), 1.42 (solvent *B*), and 1 98 (solvent *C*)

Anal Calc for $C_8H_{16}O_4$ C, 54 5, H, 9 1 Found C, 54 7, H, 9 1

*Methyl 3-O-acetyl-4,6-O-benzylidene-2-deoxy-3-C-methyl-*α-D-xylo-*hexopyranoside* — To a solution of compound **5** (190 mg) in *N,N*-dimethylaniline (0 41 ml) was added acetyl chloride (0 1 ml), and the mixture was left at room temperature for 1 h and then heated on a steam bath for 4 h The reaction mixture was poured into water and extracted with chloroform The extracts were washed with 6N sulfuric acid, sodium hydrogen carbonate solution, and water, and concentrated to yield a syrup (190 mg), which was chromatographed on silica gel with 2 3 ethyl acetate–petroleum ether (b p 60–80°) as eluent The 3-*O*-acetyl derivative was isolated as a syrup, R_F 0 75 (t l c), ν_{max}^{film} 5 75 (OAc), 13 3, 14 4 μm (Ph), n m r. data τ 2 4–2 8 (5-proton multiplet, Ph), τ 4 41 (1-proton singlet, benzylidene-methine H), τ 6 65 (3-proton singlet, C-1 OMe), τ 8 02 (3-proton singlet, OAc), τ 8 40 (3-proton singlet, C-3 Me)

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REFERENCES

1 G B Howarth, W A Szarek, and J K N Jones, *Chem Commun*, (1968) 62
2 E Gaumann, R Hutter, W Keller-Schierlein, L Neipp, V Prelog, and H Zahner, *Helv Chim Acta*, 43 (1960) 601
3 W Keller-Schierlein and G Roncari, *Helv Chim Acta*, 45 (1962) 138
4 W Keller-Schierlein and G Roncari, *Helv Chim Acta*, 47 (1964) 78
5 G Roncari and W Keller-Schierlein, *Helv Chim Acta*, 49 (1966) 705
6 G B Howarth and J K N Jones, *Can J Chem*, 45 (1967) 2253
7 A B Foster, W G Overend, and M Stacey, *J. Chem Soc*, (1951) 974
8 B T Lawton, unpublished results

9 P J. BEYNON, P. M COLLINS, P T DOGANGES, AND W G. OVEREND, *J Chem Soc (C)*, (1966) 1131
10 E L ELIEL, *Stereochemistry of Carbon Compounds*, McGraw-Hill, New York, 1962, p 69
11 S HANESSIAN, *Carbohyd Res*, 2 (1966) 86; *Advan Carbohyd Chem*, 21 (1966) 143
12 A C RICHARDSON AND K A MCLAUCHLAN, *J Chem Soc*, (1962) 2499
13 T D NEVITT AND G S HAMMOND, *J Am Chem Soc*, 76 (1954) 4124.
14 F W LICHTENTHALER AND P EMIG, *Tetrahedron Lett*, (1967) 577
15 R J FERRIER, W G OVEREND, G A RAFFERTY, H M WALL, AND N R WILLIAMS, *Proc Chem Soc*, (1963) 133, B FLAHERTY, W. G OVEREND, AND N R WILLIAMS, *J Chem Soc (C)*, (1966) 398
16 A P MACLENNAN, H M RANDLE, AND D W SMITH, *Anal Chem*, 31 (1959) 2020

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STUDIES ON CARBOHYDRATE-METABOLISING ENZYMES

PART XVII* THE ENZYMIC SYNTHESIS OF SOME DISACCHARIDES CONTAINING α-D-GLUCOSYL RESIDUES

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ABSTRACT

By the action of an enzyme preparation from *Tetrahymena pyriformis*, α-D-glucosyl residues may be transferred from phenyl α-D-glucopyranoside to various carbohydrates By this means, the following disaccharides have been prepared and characterised 6-*O*-α-D-glucopyranosyl-D-galactose and -D-mannose, 4-*O*-α-D-glucopyranosyl-L-xylose, 1-*O*-α-D-glucopyranosylribitol, 1-*O*-α-D-glucopyranosylerythritol, 1-*O*-α-D-glucopyranosyl-D-mannitol, and a mixture of 1-*O*- and 6-*O*-α-D-glucopyranosyl-D-glucitol These results show the preferential transfer of α-D-glucosyl residues to primary alcohol groups in carbohydrate acceptor substrates

INTRODUCTION

Previous studies[2,3] have shown that extracts of the ciliate *Tetrahymena pyriformis* show hydrolytic activity towards α-D-glucosides, and can also transfer an α-D-glucosyl residue from a suitable donor substrate (*e g*, maltose or phenyl α-D-glucopyranoside) to other carbohydrates For example, transfer to D-glucose and to D-xylose yields isomaltose[2] and 4-*O*-α-D-glucopyranosyl-D-xylose[3], respectively, whereas transfer to D-lyxose gives a mixture of 2-*O*-, 3-*O*-, and 4-*O*-α-D-glucopyranosyl-D-lyxose

The enzyme system shows a distinct specificity towards the acceptor substrates Preliminary studies[3] have shown that D-fructose, D-glucuronic acid, sucrose, and methyl α-D-glucopyranoside did not serve as acceptors, even though their chemical structures are not very different from that of D-glucose By contrast, other monosaccharides (*e g*, D-galactose and D-mannose) and several alditols (*e g*, erythritol and glycerol) were acceptors, and the enzymic transfer resulted in the formation of a new disaccharide. We now report on the characterisation of these products A preliminary account of this work has been given elsewhere[4]

*For Part XVI, see Ref 1

MATERIALS AND METHODS

Materials — Freeze-dried preparations of *Tetrahymena pyriformis* cells (Extracts *B* and *C*), kindly provided by Dr J F Ryley, were used as the source of enzyme Extract *C* was prepared in 1966 and showed[3] a similar but slightly stronger activity than extract *B*.

Phenyl α-D-glucopyranoside was synthesised as described previously[3] D-Mannose, D-galactose, D-glucitol, and L-xylose were commercial or laboratory samples which were purified, as required, by preparative, paper chromatography Ribitol was prepared by the borohydride reduction of D-ribose, and freed from borate by repeated evaporation of methanol at 40°

General methods — The conditions used for the partial, acid hydrolysis of disaccharides, before and after reduction with potassium borohydride, and for the periodate oxidation and micro-scale methylation of disaccharides were those used previously[3] Gas–liquid partition chromatography of methyl glycosides was carried out with a Pye 104 Chromatograph, according to the procedure of Aspinall[5]

Paper chromatography and electrophoresis — The following solvent systems were used for paper chromatograms (*A*) ethyl acetate–pyridine–water (10 4 3), (*B*) ethyl acetate–acetic acid–formic acid–water (18 3 1 4), (*C*) butyl alcohol–acetic acid–water (4 1 5 upper layer)

Electrophoresis of disaccharides was effected by using a small-scale Shandon apparatus, with 0 05M borate buffer (pH 10 0), results are expressed as M_G values (D-glucose = 1 00) For non-reducing disaccharides or for borohydride-reduced disaccharides, a molybdate buffer (pH 5 0) was used, results are expressed[3] as M_s (D-glucitol = 1 00)

The following spray reagents were used, aniline oxalate, silver nitrate, aniline–diphenylamine (a specific reagent[6] for oligosaccharides containing a 4-*O*-substituted aldohexose reducing end-group), periodate–*p*-rosaniline (a specific reagent[7] for carbohydrates, *e g*, 3-*O*-substituted hexoses and hexitols, which on periodate oxidation, give a malonaldehyde derivative)

Preparation and isolation of disaccharides — Digests containing phenyl α-D-glucopyranoside (5 mg), acceptor (5 mg), and extract *B* (1 mg) in water (0 5 ml) were incubated under toluene for 48 h at 37° Paper-chromatographic analysis (solvent *A*) showed the presence of the acceptor, glucose, isomaltose (R_G 0 46), and a new carbohydrate, which had R_G 0 57, 0 59, 0 65, 0 39, and 0 34 when D-mannose, ribitol, L-xylose, D-glucitol, and D-galactose, respectively, was the acceptor Acid hydrolysis of all of the new carbohydrates gave a mixture of equal amounts (visual estimation) of glucose and the acceptor, showing that the latter had all functioned as acceptors for trans-α-D-glucosylation

The rate of formation of the new carbohydrates was examined by using digests containing phenyl α-D-glucopyranoside (10 mg), acceptor (10 mg), and extract *B* (2 mg) in water (0 3 ml), which were incubated at 37° and examined at intervals by paper chromatography (solvent *A*, silver nitrate spray) The results showed that the

alditols, ribitol and D-glucitol, were the most efficient acceptors, since the glucosyl-alditols were produced in preference to isomaltose L-Xylose appeared to be equally as efficient an acceptor as D-glucose, and a considerably more efficient acceptor than either D-galactose or D-mannose

Except for the synthesis of α-D-glucosyl-L-xylose, large-scale digests [containing phenyl α-D-glucopyranoside (4 5 g), carbohydrate acceptor (5 0 g), extract *B* or *C* (1 0 g), and water (100 ml)] were incubated at 37° under toluene for a period of time (1 to 6 days) which would give the maximal amount of new disaccharide with minimal formation of isomaltose The disaccharide was then isolated from the enzyme digests (which had been heated for 20 min at 100° to inactivate the enzymes, cooled, centrifuged, and concentrated) by an appropriate combination of charcoal–Celite column chromatography or preparative, paper chromatography with Whatman 3 MM paper and solvent *A*

RESULTS

Properties of α-D-glucosyl-D-mannose — The chromatographic and electrophoretic properties of α-D-glucosyl-D-mannose (yield 85 mg) are given in Table I The disaccharide gave a brown colour with aniline–diphenylamine [absence of a (1→4)-linkage] and a blue colour with periodate–*p*-rosaniline [absence of a (1→3)-linkage] These conclusions are supported by the high M_s value of the borohydride-reduced disaccharide, which indicated the presence of at least four adjacent hydroxyl groups[8]

TABLE I

PROPERTIES OF α-D-GLUCOSYL DISACCHARIDES

Property	*Glucosyl-mannose*	*Glucosyl-galactose*	*Glucosyl-ribitol*	*Glucosyl-glucitol*	*Glucosyl-erythritol*	*Glucosyl mannitol*
$[\alpha]_D^{20}$, water (degrees)	+91	+124	+84	+76	+115	+101
R_G, solvent *A*	0 57	0 34	0 59	0 39	0 76	0 45
R_G, solvent *C*	0 42	0 31	0 53	0 38	0 75	—
M_s, molybdate	0 69[a]	0 69[a]	0 60	0 68	—	0 70
Colour with aniline oxalate	brown	brown	none	none	none	none
Colour with periodate–*p*-rosaniline	blue	blue	purple	purple	purple	purple
Periodate oxidation						
(*a*) formaldehyde released (mol)	—	—	0 96	0 99	—	0 94
(*b*) periodate reduced (mol)	—	—	2 8	4 0	—	—

[a]After borohydride reduction

The borohydride-reduced disaccharide gave a mixture of glucose and mannitol on acid hydrolysis The disaccharide was methylated[3], and after methanolysis, the methyl glycosides of 2,3,4,6-tetra-*O*-methyl-D-glucose and 2,3,4-tri-*O*-methyl-D-

mannose were detected by g l c These results indicate a (1→6)-linkage, and since the disaccharide had $[\alpha]_D$ +91° (*c* 1 02, water), an α-D configuration is inferred The disaccharide is therefore 6-*O*-α-D-glucopyranosyl-D-mannopyranose

Properties of α-D-*glucosyl*-D-*galactose* — This disaccharide (yield 21 mg) gave a blue coloration with periodate–*p*-rosaniline and a brown colour with aniline–diphenylamine, and the reduced disaccharide had a high electrophoretic mobility (see Table I, *cf* α-D-glucosyl-D-mannose) Methanolysis of the methylated disaccharide gave the methyl glycosides of 2,3,4,6-tetra-*O*-methyl-D-glucose and 2,3,4-tri-*O*-methyl-D-galactose Since the disaccharide had $[\alpha]_D$ +124° (*c* 0 98, water), these results characterise it as 6-*O*-α-D-glucopyranosyl-D-galactose

Properties of α-D-*glucosyl*-L-*xylose* — A digest containing phenyl α-D-glucopyranoside (0 9 g), L-xylose (1 03 g), and extract *C* (0 22 g) in water (20 ml) was incubated for 48 h Glucosylxylose (40 mg) was isolated from the digest, the chromatographic and other properties are given in Table II Both before and after borohydride reduction, the disaccharide gave a blue colour with the periodate–*p*-rosaniline reagent, suggesting the absence of a (1→2)- or a (1→3)-linkage After methylation and subsequent methanolysis, the methyl glycosides of 2,3,4,6-tetra-*O*-methyl-D-glucose and a carbohydrate having retention times similar to those of 2,3-di-*O*-methyl-D-xylose were detected by g l c , indicating a (1→4)-xylopyranoside or (1→5)-xylofuranoside structure On oxidation with 15mM sodium metaperiodate at 18°, the reduction of periodate was 3 1, 3 6, 3 8, and 3 9 (constant) mol after 1 5, 5 5, 19, and 23 h A (1→4)- or (1→5)-linked glucosylxylose would reduce 4 0 mol The presence of a (1→4)-linkage was finally indicated by the M_s value of the reduced disaccharide, which was similar to that for the product from 4-*O*-α-D-glucopyranosyl-D-xylose (see Table II), and lower than the value expected for a (1→5)-linked structure[3]

TABLE II

PROPERTIES OF α-D-GLUCOSYLXYLOSES

	α-D-*Glucosyl*-L-xylose	α-D-*Glucosyl*-D-xylose[3]
$[\alpha]_D$, water (degrees)	+62	+97
R_G value, solvent *A*	0 65	0 68
Colour with aniline oxalate	pink	pink
Colour with periodate–*p*-rosaniline reagent	blue	blue
M_G value, borate	0 31	0 28
M_s value, molybdate	0 35	0 30
Periodate reduction, mol	3 9	3 9

Properties of α-D-*glucosylribitol* — The α-D-glucosylribitol readily crystallised from ethanol (yield 160 mg) and was homogeneous by chromatography in solvents *A* and *C* It did not react with aniline oxalate, but gave an immediate purple colour with the periodate–*p*-rosaniline reagent, indicating the release of formaldehyde On oxidation at 2° with 2mM sodium metaperiodate, 0 83, 0 96, and 0 96 mol of formaldehyde were released after 5, 19, and 26 h On oxidation at 18° with the dilute

solution of metaperiodate, the periodate reduction was 2 7, 3 3, 3 5, and 3 9 mol after 2.5, 21 5, 28, and 48 h. When the amount of periodate reduced was plotted against time, extrapolation gave an initial periodate reduction of 2 8 mol Since a 3-*O*-substituted ribitol would have released 2 mol of formaldehyde, and a 2-*O*- or 4-*O*-substituted ribitol would have reduced 2 mol. of periodate under these experimental conditions (in which the linear ribitol residue would be preferentially oxidised, *cf* Ref 9), the results show the new disaccharide to be a 1-*O*-substituted ribitol This conclusion is supported by the high M_s value, which indicates the presence of at least four adjacent hydroxyl groups[8]

Properties of α-D-*glucosyl*-D-*glucitol* — This disaccharide was crystallised from ethanol (yield 178 mg), was chromatographically homogeneous, and had $[\alpha]_D$ +76° (*c* 0 37, water) The reaction with aniline oxalate and periodate–*p*-rosaniline reagents was the same as for glucosylribitol On oxidation in dilute solution at 2° with 2mM sodium metaperiodate, 0 92, 0 97, and 0 99 mol. of formaldehyde were released after 6, 24, and 27 h On oxidation at 18°, the periodate reduction was 4 1, 4 6, 4 8, 5 1, and 5 3 mol after 2 5, 21 5, 28, 45, and 52 h These results correspond to an initial reduction of 4 0 mol of periodate, and show the presence of a 1-*O*- or 6-*O*-substituted D-glucitol.

Glucosylglucitol (*ca* 5 mg) was oxidised at 2° by mM sodium metaperiodate (20 ml) for 24 h Excess periodate was then reduced by the addition of M-sodium sulphite (0 5 ml) After a further 30 min at 2°, the solution was deionised with mixed Amberlite IR-120 and IR-45 resins and then evaporated to dryness Complete acid hydrolysis of the residue produced a mixture of glucose, with smaller but approximately equal amounts of xylose and arabinose, as shown by paper chromatography Since the limited oxidation of 1-*O*-D-glucosyl-D-glucitol would give a derivative of L-xylose, and 6-*O*-D-glucosyl-D-glucitol would similarly yield a derivative of D-arabinose, these results show that a mixture of two isomeric disaccharides had been produced

Properties of α-D-*glucosylerythritol* — The purified disaccharide (yield 150 mg) was chromatographically homogeneous in three solvents, and on total hydrolysis with acid, both before and after borohydride reduction, gave a mixture of approximately equal amounts of glucose and erythritol The disaccharide, which had $[\alpha]_D$ +115° (*c* 2 72, water), was not hydrolysed by a β-glucosidase preparation from sweet almonds

Glucosylerythritol (20 mg) was oxidised with 1 4mM sodium metaperiodate solution (100 ml) for 8 h at 18° The solution was deionised with Amberlite IR-120 and IR-45 resins, before the addition of potassium borohydride (20 mg) After 12 h at 18°, the solution was deionised, and hydrolysed with acid The products were glucose and ethylene glycol, thus establishing that the α-D-glucosyl residue was attached to a primary alcohol group in erythritol Attachment to a secondary alcohol group would yield a mixture of glucose and glycerol

Properties of α-D-*glucosyl*-D-*mannitol* — From a digest of phenyl α-D-glucopyranoside and D-mannitol, a carbohydrate was isolated which, on acid hydrolysis, gave glucose and mannitol In solvent *A*, a single sugar (R_G 0 45) was present, but

in solvent *B*, a trace of isomaltose was indicated in addition to the main non-reducing sugar. The glucosylmannitol was therefore further purified by preparative, paper chromatography with solvent *B* The final product, yield 150 mg, crystallised from ethanol and had $[\alpha]_D$ +101° (*c* 0 93, water), it was not attacked by almond β-glucosidase, whereas an authentic sample of 1-*O*-β-D-glucosyl-D-mannitol (isolated from a partial, acid hydrolysate of laminarin[10] by Dr D H Hutson) was readily hydrolysed This latter disaccharide had the same M_s value in molybdate buffer as the enzymically synthesised carbohydrate, suggesting the presence of four adjacent hydroxyl groups, *i e*, the mannitol was substituted[8] at either C-1 or C-2

On oxidation with 2mM sodium metaperiodate at 2°, the release of formaldehyde was 0 94 mol (constant) after 12, 18, and 24 h In a similar experiment with 1-*O*-β-D-glucosyl-D-mannitol from laminarin, the yield was 0 96 mol α-D-Glucosyl-D-mannitol (20 mg) was oxidised with mM sodium metaperiodate (100 ml) for 24 h at 18°. The solution was deionised and concentrated By paper chromatography (solvent *A*, aniline oxalate spray), a minor, pink spot (R_G 0 88) and a major, yellow streak were formed The minor component (R_G 0 88) was isolated by preparative, paper chromatography On acid hydrolysis, glucose and arabinose were formed, on reduction, followed by electrophoresis in molybdate buffer, the M_s value of the alcohol was 0 45 This sugar is therefore probably 5-*O*-α-D-glucosyl-D-arabinofuranose, (*cf* Table I of Ref 3) The zone which gave a yellow colour with aniline oxalate was isolated and reduced The major component (*GM1*) had R_G 0 92 in solvent *A*, and gave glucose and glycerol on acid hydrolysis Further periodate oxidation of *GM1*, followed by reduction, gave a second sugar (*GM2*) having R_G 1 20 in solvent *A*, which, on acid hydrolysis, gave glucose and ethylene glycol These results indicate that the original disaccharide is 1-*O*-α-D-glucopyranosyl-D-mannitol, and that on

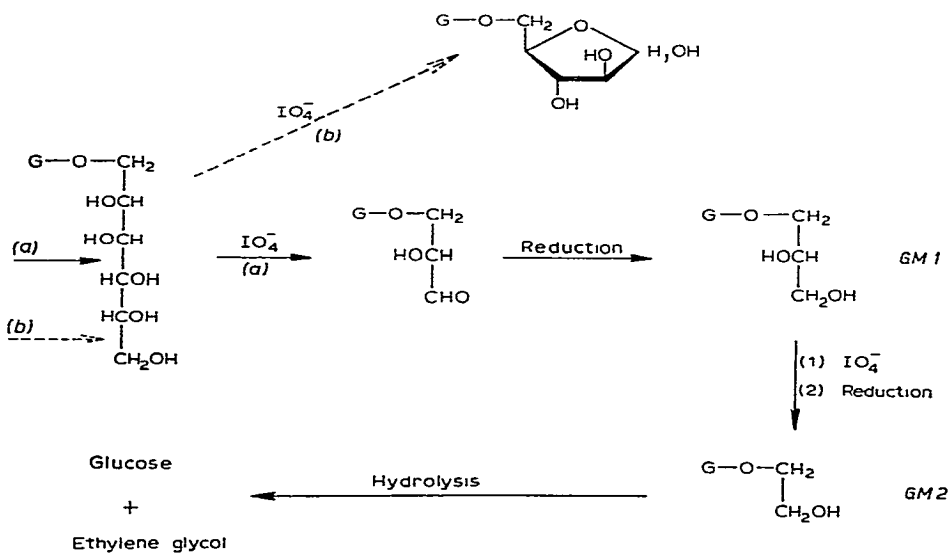

Fig 1

periodate oxidation, there is a preferential oxidation of the bond between C-3 and C-4 of mannitol, with a slower oxidation of the C-5–C-6 bond to give 5-*O*-α-D-glucosyl-D-arabinose (Fig 1) These observations are fully in agreement with the steric effects reported by Schwarz[11] on the periodate oxidation of hexitols

DISCUSSION

The present study shows clearly that the enzyme system in *Tetrahymena pyriformis* transfers α-D-glucosyl residues from phenyl α-D-glucopyranoside to various carbohydrates with retention of the anomeric configuration of the glucosyl residue This contrasts with the enzyme system in another protozoon (*Astasia ocellata*), which was examined in the previous paper[1], and found to transfer D-glucosyl residues from α-D-glucosyl phosphate to other carbohydrates with inversion of configuration

In contrast to the enzyme system in *A ocellata*, which can only use certain secondary alcohol groups as acceptor sites[1], the *T pyriformis* enzyme system shows a high preference for transfer to primary alcohol groups In the cases of D-glucose, D-mannose, and D-galactose, (1→6)-linked disaccharides are formed, and there was no evidence for the synthesis of significant amounts of disaccharides containing other types of linkage With alditols, there was also a clear requirement for primary alcohol groups However, since alditols contain two such groups, it was of interest to examine the products of enzyme action in some detail, to see if there was preferential utilisation of one of the two groups Those alditols which have a symmetrical structure, *e g*, D-mannitol and ribitol, gave, as might be expected, only one product However, D-glucitol gave a mixture of approximately equal parts of two isomeric disaccharides This mixture could not be separated by paper chromatography in two solvents or by electrophoresis The specific rotation reported in Table I therefore represents an average value and not a characteristic physical constant

When monosaccharides which do not contain a primary alcohol group (*e g*, aldopentopyranoses) are used as acceptors, transfer to the equatorial OH group at C-4 predominates[3] This situation has been observed with D-ribose, D-lyxose, and D-xylose With the availability of L-xylose, which exists in the *1C* conformation having an equatorial OH group at C-4, this specificity still holds, since the sole product was 4-*O*-α-D-glucopyranosyl-L-xylose

The enzyme system of *T pyriformis* provides a convenient method for the synthesis of a limited range of disaccharides Of the disaccharides described in Tables I and II, 6-*O*-α-D-glucopyranosyl-D-galactose ($[\alpha]_D$ +125°) has been synthesised chemically by Goldstein and Whelan[12], and isomaltitol (6-*O*-α-D-glucopyranosyl-D-glucitol), one of the two isomeric disaccharides isolated from the digest containing D-glucitol, has been prepared by the catalytic reduction of isomaltose, and had[13] $[\alpha]_D$ +89° The remainder appear to be new compounds whose synthesis is not described in two recent comprehensive publications[14,15]. The yields obtained in the present work are regarded as an order of magnitude only, since they represent the results of a compromise between the relative rate of synthesis of the new disaccharide

and of isomaltose, and of their ease of separation and isolation. In addition, there were certain unavoidable losses due to overlapping of fractions during chromatography. The new disaccharides provide a useful range of substrates for current studies on the specificity of α-glucosidases from various sources

ACKNOWLEDGMENTS

We are indebted to the Science Research Council for maintenance allowances (to J R S and I. R. P.) and a research grant, and to the Medical Research Council for an equipment grant for the g l c apparatus.

REFERENCES

1 D J MANNERS AND D C TAYLOR, *Arch Biochem Biophys*, 121 (1967) 443
2 A R ARCHIBALD AND D J MANNERS, *Biochem J*, 73 (1959) 292
3 D J MANNERS AND J R STARK, *Carbohyd Res*, 3 (1966) 1
4 J R STARK, Ph D Thesis, University of Edinburgh, 1964, I R PENNIE, Ph D Thesis, University of Edinburgh, 1967
5 G O ASPINALL, *J Chem Soc*, (1963) 1676
6 S SCHWIMMER AND A BEVENUE, *Science*, 123 (1956) 543
7 F E HARDY AND J G BUCHANAN, *J Chem Soc*, (1963) 5881
8 E J BOURNE, D H HUTSON, AND H WEIGFL, *J Chem Soc*, (1961) 35
9 M J CLANCY AND W J WHELAN, *Chem Ind* (London), (1959) 673
10 S PEAT, W J WHELAN, AND H G LAWLEY, *J Chem Soc*, (1958) 729
11 J C P SCHWARZ, *J Chem Soc*, (1957) 276
12 I J GOLDSTEIN AND W J WHELAN *J Chem Soc*, (1963) 4264
13 M L WOLFROM, A THOMPSON, A N O'NEILL, AND T T GALKOWSKI, *J Am Chem Soc*, 74 (1952) 1062
14 J STANĚK, M ČERNY, AND J PACÁK, *The Oligosaccharides*, Czechoslovak Academy of Sciences, Prague, 1965
15 R W BAILEY, *The Oligosaccharides*, Pergamon Press, Oxford, 1965

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THE REACTION OF THE PERIODATE-OXIDATION PRODUCTS OF 1,4-ANHYDROERYTHRITOL AND OF SOME RELATED PYRROLIDINE GLYCOLS WITH THIOSEMICARBAZIDES

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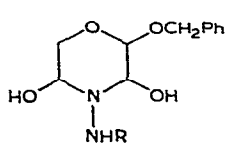
ABSTRACT

Simple analogues of the dialdehyde formed by periodate oxidation of benzyl β-L-arabinopyranoside have been prepared Oxydiacetaldehyde may be condensed with one or two moles of substituted thiosemicarbazides, whereas *N*-aryliminodiacetaldehydes give only bis(thiosemicarbazones) The oxidation of *N*-arylpyrrolidine-3,4-diols with one equivalent of periodate is straightforward if electron-attracting aromatic substituents are present, but, otherwise, some cleavage of N–C bonds in the pyrrolidine ring can be demonstrated The crystalline hemialdal, 2,6-dihydroxy-4-phenylmorpholine, was isolated The rates of conversion of some α-functionally-substituted thiosemicarbazones into glyoxal derivatives have been compared

INTRODUCTION AND DISCUSSION

In a recent study[1] of the structure of the thiosemicarbazide derivatives of periodate-oxidised polysaccharides, one of us used, as model compounds the oxidation products of some simple sugars The mode of action of certain hydrazides on these monosaccharide derivatives was determined, and one group of products, from benzyl β-L-arabinopyranoside[2], proved to have the cyclic structure **1** This led us to examine



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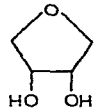

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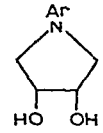

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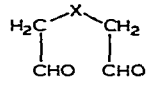
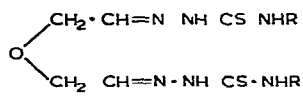
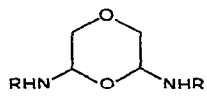


7 X = *p*-ClC$_6$H$_4$N
8 X = *p*-MeOC$_6$H$_4$N

4 X = O
5 X = S
6 X = PhN



9 R = Me
10 R = Pr
11 R = CH_2Ph



12 R = NH CS NHMe

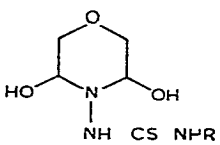
still simpler dialdehydes of comparable structure, derived, respectively, from 1,4-anhydroerythritol (**2**) and the pyrrolidine glycols (**3**), and we now report on the

reactions of these oxo-compounds with thiosemicarbazides Some preliminary work with the dialdehydes **5** and **6** was carried out earlier[3]

Oxydiacetaldehyde (**4**) was first prepared, as the bis-acetal, by means of a conventional ether synthesis[4] Periodate oxidation of the appropriate glycol **2** is more convenient Klosterman and Smith[5] compared the rate of consumption of this reagent by the *cis*- and *trans*-isomers, but isolated neither the dialdehyde nor any derivative The oxidation product has since been used[6] to prepare the oxygen analogue of Ψ-pelletierine

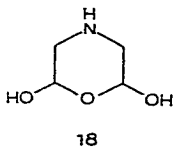
We have obtained bis(thiosemicarbazones) **10** and **11** in 80–95% yield by using the appropriate 4-substituted thiosemicarbazide The yield of 4-methylthiosemicarbazone (**9**) was somewhat lower due to its water-solubility In the presence of water, this compound exists as a monohydrate, which evidently has the cyclic structure (**12**), since the u v absorption spectrum has a maximum at 246–247 nm, and not at 270 nm which is characteristic of C=N in this context[1] The *p*-dioxane **12** can be dehydrated readily to the bis(thiosemicarbazone)

When one equivalent of thiosemicarbazide was used, the expected analogues (**13–17**) of the benzyl β-L-arabinopyranoside derivative **1** were obtained in yields



13 R=H
14 R=Me
15 R=CH_2CH_2OH

16 R=Pr
17 R=CH_2Ph



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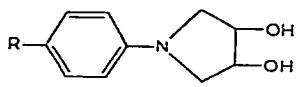


19 R=H
20 R=Cl
21 R=MeO

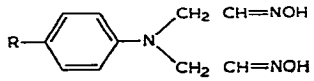
of 40–80% Like the other singly-bonded compounds **12** and **1**, these morpholines showed maximal absorption at 242 ±4 nm They could be converted readily into the bis(thiosemicarbazones), *e g* , compound **9**, by treatment with a second equivalent of the same thiosemicarbazide Treatment with a different thiosemicarbazide led to disproportionation, we were thus unable to obtain "mixed" compounds similar to those having high anti-tumour activity derived from pyruvaldehyde[7]

The benzyloxy dialdehyde obtained from benzyl β-L-arabinopyranoside gave only cyclic products (**1**) whether one or two equivalents of thiosemicarbazide were used[1] Fresh attempts to obtain a bis(thiosemicarbazone) from this dialdehyde, under the conditions used to prepare compounds **9-11**, gave intractable products Whether this difference in behaviour of the benzyloxy dialdehyde from the simple analogue **4** is due to steric or electronic effects is not yet clear In the case of the sulphur compound **5**, we have so far been able to prepare only bis-derivatives analogous to compound **9**, even with equimolecular amounts of dialdehyde and thiosemicarbazide[3]

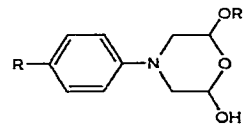
Iminodiacetaldehyde has been known as the hydrate (**18**) for many years[8] The bis-acetals of the *N*-methyl and *N*-benzyl compounds were prepared later[9], but no attempt to make *N*-aryl compounds was recorded until recently[3] Since anilinoacetal-

dehyde is much less stable than its alkylamino counterparts[10], some trouble was anticipated in attempts to oxidise the glycols **19–21**. However, when compound **19** was oxidised and treated with 2 equivalents of 4-methylthiosemicarbazide in the presence of acetic acid, the analysis figures for the product, although difficult to reproduce, were always approximately correct, and the yield of product was good, indicating that the reaction was, on the whole, proceeding as desired[3] The situation required clarification, and the first step was to study, in greater detail, the oxidation of pyrrolidine glycols

A *cis*-[12] and a *trans*-series[13] of these has been prepared, but the method of Roberts and Ross[14] is more convenient, and we used it to make the three *cis*-glycols **19–21** We found that milder conditions (10 min at 0–3°) than hitherto[3] used were quite adequate for the oxidation, although even then the reaction between compound **6** and 4-methylthiosemicarbazide led to inconsistent analytical results However, on changing the reagent from 4-methylthiosemicarbazide to hydroxylamine, we were able to isolate the dioximes **22–24** in 69, 93, and 46% yields, respectively, without



22 R = H
23 R = Cl
24 R = MeO



25 R = Cl , R' = Me
26 R = R = H

any difficulty From these results, it appears that the oxidation of the *p*-chloro compound proceeds as desired under these conditions, whereas, as the aromatic substituent becomes more electron-repelling in character, other centres in the pyrrolidine **3** are vulnerable to attack, giving rise to increasing proportions of by-products Since the pure dioximes are readily obtained, the contaminating hydroxylamine derivatives of those by-products are evidently more easily eliminated by fractional crystallisation than are the corresponding thiosemicarbazide derivatives

As well as at the glycol grouping, periodate could attack compound **3** in two ways Like other oxidants, it converts arylamines into highly-coloured products of the quinoneimine type[11a] In the special case where the amine is a 3-arylamino-1,2-glycol and excess periodate is present, cleavage readily proceeds beyond breaking of the glycol linkage, and the intermediate α-arylaminoaldehyde is further oxidised[11b] The presence of electron-attracting substituents retards the first type of oxidation, but not the second[11] It now appears that, when one equivalent of periodate is used, an electron-attracting substituent will inhibit the second type of oxidation, but that with other or no substituents this can still proceed to varying degrees We assume that, with this limited amount of periodate at the temperature employed, non-specific oxidation of the arylamine function does not take place to any significant extent, since no dark colours were observed Co-products arising from the second type of oxidation were identified in the case of compounds **19** and **21** and are discussed below.

The oxidation product from the *p*-chloro compound crystallised readily from methanol and proved to have a methyl glycoside type of structure (**25**) It could be

isolated in 63% yield When a methanolic solution of the unsubstituted compound **19** was treated with aqueous periodate in the usual way, a syrupy product always resulted However, the inverse mode of addition permitted the use of a smaller proportion of methanol in the reaction mixture, and the product was crystalline It proved to be compound **26**, but the yield, after recrystallisation, was poor Although, in general, we prefer to add the periodate solution to these glycols, the yield of dioxime **22** was independent of the mode of addition

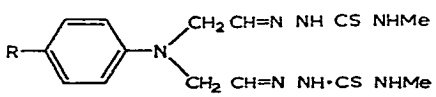
Having established that the periodate oxidation took the desired course, within limits, we returned to the study of thiosemicarbazone formation U v -monitoring provided a key to the problem of isolating pure compounds The most readily accessible dialdehyde, **7**, reacted with 4-methylthiosemicarbazide more satisfactorily in the absence of acetic acid, even though several days were required for separation of the product Successive fractions were examined spectroscopically and, since all had an absorption maximum at 270 nm, characteristic of thiosemicarbazones, they were combined and recrystallised to give compound **28** without any difficulty

TABLE I

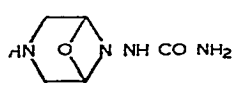
ULTRAVIOLET SPECTRAL DATA

Compound	λ_{max} (*nm*)	*log* ε_{max}
13	238[a]	4 13
14	241[b]	4 15
15	238[a]	4 13
16	243[b]	4 13
17	244[b]	4 14
12	246–247[b]	4 38
27	270[c]	4 64
28	270–271[c]	4 64
29	269–270[c]	4 59
31	244–245[c]	4 52
32	242[c]	4 54

[a]In water [b]In 95% ethanol [c]The compound (*ca* 30 mg) was dissolved in 2-methoxyethanol (20–30 ml) and diluted with 95% ethanol



27 R=H
28 R=Cl
29 R=MeO



30

The reaction with the oxidation products from the other two pyrrolidine glycols was more complex Under the same conditions as used above, a series of fractions was obtained from the unsubstituted compound **19**, these all melted in the same region, but differed spectroscopically. Only the first fraction, which had an absorption maximum at 270 nm, proved to be compound **27** Successive, further fractions had

increasing proportions of material with maximal absorption at 243–247 nm, as shown in Fig 1 This is discussed later

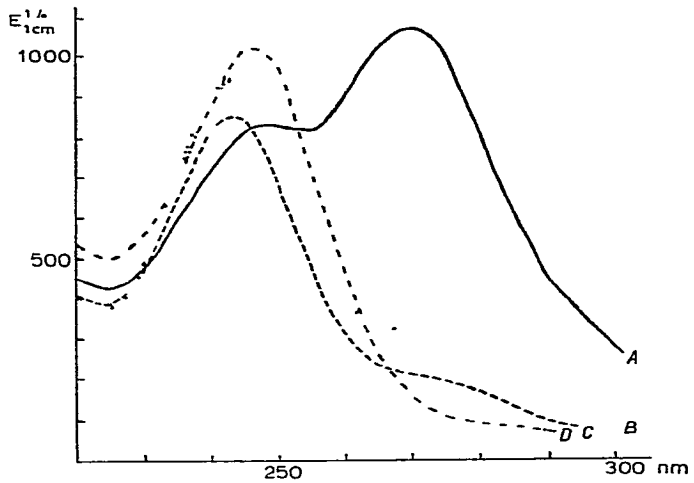


Fig 1 Ultraviolet spectra of the first four fractions (*A–D*) of product arising from the reaction of 4-methylthiosemicarbazide with periodate-oxidised **19** (————, *A*, , *B*, – – – – –, *C*, and – – – –·– , *D*)

Treatment of the *p*-anisyl derivative **21** with periodate, followed by 4-methylthiosemicarbazide, as before gave a product which showed no significant absorption at 270 nm, but had a peak at 242 nm However, when the reaction with the thiosemicarbazide was carried out in the presence of water, the small amount of thiosemicarbazone (**29**) formed, with appropriate absorption at 270 nm, separated together with the principal product The less-soluble thiosemicarbazone was isolated readily by fractional crystallisation

The yields of bis(4-methylthiosemicarbazones) obtained from the oxidation products of the three glycols paralleled the yields of the dioximes This provided further evidence that the *p*-chlorophenyl compound (**20**) was attacked by one equivalent of periodate at the glycol grouping, with a high degree of specificity. The hemiacetal (**25**) was next treated with one equivalent of 4-methylthiosemicarbazide under the conditions used to prepare the cyclic derivative **14** in the oxygen series The only isolated product, however, was the bis(4-methylthiosemicarbazone) (**28**) The dialdehyde (**7**) thus differs from the oxygen compound (**4**) and its benzyloxy derivative Wolff and Marburg[8] obtained a cyclic derivative formulated as structure **30** from the *N*-unsubstituted iminodialdehyde hydrate (**18**) and semicarbazide, although phenylhydrazine gave the normal bis-compound

Some confirmation of the course of periodate oxidation of the pyrrolidine glycols was provided by the secondary products which sometimes crystallised following treatment with thiosemicarbazides These had u v. maxima characteristic of thiosemicarbazide (C–N) as distinct from thiosemicarbazone (C=N). Fig 2 shows the spectra of the purified co-product from oxidised **19** and 4-propylthiosemicarbazide

and of the bis(thiosemicarbazone) **27**. Analysis figures obtained from several samples of two co-products were very consistent and fitted best a structure comprising one arylamine and two thiosemicarbazide residues, together with two carbon atoms Some indication of a similar loss of two carbons of the original pyrrolidine ring had also been obtained earlier[3] Molecular-weight determination precluded a dimeric formula, and of the possible structures accommodating these features, **31** and **32** seemed the most reasonable A similar ortho-derivative (**33**) has been described previously[15]

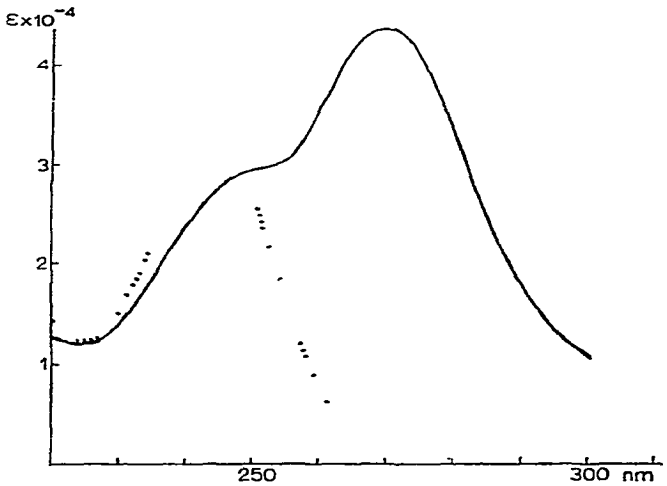
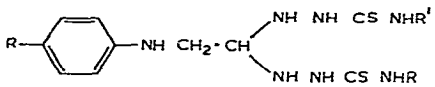
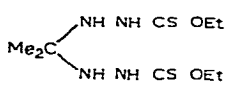


Fig 2 Ultraviolet spectra of **27** (————) and **31** ( )



31 R=H, R'=Pr
32 R=MeO, R'=Me



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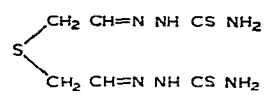

34

Glyoxal bis(2,4-dinitrophenylhydrazone) was formed readily on treatment of compounds **31** and **32** with 2,4-dinitrophenylhydrazine sulphate, in yields of the same order as afforded by the α-arylaminoaldehyde derivative **27** The appropriate glyoxal bis(thiosemicarbazone) was obtained by acid hydrolysis of compounds **31** and **32**, although in neither case could the intermediate arylaminoacetaldehyde thiosemicarbazone be isolated

It is thus evident that the 1,2-bond in the pyrrolidines **3** may be at least as susceptible to attack by periodate as the 3,4-bond, even with *cis*-hydroxyl groups The degree of specificity is determined by the substituent in the benzene ring

The ethers **12–14** and **1** all afforded glyoxal bis(2,4-dinitrophenylhydrazone) in better yields than the amines **27**, **31**, and **32**, as is shown in Table IV The sulphide **34**, in which the hetero atom is less electronegative, leading to almost completely covalent bonds with the flanking carbon atoms, behaved quite differently Here the product was thiodiacetaldehyde bis(2,4-dinitrophenylhydrazone) The reaction of 2,4-di-

nitrophenylhydrazine with several α-functionally-substituted aldehydes has been described in the literature[16], but no comparative data on the oxidation of derivatives of these compounds could be found

Further investigation of the chemistry of the dialdehydes referred to in this paper is in progress

EXPERIMENTAL

Melting points are corrected. Solutions were concentrated under diminished pressure below 40° For recrystallisation, solutions of compounds were not heated above 70°

Preparation of aqueous oxydiacetaldehyde (**4**) — 1,4-Anhydroerythritol[17] (2 08 g, 20 mmoles), dissolved in water (30 ml), was added with stirring to a solution of sodium metaperiodate (4 28 g, 20 mmoles) in water (150 ml), and the mixture was kept (24 h) in the dark at room temperature The consumption of periodate was shown[18] to be 0 98 mole/mole Iodate and excess periodate were precipitated from the resulting solution of oxydiacetaldehyde by the addition of barium chloride dihydrate (2 96 g) in water (48 ml) The mixture was neutralised ($BaCO_3$) and filtered through Celite, the insoluble material being washed with water (3 × 6 ml) Barium ions were removed by using potassium sulphate (1 86 g) in water (60 ml), and the resulting precipitate was separated and washed with water (3 × 6 ml) The solution of oxydiacetaldehyde was deionised by treatment (3 h) with Amberlite MB-1 (50 g). The resin was removed by filtration through Celite and washed with water (3 × 100 ml) This deionisation was unnecessary in the preparation of water-insoluble derivatives

Water-soluble 3,5-dihydroxy-4-thioureidomorpholines (See Table II) — Aqueous oxydiacetaldehyde, after deionisation with Amberlite MB-1, was treated with an equimolecular amount of the appropriate thiosemicarbazide (in solution), and the resulting mixture was evaporated to dryness The addition and evaporation of several portions of methanol left a solid residue which, on recrystallisation, afforded the colourless, water-soluble 3,5-dihydroxy-4-thioureidomorpholine

4-(3-Benzylthioureido)-3,5-dihydroxymorpholine (**17**) — A solution of 4-benzylthiosemicarbazide (543 mg, 3 mmoles) in ethanol (48 ml) was added to aqueous oxydiacetaldehyde, from 1,4-anhydroerythritol (3 mmoles), and the resulting mixture was concentrated to *ca* 10 ml Compound **17** (622 mg, 73%), m p 119–120°, separated initially as an oil The analytical results are given in Table II

Oxidiacetaldehyde bis(4-methylthiosemicarbazone) (**9**) — Aqueous oxydiacetaldehyde, from 1,4-anhydroerythritol (12 mmoles), was added to a solution of 4-methylthiosemicarbazide (2 52 g, 24 mmoles) in water (42 ml) containing acetic acid (0 48 ml) After 2 days at room temperature, the opaque mixture deposited crystals which were collected Further crops were obtained during the next 5 days (in all 1 82 g, 55%, after drying at room temperature/18 mm), m p 151 5–155° Crystallisation from *N,N*-dimethylformamide–water (3 4) gave colourless plates, m p 153–154°, which absorbed water (6 6%) in a hygrostat (saturated sodium nitrite)

TABLE II

3,5-DIHYDROXY-4-THIOUREIDOMORPHOLINES[a]

Compound	M p [b] (degrees)	Yield (%)		Analysis				Crystallised from
				C	H	N	S	
13	Decomposed gradually > 150[c]	79	Found	31 2	5 9	22 0	16 4	*N,N*-Dimethylformamide–ether (2 3)
			Calc	31 1	5 7	21 8	16 6	
14	152–153 5	44	Found	35 1	6 3	20 1	15 3	Methanol
			Calc	34 8	6 3	20 3	15 5	
15	131–131 5	38	Found	35 7	6 4	17 2	13 5	Methanol–chloroform (1 12)
			Calc ·	35 45	6 3	17 7	13 5	
16	140–141 5	45	Found	41 3	7 1	18 2	14 0	Methanol-ether (1 4)
			Calc	40 85	7 2	17 9	13 6	
17	130–132	73	Found	50 9	6 1	14 9	11 5	Chloroform–light petroleum (b p 40–60°) (1 1)
			Calc	50 9	6 0	14 8	11 3	

[a]These compounds are sensitive to heat [b]All melting points were with decomposition [c]Recrystallised **13**, dried at room temp /18 mm, has m p 99–100° (dec), and contains 39 6% of *N,N*-dimethylformamide which is removed at 78°/0 01 mm

to yield 3,5-bis(4-methylthiosemicarbazido)-*p*-dioxane (**12**), m p 150–153° (Found C, 32 9; H, 6 1, N, 28 5, S, 21 7 $C_8H_{18}N_6O_2S_2$ calc C, 32 7, H, 6 1; N, 28 6, S, 21 8%) The *p*-dioxane derivative **12** lost 6 3% by weight (≡ 1 mole of H_2O/mole) at room temperature/18 mm over phosphorus pentaoxide to give compound **9**

Oxydiacetaldehyde bis(4-propylthiosemicarbazone) (**10**) — Compound **10** was prepared by the dropwise addition with stirring, at 13–14°, of aqueous oxydiacetaldehyde, from 1,4-anhydroerythritol (3 mmoles), to a solution of 4-propylthiosemicarbazide (798 mg, 6 mmoles) in methanol (25 ml) containing acetic acid (1 2 ml) The product (784 mg, 79%) m p 138 5–140°, was collected after 18 hours and crystallised from *N,N*-dimethylformamide–water (2 3) to give colourless crystals, m p 138–139° (Found C, 43 7, H, 7 2, N, 25 4, S, 19 6 $C_{12}H_{24}N_6OS_2$ calc C, 43 4, H, 7 2, N, 25.3; S, 19 3%)

Oxydiacetaldehyde bis(4-benzylthiosemicarbazone) (**11**) — Oxydiacetaldehyde solution, from 1,4-anhydroerythritol (2 mmoles), was added dropwise with stirring to a solution of 4-benzylthiosemicarbazide (724 mg, 4 mmoles) in a mixture of ethanol (32 ml) and water (20 ml) containing acetic acid (1 6 ml) The initially, oily product **11** (810 mg, 95%) had m p 147–148° Crystallisation from *N,N*-dimethylformamide–water (2 1) raised the m p to 155 5–156.5° (Found C, 56 0, H, 5 8, N, 19 6, S, 15.1. $C_{20}H_{24}N_6OS_2$ calc C, 56 1, H, 5 6, N, 19 6, S, 15 0%)

Conversion of compound **14** *into compound* **12** — The morpholine **14** (207 mg, 1 mmole) and 4-methylthiosemicarbazide (105 mg, 1 mmole) were mixed in water (10 ml) containing acetic acid (0 02 ml) During 7 days, the *p*-dioxane derivative **12** (61%) was deposited

cis-4-p-*Chlorophenyl-3,4-dihydroxypyrrolidine* (**20**) — *meso*-1,4-Dichloro-

butane-2,3-diol[19] (2 862 g, 18 mmoles) and *p*-chloroaniline (8.04 g, 63 mmoles) were heated at 150° (1 5 h) The product (3.16 g, 82%), m p 146–148°, was collected after addition of water. Recrystallisation from benzene yielded colourless needles, m p 148 5–150° (Found C, 56.7, H, 5 8; Cl, 17.1; N, 6 5 $C_{10}H_{12}ClNO_2$ calc C, 56 2; H, 5 6, Cl, 16 6, N, 6 6%). The corresponding *trans* compound[13] has m p 182°.

Preparation of phenyliminodiacetaldehydes (**6–8**) — To the appropriate 1-aryl-3,4-dihydroxypyrrolidine (6 mmoles) in methanol (100 ml) was added dropwise, at 0–2° (7–9° in the case of **21**), 0 1M sodium metaperiodate (60 ml) Precipitated inorganic salts were dissolved by the addition of water (100 ml), and the resulting phenyliminodiacetaldehyde was extracted immediately with ether (100 ml and 3 × 50 ml) The extract was washed with saturated brine (40 ml) and dried ($MgSO_4$), and the solvent was removed

The crude dialdehyde **7** was crystalline When heated (10 min, 70°) in concentrated methanolic solution, it yielded colourless needles of the cyclic hemiacetal **25** (63%), m p 98–103 5° Recrystallisation from benzene–light petroleum (b p 40–60°) (5 2) raised the m p to 103 5–106° (Found C, 54 3, H, 5 8, Cl, 15 0, N, 5 7, OMe, 12 8 $C_{11}H_{14}ClNO_3$ calc · C, 54 2, H, 5 75, Cl, 14 6, N, 5 75, OMe, 12 7%). The infrared spectrum of compound **25** had a strong band at 3330 cm^{-1} (OH) and no absorption in the 1700 cm^{-1} region (C=O) Recrystallisation from aqueous acetone did not convert compound **25** into the corresponding hemialdal (*cf* Ref 20)

Dioximes (**22–24**) *of phenyliminodiacetaldehydes* (*See Table III*) — The derivatives were prepared by the procedure here given for the dioxime (**23**) of *p*-chlorophenyliminodiacetaldehyde The crude dialdehyde, prepared as above from compound **20** (2 mmoles), was dissolved in methanol (8 ml) and treated with a mixture of hydroxylamine hydrochloride (278 mg, 4 mmoles) and sodium acetate trihydrate (545 mg, 4 mmoles) in water (5 ml) The mixture, after 30 min at room temperature, was concentrated, and the dioxime which separated was recrystallised from aqueous methanol

TABLE III

DIOXIMES OF PHENYLIMINODIACETALDEHYDES

Dioxime	*Yield* (%)	*M p* [a] (*degrees*)		*Analysis*			
				C	*H*	*Cl*	*N*
22	69[b]	137 5	Found	58 1	6 7		20 2
			Calc	58 0	6 3		20 3
23	93	144–145	Found	50 1	5 2	14 9	17 5
			Calc	49 7	5 0	14 7	17 4
24	46	130–130 5	Found	55 5	6 4		17 9
			Calc	55 7	6 3		17 7

[a]All melting points were with decomposition [b]When the dialdehyde was prepared by the addition of glycol to periodate (*cf* **26**), the yield of compound **22** was 68 5%

Phenyliminodiacetaldehyde bis(4-methylthiosemicarbazone) (**27**) — The crude dialdehyde, from compound **19** (6 mmoles), was treated with a solution of 4-methylthiosemicarbazide (1 26 g, 12 mmoles) in methanol (30 ml). The resulting, almost colourless solution deposited (overnight) colourless crystals of compound **27** (918 mg, 44%), m p. 154–155° (dec) More crystalline material separated on subsequent days, but this showed no ultraviolet absorption maximum in the region (270 nm) characteristic of thiosemicarbazones Recrystallisation of the first fraction from *N,N*-dimethylformamide–methanol–water (1 5 1) raised the m p to 159.5–160° (dec) (Found C, 47 9, H, 6 3, N, 27 4, S, 17 7 $C_{14}H_{21}N_7S_2$ calc C, 47 9, H, 6 0, N, 27 9, S, 18 2%)

p-*Chlorophenyliminodiacetaldehyde bis(4-methylthiosemicarbazone)* (**28**) — 4-Methylthiosemicarbazide (630 mg, 6 mmoles), dissolved in methanol (16 ml), was added to a solution of the crude dialdehyde, from compound **20** (3 mmoles), in methanol (10 ml) During the next 5 days, 4 crops of colourless crystals of compound **28** (totalling 816 mg, 70%), m p 163 5–171°, each showing an ultraviolet absorption peak at 269–270 nm, were collected, the mother liquor gradually became yellow The combined fractions, on recrystallisation from *N,N*-dimethylformamide–methanol–water (1 4 1), had m p 170 5° (dec) (Found· C, 43 6, H, 5 5, Cl, 9 2, N, 24 8, S, 16 3 $C_{14}H_{20}ClN_7S_2$ calc C, 43 6, H, 5 2; Cl, 9 2; N, 25 4, S, 16 6%)

p-*Anisyliminodiacetaldehyde bis(4-methylthiosemicarbazone)* (**29**) — A solution of the crude dialdehyde, from compound **21** (4 mmoles), in methanol (24 ml) was treated with 4-methylthiosemicarbazide (840 mg, 8 mmoles), dissolved in water (24 ml) The mixture, after 2 h at room temperature, was concentrated The solid (863 mg) which separated had λ_{max} at 243 nm, in addition to significant absorption at 270 nm Crystallisation first from *N,N*-dimethylformamide–methanol–water (1 7 5) and then from the same solvents (2 10 1) eliminated the material with an absorption peak at 243 nm and afforded colourless crystals of compound **29** (206 mg, 14%), m p 157° (dec) The analytical sample had m p 158–159° (dec) (Found C, 47 1, H, 6 3, N, 25 6, S, 17 1 $C_{15}H_{23}N_7OS_2$ calc C, 47 35, H, 6 0, N, 25 7, S, 16 8%)

Treatment of compound **25** *with 1 equivalent of 4-methylthiosemicarbazide* — The cyclic hemiacetal **25** (243 mg, 1 mmole) and 4-methylthiosemicarbazide (105 mg, 1 mmole) were mixed in methanol (6 8 ml) The solution became dark-red overnight and deposited colourless crystals of the bis(4-methylthiosemicarbazone) (**28**) (58 mg, 30%), m p 171° (dec) No further material separated from the mother liquor

Phenyliminodiacetaldehyde hemialdal (**26**) — The pyrrolidine **19** (537 mg, 3 mmoles), in methanol (15 ml), was added dropwise with stirring, at 2–4°, to an equimolecular amount of 0 1M aqueous sodium metaperiodate The product was extracted immediately with ether From the washed (saturated brine) and dried ($MgSO_4$) extract, after removal of solvent, was obtained a pink, semi-solid residue which, on trituration with benzene (8 5 ml), yielded colourless crystals of compound **26** (190 mg, 32 5%), m p 96 5–98°. Recrystallisation from benzene–light petroleum (b p 40–60°) (2 1) raised the m.p to 100 5–102 5° (Found C, 61 3, H, 6 6, N, 7.3

$C_{10}H_{13}NO_3$ calc C, 61 5, H, 6 7, N, 7 2%) The infrared spectrum of compound **26** had a very strong band at 3330 cm^{-1} (OH) and no absorption in the 1700 cm^{-1} region (C=O)

Secondary products from the reaction of thiosemicarbazides with periodate-oxidised pyrrolidine glycols — The pyrrolidine (**19**) (716 mg, 4 mmoles) was oxidised as described for the preparation of compound **26** The crude product was treated with 4-propylthiosemicarbazide (1 064 g, 8 mmoles), dissolved in methanol (6 ml) From the resulting solution, colourless crystals (485 mg), m p 142–143 5° (dec), separated overnight Two recrystallisations, from (*a*) *N,N*-dimethylformamide–methanol–water (2 10 1) and (*b*) *N,N*-dimethylformamide–methanol (1 5), afforded compound **31** (339 mg, 22%), m p 147–148° (dec) [Found (for different samples) C, 49 2, 49 6, 50 3, H, 7 5, 7 5, 7 4, N, 25 6, 25 1, 25 7, S, 17 4, 17 5, 17 4 $C_{16}H_{29}N_7S_2$ calc C, 50 1, H, 7 6, N, 25 55, S, 16 7%]

When compound **31** (153 mg, 0 4 mmole) was stirred (18 h) in ethanol (6 1 ml) containing 2N HCl (0 61 ml), it yielded glyoxal bis(4-propylthiosemicarbazone) (11 7 mg, 10%), m p 203 5–208° (dec), undepressed by an authentic sample. The latter was made in the usual way and crystallised from aqueous pyridine as yellow crystals, m p 220–221° (dec) (Found C, 42 1, H, 7 0, N, 29 6, S. 22 8 $C_{10}H_{20}N_6S_2$ calc C, 41 7, H, 6 9, N, 29 2, S. 22 2%)

The pyrrolidine **21** (1 672 g, 8 mmoles) was oxidised as usual, *i e* , by addition of periodate to the glycol, and the resulting product was treated with a solution of 4-methylthiosemicarbazide (1 68 g, 16 mmoles) in methanol (30 ml) Fractions [406 mg, m p 128–130° (dec)] and [1 3 g, m p 135–136 5° (dec)] separated after 17 and 41 h, respectively These, having λ_{max} at *ca* 242 nm and no significant absorption at 270 nm, were combined and recrystallised twice from *N,N*-dimethylformamide–methanol–water (2 10 1) to give colourless crystals (1 08 g, 38%), m p 132 5–134 5° (dec), tentatively assigned the structure **32** [Found (for different samples) C, 41 8, 41 7, H, 6 7, 6 8, N, 26.7, 26 9, S, 18 4, 18 5; OMe, 11 7%, Mol wt , <450 $C_{13}H_{23}N_7OS_2$ calc C, 43 65, H, 6 5, N, 27 45, S, 17 95, OMe, 8 7%, Mol wt , 357 5] Hydrolysis as above afforded glyoxal bis(4-methylthiosemicarbazone) (15%), m p 240–241° (dec), undepressed by an authentic sample, m p 243° (dec)

Treatment of thiosemicarbazide derivatives with 2,4-dinitrophenylhydrazine sulphate (DNP) — The derivatives were refluxed (1 5 h) with DNP in methanol In each case, orange crystals of glyoxal bis(2,4-dinitrophenylhydrazone), m p 312–313° (dec), undepressed by an authentic sample, m p 315–316° (dec), separated

TABLE IV

YIELDS OF GLYOXAL BIS(2 4-DINITROPHENYLHYDRAZONE) FROM THIOSEMICARBAZIDE DERIVATIVES

Derivative	**31**[a]	**32**[a]	**27**[b]	**13**[b]	**14**[b]	**12**[b]	**1**[b c]
Yield (%)	18	9 5	14	50	45	36	77

[a]Treated with 2 equivalents of reagent [b]Treated with 4 equivalents of reagent [c]R=CSNHPr

Yields are given in Table IV. When the solution of compound **12** was refluxed for 15 min, the yield of derivative was 9 5%. Similar treatment of the sulphide[3] **34** for 15 min with 4 equivalents of DNP gave thiodiacetaldehyde bis(2,4-dinitrophenylhydrazone), m p 220–222°, undepressed by an authentic sample (lit.[4], m p 219–221°), in 46% yield In this case, refluxing for 1 5 h also gave largely the thiodiacetaldehyde derivative (40% yield, m p 216–223°)

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REFERENCES

1 J E McCormick, *J Chem Soc (C)*, (1966) 2121
2 J E McCormick, *Carbohyd Res*, 4 (1967) 262
3 V C Barry, M L Conalty, J E McCormick, R S McElhinney, M R McInerney, and J F O Sullivan, unpublished results
4 C L Zirkle, F R Gerns, A M Pavloff, and A Burger, *J Org Chem*, 26 (1961) 395
5 H Klosterman and F Smith, *J Amer Chem Soc*, 74 (1952) 5336
6 R D Guthrie and J F McCarthy, *J Chem Soc (C)*, (1966) 1207
7 V C Barry M L Conalty, and J F O'Sullivan, *Cancer Res*, 26 (1966) 2165
8 L Wolff and R Marburg, *Ann*, 363 (1908) 169, O Bayer, in Houben-Weyl, *Methoden der Organischen Chemie*, 4th Ed , Vol 7/1, Thieme Verlag, Stuttgart, 1954, p 426, H Spanagel and E Muller, *ibid*, Vol 6/4, 1966, p 602
9 N Vinot, *Compt Rend*, 248 (1959) 3013
10 M Chastrette, *Ann Chim* (Paris), 7 (1962) 643
11 (*a*) H Tanabe, *Chem Pharm Bull* (Tokyo), 6 (1958) 645, (*b*) K Hattori, H Harada, and Y Hirata, *Bull Chem Soc Japan*, 35 (1962) 312, (*c*) J Kawashiro, *J Pharm Soc Japan*, 73 (1953) 943, H Tanabe, *ibid*, 76 (1956) 1023 J R Clamp and L Hough, *Biochem J*, 101 (1966) 120
12 A J Hill and M G McKeon, *J Amer Chem Soc*, 76 (1954) 3548
13 A Weickmann, German Pat 805,522 (1951), *Chem Abstr*, 46 (1952) 1049
14 J J Roberts and W C J Ross, *J Chem Soc*, (1952) 4288
15 J Wangel *Arkiv Kemi* 1 (1950) 431
16 L A Jones, C K Hancock, and R B Seligman, *J Org Chem*, 26 (1961) 228
17 F H Otey and C L Mehltretter, *J Org Chem*, 26 (1961) 1673
18 G O Aspinall and R J Ferrier, *Chem Ind* (London), (1957) 1216
19 L N Owen, *J Chem Soc*, (1949) 243
20 R D Guthrie and J Honeyman, *J Chem Soc*, (1959) 2441

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MIGRATION OF THE METHYLTHIO GROUP IN THE SOLVOLYSIS OF SOME METHYL 1-THIO-6-*O*-TOLUENE-*p*-SULPHONYL-β-D-GLYCOPYRANOSIDES

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ABSTRACT

The hydrolysis and methanolysis of methyl 1-thio-6-*O*-toluene-*p*-sulphonyl-β-D-glucopyranoside proceeds with migration of the methylthio group to C-6 Acetolysis of methyl 1-thio-6-*O*-toluene-*p*-sulphonyl-β-D-glucopyranoside triacetate and of the corresponding galactoside likewise gives 6-*S*-methyl-6-thio compounds The stereochemical outcome at C-1 of these reactions is discussed

Replacement of the methylthio group of the methyl 1-thio-6-*O*-toluene-*p*-sulphonyl-α- and -β-D-glucopyranosides by an acetoxyl group, using mercuric acetate in acetic acid, proceeds mainly with inversion of configuration at C-1

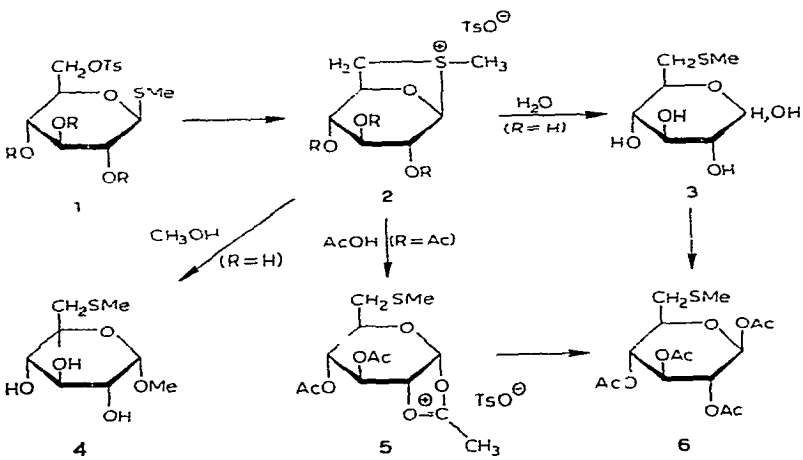
INTRODUCTION

During kinetic studies[1] of the alkaline cyclisation of glycoside 6-toluene-*p*-sulphonates to 3,6-anhydro compounds, it was found that methyl 1-thio-6-*O*-toluene-*p*-sulphonyl-β-D-glucopyranoside (**1**, R = H) decomposed with the liberation of toluene-*p*-sulphonate ions even in a neutral aqueous solution, the half-life for the reaction being about 30 h at room temperature By contrast, the corresponding α-D-thioglucoside and the oxygen analogues (the methyl 6-*O*-toluene-*p*-sulphonyl-α- and -β-D-glucopyranosides) were unreactive under the same conditions These results suggest that the reactivity of the β-thioglucoside **1** (R = H) is due to neighbouring-group participation by the methylthio group, and the behaviour of this compound was therefore examined further

As detailed below, the reaction was found to involve migration of the methylthio group to C-6, presumably *via* a 1,6-cyclic sulphonium intermediate Migration reactions through a three-membered sulphonium ring had already been observed in carbohydrate chemistry before this work was begun[2] More recently, other interesting migration and rearrangement reactions have been encountered in solvolyses of carbohydrate sulphonates, involving participation by alkylthio groups[3], alkoxyl groups[4,5], and even ring oxygen atoms[5,6]

RESULTS AND DISCUSSION

Chromatographic evidence showed that the decomposition of the toluene-*p*-sulphonate **1** (R = H) in aqueous solution yielded a single product On acetylation, this gave crystalline 6-*S*-methyl-6-thio-β-D-glucopyranose tetra-acetate (**6**), identified by its n m.r spectrum (see Experimental section) and by desulphurisation to the known 6-deoxy-β-D-glucopyranose tetra-acetate[7] The formation of 6-*S*-methyl-6-thio-D-glucopyranose (**3**) clearly confirms that the reaction of the toluene-*p*-sulphonate proceeds with neighbouring-group participation by sulphur *via* the cyclic sulphonium compound **2** (R = H) The ready occurrence of this reaction, even although it must proceed through an unstable conformation of the toluene-*p*-sulphonate, is a striking illustration of the efficacy of alkylthio groups in neighbouring-group reactions[8] A cyclic sulphide related to the above sulphonium compound has been reported[9] The fact that the sulphonium compound reacts exclusively at C-1 may be ascribed to the mesomeric effect of the ring oxygen atom



Since the reaction of the toluene-*p*-sulphonate **1** (R = H) with water gives no information about the stereochemistry of the opening of the sulphonium ring, the reaction with methanol was examined, calcium carbonate being added to prevent anomerisation by the toluene-*p*-sulphonic acid liberated The main product was methyl 6-*S*-methyl-6-thio-α-D-glucopyranoside (**4**), formed with inversion at C-1, this was isolated as its triacetate, which was characterised by n m r and by desulphurisation to the known methyl 6-deoxy-α-D-glucopyranoside triacetate[10] Chromatographic evidence indicated the presence of a second compound, presumably methyl 6-*S*-methyl-6-thio-β-D-glucopyranoside

The methanolysis of the triacetate (**1**, R = Ac) of methyl 1-thio-6-*O*-toluene-*p*-sulphonyl-β-D-glucopyranoside was then examined to see if participation by the acetoxy group at C-2 in the opening of the sulphonium ring would influence the course of the reaction Unfortunately, this compound was relatively unreactive and had

only partly reacted in boiling methanol after 5 h However, the compound reacted smoothly at 100° with anhydrous acetic acid containing sodium acetate, giving mainly 6-*S*-methyl-6-thio-β-D-glucopyranose tetra-acetate (**6**) and about 10% of a second compound The latter was chromatographically identical with the corresponding α-D tetra-acetate, an authentic sample of which was obtained by anomerisation of the β-D tetra-acetate with zinc chloride

The preferential formation of the β-D tetra-acetate suggests that the acetolysis involves the opening of the sulphonium ring of the intermediate (**2**, R = Ac) by the acetoxy group at C-2, giving the acetoxonium ion **5**, which then reacts with inversion at C-1 Formation of the acetoxonium ion was supported by the fact that, in acetic acid containing 1% of water, the main products were partially acetylated compounds (presumably triacetates of 6-*S*-methyl-6-thio-D-glucose, since they gave the tetraacetates of this compound on acetylation) Such behaviour is characteristic of acetoxonium ions[11]

Acetolysis of methyl 1-thio-6-*O*-toluene-*p*-sulphonyl-β-D-galactopyranoside triacetate also proceeded with migration of the methylthio group and gave mainly 6-*S*-methyl-6-thio-β-D-galactopyranose tetra-acetate, recognised by its n m r spectrum Since the rearrangement reactions described here must occur *via* an unstable conformation of the toluene-*p*-sulphonates, the relative reactivities of stereoisomeric methyl 1-thio-6-*O*-toluene-*p*-sulphonyl-β-D-glycopyranosides would be of interest

Attempted acetolysis of methyl 6-*O*-toluene-*p*-sulphonyl-β-D-glucopyranoside triacetate under the conditions used for the thioglycosides gave only unchanged starting material

The toluene-*p*-sulphonates mentioned above were prepared by conventional methods The position of the toluene-*p*-sulphonyl group in the methyl 1-thio-6-*O*-toluene-*p*-sulphonyl-α-D-glucopyranoside was confirmed by treatment with mercuric acetate in acetic acid, followed by acetylation of the product to the known 6-*O*-toluene-*p*-sulphonyl-β-D-glucopyranose tetra-acetate[7] Under similar conditions, methyl 1-thio-6-*O*-toluene-*p*-sulphonyl-β-D-glucopyranoside also reacted mainly with inversion at C-1, giving 6-*O*-toluene-*p*-sulphonyl-α-D-glucopyranose tetra-acetate, an authentic sample of which was prepared by anomerisation of the β-D tetra-acetate with perchloric acid in acetic anhydride Surprisingly, methyl 1-thio-6-*O*-toluene-*p*-sulphonyl-β-D-glucopyranoside triacetate was recovered unchanged when treated with mercuric acetate under the same conditions

The n m r spectrum of methyl 6-*S*-methyl-6-thio-α-D-glucopyranoside triacetate showed an interesting effect On one occasion, the peaks at τ 7 82 ($-SCH_3$) and τ 7 39 ($-SCH_2-$) were found to be greatly broadened, although the other peaks in the spectrum were quite sharp It is probable that this was due to contamination by copper ions, since addition of a trace of cupric chloride gave the same effect The selective broadening of the signals from protons α- to the sulphur atom can be ascribed to relaxation in a paramagnetic copper complex, we have observed the same phenomenon for a number of simple thioethers[12] An interesting feature is that, in the spectrum of diethyl sulphide, for example, the β-CH_3 gives a recognisable triplet even

when the α-CH_2 has been broadened by the addition of cupric chloride so as to be indistinguishable from the base-line Selective broadening by copper ions is clearly a useful adjunct in the interpretation of the spectra of thioethers, *e g*, it provided a ready distinction between SCH_3 and CH_3CO O peaks for some of the compounds studied here (see Experimental section)

EXPERIMENTAL

General — Evaporations were carried out under diminished pressure on a rotatory evaporator at *ca* 30°, unless otherwise stated Chloroform extracts were dried with anhydrous sodium sulphate The light petroleum used had b p 60–80° Optical rotations were measured at *ca* 20° in 1- or 2-dm tubes. N m r spectra were obtained at 60 MHz on a Perkin-Elmer R 10 spectrometer by using 10–20% solutions in deuterochloroform with tetramethylsilane as internal standard

Chromatographic methods — Paper chromatography was done on Whatman No 1 paper with the following solvent systems (*i*) butyl alcohol–ethanol–water (4.1 5, upper layer), (*ii*) methyl sulphoxide as stationary phase and isopropyl ether as mobile phase[13] (in the application of the stationary phase, the paper was dried at 85–90° instead of 60°) Sprays used were (*a*) silver nitrate–sodium hydroxide[14], (*b*) a saturated solution of aniline oxalate in methanol, followed by heating for 15 min at 110° (to detect free sugars)

Thin-layer chromatography was done on Kieselgel G (Merck) with benzene–ethanol (2 1) Sprays used were (*c*) anisaldehyde–sulphuric acid[15] and (*d*) 1% diphenylamine in ethanol followed by exposure to ultraviolet light[16]

Methyl 1-thio-6-O-toluene-p-sulphonyl-α-D-glucopyranoside. — Toluene-*p*-sulphonyl chloride (0 2 g) in anhydrous pyridine (2 ml) was added dropwise to methyl 1-thio-α-D-glucopyranoside[17] (0 2 g) in pyridine (2 ml) at −10° The mixture was kept at −10° for 2 h and then at room temperature overnight After addition of a little water, the pyridine was removed by repeated evaporation with ethanol The residue was dissolved in chloroform and washed with 2N sulphuric acid and sodium hydrogen carbonate solution Evaporation and crystallisation from ethyl acetate–light petroleum gave the toluene-*p*-sulphonate (0 15 g) A dried sample had m p 114° (dec), $[\alpha]_D$ +169 5° (*c* 1 3, ethanol) On standing in air, the compound (60 mg) absorbed water (1 5 mg), corresponding to the formation of a hemihydrate, m p 80–82° (dec) (Found C, 45 1, H, 5 6, S, 16 85 $C_{14}H_{20}O_7S_2$ 0 5 H_2O calc C, 45 0, H, 5.6, S, 17 2%) After drying, m p 114° (dec) was again obtained

Methyl 1-thio-6-O-toluene-p-sulphonyl-β-D-glucopyranoside — Toluene-*p*-sulphonyl chloride (2 2 g) in anhydrous pyridine (20 ml) was added dropwise, during 2 h, with stirring to methyl 1-thio-β-D-glucopyranoside[17 18] (2 1 g) in pyridine (20 ml) at −10° The mixture was kept at −10° for a further 2 h and at room temperature overnight It was then poured into light petroleum (250 ml) The clear, supernatant liquid was decanted, and the residual syrup was dissolved in chloroform (30 ml) and poured, with vigorous stirring, into ice-cold 2N sulphuric acid (100 ml) The white precipitate which separated was filtered off and washed thoroughly with water

Crystallisation from chloroform–light petroleum gave the toluene-*p*-sulphonate (1 6 g), m p 108° (dec), $[\alpha]_D$ −10° (*c* 2, ethanol) (Found C, 46 1, H, 5 5; S, 17 6 $C_{14}H_{20}O_7S_2$ calc C, 46 1, H, 5 5, S, 17 6%) The above material was fairly stable, but samples prepared by using a different work-up, which included crystallisation from ethanol, decomposed with blackening in a few days

*Methyl 1-thio-6-*O*-toluene-*p*-sulphonyl-β-*D*-glucopyranoside triacetate* — (*a*) Methyl 1-thio-β-D-glucopyranoside (2 1 g) was treated with toluene-*p*-sulphonyl chloride as described above After the reaction mixture had stood overnight, it was again cooled to −10°, and acetic anhydride (5 ml) was added dropwise After 15 h at room temperature, the solution was poured with vigorous stirring into ice-cold water (300 ml), yielding a precipitate of the triacetate, which was crystallised from ethanol to give needles (3 1 g), m p 146–148° (dec), $[\alpha]_D$ +9° (*c* 1, chloroform) (Found C, 49 0, H, 5 3, S, 12 5 $C_{20}H_{26}O_{10}S_2$ calc C, 49 0, H, 5 3, S, 13 1%)

(*b*) The same triacetate, m p and mixed m p 146–148° (dec), $[\alpha]_D$ +9° (*c* 1, chloroform) was obtained in 85% yield when methyl 1-thio-6-*O*-toluene-*p*-sulphonyl-β-D-glucopyranoside in pyridine was treated with acetic anhydride

*Treatment of methyl 1-thio-6-*O*-toluene-*p*-sulphonyl-β-*D*-glucopyranoside with water.* — The toluene-*p*-sulphonate (1 2 g) in water (50 ml) was heated for 2 h at 70–75°. T l c with spray *d* showed that the starting material (R_F 0 45) had reacted; spray *c* revealed the product (R_F 0 19) Paper chromatography (solvent *i*, spray *b*) showed the presence of a free sugar (R_G 3 0), no additional products were revealed by spray (*a*) The solution was buffered with sodium acetate (0 5 g) and evaporated The residue was dried over phosphorus pentoxide and then heated for 140 min at 100° with anhydrous sodium acetate (0 5 g) and acetic anhydride (20 ml) The solution was poured into ice-cold water, and the product obtained by using chloroform Paper chromatography (solvent *ii*, spray *a*) showed it to be mainly 6-*S*-methyl-6-thio-β-D-glucopyranose tetra-acetate (see below), together with some of the α-D anomer Four crystallisations from ethanol gave the pure β-D anomer (0 75 g), m p 103–104°

*Methanolysis of methyl 1-thio-6-*O*-toluene-*p*-sulphonyl-β-*D*-glucopyranoside* — The toluene-*p*-sulphonate (1 2 g), calcium carbonate (1 0 g), and anhydrous calcium sulphate (1 0 g) in anhydrous methanol (50 ml) were heated under reflux for 5 h T l c (sprays *c* and *d*) indicated complete conversion into a product having R_F 0 35 The product, after filtration, and evaporation of the methanol, was chromatographed[19] on a column (70 × 2 cm) of Dowex-1X2 (OH^-, 200–400 mesh) by elution with water. The fractions (5 ml) were examined polarimetrically and, in some cases, by paper chromatography in solvent (*ii*) after acetylation

Fractions 125–161 contained only the α-D-glucoside (0 40 g), acetylation of this compound (0 32 g) with acetic anhydride (5 ml) and pyridine (10 ml) gave methyl 6-*S*-methyl-6-thio-α-D-glucopyranoside triacetate, which crystallised from ethanol as needles (0.40 g), m p 119°, $[\alpha]_D$ +144° (*c* 1 6, chloroform) (Found C, 48 2, H, 6.0; S, 9 0 $C_{14}H_{22}O_8S$ calc C, 48 0, H, 6 3, S, 9 1%) The n m r spectrum included peaks at τ 6 55 (3-proton singlet, $-OCH_3$), τ *ca* 7 39 (2-proton multiplet, $-CH_2S-$), τ 7 82, 7 93, 7 97, and 8 00 [12 protons, $-SCH_3$ and three acetoxyl groups, the peak

at τ 7 82 is assigned to -SCH_3 as it is selectively broadened by Cu^{II}, see Discussion]

Fractions 162–180 (0.14 g) were acetylated to give a product which was shown by paper chromatography (solvent *u*, spray *a*) to consist of the above triacetate (R_F 0 78) and a slower-moving compound (R_F 0 62), presumably the β-D-anomer

Desulphurisation of the methyl 6-*S*-methyl-6-thio-α-D-glucopyranoside triacetate (0.4 g) with Raney nickel in boiling ethanol, followed by crystallisation of the product from light petroleum (b p 40–60°), gave methyl 6-deoxy-α-D glucopyranoside triacetate (0 2 g), m.p 76–77°, $[\alpha]_D$ +153° (*c* 1 6, chloroform) (lit [10], m p 77–78°, $[\alpha]_D$ +153 6° in chloroform) The n m r spectrum included peaks at τ 8 80 (3-proton doublet, separation 6 5 Hz, C-CH_3), τ 7 93, 7 97, and 8 00 (9 protons, three acetoxyl groups), and τ 6 59 (3 protons, OCH_3)

*Acetolysis of methyl 1-thio-6-O-toluene-*p*-sulphonyl-β-D-glucopyranoside triacetate* — (*a*) *In anhydrous acetic acid* A solution of the toluene-*p*-sulphonate (0 9o g) and anhydrous sodium acetate (0 53 g) in acetic acid (50 ml, dried by using triacetyl borate)[20] was heated on an oil-bath for 2 h at 100° with the exclusion of moisture After evaporation of the acetic acid at 50°, the residue was extracted with chloroform, and the extract was washed with sodium hydrogen carbonate solution and water Evaporation of the chloroform gave a syrup (0 73 g) which was shown by paper chromatography (solvent *u*, spray *a*) to be mainly 6-*S*-methyl-6-thio-β-D-glucopyranose tetra-acetate (R_F 0 62), together with a little of the α-D anomer (R_F 0 74, see below) and traces of two slower-moving materials The syrup had $[\alpha]_D$ +17 8° (chloroform), suggesting that the proportion of β-D anomer was >90%. Two crystallisations from ethanol gave the pure β-D-tetra-acetate as needles (0 56 g), m p 104°, $[\alpha]_D$ +10° (*c* 2 75, chloroform) (Found C, 47 6, H, 5 7, S, 8 7 $C_{15}H_{22}O_9S$ calc C, 47.6; H, 5 8, S, 8 5%) The n m r spectrum included peaks at τ 4 30 (1-proton doublet, separation 7 Hz, anomeric proton), τ *ca* 7 35 (2-proton multiplet, -CH_2S-), τ 7 87, 7 90, 7 97, 7 98, and 7 99 [15-protons, -SCH_3 and four acetoxyl groups, the peak at 7 87 τ is assigned to -SCH_3 as it is selectively broadened by Cu^{II}, see Discussion]

Desulphurisation of the β-D tetra-acetate (0 3 g) with Raney nickel in boiling ethanol, followed by crystallisation of the product from ethanol, gave 6-deoxy-β-D-glucopyranose tetra-acetate (0 18 g), m p 149–150°, $[\alpha]_D$ +22° (*c* 1 4, chloroform) (lit [7], m p 151°, $[\alpha]_D$ +22° in chloroform) N m r spectrum τ 8 77 (3-proton doublet, separation 6 Hz, C-CH_3), τ 7 90, 7 96, 7 98, and 8 00 (12-protons, four acetoxyl groups), and τ 4 28 (1-proton doublet, separation 7 5 Hz, anomeric proton)

(*b*) *In acetic acid containing 1% of water* The toluene-*p*-sulphonate (0 98 g) was treated as in (*a*), but with acetic acid containing 1% of water On working up as above, evaporation of the chloroform gave a syrup (0 55 g) which, when examined by paper chromatography (solvent *u*, spray *a*), gave intense spots having R_F less than 0 25 and only faint spots corresponding to the two tetra-acetates of 6-*S*-methyl-6-thio-D-glucopyranose After acetylation of the syrup with acetic anhydride and sodium acetate, as described above for the parent sugar, only the two tetra-acetates were present Crystallisation of the acetylation product from ethanol gave 6-*S*-methyl-6-thio-β-D-glucopyranose tetra-acetate (0 49 g), m p and mixed m p 103–104°

6-S-Methyl-6-thio-α-D-glucopyranose tetra-acetate — 6-*S*-Methyl-6-thio-β-D-glucopyranose tetra-acetate (0.4 g) and zinc chloride (0 1 g) in acetic anhydride (5 ml) were heated for 30 min at 100° The mixture was then poured, with stirring, into ice-cold water (25 ml) The product was isolated by using chloroform Crystallisation from ethanol gave the α-D-tetra-acetate as needles (0 15 g), m p 132–134°, $[\alpha]_D$ +115° (*c* 1 6, chloroform) (Found. C, 47.5; H, 5 5; S, 8 5 $C_{15}H_{22}O_9S$ calc: C, 47 6, H, 5 8, S, 8 5%) N m r spectrum· 3 70 τ (1-proton doublet, separation *ca* 3.5 Hz, anomeric proton), τ 7 39 (2-proton multiplet, $-CH_2S-$), τ 7 82, 7 86, 7 95, and 7.98 (15-protons, $-SCH_3$ and four acetoxyl groups, the peak at τ 7 86 was selectively broadened in one spectrum and is assigned to $-SCH_3$, that at τ 7 98 represents two acetoxyl groups)

Methyl 1-thio-6-O-toluene-p-sulphonyl-β-D-galactopyranoside triacetate — Methyl 1-thio-β-D-galactopyranoside[21] (4 0 g) in pyridine was treated with toluene-*p*-sulphonyl chloride as described above for the corresponding glucoside. After the reaction mixture had stood overnight at 0–5°, it was treated with acetic anhydride and worked up as described above Four crystallisations from ethanol gave the toluene-*p*-sulphonate as needles (2 9 g), m p 115°, $[\alpha]_D$ *ca* 0° (*c* 2 0, chloroform) (Found C, 49 0, H, 5 3, S, 12 9 $C_{20}H_{26}O_{10}S_2$ calc C, 49 0, H, 5 3; S, 13 1%)

Acetolysis of methyl 1-thio-6-O-toluene-p-sulphonyl-β-D-galactopyranoside triacetate — The toluene-*p*-sulphonate (0 98 g) was treated as described above for the corresponding glucoside (procedure *a*) Paper chromatography (solvent *u*, spray *a*) indicated that the resulting crude product was mainly 6-*S*-methyl-6-thio-β-D-galactopyranose tetra-acetate (R_F *ca* 0 28), together with a small amount of material having R_F *ca* 0 55, presumably the α-D anomer The crude product had $[\alpha]_D$ +7 1° (c 1 0, chloroform), calculation using Hudson's Rules of Isorotation suggests that the proportion of the α-D anomer was *ca* 8%. Three crystallisations from ethanol gave the pure β-D-tetra-acetate as prisms (0 52 g), m p 104–106°, $[\alpha]_D$ +1° (*c* 2 0, chloroform) (Found. C, 47 8, H, 5 9, S, 7 9 $C_{15}H_{22}O_9S$ calc C, 47 6, H, 5 8, S, 8 5%) The structure of the tetra-acetate was confirmed by the following signals in its n m r spectrum τ 4 20–4 80 (4-proton complex multiplet, a doublet at τ 4 27 with separation 7 Hz was assigned to the anomeric proton), τ 6 00 (1-proton triplet, H-5), 7 37 τ (2-proton quartet, $-CH_2S-$), τ 7 83, 7 89, 7 97, and 8 02 (15-protons, $-SCH_3$ and four acetoxyl groups, the signal at 7 89 τ had twice the intensity of the others)

6-O-Toluene-p-sulphonyl-α-D-glucopyranose tetra-acetate — 6-*O*-Toluene-*p*-sulphonyl-β-D-glucopyranose tetra-acetate (0 5 g) was added in portions to acetic anhydride (10 ml) containing 60% perchloric acid (0 2 ml) at 30–40° After 30 min at the same temperature, the mixture was poured into ice–water (50 ml) Processing with chloroform gave a product (0 48 g) which was dissolved in warm carbon tetrachloride Some starting material was filtered off from the cooled solution, and the filtrate was evaporated Two similar treatments of the residue gave chromatographically pure α-D tetra-acetate, which crystallised from ethanol as needles (0 37 g), m p 128–129°, $[\alpha]_D$ +92° (*c* 2 0, chloroform) (Found C, 50 4, H, 5 3; S, 6 4 $C_{21}H_{26}O_{12}S$ calc C, 50 2, H, 5 2, S, 6.4%) Ohle and Vargha[22] described

a hydrate, m p 129–130°, $[\alpha]_D$ +105.7°, obtained from the mother liquors from the acetylation of 6-*O*-toluene-*p*-sulphonyl-D-glucose

*Treatment of methyl 1-thio-6-*O*-toluene-*p*-sulphonyl-β-D-glucopyranoside with mercuric acetate.* — The toluene-*p*-sulphonate (0 34 g) was dissolved at room temperature in acetic acid (10 ml, anhydrous) containing mercuric acetate (1.1 g) After 1 h at room temperature, the acetic acid was evaporated at 50° The dried residue in pyridine (10 ml) was treated at 0° with acetic anhydride (5 ml) added dropwise The reaction mixture was left overnight at room temperature when a yellow precipitate separated The entire mixture was poured into ice-cold potassium iodide (10 g) in water (50 ml), most of the precipitate dissolved Processing with chloroform gave a syrup (0 4 g) which was shown by paper chromatography (solvent *u*, spray *a*) to be mainly 6-*O*-toluene-*p*-sulphonyl-α-D-glucopyranose tetra-acetate (R_F 0 24), with a small proportion of the β-D anomer (R_F 0 12) The syrup was dissolved in warm carbon tetrachloride and, after cooling, the solution was filtered to remove some β-D anomer. The filtrate was evaporated, and the above process was repeated until the product dissolved completely in carbon tetrachloride Evaporation and crystallisation from ethanol then gave the pure α-D-tetra-acetate as needles (0 30 g), m p and mixed m p 128–129°, $[\alpha]_D$ +91 5° (*c* 1 2, chloroform)

*Treatment of methyl 1-thio-6-*O*-toluene-*p*-sulphonyl-α-D-glucopyranoside with mercuric acetate* — The toluene-*p*-sulphonate (0 1 g), dissolved in anhydrous acetic acid (10 ml) containing mercuric acetate (0 4 g), was treated as described above for the β-D anomer The yellow precipitate which formed during the acetylation was filtered off, and the filtrate was poured with stirring into ice-cold water Chromatography (solvent *u*, spray *a*) of the crude product showed it to be mainly 6-*O*-toluene-*p*-sulphonyl-β-D-glucopyranose tetra-acetate, with traces of the α-D anomer. One crystallisation from ethanol gave the pure β-D anomer as needles (0 09 g), m p 204–205° (dec), $[\alpha]_D$ +23 7° (*c* 1 1, chloroform) (lit [7,23], m p 203–205°, $[\alpha]_D$ +23 9°)

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REFERENCES

1 R BAKER, G R INGLIS, E V E ROBERTS, AND J C P SCHWARZ, unpublished work
2 C D ANDERSON, L GOODMAN, AND B R BAKER, *J Am Chem Soc*, 81 (1959) 3967, C D ANDERSON, W W LEE, L GOODMAN, AND B R BAKER, *ibid*, 83 (1961) 1900, G CASINI AND L GOODMAN, *ibid*, 86 (1964) 1427
3 N A HUGHES AND R ROBSON, *J Chem Soc (C)*, (1966) 2366
4 N A HUGHES AND P R H SPEAKMAN, *J Chem Soc (C)*, (1967) 1182
5 C L STEVENS, R P GLINSKI, K G TAYLOR, P BLUMBERGS, AND F SIROKMAN, *J Am Chem Soc*, 88 (1966) 2073
6 P W AUSTIN, J G BUCHANAN, AND R M SAUNDERS, *J Chem Soc (C)*, (1967) 372 P W AUSTIN J G BUCHANAN, AND D G LARGE, *Chem Commun*, (1967) 418, N A HUGHES, *ibid*, (1967) 1072, S HANESSIAN, *ibid*, (1966) 796

7 E HARDEGGER AND R M MONTAVON, *Helv Chim Acta*, 29 (1946) 1199
8 B CAPON, *Quart Rev* (London), 18 (1964) 65, K D GUNDERMANN, *Angew Chem, Intern Ed Engl*, 2 (1963) 674
9 M AKAGI, S TEJIMA, AND M HAGA, *Chem Pharm Bull* (Tokyo), 11 (1963) 58
10 J COMPTON, *J Am Chem Soc*, 60 (1938) 395
11 R M ROBERTS, J CORSE, R BOSCHAN, D SEYMOUR, AND S WINSTEIN, *J Am Chem Soc*, 80 (1958) 1247, R U LEMIEUX AND J D T CIPERA, *Can J Chem*, 34 (1956) 906
12 J C P SCHWARZ AND P F SWINTON, unpublished work
13 B WICKBERG, *Acta Chem Scand*, 12 (1958) 615
14 W E TREVELYAN, D P PROCTER, AND J S HARRISON, *Nature*, 166 (1950) 444
15 E STAHL AND U KALTENBACH, *J Chromatogr*, 5 (1961) 351
16 M JACKSON AND L D HAYWARD, *J Chromatogr*, 5 (1961) 166
17 J F CLAPPERTON, E V E ROBERTS, AND J C P SCHWARZ, unpublished work
18 W SCHNEIDER, J SEPP, AND O STIEHLER, *Ber*, 51 (1918) 220, M ČERNY AND J PACÁK, *Collection Czech Chem Commun*, 24 (1959) 2566, 26 (1961) 2084
19 P W AUSTIN, F E HARDY, J G BUCHANAN, AND J BADDILEY, *J Chem Soc*, (1963) 5350, J F CLAPPERTON, E V E ROBERTS, AND J C P SCHWARZ, unpublished work
20 A PICTET AND A GELEZNOFF, *Ber*, 36 (1903) 2219
21 B HELFERICH, H GRUNEWALD, AND F LANGENHOFF, *Ber*, 86 (1953) 873, M ČERNY, J STANĚK, AND J PACÁK, *Monatsh Chem*, 94 (1963) 290
22 H OHLE AND L v VARGHA, *Ber*, 62 (1929) 2425
23 B HELFERICH AND W KLEIN, *Ann*, 450 (1926) 219

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STUDIES ON URONIC ACID MATERIALS

PART XXIX* SOME STRUCTURAL FEATURES OF *Acacia laeta* GUM

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ABSTRACT

The gum polysaccharide exuded by *Acacia laeta* trees contains D-galactose, L-arabinose, L-rhamnose, D-glucuronic acid, and 4-*O*-methyl-D-glucuronic acid Linkage analysis has yielded 3-*O*-β-L-arabinopyranosyl-L-arabinose, 3-*O*-β-L-arabinofuranosyl-L-arabinose, 3-*O*-α-D-galactopyranosyl-L-arabinose, 3-*O*-β-D-galactopyranosyl-D-galactose, and 6-*O*-β-D-galactopyranosyl-D-galactose The *O*-methyl derivatives of *A laeta* gum and autohydrolysed *A laeta* gum were analysed, after methanolysis, by g l c The autohydrolysed gum was subjected to a Smith degradation, and the product examined by linkage and methylation analysis *A laeta* gum was subjected to eight successive Smith-degradations, the *O*-methyl derivatives of each of the polysaccharides obtained from the first six degradations and from the eighth degradation were analysed, after methanolysis, by g l c The structural evidence obtained does not give unequivocal proof that the gum contains a "main chain" of β-(1→3)-linked D-galactose residues

INTRODUCTION

A laeta R Br ex Benth is a natural hybrid of *A senegal* (L) Willd and *A mellifera* (Vahl) Benth , and it occurs in two varieties *A laeta* var *hashab* (which resembles *A senegal*) and *A laeta* var *mellifera* (which resembles *A mellifera*) The results of a detailed examination of *A laeta* var. *hashab* gum are reported in this Paper, and the structural features of this gum are compared with those of *A senegal* gum (gum arabic) The characterisation of two aldobiouronic acids, 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose and 6-*O*-(4-*O*-methyl-β-D-glucopyranosyluronic acid)-D-galactose, from *A laeta* var *hashab* gum has been reported[2], together with the results of an analytical study of the extensive inter-nodule variation shown by this species, from which average values for the analytical parameters expressing the composition of the gum were calculated

RESULTS AND DISCUSSION

The gum nodules from *A laeta* var *hashab* were collected, by the Gum Research Officer to the Republic of Sudan, from botanically authenticated trees at the El

*For Part XXVIII, see Ref 1

Ain Forest Reserve, near El Obeid, in November 1962 After purification, the gum polysaccharide was shown to have the specific, rotation and sugar composition shown in Table I Samples of the gum were examined by zone electrophoresis on cellulose-acetate film, and by ion-exchange chromatography on DEAE-cellulose[3]. No sharp discontinuities in the properties of the molecular species were indicated by these experiments. It therefore seems probable that *A laeta* var *hashab* gum exhibits the same kind of heterogeneity as *A senegal* gum[4,5], and may be considered to contain a continuous spectrum of related molecular species

Two disaccharides were isolated by thick-paper chromatography of the combined neutral syrups obtained from the graded hydrolysis of the gum outlined in Fig 1 The first disaccharide had the same paper-chromatographic mobility as 3-*O*-β-D-galactopyranosyl-D-galactose and was characterised as its crystalline monohydrate Methylation evidence confirmed the presence of a (1→3) link Methylation data for the second disaccharide and preparation of the phenylosazone confirmed that the disaccharide was 6-*O*-β-D-galactopyranosyl-D-galactose Partial hydrolyses with acid have shown that 3-*O*-β-D-galactopyranosyl-D-galactose[6] and 6-*O*-β-D-galactopyranosyl-D-galactose[4] are constituent units of *A senegal* gum

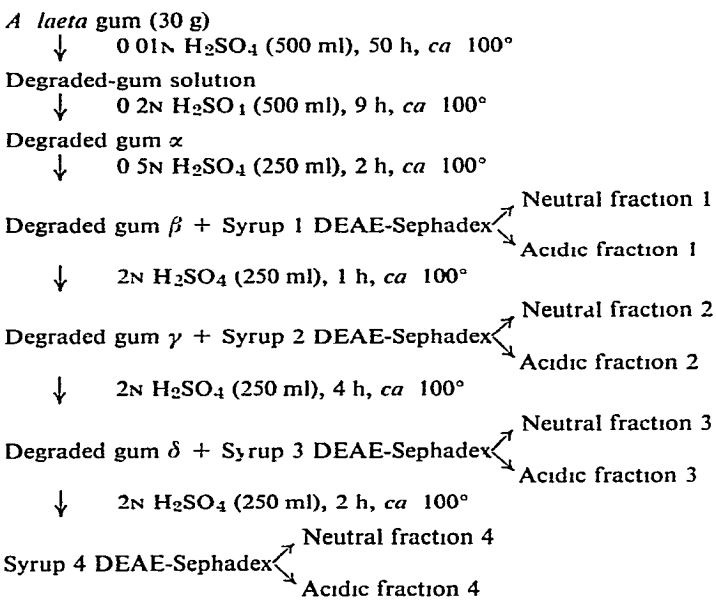


Fig 1 Diagramatic representation of stepwise hydrolysis of *A laeta* gum

A disaccharide, obtained by thick-paper chromatography of the diffusate from an autohydrolysis (80° for 6 h) in a dialysis bag placed in distilled water, had the same paper-chromatographic mobility as 3-*O*-β-L-arabinofuranosyl-L-arabinose Confirmation of this structure was obtained by acid hydrolysis, methylation data, and preparation of the phenylosazone The fact that the disaccharide is released under

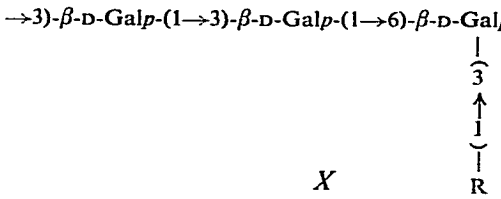
such mild conditions suggests that the reducing L-arabinose residue was initially present (in the gum) in the furanose form This disaccharide has been characterised from the gums of *A arabica*[7], *A drepanolobium*[1], and *A nubica*[8] Paper chromatography of a mild, acid hydrolysate has indicated that it is a constituent unit of *A senegal* gum[9].

Autohydrolysis (60 g on a boiling-water bath) of a 4% gum solution released all of the rhamnose, nearly all of the arabinose, and some galactose Degraded-gum *A*, isolated after dialysis, contained galactose (76%), arabinose (3%), and uronic acid (21%) The specific rotations of the whole gum and degraded-gum *A* from *A laeta* are −42° and −28°, respectively, *cf* values of −31 5° and −11°, respectively, for *A senegal*[5]. Molecular-sieve chromatography[4,5 10,11] gave estimated values of 5,700 and >200,000, respectively, for the number-average molecular weights of degraded-gum *A* and *A laeta* gum This degradation is much greater than can result from the simple removal of arabinose and rhamnose from the periphery, and does not appear to have been caused by the presence of any internal, acid-labile residues of arabinofuranose, since acid hydrolysis of borohydride-reduced, degraded-gum *A* yielded only galactitol and no arabinitol There is no evidence for the presence of any galactofuranose residues in the gum, thus, as in the case of *A senegal* gum[4,5], certain galactopyranosidic bonds must be unusually sensitive towards the mild conditions of autohydrolysis

An examination of methylated, degraded-gum *A* showed the presence of 2,3,4,6-tetra-(+ +), 2,4,6-(+ +), 2,3,4-tri-(+ +), 2,4-di-(+ + +), and 2-*O*-methyl-D-galactose (+), 2,3,4-tri-*O*-methyl-D-glucuronic acid (+ + +), and trace amounts of 2,6-di-*O*-methyl-D-galactose and 2,3,4- and 2,3,5-tri-*O*-methyl-L-arabinose The presence of 2,6-di- and 2-*O*-methyl-D-galactose is ascribed to undermethylation Methylation has revealed the presence of traces of terminal, non-reducing L-arabinopyranose and L-arabinofuranose residues in the degraded-gum *A* of *A drepanolobium*[1] Only terminal, non-reducing L-arabinopyranose residues are present in degraded-gum *A* of *A arabica*[7], and only terminal, non-reducing L-arabinofuranose residues in the autohydrolysed gum of *A senegal*[4]. The proportions of the other *O*-methyl sugars obtained from methylated, degraded-gum *A* of *A laeta* are similar to those obtained from methylated, autohydrolysed gum of *A senegal*[4], except that the former contains a larger proportion of 2,4,6-tri-*O*-methyl-D-galactose This suggests that degraded-gum *A* of *A laeta* has a larger proportion of β-D-(1→3)-glycosidic links than has autohydrolysed gum of *A senegal* This could be the reason for the lower specific rotation of degraded-gum *A* of *A laeta*

Smith degradation[12] of degraded-gum *A* yielded degraded-gum *B* Partial, acid hydrolysis of degraded-gum *B* gave 3-*O*-β-D-galactopyranosyl-D-galactose and a trace of 6-*O*-β-D-galactopyranosyl-D-galactose An examination of methylated, degraded-gum *B* indicated the presence of 2,3,4,6-tetra-(+), 2,4,6-(+ + + + + + +), 2,3,4-(trace) and 2,3,6-tri-(trace), 2,6-(+) and 2,4-di-(+ +), and 2-*O*-methyl-D-galactose (+) The presence of 2,3,6-tri-, 2,6-di-, and 2-*O*-methyl-D-galactose is probably due to undermethylation The proportion of the *O*-methyl-D-galactose is

evidence that degraded-gum *B* is a galactan composed predominantly of β-D-(1→3)-glycosidic linkages, with a few β-D-(1→6) branch-points The identification of a trace of 2,3,4-tri-*O*-methyl-D-galactose from methylated, degraded-gum *B* indicates that *X* is a possible structural fragment of degraded-gum *A*



where *R* = L-Ara*p*-(1→, L-Ara*f*-(1→, D-Gal*p*-(1→, or →6)-D-Gal*p*-(1→

Two disaccharides were obtained from the diffusate of the autohydrolysate of *A laeta* gum by thick-paper chromatography The first was identified as 3-*O*-β-L arabinopyranosyl-L-arabinose by paper chromatography, acid hydrolysis, methylation evidence, and preparation of the crystalline phenylosazone The second disaccharide was identified as 3-*O*-α-D-galactopyranosyl-D-arabinose, by using the same methods The fact that both disaccharides are released on autohydrolysis suggests that, in each case, the reducing arabinose residue was initially present in the furanose form Partial hydrolyses with acid have shown that 3-*O*-β-L-arabinopyranosyl-L-arabinose[13] and 3-*O*-α-D-galactopyranosyl-L-arabinose[6 14] are constituent units of *A senegal* gum.

The methylated gum was methanolysed, and analysed by g l c , the methyl glycosides of 2,3,4-tri-*O*-methyl-L-rhamnose, 2,3,5- and 2,3,4-tri- and 2,5-di-*O*-methyl-L-arabinose, 2,3,4,6-tetra-, 2,4,6- and 2,3,4-tri-, and 2,4-di-*O*-methyl-D-galactose, and 2,3,4-tri- and 2,3-di-*O*-methyl-D-glucuronic acid were identified The g l c traces were virtually identical with those obtained[4] for a methanolysate of methylated *A senegal* gum, and are indicative of a strong structural similarity between the gums of *A senegal* and *A laeta* var *hashab*

A sample of *A laeta* gum was subjected to eight successive Smith-degradations[12] The relative data are given in Table I The first Smith-degradation removed all of the rhamnose and 4-*O*-methylglucuronic acid residues, together with some of the galactose, arabinose, and glucuronic acid Complete oxidation of all of the glucuronic acid residues would be expected during the first treatment with periodate, the incomplete oxidation observed may result from steric hindrance[15]

On methanolysis, the *O*-methyl derivative of polysaccharide **1** gave the methyl glycosides of the methylated sugars detailed in Table II Thus, all of the arabinopyranose and rhamnopyranose residues are removed by one Smith-degradation The presence of 2,6-di-*O*-methyl-D-galactose may result from undermethylation at 0-4 of certain D-galactose residues On methanolysis, the *O*-methyl derivatives of polysaccharides **2** and **3** gave rise, with the exception of 2,3,4-tri-*O*-methyl-D-glucuronic acid, to the same *O*-methyl sugars Similarly, the *O*-methyl derivative of

TABLE I

DATA FOR *A laeta* GUM AND SMITH-DEGRADED POLYSACCHARIDES **1–8**

Polysaccharide	$[\alpha]_D$ (*degrees*)	*Constituent sugars* (%)					*Recovery* (%)	*Periodate reduced* (*mmoles/g*)	*Formic acid released* (*mmoles/g*)
		Gal	*Ara*	*Rha*	*Glu A*	*4-O-Methyl-Glu A*			
A laeta gum	−42	44	29	13	10 5	3 5	—	5 24	1 40
1	−18	68	28	—	4	—	60	5 00	2 06
2	+ 4	91	9	—	—	—	53	4 14	1 62
3	+20	97	3	—	—	—	60	2 81	1 25
4	+26 5	97	3	—	—	—	65	1 47	0 69
5	+28	100	—	—	—	—	73	1 43	0 63
6	+28	100	—	—	—	—	77	1 44	0 67
7	—	100	—	—	—	—	79	0 95	0 43
8	+27	100	—	—	—	—	78	—	—

polysaccharide **4** gave the same methyl glycosides, with the exception of 2,5-di-*O*-methyl-L-arabinose Polysaccharides **5**, **6**, and **8** contained only galactose, and, on methylation and methanolysis, gave rise to all of the methyl galactosides detected in the earlier methanolysates

Acid hydrolysis of polysaccharide **1** yielded a small proportion of 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose Partial, acid hydrolysis of each of the degraded polysaccharides gave 3-*O*-β-D-galactopyranosyl-D-galactose and 6-*O*-β-D-galactopyranosyl-D-galactose, together with the β-(1→3)-linked D-galactose trisaccharide in the partial hydrolysates of polysaccharides **2–8** In addition, the β-(1→6)-linked D-galactose trisaccharide was present in the hydrolysates of polysaccharides **2**, **3**, and **4** The detected amounts of the β-(1→6)-linked D-galactose disaccharide decreased from polysaccharides **2** to **8**. The (1→3)-linked trisaccharide was isolated from a large-scale partial hydrolysate of polysaccharide **5′** (obtained after five successive Smith-degradations in a duplicate experiment) and was characterised by crystallisation from aqueous ethanol

On molecular-sieve chromatography[4 5 10 11] with "Bio-Gel P300", *A laeta* gum was eluted at the void volume Since the viscosity properties of *A laeta* gum are similar to those of *A senegal* gum, its number-average molecular weight, $\bar{M}_n$, is probably close to the value of 250,000 which Oakley[16] obtained for *A. senegal* gum Molecular-sieve chromatography on a previously calibrated "Bio-Gel P300" column enabled the approximate, number-average molecular-weights, $\bar{M}_n$, of polysaccharides **1–8** to be estimated There is a significant drop in $\bar{M}_n$ to *ca* 91,000 for polysaccharide **1** after the first Smith-degradation, and subsequently to 14,000, 12,000, and 9,500 for polysaccharides **6**, **7**, and **8**, respectively

On the basis of the available evidence, the structural interpretations for polysaccharides **5–8** are those that have been put forward for polysaccharide *E*, the fifth Smith-degraded product of *A. senegal* gum[4] Methylation evidence indicates that

polysaccharides **5–8** are not simple, linear β-D-(1→3)-linked galactans Although 2,4,6-tri-*O*-methyl-D-galactose is the preponderant *O*-methyl sugar obtained from methylated polysaccharides **5–8**, the identification of some 2,4-di-*O*-methyl-D-galactose and 2,3,4,6-tetra-*O*-methyl-D-galactose indicates occasional branching at the C-3 and C-6 positions The presence of small amounts of the β-(1→6)-linked-D-galactose disaccharide in the partial, acid hydrolysates of these polysaccharides, and the relatively low, intrinsic viscosity of the gum, give further support for a branched structure If, however, there is a "main chain" of β-(1→3)-linked D-galactose residues in polysaccharides **5–8**, it must carry short side-chains of β-(1→3)-linked D-galactose residues, the branch points for these would be through β-D-(1→6)-linkages (Fig 2*A*) Alternatively, there could be occasional β-D-(1→6)-linkages along a "main chain" of β-D-(1→3)-linked D-galactose residues, here the branch points for the short side-chains would be through β-D-(1→3)-linkages to β-D-(1→6)-linked residues in the "main chain" (Figure 2*B*) A randomly branched structure of β-D-(1→3)-linked chains with β-D-(1→6) branch points would also satisfy the available structural evidence (Fig 2*C*)

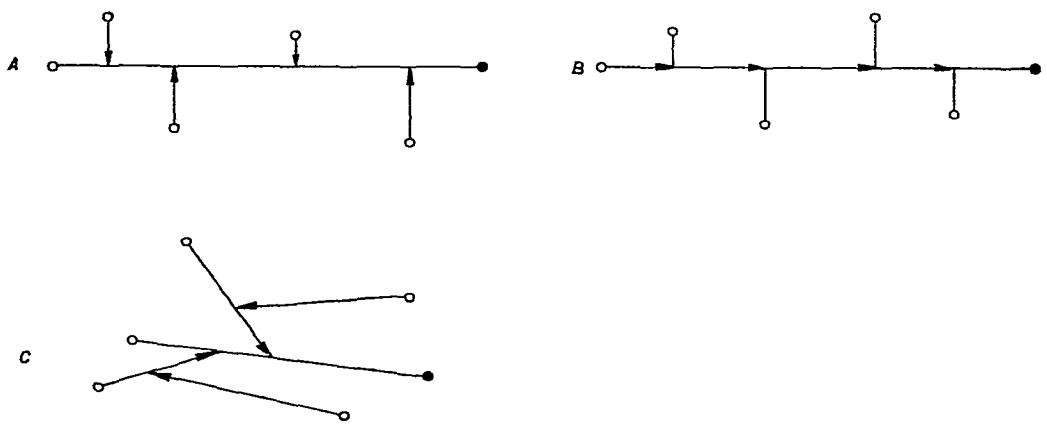


Fig 2 Diagramatic representation of some of the possible structures for the galactan framework of *A laeta* var *hashab* gum

— are chains of β-(1→3)-linked D-Gal*p* residues, ↓ are β-(1→6)-linkages at branch points, ● are reducing end-groups, ○ are terminal, non-reducing groups

The ratio of the amounts of 2,4,6-tri-*O*-methyl-D-galactose to 2,3,4,6-tetra-*O*-methyl-D-galactose and 2,4-di-*O*-methyl-D-galactose (g l c of methyl glycosides) increased from polysaccharides **5** to **8**, indicative of a progressive decrease in branching Similarly, the ratio of 2,4,6-tri-*O*-methyl-D-galactose to 2,3,4,6-tetra-*O*-methyl-D-galactose and 2,4-di-*O*-methyl-D-galactose is greater for polysaccharide **5** than for polysaccharide *E* of *A senegal* gum[4] This suggests that the galactan framework of *A laeta* gum has a higher proportion of β-D-(1→3)-linkages than *A senegal* gum This could be one of the factors contributing to the lower specific rotation of *A laeta* gum.

Four Smith-degradations were required to remove all of the arabinose residues from *A senegal* gum[4]. Thus, the longest arabinose-containing side-chains in *A senegal* gum contain four residues However, five Smith-degradations were required to remove all of the arabinose residues from *A laeta* gum, and therefore some of the arabinose-containing side-chains of *A laeta* gum must contain five residues Methylation analysis of autohydrolysed *A. senegal* gum showed that arabinofuranose units were the only arabinose residues attached to the galactan framework[4]. In contrast, both arabinofuranose and arabinopyranose units have been shown to be attached to the galactan framework of *A laeta* gum

Apart from these differences, *A laeta* and *A senegal* gums are closely similar on an overall molecular basis As in the case of *A senegal* gum[4], there is no unequivocal proof for a "main chain" or "backbone" in *A laeta* gum

EXPERIMENTAL

The analytical methods have been described in detail[5] Paper chromatography was carried out on Whatman Nos 1 and 3MM papers with the following solvent systems (v/v), (*a*) benzene–butyl alcohol–pyridine–water (1 5 3 3, upper layer), (*b*) ethyl acetate–acetic acid–formic acid–water (18 3 1 4), (*c*) ethyl acetate–pyridine–water (10 4 3), (*d*) butyl alcohol–ethanol–water (4 1 5, upper layer), (*e*) butanone–acetic acid–water (9 1·1, saturated with boric acid), (*f*) butanone–water–ammonia (sp gr 0 88) (200 17 1), (*g*) ethyl acetate–acetic acid–water (9 2 2), (*h*) butanone half saturated with water R_{Gal} values refer to distances moved relative to that of galactose R_G values of *O*-methyl sugars refer to distances moved relative to that of 2,3,4,6-tetra-*O*-methyl-D-glucose (see Table II) G l c (Chromatograph Type S3A, flame-ionisation detection, Gas Chromatography Ltd) of mixtures of *O*-methyl sugars was carried out at nitrogen flow-rates of ca. 100 ml/min on columns of (*i*) 15% by weight of poly(butane-1,4-diol succinate) on 60-70 mesh Celite (5 ft × 0 25 in) at 175° and (*ii*) 15% by weight of poly(ethylene glycol adipate) on 60–70 mesh Celite (3 ft × 0 25 in) at 160°. Retention times (*T*) (Table II) are given relative to methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucopyranoside as standard Unless otherwise stated, polysaccharides were methylated successively with methyl sulphate and sodium hydroxide, and with methyl iodide and silver oxide, and methanolyses were carried out under reflux for 7 h with 5% methanolic hydrogen chloride Disaccharides were methylated first by Haworth's method[18] and then by Kuhn's method[19]

Purification of A laeta *gum* — The gum (220 g) was dissolved in water (4 l), filtered, dialysed, and electrodialysed The polysaccharide was isolated as the freeze-dried product (208 g), $[\alpha]_D$ −42° (*c* 1 0) (Found limiting-viscosity number, 20 7, N, 0 65, OMe, 0 35%; equivalent weight, 1250, uronic acid, 14 0, galactose, 44, arabinose, 29, rhamnose, 13% All sugar percentages are for the "anhydro" forms and are corrected for moisture and protein contents

The gum migrated as a single band on electrophoresis on strips (18 × 5 cm) of cellulose-acetate film in both 0 1M ammonium carbonate buffer (pH 8.9) and 0 1M acetate buffer (pH 4 7) at field strengths between 15 and 20 volts/cm for 2–4 h

Polysaccharide bands were stained by a modification of the periodate–rosaniline hydrochloride method[17] The gum was also chromatographed[3] on a DEAE-cellulose column (35 × 1 5 cm). Gradient elution with sodium chloride solution (0 02→0 50M), buffered at pH 6, yielded a single, slightly asymmetric peak

Separation and characterisation of neutral sugars —The gum (5 g) was hydrolysed with 2N sulphuric acid (500 ml) for 12 h on a boiling-water bath, and the cooled solution was neutralised with barium carbonate, filtered, treated with Amberlite resin IR-120 (H^+), and concentrated to a syrup The syrup was fractionated on a Duolite A-4 resin column (20 × 2 cm) in the formate form, the neutral sugars being eluted with water (2 l) The neutral fraction was concentrated to a syrup (3 3 g) and chromatographed on a cellulose column (60 × 3 cm) with solvent (*a*), to give three pure monosaccharides

Fraction 1 (900 mg) was D-galactose, m p and mixed m p 118–119°, $[\alpha]_D$ +80° (equil) (*c* 4 0) It gave mucic acid, m p 213–214°

Fraction 2 (1 04 g) was L-arabinose, m p (from aqueous ethanol) and mixed m p 159°, $[\alpha]_D$ +105° (equil) (*c* 3 0) The X-ray diffraction pattern was identical with that of an authentic specimen of L-arabinose The derived phenylosazone had m p 164–165°

Fraction 3 (275 mg) was L-rhamnose, m p (from aqueous ethanol) and mixed m p 93-94°, $[\alpha]_D$ +9° (equil) (*c* 3 0) The X-ray diffraction pattern was identical with that of an authentic specimen of L-rhamnose The derived phenylosazone had m p 181–182° (from water)

Isolation and characterisation of D-*galactose disaccharides* — The gum (30 g) was hydrolysed with 0 01N sulphuric acid (500 ml) for 60 h on a boiling-water bath After dialysis against tap water for 48 h, the solution was adjusted to 0 2N with respect to sulphuric acid and hydrolysed for 4 h on a boiling-water bath After neutralisation, degraded-gum α (6 5 g) was isolated by alcohol precipitation Fig 1 shows the stepwise hydrolysis of degraded-gum α Syrups 1-4 were separated into a neutral fraction and an acidic fraction by ion-exchange chromatography on a column (15 × 2 5 cm) of DEAE-Sephadex G-25 in the formate form The four neutral fractions were combined and concentrated to a syrup (2 6 g), which was chromatographed on Whatman 3MM papers in solvent (*a*) to give two pure disaccharides

Fraction 1 (118 mg) had $[\alpha]_D$ +62° (*c* 2 36) and yielded galactose on acid hydrolysis It had the same R_{Gal} values [0 47 in solvent (*a*) and 0 53 in solvent (*c*)] as 3-*O*-β-D-galactopyranosyl-D-galactose A portion (50 mg) of the disaccharide was methylated G l c examination of the methanolysis products revealed peaks with retention times corresponding to the methyl glycosides of 2,3,4,6-tetra-, and 2,4,6- and 2,5,6-tri-*O*-methyl D-galactose [(*i*) *T* 3 99, (*ii*) *T* 3 22] Hydrolysis of the methyl glycosides, followed by paper-chromatographic examination in solvent (*d*), confirmed the presence of 2,3,4,6-tetra-, and 2,4,6- and 2,5,6-tri-*O*-methyl-D-galactose (R_G 0 86) The disaccharide was crystallised from aqueous acetone to give needles of 3-*O*-β-D-galactopyranosyl-D-galactose monohydrate, m p and mixed m p 157°, its X-ray diffraction pattern was identical with that of authentic material

Fraction 2 (87 5 mg) had $[\alpha]_D$ +28° (*c* 1 70) and yielded galactose on acid hydrolysis It had the same R_{Gal} values [0 29 in solvent (*a*) and 0 38 in solvent (*c*)] as 6-*O*-β-D-galactopyranosyl-D-galactose A portion (40 mg) was methylated and methanolysed to give products corresponding (g l c) to the methyl glycosides of 2,3,4,6-tetra-, and 2,3,5- [(*i*) *T* 4 03, 5 37, (*ii*) *T* 3 32, 4 35] and 2,3,4-tri-*O*-methyl-D-galactose Hydrolysis of the methyl glycosides, followed by paper chromatography in solvent (*d*), confirmed the presence of 2.3.4,6-tetra-, and 2,3,5- (R_G 0 83) and 2,3,4-tri-*O*-methyl-D-galactose The phenylosazone had m p 201–203° (from water)

Isolation and characterisation of 3-O-β-L-arabinofuranosyl-L-arabinose — Chromatography in solvent (*b*) and iodometric titration during a small-scale autohydrolysis at 100° showed that the reaction was essentially complete after 50 h However, a disaccharide released at the beginning of the autohydrolysis had been almost totally broken down after 50 h In order to isolate this disaccharide, the gum (5 g) in water (250 ml) was heated at 80° in a dialysis bag placed in distilled water (1 liter), which was changed at 2-h intervals After 6 h, the autohydrolysate was cooled, and dialysed against distilled water (1 liter) for a further 24 h The combined dialysates were concentrated to a syrup (1 3 g) which was chromatographed on Whatman 3MM papers in solvent (*b*) to give the disaccharide (28 mg), $[\alpha]_D$ +90° (*c* 0 56), which yielded arabinose on acid hydrolysis It had the same paper-chromatographic mobility [R_{Gal} 1 25 in solvent (*b*)] as 3-*O*-β-L-arabinofuranosyl-L-arabinose A portion (10 mg) was methylated and methanolysed to give products corresponding (g l c) to the methyl glycosides of 2,3,5-tri-, and 2,5- and 2,4-di-*O*-methyl-L-arabinose [(*i*) *T* 2 19, 2 26, (*ii*) *T* 1 52, 1 63] Hydrolysis of the methyl glycosides, followed by paper chromatography in solvent (*d*), confirmed the presence of 2,3,5-tri-, and 2,5- and 2,4-di-*O*-methyl-L-arabinose (R_G 0 66) The phenylosazone of the disaccharide had m p 198–199° (from water)

Preparation of degraded-gum A — The gum (60 g) was dissolved in water (1 5 l) and autohydrolysed for 60 h on a boiling-water bath The cooled solution was filtered, and dialysed against distilled water (8 l) Freeze-drying gave degraded-gum *A* (25 g), $[\alpha]_D$ −28° (*c* 1 0) (Found uronic acid, 21, galactose, 76, arabinose, 3%)

Degraded-gum *A* (500 mg) was dissolved in water (100 ml) and reduced with sodium borohydride (500 mg) After dialysis, the freeze-dried product was hydrolysed Paper-chromatographic examination of the hydrolysate in solvent (*e*) indicated a trace of galactitol, but no arabinitol

The diffusate was concentrated to a syrup (33 g) A portion (5 g) was fractionated on a cellulose column (60 × 3 cm) with solvent (*i*) to give two disaccharides, which were purified by further fractionation on Whatman 3MM paper in solvent (*b*)

Fraction 1 (271 mg), $[\alpha]_D$ +148° (*c* 3 0), yielded galactose and arabinose on acid hydrolysis It had the same paper-chromatographic mobility [R_{Gal} 0 71 in solvent (*a*) and 0 51 in solvent (*b*)] as 3-*O*-α-D-galactopyranosyl-L-arabinose A portion (50 mg) was methylated and methanolysed to give products corresponding (g l c) to the methyl glycosides of 2,3,4,6-tetra-*O*-methyl-D-galactose, and 2,5- and 2,4-di-*O*-methyl-L-arabinose [(*i*) *T* 2 20, 2 25, (*ii*) *T* 1 53, 1 61] Hydrolysis of the methyl

glycosides, followed by paper chromatography in solvent (*d*), confirmed the presence of 2,3,4,6-tetra-*O*-methyl-D-galactose, and 2,5- and 2,4-di-*O*-methyl-L-arabinose (R_G 0 67) The phenylosazone of the disaccharide had m p 235° (decomp) (from water)

Fraction 2 (35 mg), $[\alpha]_D$ +180° (*c* 0 70), yielded arabinose on acid hydrolysis It had the same paper-chromatographic mobility [R_{Gal} 0 71 in solvent (*b*) and 0 86 in solvent (*c*)] as 3-*O*-β-L-arabinopyranosyl-L-arabinose A portion (15 mg) was methylated and methanolysed to give products corresponding (g l c) to the methyl glycosides of 2,3,4-tri-, and 2,5- and 2,4-di-*O*-methyl-L-arabinose [(*i*) *T* 2 18, 2 24, (*ii*) *T* 1 50, 1 59] Hydrolysis of the methyl glycosides, followed by paper chromatography in solvent (*d*), confirmed the presence of 2,3,4-tri-, and 2,5- and 2,4-di-*O*-methyl-L-arabinose (R_G 0 68) The phenylosazone of the disaccharide had m p 233–234° (decomp) (from ethanol)

Methylation of degraded-gum A — Degraded-gum *A* (5 g) was methylated to give a product (3 8 g) (Found OMe, 39 1%, not raised by further attempted methylation) A portion (50 mg) was methanolysed to give the *O*-methyl sugars listed in Table II Hydrolysis of the methyl glycosides, followed by paper-chromatographic examination in solvents (*d*) and (*f*), indicated the presence of 2,6-di- and 2-*O*-methyl-D-galactose, in addition to those *O*-methyl sugars already characterised as their methyl glycosides

TABLE II

EXAMINATION OF METHANOLYSIS PRODUCTS FROM METHYLATED, DEGRADED-GUM *A*

O-Methyl sugars	*Approximate, relative molar proportions*	*Relative retention times* (T) *of methyl glycosides*				R_G *in solvent* (f)
		Column (i)		*Column* (ii)		
2,3,5-Tri-*O*-methyl-L-arabinose	Trace	0 58,	0 72	0 46	0 64	—
2,3,4-Tri-*O*-methyl-L-arabinose	Trace	0 98		0 85		—
2,3,4,6-Tetra-*O*-methyl-D-galactose	+ +	1 67		1 64		0 78
2,4,6-Tri-*O*-methyl-D-galactose	+ +	3 75	4 20	2 98	3 46	0 39
2,3,4-Tri-*O*-methyl-D-galactose	+ +	6 45		5 20		0 33
2,4-Di-*O*-methyl-D-galactose	+ + +	14 6	16 4	9 9	11 3	0 11
2,6-Di-*O*-methyl-D-galactose	Trace	—		—		0 18
2,3,4-Tri-*O*-methyl-D-glucuronic acid[a]	+ + +	2 21	2 97	2 09	2 70	—
2-*O*-Methyl-D-galactose	+	—		—		0 05

[a]As methyl ester methyl glycoside

Smith degradation of degraded-gum A — Degraded-gum *A* (15 g) was dissolved in 0 25M sodium metaperiodate solution (1 5 l) and left in darkness After 96 h, the reaction was stopped by addition of ethylene glycol (30 ml) The solution was dialysed against running tap-water for 2 days Sodium borohydride (4 g) was added, and the mixture kept at room temperature for 30 h After dialysis for a further 2 days, the

polyalcohol was hydrolysed in N sulphuric acid for 2 days at room temperature The solution was then neutralised with barium carbonate, filtered, deionised with Amberlite resin IR-120 (H^+), and dialysed against distilled water (2 l) The syrup obtained from the dialysate was shown by paper chromatography in solvent (*d*) to contain glycerol and glycolaldehyde After further dialysis against running tap-water for 2 days, degraded-gum *B* (1.25 g, 8 4%) was isolated as the freeze-dried product.

Partial acid hydrolysis and methylation of degraded-gum B — Degraded-gum *B* (20 mg) was hydrolysed with 0 5N sulphuric acid (10 ml) for 1 h on a boiling-water bath The solution was neutralised with barium carbonate, filtered, deionised with Amberlite resin IR-120 (H^+), and concentrated Paper-chromatographic examination in solvent (*a*) indicated the presence of galactose, 3-*O*-β-D-galactopyranosyl-D-galactose, and a trace of 6-*O*-β-D-galactopyranosyl-D-galactose

Degraded-gum *B* (500 mg) was methylated and methanolysed to give the methyl glycosides shown in Table III Hydrolysis of the methyl glycosides, followed by paper chromatography in solvents (*d*) and (*f*), indicated the presence of 2,6-di- and 2-*O*-methyl-D-galactose, in addition to those *O*-methyl sugars already characterised by g l c of their methyl glycosides

TABLE III

EXAMINATION OF METHANOLYSIS PRODUCTS FROM METHYLATED, DEGRADED-GUM *B*

O-Methyl sugars	*Approximate, relative molar proportions*	*Relative retention times* (T) *of methyl glycosides*		R_G *in solvent* (f)
		Column (i)	*Column* (ii)	
2,3,4,6-Tetra-*O*-methyl-D-galactose	+	1 68	1 67	0 78
2,4,6-Tri-*O*-methyl-D-galactose	+ + + + + +	3 72, 4 16	2 99, 3 44	0 39
2,3,6-Tri-*O*-methyl-D-galactose	Trace	—	—	0 49
2,3,4-Tri-*O*-methyl-D-galactose	Trace	6 40	5 26	0 33
2,4-Di-*O*-methyl-D-galactose	+ +	14 6 16 4	9 8 11 1	0 11
2,6-Di-*O*-methyl-D-galactose	+	—	—	0 18
2-*O*-methyl-D-galactose	+	—	—	0 05

Methylation of A laeta *gum* — The gum (500 mg) was methylated to give a product (410 mg), $[\alpha]_D$ −49° (*c* 1 0, chloroform) (Found OMe, 42 1%) Methanolysis gave the methyl glycosides shown in Table IV Hydrolysis of the methyl glycosides, followed by paper chromatography in solvent (*d*), indicated the presence of 2-*O*-methylgalactose, in addition to those *O*-methyl sugars already characterised by g l c of their methyl glycosides

Preparation, partial acid hydrolysis, and methylation of polysaccharide **1** — The gum (28 3 g of polysaccharide) was dissolved in water (700 ml), mixed with 0 25M sodium metaperiodate (700 ml), and left for 48 h The amount of formic acid released in this time was 1 40 mmoles/g, and the periodate consumed was 5 24 mmoles/g The reaction was stopped by addition of ethylene glycol (15 ml), and the solution

was dialysed against running tap-water for 48 h Sodium borohydride (7 5 g) was added, and the mixture kept for 30 h at room temperature, and then dialysed for a further 48 h The polyalcohol was hydrolysed in N sulphuric acid for 48 h at room temperature

TABLE IV

EXAMINATION OF METHANOLYSIS PRODUCTS FROM METHYLATED *A laeta* GUM

O-*Methyl sugars*	*Relative retention times* (T) *of methyl glycosides*		R$_G$ *in solvent* (d)
	Column (I)	*Column* (II)	
2,3,4-Tri-*O*-methyl-L-rhamnose	0 50	0 45	1 01
2,3,5-Tri-*O*-methyl-L-arabinose	0 60, 0 72	0 52, 0 62	0 98
2,3,4-Tri-*O*-methyl-L-arabinose	1 03	0 82	0 78
2,5-Di-*O*-methyl-L-arabinose	1 81, 3 18	1 27,(2 16)[a]	0 80
2,3,4-6-Tetra-*O*-methyl-D-galactose	1 70	1 67	0 88
2,4,6-Tri-*O*-methyl-D-galactose	3 83 4 27	2 92 3 45	0 72
2,3,4-Tri-*O*-methyl-D-galactose	6 66	5 09	0 72
2,4-Tri-*O*-methyl-D-galactose	14 7, 16 6	9 8, 11 2	0 52
2,3,4-Tri-*O*-methyl-D-glucuronic acid[b]	2 34 2 91	(2 16),2 70	—
2,3-Di-*O*-methyl-D-glucuronic acid[b]	7 8 9 5	5 8, 6 8	—
2-*O*-methyl-D-galactose	—	—	0 32
Unknown peaks	1 19, 1 45	0 98, 1 13	—

[a]Figures in parentheses indicate *T* values of components not completely resolved [b]As methyl ester methyl glycoside

After dialysis against running tap-water for 48 h, polysaccharide **1** was isolated as the freeze-dried product (17 0 g, 60%), $[\alpha]_D$ −18° (*c* 0 82) (Found uronic acid, 4, galactose, 68, arabinose, 28%) Hydrolysis of polysaccharide **1** with N sulphuric acid for 7 h on a boiling-water bath gave a trace of 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose [R_{Gal} 0 22 in solvent (*b*)] in addition to galactose and arabinose Molecular-sieve chromatography[4 5 10 11] of polysaccharide **1** (20 mg) on a previously calibrated "Bio-Gel P 300" column (45 × 4 8 cm) gave $\bar{M}_n$ ~91,000

Polysaccharide **1** (20 mg) was hydrolysed with 0 5N sulphuric acid (10 ml) for 1 h on a boiling-water bath Paper-chromatographic examination of the hydrolysate indicated the presence of galactose, arabinose, and two neutral disaccharides having the mobilities of 3-*O*-β-D-galactopyranosyl-D-galactose [R_{Gal} 0 49 in solvent (*a*) and 0 54 in solvent (*c*), minor component] and 6-*O*-β-D-galactopyranosyl-D-galactose [R_{Gal} 0 31 in solvent (*a*) and 0 38 in solvent (*c*), major component]

Polysaccharide **1** (200 mg) was methylated to give a product (125 mg), $[\alpha]_D$ −43° (*c* 1 0, chloroform) (Found OMe, 39 5%, not raised on further methylation) (see Table VI) Methanolysis of a sample of this product, followed by g l c examination of the mixture of methyl glycosides, gave the results shown in Table V

Preparation, partial acid hydrolysis, and methylation of polysaccharides **2–8** — The following weights of polysaccharide were periodate oxidised, reduced with

borohydride, hydrolysed, and the corresponding degraded-polysaccharides were recovered as for the first Smith-degradation Polysaccharide **1** (16 5 g) gave polysaccharide **2** (8 75 g), **2** (8 35 g) → **3** (5 0 g), **3** (4 6 g) → **4** (3 0 g), **4** (2 6 g) → **5** (1 9 g); **5** (1 55 g) → **6** (1.2 g); **6** (0 86 g) → **7** (0 68 g), **7** (0 63 g) → **8** (0 49 g) The relevant data are given in Table I

TABLE V

O-METHYL SUGARS PRESENT IN METHYLATED, SMITH-DEGRADED POLYSACCHARIDES **1–6** AND **8**

O-Methyl sugars	**1**	**2**	**3**	**4**	**5**	**6**	**8**	*Approximate relative molar proportions in* **5**	**6**	**8**
2,3,5-Tri-*O*-methyl-L-arabinose	+	+	+	+						
2,5-Di-*O*-methyl-L-arabinose	+	+	+							
2,3,4,6-Tetra-*O*-methyl-D-galactose	+	+	+	+	+	+	+	1	1	1
2,4,6-Tri-*O*-methyl-D-galactose	+	+	+	+	+	+	+	14	16	18
2,3,4-Tri-*O*-methyl-D-galactose	+	+	+	+	+	+	+	Trace	Trace	Trace
2,6-Di-*O*-methyl-D-galactose	+	+	+	+	+	+	+	3	1	Trace
2,4-Di-*O*-methyl-D-galactose	+	+	+	+	+	+	+	1	1	1
2 3,4-Tri-*O*-methyl-D-glucuronic acid	+									

Partial, acid hydrolysis of polysaccharide **2** (20 mg), followed by paper-chromatographic examination of the hydrolysate, indicated the presence of equal amounts of 3-*O*-β-D-galactopyranosyl-D-galactose and 6-*O*-β-D-galactopyranosyl-D-galactose, and equal amounts of the β-(1→3)-linked [R_{Gal} 0 22 in solvent (*a*) and 0 23 in solvent (*c*)] and the β-(1→6)-linked D-galactose trisaccharide [R_{Gal} 0 15 in solvent (*a*) and 0 18 in solvent (*c*)] Partial, acid hydrolysis of polysaccharides **3** and **4** gave the same oligosaccharides as obtained from polysaccharide **2** However, the proportions of the β-(1→6)-linked di- and tri-saccharides decreased steadily from polysaccharides **2** to **4** Partial, acid hydrolysis of polysaccharides **5** to **8** indicated the presence of the β-(1→3)-linked di- and tri-saccharides, and the β-(1→6)-linked disaccharide The proportion of the latter disaccharide continued to fall from polysaccharides **5** to **8**, only a trace was obtained from polysaccharide **8**

Polysaccharides **1–6** and **8** were methylated (Table VI) Methanolysis of a sample of the products, followed by g l c examination of the mixture of methyl glycosides, gave the results shown in Table V

Preparation, and partial acid hydrolysis of polysaccharide **5′** — *A laeta* gum (30 g) was subjected to five successive Smith-degradations, without isolation of the intermediate polysaccharides, to obtain polysaccharide **5′** (2 2 g) The polysaccharide was hydrolysed with 0 5N sulphuric acid (500 ml) for 1 h on a boiling-water bath The hydrolysate was chromatographed on Whatman 3MM papers in solvent (*a*), and a pure trisaccharide was isolated

The trisaccharide (44 mg), $[\alpha]_D$ +51° (*c* 0 88), yielded galactose and 3-*O*-β-D-galactopyranosyl-D-galactose, as the only disaccharide, on partial hydrolysis with

TABLE VI

METHYLATION OF SMITH-DEGRADED POLYSACCHARIDES

	Polysaccharide						
	1	**2**	**3**	**4**	**5**	**6**	**8**
Amount of polysaccharide used (mg)	200	193	165	158	184	180	250
Amount of product (mg)	125	120	80	85	76	65	81
$[\alpha]_D$ of product (degrees)	−43	−33	−11	−1	−1	+1	+1
OMe (%)	39 5	41 3	39 1	37 2	37 1	36 8	36 5

acid The trisaccharide (crystallised from aqueous ethanol) had m p and mixed m p 237–242° (decomp), and R_{Gal} values of 0 22 in solvent (*a*) and 0 23 in solvent (*c*)

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REFERENCES

1 Part XXVIII D M W ANDERSON AND I C M DEA, *Carbohyd Res* , 7 (1968) 109
2 D M W ANDERSON AND R N SMITH, *Carbohyd Res* , 4 (1967) 55
3 M A JERMYN, *Austral J Biol Chem* , 15 (1962) 789
4 D M W ANDERSON, SIR EDMUND HIRST, AND J F STODDART, *J Chem Soc* (*C*), (1966) 1959
5 D M W ANDERSON AND J F STODDART, *Carbohyd Res* 2 (1966) 104
6 F SMITH, *J Chem Soc* , (1939) 744, 1724, (1940) 1035, J JACKSON AND F SMITH, *ibid*, (1940) 74, 79
7 D M W ANDERSON, SIR EDMUND HIRST AND J F STODDART, *J Chem Soc* (*C*), (1967) 1476
8 D M W ANDERSON AND G M CREE, *Carbohyd Res* , 6 (1968) 385
9 D M W ANDERSON AND I C M DEA unpublished results
10 D M W ANDERSON, I C M DEA, S RAHMAN, AND J F STODDART, *Chem Commun* , (1965) 145
11 D M W ANDERSON AND J F STODDART, *Anal Chim Acta*, 34 (1966) 401, *Lab Pract* , 16 (1967) 841
12 I J GOLDSTEIN, G W HAY, B A LEWIS, AND F SMITH, *Methods Carbohyd Chem* , 5 (1965) 361
13 P ANDREWS AND J K N JONES, *J Chem Soc* , (1955) 583
14 J K N JONES, *J Chem Soc* , (1953) 1672
15 H KLOSTERMAN AND F SMITH, *J Amer Chem Soc* , 74 (1952) 5336
16 H B OAKLEY, *Trans Faraday Soc* , 31 (1935) 136, 32 (1936) 1360, 33 (1937) 372
17 A B S CONACHER AND D I REES, *Analyst*, 91 (1966) 55
18 W N HAWORTH, *J Chem Soc* , 107 (1915) 8
19 R KUHN, H TRISCHMANN, AND I LOW, *Angew Chem* , 67 (1955) 32
20 T PURDIE AND J C IRVINE, *J Chem Soc* , 83 (1903) 1021

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CONFORMATIONAL ANALYSIS OF CELLOBIOSE, CELLULOSE, AND XYLAN

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ABSTRACT

All of the likely conformations of cellobiose, cellulose, and xylan have been explored systematically by using an electronic computer, assuming the ring conformations and (C-1)–O–(C-4′) angle for each pair of residues to be fixed and derivable from known crystal structures The absolute van der Waals energies, but not the relative energies of different conformations, are sensitive to the choice of energy functions and atomic coordinates The conformation of cellobiose in the crystal occurs near the minimum for intramolecular van der Waals interactions, perhaps slightly displaced to allow formation of an inter-residue hydrogen bond The Hermans chain conformation for cellulose corresponds to a somewhat higher van der Waals energy, but this is probably offset by efficient crystal packing resulting from the two fold screw axis, and the hydrogen bond remains. The Meyer and Misch conformation is unlikely, because of its high van der Waals energy and for other reasons The results lead to possible explanations of the known conformational stiffness of cellulose and its solubility properties in alkali The characteristics of xylan conformations have been compared with cellulose

INTRODUCTION

The molecular structure of cellulose was determined over thirty years ago, to a large extent through the brilliant researches of Sir Edmund Hirst and his colleagues[1] It was one of the first polymer structures to be established and was important for subsequent development of polymer theory In contrast, and despite much research effort, the conformation and crystal structure of cellulose are still unsolved Most workers favour the "Hermans" or "bent chain" conformation[2] rather than the conformation proposed earlier by Meyer and Misch[3], but other forms have also been suggested[4], and it is possible that none is correct because each is based on the assumption that the cellulose chain has a two-fold screw axis which, strictly, is unnecessary[5] Outside carbohydrate chemistry, the calculation of conformational energies is becoming accepted as a valid approach to the determination of conformations Except for very simple molecules, such as hydrocarbons[6], the methods are not yet refined to the extent that they can give final answers without the need to invoke

experimental evidence, but accurate predictions have been possible for some synthetic polymers[7] and polypeptides[8–12] Pioneer work with polysaccharides by Ramachandran *et al.*[13 14], and related approaches[15,16], are described in more detail below In this paper, we report on calculations which confirm that the method has promise for polysaccharides and which add further evidence in favour of the Hermans conformation for cellulose. Some unusual physical properties of cellulose are discussed in the light of the results

PRINCIPLE OF THE METHOD

We assume that solid-state and solution conformations can be understood from consideration of intramolecular interactions This is reasonable, because the same assumption has led to useful results for other polymers[7–12] and because the properties of cellulose suggest that intramolecular interactions are especially important[17] Present evidence suggests that there are standard values which can be assumed for bond angles and bond lengths in carbohydrates[13 18], and that the common sugar residues in polysaccharides will favour the *C1* conformation The overall conformation of the chain would then depend only on the angles of rotation about the two bonds to each glycosidic oxygen atom The rotational states about these bonds were therefore explored systematically, as described below, as an aid to selecting the most likely conformations for testing against experimental evidence

A set of coordinates was chosen for cellobiose, based on crystal structure data (see below) New coordinates were then calculated from these to correspond to various angles of rotation about the (C-1)–O and O–(C-4′) bonds This was done by computer, in such a way that all possible conformations were sampled The new sets of coordinates were used to characterise each conformation in several ways (*i*) The distances between all relevant pairs of atoms were calculated, and, on this basis, each conformation was classified as either *fully allowed* (implying no infringement of van der Waals radii), *marginally allowed* (implying slight compression of some atoms or groups), or *disallowed* (implying that atoms or groups are brought so close that severe van der Waals repulsion is expected) (*ii*) To find the van der Waals energy minimum, each interatomic distance was used to calculate the corresponding energies of van der Waals attraction and repulsion, and these values were summed over all interactions for each allowed conformation (*iii*) The possibility of intramolecular hydrogen bonding was assessed from the (O-5)–(O-3′) distance (*iv*) For all conformations, the two D-glucose residues can be considered to be related by an *n*-fold screw axis, where *n* need not be an integer Values of *n* were calculated for selected conformations (*v*) The length of the disaccharide projected on the screw axis, *h*, was also calculated for selected conformations

PARAMETERS AND MATHEMATICAL METHODS

The sensitivity of the results to inaccuracy in the atomic coordinates was assessed by repeating the calculations for six sets which were as follows *Set 1* was

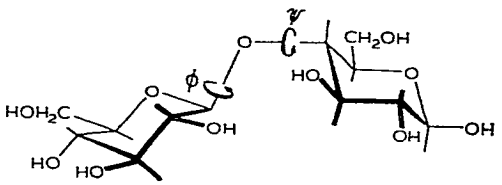
obtained by averaging the coordinates listed by Ramachandran *et al*[13] for various β-D-glucose residues, and taking a value of 117° for the (C-1)–O–(C-4′) angle. *Set 2* was derived from the crystal structure coordinates of Brown[19], for which the (C-1)–O–(C-4′) angle is 116 7° *Set 3* was obtained by modification of Brown's coordinates[19] to correspond to a (C-1)–O–(C-4′) angle of 117°. *Set 4* was derived from the refined crystal structure coordinates of Chu and Jeffrey[20], for which the (C-1)–O–(C-4′) angle is 116 1° *Set 5* was obtained by modification of Chu and Jeffrey's coordinates to correspond to a (C-1)–O–(C-4′) angle of 116 7° *Set 6* was obtained by modification of Chu and Jeffrey's coordinates to correspond to a (C-1)–O–(C-4′) angle of 117°.

Each set was transferred to new axes which had the bridge oxygen as origin, the O–(C-4′) bond was the x axis for the reducing residue and the (C-1)–O bond produced was the x axis for the nonreducing residue The xz planes were defined by (O-4′)–(C-4′)–(H-4′) and (O-4′)–(C-1)–(H-1), respectively, with the y axes perpendicular to these planes to form right-handed systems of axes These transformations were done in stages by using standard expressions for the rotation and translation of axes The systematic rotations about bonds to the bridge oxygen, followed by calculation of all interatomic distances and the projected residue length, h, were performed on the English Electric KDF9 computer with programs written in Atlas Autocode These calculations, and those for n, were based on the principles outlined by Ramakrishnan[12] For calculations of n and h, (C-1)–(O-1) in each residue was taken to be parallel to (C-4)–(O-4), and (C-1)–H to be coplanar with (C-4)–H This is inexact, but leads to simple expressions for n and h and makes no difference to the energy maps The exact approach is, in any case, impossible when coordinates are taken, as in this work, from disaccharide structures in which the residues differ in detailed geometry Comparison with results derived rigorously showed that any error was likely to be within the accuracy of experimental data for polysaccharide fibres, at least for conformations examined here From their published results[13], it would seem that Ramachandran *et al* also used this approximation. The exact expressions would have to be used for higher screw symmetries, because serious errors would otherwise result

The increments of rotation were 2° for energy calculations, but otherwise 10° The angles of rotation ϕ and ψ (see **1**), were the dihedral angles between (C-1)–(H-1) and (O-4′)–(C-4′), and between (O-4′)–(C-1) and (C-4′)–(H-4′), respectively. The conformation $\phi = \psi = 0$ is defined as having H-4′ and H-1 in the same plane as (C-1)–(O-4′)–(C-4′), and the apex of the (C-1)–(O-4′)–(C-4′) angle pointing in the same sense as the (C-4′)–(H-4′) bond and in the opposite sense from (C-1)–(H-1) To convert a model of cellobiose, in this form, into any conformation (ϕ, ψ), the reducing residue is held stationary and, viewed from the nonreducing end, the nonreducing residue is rotated anticlockwise through ψ about (C-4′)–(O-4′) and clockwise through ϕ about (O-4′)–(C-1)

The interatomic distances, which were used to classify the conformations, are listed in Table I The computer program was written in such a way that *one* inter-

atomic distance within the "fully allowed" limit was sufficient to classify a conformation as marginally allowed, unless any distance was within the "marginally allowed" limit, when it was classified as disallowed



Scheme for variation of bridge-angle geometry in cellobiose
$\phi = \psi = 0°$
Rotations are positive when each residue is rotated in the direction shown about its bond to the bridge oxygen

TABLE I

INTERATOMIC DISTANCES USED FOR THE HARD SPHERE CALCULATIONS[a]

Atom pair		*Minimum distance (Å) allowed for*	
		Fully allowed contact	*Marginally allowed contact*
C	C	3 20	3 00
C	O	2 80	2 70
C	H	2 40	2 20
O	O	2 80	2 70
O	H	2 40	2 20
H	H	2 00	1 90
H	CH_2OH	2 42	2 42
O	CH_2OH	2 90	2 90
C	CH_2OH	3 20	3 20

[a]Hydrogen bonding distances are given separately in the Figure captions

Van der Waals interactions are normally expressed by functions of either the Lennard-Jones type (potential energy, $V = -A/r^6 + B/r^{12}$, where r is the interatomic distance, and A and B are constants), or the Buckingham type ($V = -A/r^6 + Be^{-\mu r}$, where A, B, and μ are constants) The constants may be different for each atom pair and have to be derived empirically We have made the additional approximation that the hydroxymethyl group is treated as equivalent to a methyl group in its van der Waals interactions, on the grounds that the hydroxyl could probably rotate away from any region of steric overcrowding The following three sets of functions were used to calculate the conformational energies reported in this paper. (*i*) Those given by Liquori and his coworkers[8], which include functions of both types These are referred to as "Liquori functions" (*ii*) The functions proposed by Brant and Flory[21], which are of the Buckingham type with $\mu = 4\,6$ and A and B derived for each atom pair.

We used the constants given by Rao *et al* [14], together with the following values which were calculated using the methods of earlier workers

for H CH_3 interactions, $A = 718\,5$, $B = 5\,31 \times 10^5$
for O CH_3 interactions, $A = 2016\,0$, $B = 3\,10 \times 10^6$
for C CH_3 interactions, $A = 2605\,0$, $B = 6\,75 \times 10^6$

These will be referred to as the "Flory functions" (*iii*) The functions proposed by Kitaygorodsky[22], which are of the Buckingham type, but expressed thus

$$V = 3\,5(-0\,04/z^6 + 8\,6 \times 10^3 e^{-13z}), \text{ where } z = r/r_0$$

Only one constant, r_0, is dependent on the particular atom pair. The values of r_0 listed by Rao *et al* [14] were used, together with the following values which were derived geometrically

for H . CH_3 interactions, $r_0 = 3\,33$
for O CH_3 interactions, $r_0 = 3\,76$
for C CH_3 interactions, $r_0 = 3\,92$

These will be referred to as the "Kitaygorodsky functions".

The dihedral angles ϕ and ψ calculated for cellobiose in the crystalline state from the data of Brown[19], and of Chu and Jeffrey[20], will be referred to as the cellobiose (*B*) and cellobiose (*J*) conformations, respectively The values for a cellulose chain with a two-fold screw axis and projected residue repeat of 5 15 Å were taken as those at the intersections of the curves which represent $n = 2$ and $h = 5\,15$, respectively (see below) Two solutions exist for each set of coordinates One resembles the bent-chain conformation[2] in general features and will be referred to as cellulose (*H*) The Meyer and Misch conformation[3] is referred to as cellulose (*M*), although this is quite close to the second solution, the dihedral angles are taken as (0°, 180°) for all sets of coordinates, for reasons given below

All potential energies are given in Kcal/mole per disaccharide residue

RESULTS AND DISCUSSION

Several attempts at exploration of polysaccharide conformations have been reported previously Jones[15] attempted to refine the crystal structure of cellulose by an ingenious approach to systematic model building Ramachandran *et al.*[13] have used mathematical methods to examine some of the possible cellulose conformations, with interatomic distances as criteria Calculation was by hand, however, and it was therefore feasible to include only a few atoms and to cover only part of the conformational map More recently, the same group[14] has made an important study of amylose, in which conformational energies were calculated by computer Setterneri and Marchessault[16] have used computer methods to search for the xylan conformation which best satisfies stereochemical criteria and experimental evidence from X-ray diffraction and infrared dichroism.

A preliminary assessment of the types of conformation which are possible for a given polymer is most easily and quickly made by treating the atoms as "hard spheres" which are incompressible beyond certain limits The results are expressed in the form of a "conformational map" which shows the combinations of ϕ and ψ permitted by these criteria The map may also include the calculated properties of each range of allowed conformations, such as screw symmetry, projected repeat distance, and likelihood of hydrogen bonding within the chain Fig 1 shows results which represent the restriction of possible cellulose conformations by interactions between C-1 and C-3′, C-1 and C-5′, O-5 and C-4′, and C-2 and C-4′ The same contacts were examined over a limited region of the map by Ramachandran *et al*[13], and our results agree with theirs When *all* relevant contacts were included, the allowed conformations occupied smaller areas (Fig 2) Conformations were sampled at 10° intervals about each of the C-O bonds, so that a total of $36^2 = 1296$ representative conformations were examined Only 24 fully allowed conformations were found, and only a further 21 were marginally allowed, *i e*, 96% of the conformations are excluded! The results seem to explain the stiffness of the cellulose chain, which has often been noted[17], and are consistent with other available evidence The accepted crystal conformation of chitin[23] and the most-favoured cellulose conformation[2] each correspond to (−40°, 140°), which is almost within the allowed area In Fig 2, this point is outside by only one 10° increment in ϕ, and recalculation at 2° intervals showed it to be less than 2° outside With other sets of coordinates, it was actually inside The crystal conformation of cellobiose[19 20] is in the allowed area (Fig 2) Although atomic coordinates have not been published, inspection of models and photographs[24] of models of the lysozyme complex with tri-*N*-acetylchitotriose [*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamido-2-deoxy-D-glucose] shows that each glycosidic linkage in the trisaccharide has a conformation which is close to cellobiose in the crystal and therefore within the allowed region Finally, p m r spectroscopy[25] suggests an intramolecular hydrogen bond between O-5 and H-3′ for cellobiose in methyl sulphoxide solution, and this is also consistent with calculation because this hydrogen bond is possible for allowed conformations (compare Figs. 1 and 2) Reasons why all of these conformations should be "marginally allowed", rather than "fully allowed", are discussed below

The scope of the hard-sphere approach is limited because it does not show which parts of an allowed area are likely to be preferred, and therefore the polymer conformation is only defined approximately To go further, it would be desirable to search for free energy minima, by estimating and summing the free energies of all intramolecular interactions for each conformation, including dipole interactions, hydrogen bonding, torsional strain, and van der Waals attraction and repulsion Such intermolecular interactions as solvation and crystal packing ought also to be considered This is a formidable undertaking which, it seems to us, would best be approached by successive approximation The severe steric restrictions indicated by hard-sphere calculations and by experiment[17] suggest that, for cellulose, intramolecular van der

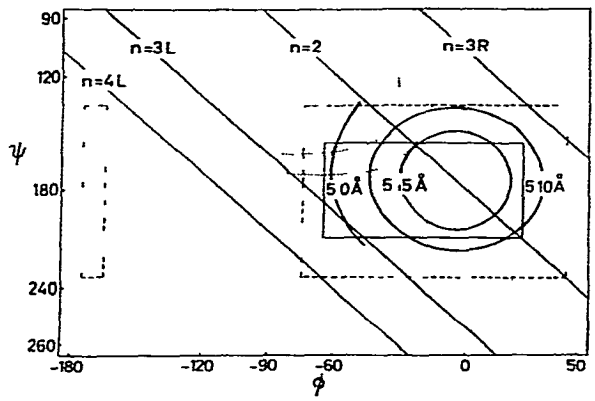


Fig 1 Restriction of cellulose conformations by interactions between C-1 and C-3 , C-1 and C-5′, O-5 and C-4′, and C-2 and C-4′, based on *Set 1* coordinates modified to correspond to a (C-1)–O–(C-4′) angle of 117 5° Fully and marginally allowed conformations lie within the solid and broken rectangles respectively The limits of (O-5) (O-3′) hydrogen bonding, assuming that this requires the O O distance to be 2 50–2 80 Å, are shown by dotted curves Conformations with the same screw axis lie on the diagonals and those with the same projected repeat distance on the solid curves The coordinates differ slightly from those used earlier[13] (see text) The angle of rotation, ϕ, is equivalent to the ϕ of Ramachandran *et al* [13], and $\psi = 360° - \phi$

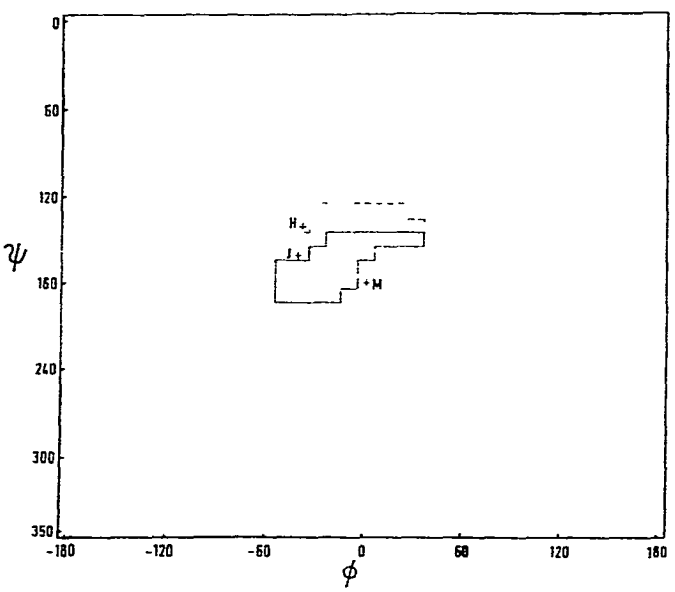


Fig 2 Allowed (enclosed by solid line) and marginally allowed (broken lines) cellulose conformations, using *Set 4* coordinates with all relevant contacts considered Cellobiose (*J*), cellulose (*H*), and cellulose (*M*) are marked as *J*, *H*, and *M*, respectively

Waals interactions (especially repulsion) might be particularly important. At this stage of the investigation, we have therefore attempted a quantitative approach to van der Waals potential energy, while considering other interactions qualitatively

All of the available van der Waals functions are semi-empirical, having been derived by fitting the constants in a theoretical equation to experimental data The three sets of equations used here were derived by somewhat different fitting procedures and are therefore to some extent independent Each has been used successfully in conformational analysis before[7 8 14 21 26] Calculations for cellulose, with each function in turn, gave the results shown as energy maps in Figs 4, 5, and 6 The positions of Flory and Kitaygorodsky minima agree tolerably (though not exactly), and also seem reasonable because they lie well within the allowed region The outer energy contours calculated with these functions are similar in shape to each other and to the outline of the allowed region (Fig. 3), which also makes the results seem plausible In contrast, the Liquori minima lie on the edge of the allowed region (Fig 6), and the contours have an unexpected shape (Fig 3), these results therefore seem less reliable The most serious discrepancies between the Flory and Kitaygorodsky functions are in absolute energies at the minima and other conformations (Table II) Earlier workers have also found that Flory functions give substantially lower energies, both for carbohydrate and peptide conformations[14 26] By comparison with experiment, energies calculated with Kitaygorodsky functions would seem to be more accurate for carbohydrates[14]. However, our chief concern at present is with *relative* energies of different conformations, for which all three sets of functions fortunately lead to similar predictions (see below) The results are also influenced by the choice of atomic coordinates With different sets of crystal coordinates and a given set of functions, the energy minimum varied in position by up to 8° in ϕ and ψ, and energies corresponding to particular conformations varied by several tenths of 1 Kcal and sometimes more (Table II) The trends in the results do not suggest that the variations could be attributed simply to changes in the (C-1)–O–(C-4′) bond angle (Table II) Several conclusions emerge irrespective of the coordinates and energy functions however, and will now be discussed

There seem to be two minima within the allowed area, and these have similar energies (Figs 4–6, Table II) The cellobiose conformation of Chu and Jeffrey[20] and that of Brown, which is only slightly different[19], both correspond to energies that are above the minimum by small amounts, which are calculated with the Flory and Kitaygorodsky functions as a fraction of 1 Kcal. Estimates with the Liquori functions are larger, but otherwise in agreement with this trend The equivalent result with the hard-sphere method was that crystal conformations occurred in the "marginally" rather than "fully" allowed region However, calculation also showed (*e g*, Fig 7) that O-3′ and O-5 are sufficiently close to hydrogen bond, the equilibrium distance between these atoms is therefore smaller than for the general case, and the conformational energy should be corrected accordingly Although the correction turns out to be negligible for the Flory functions, it is -0.2 to -0.5 Kcal for the Kitaygorodsky functions, and this brings the van der Waals energy very close to the calculated minimum With the added advantage of a strong, inter-residue hydrogen bond, this conformation would seem to have more favourable intramolecular interactions than any alternative It can also form a fully hydrogen-bonded lattice[19 20] It seems likely

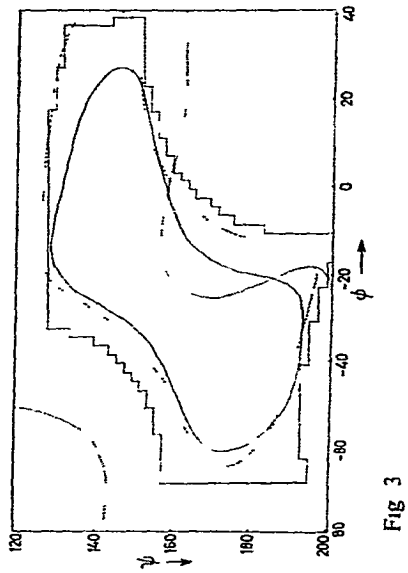


Fig 3

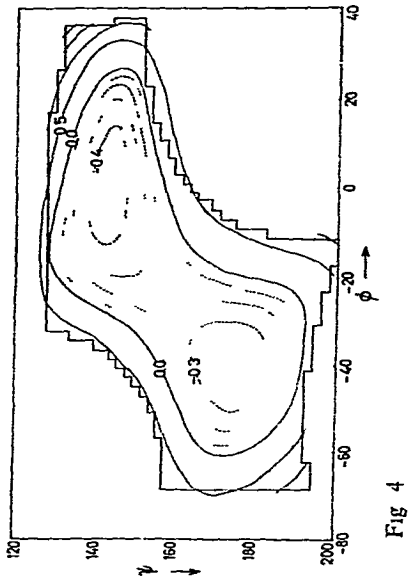


Fig 4

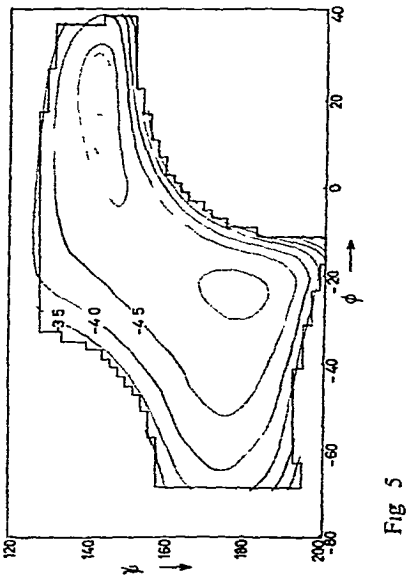


Fig 5

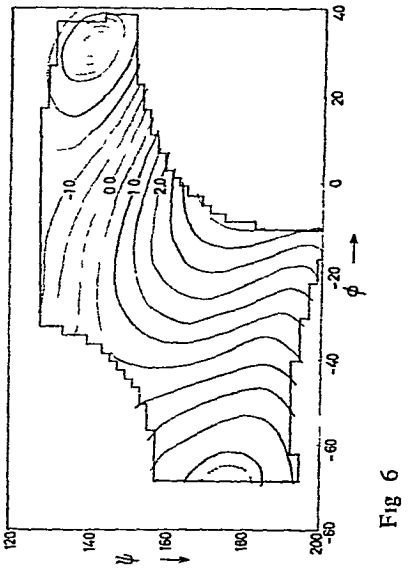


Fig 6

Fig 3 Entire, allowed area for cellulose conformations (boxed by solid straight lines), compared with 0 Kcal Kitaygorodsky contour (solid curve), −4 Kcal Flory contour (broken curve), and +2 Kcal Liquori contour (dotted curve), for *Set 4* coordinates

Fig 4 Van der Waals energy map for cellulose, using Kitaygorodsky functions and *Set 4* coordinates The entire allowed area predicted by hard-sphere calculations, is boxed by solid straight lines Broken curves represent contours at intervals of 0 1 Kcal Solid curves represent contours at intervals of 0 5 Kcal

Fig 5 Van der Waals energy map for cellulose, using Flory functions and *Set 4* coordinates Other details as for Fig 4

Fig 6 Van der Waals energy map for cellulose, using Liquori functions and *Set 4* coordinates Other details as for Fig 4

TABLE II

DIHEDRAL ANGLES AND VAN DER WAALS ENERGIES CALCULATED FOR CELLOBIOSE AND CELLULOSE CONFORMATIONS

Energy functions	*Conformation*	*Coordinates used*																		
		Set 1			Set 2			Set 3			Set 4			Set 5			Set 6			
		ϕ,°	ψ,°	V (*Kcal*)	ϕ,°	ψ,°	V (*Kcal*)	ϕ,°	ψ,°	V (*Kcal*)	ϕ,°	ψ,°	V (*Kcal*)	ϕ,°	ψ,°	V (*Kcal*)	ϕ,°	ψ,°	V (*Kcal*)	
Flory	Minima	−31	+181	−4 9	−27	+184	−5 2	−29	+184	−5 2	−24	+175	−5 0	−24	+181	−5 1	−24	+181	−5 1	
		+19	+149	−5 0	+21	+147	−5 1	+22	+148	−5 2	+23	+141	−5 2	+23	+142	−5 3	+24	+141	−5 3	
Kitay-gorodsky	Minima	−46	+180	0	−41	+181	−0 5	−43	+181	−0 6	−41	+175	−0 3	−40	+174	−0 4	−40	+175	−0 5	
		+ 5	+147	+ 0 4	+ 1	+147	−0 3	+ 1	+147	−0 4	0	+143	−0 4	0	+143	−0 5	0	+143	−0 6	
Liquori	Minima	−76	+175	−1 1	−70	+182	−2 2				−72	+176	−1 7							
		+31	+147	−1 0	+32	+146	−2 5				+32	+139	−2 8							
Flory	Cellobiose (*B*)	−44	+168	−4 4	−44	+168	−4 6	−44	+168	−4 6	−44	+168	−4 6	−44	+168	−4 6	−44	+168	−4 6	
	Cellobiose (*J*)	−42	+162	−4 1	−42	+162	−4 3	−42	+162	−4 3	−42	+162	−4 4	−42	+162	−4 5	−42	+162	−4 5	
Kitay-gorodsky	Cellobiose (*B*)	−44	+168	+ 0 2	−44	+168	−0 3	−44	+168	−0 4	−44	+168	−0 3	−44	+168	−0 4	−44	+168	−0 4	
	Cellobiose (*J*)	−42	+162	+ 0 5	−42	+162	−0 1	−42	+162	−0 2	−42	+162	−0 2	−42	+162	−0 3	−42	+162	−0 4	
Liquori	Cellobiose (*B*)	−44	+168	+ 2 0	−44	+168	+0 1				−44	+168	+0 2							
	Cellobiose (*J*)	−42	+162	+ 2 6	−42	+162	+0 5				−42	+162	+0 5							
Flory	Cellulose (*H*)	−32	+148	−3 6	−33	+147	−4 1	−33	+147	−4 0	−39	+141	−3 0	−39	+141	−3 3	−40	+140	−3.5	
	Cellulose (*M*)[a]	0	+180	+14 5	0	+180	+6 9	0	+180	+6 6	0	+180	+8 9	0	+180	+8 2	0	+180	+7 9	
Kitay-gorodsky	Cellulose (*H*)	−32	+148	+ 0 9	−33	+147	+0 7	−33	+147	+0 1	−39	+141	+0 8	−39	+141	+0 5	−40	+140	+0 4	
	Cellulose (*M*)[a]	0	+180	+ 5 2	0	+180	+2 1	0	+180	+2 2	0	+180	+2 6	0	+180	+2 4	0	+180	+2 3	
Liquori	Cellulose (*H*)	−32	+148	+ 3 3	−33	+147	+1 0				−39	+141	+0 3							
	Cellulose (*M*)[b]	0	+180	+11 5	0	+180	+6 5				0	+180	+6 5							

[a]The values of h calculated for these conformations were, from left to right across the Table, 5 25, 5 25, 5 26, 5 26, 5 27, and 5 27 Å, respectively

[b]For the values of h calculated for these conformations, see the corresponding entry for the Flory or Kitaygorodsky functions.

that, for this and perhaps other di- and oligo-saccharides, possibilities for favourable intra- and inter-molecular interaction are so restricted that the *structure* must allow each to be simultaneously near its minimum if crystallisation is to occur with a favourable free-energy change From this point of view, it is not surprising that some isomers should be syrups which obstinately resist crystallisation

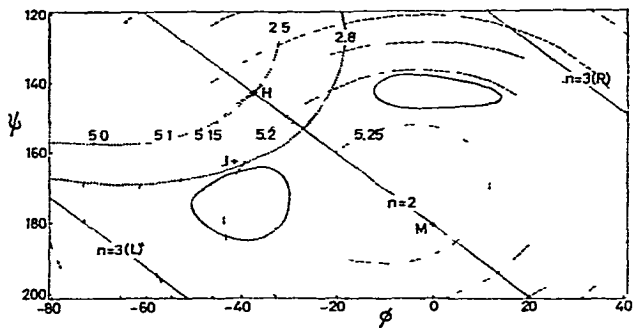


Fig 7 Van der Waals energies and other properties of cellulose and cellobiose conformations The lowest Kitaygorodsky contours (compare Fig 4) are represented by solid curves, and the limits of (O-3′) (O-5) hydrogen bonding are represented by dotted curves, assuming that the O O distance must be 2 50–2 80 Å Broken curves define conformations with the same projected repeat distance, and the diagonals define those with the same screw symmetry. $H \equiv$ cellulose (H), $M \equiv$ cellulose (M), $J \equiv$ cellobiose (J)

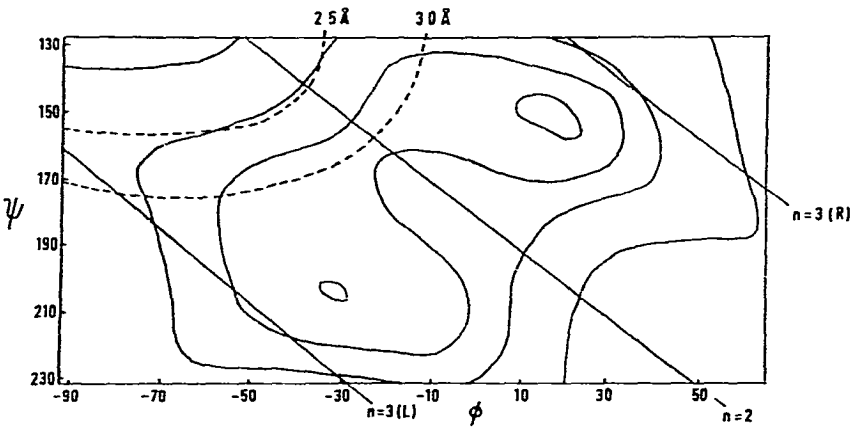


Fig 8 Conformational energy map for xylan based on *Set 4* coordinates and Kitaygorodsky functions The limits of (O-3′) (O-5) hydrogen bonding are shown by the dotted curves, assuming that the O O distance must be 2 50–3 00 Å Van der Waals energy contours are shown by solid curves for −0 4, 0, 1 0, and 10 0 Kcal Conformations having integral screw axes lie on the diagonals

With a given set of coordinates, there are two cellulose conformations having a two-fold screw axis together with the observed fibre-repeat distance Mathematically, these correspond to the intersections of the curves which represent $n = 2$ and $h = 5\ 15$Å (see Fig 7) As Jones has pointed out[15], other solutions would be possible with a variable (C-1)–O–(C-4′) bond angle, but indications from disaccharide[19,20,27 28]

and other[16 29] structures are that this does not deviate far from 117°. One solution (19°, 199°) is quite close to the Meyer and Misch conformation[3], which is known[23] to be sterically unfavourable The energy calculated with Kitaygorodsky functions and *set 4* coordinates is 49.5 Kcal per disaccharide residue, compared with 0 7 Kcal for the other solution which resembles the Hermans[2] conformation ! A more lenient assessment of the Meyer and Misch conformation was made by using the original definition, according to which the glycosidic oxygens lie on the two fold screw axis [*i e* , it is (0°, 180°)], and ignoring the discrepancy of about 0 1 Å in *h* (see Table II) Even so, a series of calculations with fifteen different combinations of coordinates and energy functions showed this conformation to be consistently less stable (by several Kcal) than the alternative (Table II) Another energetic disadvantage is the absence of an (O-5) (O-3′) hydrogen bond which occurs in the Hermans conformation, and for which there is evidence from infrared dichroism[30] Our calculations therefore support the view[23] that the Hermans conformation is more likely than the Meyer and Misch version for cellulose The Hermans conformation also seems very likely in itself it represents a displacement of the experimentally proved cellobiose conformation to slightly higher van der Waals energy, without sacrifice of the hydrogen bond The increase in intramolecular energy is estimated, without correction for the changed (O-5) (O-3′) contribution, as about 0 8 Kcal (see Table II) This could easily be offset by more efficient packing such as might be possible with an integral screw axis along the chain Chain folding of the type proposed by St John Manley[4] would be so expensive energetically (Figs 4–6) that it is difficult to imagine any source of sufficient intermolecular compensation this structure would therefore seem unlikely

Some comments can also be made on Setterineri and Marchessault's[16] xylan hydrate structure Fig 8 shows the map with Kitaygorodsky functions, and xylan coordinates derived from cellobiose by replacement of each hydroxymethyl group by hydrogen, with appropriate shortening of the bond length The Flory map was similar in its main features The conformation proposed[16] corresponds to (−80°, 172°), which is certainly more likely than any alternative with $n = 3$ and $h = 4\,93$ Å, both in van der Waals energy (see Table III) and because no other solution has the (O-5) (O-3′) hydrogen bond. It has several unfavourable features, however The van der Waals energy is 1 95 Kcal above the Kitaygorodsky minimum and 0 66 Kcal above a xylan conformation of the Hermans type (−40°, 140°), and the hydrogen bond is rather long (3 0 Å) These disadvantages might be partly offset by favourable interactions between the atomic dipoles of (O-5) and (O-4′)[31], furthermore, the 3 0 Å hydrogen bond might be stronger than would first appear, because models show the (O-3′)–H bond to be collinear with the atomic dipole of O-5, and that shortening of the O O distance causes distortion of this favourable geometry We presume that crystal packing favours a structure having an integral screw axis for both cellulose and xylan There is no obvious reason, in terms of intramolecular interactions, why these polymers should crystallise with different screw symmetries. Hydrogen bonding and dipole effects should be similar for each and calculations with

TABLE III

XYLAN CONFORMATIONS DERIVED FROM *Set 4* COORDINATES TO FIT $n = 3$, $h = 4.93$ Å

Helix sense	ϕ,°	ψ,°	V (*Kcal, from Kitaygorodsky functions*)
Left handed	−80	+172	+ 1 6
Left handed	− 6	+246	+ 4 6
Right handed	− 5	+104	+ 6 7
Right handed	+65	+174	+15 2

Kitaygorodsky functions and *Set 4* coordinates suggest that the van der Waals energy differences between (−40°, 140°) and (−80°, 172°) are also similar (0 70 Kcal for cellulose and 0 66 Kcal for xylan) Crystal packing must be the decisive influence, even though it seems probable that the conformation having a two fold screw axis is inherently more stable. In xylan diacetate, intermolecular hydrogen bonding would be replaced by weaker van der Waals and dipole interactions, and this polymer seems to crystallise with a two fold screw axis[32] in agreement with our conclusions

In solution, configurational (= conformational) entropy effects will cause both xylan and cellulose conformations to be more disordered than in the crystal[33] Even apart from the effect of the lower molecular weight of xylan and the weakening of its crystal lattice by occasional branching, it might be expected to be more soluble than cellulose for two reasons Firstly, the xylan hydrate crystal is probably less favoured by potential energy, relative to an unconstrained state such as solution, because there are fewer intermolecular hydrogen bonds per sugar residue, and the chain conformation seems inherently less stable, and, secondly, hard-sphere calculations show a larger allowed area for xylan, which should allow a bigger entropy gain when it dissolves It is well known that xylans dissolve in alkali, whereas cellulose does not normally do so, in agreement with these predictions Nevertheless, the extent of the insolubility of cellulose is, at first, surprising The *swelling* reactions of cellulose in alkali can be understood in terms of the important concepts introduced by Warwicker and Wright[34]. The cellulose chains, each in a ribbon-like conformation with equatorial bonds close to the plane of the ribbon, are considered to be laid on top of each other in stacks (called "sheets" by Warwicker and Wright) In the intact crystal structures of cellulose I and cellulose II these stacks are joined by hydrogen bonding between equatorial hydroxyl groups Evidence from X-ray diffraction indicates that swelling in sodium hydroxide causes rupture of hydrogen bonds and separation or relative movement of the stacks The reason why cellulose of high molecular weight does not dissolve at room temperature is therefore that the stacks themselves remain intact In the dry state, cohesion within each stack would be chiefly owing to van der Waals attraction[34]. However, forces of this type are so weak that, on this basis alone, it is difficult to imagine why chains should not separate completely and enter solution, because the conditions cause ionisation of the hydroxyl groups and therefore

electrostatic repulsion, and our calculations suggest that van der Waals repulsion would be significantly relieved in solution An explanation of the stability of the stacks can be deduced from the observation that cellulose fractions of low molecular weight are more soluble in alkali if the temperature is lowered[35] This implies that the stacks are *less* stable at lower temperature, and therefore that $T\Delta S°$ favours stacks rather than separated chains. Such positive entropy changes for association reactions are rare, except[36,37] where "hydrophobic interactions" stabilise the product. This type of interaction arises from the unique properties of water as solvent and is quite distinct from van der Waals forces During the past few years, it has become accepted as an important source of molecular cohesion in biological and other systems[38], but there are, as yet, no proved examples that sugar rings can associate in this way We suggest that the (H-1)–(H-3)–(H-5) surfaces of D-glucose residues in cellulose, and perhaps the (H-2)–(H-4)–(O-5) surfaces, can interact with water in a similar fashion to, for example, the nonpolar side groups of proteins or the hydrocarbon chains of detergents The result is that their association is entropically favoured and that cellulose crystallites gain stability in the presence of water*

ACKNOWLEDGMENTS

It is a pleasure to be able to thank Sir Edmund Hirst, in a special way, by contributing this paper to mark his seventieth birthday His constant encouragement, interest, and material help have sustained our research for the past eight years We thank the Pioneering Research Committee of the Institute of Paper Chemistry, Appleton, Wisconsin, U S A , for a research grant, and Dr N S Anderson and Mr J W Campbell for their collaboration in preliminary investigations Drs R H Marchessault and G A Jeffrey kindly gave us copies of important papers in manuscript

*Note added in proof (14 May 1968)—In this Department, C A Beevers and H N Hansen have recently solved the crystal structure of α-lactose monohydrate and very kindly made the preliminary coordinates available as a further test of our calculations This disaccharide is extremely interesting in relation to cellobiose intramolecular influences on conformation are expected to be identical because of the structural similarity around the bridge oxygen but packing influences should be quite different because configuration is inverted at C-1′ and C-4 and the crystal is a monohydrate Hydrogen atoms have not yet been placed by Beevers and Hansen but we estimate from the ring atom positions that the conformation is (−31°, 155°) In Fig 7, this corresponds to a rather small displacement of the cellobiose conformation ("*J*") with little change of intramolecular energy, in the direction of the two fold axis and the second minimum and approximately following the 2 8 Å hydrogen bond contour (actual O-5 O-3′ distance, 2 84 Å) The (C-1)-O-(C-4′) angle is 118 5° The lactose structure therefore provides further support for our assumptions and conclusions

A very sophisticated stereochemical study of amylose triacetate has been published[39], and evidence now exists that, as expected from our calculations, cellodextrins have rather rigid extended conformations in aqueous solutions[40]

REFERENCES

1 J C IRVINE AND E L HIRST, *J Chem Soc*, (1923) 518, W N HAWORTH, E L HIRST, AND H A THOMAS, *ibid*, (1931) 824
2 P H HERMANS, *Physics and Chemistry of Cellulose Fibres*, Elsevier, New York, 1949, C Y LIANG AND R H MARCHESSAULT, *J Polymer Sci*, 37 (1959) 385
3 K H MEYER AND L MISCH, *Helv Chim Acta*, 20 (1937) 232
4 R ST J MANLEY, *Nature*, 204 (1964) 1155
5 K C ELLIS AND J O WARWICKER, *Nature*, 181 (1958) 1614
6 N L ALLINGER, M A MILLER, F A VANCATLEDGE, AND J A HIRSCH, *J Am Chem Soc*, 89 (1967) 4345
7 A M LIQUORI, *J Polymer Sci, Part C*, 12 (1966) 209
8 P DE SANTIS, E GIGLIO, A M LIQUORI, AND A RIPAMONTI, *Nature*, 206 (1965) 456
9 T OOI, R A SCOTT, G VANDERKOOI, AND H A SCHERAGA, *J Chem Phys*, 46 (1967) 4410
10 P R SCHIMMEL AND P J FLORY, *Proc Natl Acad Sci U S*, 58 (1967) 52
11 M GOODMAN AND M FRIED, *J Am Chem Soc*, 89 (1967) 1264, J E MARK AND M GOODMAN, *ibid*, p 1267
12 C RAMAKRISHNAN, *Proc Indian Acad Sci, Sect A*, 12 (1964) 327, C RAMAKRISHNAN AND G N RAMACHANDRAN, *Biophys J*, 5 (1965) 909
13 G N RAMACHANDRAN, C RAMAKRISHNAN, AND V SASISEKHARAN, in G N RAMACHANDRAN (Ed), *Aspects of Protein Structure*, Academic Press, New York, 1963, p 121
14 V S R RAO, P R SUNDARARAJAN, C RAMAKRISHNAN, AND G N RAMACHANDRAN, in G N RAMACHANDRAN (Ed), *Conformation of Biopolymers*, Academic Press, New York, 1967, p 721
15 D W JONES, *J Polymer Sci*, 32 (1958) 371
16 W J SETTINERI AND R H MARCHESSAULT, *J Polymer Sci, Part C*, 11 (1965) 253
17 P J FLORY, *Proc Roy Soc* (London), *Ser A*, 234 (1956) 60 H MORAWETZ, *Macromolecules in Solution*, Wiley New York, 1965, p 111
18 G A JEFFREY AND R D ROSENSTEIN, *Advan Carbohyd Chem*, 19 (1964) 7
19 C J BROWN, *J Chem Soc (A)*, (1966) 927
20 S S C CHU AND G A JEFFREY, *Acta Cryst*, in press
21 D A BRANT AND P J FLORY, *J Am Chem Soc*, 87 (1965) 2791
22 A I KITAYGORODSKY, *Tetrahedron*, 14 (1961) 230
23 R H MARCHESSAULT AND A SARKO *Advan Carbohyd Chem*, 22 (1967) 421
24 C C F BLAKE, L N JOHNSON, G A MAIR, A C T NORTH, D C PHILLIPS, AND V R SARMA, *Proc Roy Soc* (London), *Ser B*, 167 (1967) 378
25 B CASU, M REGGIANI, G G GALLO, AND A VIGEVANI, *Tetrahedron*, 22 (1966) 3061
26 C M VENKATACHALAM AND G N RAMACHANDRAN, in G N RAMACHANDRAN (Ed), *Conformation of Biopolymers*, Academic Press, New York, 1967, p 83
27 C A BEEVERS AND W COCHRAN, *Proc Roy Soc* (London), *Ser A*, 190 (1947) 257
28 S S C CHU AND G A JEFFREY, *Acta Cryst*, 23 (1967) 1038
29 A HYBL, R E RUNDLE, AND D E WILLIAMS, *J Am Chem Soc*, 87 (1965) 2779
30 J MANN AND H J MARRINAN *J Polymer Sci* 27 (1958) 595
31 R H MARCHESSAULT AND C Y LIANG, *J Polymer Sci*, 59 (1962) 357
32 R H MARCHESSAULT, *Chimie et Biochimie de la Lignine, de la Cellulose et des Hemicelluloses*, Les Imprimeries Réunies de Chambéry, 1965, p 287
33 P J FLORY, *Principles of Polymer Chemistry*, Cornell University Press, Ithaca, New York, 1953
34 J O WARWICKER AND A WRIGHT, *J Appl Polymer Sci*, 11 (1967) 659
35 H STAUDINGER AND I JURISCH, *Kunstseide und Zellwolle*, 21 (1939) 6
36 W KAUZMANN, *Advan Protein Chem*, 14 (1959) 1
37 F FRANKS AND D J G IVES, *Quart Rev* (London), 20 (1966) 1
38 G NEMETHY, *Angew Chem, Intern Ed Engl*, 6 (1967) 195
39 A SARKO AND R H MARCHESSAULT, *J Am Chem Soc*, 89 (1967) 6454
40 M IIJIMA AND D. A I GORING, *Canad J Chem*, 45 (1967) 2353, 2363

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THE CONFORMATION OF AMYLOSE IN NEUTRAL, AQUEOUS SALT SOLUTION*

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ABSTRACT

Linear amylose has been subfractionated, and the hydrodynamic properties of the fractions examined From measurements of the limiting-viscosity number, $[\eta]$, in 0 33M potassium chloride, and of the weight-average molecular weight, $\overline{M}_w$, by light scattering on the acetate, the relation

$$[\eta] = 1\ 15 \times 10^{-1}\ \overline{M}_w^{0\ 50}$$

was found The significance of the exponent in this relation and others in the literature is discussed Estimations of the "stiffness" of the amylose molecule in aqueous solution have been obtained for various model systems, *i e*, the Kuhn statistical length, the Kratky-Porod persistence length, and Nagai's theory for a mixture of helical and randomly coiled segments The results of each individual theory are ambiguous It is shown, on the basis of Nagai's theory, that there is definite evidence for the *absence* of rigid helical segments in amylose in aqueous solution The temperature coefficient of $[\eta]$ for amylose in aqueous solution is also discussed

INTRODUCTION

The conformation of the amylose molecule in aqueous solution is currently of much interest Although it has been suggested[1 2] that the polysaccharide undergoes a helix–coil transformation similar to that of proteins, our results[3 4] are not consistent with this viewpoint

In this paper, we have extended our earlier hydrodynamic measurements on amylose subfractions, and have critically analysed our results in terms of recent theoretical treatments which involve helical models

EXPERIMENTAL

Linear amylose, *i e*, a sample which was completely hydrolysed into maltose

*Part 43 in the series "Physicochemical Studies on Starches", for Part 42, see *European Polymer Journal*, in the press

by crystalline β-amylase, was fractionated from methyl sulphoxide solution by the addition of butyl alcohol, as previously described[5] Each fraction was dissolved in boiling water, and reprecipitated as the butyl alcohol-complex. The complexes were dehydrate by repeated stirring with butyl alcohol

Solutions in 0 33M potassium chloride were prepared by dissolving the amylose in 0 5M potassium hydroxide at 0°, and then neutralizing with M hydrochloric acid Limiting-viscosity numbers, $[\eta]$, of the native amylose were determined by using a modified Ubbelohde viscometer, as previously described[3].

Weight-average molecular weights, $\overline{M}_w$, were obtained from light-scattering measurements In order to eliminate the possibility that solutions of the native amylose were unstable, these measurements were made on the triacetates dissolved in nitromethane, as detailed elsewhere[5] The amylose subfractions were acetylated by using pyridine and acetic anhydride[6] under conditions which have been shown to cause no degradation[7]

RESULTS AND DISCUSSION

The hydrodynamic properties of amylose in neutral, aqueous potassium chloride solution —Table I shows the results of the light-scattering and viscosity measurements

TABLE I

VISCOSITY AND MOLECULAR-WEIGHT DATA FOR AMYLOSE IN AQUEOUS POTASSIUM CHLORIDE

Fraction	$\overline{M}_w \times 10^{-6}$	$[\eta]^a$	$[\eta]/\overline{M}_w$	*Fraction*	$\overline{M}_w \times 10^{-6}$	$[\eta]$	$[\eta]/\overline{M}_w$
1	1 75	154	0 117	7	0 460	78	0 115
2	1 22	126	0 113	8	0 424	74	0 114
3	0 945	113	0 116	9	0 380	71	0 115
4	0 754	100	0 115	10	0 320	67	0 117
5	0 613	90	0 115	11	0 211	52	0 113
6	0 488	81	0 117	12	0 083	33	0 114

[a] In ml/g

carried out on the 12 amylose fractions The data, together with data from the literature, are plotted as logarithmic functions in Fig 1

Our present results obey the relation.

$$[\eta] = 1\ 15 \times 10^{-1} \overline{M}_w^{0\ 50} \qquad (1)$$

Fig 1 shows that there is very good agreement between this equation, our earlier results[3], and also those of Everett and Foster[8] The relation obtained by Cowie[9] is virtually parallel to our own, but displaced to higher molecular weights Burchard's

results[10] are apparently anomalous, as the slope of his graph is very much higher, but these measurements were carried out in pure water, whereas all of the others were performed in aqueous potassium chloride

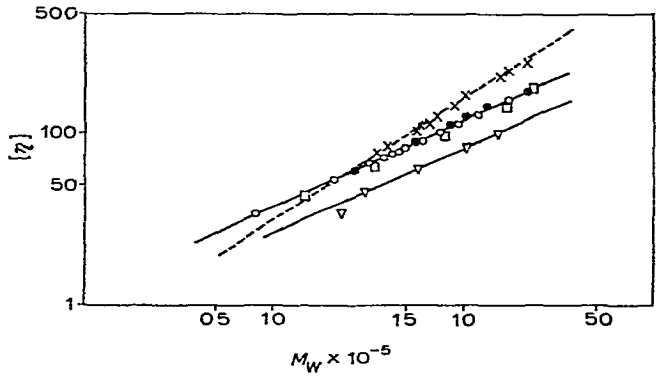

Fig 1 A logarithmic graph of $[\eta]$ as a function of M_w for amylose in neutral, aqueous solution ○ this work, □ Banks and Greenwood[3] ● Everett and Foster[8] ▽ Cowie[9], × Burchard[10]

The value of the exponent in equation (*1*) is that expected for a random coil in a θ-solvent[11]. Indeed, this is in agreement with our previous light-scattering measurements[3], in which the second virial coefficient was found to be zero It is therefore possible to obtain a direct measure of the "unperturbed" dimensions $(\bar{r_0^2}/M)^{\frac{1}{2}}$, of amylose, where $\bar{r_0^2}$ is the mean-square end-to-end distance of the polymer chain of molecular weight M, using

$$[\eta] = K_\theta M^{\frac{1}{2}}, \qquad (2)$$

where $K_\theta = \Phi_0(\bar{r_0^2}/M)^{\frac{1}{2}}$, and Φ_0, the viscosity constant, is $2\,5 \times 10^{23}$. This calculation gives a value for $(\bar{r_0^2}/M)^{\frac{1}{2}}$ of 770×10^{-11} cm For amylose composed of D-glucose units in the *C1* conformation, the calculated value for the unperturbed dimensions, assuming free rotation about the glycosidic bonds, *i e*, $(\bar{r_{of}^2}/M)^{\frac{1}{2}}$, is 390×10^{-11} cm[12 13] A measure of the short-range (steric) forces which control the unperturbed dimensions is given by the parameter, σ, where

$$\sigma = (\bar{r_0^2}/M)^{\frac{1}{2}}/(\bar{r_{of}^2}/M)^{\frac{1}{2}}. \qquad (3)$$

The derived value of σ is approximately 2, which is quite comparable with the values obtained for the flexible vinyl polymers[14]

The value of the exponent in equation (*1*) suggests that the amylose molecule in aqueous solution cannot have an extended, helical, rod-like structure, as, in that case, the exponent would be about 1.8 However, as stressed by Flory[11], it is quite possible to have a macromolecule which, although basically a random coil, is composed of a number of rigid segments It has been suggested[1 2] that amylose is such a molecule, in which the segments are rigid as a result of a helical conformation which is stabilized by intramolecular hydrogen bonding Between these rigid segments are flexible portions which enable the molecule as a whole to conform to the random-

coil model To characterise such a structure, it is necessary to obtain a measure of the lengths of the rigid segments Some calculations of this type are outlined below

One parameter often used as a measure of "stiffness" is the Kuhn statistical-segment length, A_m, defined[15] by

$$(\overline{r_0^2}/M)^{\frac{1}{2}} = (A_m b_0/M_0)^{\frac{1}{2}}, \tag{4}$$

where b_0 is the length of the monomer unit of molecular weight M_0 Unfortunately, the value of b_0 used in equation (4) is rather ambiguous Thus, if the polysaccharide is assumed to have no helical structure, $b_0 = 4\,41$ Å, on the other hand, if there is extensive helical formation, $b_0 = 1\,33$ Å [This is calculated from X-ray diffraction measurements on solid amylose-complexes, which have been shown to be a helix having a pitch of about 8 Å and 6 monomer residues per turn[16] Thus, the projected bond length, $b_0 = 8/6$ Å] In both cases, the D-glucose residues are assumed to be in the *CI* conformation, calculations by Rao and his co-workers[17] have shown that this conformation has much the lowest energy

Another much-quoted measure of stiffness is the Kratky–Porod persistence length[18], q, defined as[19]

$$\overline{r_0^2} = 6q^2\left\{\frac{x}{3} - 1 + \frac{2}{x} - \frac{2}{x^2}[1 - \exp(-x)]\right\}, \tag{5}$$

where x, the number of Kratky–Porod units per molecule, is given by

$$x = r_{max}/q \tag{6}$$

In equation (6), r_{max} is the projected contour-length of the macromolecule, and is related to the bond length of the monomer unit by

$$r_{max} = b_0 M/M_0 \tag{7}$$

For sufficiently large x, equation (5) reduces to

$$(\overline{r_0^2}/M) = 2q\,b_0/M_0 \tag{8}$$

Comparison of equations (4) and (8) shows that, in fact, $A_m = 2q$, and so equation (8) must suffer from the same ambiguities as does equation (4).

Nagai[20] has recently developed a theory specifically for a model containing helical and randomly coiled segments Assuming that each helical sequence behaves as a rigid rod without correlation in direction to the adjacent, randomly-coiled sections, he obtained

$$\overline{r_0^2}/M = (1-\xi)\,l^2 s/M_0 + \xi b_0^2 n/M_0\,, \tag{9}$$

where ξ is the fraction of monomer units in the helical form, s, the skeletal factor, is $(\overline{r_0^2}/M)\,(M_0/l^2)$, l is the bond length of the random-coil sections; b_0 is the length per monomer unit of the helix, and n is the weight-average number of monomer units in the helical segments If the helical content is sufficiently large, equation (9) reduces to

$$(\overline{r_0^2}/M) = nb_0^2/M_0 \tag{10}$$

TABLE II

DIMENSIONS OF HELICAL SEGMENTS OBTAINED BY DIFFERENT THEORETICAL TREATMENTS

	Equation (4)		*Equation* (8)		*Equation* (10)
	$b_0 = 4.41$Å,	$b_0 = 1.33$Å	$b_0 = 4.41$Å,	$b_0 = 1.33$Å	
Segment length (Å)	22	73	11	37	73
No. of monomer units/segment	5	55	2–3	28	55
Molecular weight of segment	800	9,000	400	4,500	9,000

Table II shows the various measures of segment length calculated from equations (*4*), (*8*), and (*10*). Assuming predominance of a helical structure, it is obvious that the number of monomer units per helix varies between 28 and 55. Since the size of the segment is an inverse measure of the flexibility of the molecule, these values would suggest that amylose is an inherently stiff macromolecule. On the other hand, if the polysaccharide is assumed to have no helical content, *i.e.*, $b_0 = 4.41$ Å, the derived segment-lengths are quite comparable to those of the flexible vinyl polymers[15].

Thus, none of the above treatments can show unambiguously whether or not there is extensive helix-formation in the amylose molecule. However, when these segment lengths are considered in relation to other hydrodynamic properties, it is possible to decide on the significance of the values recorded in Table II.

Two of the fundamental postulates of the Flory theory of theta-solution behaviour are that the distribution of chain segments about the centre of mass of the molecule is Gaussian, and that the random coil entrains solvent molecules within it self when it moves. These two basic factors are fairly closely related, and both will break-down in the same region of low molecular weight. The exact value at which break down occurs must depend on the segment length, and if the helical sections are very long, the onset of free-draining and the departure from Gaussian statistics will occur at relatively high molecular weights. An approximate measure of the molecular weight at which these effects become apparent may be obtained from the reciprocal extension ratio, R, defined as

$$R = r_{max}/(\overline{r_0^2})^{\frac{1}{2}}, \qquad (11)$$

where r_{max} is given by equation (*7*). This is a direct measure of the degree of coiling of the molecule, and for values greater than about 10, the macromolecule should be approximately spherical[21]. Above the molecular weight corresponding to this value, the polymer would be expected to behave as a random coil which is non-draining, at lower values, the departure from Gaussian statistics and the onset of partial free-draining should become apparent.

When the conformation is assumed to be predominantly helical (*i.e.*, $b_0 = 1.33$ Å), it can be easily shown that this critical molecular weight is approximately 9×10^5, and hence, at lower molecular weights, a downward curvature in the

$[\eta]$–$\bar{M}_w$ relation might be expected This does not occur. A more sensitive test for absence of free-draining characteristics is that the value of $[\eta]/M^{\frac{1}{2}}$ should be a constant for measurements carried out in a θ-solvent This is, in fact, observed (see Table I), and therefore the helical model is not applicable This conclusion fully supports our earlier suggestion[3] that the helical character of amylose is not pronounced in neutral, aqueous solution

Although equation (9) is applicable to a model in which there is an appreciable fraction of randomly coiled segments as well as helical portions, it contains, in addition to n, two other unknown parameters—the fraction of monomer units in the helical sections, ξ, and the skeletal factor, s. Now, if the helical-content of amylose is negligible, s can be calculated to have a value of about 5 The presence of extensive helices would increase the experimental value of $\overline{r_0^2}/M$, and so this value of s is a maximum But s is the sum of all the short-range interactions due to fixed bond angles and hindered rotation, and hence the minimum value must still be fairly high—certainly not lower than 2

Fig 2 shows n calculated from equation (9) as a function of ξ for various s-values in the range of 2 to 5 Since the low s-values must be associated with high ξ, and

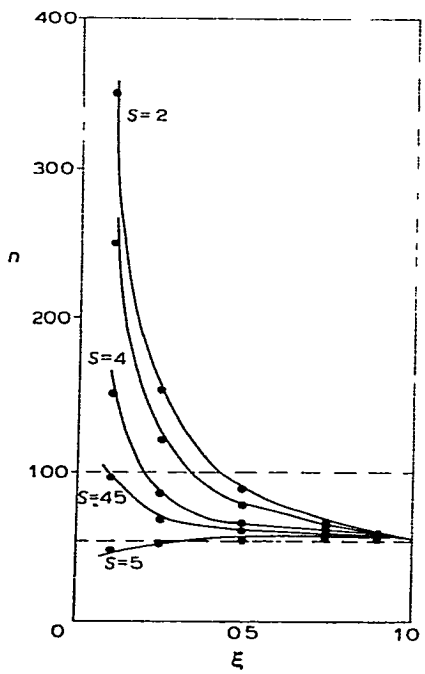

Fig 2 Number of monomer units/helical segment (n) as a function of the fraction of D-glucose residues in the helices (ξ) for different values of the skeletal parameter(s)

vice versa, the minimum value of n must lie within the dotted lines shown in Fig 2, *i e*, it must lie between 50 and 100 units

Experimentally measured radii of gyration[3] show that the Gaussian model

holds down to molecular weights of at least 1.5×10^5 Assuming that, at this level, $R = 10$ [see equation (*11*)], the maximum fraction of D-glucose residues which can be accomodated in the helices [calculated from equation (*11*)] is of the order of 0.4 Thus, one obtains a model which is composed of essentially equally-long segments (50–80 monomer units) of helix and random coil This is physically unreal, since there is no reason why the forces which stabilized the helix should be absent over such extended lengths

In fact, the use of $b_0 = 1.33$ Å, invariably leads to a model which is inconsistent with other hydrodynamic evidence We therefore suggest that, although there may be some helical character in amylose, the compact helical segments previously postulated[1,2] do not exist, that is, the helices are very much "looser" than has been suggested, and b_0 must be greater than 1 33 Å The helical nature of the macromolecule in solution is thus not a result of intramolecular hydrogen bonding, but merely a consequence of the α-D-(1→4)-linkages

Indirect evidence for rigid, helical segments in amylose is provided by the temperature-dependence of $[\eta]$ in aqueous potassium chloride If, as we have stressed, this is a θ-solvent for the polysaccharide, $[\eta]$ should be very sensitive to temperature[11] But this is not the case Over a range of 20–30°, there is virtually no change in $[\eta]$ Rao and Foster[1] have suggested that the increase in temperature has two opposing and equal effects the increased solvent-power of the water causes the amylose to expand, whilst the helical segments break down, making the amylose less rigid and therefore able to take up a more compact conformation This hypothesis implies that an increase in temperature necessarily leads to an increase in solvent-power, and that consequently solvent–solute interaction is favoured at the expense of solute–solute interaction

With aqueous solutions of amylose, we suggest that the situation is rather more complex In this case, the polymer is held in solution by hydrogen bonding with the solvent, but, at the same time, polymer–polymer interaction is also dependent on hydrogen bonding As the temperature is raised, both types of interaction will tend to become less effective Again, these tendencies will oppose each other, so that it is possible for $[\eta]$ to remain unchanged over a fairly wide range of temperature This suggestion satisfactorily accounts for the solution behaviour of amylose in aqueous potassium chloride as a function of temperature *without* introducing a helical model

We conclude that the view of amylose as a molecule composed of a number of rigid, helical segments has no experimental basis

ACKNOWLEDGMENTS

We have been fortunate enough to have worked in association with Professor Sir Edmund Hirst, C B E , F R S , for the past 16 years, and wish to express our indebtedness to him for his continued counsel and the keen interest that he has always taken in our work

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REFERENCES

1 V S R RAO AND J F FOSTER, *Biopolymers*, 1 (1963) 527
2 J SZEJTLI AND S AUGUSTAT, *Stärke*, 18 (1966) 38
3 W BANKS AND C T GREENWOOD, *Makromol Chem*, 67 (1963) 49
4 W BANKS AND C T. GREENWOOD, in *Conformation of Biopolymers*, G N RAMACHANDRAN (Ed), Academic Press, London, 1967, p 739
5 W BANKS, C T GREENWOOD, AND D J HOURSTON, *Trans Faraday Soc*, 64 (1968) 363
6 R S HIGGINBOTHAM AND G A MORRISON, *Shirley Inst Mem*, 22 (1948) 148
7 C T GREENWOOD AND J S M ROBERTSON, *J Chem Soc*, (1954) 3769
8 W W EVERETT AND J F FOSTER, *J Am Chem Soc* 81 (1959) 3464
9 J M G COWIE, *Makromol Chem*, 42 (1961) 230
10 W BURCHARD, *Makromol Chem*, 64 (1963) 110
11 P J FLORY, *Principles of Polymer Chemistry*, Cornell University Press, Ithaca, New York, 1953
12 J ELIEZER AND H J G HAYMAN, *J Polymer Sci*, 23 (1957) 387
13 W BURCHARD, *Z Physik Chem*, 42 (1964) 193
14 M KURATA AND W H STOCKMAYER, *Fortschr Hochpolymer Forsch*, 3 (1963) 196
15 H KUHN, W KUHN, AND A SILBERBERG, *J Polymer Sci*, 14 (1954) 193
16 R E RUNDLE AND D FRENCH, *J Am Chem Soc*, 65 (1943) 558.
17 V S R RAO, P R SUNDARARAJAN, C RAMAKRISHNAN AND G N. RAMACHANDRAN, in *Conformation of Biopolymers*, G N RAMACHANDRAN (Ed), Academic Press, London, 1967, p 721
18 O KRATKY AND G POROD, *Rec Trav Chim*, 68 (1949) 1106
19 H BENOIT AND P DOTY, *J Phys Chem*, 57 (1953) 958
20 K NAGAI, *J Chem Phys*, 34 (1961) 887
21 W BROWN, *Arkiv Kemi* 18 (1961) 227

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NEW NUCLEOSIDE ANALOGUES β-D-GLUCOPYRANOSYL DERIVATIVES OF TETRAHYDRO-1,3-OXAZINE* **

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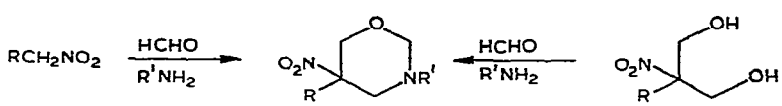
(Received January 24th, 1968)

ABSTRACT

5-Alkyl-3-(β-D-glucopyranosyl)-5-nitrotetrahydro-1,3-oxazines (**1–3**) were obtained by treating D-glucopyranosylamine with 2-alkyl-2-nitropropane-1,3-diols and formaldehyde in methanol–butyl alcohol solution Compounds **1–3** are readily hydrolyzed to yield 5-alkyl-5-nitrotetrahydro-1,3-oxazines (**4–6**) and D-glucose Compounds **4–6** react with tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate to yield derivatives of thiourea, *viz*, 5-alkyl-5-nitro-3-(tetra-*O*-acetyl-β-D-glucopyranosylthiocarbamoyl)tetrahydro-1,3-oxazines

INTRODUCTION

It has been found in previous papers[1–4] that primary amines react with primary nitroparaffins and formaldehyde to yield 5-alkyl-5-nitrotetrahydro-1,3-oxazine derivatives (*A*) Similarly, 2-alkyl-2-nitropropane-1,3-diols react with amines and formaldehyde.



A

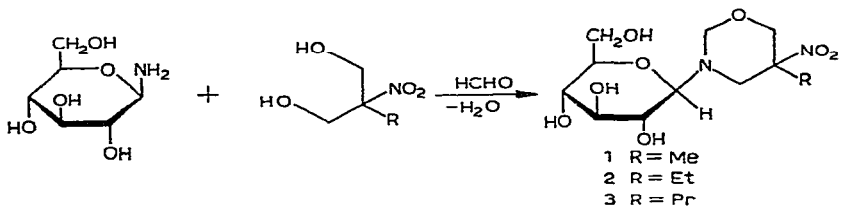
The present work was carried out to obtain 5-alkyl-3-(β-D-glucopyranosyl)-5-nitrotetrahydro-1,3-oxazines

RESULTS AND DISCUSSION

β-D-Glucopyranosylamine[5] reacted with 2-alkyl-2-nitropropane-1,3-diols and formaldehyde to yield 5-alkyl-3-(β-D-glucopyranosyl)-5-nitrotetrahydro-1,3-oxazines (**1–3**)

*Reported at XXIst IUPAC Congress in Prague, September 1967
**Chemistry of Nitroparaffins Part LXXII

Some difficulties were encountered in the isolation of the 3-(β-D-glucopyranosyl)-5-nitrotetrahydro-1,3-oxazines due to their hygroscopicity and ease of hydrolysis Hydrolysis of the β-D-glucopyranosyloxazine derivatives led to formation of 5-alkyl-



5-nitrotetrahydro-1,3-oxazines (**4–6**) and D-glucose which underwent upward mutarotation Compounds **1**, **2**, and **3** had low, positive, optical rotations, also suggestive of β-D anomeric configurations

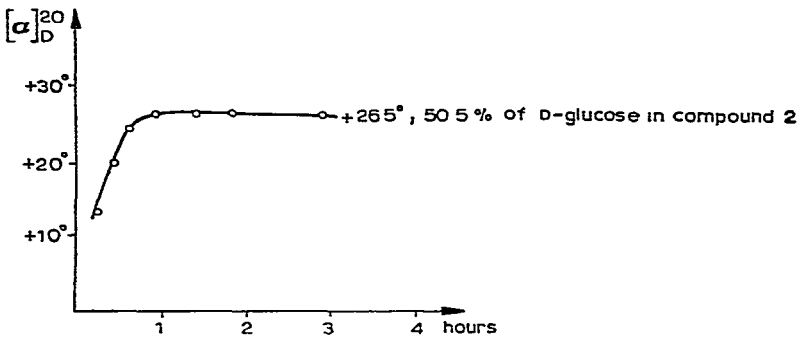
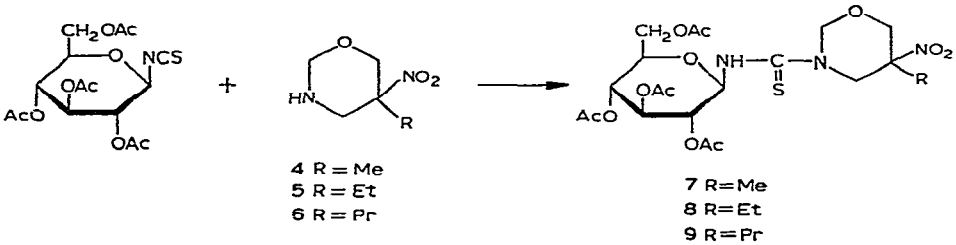


Fig 1 Mutarotation of compound **2** in 50% aqueous ethanol, ethanol was added to dissolve precipitated compound **5** and prevent turbidity

Compounds **4–6** were isolated, and characterized as their crystalline hydrochlorides They were also characterized through their crystalline *N*-β-D-glucopyranosylthiocarbamoyl derivatives (**7–9**) prepared from tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate[6,7]



The infrared spectra of substances **1–9** showed, in each case, a strong absorption at 1550 and 1360 cm^{-1}, assigned to the nitro group

EXPERIMENTAL

General — Melting points are uncorrected Specific rotations were measured

with a 2-dm tube Infrared spectra were measured with a Unicam SP-200 spectrophotometer

5-Alkyl-3-(β-D-glucopyranosyl)-5-nitrotetrahydro-1,3-oxazine (**1–3**) — To a stirred solution of D-glucopyranosylamine (9 g, 0 05 mole) and 2-ethyl-2-nitropropane-1,3-diol (7 5 g, 0 05 mole) in 95% methanol (100 ml) was added a solution of paraformaldehyde (1 8 g, 0 06 mole) in butyl alcohol (30 ml) The mixture was boiled for 5 h, and the water formed during the reaction was removed azeotropically with butyl alcohol The remaining butyl alcohol was evaporated under diminished pressure to give a syrup that crystallized on trituration with ethanol Recrystallisation from methanol–acetone gave compound **2**

Compounds **1** and **3** were prepared in an analogous manner The physical properties are given in Table I

TABLE I

DATA FOR 5-ALKYL-3-(β-D-GLUCOPYRANOSYL)-5-NITROTETRAHYDRO-1,3-OXAZINES (1–3)

Compound	*M p* (*degrees*)	$[\alpha]_D^{22}$(*degrees*)		*Yield*	*Found* (%)	*Calc* (%)
		(c 2, *pyridine*)	(c *2* , 50% *EtOH*)	(%)	*N*	*N*
1 R = Me	118–121	+13 0	+13 5→+27 8	10–13	8 86	9 09
2 R = Et	127–129	+12 5	+13 0→+26 5	9–12	8 45	8 69
3 R = Pr	123–129	+14 5	+15 0→+25 5	7–8	7 97	8 33

5-Methyl-5-nitrotetrahydro-1,3-oxazine (**4**) —3-(β-D-glucopyranosyl)-5-methyl-5-nitrotetrahydro-1,3-oxazine (6 16 g, 0 02 mole) was dissolved in water (20 ml), and the solution was extracted with ether. The extract was dried ($MgSO_4$), and evaporated under diminished pressure (rotary evaporator), and the residue was distilled to give compound **4** (2 5 g, 86%), b p 58–60°/0 2 mm

The oxazines **5** and **6** were prepared in a similar manner The physical properties are given in Table II

TABLE II

5-ALKYL-5-NITROTETRAHYDRO-1 3-OXAZINES (4–6) DERIVED FROM THE HYDROLYSIS OF 5-ALKYL-3-(β-D-GLUCOPYRANOSYL)-5-NITROTETRAHYDRO-1,3-OXAZINES

Compound	*B p* (*degrees*)	n_D,	*M p of hydrochloride* (*degrees*)	*Found* (%)	*Calc* (%)	*Ref*
				N	*N*	
4 R = Me	58–60/0 2 mm	n_D^{18} = 1 4768	168–169	15 32	15 08	—
5 R = Et	81–82/0 3 mm	n_D^{25} = 1 4753	194–195			1
6 R = Pr	87–88/0 2 mm	n_D^{19} = 1 4742[a]	190–192			8, 9, 10

[a]Not reported until now

5-Ethyl-5-nitro-3-(tetra-O-acetyl-β-D-glucopyranosylthiocarbamoyl)tetrahydro-1,3-oxazine (**8**) — To a solution of the 5-ethyl-5-nitrotetrahydro-1,3-oxazine (**5**)

(1.6 g, 0.01 mole) in chloroform (30 ml), tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (3.8 g, 0.01 mole) was added. The mixture was boiled for 3 h, and the cooled solution was evaporated under diminished pressure (rotary evaporator). The residue was crystallized from ethanol to give compound **8**.

The oxazine derivatives **7** and **9** were prepared in an analogous way, their properties are given in Table III.

TABLE III

5-ALKYL-5-NITRO-3-(TETRA-*O*-ACETYL-β-D-GLUCOPYRANOSYLTHIOCARBAMOYL)TETRAHYDRO-1,3-OXAZINES

Compound	*M.p.* (*degrees*)	$[\alpha]_D^{20}$ (*degrees*) (c 2, *chloroform*)	*Yield* (%)	*Found* (%)	*Calc.* (%)
				N	*N*
7 R = Me	136–137	−4.0	60	7.70	7.84
8 R = Et	117–119	−1.5	50	7.45	7.64
9 R = Pr	145–146	−7.5	55	7.15	7.45

REFERENCES

1 E. L. HIRST, J. K. N. JONES, S. MINAHAN, F. W. OCHYNSKI, A. T. THOMAS, AND T. URBANSKI, *J. Chem. Soc.* (1947) 924
2 M. SENKUS, *J. Am. Chem. Soc.*, 72 (1950) 2967
3 D. GURNE AND T. URBANSKI, *Roczniki Chem.*, 31 (1957) 855, 869
4 Z. ECKSTEIN, P. GLUZINSKI, W. HOFMAN, AND T. URBANSKI, *J. Chem. Soc.*, (1961) 489
5 C. A. LOBRY DE BRUYN, *Rec. Trav. Chim.*, 14 (1895) 98, 134, 145
6 E. FISCHER, *Ber.*, 47 (1914) 1382
7 K. M. HARING AND T. B. JOHNSON, *J. Am. Chem. Soc.*, 55 (1933) 395
8 T. URBANSKI, J. KOLESINSKA, AND H. PIOTROWSKA, *Bull. Acad. Polon. Sci., Cl. III*, 3 (1955) 179
9 T. URBANSKI AND H. PIOTROWSKA, *Roczniki Chem.*, 31 (1957) 553
10 D. GURNE, L. STEFANIAK, T. URBANSKI, AND M. WITANOWSKI, *Tetrahedron*, 20 (1964) 21

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A NEW METHOD FOR QUANTITATIVE, MICROSCALE DETERMINATION OF THE SULPHATE CONTENT OF CARBOHYDRATES

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(Received January 29th, 1968)

ABSTRACT

A novel method is described for the accurate, quantitative determination of sulphate on a micro-scale by using flame photometry The effects of various classes of carbohydrates, amino acids, and inorganic ions on the method have been investigated The method has been successfully applied to the determination of the sulphate ester content of sulphated carbohydrates, and a standard procedure has been established for a scale of 0–30 μg of sulphate

INTRODUCTION

The quantitative determination of sulphate is generally performed by the method of Jones and Letham[1], which involves precipitation of inorganic sulphate as the salt of 4-amino-4′-chlorobiphenyl, the amount precipitated being estimated by spectrophotometric determination of unused reagent Although one of the most insoluble of sulphate salts, the precipitate is not completely insoluble, and the method therefore relies upon precipitation of the sulphate at a concentration that leaves a negligible proportion unprecipitated The lower limit of the assay is 30 μg of sulphate

Gravimetric[2] and titrimetric methods[3] are less sensitive, whilst turbidimetric[4] and nephelometric[5] determinations can be made no more sensitive than the spectrophotometric method, since they depend essentially upon precipitation Antonopoulos[6] has improved a method similar to that of Jones and Letham, but the somewhat laborious technique has a lower limit of 10 μg of sulphate Another, similar method[8] involves exchange of the sulphate with insoluble barium chloroanilate, and spectrophotometric determination of the anion released into solution, but the procedure is very susceptible to cationic interference Although the oxygen-flask method[9] is suitable[10] for the determination of sulphate down to 1 μg, the method is based on total sulphur, such a method would not be suitable for carbohydrate–protein complexes

Since the existing methods could not be modified for use on a smaller scale, it was necessary to devise a convenient, reproducible method for quantitative determination of sulphate, which would consume little carbohydrate For this purpose, flame photometry was investigated.

METHODS AND RESULTS

Demonstration of the feasibility of sulphate determination — Various volumes of microanalytical-grade sulphuric acid (diluted to 450 p p m sulphate and standardised by titrimetric analysis, 0–2,000 μl) were added, using automatic-levelling micropipettes, to aliquots (2 ml) of calcium chloride solution (equivalent to 150 p p m calcium) to give final sulphate concentrations covering the range 0–150 p p m when the solutions were diluted to a fixed volume (6 ml) An EEL flame photometer was adjusted to give zero deflection with distilled water, and full-scale deflection when the calcium chloride standard (50 p p m Ca^{2+}) was sprayed The prepared solutions were sprayed, and the galvanometer reading (θ) was noted In this and subsequent experiments, determinations were made in duplicate, and the pairs of results were identical Zero and full-scale deflections were checked between each determination by spraying water and the calcium standard A calibration curve was obtained (Fig 1)

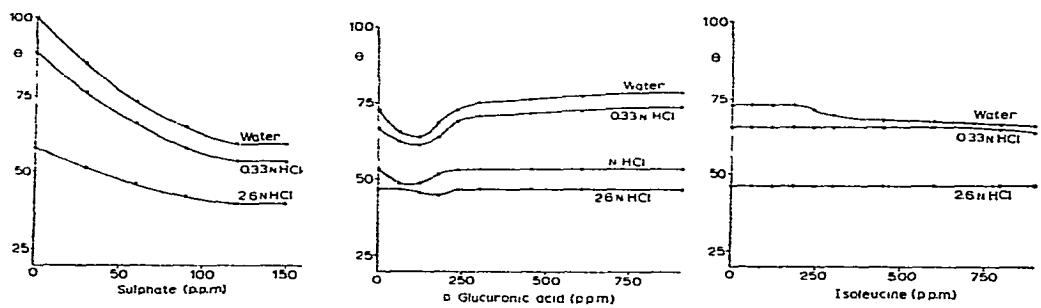

Fig 1 The flame-photometric assay of sulphate

Fig 2 Effect of D-glucuronic acid on the intensity of the calcium flame

Fig 3 Effect of isoleucine on the intensity of the calcium flame

Comparison of calibrations with sulphuric acid and ammonium sulphate as standards — When the preceding experiment was repeated with analytical-grade ammonium sulphate solution in place of sulphuric acid, an identical calibration curve was obtained

Investigation of the effect of various species on the calibration curve — (*a*) *Hydrochloric acid* When the preceding experiments were performed in the presence of hydrochloric acid, it was observed that, whilst the acid diminished the intensity of the calcium flame, a calibration curve could still be achieved. The reduction in flame intensity became greater as the acid concentration was increased (Fig 1)

(*b*) *Cations* Concentrations of sodium greater than 50 p p m. increased the transmitted flame intensity, but calibrations performed in the presence of smaller concentrations of sodium showed no deviation from the corresponding calibration performed in its absence

(*c*) *Aldoses* Calibrations performed in the absence or presence (concentrations up to 900 p p m) of D-glucose were identical, whether or not carried out in the presence of hydrochloric acid

(*d*) *2-Amino-2-deoxyhexoses* Calibrations performed in the absence and presence (concentrations up to 900 p p m) of 2-acetamido-2-deoxy-D-glucose were identical, whether or not carried out in the presence of hydrochloric acid

(*e*) *Uronic acids.* Calibrations performed in the presence of D-glucuronic acid were abnormal Determinations of one sulphate concentration (60 p p m), made over a range of concentrations of D-glucuronic acid, showed that the depressive effect of the carbohydrate upon flame intensity varied with its concentration (Fig 2)

Whilst it was possible to extend the Ruff[11 12] method for degradation of aldonic acids to a similar degradation of uronic acids, use of this method to eliminate the uronic acid effect became a very involved procedure, since the reagents required were found to affect the calibration For similar reasons, elimination of the uronic acid effect by esterification of the carboxyl group with ethylene oxide and subsequent reduction with sodium borohydride was found to be impractical

Calibrations performed in the absence or presence (270 p.p m) of D-glucuronic acid were identical when carried out in the presence of N hydrochloric acid

(*f*) *Amino acids* Calibrations performed in water and in the presence of isoleucine proved to be abnormal Determinations of one sulphate concentration (60 p p m) performed over a range of isoleucine concentrations showed that the depressive effect of the amino acid on flame intensity varied with concentration (Fig 3) However, calibrations performed in the presence of isoleucine at concentrations up to at least 600 p p m and in the presence of hydrochloric acid (0 33N or greater) showed no deviation from the corresponding control calibrations

Effect of hydrolysis vessel upon subsequent determination of sulphate — Soda, borosilicate, and silica ('Spectrosil', Thermal Syndicate Ltd , Wallsend, Northumberland) glass-tubes, and sealable nylon and Teflon tubes were individually subjected to hydrolysing conditions (0 5N or 3 9N hydrochloric acid for 2–16 h at 100°) After addition of the appropriate amount of calcium chloride solution, the solutions were tested (for contamination) against standard calcium chloride in the flame photometer Whilst acid in high concentration continually leached contaminants from all of the materials, at low concentrations, only soda glass, borosilicate glass, and nylon were attacked, silica and Teflon did not contaminate the solutions It was demonstrated that the contaminating effect of borosilicate glass at low concentrations of acid could be temporarily removed by extensive pretreatment with acid

Standard method for sulphate determination — As established in previous experiments, the presence of a uronic acid in the sample demands special treatment in order to overcome the uronic acid effect Therefore, before sulphate determination was performed, the carbazole[13] assay was applied to the sample to determine its uronic acid content Before proceeding further, samples containing high concentrations of alkali metal, alkaline earth metal, or other interfering cations, were loaded (sample wt , $\lesssim$ 5 mg, sample vol , $\gtrsim$ 500 μl) on to glass columns (80 × 6 mm) containing

AG50WX8 cation-exchange resin (H^+ form, 200-400 mesh, 1-ml settled bed vol) The sample was then washed in by successive volumes of distilled water (3×250 µl), eluted with water, and concentrated appropriately by rotary evaporation at 30° *in vacuo*

(*a*) *Procedure for samples not containing uronic acids* — Sample solutions (100 µl) containing 1–30 µg of sulphate were introduced into clean, silica tubes, and N hydrochloric acid (100 µl) was added After brief centrifugation to bring all of the liquid to the bottom of the tube, the tubes were sealed, shaken, and immersed for 2 h in a water bath at 100°. The cooled tubes were opened after centrifugation Standard calcium chloride solution (150 p p m Ca^{2+}, 100 µl) was added, and the solutions were well mixed A set of standard sulphate concentrations (0–150 p p m) in a final concentration of 0 33N hydrochloric acid was also prepared with ammonium sulphate solution Standards and samples were sprayed into the flame photometer in the usual fashion, the sulphate contents of the samples being derived from the calibration curve

(*b*) *Procedure for samples containing uronic acids* Sample solutions (50 µl) containing 1–30 µg of sulphate were hydrolysed as outlined in (*a*) After the tubes had been opened, D-glucuronic acid solution (100 µl, to give a final concentration of 270 p p m), 5N hydrochloric acid (50 µl), and calcium chloride solution (300 p p m Ca^{2+}, 50 µl) were added to give a final acid concentration of N, and calcium concentration of 50 p p m After mixing, the samples were sprayed into the flame photometer along with a set of standards as before

Assessment of the standard procedure — (*a*) *Application to authentic monosaccharide sulphates* Authentic samples of D-glucose 6-(barium sulphate), D-galactose 6-(barium sulphate), and methyl α-D-galactoside 4-(barium sulphate), were dried to constant weight *in vacuo* over phosphorus pentoxide at 61° Portions (*ca* 3 mg) were accurately weighed, and dissolved in water (250 µl) Aliquots of the solutions (200 µl) were transferred to individual AG50WX8 resin columns (H^+ form, bed vol, 1 ml) The samples were eluted with distilled water, and the first parts (5 00 ml) of the eluates were collected Aliquots (100 µl) of the solutions were hydrolysed for 2 h at 100°, in triplicate, in silica tubes at a final hydrochloric acid concentration of 0 5N, and their sulphate contents were determined (Table I)

TABLE I

SULPHATE CONTENT OF CARBOHYDRATE SULPHATES

	Sulphate content in µg of SO_4^{2-}/ml of solution	
	Calc	*Found*[a]
D-Glucose 6-(barium sulphate)	162	168
D-Galactose 6-(barium sulphate)	142	132
Methyl α-D-galactoside 4-(barium sulphate)	138	132
Chondroitin 4-(sodium sulphate)	72 5	68 3

[a]Identical values obtained in triplicate

(*b*) *Application to a sulphated polysaccharide* An authentic sample of chondroitin 4-(sodium sulphate) was dried to constant weight *in vacuo* over phosphorus pentoxide at 61° A portion (*ca* 5 mg) was accurately weighed, and dissolved in water (500 μl) An aliquot (400 μl) was loaded on to an AG50WX8 resin column (H^+ form, 1 ml) and eluted with distilled water as before The first part (5 ml) of the eluate was collected, and aliquots (50 μl) were hydrolysed in triplicate with N hydrochloric acid (50 μl) 5N Hydrochloric acid (50 μl) and calcium chloride solution (300 p p.m Ca^{2+}, 50 μl) were added D-Glucuronic acid solution (100 μl) was added to give a concentration of 270 p p m The sulphate contents were then determined in the usual way (Table I) and compared with the theoretical content of the polysaccharide based on molar ratios of unity for sulphate, D-glucuronic acid, and 2-acetamido-2-deoxy-D-glucose

DISCUSSION

The determination of metallic ions which have a characteristic colour when introduced into an oxidising flame can be achieved by flame photometry The actual intensity of the colour produced is dependent upon the following criteria the metallic ion, the concentration of the metallic ion, the chemical state of the metallic ion, the flame temperature, the rate of introduction of the metallic ion into the photometer flow-system, and the rate of passage of the metallic ion through the flame.

Where the metallic ion can exist in two forms, the preferred state of the cation will be that in which the resulting salt has the lower solubility product Therefore, if the metallic ion is added as one salt to a solution of the other anion, its chemical environment may alter to the preferred form, and, assuming that the two salts dissociate at different rates, this change would be detected by the flame photometer Since, in the experimental procedure, the only variable is the chemical state of the metallic ion such a change was made the basis for devising a new method for the determination of sulphate

It was demonstrated that a reproducible calibration curve for sulphate could be obtained in this way by conversion of calcium chloride into calcium sulphate Although the behaviour of the corresponding barium salts is similar, this ion was not chosen, since its flame intensity is much weaker than that of calcium When theoretical equivalence between calcium and sulphate was reached, further additions of sulphate, as expected, did not alter the flame intensity Ammonium sulphate is a suitable, primary sulphate standard, and calibrations could be performed in hydrochloric acid as well as in water The depressive effect of hydrochloric acid upon the flame intensity is explained in terms of diminished decomposition of the calcium salt as a result of the mass action of chloride ion Since the method depends upon the relative depression of calcium flame intensity, the fact that calcium chloride is very deliquescent (and therefore cannot be weighed with great accuracy) has no influence on the calibration, provided that the same solution is used throughout for a set of determinations

The calibration curve was applicable over the range 3–120 p p m sulphate,

and, since a sample of 300 μl was the smallest that was sufficient to allow time for the galvanometer reading to become stabilised and a reading to be taken, this corresponds to a range of 1–36 μg of sulphate If desired, the upper limit could be extended up to concentrations of sulphate equivalent to the saturation concentration of calcium sulphate.

The effect of other materials likely to be present in carbohydrate hydrolysates was then investigated Since ammonium sulphate is not normally present in such hydrolysates, standard sulphuric acid was used as the source of sulphate in preference to this salt

Since the EEL flame photometer relies upon broad-spectrum, optical filters for elimination of interference, the wavelength of the emission from interfering ions is important Sodium was chosen to represent the behaviour of extraneous metallic ions, since it has the most intense flame colour Its presence affected a calibration, and, since polysaccharides containing uronic acids are usually prepared as salts, cations were removed by ion exchange before making a sulphate determination

Organic matter in smaller concentrations does not normally affect flame photometric determination and, accordingly, the calibrations were not affected by D-glucose and 2-acetamido-2-deoxy-D-glucose In contrast, the effect of D-glucuronic acid in water, was to give, initially, a diminished flame intensity As the uronic acid concentration was further increased, the intensity steadily increased up to concentrations of 900 p p m , and passed through a normal value at 240 p p m These effects, although less marked, were still significant when the determinations were made in the presence of hydrochloric acid and can be explained in terms of competition between sulphate and uronate ions for calcium At low concentrations, both sulphate and uronate could be associated with the same calcium ion This could give place to calcium uronate alone at higher concentrations of uronate. Calcium glucuronate would be more volatile than calcium sulphate In order to eliminate the uronic acid effect, esterification and borohydride reduction, and Ruff degradation were attempted, but were rejected on the basis of their being laborious and time-consuming, and their contribution of contaminating species to the samples The problem was finally overcome by adding, to uronic acid-containing samples, after hydrolysis, D-glucuronic acid to give a total concentration of 270 p p m , and performing the determination in N hydrochloric acid Under these conditions, uronic acid was found to be without effect on the sulphate determination The margin between 240 and 270 p p m D-glucuronic acid allowed for some decarboxylation during hydrolysis of the uronic acid present in the carbohydrate sample

Although isoleucine affected the calibrations made in water, this effect was largely absent when the determination was performed in acid Since organic matter does not normally have an effect, this phenomenon was also attributed to the ionic nature of the added species It is suggested that, at nearly neutral pH-values, the carboxyl function of the amino acid associates with the calcium ion and decreases its volatility, but that the presence of acid prevents the formation of such a species Although amino acids would be expected to differ in their behaviour, the character-

istics of isoleucine were accepted as being representative of those of all amino acids. The presence of acid is necessary for the analysis of samples containing protein, but, under the conditions subsequently chosen for the liberation of sulphate from polysaccharides, proteins would be only partially hydrolysed Since the carboxyl groups of amino acids are involved in peptide linkages, the effect of free amino-acids was presumed to be greater than that of those forming part of oligopeptide or protein chains

Several materials were investigated for their resistance to leaching under hydrolysing conditions, since ordinary glass was attacked extensively. Although no material was entirely satisfactory under strongly acidic conditions, it has been demonstrated[14] that complete cleavage of *O*-sulphate bonds in acidic polysaccharides occurs after treatment for two hours with 0 5N hydrochloric acid at 100°. Since only mild conditions are required, silica or Teflon tubes could be used for the hydrolysis Silica tubing was selected in preference to Teflon, since it is sealed more easily and is commercially available Use of pretreated soda or borosilicate glass was rejected on the grounds that their contaminating effects might return as the glasses aged

Thus, a standard procedure was devised for the simple and reproducible quantitative determination of free sulphate on a micro-scale The method was finally assessed by determination of the sulphate content of authentic carbohydrate sulphates Within experimental limits, the results obtained were in excellent agreement with the theoretical values Since the *N*-sulphate bond is more labile than the *O*-sulphate bond, the method would be equally applicable to the determination of *N*-sulphate

The moderately sensitive 'EEL' flame-photometer relies solely upon a coarse, optical filter to eliminate interference Use of a diffraction grating to resolve the emitted light, and determination of its intensity at a wavelength characteristic of calcium would eliminate interference by emission from extraneous matter and further increase the sensitivity

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REFERENCES

1 A S Jones and D S Letham, *Analyst*, 81 (1956) 15
2 R Belcher, A J Nutten, and W I Stephen, *Mikrochim Acta*, (1953) 51
3 R Belcher, A J Nutten, and W I Stephen, *J Chem Soc*, (1953) 1334
4 K S Dodgson, *Biochem J*, 78 (1961) 312
5 J M Martin, M Sc Thesis, University of Birmingham, 1966
6 C A Antonopoulos, *Acta Chem Scand*, 16 (1962) 1521
7 P W Kent and M W Whitehouse, *Analyst*, 80 (1955) 630
8 I T Sutherland, *Clin Chim Acta*, 14 (1966) 554

9 W SCHONIGER, *Mikrochim Acta*, (1956) 869
10 R MCGILLIVRAY AND S C. WOODGER, *Analyst* 91 (1966) 611
11 O RUFF, *Ber*, 31 (1898) 1573
12 O RUFF AND G OTTENDORF, *Ber*, 33 (1900) 1798
13 T BITTER AND H M MUIR, *Anal Chem*, 4 (1962) 330
14 J. A CIFONELLI, personal communication

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A PROTON MAGNETIC RESONANCE SPECTRAL STUDY OF HEPARIN L-IDURONIC ACID RESIDUES IN COMMERCIAL HEPARINS*

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ABSTRACT

The p m r spectra of commercial heparin samples from a variety of sources indicate that there are at least two types of heparin These types are differentiated by small, but distinct, variations in the relative intensities of their proton signals and, most noticeably, by the presence of acetyl groups in samples of one of the types The spectral data also indicate that approximately equimolar proportions of three kinds of constituent sugar residues are present in all of the heparin samples Deaminative degradation of a heparin, followed by exhaustive reduction of the carboxyl groups, acetolysis, and deacetylation, yielded L-idose as a major product It is tentatively concluded that L-idosyluronic acid residues constitute about one-third of the constituent sugars of the commercial heparins, and that the aminodeoxy-D-hexosyl and D-glucosyluronic residues (already recognized as constituents) each constitute one-third of the polymeric material

INTRODUCTION AND DISCUSSION

Heparin contains 2-deoxy-2-(sulfoamino)-α-D-glucopyranosyl 6-sulfate (GA) and α-D-glucopyranosyluronic acid 2-sulfate (GU) residues as major constituent sugars[1] These are joined *via* (1 → 4)-glycosidic bonds in alternating sequences[2,3], *i e*, GA-(1 → 4)-GU and GU-(1 → 4)-GA In addition, minor proportions of D-xylose and D-galactose residues are encountered towards the reducing end of the polymer chain which, in deproteinized preparations of heparin, is sometimes terminated by a residue of L-serine[4,5] Also, there are several reports that an iduronic acid is a component sugar acid[6–8]

It is well known that heparin samples may differ widely in anticoagulant activity, but the reason for such variability is not clearly understood Present chemical

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methods of analysis do not appear to provide an adequate quantitative basis for comparing heparins[1,9,10], and the current study has been untertaken to see if proton magnetic resonance (p m r) spectroscopy, which generally furnishes excellent quantitative data, might be useful for this purpose. Accordingly, a number of commercial heparins representing a wide range of activities (Table I) have been examined It is now found that these samples also show distinct differences in their p m r. spectra, as we have already noted briefly[11], although so far no correlation has been detected between biological activity and spectral characteristics

TABLE I

RELATIVE INTENSITIES OF SIGNALS IN P M R SPECTRA OF HEPARINS

Heparin sample	*Signals (area ratios)*								*Acetyl*	*Anticoagulant activity, per mg*	
	a	b	c	d	e	f	g	h			
Group A										*a*	*b*
1 NBC-5717	1	0 8	1 2	2 9	1 1	3 7	6 1	1 6	0 8	62	114
2 NBC-8947	1	0 8	0 8	2 6	1 0	3 5	6 4	2 8	0 8	42	127
3 NBC-8242	1	0 9	1 2	2 6	1 1	3 2	5 2	1 7	0 7	125	131
4 NBC-8941	1	0 9	1 3	2 6	1 2	3 6	5 7	1 6	1 0	123	120
5 Abbott-724-7380	1	1 0	1 2	2 7	0 9	3 2	4 8	1 7	0 4	165	169
6 Lederle-1804-4160	1	0 9	1 1	2 8	1 2	3 5	5 9	2 3	0 6	139	156
Average	1	0 9	1 1	2 7	1 1	3 5	5 7	2 0	0 7		
Group B[c]											
7 NBC-2590	1	1 0	1 2	2 9	1 2	3 2	4 1	1 6		15	101
8 Abbott-1958	1	1 2	1 3	3 3	1 4	4 1	4 6	2 3		92	145
9 Lederle-7-8738	1	1 1	1 2	3 0	1 3	3 5	4 4	2 1		136	160
10 Upjohn-SH-042	1	1 2	1 3	2 9	1 3	3 0	3 5	1 4		172	168
11 Upjohn-WD-010	1	1 1	1 3	3 0	1 2	2 8	3 4	1 2		—	140
12 Dog heparin	1	1 1	1 4	2 9	1 3	3 4	5 3	1 5		285	198
Average	1	1 1	1 3	3 0	1 3	3 3	4 2	1 7			

[a]By Howell assay[10] [b]By U S P assay [c]Most of the samples in this group appear to contain a trace of acetyl, but less than 0 1%

There appear to be at least two types of heparin marketed currently for medicinal purposes This conclusion is suggested by the difference between spectra 1A and 1B (Fig 1*), which are representative of the heparins examined, about half of the samples afford spectra resembling 1A, and the other half, 1B** Each spectrum appears to be derived from the same *kinds* of protons—inasmuch as signals having the same chemical shifts are common to all— and hence all samples are presumed to be constructed of the same types of monosaccharide residue However, the relative intensities of the signals vary noticeably for the two groups (Fig 1 and Table I) and

*In common with those of other polymers, these spectra are characterized by broad signals that, due to time-averaging effects, do not exhibit the splittings produced by protons of compounds of low molecular weight

**The 60-MHz spectrum reported for a heparin sample by Inoue and Inoue[12] resembles 1B

to a lesser extent, among the samples within each group (Table 1) These differences are especially pronounced for two or three of the signals, and are most strikingly evident in the presence (Fig 1A) or virtual absence (Fig 1B) of a signal (at 2 0 ppm) attributed to protons of *N*-acetyl groups[11]

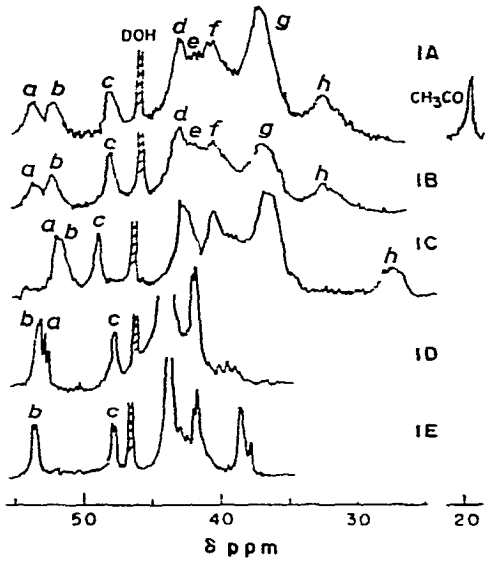

Fig 1 P m r spectra at 100 MHz of (1A and 1B) representative heparin samples A and B, respectively, (1C) sample B, after partial hydrolysis with acid, and neutralization (1D) sample B after deamination by nitrous acid, (1E) deamination product represented by 1D, after reduction with sodium borohydride

Since other mucopolysaccharides occur in tissue from which heparin is extracted, the question of homogeneity of the heparin preparations arises Dermatan sulfate appears to be a most likely contaminant[13] It contains 2-acetamido-2-deoxy-D-galactose as a major constituent sugar, a fact that accounts for the strong acetyl-proton signal present in its spectrum[11 12] Hence, those heparin samples (Fig 1B and Table I, group B) that show no evidence of the presence of such *N*-acetyl groups can be regarded as free from dermatan sulfate Although the samples of group A give significant acetyl-proton signals, other features of their spectra are sufficiently different from that of dermatan sulfate to make it unlikely that they contain the latter as a substantial contaminant Thus, artificial mixtures of an acetyl-free heparin with dermatan sulfate give spectra distinctly different from those of the heparin samples that do produce acetyl-proton signals These differences are readily noticeable at 4 85, 4 65, 3 95, and 3 50 ppm, where signals due to dermatan sulfate become apparent in the spectra of the mixtures, but are absent from the 1A type of heparin spectrum.

These data do not establish that the heparins examined in the current study are homogeneous They do indicate, however, that methods currently used commercially for extraction and purification of these mucopolysaccharides yield products having a relatively high degree of uniformity in composition

P m r. spectroscopy provides information about the relative proportions of the various kinds of protons within the molecule, data that are little affected by the presence of such extraneous materials as inorganic salts and residual moisture or solvents Such contaminants must, however, be accounted for in chemical analyses, a problem of considerable difficulty with heparin samples[10] Hence, if the spectral signals are satisfactorily assigned to specific protons, their relative intensities furnish an independent assay of the ratio of the monosaccharide constituents

In the current study, the most useful approach to analysis of the heparin spectra has been based on known properties of the (sulfoamino)hexose constituent The anomeric proton (H-1) signal of the latter, as well as that of the glycosyluronic (glycuronosyl, uronide) component, would be expected to be one of the three signals (*a*, *b*, and *c*, Fig 1A and B) located at the low-field side of the deuterium hydroxide (DOH) signal, that is, at about 5 4, 5 2, and 4 8 ppm Since the sulfoamino group is highly labile[14], heparin subjected to mild hydrolysis with acid has been examined Its spectrum (Fig 1C) shows that the signal at lowest field originally (*a* in 1A or 1B) and also signal *h* are displaced upfield By contrast, signal *b* and much of the rest of the spectrum have undergone relatively little change, whereas signal *c* has suffered a downfield displacement The properties of signals *a* and *h* can be simulated with model compounds Thus, the chemical shifts of H-1 and H-2 of methyl 2-deoxy-2-sulfoamino-α-D-glucopyranoside[15] are 5 1 and 3 3 ppm, respectively, and, for the *N*-desulfated amino glycoside, they are 4 8 and 2 8 ppm On this basis, the signals *a* and *h* are ascribed to H-1 and H-2, respectively, of the (sulfoamino)hexose residues of the heparin* Furthermore, *N*-acetylation of the partially hydrolyzed heparin restores the appearance of the spectrum almost to its original form, although a very strong signal attributable to the newly introduced *N*-acetyl group is now detected

Additional information about the origin of signal *a* is provided by deamination with nitrous acid The product obtained on treating heparin with nitrous acid[16] for 6 days produces a signal at 5 4 ppm (Fig 1D)** ascribable to a proton of a hydrated aldehyde[16], which is also produced by treating glycoside 3 with nitrous acid After reduction of the compound with sodium borohydride (Fig 1E), this signal is no longer detected, although a strong signal, undoubtedly due to the newly formed carbinol group, now appears at 3 8 ppm*** The reduced degradation product shows only two low-field signals, the chemical shifts of which are consistent with the probability that these correspond to *b* and *c* of the original heparin spectra

As pointed out previously[11], the chemical shift of signal *b* is close to that of H-1 of α-D-glucopyranosyl 2-sulfate residues of partially sulfated dextran[17], and also to that expected from the results of a spectral examination of some α- and β-D-

*This contrasts with the suggestion of Inoue and Inoue[12] that a low-field signal corresponding to *c* (Fig 1A or 1B) is due to H-2

**The overall improved resolution found for spectrum 1D (and also 1E) is attributable to the relatively low molecular weight of these deamination products

***This result was confirmed by reducing with borodeuteride the signal at 3 8 ppm was now much smaller, but the spectrum was altered little in other respects

glucopyranosyluronic disaccharides Hence, it appears valid to ascribe the H-1 signals of the amino-D-hexosyl and D-glucosyluronic constituents of heparin to signals *a* and *b*, respectively, and, based on their relative intensities (Table 1), to conclude that these two constituents are present in the ratio of about 1 1

Although this ratio agrees with the results of chemical analyses which give a hexosamine to uronic acid ratio of 1 1 for heparins[1,9,10], the observed intensities of signals *a* and *b* (relative to those of the other signals) appear inadequate to account for the total material present in these commercial samples Thus, if the spectra were to accord with chemical analyses, the area *a* plus *b*, relative to the overall area of the other proton signals (*c* to *h*), should be 1 5 — that is, a hexosamine residue contains seven non-exchangeable protons, and a hexuronic acid residue, five, giving an average of six per residue of the disaccharide unit.

The ratios observed are, however, much higher Measured with the spectrometer integrator, the ratio obtained from Fig 1A is about 1 6 8 and for Fig 1B, about 1 6 2 (with an allowance being approximated for the DOH signal) Even higher ratios are obtained when the intensities are measured by planimetry (see Experimental section) Thus, according to Table I, the average ratio is about 1 8 for the group A heparins and about 1 7 for the group B samples At 60 MHz, the ratio calculated[12] from the intensities of corresponding signals is 1 5 2 The smaller relative areas of *a* and *b* in the 100-MHz spectra, compared with those at 60 MHz, are probably due to less overlap of these signals by the more-intense, upfield signals, including the DOH peak Since some overlap is also evident in the spectra now described, the actual ratios may be even higher Hence, signals *a* and *b* do not appear to account entirely for the anomeric protons of the molecules

A more self-consistent group of data is obtained by assuming that *all three* low-field signals are due to anomeric protons—that is, that *c* is also an H-1 signal—and by comparing the intensity values in Table I Thus, the ratio of $(a+b+c)$ to the total of zones *d*–*h* is 3 0 15 0 for the group A heparins (Table I) In agreement with this result, the expected ratio is 3 0 14 0, if *b* and *c* are regarded as uronide H-1 signals and the hexosamine uronide ratio as 1 2 For the type B heparins, the observed and calculated ratios are 3 4 14 5 and 3 4 15 6, respectively Taking into account the fact that the measured areas of signal *c* are disproportionately large (Experimental section), the overall agreement is probably better than these ratios indicate According to these findings, then, all of the heparin samples contain only about half as much hexosamine as uronic acid

If signals *b* and *c* are both due to D-glucuronosyl residues, the large difference between them in chemical shift must be attributed to other structural differences It was suggested above that the chemical shift of signal *b* is close to that expected for an α-D-glucopyranosyl residue bearing a 2-sulfate group Hence signal *c* could be due to a residue containing the *O*-sulfate group more remote from H-1, or to a non-sulfated residue, structures that have previously been suggested as possibilities[1] An alternative possibility, namely, that the relatively high field shift for *c* is indicative of a β-D anomeric configuration (axial[18] H), is unlikely in view of the small spacing

that characterizes the corresponding signal in the deaminatively degraded product (Fig 1D or 1E) (Thus, the H-1 signal of 6-*O*-*β*-D-glucopyranuronosyl-D-galactose is found to have a spacing of 7 2 Hz, in common with that of other *β*-D-glucopyranosides) An unusual characteristic of signal *c* is its markedly lower shift after the heparin has been subjected to mild hydrolysis with acid (Fig 1C) This shift downfield is also observed if a heparin solution is acidified, but, in this instance, the effect is reversed if the pH of the solution is re-adjusted to 8–9

It appeared possible that signal *c* is produced by a *third* kind of monosaccharide residue, especially in view of the several reports of an iduronic acid in heparin samples, as noted above Therefore, it became of interest to check our samples for the possible presence of a sugar constituent other than the hexosamine and D-glucuronic acid residues For this purpose, chemical degradative methods described by earlier workers have been utilized, and some of the results obtained are given below

A sample of commercial heparin (type B, sample no 11, Table I) was subjected to deamination with nitrous acid, followed by borohydride reduction, as described above, to give the results depicted in Fig 1D and 1E, respectively, this product was sparingly soluble in methanolic hydrogen chloride A portion of it, extracted by repeated treatment with this solvent (and spectroscopically indistinguishable from the total material, 1E), was treated first with diazomethane to esterify uronic acid residues, and then with aqueous sodium borohydride to reduce ester groups The product was then re-treated several times in the same sequence—by dissolution in methanolic hydrogen chloride, esterification with diazomethane, and reduction with borohydride—which finally afforded uronide-free material The latter, on partial acetolysis with acetic anhydride–sulfuric acid[19], followed by catalytic deacetylation, yielded at least six products having a wide range of mobilities on paper chromatograms Among these were two reducing sugars having the respective mobilities of a glucose and an idose Treatment with D-glucose oxidase showed that the former was D-glucose The second sugar was isolated as a syrup by column chromatography on cellulose, and was characterized as L-idose by conversion into L-iditol hexaacetate, the yield of the crude syrup was about 20%, based on the weight of the neutral degradation product that had been subjected to acetolysis Two different samples (5 and 9), examined by the same general procedure in small-scale experiments, have also yielded an idose and a glucose, as indicated by paper chromatography

Since all of the heparin preparations are so similar in their spectral properties, particularly insofar as they appear to contain three constituent monosaccharides, it is reasonable to assume that each contains L-iduronosyl residues If H-1 of this latter kind of residue gives rise to signal *c*, the proportion of L-iduronic acid in each sample listed in Table I is about one-third of the total

Although L-idose has been found in the current study, it had not been detected by others[15,20] in carboxyl-reduced products derived from heparin However, acid hydrolysis, not acetolysis, was used by these workers, and this would have yielded mainly 1,6-anhydro-L-idose[21], thus lessening the likelihood of detecting L-idose itself Nevertheless, our conditions of reduction were more strongly alkaline, so that

at least part of the L-idose isolated could have been formed spuriously, for instance by isomerization of D-glucuronosyl residues at C-5 or *via* hydration of an α,β-unsaturated, uronic acid intermediate Against this possibility is the apparent absence of such side-reactions in the many other hydride reductions of uronosides reported in the literature, as well as the precedent afforded by the earlier reports of an iduronic acid in heparins In addition, depending on the reliability of the intensity measurements, the p m r spectra appear to suggest that the heparins contain a third major type of sugar residue

An alternative possible source of these divergent results is that the commercial heparins we have examined consist of a mixture of polysaccharides Thus, the heparin examined in ref 20 had been subjected to an extensive preliminary fractionation Hence, the L-iduronosyl residues could form part of a polysaccharide closely associated with heparin, but as yet uncharacterized If this is so, it remains to be determined whether the L-iduronosyl residues do or do not contribute to some of the physiological properties of commercial heparins

EXPERIMENTAL

Measurement of proton magnetic resonance spectra — Solutions for p m r. spectral examination, about 10% (w/v), were prepared by dissolving the sample in deuterium oxide and lyophilizing, and then repeating this deuterium-exchange process 2 or 3 times

Spectra were recorded with a Varian HA-100 high-resolution spectrometer at a sweep width of 500 Hz Tetramethylsilane, contained in a coaxial capillary tube mounted inside the sample tube, was used as the reference Particular care was taken to optimize the homogeneity and phase settings, to ensure the most satisfactory integral measurements Each spectrum was subdivided into a number of zones based on the signals most readily apparent, as shown in Fig 2 for a typical example, and the areas of these zones were measured with a planimeter Their relative areas are given in Table I

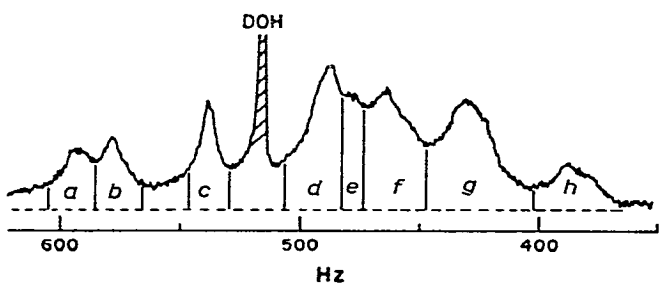

Fig 2 Regions of the spectrum p m r of heparin that have been integrated by planimetry Sagments *a* to *h* correspond to the signals designated similarly in Fig 1 and Table I

By recording the spectrum at 75°, the peak due to deuterium hydroxide (DOH) was shifted upfield permitting a more satisfactory measurement of the area of

signal *c* Some comparative values for relative areas of *a*, *b*, and *c* at the normal probe-temperature (35°) and at 75° are as follows

Sample	*Relative peak areas*					
	at 35°			*at 75°*		
	a	b	c	a	b	c
NBC-5717	1	0 8	1 2	1	0 8	0 8
Dog heparin	1	1 1	1 4	1	1 1	1 1

In some instances, integrals were recorded with the integrator facility of the spectrometer However, because of the disproportionate effect of baseline rise on signals *a*, *b*, and *c* by the strong group of upfield signals (including the DOH signal), these integrals are regarded as less satisfactory for the present purpose than are the planimeter measurements

Deaminative degradation and exhaustive reduction of heparin — A Deamination Sodium heparinate (sample 11, Table I) (30 g) was dissolved in water (50 ml) and treated with a solution composed of sodium nitrite (30 g), water (750 ml), and M hydrochloric acid (240 ml) (reaction conditions of Foster *et al*[15]) After 110 h, the pH of the solution was adjusted to about 9 with sodium carbonate After lyophilization, and deuterium exchange with deuterium oxide, a sample of this reaction mixture produced the spectrum represented by Fig 1D (spectral examination of samples at earlier stages indicated that the reaction was complete in 90–100 h)

The reaction mixture was added to a vigorously stirred slurry of potassium borohydride (7 g) in ice–water (50 ml) After 18 h at room temperature, the solution was rendered neutral with acetic acid, and concentrated *in vacuo* at 50° A sample of the residue, after deuterium exchange, gave the spectrum shown in Fig 1E

B Extraction with methanolic hydrogen chloride The residue was suspended in methanol, the solvent was evaporated off, and this treatment was repeated Methanolic hydrogen chloride (0 05M, 200 ml) was added, the suspension was shaken for 48 h, and filtered, and the solid was washed with the solvent, the liquid being retained A second extraction of the solid was conducted for 60 h, and the extracts were combined, neutralized with ethereal diazomethane, and evaporated to dryness, affording a yellow solid (16 g)

The p m r spectra of this extracted material and of the insoluble residue in deuterium oxide were closely similar, indicating that both fractions were essentially the same and, hence, that although a partial, non-preferential extraction had been effected From the intensities of these spectra, it was estimated that the methanol extract contained perhaps half of the degraded heparin, and also that it was heavily contaminated with extraneous salts

C *Borohydride reductions* An aqueous solution (50 ml) of the methanolic extract was added dropwise to a vigorously stirred solution of sodium borohydride

(5 g) in ice–water (50 ml) After 18 h, the solution was neutralized with 3M sulfuric acid, and concentrated, 0 02M methanolic hydrogen chloride was added and distilled off, and this treatment with methanol was repeated twice to remove methyl borate The residue was shaken with 0 05M methanolic hydrochloric acid (200 ml) for 18 h, and the extract was neutralized with ethereal diazomethane, and evaporated; yield, 9.5 g of a yellow solid

A solution of this solid in water (40 ml) was added dropwise to a stirred solution of sodium borohydride (2 g in 25 ml of ice–water) After 18 h, Amberlite IR-120 ion-exchange resin was added to neutrality, the solution was evaporated to dryness, and the methyl borate was removed by evaporation after treatment with acidified methanol as above This reduction product was, in turn, extracted with 0 05M methanolic hydrogen chloride, the extract was neutralized with ethereal diazomethane, and concentrated, and a third reduction with sodium borohydride was performed

The same sequence of procedures was repeated, to effect a fourth borohydride reduction; the final product was deionized with a mixed-bed resin, and the neutral material was recovered as a light-brown syrup; yield, 1 6 g On a paper chromatogram (solvent, 10 4 3 ethyl acetate–pyridine–water[21], spray, ammoniacal silver nitrate[22]), the syrup showed components having R_G 0 85 (strong), 1 0 (faint), 1 24 (moderate), and 1 51 (faint), respectively

Partial acetolysis — To the neutral syrup (1 5 g) was added 200 l cold acetic anhydride–sulfuric acid[19], and the suspension was stored for 72 h at 3°. Ice was then added, and the reaction mixture was carefully neutralized with sodium hydrogen carbonate, and extracted several times with chloroform On evaporation, the extract yielded a syrup (2 2 g) that was deacetylated catalytically with cold, methanolic sodium methoxide A mixed-bed, ion-exchange resin was added to the methanol solution, followed by water, and the solution was evaporated, affording a pale-yellow syrup (1 2 g) Paper-chromatographic examination in several solvents (spray, aniline hydrogen oxalate[23]) showed the presence of a major reducing component (Product A) travelling at the same rate as an idose Also detected was a minor component, identified as D-glucose by the fact that it was oxidized by D-glucose oxidase Unidentified, nonreducing spots were also detected with the silver nitrate spray

Product A — The syrupy sugar mixture (1 1 g) obtained after partial acetolysis was applied to a cellulose column (35 × 4 cm), and the chromatogram was developed with one-quarter-saturated, aqueous butanol Fractions of the effluent that, by paper chromatography contained only the component identified as an idose, were combined and evaporated The colorless syrup obtained (0 28 g) showed $[\alpha]_D^{25}$ −14 7° (*c* 6 3, water) (L-idose shows[24] $[\alpha]_D$ −17°) The p m r spectra of this syrup in deuterium oxide and in methyl sulfoxide-d_6 were virtually indistinguishable from those of L-idose in the same two solvents

A portion of the syrup (70 mg) in water (3 ml) was heated with phenylhydrazine hydrochloride (0 5 g) and sodium acetate (0 4 g) for 2 h on a steam bath A small quantity of a brown-yellow solid that separated on cooling was recrystallized several times from ethyl acetate–benzene, affording yellow needles, m p 161–163° The X-ray

powder diagram of this product was indistinguishable from that of authentic L-*xylo*-hexulose phenylosazone (m p 162–164°)

L-*Iditol hexaacetate* — An aqueous solution (2 ml) of the crude L-idose (100 mg) was added dropwise to a stirred solution of sodium borohydride (50 mg) in ice–water (2 ml) After a reaction time of 18 h, an excess of Amberlite IR-120 was added, the solution was evaporated to dryness, and methanol was added and then distilled off, this procedure being repeated to remove methyl borate Acetic anhydride (2 ml) and anhydrous sodium acetate (200 mg) were added, and the mixture was heated for 1 5 h on a steam bath, and evaporated to dryness The product was extracted into chloroform, the solvent was removed, and crystallization occurred when the residue was dissolved in ethanol After recrystallization, the product had m p 122–124° It gave an X-ray diffraction powder diagram and a *negative* optical rotatory dispersion curve indistinguishable from those of authentic L-iditol hexaacetate

Anal Calc for $C_{18}H_{26}O_{12}$· C, 49 8; H, 6 0 Found C, 50.1, H, 6 1

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REFERENCES

1 Detailed accounts of the chemistry of heparin up to 1964 are given by (*a*) A B FOSTER AND A J HUGGARD, *Adv an Carbohyd Chem*, 10 (1955) 335, (*b*) J S BRIMACOMBE AND J M WEBBER *Mucopolysaccharides* Elsevier Publishing Co , Amsterdam 1964, p 92
2 M L WOLFROM, J R VERCELLOTTI, AND D HORTON *J Org Chem*, 29 (1964) 540
3 M L WOLFROM, H TOMOMATSU, AND W A SZAREK, *J Org Chem*, 31 (1966) 1173
4 U LINDAHL, J A CIFONELLI, B LINDAHL, AND L RODEN, *J Biol Chem*, 240 (1965) 2817
5 U LINDAHL AND L RODEN, *J Biol Chem*, 240 (1965) 2821
6 J CIFONELLI AND A DORFMAN, *Biochem Biophys Res Commun*, 7 (1962) 41
7 A A HORNER, *Proc Can Fed Biol Soc*, (1966) 28
8 U LINDAHL, *Biochim Biophys Acta*, 130 (1966) 368
9 L B JAQUES, *Progr Med Chem*, 5 (1967) 139
10 L B JAQUES L W KAVANAGH, AND A LAVALLEE, *Arzneimittelforsch*, (1967) 774
11 L B JAQUES L W KAVANAGH, M MAZUREK, and A S PERLIN *Biochem Biophys Res Commun*, 24 (1966) 447
12 S INOUE AND Y INOUE, *Biochem Biophys Res Commun*, 23 (1966) 513
13 R MARBET AND A WINTERSTEIN, *Helv Chim Acta*, 34 (1951) 2311
14 M L WOLFROM, R A GIBBONS, AND A J HUGGARD, *J Am Chem Soc*, 79 (1957) 5043
15 A B FOSTER, R HARRISON, T D INCH, M STACEY, AND J M WEBBER, *J Chem Soc*, (1963) 2279
16 A S PERLIN, *Can J Chem*, 42 (1964) 1365
17 W M PASIKA AND L H CRAGG, *Can J Chem*, 41 (1963) 777
18 R U LEMIEUX, R K KULLNIG, H J BERNSTEIN, AND W G SCHNEIDER, *J Am Chem Soc*, 80 (1958) 6098
19 N K RICHTMYER AND C S HUDSON, *J Am Chem Soc*, 63 (1941) 1727

20 M L WOLFROM, J R VERCELLOTTI AND G H S THOMAS *J Org Chem*, 29 (1964) 536
21 E SORKIN AND T REICHSTEIN *Helv Chim Acta*, 28 (1945) 1
22 M. A JERMYN AND F A ISHERWOOD, *Biochem J* 44 (1949) 402
23 S M PARTRIDGE, *Nature*, 158 (1946) 270
24 R H HORROCKS, *Nature*, 164 (1949) 444
25 L VON VARGHA, *Chem Ber*, 87 (1954) 1351

Carbohyd Res, 7 (1968) 369–379

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UNTERSUCHUNGEN ZUR INDUKTION DER LAC-ENZYME

TEIL III. SYNTHESE RADIOAKTIV MARKIERTER INDUKTOREN DURCH ENZYMATISCHEN GALAKTOSYLTRANSFER UND DIREKTE ENZYMATISCHE VERKNÜPFUNG VON GLYCON UND AGLYCON MIT β-GALAKTOSIDASE

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(Eingegangen am 11. Januar, 1968)

ABSTRACT

By using ^{14}C-labelled D-glucose, D-galactose, or glycerol, a number of β-D-galactosides were synthetisized by β-galactosidase catalysis. The label can be specifically incorporated into either the glyconic or the aglyconic moiety. The enzymic syntheses were carried out either by transgalactosylation (kinetic control), or by direct combination of free D-galactose with the acceptor D-glucose or glycerol (thermodynamic control), in the presence of β-galactosidase.

In the case of β-D-galactosyl-D-glucoses, all possible combinations could be detected in different amounts, depending on which method was used. The combination of D-galactose with glycerol yields mainly 1-*O*-β-D-galactosyl-D-glycerol, together with a small proportion of 2-*O*-β-D-galactosylglycerol, when the method of transgalactosylation is used. The direct combination yields additional 1-*O*-β-D-galactosyl-L-glycerol in minor quantity.

ZUSAMMENFASSUNG

Unter Verwendung von ^{14}C-markierter D-Glucose, D-Galaktose, und Glycerin werden durch enzymatische Katalyse durch β-Galaktosidase eine Reihe von radioaktiv markierten β-D-Galaktosiden hergestellt. Je nach Verwendung der radioaktiven Ausgangsprodukte ist das gebildete β-D-Galaktosid spezifisch im glyconischen oder aglyconischen Teil markiert. Die enzymatische Synthese erfolgt entweder durch Transgalaktosylierung (kinetisch kontrolliert) oder durch direkte Verknüpfung von freier D-Galaktose mit dem Akzeptor D-Glucose oder Glycerin in Gegenwart von β-Galaktosidase (thermodynamisch kontrolliert). Je nach angewendeter Methode können in veränderlichen Ausbeuten alle möglichen β-D-Galaktosyl-D-glucosen nachgewiesen werden. Die Kombination von D-Galaktose mit Glycerin liefert bei Anwendung der Transgalaktosylierung hauptsächlich 1-*O*-β-D-Galaktosyl-D-glycerin neben wenig 2-*O*-β-D-Galaktosylglycerin. Bei der direkten enzymatischen Verknüpfung mit freier D-Galaktose als Substrat wird zusätzlich noch 1-*O*-β-D-Galaktosyl-L-glycerin in geringer Menge gebildet.

EINLEITUNG

Bei der Untersuchung des Induktionsphänomens in Bakterien ist die Anreicherung des Induktors im Zellinnern, sowie sein Metabolismus von Bedeutung. Beides läßt sich mit Hilfe von radioaktiven Induktoren untersuchen. Wir berichten in dieser Arbeit über die Möglichkeiten, radioaktive Induktoren der Lac-Enzyme präparativ auf einfache Weise zu gewinnen.

Als Induktoren wirksam sind β-D-Galaktoside mit verschiedenen aglyconischen Resten. Je nach Problematik sollte die radioaktive Markierung im glyconischen oder aglyconischen Teil der β-D-Galaktoside enthalten sein. Prinzipiell wurden zwei verschiedene enzymatische Verfahren benutzt: Der Transfer des Galaktosylrestes[1-7] auf markierte Akzeptorverbindungen, D-[^{14}C]Glucose und [^{14}C]Glycerin, sowie die direkte enzymatische Synthese[8-13] aus D-[^{14}C]Galaktose und dem entsprechenden nicht markierten Aglycon. Beide Reaktionstypen werden durch β-Galaktosidase katalysiert. Durch diese enzymatischen Synthesen erhält man in einer Stufe die gewünschten markierten Verbindungen mit der gleichen spezifischen Aktivität wie die Ausgangsverbindungen.

ERGEBNISSE UND DISKUSSION

A. Enzymatische Synthese von β-D-Galaktosiden durch Transgalaktosylierung auf D-[^{14}C]Glucose oder [^{14}C]Glycerin (Markierung im Aglyconteil)

Bei der Einwirkung von β-Galaktosidase auf *o*-Nitrophenyl-β-D-galaktopyranosid (ONPGal) in Gegenwart von D-[^{14}C]Glucose entstehen eine Reihe von Oligosacchariden (**1–3**), die nach vollständiger Spaltung des ONPGal allmählich wieder hydrolysiert werden. Tabelle I gibt die R_{Gal}-Werte und Ausbeuten an neugebildeten β-D-Galaktosiden wieder, die nach 80%iger Spaltung von ONPGal unter den

TABELLE I

R_{Gal}-WERTE UND AUSBEUTEN, DER BEI DEM ENZYMATISCHEN GALAKTOSYLTRANSFER AUF D-[^{14}C]GLUCOSE GEBILDETEN β-D-GALAKTOSIDE

Substanz	**1**	**2**	**3**
R_{gal}	0.51	0.59	0.78
Chromatographisch vergleichbar	Allolactose	Lactose	3-*O*-β-D-Galaktosyl-D-glucose
% Ausbeute, bezogen auf eingesetzte D-[^{14}C] Glucose	25.6	0.65	1.17

Versuchsbedingungen erhalten wurden. Durch den Vergleich der R_{Gal}-Werte mit denen von authentischen Verbindungen lassen sich die Substanzen **1–3** zuordnen.

Es verhalten sich **1** wie Allolactose, **2** wie Lactose und **3** wie 3-*O*-β-D-Galaktosyl-D-glucose. Die Cokristallisation von **1** in acetylierter Form mit Allolactoseacetat, sowie die Cokristallisation von rechromatographiertem **2** mit authentischer Lactose ist in Tabelle II wiedergegeben. Bei der Cokristallisation des acetylierten **1** mit Allolactoseacetat kann man auch bei reiner Allolactose keinen 100%igen Einbau in das authentische kristalline β-Acetat erwarten, da bei der Acetylierung der radioaktiven Substanz **1** die definierte β-Form nicht quantitativ gebildet wird. Sie allein kann aber nur mit dem β-Acetat cokristallisieren. Der gefundene Wert von 66.5% spricht daher für eine Identität von **1** mit Allolactose. Im Falle von **2** sollte man allerdings bei Identität mit Lactose einen 100%igen Einbau erwarten. Der nur 57%ige Einbau läßt vermuten, daß diese Verbindung offenbar noch eine oder mehrere andere Substanzen enthält, die sich chromatographisch von Lactose nicht unterscheiden lassen. Wie im weiteren Verlauf der Arbeit gezeigt werden soll, handelt es sich hierbei um nicht reduzierende Disaccharide.

TABELLE II

COKRISTALLISATION VON ACETYLIERTEM **1** MIT AUTHENTISCHEM β-ALLOLACTOSEACETAT

	Ausgangs-aktivität d. Mischg.	*Kristallisation*		
		1.	*2.*	*3.*
cpm/mg	3920	3060	2620	2600
% Einbau	—	78	67.0	66.5
Cokristallisation von rechromatographiertem **2** mit authentischer α-Lactose				
cpm/mg	905	469	526	521
% Einbau	—	51.7	58	57

Führt man die enzymatische Spaltung von ONPGal in Gegenwart von sehr geringen Mengen [^{14}C]Glycerin durch, entstehen ebenfalls mehrere Produkte. Das

TABELLE III

R_{Gal}-WERTE UND AUSBEUTEN, DER BEIM ENZYMATISCHEN GALAKTOSYLTRANSFER AUF [^{14}C]GLYCERIN GEBILDETEN β-D-GALAKTOSIDE

Substanz	**4**	**5**	**6**	**7**
R_{Gal}	1.00	1.15	—	—
Chromatographisch vergleichbar	1-*O*-β-D-Galaktosyl-D-glycerin 1-*O*-β-D-Galaktosyl-L-glycerin	2-*O*-β-D-Galaktosyl-glycerin	—	—
% Ausbeute bezogen auf eingesetztes [^{14}C]Glycerin	55.9	3.2	29.3	2.4

Autoradiogramm des chromatographierten Ansatzes, bei dem 80% des ONPGal gespalten sind, zeigt Abb. 1. Die Ausbeuten der gebildeten Substanzen **4**+**5**, **6** und **7** sowie die R_{Gal}-Werte, der in einem anderen Laufmittel rechromatographierten Verbindungen **4** und **5** sind in Tabelle III wiedergegeben.

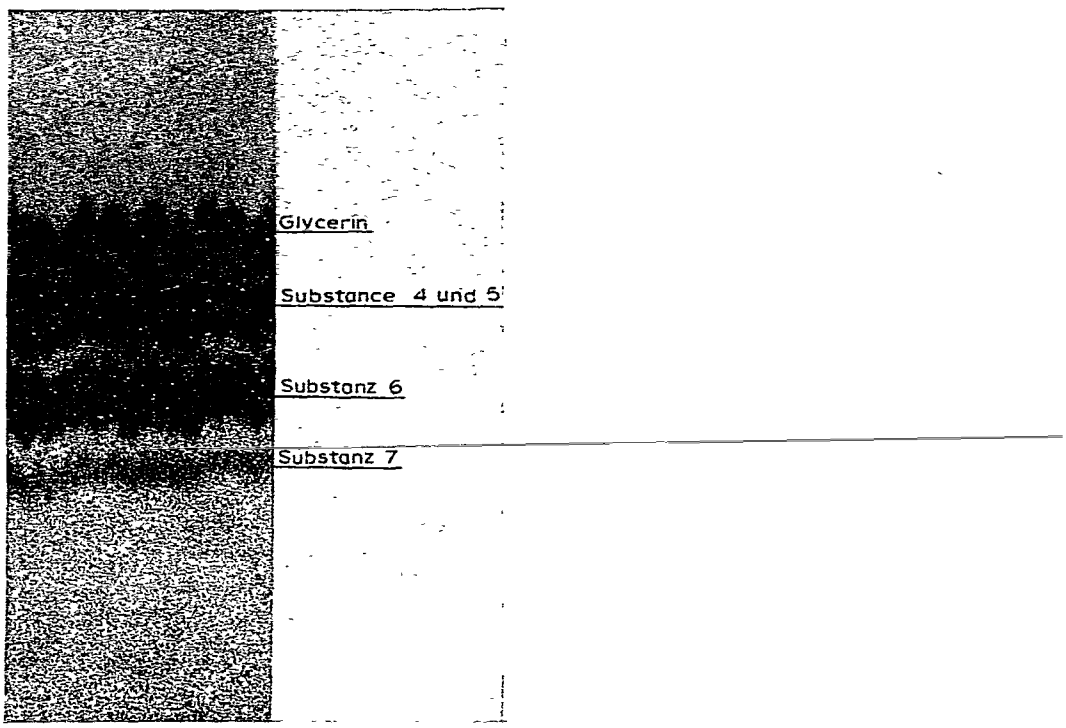

Abb. 1. Enzymatischer Galaktosyltransfer auf [^{14}C]Glycerin. Spaltansatz: 0.45 ml Puffer (10^{-1}M Kaliumnatriumphosphat, pH 7.0, Mg^{2+} 10^{-3}M), 10 μg [^{14}C]Glycerin (12.1 mC/mMol) entsprechend 2×10^{-4}M; 20.1 mg ONPGal entsprechend 1.4×10^{-1}M; 0.05 ml Enzymlösung (20,000 E). Inkubationszeit 2.8 min. Whatman Nr. 3 Chromatographiepapier. Lösungsmittel: Phenol–Wasser, 4:1. Belichtungszeit 3 Tage.

Durch den Vergleich mit den R_{Gal}-Werten der authentischen Verbindungen läßt sich Substanz **4** 1-*O*-β-D-Galaktosyl-D-glycerin und Substanz **5** 2-*O*-β-D-Galaktosylglycerin zuordnen. Die Ergebnisse der Cokristallisation von **4**+**5** (Abb. 1) mit 1-*O*-β-D-Galaktosyl-D-glycerin und 2-*O*-β-D-Galaktosylglycerin gibt Tabelle IV wieder; **4**+**5** besteht demnach zu 93–95% aus dem 1-verknüpften und zu 5.6–5.3% aus dem 2-verknüpften Glyceringalaktosid. Die beiden Werte ergänzen sich gut zu 100%, sodaß die Anwesenheit von 1-*O*-β-D-Galaktosyl-L-glycerin ausgeschlossen werden kann[14].

Bei den restlichen markierten Verbindungen **6** und **7** (Abb. 1) handelt es sich um Produkte, die durch Transfer eines oder mehrerer Galaktosylreste auf schon

TABELLE IV

COKRISTALLISATION VON **4** + **5** (BILD *1*) MIT *1-O-β*-D-GALAKTOSYL-D-GLYCERIN (*a*) UND *2-O-β*-D-GALAKTOSYLGLYCERIN (*b*)

(*a*)	*Ausgangs-aktivität d. Mischg.*	*Kristallisation*		
		1	*2*	*3*
cpm/mg	5240	4860	4870	5070
% Einbau	—	92.8	92.9	96.5

(*b*)	*Ausgangs aktivität d. Mischg.*	*Kristallisation*					
		1	*2*	*3*	*4*	*5*	*6*
cpm/mg	3320	2208	800	403	257	188	178
% Einbau	—	66.5	24.2	12.1	7.8	5.6	5.3

entstandenes Galaktosylglycerin gebildet werden. Ihre Struktur wurde nicht näher untersucht.

so entstehen neben **4** und **5** keine Transferprodukte höheren Molekulargewichts.

B. Enzymatische Synthese von β-D-Galaktosiden durch direkte Verknüpfung von D-[^{14}C]*Galaktose mit* D-*Glucose,* D-*Galaktose und Glycerin (Markierung im Glyconteil).*

Die Inkubation von D-[^{14}C]Galaktose und D-Glucose, beide in 40%iger Lösung, mit β-Galaktosidase ergibt ein Gleichgewichtsgemisch. Das Autoradiogramm der papierchromatographischen Auftrennung zeigt Abb. 2. Neben unveränderter D-Galaktose sind eine Reihe von Disacchariden zu erkennen, die durch direkte Verknüpfung von D-Galaktose mit den verschiedenen Hydroxylgruppen der D-Glucose und D-Galaktose entstanden sind. Ändert man die Versuchsbedingungen und inkubiert sehr wenig D-[^{14}C]Galaktose hoher spezifischer Aktivität mit einem großen Überschuß nicht markierter D-Glucose in 60–70%iger Lösung mit β-Galaktosidase, so ist nur eine direkte Verknüpfung mit D-Glucose zu beobachten. Das Chromatogramm eines solchen Ansatzes (Abb. 3) zeigt neben unveränderter D-Galaktose vier neu gebildete Disaccharide (**8**–**11**), die sich durch chromatographischen Vergleich mit authentischen Substanzen zuordnen lassen. Ausbeuten und R_{Gal}-Werte der gebildeten *O-β*-D-[^{14}C]Galaktosyl-D-glucosen zeigt Tabelle V. Es verhalten sich **8** wie Allolactose, **9** wie Lactose, **10** wie 2-*O-β*-D-Galaktosyl-D-glucose und **11** wie 3-*O-β*-D-Galaktosyl-D-glucose. Die einzelnen Substanzen werden in drei verschiedenen Lösungsmitteln (*A*, *B*, *C*) rechromatographiert und so gereinigt. Dabei läßt sich Substanz **9** in zwei nahe zusammen laufende Substanzen (**9a** und **9b**) auftrennen. Beide werden mit authentischer Lactose cokristallisiert (Tabelle VI), **9a** cokristallisiert zu 62%, **9b** zu weniger als 14% mit authentischer Lactose. Die radioaktiven Substanzen **12** und **13**, die nach der Cokristallisation in den Mutterlaugen zurückbleiben

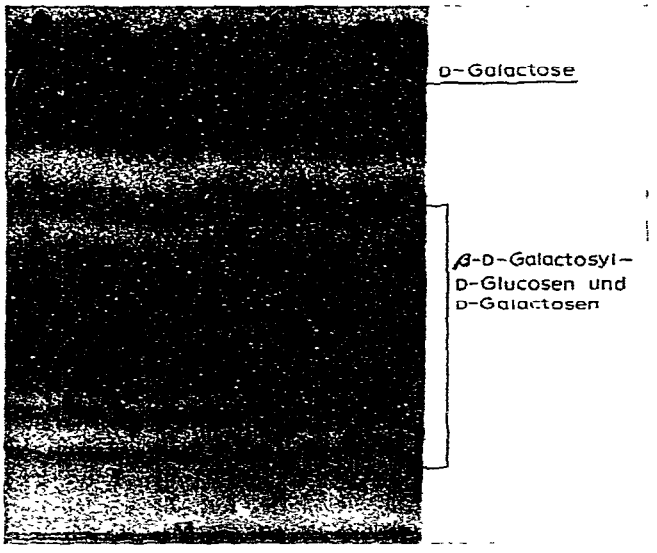

Abb. 2. Direkte enzymatische Verknüpfung von D-[^{14}C]Galaktose mit D-Glucose und D-Galaktose. Ansatz : 0.25 ml Puffer (10^{-1}M Kaliumnatriumphosphat, pH 7.0, Mg^{2+} 10^{-3}M), 100 mg D-[^{14}C]-Galaktose (0.05 mC); 100 mg D-Glucose; 0.05 ml Enzymlösung (1 mg β-Galaktosidase). Inkubationszeit 8 Tage. Whatman Nr. 3 Chromatographiepapier. Lösungsmittel: 1-Butanol–Pyridin–Wasser, 6:3:4. Belichtungszeit 2 Tage.

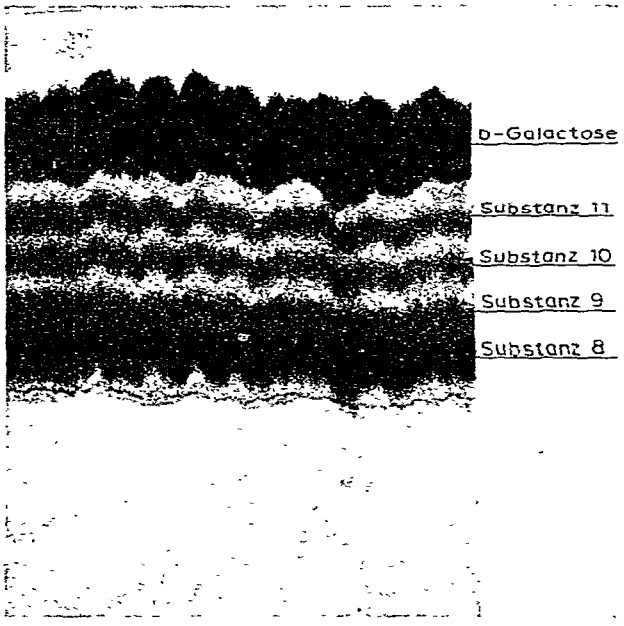

Abb. 3. Direkte enzymatische Verknüpfung von D-[^{14}C]Galaktose mit D-Glucose.
Ansatz : 0.1 ml Puffer (10^{-1}M Kaliumnatriumphosphat, pH 7.0, Mg^{2+} 10^{-3}M); 0.6 mg D-[^{14}C]-Galaktose (0.1 mC); 80 mg D-Glucose; 0.05 ml Enzymlösung (1 mg β-Galaktosidase). Inkubationszeit 5 h. Whatman Nr. 3 Chromatographiepapier. Lösungsmittel : 1-Butanol–Pyridin–Wasser, 6:4:3. Belichtungszeit 1 Tag.

TABELLE V

R_{Gal}-WERTE UND AUSBEUTEN DER BEI DER DIREKTEN ENZYMATISCHEN VERKNÜPFUNG VON D-[^{14}C]GALAKTOSE MIT D-GLUCOSE GEBILDETEN RADIOAKTIVEN D-[^{14}C]GALAKTOSYL-D-GLUCOSEN

Substanz	**8**	**9**	**10**	**11**
R_{Gal}	0.51	0.59	0.68	0.76
Chromatographisch vergleichbar	Allolactose	Lactose	2-*O*-β-D-Galaktosyl-D-glucose	3-*O*-β-D-Galaktosyl-D-glucose
% Ausbeute bezogen auf eingesetzte D-[^{14}C]Galaktose	9.2	2.3	2.2	2.3

TABELLE VI

COKRISTALLISATION VON **9a** UND **9b** MIT AUTHENTISCHER α-LACTOSE

	Ausgangs-aktivität d. Mischg.	*Kristallisation*			
		1	*2*	*3*	*4*
9a					
cpm/mg	2880	2290	2050	1750	1810
% Einbau	—	79.5	71	61	63
9b					
cpm/mg	2910	620	460	405	—
% Einbau	—	21.3	15.7	13.9	—

und keine Radioaktivität in Form von Lactose mehr enthalten, reagieren nicht mit *p*-Bromphenylhydrazin und werden durch Umsatz mit $NaBH_4$ nicht reduziert. Die Substanz **12** wird durch α-D-Glucosid-spezifische Maltase aus Pferdeserum (α-Maltase) hydrolysiert, **13** hingegen nicht. Wir schließen daraus, daß die Substanz **12** 1-*O*-α-D-Glucosyl-β-D-[^{14}C]galaktosid und Substanz **13** 1-*O*-β-D-Glucosyl-β-D-[^{14}C]galaktosid darstellt.

Das Haupttransferprodukt **8**, das sich chromatographisch wie Allolactose verhält, kann in seiner acetylierten Form zu 70% mit authentischem β-Allolactose-acetat cokristallisiert werden (Tabelle VII), was nach dem unter *A*. Erwähnten einer Identität gleichkommt. Bei der Cochromatographie der markierten Substanz **10** und **11** mit einem Überschuß von authentischer 2-*O*-β-D-Galaktosyl-D-glucose und 3-*O*-β-D-Galaktosyl-D-glucose sind die Autoradiogramme und die chemisch entwickelten Chromatogramme deckungsgleich. Die Substanz **10** wird demnach als 2-*O*-β-D-[^{14}C]Galaktosyl-D-glucose und **11** als 3-*O*-β-D-[^{14}C]Galaktosyl-D-glucose angesehen.

Zur Herstellung einer größeren Menge radioaktiv markierter β-D-[^{14}C]Galaktosyl-D-glucosen wird der enzymatische Ansatz nach Elution der jeweils nicht umgesetzten D-[^{14}C]Galaktose unter erneuter Zugabe von nicht markierter D-Glucose und

β-Galaktosidase sechsmal wiederholt. Die getrennt eluierten Banden werden gesammelt und dreimal in verschiedenen Lösungsmitteln (*A*, *B*, *C*) chromatographiert. Auf diese Weise läßt sich 6-*O*-β-D-[^{14}C]Galaktosyl-D-glucose (Allolactose), 2-*O*-β-D-[^{14}C]Galaktosyl-D-glucose und 3-*O*-β-D-[^{14}C]Galaktosyl-D-glucose in einer spezifischen Aktivität von 35 mC/mMol in radiochemisch reiner Form gewinnen. Zur Verwendung bei Induktions und Permeationsversuchen an Bakterien müssen diese Verbindungen durch Kohle filtriert werden, um Verunreinigungen zu entfernen, die aus dem Chromatographiepapier stammen und das Wachstum der Bakterien in unkontrollierbarer Weise hemmen.

TABELLE VII

COKRISTALLISATION VON **8** IN ACETYLIERTER FORM MIT AUTHENTISCHEM β-ALLOLACTOSEACETAT

	Ausgangs-aktivität d. Mischg.	*Kristallisation*			
		1	*2*	*3*	*4*
cpm/mg	19,250	20,300	13,150	14,650	13,550
% Einbau	—		68.8	76.0	70.5

Substanz **9a** besteht, wie bereits gezeigt, zu 63% aus 4-*O*-β-D-[^{14}C]Galaktosyl-D-glucose (Lactose). Zur Reindarstellung dieser [^{14}C]Lactose wird mit authentischer Lactose verdünnt, cokristallisiert und sechsmal umkristallisiert. Die spezifische Aktivität der so erhaltenen [^{14}C]Lactose beträgt, 46,000 dpm/mg.

Auf gleiche Weise wie die D-[^{14}C]Galaktose-markierten β-Galaktosylglucosen lassen sich durch direkte Verknüpfung auch die *O*-β-D-[^{14}C]Galaktosyl-glycerine synthetisieren. Nach papierchromatographischer Analyse und Autoradiographie läßt sich neben unumgesetzter D-[^{14}C]Galaktose eine starke Bande erkennen, die die Syntheseprodukte (**14**, **15**) enthält. R_{Gal}-Werte der rechromatographierten Substanzen sowie ihre Ausbeute bei der Synthese sind Tabelle VIII zu entnehmen.

TABELLE VIII

R_{Gal}-WERTE UND AUSBEUTEN DER BEI DER DIRFKTEN ENZYMATISCHEN VERKNÜPFUNG VON D-[^{14}C]GALAKTOSE MIT GLYCERIN GEBILDETEN β-D-[^{14}C]GALAKTOSYLGLYCERINE

Substanz	**14**	**15**
R_{Gal}	1.00	1.15
Chromatographisch vergleichbar	1-*O*-β-D-Galaktosyl D-glycerin 1-*O*-β-D-Galaktosyl-L-glycerin	2-*O*-β-D-Galaktosyl-glycerin
% Ausbeute bezogen auf D-[^{14}C]Galaktose	17.4	4.4

Durch den Vergleich der R_{Gal}-Werte mit denen von authentischen Verbindungen läßt sich **14** 1-*O*-β-D-Galaktosyl-D-glycerin oder 1-*O*-β-D-Galaktosyl-L-glycerin und **15** 2-*O*-β-D-Galaktosylglycerin zuordnen. Die Cokristallisation mit den authentischen Verbindungen (Tabelle IX) zeigt, das **14**+**15** zu 60.5% aus 1-*O*-β-D-[^{14}C]Galaktosyl-D-glycerin und zu 14.7% aus 2-*O*-β-D-[^{14}C]Galaktosylglycerin besteht. Da Synthese-

TABELLE IX

COKRISTALLISATION VON **14**+**15** MIT 1-*O*-β-D-GALAKTOSYL-D-GLYCERIN (*a*) UND 2-*O*-β-D-GALAKTOSYLGLYCERIN (*b*)

	Ausgangs-aktivität d. Mischg.	*Kristallisation*						
		1	*2*	*3*	*4*	*5*	*6*	*7*
(*a*)								
cpm/mg	3770	3280	3085	2950	2760	2590	2280	2280
% Einbau	—	87.0	82.0	78.1	73.2	68.6	60.5	60.5
(*b*)								
cpm/mg	5732	3030	1300	984	805	845		
% Einbau	—	52.9	22.7	17.2	14.1	14.7		

produkte mit zwei und mehr Galaktosylresten, wie sie bei der Transgalaktosylierung auf Glycerin bei hohem Überschuß an ONPGal entstehen (Abb. 1), geringere R_{Gal}-Werte aufweisen, muß es sich bei den restlichen 24.8% Radioaktivität, die bei der Cokristallisation weder in das 1-*O*-β-D-Galaktosyl-D-glycerin noch in das 2-*O*-β-D-Galaktosylglycerin eingebaut werden, um das dritte der drei theoretisch möglichen Isomeren, nämlich das 1-*O*-β-D-[^{14}C]Galaktosyl-L-glycerin handeln, das den gleichen R_{Gal}-Wert wie **14** hat. Der endgültige Beweis für dessen Identität durch Cokristallisation mit der authentischen Verbindung konnte nicht erbracht werden, da es nicht gelang, das chemisch synthetisierte 1-*O*-β-D-Galaktosyl-L-glycerin zu kristallisieren. Zur präparativen Herstellung von reinem 1-*O*-β-D-[^{14}C]Galaktosyl-D-glycerin und 2-*O*-β-D-[^{14}C]Galaktosylglycerin wird mit den entsprechenden Verbindungen cokristallisiert und bis zur konstanten spezifischen Aktivität umkristallisiert. Es werden dabei Präparate einer spezifischen Aktivität von 1.8×10^6 bzw. 0.5×10^6 dpm/mg erhalten.

Die in den Tabellen wiedergegebenen Ergebnisse zeigen, daß die direkte Verknüpfung von D-Galaktose als Substrat mit Glycerin oder D-Glucose im Überschuß durch β-Galaktosidase weit geringere Stereoselektivität aufweist, als der enzymatische Galaktosyltransfer von einem Substrat wie ONPGal auf die genannten Akzeptoren im Überschuß. So wird bei der enzymatischen ONPGal-Spaltung in Gegenwart von D-Glucose fast ausschließlich Allolactose gebildet. Unter den Bedingungen der direkten Verknüpfung hingegen werden sämtlich mögliche *O*-β-D-Galaktosylglucosen in guter Ausbeute erhalten werden. Allerdings überwiegt auch hier die Bildung von Allolactose.

Ganz entsprechende Verhältnisse findet man bei der Verwendung von Glycerin als Akzeptor. Unter den Bedingungen der Transgalaktosylierung findet die Über-

tragung des Galaktosylrestes zu über 90% auf die primäre OH-Gruppe statt und noch dazu in stereoselektiver Reaktion in Bezug auf das Mesokohlenstoffatom des Glycerins. Unter den Bedingungen der direkten Verknüpfung jedoch wird das sekundäre OH in merklichem Ausmaß galaktosyliert. Außerdem scheint die Galaktosylierung der primären OH-Gruppe des Glycerins weniger stereospezifisch zu erfolgen, da offensichtlich neben dem Hauptprodukt 1-*O*-*β*-D-Galaktosyl-D-glycerin in erheblicher Menge noch 1-*O*-*β*-D-Galaktosyl-L-glycerin gebildet wird.

Der Unterschied in der Zusammensetzung der Reaktionsprodukte bei der direkten Verknüpfung von Monomeren im Gegensatz zur Transgalaktosylierung ist auf die Verschiedenheit der Reaktionsbedingungen zurückzuführen. Bei der direkten Verknüpfung wird das Gleichgewicht zwischen den Monomeren, den Syntheseprodukten und Wasser mit Hilfe des Enzyms eingestellt. Die Reaktion ist demnach thermodynamisch kontrolliert. Die Transgalaktosylierung ist hingegen kinetisch kontrolliert. Die Einstellung des Gleichgewichts wird hierbei nicht abgewartet.

Zu diskutieren ist der Unterschied vor allem bei der stereoselektiven Galaktosylierung der primären OH-Gruppe des Glycerins, die in Bezug auf das C-Atom 2 des Glycerins über einen diastereomeren Übergangszustand führen muß. Eine analoge Reaktion kennt man in der stereoselektiven enzymatischen Phosphorylierung von Glycerin mit Glycerokinase[15]. Schwartz und Carter[16] führen die stereoselektive enzymatische Reaktion eines Mesokohlenstoffatoms auf solche unterschiedlichen diastereomeren Übergangszustände zurück.

So kann auch die verschiedene Stereoselektivität, die bei der Galaktosylierung des Glycerins unter den oben angeführten Bedingungen auftritt, verstanden werden. In der kinetisch kontrollierten Reaktion wird der Energieinhalt des Übergangszustandes zum bestimmenden Faktor, während bei der thermodynamisch kontrollierten Reaktion die Energieinhalte der Produkte mit ins Gewicht fallen. Ganz entsprechend diesen Vorstellungen fand Yermyn[17] bei der Untersuchung der enzymatischen Transglycosylierungsreaktion mit einer Aryl-Glucosidase unterschiedliche Akzeptoreigenschaften bei Anwendung optisch aktiver Akzeptoren.

EXPERIMENTELLER TEIL

Allgemeine Arbeitsmethoden. — 1. Papierchromatographie: Papierchromatogramme wurden absteigend auf Whatman Nr. 3 und Nr. 1 Chromatographiepapier angefertigt. Als Lösungsmittel dienten: 1-Butanol–Propionsäure–Wasser (142:71:100, v/v/v) (*A*), 1-Butanol–Pyridin–Wasser (6:4:3, v/v/v) (*B*), oder Phenol–Wasser (4:1, w/w) (*C*).

Die Autoradiogramme wurden durch Belichten von Kodak-Röntgenfilm (blauempfindlich, einfach beschichtet) in entsprechenden Kassetten erhalten. Die Belichtungszeit beträgt je nach Aktivität der chromatographierten radioaktiven Substanzen 2–20 Tage.

2. Die quantitative Bestimmung der Radioaktivität erfolgt im Scintillationszähler (720 Series, Liquid Scintiliation System der Nuclear Chicago Company).

3. Cokristallisation von radioaktiven Galaktosiden mit authentischen Verbindungen.

50–60,000 cpm der zu untersuchenden Substanz werden in 25 ml Spitzkölbchen eluiert. Die Lösung wird zur Trockene gedampft. In die Kölbchen wird die reine kristalline Substanz eingewogen (15–30 mg). Dann werden 2 ml Wasser zugegeben und die Substanz aufgelöst. Im Falle des Allolactoseacetats wird Äthanol als Lösungsmittel verwendet.

Aus dieser Ausgangslösung wird 0.05 ml entnommen und im Scintillationszähler gezählt. Zusammensetzung der Scintillationslösung: 15 ml einer Lösung von 0.4% 2,5-Diphenyloxazol und 0.005% 2-*p*-Phenylen-bis-(phenyloxazol) in Toluol, 1.5 ml Solubilizer (CS-3 der Fa. Sentol, Littleton, Colorado) und 0.05 ml der zu untersuchenden Lösung, die ungefähr 1 mg Zucker enthält. Der Zusatz von Solubilizer ist nur zum Zählen wäßriger Lösungen nötig. Die Zählausbeute beträgt dann zwischen 60 und 80%. Die Ausgangslösung wird eingedampft. Der sirupöse Rückstand wird in der Wärme mit wenig Äthanol versetzt und zur Kristallisation bei Zimmertemperatur ausgestellt. Impft man die Lösung mit einem kleinen Kristall der authentischen Verbindung an, so haften die wachsenden Kristalle meist fest an der Innenseite des Kölbchens. Wenn die Kristalle nicht mehr weiterwachsen, wird die Mutterlauge vorsichtig abgesaugt und die an der Glaswand haftenden Kristalle zweimal mit 0.5 ml Äthanol gewaschen. Dann wird der restliche Alkohol am Rotationsverdampfer bei einer Badtemperatur von 50–60° entfernt.

Von den trockenen Kristallen wird ungefähr 1 mg auf der Mikrowaage abgewogen und in ein Zählgläschen gegeben. Die Substanz wird in 0.05 ml Wasser bzw. im Falle des Allolactoseacetats in 0.05 ml Äthanol gelöst. Es wird 1.5 ml Solubilizer und 15 ml Scintillationslösung zugegeben. Die Kristalle werden erneut gelöst und wie oben beschrieben zur Kristallisation gebracht.

Enzymatische Synthesen und Spaltansätze

1. *Enzymatischer Galaktosyltransfer von ONPGal auf* D-[^{14}C]*Glucose und* [^{14}C]*Glycerin mit β-Galaktosidase.* — (*a*) *Galaktosyltransfer auf* D-[^{14}C]*Glucose.* Spaltansatz: 4.5 ml Puffer (10^{-1}M Kaliumnatriumphosphat, pH 7.0, Mg^{2+} 10^{-3}M); 40.3 mg D-[^{14}C]Glucose (uniform markiert, spezifische Aktivität 0.05 mC/mMol) entsprechend 4.5×10^{-2}M; 198.9 mg ONPGal entsprechend 1.39×10^{-1}M. Temperatur 40°. Die Reaktion wird gestartet durch Zugabe von 0.5 ml Enzymlösung (1.6×10^5E). Die Einheit der β-Galaktosidase ist definiert als diejenige Menge Enzym, die unter den Versuchsbedingungen ein nMol ONPGal in der Minute spaltet. Nach 3.5 min (80%ige Spaltung des ONPGal) wird das Enzym 3 min bei 100° denaturiert. Die Lösung wird mit einer Kapillare in Streifenform auf 4 Bogen Whatman Nr. 3 Chromatographiepapier gebracht und mit Lösungsmittel *B* chromatographiert. Zur Ausbeutebestimmung wird aus dem Chromatogramm ein 1 cm breiter Streifen in Laufrichtung herausgeschnitten und die Aktivität der ausgeschnittenen Zonen im Scintillationszähler bestimmt.

(*b*) *Galaktosyltransfer auf* [^{14}C]*Glycerin.* Spaltansatz: 0.45 ml Puffer (10^{-1}M

Kaliumnatriumphosphat, pH 7.0, Mg^{2+} 10^{-3}M);10 µg[^{14}C]Glycerin (12.1 mC/mMol) entsprechend 2×10^{-4}M; 20.1 mg ONPGal entsprechend 1.4×10^{-1}M. Temperatur 40°. Die Reaktion wird gestartet durch Zugabe von 0.05 ml Enzymlösung (20,000 E). Nach 2.8 min (80%ige Spaltung des ONPGal) wird das Enzym 3 min bei 100° denaturiert. Die Lösung wird auf Whatman Nr. 3 Chromatographiepapier mit Lösungsmittel *C* chromatographiert. Die Ausbeute der gebildeten Produkte wird durch lichtoptische Auswertung des Autoradiogramms und Planimetrieren der erhaltenen Peaks errechnet.

Ein entsprechender Ansatz wurde mit 30.2 mg [^{14}C]Glycerin (0.004 mC/mMol) entsprechend 3.1×10^{-1}M und 20.1 mg ONPGal 1.4×10^{-1}M durchgeführt.

2. *Direkte Verknüpfung von* D-[^{14}C]*Galaktose mit* D-*Glucose,* D-*Galaktose und Glycerin mit Hilfe von β-Galaktosidase.* — (*a*) *Synthese von* O-*β*-D-[^{14}C]*Galaktosyl*-D-*glucosen.* Ansatz: 0.1 ml Puffer (10^{-1}M Kaliumnatriumphosphat, pH 7.0, Mg^{2+} 10^{-3}M); 0.6 mg D-[^{14}C]Galaktose (35 mC/mMol); 80 mg D-Glucose und 0.04 ml Enzymlösung (1 mg β-Galaktosidase) werden 5 h bei Zimmertemperatur inkubiert. Dann wird das Enzym 3 min bei 100° denaturiert und die Mischung in wenig Wasser aufgenommen. Die Lösung wird samt Niederschlag mit einer Kapillare auf zwei Bogen Whatman Nr. 3 Chromatographiepapier aufgetragen und in Lösungsmittel *B* chromatographiert. Das Autoradiogramm eines solchen Chromatogramms zeigt Abb. 3.

Bei Verwendung größerer Mengen D-[^{14}C]Galaktose (100 mg) findet auch Synthese von markierten β-Galaktosylgalaktosen statt (Abb. 2).

Die Ausbeute der im ersten Versuch gebildeten *O*-*β*-D-[^{14}C]Galaktosyl-D-glucosen wird wie unter 1(*a*) beschrieben, bestimmt. Die nicht umgesetzte D-[^{14}C]-Galaktose wird eluiert und eingedampft. Dabei bleibt ein brauner Rückstand. Ohne weitere Reinigung werden hierzu 50 mg D-Glucose, 0.1 ml Puffer und 0.04 ml Enzymlösung (1 mg β-Galaktosidase) gegeben und die Inkubation sowie die chromatographische Trennung sechsmal wiederholt. Die einzelnen Transferprodukte werden eluiert und nacheinander in Lösungsmittel *A*, *B*, *C* rechromatographiert.

(*b*) *Synthese von* O-*β*-D-[^{14}C]*Galaktosyl-glycerinen.* Ansatz: 0.1 ml Puffer (10^{-1}M Natriumkaliumphosphat, pH 7.0, Mg^{2+} 10^{-3}M); 0.25 mg D-[^{14}C]Galaktose (35 mC/mMol); 80 mg Glycerin und 0.04 ml Enzymlösung (1 mg β-Galaktosidase) werden 5 h bei Zimmertemperatur inkubiert. Die Lösung wird 3 min bei 100° denaturiert und auf Whatman Nr. 3 Chromatographiepapier in Lösungsmittel *C* durchgeführt. Die Ausbeute an markierten Transferprodukten wird wie unter 1(*a*) beschrieben, bestimmt. Die eluierten Transferprodukte werden nacheinander in Lösungsmittel *A*, *B*, *C* rechromatographiert.

3. *Umsetzungen mit radioaktiven β-*D-*Galaktosiden.* — (*a*) *Enzymatische Spaltung mit β-Galaktosidase.* Etwa 2–10,000 cpm von der zu untersuchenden Substanz werden mit Puffer (10^{-1}M Kaliumnatriumphosphat, pH 7.0, Mg^{2+} 10^{-3}M) eluiert ungefähr 0.08 ml). Dazu werden 20 µl einer β-Galaktosidaselösung (6000 E) gegeben. Nach 30 min Inkubationszeit werden die Lösungen auf Whatman nr. 1 Chromatographiepapier gegeben und zusammen mit den Ausgangsverbindungen in Lösungs-

mittel *B* chromatographiert. Die Chromatogramme werden autoradiographisch entwickelt.

(*b*) *Enzymatische Spaltung mit α-Maltase aus Pferdeserum.* 2–10,000 cpm der zu untersuchenden Substanz wird mit 10^{-1}M Natriumacetatpuffer, pH 5.0 eluiert (etwa 0.08 ml). Dazu werden 0.05 ml einer α–Maltaselösung zugegeben. Nach 2 h Inkubationszeit wird der Ansatz auf Whatman Nr. 1 Chromatographiepapier in Lösungsmittel *B* chromatographiert. Die Chromatogramme werden autoradiographisch entwickelt.

(*c*) *Acetylierung von 6-O-β-D-[*^{14}*C]Galaktosyl-D-glucose.* Ungfähr 200,000 cpm von **8** bzw. **1** werden in ein kleines Reagenzglas eluiert und die Lösung (0.5 ml) lyophilisiert. In das Reagenzglas kommen eine Spatelspitze frisch geschmolzenes Natriumacetat und 0.2 ml destilliertes Essigsäureanhydrid. Das Reagenzglas wird abgeschmolzen und 3 h auf 120° erhitzt. Nach Erkalten wird das Glas geöffnet und 0.5 ml Chloroform zugegeben. Anschließend wird längere Zeit mit Wasser gewaschen. Dazu wird langsam mit einer Kapillare Wasser durch die Chloroformschicht gedrückt. Das überschüssige Wasser läuft über und wird verworfen. Die Chloroformschicht wird eingedampft und der Rückstand mit authentischem Allolactoseacetat cokristallisiert.

(*d*) *Umsetzung mit* p-*Bromphenylhydrazin.* 0.05 g *p*-Bromphenylhydrazin werden in 1 ml Äthanol gelöst. Davon werden je 0.25 ml zu 5 Tropfen wäßrigem Zuckereluat (10–20,000 cpm) gegeben. Die Lösungen werden 10 min bei 50–60° stehengelassen und anschließend auf Whatman Nr. 1 Chromatographiepapier in Lösungsmittel *B* chromatographiert.

(*e*) *Reduktion mit* $NaBH_4$. Die radioaktive Substan z(10–20,000 cpm) wird mit Wasser eluiert (0.1 ml). Hierzu werden 2 mg $NaBH_4$ in 0.05 ml Wasser gegeben. Die Lösung bleibt 2 h bei Zimmertemperatur stehen und wird dann auf Whatman Nr. 1 Chromatographiepapier in Lösungsmittel *B* chromatographiert. Bei der Chromatographie tritt ein starker Salzeffekt auf, sodaß die radioaktiven Zonen eluiert und rechromatographiert werden müssen. Die Chromatogramme werden autoradiographisch entwickelt.

DANK

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LITERATURVERZEICHNIS

1 K. WALLENFELS, *Naturwissenschaften*, 38 (1951) 306.
2 K. WALLENFELS UND E. BERNT, *Angew. Chem.*, 64 (1952) 28.
3 K. WALLENFELS, E. BERNT UND G. LIMBERG, *Ann.*, 584 (1953) 63.
4 M. ARONSON, *Arch. Biochem. Biophys.*, 39 (1952) 370.
5 J. H. PAZUR, *J. Biol. Chem.*, 208 (1954) 439.
6 J. EDELMANN, *Advan. Enzymol.*, 17 (1956) 189.
7 R. KUHN, H. H. BAER UND A. GAUHE, *Ber.*, 88 (1955) 1730.
8 A. CROFT-HILL, *J. Chem. Soc.*, 73 (1898) 634.

9 E. FISCHER UND E. F. ARMSTRONG, *Ber.*, 35 (1902) 3144.
10 E. BOURQUELOT, H. HÉVESSY UND J. COIRE, *Compt. Rend.*, 157 (1913) 732.
11 E. BOURQUELOT UND A. AUBRY, *Compt. Rend.*, 163 (1916) 60.
12 G. ZEMPLÉN, *Ber.*, 48 (1915) 233.
13 H. PRINGSHEIM UND J. LEIBOWITZ, *Ber.*, 57 (1924) 1576.
14 W. BOOS, J. LEHMANN UND K. WALLENFELS, *Carbohyd. Res.*, 1 (1966) 419.
15 H. KALCKAR, *Biochem. J.*, 33 (1939) 631.
16 P. SCHWARTZ UND H. E. CARTER, *Proc. Natl. Acad. Sci. U.S.*, 40 (1954) 499.
17 M. A. YERMYN, *Austral. J. Biol. Sci.*, 27 (1966) 903.

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REACTION OF AMMONIA WITH SOME ACETYLATED AND BENZOYLATED MONOSACCHARIDES

PART XII*. *N*-ACYL-D-GLUCOSYLAMINES AND 1,1-BIS(ACYLAMIDO)-1-DEOXY-D-GLUCITOLS ISOLATED FROM THE AMMONOLYSIS OF DIFFERENT PENTA-*O*-ACYL-D-GLUCOSES

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ABSTRACT

New *N*-acyl-D-glucosylamines (**2,3**) and 1,1-bis(acylamido)-1-deoxy-D-glucitols (**1**) have been isolated by applying chromatographic and ionophoretic techniques to the separation of the products from the ammonolysis of penta-*O*-acetyl-, penta-*O*-propionyl-, and penta-*O*-benzoyl-D-glucopyranose. It has been found that, in all cases, acyclic (**1**) and cyclic (**2, 3**) products are formed simultaneously. The molar ratio of the cyclic products formed (**2**+**3**) to the acyclic ones (**1**) is higher if the acyl group is aliphatic. The yield of *N*-benzoyl-β-D-mannopyranosylamine obtained in the ammonolysis of penta-*O*-benzoyl-D-mannopyranose is higher than that of *N*-benzoyl-β-D-glucopyranosylamine from penta-*O*-benzoyl-D-glucopyranose, showing the influence of inversion of configuration at C-2. This influence is less pronounced for other acylated monosaccharides that belong to the *manno* series.

INTRODUCTION AND DISCUSSION

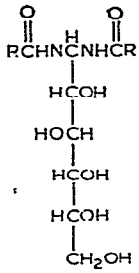
The action of ammonia in methanolic solution on acylated monosaccharides involves a complex series of reactions. The process has mostly been studied with fully acylated aldopyranoses, from which, among other products, two types of *N*-acyl derivatives of the aldose (resulting from an intramolecular migration of the acyl groups) are produced. In the D-series, one type consists of the acyclic 1,1-bis-(acylamido)-1-deoxy-D-alditols (**1**), and the other, of the cyclic *N*-acyl-D-aldosylamines, which can have an α-D-aldofuranoid (**2**) or a β-D-aldopyranoid (**3**) structure[2].

The reaction has been applied to acylated disaccharides[3], and studied extensively under different conditions.

In most of the work reported, ammonolysis of the acylated aldopyranoses has afforded only one type of *N*-acyl derivative. The known exceptions are the ammonolysis of 1,2,3,4,6-penta-*O*-benzoyl-D-mannose[4] and 1,2,3,4-tetra-*O*-benzoyl-L-rhamnose[5], from which, not only the acyclic compounds, but also the cyclic *N*-benzoyl-β-(D or L)-aldopyranosylamines were isolated. In addition, Deferrari and Lederkremer[6] recently reported that, in the ammonolysis of hexa-*O*-benzoyl-D-*glycero*-L-*manno*-heptose, both types of product were isolated. It is interesting that all of the monosaccharides thus far known to show this behavior have the *manno* configuration.

*Part XI, see Ref. 1.

In contrast, the only product isolated from the ammonolysis of 1,2,3,4,6-penta-*O*-benzoyl-β-D-glucose was the acyclic 1,1-bis(benzamido)-1-deoxy-D-glucitol (**1**; R = Ph).



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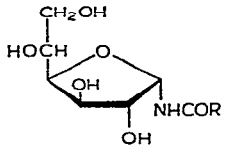

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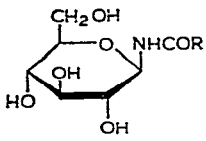

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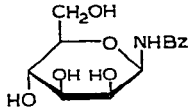

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The production of *N*-acetyl-D-glycosylamines was also exceptional in the ammonolysis of acetylated monosaccharides. When this work was started, the only case known was the isolation of *N*-acetyl-α-D-glucofuranosylamine (**2**; R = Me) as the only product from the ammonolysis of 2,3,4,5,6-penta-*O*-acetyl-D-glucose[7] and from 1,2,3,4,6-penta-*O*-acetyl-β-D-glucose[8]. Later, it was found that, when the 1,2,3,4-tetraacetate of β-D-xylose (a sugar homomorphous with D-glucose), was submitted to the reaction, it afforded only the two cyclic derivatives, namely, *N*-acetyl-β-D-xylopyranosylamine and *N*-acetyl-α-D-xylofuranosylamine[2].

As chromatographic techniques had not been employed in processing of any of the ammonolyses of acylated D-glucoses, there was a reasonable doubt as to whether other *N*-acylated derivatives had been produced, but had remained among the products of the reaction in discarded mother liquors. Consequently, it was felt that a re-investigation of the work could be of interest. Therefore, ammonolysis of the fully acetylated and benzoylated D-glucopyranoses was repeated, and that of a new ester, namely, 1,2,3,4,6-penta-*O*-propionyl-D-glucose was also studied. In each instance, the mixture of the reaction products was submitted to chromatographic separation, with emphasis on the isolation of the 1,1-bis(acylamido)-1-deoxy-D-glucitols (**1**) and the *N*-acyl-D-glucosylamines (**2** and **3**).

As described in the experimental part, the ammonolysis of 1,2,3,4,6-penta-*O*-acetyl-β-D-glucose afforded, not only the expected *N*-acetyl-α-D-glucofuranosylamine (**2**; R = Me), but also the unknown 1,1-bis(acetamido)-1-deoxy-D-glucitol (**1**; R = Me). Moreover, traces of a product that behaved as *N*-acetyl-β-D-glucopyranosylamine (**3**; R = Me) were detected.

Because of the small differences in reactivity between the acetyl and the propionyl group, the ammonolysis of 1,2,3,4,6-penta-*O*-propionyl-D-glucose was expected to yield similar products, and this was found to be the case. *N*-Propionyl-α-D-glucofuranosylamine (**2**; R = Et), *N*-propionyl-β-D-glucopyranosylamine (**3**; R = Et), and 1,1-bis(propionamido)-1-deoxy-D-glucitol (**1**, R = Et) were isolated in crystalline condition.

TABLE I

AMMONOLYSIS OF SOME FULLY ACYLATED GLYCOPYRANOSES

Monosaccharide	*Acyl group*	*Products isolated*						*Molar ratio (A/B)*
		N-*Acyl*-β-D-*glycopyranosylamines*	N-*Acyl*-α-D-*glycofuranosylamines*	*Cyclic products*[c]		*1,1-Bis(acylamido)-1-deoxy*-D-*glucitols*		
		Yield, %	*Yield*, %	*Total yield*		*Yield*		
				%	(*moles/mole*)*100* (*A*)	%	(*moles/mole*)*100* (*B*)	
D-Glucose	acetyl	traces	12.1	12.1	22.0	8.0	11	2.00
	propionyl	0.9	5.6	6.5	12.1	3.8	5.7	2.12
	benzoyl	1.0	[b]	1.0	2.5	21.4	37.1	0.07
D-Xylose	acetyl[2]	3.1	9.2	12.3	20.5	[b]	—	—
D-Mannose	benzoyl[4,a]	8.0	[b]	8.0	19.8	20.8	36.0	0.55
L-Rhamnose	benzoyl[5,a]	1.84	[b]	1.84	4.0	18.6	27.8	0.14
D-*glycero*-L-*manno*-Heptose	benzoyl[6,a]	2.5	[b]	2.5	6.6	35.0	67.5	0.10

[a]Chromatographic techniques were not employed to separate the products from the ammonolysis. [b]Not detected. [c]*N*-Acyl-β-D-glycopyranosylamines plus *N*-acyl-α-D-glycofuranosylamines.

When the ammonolysis of 1,2,3,4,6-penta-*O*-benzoyl-β-D-glucose was repeated, the most interesting finding was that a small proportion of *N*-benzoyl-β-D-glucopyranosylamine (**3**; R = Ph) is formed in the reaction. The same compound had been isolated from the products of ammonolysis of hexa-*O*-benzoyl-D-*glycero*-D-*gulo*-heptononitrile[9].

Table I gives the yields of the products obtained from each ammonolysis. It shows clearly that the cyclic *N*-acyl-D-glucosylamines (**2** and **3**) are produced in significantly higher yields from the acetylated and propionylated D-glucoses than from 1,2,3,4,6-penta-*O*-benzoyl-D-glucose. The *N*-acyl-α-D-glucofuranosylamines (**2**; R = Me or Et) preponderate over the β-D-pyranoid analogs (**3**; R = Me or Et). These results are similar to those obtained in the ammonolysis of tetra-*O*-acetyl-β-D-xylopyranose[2], which are also included in Table I. In the last case, however, a search for the corresponding acyclic bis(acylamido) compound was fruitless.

It is as yet not possible to give an explanation for the low yield of *N*-benzoyl-β-D-glucopyranosylamine (**3**; R = Ph) obtained from the ammonolysis of the parent compound. Both the larger bulk of the benzoyl group and its lower reactivity to nucleophilic reagents may play a role.

On comparison of the results of ammonolysis of 1,2,3,4,6-penta-*O*-benzoyl-β-D-glucose with those obtained from ammonolysis of 1,2,3,4,6-penta-*O*-benzoyl-D-mannose[4], it becomes evident that the configuration of the monosaccharide has an influence. The inversion of the configuration at C-2 of D-glucose to give the latter hexose favors the production of *N*-benzoyl-β-D-mannopyranosylamine (**4**), as indicated by the larger ratio of A/B (see Table I).

Although this ratio is also greater for the other benzoylated monosaccharides having the *manno* configuration that are listed in Table I, the difference, when compared to D-glucose, is not large enough to allow of a generalization regarding the influence of the configuration at C-2, without further analytical work on the products of their ammonolysis.

In the course of this work, two new *N*-acyl-β-D-glucopyranosylamines (**3**; R = Et or Ph) were prepared for use as standards. Ammonolysis of their *O*-acylated derivatives gave high yields of the parent compounds, showing that they are stable under the conditions of the reaction.

When strong, electron-attracting groups are present in the acylamido residue, the electrophilicity of C-1 of the monosaccharide moiety is increased, and it becomes more sensitive to nucleophilic reagents; this is exemplified by the formation of 1-acetamido-1-deoxy-1-*p*-tolylsulfonamido-D-glucitol on treatment of tetra-*O*-acetyl-*N*-*p*-tolylsulfonyl-β-D-glucopyranosylamine with methanolic ammonia[10]. The higher reactivity of C-1 in *N*-*p*-tolylsulfonyl-β-D-glucopyranosylamine is also the cause of its so-called mutarotation[11]; the compound decomposes in aqueous solution, producing D-glucose and *p*-toluenesulfonamide, as shown by paper chromatography; the final rotation is almost identical with that expected for the equilibrium value of the D-glucose liberated.

EXPERIMENTAL

General. — All evaporations were conducted under diminished pressure. Melting points are uncorrected. Methanolic ammonia (16%, w/v) was used in all cases. Whatman No. 1 paper was employed for analytical chromatography, and Whatman No. 3MM paper impregnated with 0.01M sodium borate was used for preparative purposes. The following solvent systems were used: (A) 10:4:3 (v/v) ethyl acetate–pyridine–water, (B) 5:3:1:3 (v/v) ethyl acetate–pyridine–benzene–water, (C) 3:1:1 (v/v) isobutyl alcohol–petroleum ether (b.p. 60–70°)–water, (D) 10:4:3 (v/v) ethyl acetate–pyridine–0.01M sodium borate, and (E) 5:3:1:3 (v/v) ethyl acetate–pyridine–benzene–0.01M sodium borate. The spray reagents used were: (A) potassium periodatocuprate[12], and (B) aniline hydrogen phthalate in butanol saturated with water. R_N values are referred to *N*-benzoyl-β-D-glucopyranosylamine as unity. Ionophoresis was performed on Whatman No. 3MM paper, with 0.01M sodium borate as the complexing agent, under a potential gradient of 15 volt/cm.

When not specified, identification of known compounds was made by mixed m.p., i.r. spectra, and paper chromatography.

The ammonolyses were conducted by dissolving the acylated D-glucoses in methanolic ammonia (10 ml per g), keeping the solution for 5 h at room temperature, and evaporating to dryness. In each instance, the residue was thoroughly dried *in vacuo* at room temperature, and then extracted with cold (unless otherwise stated) ethyl acetate. Paper chromatography of the extract (solvent B) revealed that, for the penta-*O*-acetyl- and penta-*O*-benzoyl-D-glucopyranoses, it contained only the acylamide and traces of D-glucose and D-glucosylamine. With pentapropionyl-D-glucopyranose, some **1** (R = Et) passed into the extract, and was recovered as described later.

Ammonolysis of 1,2,3,4,6-penta-O-acetyl-β-D-glucose. — The residue (27.6 g) from the ammonolysis of 50 g of the title compound, after extraction with ethyl acetate, was submitted to paper chromatography. Chromatograms showed an elongated spot, R_F *ca.* 0.40 [*N*-acetyl-β-D-glucopyranosylamine (**3**; R = Me) has R_F 0.38, and *N*-acetyl-α-D-glucofuranosylamine (**2**; R = Me) has R_F 0.40], and another spot, R_F 0.53, produced by the compound later characterized as 1,1-bis(acetamido)-1-deoxy-D-glucitol (**1**; R = Me). D-Glucose (R_F 0.20) and D-glucosylamine (R_F 0.17) were also detected (solvent A).

Most of the residue dissolved on treatment with 50 ml of cold, absolute ethanol, giving a solution (X) and leaving crystals (2.9 g), m.p. 190–191°. Recrystallized from the same solvent, the compound had m.p. 192–193°, and $[\alpha]_D^{28}$ +85.9°, and was identified as **2** (R = Me). This compound (0.8 g) was benzoylated with benzoyl chloride (4.2 ml) and pyridine (20 ml) for 2 h at 20°, to give *N*-acetyl-2,3,5,6-tetra-*O*-benzoyl-α-D-glucosylamine, which was isolated in the usual way. It was recrystallized from chloroform–petroleum ether (b.p. 60–80°), yield 1.1 g (45%), m.p. 150–152°, $[\alpha]_D^{21}$ −19.1° (*c* 1.0, chloroform). [On ammonolysis, 90% of the original **2** (R = Me) was recovered.]

Anal. Calc. for $C_{36}H_{31}NO_{10}$: C, 67.82; H, 4.82. Found: C, 67.81; H, 4.78.

The ethanolic mother liquors (X) were evaporated to dryness, and the residue was chromatographed (solvent A) on a cellulose column (8 × 120 cm). Fractions of 200 ml were collected, and these were monitored by paper chromatography.

A small amount of acetamide was eluted in the first fractions. Fractions 3–7 contained **2** (R = Me) and a product characterized as **1** (R = Me). On evaporation of the combined fractions, a residue (2.6 g) was obtained which, on treatment with cold ethanol, gave a solution (Y) and an insoluble fraction consisting of **2** (R = Me). Recrystallization from ethanol gave 0.45 g of the pure product, the total yield being 12.1%.

Concentration of the ethanolic solution (Y) gave the other compound, which, when recrystallized from absolute ethanol, yielded 1.9 g of small plates, m.p. 162–164°. Further recrystallization gave pure **1** (R = Me), m.p. 165–167°, $[\alpha]_D^{23}$ +3.7° (*c* 1.1, water), $[\alpha]_D^{22}$ +23.0° (*c* 1.5, pyridine).

Anal. Calc. for $C_{10}H_{20}N_2O_7$: C, 42.85; H, 7.14; N, 10.00. Found: C, 43.05; H, 7.07; N, 9.71.

On periodate oxidation, 3.98 moles of periodic acid were consumed per mole of compound, producing 3.0 moles of formic acid and 1.06 moles of formaldehyde.

On acetylation with acetic anhydride–pyridine, 1,1-bis(acetamido)-2,3,4,5,6-penta-*O*-acetyl-1-deoxy-D-glucose was obtained in a yield of 90%. Recrystallized from chloroform–petroleum ether, it had m.p. 189–191°, $[\alpha]_D^{22}$ +21.1° (*c* 1.9, chloroform).

Anal. Calc. for $C_{20}H_{30}N_2O_{12}$: C, 48.98; H, 6.13; N, 5.71. Found: C, 48.94; H, 6.05; N, 5.60.

Fractions 9–10 contained mainly **1** (R = Me), and, from them, 0.9 g (total yield 8.0%) of pure product was isolated. Examination of the ethanolic mother liquors by paper chromatography (solvents D and E), and by ionophoresis, indicated the presence of small amounts of a compound which behaved as **3** (R = Me) (on comparison with an authentic sample prepared by the method of Onodera and Kitaoka[13]).

In the subsequent fractions, only D-glucose and D-glucosylamine were present.

*Ammonolysis of 1,2,3,4,6-penta-*O*-propionyl-*D*-glucose.* — (*a*) *1-Deoxy-1,1-bis(propionamido)-*D*-glucitol* (**1**; *R* = *Et*). The crude mixture obtained from the ammonolysis of 84.0 g of the title compound was extracted with warm (60°) ethyl acetate (giving residue Z). Paper chromatography of the extract (solvent B) revealed that it contained propionamide, D-glucose, D-glucosylamine, and a compound giving a strong spot, R_F 0.63. On standing at 5°, the last compound crystallized (m.p. 115–127°). Recrystallization (absolute ethanol) gave 1.29 g (2.6%) of **1** (R = Et); needles, m.p. 155–156°, $[\alpha]_D^{20}$ +6.8° (*c* 1.6, water), $[\alpha]_D^{22}$ +16.2° (*c* 1.1, pyridine).

Anal. Calc. for $C_{12}H_{24}N_2O_7$: C, 46.75; H, 7.79; N, 9.09. Found: C, 46.90; H, 7.92; N, 9.11.

On periodate oxidation, 4.02 moles of periodic acid were consumed per mole of compound, producing 2.92 moles of formic acid and 1.01 moles of formaldehyde.

Acetylation with pyridine–acetic anhydride gave 2,3,4,5,6-penta-*O*-acetyl-1-deoxy-1,1-bis(propionamido)-D-glucitol, m.p. 169–171°, $[\alpha]_D^{22}$ +25.3° (*c* 0.9, chloroform).

Anal. Calc. for $C_{22}H_{34}N_2O_{12}$: C, 50.96; H, 6.65; N, 5.45. Found: C, 51.06; H, 6.44; N, 5.39.

(*b*) N-*Propionyl*-α-D-glucofuranosylamine (**2**; R = Et). The residue (**Z**) (33.5 g) left after extraction with ethyl acetate was chromatographed on a column of cellulose (4.5 × 130 cm), with solvent B as eluant.

A small amount of propionamide was first eluted; fractions 46–123 gave only one spot, R_F 0.80, and fractions 136–188 showed an elongated spot centered at R_F 0.63 (**1**; R = Et). In subsequent fractions, only D-glucose and D-glucosylamine were detected.

Fractions 46–123 were concentrated to a small volume and, on standing at room temperature, this yielded 1.80 g of crystalline *N*-propionyl-α-D-glucofuranosylamine, m.p. 144–146°; recrystallized from isopropyl alcohol, it had m.p. 146–147°; $[\alpha]_D^{18}$ +82.7° (*c* 0.8, water). The mother liquors afforded a further crop, weighing 0.63 g (total yield 5.6%),

Anal. Calc. for $C_9H_{17}NO_6$: C, 45.95; H, 7.23; N, 5.95. Found: C, 46.36; H, 7.37; N, 5.67.

This compound consumed 2.01 moles of periodic acid per mole and produced 0.98 mole of formaldehyde. On oxidation with sodium periodate, the rotation (calculated on the original weight of the compound employed) changed to +11.3°, and on reduction with sodium borohydride, this value changed to +9.8°, showing that the compound was the α-D anomer[2].

On evaporation of fractions 136–188, a residue (1.26 g) was obtained which could not be induced to crystallize. It was therefore resubmitted to chromatography under the same conditions. The first fractions yielded 0.67 g of **1** (R = Et), m.p. 154–155° (total yield 3.9%). On evaporation, the next fractions gave uncrystallizable residues which, on ionophoresis, gave two spots; one, having M_G 0.72, corresponded to **1** (R = Et), and the other, with M_G 0.17, was later identified as **3** (R = Et) and was isolated in crystalline condition from a second ammonolysis.

(*c*) N-*Propionyl*-β-D-*glucopyranosylamine* (**3**; R = Et). Another ammonolysis of 1,2,3,4,6-penta-*O*-propionyl-D-glucose (55.0 g) was performed under identical conditions, except that solvent A was used for all of the chromatography. The total yield of **1** (R = Et) was 1.23 g (3.7%).

By column chromatography, a series of fractions was separated which, although giving only one spot, R_F 0.43, on plain paper, were found to contain two products, having R_F 0.16 (**2**; R = Et) and R_F 0.53 (**3**; R = Et), when paper impregnated with 0.01M sodium borate and solvent D was employed. On ionophoresis, two spots were also observed: M_G 0.16 (**3**; R = Et) and M_G 0.62 (**2**; R = Et). These fractions were combined, and, on evaporation, gave 1.4 g of a syrup.

When 0.5 g of the syrup was submitted to preparative, paper chromatography (sodium borate), two zones were obtained; these were eluted with water, the solutions were passed through a column of Amberlite IR-120 (H^+) ion-exchange resin, and the boric acid was removed by repeated evaporation with methanol. The eluate from the band having R_F 0.53 gave a syrup (0.13 g); this was dissolved in absolute ethanol,

and the solution was nucleated with pure **3** (R = Et), and kept at room temperature. A crystalline precipitate of *N*-propionyl-β-D-glucopyranosylamine, m.p. 186–189°, was formed. Recrystallization from the same solvent gave 0.1 g (total yield 0.9%), m.p. 191–192°, $[\alpha]_D^{29}$ −21.8° (*c* 0.3, water); the compound was identified by comparison with a sample prepared as indicated later.

Acetylation with acetic anhydride–pyridine gave 2,3,4,6-tetra-*O*-acetyl-*N*-propionyl-β-D-glucosylamine (75%), m.p. 151–152° (from ethanol), $[\alpha]_D^{20}$ +16.1° (*c* 0.7, chloroform). On ammonolysis, 97% of the original compound was recovered.

The eluate of the zone having R_F 0.16, which contained compound **2** (R = Et), although chromatographically pure, could not be crystallized from this ammonolyzate.

N-*Propionyl-2,3,4,6-tetra*-O-*propionyl-β-*D-*glucosylamine.* — β-D-Glucosylamine (5 g) was added to a mixture of propionic anhydride (25 ml) and pyridine (25 ml) precooled to 0°. The suspension was shaken until dissolution occurred, and was then kept for 2 h at 5°. The solution was poured into ice–water, giving a syrup which crystallized on being washed with cold water. Recrystallization from 50% methanol yielded 5.0 g (39%) of the title compound, m.p. 103–104°, $[\alpha]_D^{21}$ +19.7° (*c* 1.0, chloroform).

Anal. Calc. for $C_{21}H_{33}NO_{10}$: C, 54.89; H, 7.23; N, 3.04. Found: C, 54.89; H, 7.23; N, 3.38.

On ammonolysis, a 90% yield of *N*-propionyl-β-D-glucopyranosylamine was obtained, m.p. 190–191°, $[\alpha]_D^{27}$ −22.9°. The same compound was also obtained by *N*-propionylation of β-D-glucopyranosylamine, following the general method described by Onodera and Kitaoka[13].

Ammonolysis of 1,2,3,4,6-penta-O-*benzoyl-β-*D-*glucose.* — The residue (26.5 g) (from the ammonolysis of 60.0 g of the title compound) that was left after extraction with ethyl acetate was dissolved in 300 ml of boiling methanol; on cooling the solution, 7.3 g of crystals, m.p. 195–198°, was obtained. Recrystallization from ethanol yielded 6.7 g of pure 1,1-bis(benzamido)-1-deoxy-D-glucitol (**1**; R = Ph), m.p. 201–202°, $[\alpha]_D^{20}$ +1.6°.

The ethanolic mother liquors were evaporated to dryness, and the residue (15.2 g) was chromatographed on a cellulose column (4.6 × 120 cm) with solvent A as the eluant. Fractions (20 ml) were collected, and monitored by paper chromatography. After elution of a small amount of benzamide in the first fractions, the next eluates afforded 0.31 g of pure **1** (R = Ph), the total yield being 21.4%.

The subsequent fractions, which were chromatographically identical, were evaporated to dryness, affording a syrup (0.32 g) which, when submitted to chromatography on paper impregnated with 0.01M sodium borate (solvent D) or to ionophoresis, gave two spots. The major component, having R_N 1.0 and M_G 0.14, behaved like *N*-benzoyl-β-D-glucopyranosylamine (**3**; R = Ph), and the minor component, present in traces, had R_F 0.39 and M_G 0.45. The syrup was dissolved in a small volume of warm ethanol, and the solution was nucleated with **3** (R = Ph). On standing at room temperature, a crystalline precipitate was formed which yielded 0.27 g (1.0%) of *N*-benzoyl-β-D-glucopyranosylamine (**3**; R = Ph), m.p. 230–232°

(ethanol); $[\alpha]_D^{20}$ −11.6° (*c* 0.6, water). It was identified by direct comparison with a sample prepared as described later.

On acetylation with acetic anhydride–pyridine, it gave a 90% yield of 2,3,4,6-tetra-*O*-acetyl-*N*-benzoyl-β-D-glucosylamine, m.p. 196–198°; $[\alpha]_D^{18}$ −7.9° (*c* 0.6, chloroform). (On ammonolysis, the parent compound was recovered in 96% yield.)

Anal. Calc. for $C_{21}H_{25}NO_{10}$: C, 55.87; H, 5.54. Found: C, 56.10; H, 5.58.

The compound responsible for the second spot has not yet been identified.

N-*Benzoyl-2,3,4,6-tetra*-O-*benzoyl-β-*D-*glucosylamine.* — Benzoyl chloride (13.5 ml) was slowly added to a suspension of β-D-glucosylamine (1.8 g) in pyridine (15 ml) precooled to 0°. After being kept for 3 h, the mixture was poured into ice–water; an oil was precipitated, and this solidified on being washed with water. Recrystallization from ethanol gave 3.67 g (52.3%) of a product having m.p. 113–115°, which, on further heating, solidified at 140–150° and melted again sharply at 184°; $[\alpha]_D^{25}$ +23.2° (*c* 0.6, chloroform). The m.p. of 113–115° was not changed by drying *in vacuo* for analysis.

Anal. Calc. for $C_{41}H_{33}NO_{10}$: C, 70.18; H, 4.75; N, 2.00. Found: C, 70.16; H, 4.85; N, 2.15.

When 0.5 g of the product having m.p. 113–115° was slowly heated to 180°, the same sequence of changes was observed. The final solid melted at 184°, and this melting point was not changed by further recrystallization from ethanol, $[\alpha]_D^{22}$ +22.7°.

When a solution, in warm ethanol, of the product having m.p. 113–115° was nucleated with crystals having m.p. 184°, the higher-melting form crystallized out. An ethanolic solution of the product having m.p. 184° was nucleated with the low-meting form, and gave crystals which melted over a range starting at *ca.* 120°. On further recrystallization with repeated nucleation, the form melting at 113–115° was again obtained. Ammonolysis of both forms produced *N*-benzoyl-β-D-glucopyranosylamine (**3**; R = Ph) in 97% yield, m.p. 230–232°, $[\alpha]_D^{26}$ −12.0°; this was identical with the minor product obtained by the ammonolysis of 1,2,3,4,6-penta-*O*-benzoyl-β-D-glucose.

N-Benzoyl-β-D-glucopyranosylamine was also prepared by adapting the method of Onodera and Kitaoka[13] to the *N*-benzoylation of β-D-glucosylamine.

Anal. Calc. for $C_{13}H_{16}NO_6$: C, 55.12; H, 6.05; N, 4.95. Found: C, 55.12; H, 6.08; N, 4.98.

On oxidation with sodium periodate, 1.94 moles of oxidant were consumed per mole of compound 1.9 moles of formic acid were produced.

Ammonolysis of 2,3,4,6-tetra-O-*acetyl*-N-p-*toluenesulfonamido-β-*D-*glucosylamine.* — This compound was ammonolyzed as described by Helferich and Mitrowsky[10]. The resulting solution was evaporated to dryness, and the residue was extracted with warm methanol (30°), giving a solution (Q) and an insoluble fraction (0.21 g), m.p. 193–196°. Recrystallization from 50% methanol gave 0.2 g (18%) of the known 1-acetamido-1-deoxy-1-*p*-toluenesulfonamido-D-glucitol[10]; m.p. 195–197°; $[\alpha]_D^{17}$ −61.9° (water); $[\alpha]_D^{22}$ −19.6° (*c* 0.6, pyridine).

On oxidation with sodium periodate, 4.09 moles of reagent were consumed per

mole of compound, and 1.00 mole of formaldehyde and 3.00 moles of formic acid were produced.

Paper chromatography of the methanol extract (Q) by use of solvent C revealed that, besides the formerly isolated bis(acylamido) compound (R_F 0.69), it contained acetamide (R_F 0.86), *p*-toluenesulfonamide (R_F 0.78), *N*-*p*-tolylsulfonyl-β-D-glucopyranosylamine (R_F 0.59), D-glucose (R_F 0.15), and D-glucosylamine (R_F 0.10).

The residue obtained on evaporation of the methanolic solution (Q) was chromatographed on a cellulose column (2×45 cm), with elution with solvent C. Fractions (10 ml) that contained the same substance were combined, and were processed as usual. A small amount of *p*-toluenesulfonamide, a further quantity (0.22 g, total yield 36%) of 1-acetamido-1-deoxy-1-*p*-toluenesulfonamido-D-glucitol, and 0.06 g (6%) of *N*-*p*-tolylsulfonyl-β-D-glucopyranosylamine were isolated. The last compound had m.p. 195–197°, $[\alpha]_D^{20}$ +16.6° (1 h)→ +28.3° (24 h)→ +30.0° (48 h) (in water); lit.[11], m.p. 195–197° and $[\alpha]_D^{20}$ +6.9° (25 min)→ +29.7° (48 h). Paper chromatography (solvent C) showed that only D-glucose and *p*-toluenesulfonamide were present in the solution. When the rotation was calculated on the basis of the weight of D-glucose formed, the value $[\alpha]_D^{20}$ +50.0° was obtained; this is almost identical with the equilibrium value for free D-glucose.

REFERENCES

1 A. LEZEROVICH, E. G. GROS, J. F. SPROVIERO, AND V. DEULOFEU, *Carbohyd. Res.*, 4 (1967) 1.
2 A. S. CEREZO AND V. DEULOFEU, *Carbohyd. Res.*, 2 (1966) 35, and references cited therein.
3 I. M. E. THIEL, J. O. DEFERRARI, AND R. CADENAS, *J. Org. Chem.*, 31 (1966) 3704, and references cited therein.
4 J. O. DEFERRARI AND V. DEULOFEU, *J. Org. Chem.*, 17 (1952) 1093.
5 J. O. DEFERRARI AND V. DEULOFEU, *J. Org. Chem.*, 22 (1957) 802.
6 J. O. DEFERRARI AND R. M. DE LEDERKREMER, *Carbohyd. Res.*, 4 (1967) 365.
7 R. C. HOCKETT AND R. L. CHANDLER, *J. Amer. Chem. Soc.*, 66 (1944) 957.
8 C. NIEMANN AND J. T. HAYS, *J. Amer. Chem. Soc.*, 62 (1940) 2960.
9 J. O. DEFERRARI AND B. MATSUHIRO, *J. Org. Chem.*, 31 (1966) 905.
10 B. HELFERICH AND A. MITROWSKY, *Chem. Ber.*, 85 (1952) 1.
11 B. HELFERICH, K. H. SCHMIDT, AND D. NACHSHEIM, *Ann.*, 605 (1957) 182.
12 T. G. BONNER, *Chem. Ind.* (London), (1960) 345.
13 K. ONODERA AND S. KITAOKA, *J. Org. Chem.*, 25 (1960) 1322.

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DETERMINATION OF PYRANOID AND FURANOID STRUCTURES OF CYCLIC ACETALS OF HEXULOSES BY INFRARED SPECTROSCOPY*

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ABSTRACT

Infrared spectral studies of various cyclic acetals of some hexuloses are described. Examination of the lower-frequency region of absorption revealed the possibility of differentiating between furanoid and pyranoid structures of hexuloses on the basis of the position of a fairly sharp band appearing in the region of 725–680 cm^{-1}.

INTRODUCTION

It has been reported[1,2] that alcohols usually show an infrared absorption band in the region of 750–650 cm^{-1}, but this has not found much application in correlation work. In studies on long-chain primary alcohols, Tasumi and Shimanouchi[3] pointed out that an OH out-of-plane bending frequency is found at about 687 cm^{-1} for the β-form and at 612 cm^{-1} for the γ-form. Barker and Stephens[4] have shown that compounds containing a furanose or a hydrofuranol ring exhibit infrared absorptions of the A, B, C, and D types at 924 $\pm$13, 879 $\pm$7, and 799 $\pm$17 cm^{-1}, respectively. Tokuyama *et al.*[5] similarly found four types of absorption bands in the infrared spectra of a number of isopropylidene acetals of sorbofuranose, but the positions are at the higher-frequency regions of 958 $\pm$24, 895 $\pm$7, 864 $\pm$6, and 823 $\pm$7 cm^{-1}, respectively. Tipson *et al.*[6] made a fairly thorough investigation of the infrared absorption spectra of some cyclic acetals of sugars, and pointed out that the compounds having a hydroxyl group at C-1 show a band at 893.7–892.1 cm^{-1} and at 683.1–680.7 cm^{-1}. Verstraeten[7] differentiated ketofuranose from ketopyranose structures by their infrared absorption bands in the 850 $\pm$6 cm^{-1} and 834 $\pm$4 cm^{-1} regions, respectively.

These observations have been confirmed in connection with the present work on cyclic acetals of some ketopyranoses and ketofuranoses, although the range of absorption at the lower-frequency region of 683.1–680.7 cm^{-1} does not appear to be quite so much restricted as reported[6]. The region of 893.7–892.1 cm^{-1} usually contains a complex absorption pattern and, therefore, in the present work, more attention was paid to the lower-frequency region. As a result of these studies, it has

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been possible to differentiate between the furanoid and pyranoid structures of hexulose derivatives on the basis of the position of a fairly sharp band appearing in the 725–680 cm^{-1} region.

EXPERIMENTAL

Materials. — All of the sugars and their derivatives prepared in the course of the present work were of analytical purity. The solvents and the acidic dehydrating agents used for the acetylation were reagent grade (Merck and Co.).

Procedure. — The spectra (except those for which references are given) were recorded on a Perkin–Elmer Infracord Spectrophotometer, calibrated with polystyrene, by the Nujol mull technique.

Results. — The infrared bands of compounds **1** to **14** in the region of 725–680 cm^{-1} are given in Table I.

TABLE I

BANDS IN THE INFRARED SPECTRA OF ACETALS OF HEXULOSES IN THE 725–680 cm^{-1} REGION

	Compound	*Free OH at C-1*	*Band[a] (cm^{-1})*	*References*
1	2,3:4,6-Di-*O*-isopropylidene-α-L-sorbofuranose	yes	689 (s)	6
2	2,3:4,6-Di-*O*-isopropylidene-1-*O*-methyl-α-L-sorbofuranose	no	692 (w)	
3	1-*O*-Acetyl-2,3:4,6-di-*O*-isopropylidene-α-L-sorbofuranose	no	697 (m)	
4	2,3:4,6-Di-*O*-isopropylidene-L-*xylo*-hexulofuranonic acid (di-*O*-isopropylidene-2-keto-L-gulonic acid)	no	no band	6
5	1,2:4,6-Di-*O*-isopropylidene-α-L-sorbofuranose	no	no band	
6	2,3:4,6-Di-*O*-isobutylidene-α-L-sorbofuranose	yes	685 (m)	
7	4,6-*O*-Cyclohexylidene-2,3-*O*-isopropylidene-α-L-sorbofuranose	yes	690 (s)	
8	2,3:4,6-Di-*O*-cyclohexylidene-α-L-sorbofuranose	yes	683 (m)	
9	1-*O*-Acetyl-2,3:4,6-di-*O*-cyclohexylidene-α-L-sorbofuranose	no	694 (w)	
10	2,3-*O*-Isopropylidene-α-L-sorbofuranose	yes	686 (s), 719 (m)	
11	2,3:4,5-Di-*O*-isopropylidene-β-D-fructopyranose	yes	714 (m)	6
12	1,2-*O*-Isopropylidene-α-L-sorbopyranose	no	no band	
13	1,2:4,5-Di-*O*-isopropylidene-α-D-fructopyranose	no	no band	6
14	L-Sorbopyranose	yes	725 (s)	8

[a]Key: m = moderate; s = strong; w = weak.

DISCUSSION

Compounds **1**, **6**, **7**, **8**, and **10** (see Table I) show strong to moderate absorption at 686 ± 4 cm^{-1}, and the only common feature in the structure of these compounds is the presence of an OH group at C-1; in particular, 2,3:4,6-di-*O*-isopropylidene-α-L-sorbofuranose (**1**), in which there is only one free OH group (at C-1), shows a fairly strong absorption at 689 cm^{-1}. When this hydroxyl group is converted into

the corresponding methyl ether, to give 2,3:4,6-di-*O*-isopropylidene-1-*O*-methyl-α-L-sorbofuranose (**2**), the band under discussion vanishes, and only a very weak absorption at 692 cm^{-1} is apparent. The band is also absent from the spectrum of 1-*O*-acetyl-2,3:4,6-di-*O*-isopropylidene-α-L-sorbofuranose (**3**).

When the hydroxymethyl group on C-2 is oxidized to a carboxyl group, to give 2,3:4,6-di-*O*-isopropylidene-L-*xylo*-hexulofuranonic acid (**4**), no absorption is apparent in the region under discussion. When the hydroxyl group on C-1 is involved in the formation of a 1,3-dioxolane ring (compound **5**), the region is again free from any absorption. 2,3:4,6-Di-*O*-isobutylidene-α-L-sorbofuranose (**6**), 4,6-*O*-cyclohexylidene-2,3-*O*-isopropylidene-α-L-sorbofuranose (**7**), and 2,3:4,6-di-*O*-cyclohexylidene-α-L-sorbofuranose (**8**) all show absorption in this region, presumably because of the presence of a hydroxyl group on C-1.

On acetylation, compound **8** gives 1-*O*-acetyl-2,3:4,6-di-*O*-cyclohexylidene-α-L-sorbofuranose (**9**), and the absorption at 683 cm^{-1} in the spectrum of **8** is replaced by a very weak absorption at 694 cm^{-1} for **9**. In 2,3-*O*-isopropylidene-α-L-sorbofuranose (**10**), absorption (presumably due to the free hydroxyl group on C-1) is observed at 685 cm^{-1}; the hydroxyl groups on C-4 and C-6 probably give the absorption at higher frequency, namely, 719 cm^{-1} (Nujol gives very weak absorption in this region). This conclusion is corroborated by the presence of a doublet in the OH stretching frequency region; the peak at 3200 cm^{-1} is probably due to the hydroxyl groups on C-4 and C-6.

The compounds discussed so far have a furanoid ring in common. In contrast, compounds **11** to **14** contain a pyranoid ring. Compound **11** shows moderate absorption at 714 cm^{-1}, which must be due to the presence of the hydroxyl group on C-1. This absorption is not observed when the hydroxyl group at C-1 of the hexulopyranose is involved in the formation of a 1,3-dioxolane ring, as in 1,2-*O*-isopropylidene-α-L-sorbopyranose (**12**) and 1,2:4,5-di-*O*-isopropylidene-α-D-fructopyranose (**13**). Both of these compounds contain other hydroxyl group(s), none of which absorb in the region under discussion (714 cm^{-1}), and, therefore, absorption in this region can be attributed to the hydroxyl group at C-1 of the hexulopyranose. This absorption is also seen at 725 cm^{-1} in the spectrum of L-sorbopyranose (**14**), in which all of the hydroxyl groups are free. In this case, the shift of the absorption band under discussion is probably due to stronger hydrogen-bonding, and corresponds with the shift of the absorption band in the OH stretching region, where the absorption is at 3300 cm^{-1}.

Thus, the hexulofuranose derivatives containing a free hydroxyl group at C-1 always give an absorption band at 686 ± 4 cm^{-1}, which is probably associated with OH out-of-plane deformation, whereas, in a hexulopyranose derivative, such as 2,3:4,5-di-*O*-isopropylidene-β-D-fructopyranose, the band is found to be shifted to 714 cm^{-1}. The generalization made for the hexulopyranoses could not be substantiated by other examples, since not many ketopyranoses having a free hydroxyl group at C-1 are yet available. This generalization, however, seems to be logical from a study of models of these hexulofuranoses and hexulopyranoses. The hydroxyl hydrogen

atom at C-1 is found to be closer to the hydrogen atom at C-3 in the furanose derivatives, and this situation may influence the OH deformation of sugars having the furanose structure. This shift could also be due to the difference in the neighboring ring-structures. The furanose ring is essentially planar, whereas pyranose compounds favor a chair conformation. As pointed out by Tipson *et al.*[6], in the isopropylidene derivatives of certain pyranose sugars, equatorial and axial hydroxyl groups on adjacent carbon atoms are involved in the 1,3-dioxolane ring-formation; and spanning of these groups with a planar dioxolane ring would introduce a strain in the pyranose ring, inducing it to assume a nonchair shape, so that either, or both, of the rings would be somewhat distorted. In contrast, both the dioxolane and the furanose rings in an isopropylidene derivative of a furanose sugar are essentially planar.

Table II contains a list of the compounds prepared for the first time in the course of this work; their structures have been confirmed by other physicochemical properties and by chemical methods. Their spectral absorption in the region under discussion further confirms the furanose or pyranose structures already assigned to them.

TABLE II

BANDS IN THE INFRARED SPECTRA OF SOME NEW ACETALS OF HEXULOSES IN THE 725–685 CM^{-1} REGION

	Compound	*Free OH at C-1*	*Band*[a] *(cm^{-1})*
15	2,3-*O*-Isobutylidene-α-L-sorbofuranose	yes	690 (m)
16	2,3:4,6-Di-*O*-methylene-α-L-sorbofuranose	yes	687 (m)
17	2,3-*O*-Cyclohexylidene-α-L-sorbofuranose	yes	690 (m)
18	2,3:4,6-Di-*O*-Isobutylidene-α-L-sorbofuranose (m.p. 105°)	yes	690 (m)
19	1,2:3,4-Di-*O*-Isopropylidene-β-L-sorbofuranose	no	699 (m)
20	1,2-*O*-Isobutylidene-α-L-sorbopyranose	no	no band
21	1,2-*O*-Cyclohexylidene-α-L-sorbopyranose	no	no band
22	1,2-*O*-Isopropylidene-α-L-sorbofuranose	no	no band

[a]Key: m = moderate.

Compounds **15**, **16**, **17**, and **18** (see Table II) contain a furanose ring and a primary hydroxyl group on C-1; in conformity with the previous discussion, there is, in each case, moderate absorption in the range 686 ± 4 cm^{-1}. Compound **19** has a hydroxyl group on C-5, and shows an absorption at higher frequency, *viz.*, 699 cm^{-1}. Finally, compounds **20**, **21**, and **22** do not have a free hydroxyl group on C-1, and their spectrograms are free from any absorption in this region.

Similar extensive studies could not be made with the acetals of aldohexoses, as very few pyranoid and furanoid derivatives that have a free hydroxyl group on C-1 are available in this series. The spectra of two aldopentoses and their acetals, however, gave some interesting results; the bands in the region 683–680 cm^{-1} for these compounds are given in Table III.

TABLE III

BANDS IN THE INFRARED SPECTRA OF TWO ALDOPENTOSES AND THEIR ISOPROPYLIDENE ACETALS IN THE REGION OF 683–680 cm^{-1}

Compound	*Band*[a] (cm^{-1})	*References*
23 β-D-Arabinopyranose	683 (m)	7, 8
24 1,2:3,4-Di-*O*-isopropylidene-β-D-arabinopyranose	683 (m)	6
25 α-D-Xylopyranose	680 (m)	7, 8
26 1,2:3,5-Di-*O*-isopropylidene-α-D-xylofuranose	680 (m)	6

[a]Key: m = moderate.

It is important to note that, despite the differences in the structures of these compounds, they all absorb in the region 680–683 cm^{-1}. These absorptions *cannot* be attributed to any hydroxyl group(s), because, in the spectrum of β-D-arabinopyranose (**23**), there are four free hydroxyl groups, whereas its di-*O*-isopropylidene derivative (compound **24**), which is pyranoid, is devoid of free hydroxyl groups, but also shows the band. Again, α-D-xylopyranose (**25**) has four free hydroxyl groups, whereas its di-*O*-isopropylidene derivative (compound **26**), which is furanoid, has no free hydroxyl group. Yet, all of these four compounds absorb in the same region. This result indicates that the absorption in this region arises, in all probability, from the ring-breathing mode.

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REFERENCES

1 L. J. BELLAMY, *Infra-red Spectra of Complex Molecules*, Wiley, New York, 1962, p. 3.
2 C. N. R. RAO, *Chemical Applications of Infrared Spectroscopy*, Academic Press, New York and London, 1963, p. 188.
3 M. TASUMI AND T. SHIMANOUCHI, *Spectrochim. Acta*, 20 (1964) 629.
4 S. A. BARKER AND R. STEPHENS, *J. Chem. Soc.*, (1954) 4550.
5 K. TOKUYAMA, M. KIYOKAWA, AND N. HOKI, *Bull. Chem. Soc. Japan*, 36 (1963) 1392.
6 R. S. TIPSON, H. S. ISBELL, AND J. E. STEWART, *J. Res. Natl. Bur. Std.*, 62 (1959) 2960.
7 L. M. J. VERSTRAETEN, *Anal. Chem.*, 36 (1964) 1040; *Carbohyd. Res.*, 1 (1966) 481.
8 R. S. TIPSON AND H. S. ISBELL, *J. Res. Natl. Bur. Std.*, 66A (1962) 31.

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SOME OBSERVATIONS ON THE SYNTHESIS OF SOPHOROSE

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ABSTRACT

Condensation of tetra-*O*-acetyl-α-D-glucopyranosyl bromide with 1,3,4,6-tetra-*O*-acetyl-α-D-glucopyranose in acetonitrile solution containing mercuric cyanide and mercuric bromide affords the octa-acetates of both kojibiose (2-*O*-α-D-glucopyranosyl-D-glucose) and sophorose (2-*O*-β-D-glucopyranosyl-D-glucose). Each of these products can be isolated in approximately 28% yield. Although this method results in a lower over-all yield of sophorose from D-glucose than an alternative procedure, it has the advantage of being more rapid and direct, and is particularly well-suited to preparation of the acetylated glycosyl bromide.

INTRODUCTION AND DISCUSSION

Previous authors[1-3] have employed the Koenigs–Knorr procedure for the synthesis of sophorose (2-*O*-β-D-glucopyranosyl-D-glucose). The original method[1] involved condensation of methyl 4,6-*O*-benzylidene-α-D-glucopyranoside with tetra-*O*-acetyl-α-D-glucopyranosyl bromide to form methyl 4,6-*O*-benzylidene-2-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-α-D-glucopyranoside. The free sugar was only obtained from this compound *via* a series of intermediate derivatives, and the over-all yield of sophorose was less than 5%. A 65% yield of sophorose octa-acetate was obtained[2] by condensation of 1,3,4,6-tetra-*O*-acetyl-D-glucose with tetra-*O*-acetyl-α-D-glucopyranosyl bromide. More recently, a modification[3] of the Freudenberg procedure[1,4] gave a 30% yield of crystalline sophorose from the benzylidene compound.

The 1,3,4,6-tetra-*O*-acetyl-D-glucose employed by Gakhokidze[2] would appear to be the inaccessible[5] β-anomer. However, 1,3,4,6-tetra-*O*-acetyl-α-D-glucose can be readily prepared[6], and a simple and direct synthesis of sophorose from this compound therefore appeared possible. Condensation with tetra-*O*-acetyl-α-D-glucopyranosyl bromide was carried out by the improved[3] Koenigs–Knorr procedure, but t.l.c. revealed that only a small amount of disaccharide was formed. This was recovered, in 3–5% yield, as the relatively insoluble and readily crystallizable glycosyl bromide hepta-acetate ("α-acetobromosophorose"). Contrary to expectation and to the findings of Gakhokidze[2], use of the anomeric tetra-*O*-acetyl-β-D-glucose gave

essentially the same results. No β-sophorose octa-acetate could be crystallized from the reaction mixture, and bromination did not lead to a higher yield of α-acetobromosophorose. Control experiments with methyl 4,6-*O*-benzylidene-α-D-glucopyranoside always gave the expected product in the reported[3] 40% or higher yield.

In view of the above results, further experiments were directed towards a synthesis of sophorose by the method of Helferich and Zirner[6], involving reaction in the presence of mercuric cyanide and mercuric bromide in acetonitrile solution. Reaction under these conditions has been reported[6] to result in the formation of the α-octa-acetate of kojibiose (2-*O*-α-D-glucopyranosyl-D-glucose), which was isolated in 21% yield. However, as condensation proceeds *via* an intermediate glycosyl cation formed by loss of halogen from the acetylated glycosyl halide, formation of both α- and β-D-glycosides must be considered possible[6]. Examination by t.l.c. of the reaction mixture from the condensation of 1,3,4,6-tetra-*O*-acetyl-α-D-glucopyranose and tetra-*O*-acetyl-α-D-glucopyranosyl bromide under the exact conditions of Helferich and Zirner[6] revealed that two major products, differing only slightly in chromatographic behaviour, were formed. α-Kojibiose octa-acetate crystallized from ethanolic solution in the expected 21% yield, and bromination of the dry residue from the mother liquor led to the isolation of α-acetobromosophorose in 25% yield. Modification of the reaction conditions, by employing a 30% excess of glycosyl halide instead of the molar equivalent and extending the reaction period from 2 to 6 h, increased the yield of α-kojibiose octa-acetate to 27.6% and that of α-acetobromosophorose to 31.3%. The non-crystalline α-sophorose octa-acetate could also be converted *via* the bromide to the β-octa-acetate which was isolated in an over-all yield from D-glucose of 28.2%. Deacetylation of this compound gave α-sophorose monohydrate in 62.8% yield. Since pure 1,3,4,6-tetra-*O*-acetyl-α-D-glucopyranose can be prepared from D-glucose in 28% yield[5,6], the over-all yield of sophorose from D-glucose is calculated to be 4.96%. Sophorose can also be prepared by direct deacetylation of the α-octa-acetate in the kojibiose mother liquor, the yield in this case being slightly lower (4.27% from D-glucose) owing to the presence of various impurities which interfere with crystallization. If it is considered that methyl α-D-glucopyranoside can be prepared in 50% yield from D-glucose[7] and that the yield[8] of the 4,6-*O*-benzylidene derivative from the methyl glycoside is 70%, the over-all yield of sophorose from D-glucose by the method of Coxon and Fletcher[3] is 10.5%. However, despite the lower yield by the Helferich and Zirner procedure[6], its directness and simplicity make it the method of choice. In addition, the acetylated glycosyl halide is of particular value for the synthesis of glycosides, and this compound is very readily preparable by the latter procedure.

EXPERIMENTAL

All m.ps. are uncorrected and were determined by the Kofler method. Merck Kieselgel G was employed for t.l.c., with 15% (v/v) methanol in benzene as irrigant and sulphuric acid as spray reagent.

1,3,4,6-Tetra-O-acetyl-2-O-(2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl)-α-D-glucopyranose (α-kojibiose octa-acetate). — 1,3,4,6-Tetra-*O*-acetyl-α-D-glucopyranose[6] (1.75 g) was dissolved in a solution of mercuric cyanide (0.64 g) and mercuric bromide (0.90 g) in acetonitrile (14 ml), and 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide (2.75 g) was added. After 6 h at room temperature, the solvent was evaporated, and the residue was treated with chloroform (50 ml). The filtered extract was washed with aqueous M potassium bromide (3 × 10 ml) and water (2 × 20 ml). The chloroform was evaporated, and the residue was crystallized from ethanol to yield α-kojibiose octa-acetate (0.94 g, 27.6%), m.p. 168–169°, $[\alpha]_D^{22}$ +153.2° (*c* 2.5, chloroform); lit.[6], m.p. 168–168.5°, $[\alpha]_D^{18}$ +152.5° (chloroform).

3,4,6-Tri-O-acetyl-2-O-(2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl)-α-D-glucopyranosyl bromide (α-acetobromosophorose). — The ethanolic mother liquor from the preparation of α-kojibiose octa-acetate was evaporated to dryness, and the residue was dissolved in dichloromethane (2 ml) and treated with a solution of 40% hydrogen bromide in glacial acetic acid (15 ml) for 20 min at room temperature. The solution was diluted with chloroform (75 ml), washed with iced water, saturated aqueous sodium hydrogen carbonate, and water until the washings were neutral to litmus, filtered through anhydrous sodium sulphate, and evaporated to dryness at 30°. Crystallization of the residue from dichloromethane–ether yielded α-acetobromosophorose (1.1 g, 31.3%), m.p. 189–191°, $[\alpha]_D^{26}$ +97.7° (*c* 1.6, chloroform); lit.[3], m.p. 190–191°, $[\alpha]_D^{20}$ +97.4° (chloroform).

1,3,4,6-Tetra-O-acetyl-2-O-(2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl)-β-D-glucopyranose (β-sophorose octa-acetate). — The chloroform solution of crude α-acetobromosophorose, prepared as described above, was evaporated to dryness at 30° and treated with a solution of mercuric acetate (3.0 g) in glacial acetic acid (40 ml) for 2 h at room temperature. The solution was diluted with chloroform (150 ml), washed with water (4 × 50 ml), and evaporated to dryness. Crystallization of the residue from ethanol afforded β-sophorose octa-acetate (0.96 g, 28.2%), m.p. 191–192°, $[\alpha]_D^{22}$ −3.5° (*c* 2.5, chloroform); lit.[4], m.p. 192°, $[\alpha]_D^{20}$ −4° (chloroform).

2-O-β-D-Glucopyranosyl-α-D-glucopyranose (α-sophorose). — β-Sophorose octa-acetate (0.96 g) in absolute methanol (10 ml) was treated with 1.2% methanolic sodium methoxide (6 ml) for 30 min at room temperature. The solution was adjusted to pH 7.0 with dilute hydrochloric acid, desalted electrolytically (Shandon Mark II desalter), and evaporated almost to dryness at 35°. The residue was crystallized from aqueous ethanol–ether to yield α-sophorose monohydrate (0.32 g, 62.8%), m.p. 195–197°, $[\alpha]_D^{22}$ +33.6→ +19.0° (equil.; *c* 3.2, water); lit.[9], m.p. 196–198°, $[\alpha]_D^{18}$ +19° (equil., water).

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REFERENCES

1 K. FREUDENBERG AND K. SOFF, *Ber.*, 69 (1936) 1245.
2 A. M. GAKHOKIDZE, *Zh. Obshch. Khim.*, 11 (1941) 117; *Chem. Abstr.*, 35 (1941) 5467.
3 B. COXON AND H. G. FLETCHER, JR., *J. Org. Chem.*, 26 (1961) 2892.
4 O. TH. SCHMIDT, *Methods Carbohyd. Chem.*, 1 (1962) 349.
5 B. H. KOEPPEN, *Tetrahedron*, in press.
6 B. HELFERICH AND J. ZIRNER, *Ber.*, 95 (1962) 2604.
7 G. N. BOLLENBACK, *Methods Carbohyd. Chem.*, 2 (1963) 326.
8 N. K. RICHTMYER, *Methods Carbohyd. Chem.*, 1 (1962) 107.
9 M. J. CLANCY, *J. Chem. Soc.*, (1960) 4213.

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THE HYDRODYNAMIC BEHAVIOUR OF NATIVE AMYLOSE IN GOOD SOLVENTS*

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ABSTRACT

Viscosity measurements on 12 fractions of linear amylose have been made in three thermodynamically good solvents—formamide (F), methyl sulphoxide (Me_2SO), and aqueous potassium hydroxide (KOH). The following Mark–Houwink relations were obtained:

$$[\eta]_F = 3.05 \times 10^{-2} \bar{M}_w^{0.62},$$
$$[\eta]_{Me_2SO} = 1.51 \times 10^{-2} \bar{M}_w^{0.70}, \text{ and}$$
$$[\eta]_{KOH} = 8.36 \times 10^{-3} \bar{M}_w^{0.77}.$$

The Kamide–Inamoto and Stockmayer–Fixman–Burchard theories show that the amylose molecules in these solvents are extended as a result of solute–solvent interaction and there is little evidence of any free-draining characteristics. The graph of $[\eta]/M^{1/2}$ against $M^{1/2}$ for all three good solvents is linear with a common intercept. Incidently, this intercept is the same as that for an aqueous theta-solvent, showing that the basic skeletal structure of amylose is the same in each of these solvents. The conformation of amylose in these three good solvents is regarded as being that of a random coil with negligible helical character.

INTRODUCTION

In the previous part of this series[1], we discussed the conformation of native amylose in neutral, aqueous salt solution, and showed that this solvent is a Flory θ-solvent[2], in which amylose exhibits its unperturbed dimensions. We have now carried out measurements on amylose dissolved in three good solvents, *i.e.*, formamide, methyl sulphoxide, and aqueous potassium hydroxide, and report here our conclusions regarding the hydrodynamic behaviour of the polysaccharide in these solvents.

EXPERIMENTAL

The preparation of the twelve amylose fractions used in this work, and the determination of their weight-average molecular weight, $\bar{M}_w$, has been described previously[1,3].

*This is Part 44 in the series "Physicochemical Studies on Starches"; Part 43 is Ref. 1.

Solutions were prepared by weight from the dried, dehydrated, butyl alcohol–amylose complex by shaking gently overnight at room temperature with formamide (F) and methyl sulphoxide (Me_2SO). In the case of the 0.15M potassium hydroxide (KOH), 2 h at 0–2° with gentle agitation was sufficient to give stable, gel-free solutions. Concentrations in this solvent, determined by hydrolysis to D-glucose (1.5N sulphuric acid for 2 h at 100°) and subsequent estimation of the reducing sugar by alkaline ferricyanide[4], agreed to within 1% with those from the dry-weight.

Limiting-viscosity numbers, $[\eta]$, were determined in modified Ubbelohde viscometers (average shear-rate = 1200 sec^{-1}) for which the kinetic energy correction was negligible. The value of $[\eta]$ for the fraction of largest molecular weight (Fraction 1) in 0.15M potassium hydroxide was also measured in a rotating cylinder viscometer, based on the design of Ogston and Stanier[5], at shear rates in the range 0.5–5 sec^{-1}. No difference was found in the $[\eta]$-value, and so no correction for shear effects was necessary for measurements made in the Ubbelohde viscometer.

RESULTS AND DISCUSSION

Limiting-viscosity number/molecular weight relations.

The results of the viscosity measurements in the three solvents, and also the molecular weights of the twelve fractions, are shown in Table I. The corresponding logarithmic functions (Fig. 1) yield the relations

$$[\eta]_F = 3.05 \times 10^{-2} \bar{M}_w^{0.62} \quad (1)$$

$$[\eta]_{Me_2SO} = 1.51 \times 10^{-2} \bar{M}_w^{0.70} \quad (2)$$

$$[\eta]_{KOH} = 8.36 \times 10^{-3} \bar{M}_w^{0.77} \quad (3)$$

TABLE I

MOLECULAR WEIGHTS AND LIMITING-VISCOSITY NUMBERS FOR AMYLOSE FRACTIONS IN DIFFERENT SOLVENTS

Fraction	$\bar{M}_w \times 10^{-6}$	$[\eta]_F$	$[\eta]_{Me_2SO}$	$[\eta]_{KOH}$
1	1.75	230	365	495
2	1.22	182	268	395
3	0.954	155	232	340
4	0.754	132	197	285
5	0.613	118	172	250
6	0.488	102	145	205
7	0.460	96	133	190
8	0.424	94	125	180
9	0.380	88	121	165
10	0.320	78	104	145
11	0.211	61	79	105
12	0.083	36	44	54

The values of the exponents in equations (*1*)–(*3*) reflect the hydrodynamic shape of the dissolved macromolecule, and also the extent of solvent–solute interaction.

The deviation of the value of our exponents from that of 0.5 for a θ-solvent can be described in terms of models which involve the effect of either partial free-draining (Kirkwood and Riseman[6], Debye and Bueche[7]), or solute–solvent interaction (Flory[2]). Methods of deciding between these two models are considered below.

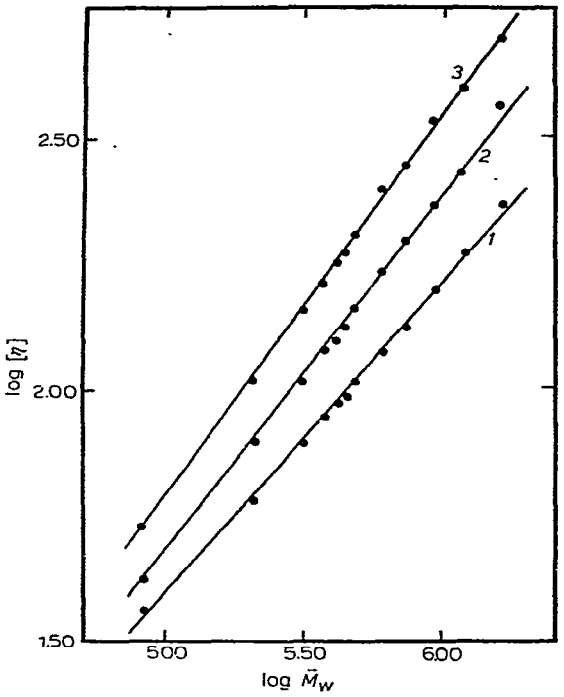

Fig. 1 — Graph of log $\bar{M}_{\eta o}$ against log [η] for the amylose fractions dissolved in formamide (*1*), methyl sulphoxide (*2*), and 0.15M potassium hydroxide (*3*).

Tests for partial free-draining characteristics

1. Kamide and Inamoto theory[8]. This theory makes use of the parameters in the Mark–Houwink equation,

$$[\eta] = KM^a, \tag{4}$$

which, according to Flory[2], may be written as

$$[\eta] = K_\theta M^{1/2}\alpha^3, \tag{5}$$

where $K_\theta = \Phi_o\,(\bar{r}_o^2/M)$. Φ_o is the viscosity constant, $\bar{r}_o^2$ is the mean-square end-to-end distance of the polymer coil, and α is an expansion factor. Kurata, Stockmayer, and Roig[9] related α to M by

$$\alpha^3 - \alpha = (1 + 1/3\alpha^{-2})^{-3/2} cM^{1/2}, \tag{6}$$

where c is a constant for a given polymer–solvent system. Substitution of (*6*) in (*5*) gives

$$-\log K + 1.5 \log [1 + 4/3\ \{(a-0.5)^{-1} - 2\}^{-1}] = (a-0.5) \log M_o - \log K_\theta, \tag{7}$$

where M_o is the geometric mean molecular weight of the range over which the Mark–Houwink parameters were determined. If the draining factor is negligible, a plot of $(-\log K + 1.5 \log [1+4/3\ \{(a-0.5)^{-1}-2\}^{-1}])$ against $(a-0.5)$ should be linear, with an intercept of $-\log K_\theta$. Fig. 2 shows a graph of our data for amylose according to equation (7). The value of K_θ obtained earlier[1] (11.6×10^{-2}) has also been inserted. A satisfactorily linear relation is obtained, suggesting that draining effects are absent in all of the three solvents.

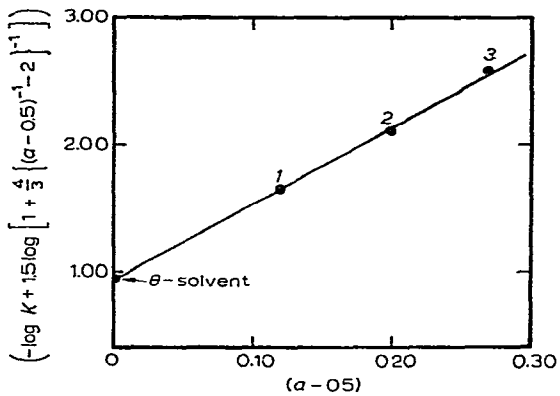

Fig. 2 — Kamide and Inamoto theory[8], graph of $(-\log K+1.5 \log [1+4/3\ \{(a-0.5)^{-1}-2\}^{-1}]$ against $(a-0.5)$ for the amylose fractions dissolved in formamide (*1*), methyl sulphoxide (*2*), and 0.15M potassium hydroxide (*3*).

2. Fixman–Stockmayer–Burchard theory. Another method of showing whether free-draining is operative is to use the relation

$$[\eta]/M^{1/2} = K_\theta + c' K_\theta M^{1/2}, \tag{8}$$

where c' is a constant for a given polymer–solvent system. This relation is obtained by substituting the Fixman[10] closed expression for the expansion factor, α,

$$\alpha^3 = 1 + c' M^{1/2} \tag{9}$$

into equation (5). Equation (8) is known as the Stockmayer–Fixman[11] relation, but was, in fact, first advanced by Burchard[12]. Thus, a graph of $[\eta]/M^{1/2}$ against $M^{1/2}$ should be linear, if the drainage factor for amylose is negligible. Fig. 3 shows the present data graphed according to equation (8). For all three good solvents, linear relations may be drawn through the points for all except the fractions of highest molecular weight. (For discussion of this, see later).

Equation (8) is based on the Kurata–Yamakawa[13] extension of the Kirkwood–Riseman[6] model to non-ideal solvents, giving

$$[\eta] = (\pi^{3/2} N_A)\,[XF(X)]\,[(\bar{r}_o^2)^{3/2}/6^{3/2} M]\,[1 + p(X)z - \ldots], \tag{10}$$

where $X = (3/2\pi M_o)^{1/2}(d/b)M^{1/2}$, and $z = (3/2\pi)^{3/2} BM^{1/2}/(\bar{r}_o^2/M)^{3/2}$.

N_A is Avogadro's Number, $F(X)$ and $\rho(X)$ are tabulated functions[13] of the drainage parameter X, M_o is the molecular weight of the monomer unit of length b and having a Stokes diameter d, and B is a solute–solvent interaction parameter. The functions $F(X)$ and $\rho(X)$ tend to limiting values as the extent of free draining becomes vanishingly small; this is the condition for the use of equation (*8*). In agreement with Baumann[14], we think that the deviation of values at high molecular weights arises from the approximation by which equation (*10*) is simplified to (*8*), namely that the final factor in (*10*) has to be interrupted after the linear term. This approximation will lose its validity as the molecular weight increases, and consequently the values of $[\eta]/M^{1/2}$ will deviate from a linear relation with $M^{1/2}$ in this region. Fig. 3 therefore again shows that the effect of draining is negligible in these solvents.

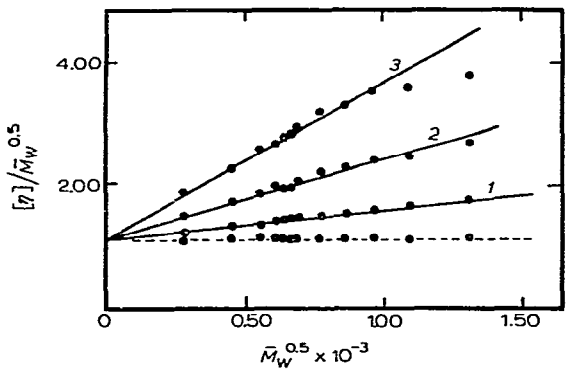

Fig. 3 — Stockmayer–Fixman–Burchard theory[11,12]; graph of $[\eta]/\bar{M}_w^{1/2}$ against $\bar{M}_w^{1/2}$ for the amylose fractions dissolved in formamide (*1*), methyl sulphoxide (*2*), and 0.15M potassium hydroxide (*3*). Previous results[1] for the fractions dissolved in 0.15M aqueous potassium chloride are shown as a dotted line.

3. Sedimentation measurements. The extent of free draining may also be obtained independently from measurement of the sedimentation coefficient (S_o). For a series of fractions, an equation analogous to (*4*) may be written,

$$S_o = K_s M^b. \qquad (11)$$

The value of the exponent b depends on the model used to describe the dissolved polymer molecule. In the partially free-draining model, b decreases from 0.50 (impermeable coil) to 0 (free-draining coil); for the model based on expansion as a result of polymer–solvent interaction, b decreases from 0.50 (θ-solvent) to a limiting value of 0.40 (according to Flory[2]), or to 0.33 (according to Kurata and Stockmayer[9]) for very good solvents. We have reported[15] sedimentation studies carried out on amylose fractions dissolved in 0.15N potassium hydroxide. The value of b was found to be 0.41. The corresponding value of the viscosity exponent a is 0.78. According to the Flory theory, a and b are related by $b = (2-a)/3$. Using the experimental value of $a = 0.78$, the calculated value of b is 0.41, *i.e.*, identical to the experimental figure. On the other hand, if the Kirkwood–Riseman model is valid, the theoretical

value of b can be shown to be 0.35. We believe that the difference between this calculated value and that determined experimentally is significant.

Conclusions regarding free draining

It is our conclusion, therefore, that there is no evidence for any measurable degree of free draining in amylose of molecular weight greater than *ca.* 80,000, in any of the solvents employed. In fact, the available evidence confirms that the different exponents in (*1*)–(*3*) result directly from varying degrees of solvent–solute interaction.

Conformation of the amylose molecule in solution

Equation (*8*) has been widely used to determine the unperturbed dimensions of polymers from measurements carried out in good solvents—the intercept is equal to K_θ when $[\eta]/M^{\frac{1}{2}}$ is plotted against $M^{\frac{1}{2}}$.

The results for the three good solvents in Fig. 3 pass, without strain, through a common intercept, and hence the basic skeletal structure of the amylose molecule must be the same in each solvent. But when we consider also our results[1] for amylose in neutral, aqueous potassium chloride, a θ-solvent, the dotted line in Fig. 3 results, and it can be seen that the intercept is again the same as for the good solvents. The basic skeletal structure for the amylose is thus the same in a θ-solvent as in a good solvent. The only molecular configuration consistent with this experimental fact is that the amylose molecule is behaving as a random coil in solution with no helical character.

Elsewhere[1,16], we have postulated that amylose in neutral, aqueous solution behaves as such a random coil. The above evidence directly substantiates this. Helical character is *impossible* to reconcile with the facts that the skeletal structure of amylose is the same in neutral and alkaline aqueous solution, whilst the limiting-viscosity number may increase some 300% in the process[17].

Comparisons with earlier results in the literature

The values for our exponents in equations (*1*)–(*3*) are not the same as earlier results in the literature, but direct comparisons are difficult. Burchard[18] found an exponent of 0.67 for formamide, and, moreover, considered that solutions in this solvent were metastable. However, we could detect no change in $[\eta]$ for our fractions that had been kept in formamide solution for up to two weeks, and hence we conclude that formamide solutions of amylose are quite stable. Values for the exponents of 0.82 (Ref. 18), 0.64 (Ref. 19), and 0.87 (Ref. 20) have been reported for methyl sulphoxide, in contrast to our value of 0.70. However, other workers have used *dried* methyl sulphoxide, which is hygroscopic. We have found that the experimental difficulties caused thereby can be avoided by using a solvent with 0.2% water-content. Earlier results obtained from measurements in dilute alkali cannot be compared because various normalities have been used; the limiting-viscosity number of an amylose fraction is very dependent on the concentration of alkali[17], and hence it is probable that the exponent will also vary.

Both Burchard[18] and Cowie[20] have suggested that there is a fairly large degree of free draining for amylose in these solvents, but it should be noted that Kamide and Inamoto[8] have re-analysed the results of these authors and have also concluded that the draining effect is negligibly small.

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REFERENCES

1 W. BANKS AND C. T. GREENWOOD, *Carbohyd. Res.*, 7 (1968) 349.
2 P. J. FLORY, *Principles of Polymer Chemistry*, Cornell University Press, Ithaca, New York, U.S.A., 1953.
3 W. BANKS, C. T. GREENWOOD, AND D. J. HOURSTON, *Trans. Faraday. Soc.*, in press.
4 L. H. LAMPITT, C. H. F. FULLER, AND L. COTON, *J. Sci. Food Agr.*, 6 (1955) 656.
5 A. G. OGSTON AND J. E. STANIER, *Biochem. J.*, 53 (1953) 4.
6 J. C. KIRKWOOD AND J. RISEMAN, *J. Chem. Phys.*, 16 (1948) 565.
7 P. DEBYE AND A. M. BUECHE, *J. Chem. Phys.*, 16 (1948) 573.
8 K. KAMIDE AND Y. INAMOTO, *Chem. High Polymers* (Tokyo), 22 (1965) 691.
9 M. KURATA, W. H. STOCKMAYER, AND A. ROIG, *J. Chem. Phys.*, 33 (1960) 151.
10 M. FIXMAN, *J. Chem. Phys.*, 36 (1962) 3123.
11 W. H. STOCKMAYER AND M. FIXMAN, *J. Polymer Sci. C*, 1 (1963) 137.
12 W. BURCHARD, *Makromol. Chem.*, 50 (1961) 20.
13 M. KURATA AND H. YAMAKAWA, *J. Chem. Phys.*, 29 (1958) 311.
14 H. BAUMANN, *J. Polymer Sci. B*, 3 (1965) 1069.
15 W. BANKS AND C. T. GREENWOOD, *Stärke*, in press.
16 W. BANKS AND C. T. GREENWOOD, in *Conformation of Biopolymers*, G. N. RAMACHANDRAN (Ed.), Academic Press, London, 1967, p. 739.
17 R. GEDDES, C. T. GREENWOOD, AND A. R. PROCTER, *Abstr., Intern. Symp. Carbohyd. Chem.*, Münster, July 1964, pp. 26-27.
18 W. BURCHARD, *Makromol. Chem.*, 64 (1963) 110.
19 W. W. EVERETT AND J. F. FOSTER, *J. Amer. Chem. Soc.*, 81 (1959) 3464.
20 J. M. G. COWIE, *Makromol. Chem.*, 42 (1961) 230.

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ACACIA MEARNSII GUM

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(Received February 26th, 1968)

ABSTRACT

The exudate gum from *Acacia mearnsii* contains residues of D-galactose, L-arabinose, L-rhamnose, and D-glucuronic acid (in part, as the 4-methyl ether). Graded hydrolysis with acid affords mixtures of neutral and acidic oligosaccharides, including 6-*O*-(*β*-D-glucopyranosyluronic acid)-D-galactose, 6-*O*-(4-*O*-methyl-*β*-D-glucopyranosyluronic acid)-D-galactose, 3- and 6-*O*-*β*-D-galactopyranosyl-D-galactose, and 3-*O*-*β*-L-arabinopyranosyl-L-arabinose. A structure is proposed for the gum in the light of these results and of the examination of cleavage products from methylated derivatives of the native polysaccharide, a degraded polysaccharide formed on autohydrolysis, and a degraded galactan formed on further degradation of the periodate-oxidised, degraded polysaccharide. A structural comparison is made between this gum collected in Jamaica and the gum from the same species collected in South Africa. No major structural differences have been detected between the two gum samples.

INTRODUCTION

Through the kindness of Dr. Jean Tyler, we were supplied with a sample of the exudate gum from *Acacia mearnsii* (syn. *A. decurrens* var. *mollis*, *A. mollissima*), collected from a single tree in Jamaica. Since significant differences in detailed structure have been demonstrated amongst gums from various *Acacia* species[1,2]. It was of interest to compare this sample of gum with that of a tree of the same species from a very different geographical location is South Africa. A preliminary examination of the latter gum was reported some years ago[3,4], and a further sample of gum of similar origin has been examined recently[5].

RESULTS AND DISCUSSION

Acacia mearnsii gum of Jamaican origin furnished a polysaccharide which was homogeneous by the criterion of chromatography on *O*-(2-diethylaminoethyl)-cellulose. The polysaccharide contained 12% of uronic acid, and was composed of

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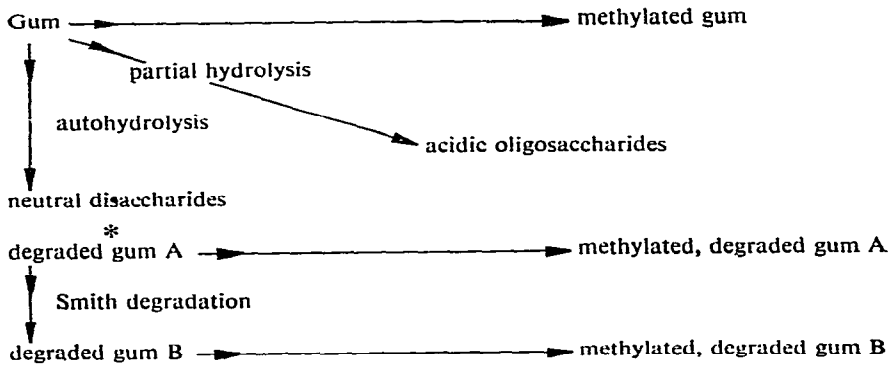
galactose*, arabinose, rhamnose, and glucuronic acid (including, in part, the 4-methyl ether) in the approximate molar ratios of 5.6:4.9:1.0:1.6. The experiments performed on the polysaccharide are summarised in the following flowsheet.



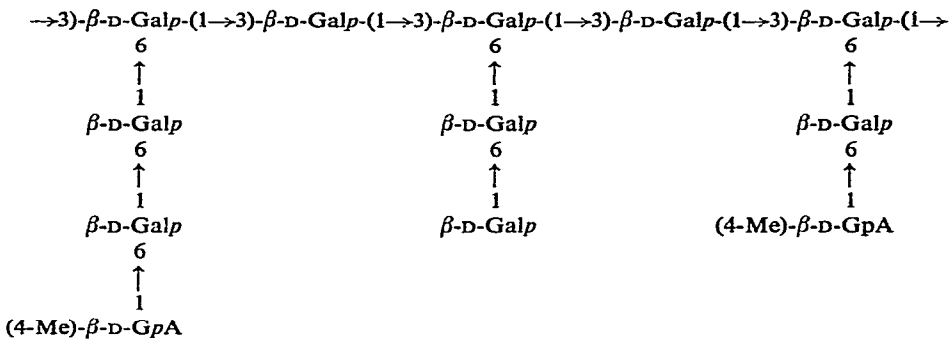
Partial, acid hydrolysis of the gum gave a mixture of acidic oligosaccharides, including the two aldobiouronic acids, 6-*O*-(*β*-D-glucopyranosyluronic acid)-D-galactose (**1**) and 6-*O*-(4-*O*-methyl-*β*-D-glucopyranosyluronic acid)-D-galactose (**2**). Two trisaccharides (**3** and **4**) in which residues of the disaccharides (**1** and **2**) were probably further linked to galactose by (1→6)-linkages were partially characterised. Less drastic degradation of the polysaccharide, involving autohydrolysis, led mainly to the liberation of arabinose and rhamnose, and to the isolation of degraded gum A, which was composed solely of residues of glucuronic acid and galactose. The degraded gum was probably still qualitatively representative of the interior structure of the parent polysaccharide; but, that some fragmentation of the inner portions of the molecule had occurred was indicated by the characterisation of 3- (**5**) and 6-*O*-*β*-D-galactopyranosyl-D-galactose (**6**) amongst the products of low molecular weight from the autohydrolysis. A further disaccharide, namely 3-*O*-*β*-L-arabinopyranosyl-L-arabinose (**7**), which was characterised as an autohydrolysis product, presumably arose from peripheral units in the gum structure.

β-D-G*p*A-(1→6)-D-Gal	**1**	4-Me-*β*-D-G*p*A-(1→6)-D-Gal	**2**
β-D-Gal*p*-(1→3)-D-Gal	**5**	*β*-D-Gal*p*-(1→6)-D-Gal	**6**
β-L-Ara*p*-(1→3)-L-Ara	**7**		

The highly branched nature of the degraded gum was established by the detection of methyl glycosides of 2,3,4-tri-*O*-methylglucuronic acid, and 2,3,4,6-tetra-, 2,3,4- and 2,4,6-tri-, and 2,4-di-*O*-methylgalactose on methanolysis of the methylated gum An indication of the general distribution of (1→3)- and (1→6)-linkages between galactose residues was obtained by subjecting degraded gum A to further degradation by Smith's procedure[6], involving sequential periodate oxidation, reduction, and mild hydrolysis with acid, to give degraded gum B. Partial hydrolysis and methylation

*Configurational symbols are omitted where sugars were identified by chromatographic methods.

studies showed that degraded gum B was a D-galactan consisting mainly of (1→3)-linked sugar residues. On the basis of these results, the following structure (**8**), which is not unique, but accommodates the main structural features, may be proposed for degraded gum A.

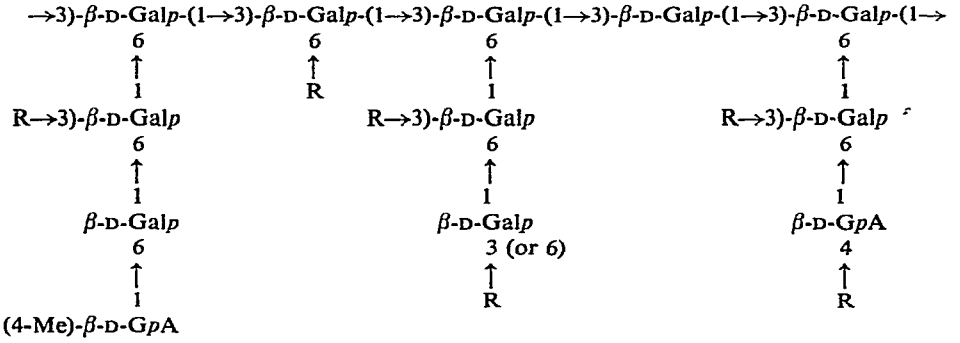


8

A sample of the gum was converted into the methylated derivative. Through the kindness of Professor A. M. Stephen, we were also provided with a sample of methylated *Acacia mearnsii* gum of South African origin. Both of the methylated polysaccharides were reduced with lithium aluminium hydride, samples of both of the methylated gums and of their reduction products were treated with methanolic hydrogen chloride, and the resulting mixtures of methyl glycosides were examined semi-quantitatively by gas chromatography. The results showed that both of the methylated gums contained residues of the following sugars: 2,3,4-tri-*O*-methylrhamnose, 2,3,5-tri- and 2,3- and 2,5-di-*O*-methylarabinose, 2,3,4,6-tetra-, 2,3,4- and 2,4,6-tri-, and 2,4-di-*O*-methylgalactose, and 2,3,4-tri- and 2,3-di-*O*-methylglucuronic acid. In addition, residues of 2,3,4-tri- or 3,5-di-*O*-methylarabinose, or both, were also present, but, in our hands, the methyl glycosides of these sugars could not be distinguished from each other by gas chromatography. The 3,5-dimethyl ether was, however, detected in the investigation by Stephen *et al.*[5]. No substantial differences in the relative proportions of the constituent sugar units in the two methylated polysaccharides were observed.

A comparison of the sugar constituents of methylated *Acacia mearnsii* gum with those from methylated degraded gum A indicates that the peripheral residues removed during the autohydrolysis of the gum include end-groups of L-rhamnopyranose, L-arabinofuranose, and (in small proportion) L-arabinopyranose, together with 3- and 5-*O*-substituted L-arabinofuranose residues. For the presence of contiguous arabinose residues in the gum, direct evidence based on the characterisation of partial-hydrolysis products is available only for 3-*O*-β-L-arabinopyranosyl-L-arabinofuranose residues, but the presence of differently linked arabinose residues may be inferred from the methylation data. The exact points of attachment of the various outer-chain units to the inner parts of the polysaccharide structure are not yet known, but it is clear that these include C-4 of the unmethylated D-glucuronic acid residues and

C-3 or C-6, or both, of the D-galactose residues. Structure **9** may, therefore, be proposed to accommodate the known structural features of *Acacia mearnsii* gum. Although a comb-like structure is indicated for the basal D-galactan core, it should be noted that a small degree of multiple branching has not been excluded[7].



9

Proposed structure for *Acacia mearnsii* gum; R = L-Ara*f*-(1→, L-Ara*f*-(1→3)-L-Ara*f*-(1→, L-Ara*f*-(1→5). L-Ara*f*-(1→, β-L-Ara*p*-(1→3)-L-Ara*f*-(1→, or L-Rha*p*-(1→

The structure of *Acacia mearnsii* gum has been established in adequate detail for comparison to be made with the structures of gums of certain other *Acacia* species. The gum, like those from *A. senegal* (gum arabic)[1,7] and *A. pycnantha*[8], contains a branched D-galactan core in which (1→3)-linkages preponderate in the inner chains and (1→6)-linkages in the outer chains. Like these two gums, *A. mearnsii* gum contains residues of β-D-glucuronic acid (or its 4-methyl ether) (1→6)-linked to those of D-galactose. Residues of α-D-glucuronic acid (1→4)-linked to D-galactose, as found in *A. karroo*[4] and *A. arabica*[2] gums, have not been detected. The general nature of the peripheral residues of L-arabinose and L-rhamnose is similar for all *Acacia* gums that have been examined in reasonable detail, but, with the exception of the L-rhamnose residues in gum arabic[9], the exact points of attachment to D-glucuronic acid or D-galactose residues have not yet been ascertained. It is, however, probable that the various *Acacia* gums differ substantially in the detailed sequences of sugar residues in these peripheral units, and this feature has been indicated, in part, by the characterisation of disaccharides that are liberated on controlled hydrolysis with acid. 3-*O*-β-L-Arabinopyranosyl-L-arabinose (**7**) has been isolated from gum arabic[1], *A. karroo*[4], and *A. arabica*[2] gums, as well as from *A. mearnsii* gum. Although 3-*O*-β-L-arabinofuranosyl-L-arabinose (**10**), which has been obtained from *A. pycnantha*[8] and *A. arabica*[2] gums, has not been detected as a partial-hydrolysis product from *A. mearnsii* gum, the presence of such residues may be inferred from methylation results. In contrast, 3-*O*-α-D-galactopyranosyl-L-arabinose (**11**), which has been isolated from gum arabic[1] and *A. cyanophylla* gum[10], and would be much less likely to escape detection, is not formed from *A. mearnsii* gum.

β-L-Ara*f*-(1→3)-L-Ara **10** α-D-Gal*p*-(1→3)-L-Ara **11**

The main structural differences that have been recognised amongst *Acacia* gums have been those between gums of different species. In contrast, our present conclusions, base on (*a*) a comparison of the cleavage products from the methylated gums, and (*b*) a comparison of the partial-hydrolysis products from the present work on *Acacia mearnsii* gum from Jamaica, with those characterised previously from gum from South Africa[3,4], are: that the samples of gum from the same botanical species show no apparent differences in the nature of the sugar residues or in the linkages between them, although there may be some small differences in the relative proportions of these residues.

EXPERIMENTAL

General methods. — Paper chromatography was performed with Whatman Nos. 1 and 3MM papers and the following solvent-systems (v/v): (*A*) 10:4:3 ethyl acetate–pyridine–water; (*B*) 18:3:1:4 ethyl acetate–acetic acid–formic acid–water; (*C*) 18:8:3:9 ethyl acetate–acetic acid–formic acid–water; (*D*) 8:2:1 ethyl acetate–pyridine–water. Gas-liquid chromatography (g.l.c.) was performed on columns of (*a*) 15% by weight of butan-1,4-diol succinate polyester (175°), (*b*) 10% by weight of polyphenyl ether [*m*-di-(*m*-phenoxyphenoxy)benzene] (200°), and (*c*) 3% by weight of neopentyl glycol adipate polyester (150°) on Celite. Unless otherwise stated, optical rotations were observed for aqueous solutions at *ca.* 18°.

Isolation and examination of gum acid. — Powdered *Acacia mearnsii* gum (18 g) was stirred in water (3 liters) for 2 days, insoluble material was removed by centrifugation, and the supernatant liquid was concentrated (to 250 ml), acidified by the addition of concentrated hydrochloric acid (2.5 ml), and poured, with stirring, into ethanol (1 liter) to give gum acid (13.7 g), $[\alpha]_D$ −61° (*c* 0.9) [Found: uronic acid (by decarboxylation), 12.1%]. A portion of the gum remained insoluble in water, but it dissolved in aqueous ammonia and afforded a further quantity of polysaccharide of similar composition.

A portion of the gum (50 mg) was chromatographed on *O*-(2-diethylaminoethyl)-cellulose (phosphate form)[11] with sodium dihydrogen phosphate buffer (pH 6) of increasing concentration as the eluant. A small proportion of material (*ca.* 4%) was eluted with the starting buffer of 0.05M phosphate, and was shown by paper chromatography to consist of galactose and arabinose. These sugars may have been formed by adventitious hydrolysis during handling of the gum acid. Polysaccharide (*ca.* 80% of the material applied to the column) was eluted in a single band with 0.25M buffer.

Gum acid (5 g) was hydrolysed wiht 0.5M sulphuric acid for 5 h on a boiling-water bath, and the hydrolysate was rendered neutral with barium hydroxide and barium carbonate, passed through Amberlite IR-120 (H^+) resin to remove barium ions, and adsorbed on Amberlite CG-45 (formate form) resin. Elution of the latter resin with water afforded a mixture of neutral sugars, a portion (500 mg) of which was separated by filter-sheet chromatography in solvent *B* to give rhamnose (78 mg),

which was characterised by conversion into the 2-benzoylhydrazone, m.p. and mixed m.p. 178–179°; arabinose (244 mg), characterised as the benzoylhydrazone, m.p. and mixed m.p. 186°, and galactose (106 mg), which was characterised by conversion into the β-pentaacetate, m.p. and mixed m.p. 142°.

Gum acid (50 mg) was hydrolysed with 0.05M sulphuric acid for 1 h on a boiling-water bath, and furnished degraded gum, which contained galactose and only a trace of arabinose as neutral-sugar constituents, and a mixture of soluble sugars. Chromatographic separation of the soluble, neutral sugars, and estimation by the phenol–sulphuric acid reagent, showed the presence of arabinose and rhamnose in the molar ratio of 5:1, and a trace of galactose. A further quantity of gum acid was hydrolysed with 0.5M sulphuric acid, and estimation of neutral sugars liberated showed the presence of galactose, arabinose, and rhamnose (corrected for preponderant destruction of the latter sugar under the more drastic conditions of hydrolysis) in the molar ratios of 4:5:1. Assuming that acidic sugars were liberated mainly in the form of aldobiouronic acids containing glucuronic acid (or its 4-methyl ether) and galactose residues, the gum has the following estimated molar composition: galactose: arabinose:glucuronic acid:rhamnose in the ratio of 5.6:5:1.6:1.

Partial hydrolysis of gum, and identification of acidic oligosaccharides. — Gum acid (3 g) was hydrolysed in 0.5M sulphuric acid (300 ml) for 6 h at 90–95°, and the cooled solution was rendered neutral with barium hydroxide and barium carbonate: the suspension was filtered, and the filtrate was passed through a column of Amberlite IR-120 (H^+) resin, to remove barium ions. The eluate was concentrated, and the product was adsorbed on a column (12 g, 15×2 cm) of *O*-(2-diethylaminoethyl)-Sephadex (formate form). Elution of the column with water gave a mixture (2.15 g) of neutral sugars containing rhamnose, arabinose, galactose, and traces of 3- and 6-*O*-galactopyranosylgalactose. Elution with 3% formic acid gave a mixture of acidic sugars (0.65 g), which was fractionated by filter-sheet chromatography in solvent *B* to give glucuronic acid (40 mg), 4-*O*-methylglucuronic acid (20 mg), and oligosaccharides **1** (224 mg), **2** (102 mg), **3** (24 mg), and **4** (10 mg).

Oligosaccharide **1**. — The sugar, R_{Gal} 0.15 in solvent *B*, had $[\alpha]_D$ −7.5° (*c* 1.1), was chromatographically indistinguishable from 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose, and gave glucuronic acid, glucurono-1,4-lactone and galactose on hydrolysis. Reduction of the derived methyl ester methyl glycosides with potassium borohydride, followed by hydrolysis, gave glucose and galactose. G.l.c. of the methanolysis products from the methylated disaccharide, on columns *a* and *b*, showed the presence of methyl glycosides of 2,3,4-tri-*O*-methylglucuronic acid, and 2,3,4- and 2,3,5-tri-*O*-methylgalactose.

Oligosaccharide **2**. — The sugar, R_{Gal} 0.49 in solvent *B*, had $[\alpha]_D$ +1° (*c* 1.0), was chromatographically indistinguishable from 6-*O*-(4-*O*-methyl-β-D-glucopyranosyluronic acid)-D-galactose, and gave 4-*O*-methylglucuronic acid and galactose on hydrolysis. Reduction of the derived methyl ester methyl glycosides with potassium borohydride, followed by hydrolysis, gave 4-*O*-methylglucose and galactose. The identity of the 4-methyl ether was established by chromatography of its periodate-

oxidation products[13]. G.l.c. of the methanolysis products from the methylated disaccharide, on columns *a* and *b*, showed the presence of methyl glycosides of 2,3,4-tri-*O*-methylglucuronic acid, and 2,3,4- and 2,3,5-tri-*O*-methylgalactose.

Oligosaccharide **3**. — The sugar, R_{Gal} 0.04 in solvent *B*, had $[\alpha]_D$ +25° (*c* 1.2) and gave glucuronic acid, galactose, and oligosaccharide **1** on partial hydrolysis. Reduction of the derived methyl ester methyl glycosides with potassium borohydride, followed by hydrolysis, gave glucose and galactose. Determination of the content of uronic acid by the carbazole method[14], and of total sugar by the phenol–sulphuric acid reagent[12], indicated that the sugar was a trisaccharide. G.l.c. of the methanolysis products from the methylated trisaccharide, on columns *a* and *b*, showed the presence of methyl glycosides of 2,3,4-tri-*O*-methylglucuronic acid, and 2,3,4- and 2,3,5-tri-*O*-methylgalactose.

Oligosaccharide **4**. — The sugar, R_{Gal} 0.09 in solvent *B*, had $[\alpha]_D$ +8° (*c* 1.0) and gave 4-*O*-methylglucuronic acid, galactose, and oligosaccharide **2** on partial hydrolysis. Reduction of the derived methyl ester methyl glycosides with potassium borohydride, followed by hydrolysis, gave 4-*O*-methylglucose and galactose. Partial hydrolysis of the above reduction products gave, *inter alia*, a disaccharide having the chromatographic mobility of 6-*O*-β-D-galactopyranosyl-D-galactose. Determination of the content of uronic acid by the carbazole method[14], and of total sugar by the phenol–sulphuric acid reagent[12], indicated that the sugar was a trisaccharide.

Autohydrolysis of gum acid, and isolation of degraded gum A. — Gum acid (8 g) in water (400 ml) was heated for 64 h on a boiling-water bath. The cooled solution was poured into ethanol (1.5 liter) to give degraded gum A (1.8 g), $[\alpha]_D$ −33° (*c* 0.85) [Found: uronic acid (by decarboxylation), 19%], and concentration of the supernatant liquid gave a syrupy mixture of sugars (6.5 g).

A sample of degraded gum A was methylated successively with methyl sulphate–sodium hydroxide, and methyl iodide–silver oxide, to give methylated, degraded gum A, $[\alpha]_D$ −46° (*c* 0.84, chloroform). [Found: OMe, 43.1%]. The methylated polysaccharide was heated with methanolic hydrogen chloride, and g.l.c. of the products on columns *a* and *b* showed the presence of methyl glycosides of 2,3,4-tri-*O*-methylglucuronic acid, and 2,3,4,6-tetra-, 2,3,4- and 2,4,6-tri-, and 2,4-di-*O*-methylgalactose.

The above syrup (6.5 g) was adsorbed on charcoal–Celite, monosaccharides that were eluted with water were discarded, and gradient elution with water containing 2.5→10% of ethanol gave fractions containing oligosaccharides **5**–**7**. Chromatographically pure oligosaccharides were obtained by filter-sheet chromatography in solvent *B*.

Oligosaccharide **5**. — The sugar (20 mg), R_{Gal} 0.51 in solvent *A*, $[\alpha]_D$ +50° (*c* 0.2), crystallised from acetone–water and had m.p. 225°. The disaccharide gave galactose, only, on hydrolysis, and was chromatographically indistinguishable from 3-*O*-β-D-galactopyranosyl-D-galactose. Widely varying melting points have been recorded for different samples of this disaccharide[15], and no samples having the above melting point were available for comparison. However, the disaccharide gave an X-ray powder photograph identical with that of another sample having m.p. 168°.

Oligosaccharide 6. — The sugar (16 mg), R_{Gal} 0.3 in solvent *A*, $[\alpha]_D$ +45° (*c* 0.16), was chromatographically indistinguishable from 6-*O*-β-D-galactopyranosyl-D-galactose, and gave galactose, only, on hydrolysis. The disaccharide was characterised by conversion into the phenylosazone, identified by m.p. 179° and mixed m.p. (with sample, m.p. 181°) 180°, and by X-ray powder photography.

Oligosaccharide 7. — The sugar (24 mg), R_{Gal} 0.91 in solvent *A*, $[\alpha]_D$ +183° (*c* 0.24), was chromatographically indistinguishable from 3-*O*-β-L-arabinopyranosyl-L-arabinose, and gave arabinose, only, on hydrolysis. G.l.c. of the methanolysis products from the methylated derivative, on columns *a* and *b*, showed the presence of methyl glycosides of 2,3,4-tri-, and 2,4- and 2,5-di-*O*-methylarabinose. The disaccharide was characterised by conversion into the phenylosazone, which was identified by m.p. 221° and mixed m.p. 223°, and by X-ray powder photography.

Formation and examination of degraded gum B. — Degraded gum A (1 g) was oxidised with sodium metaperiodate (4.5 g) in water (100 ml) for 48 h (consumption of reagent, determined spectrophotometrically[16], was constant and corresponded to 1.5 molecules per sugar residue). A calculated quantity (1.34 g) of ethylene glycol was added to decompose excess of periodate, sodium ions were removed by passage through Amberlite IR-120 (H^+) resin, and the resulting effluent was rendered neutral with barium carbonate. Insoluble barium salts were removed by centrifugation, and washed with water (4 × 50 ml), the centrifugate and washings were combined and concentrated (to 50 ml), and the solution was treated with potassium borohydride (1 g) for 2 days. The resulting solution was treated with Amberlite IR-120 (H^+) resin, to decompose excess of hydride and to remove potassium ions, the suspension was filtered, and the filtrate was evaporated, with repeated additions of methanol, to remove boric acid as methyl borate. The residue was treated with 0.5M sulphuric acid (80 ml) for 12 h at room temperature, neutralised, concentrated, and poured into ethanol (10 vol), tc give degraded gum B (75 mg), $[\alpha]_D$ 0° (*c* 1.0). Evaporation of the supernatant liquid gave a syrup (257 mg), from which no sugars were detected on hydrolysis.

Partial hydrolysis of degraded gum B with 0.25M sulphuric acid for 0.5 h on the boiling-water bath gave galactose, 3-*O*-galactopyranosylgalactose (oligosaccharide 5), and the polymer-homologous tri- and higher oligo-saccharides, but 6-*O*-galactopyranosylgalactose was not detected. Successive treatments of degraded gum B (40 mg) with methyl sulphate–sodium hydroxide, and methyl iodide–silver oxide gave methylated, degraded gum B (10 mg), $[\alpha]_D$ −6° (*c* 1.0, chloroform) [Found: OMe, 42.5%, not raised on further methylation]. A sample of methylated, degraded gum B was heated with methanolic hydrogen chloride, and a portion of the resulting mixture of methyl glycosides was shown by g.l.c. on columns *b* and *c* to contain derivatives of 2,3,4,6-tetra- and 2,4,6-tri-*O*-methylgalactose. The remaining methyl glycosides were hydrolysed, and paper chromatography of the hydrolysate showed the presence of 2,3,4,6-tetra- and 2,4,6-tri-*O*-methylgalactose as major components, together with a trace of 2,6-di-*O*-methylgalactose.

Preparation and examination of methylated Acacia mearnsii *gum.* — Successive

treatments of gum with methyl sulphate–sodium hydroxide and methyl iodide–silver oxide gave methylated gum, $[\alpha]_D$ −66° (*c* 0.97, chloroform) [Found: OMe, 42.4%]. Lithium aluminium hydride (250 mg) in tetrahydrofuran (8 ml) was added to a sample (250 mg) of the methylated gum in tetrahydrofuran (8 ml) during 0.5 h, and the solution was refluxed for 3 h. Excess of hydride was decomposed by the addition of ethyl acetate and water, and the resulting mixture was shaken with dilute sulphuric acid and chloroform. The chloroform extract furnished reduced, methylated gum (200 mg), $[\alpha]_D$ −66° (*c* 0.8, chloroform) [Found: OMe, 40.5%].

Through the courtesy of Professor A. M. Stephen, a sample was supplied of methylated gum[5] obtained from *Acacia mearnsii* of South African origin, $[\alpha]_D$ −55° (*c* 1.4 at pH 8) [Found: equiv. (by titration), 1920]. The methylated gum, after a further treatment with methyl iodide–silver oxide to ensure similar, final-methylation conditions, had $[\alpha]_D$ −66° (*c* 0.99, chloroform) [Found: OMe, 42.6%]. Reduction of a portion of the methylated gum with lithium aluminum hydride, as already described, gave reduced, methylated gum, $[\alpha]_D$ −66° (*c* 1.0, chloroform) [Found: OMe, 40.8%].

Samples of both of the methylated gums, and of their reduction products, were treated with methanolic hydrogen chloride, and the resulting mixtures of methyl glycosides were examined by g.l.c. on columns *a* and *b*. The relative retention times of methyl glycosides that were formed from both of the methylated gums are shown in following Table I. Small differences in the relative proportions of certain components were observed, but it was not possible to perform accurate quantitative analyses on such complex mixtures of glycosides.

TABLE I

GAS–LIQUID CHROMATOGRAPHIC DATA FOR METHYLATED DERIVATIVES

Sugar	*Relative retention-times of methyl glycosides in:*			
	column a		*column* b	
2,3,4-Tri-*O*-methylrhamnose	0.45		(0.46)[a]	
2,3,4-Tri-*O*-methylarabinose (and/or 3,5-di-*O*-methylarabinose)	1.04		0.81	
2,3,5-Tri-*O*-methylarabinose	0.55	0.72	(0.46)	0.58
2,3-Di-*O*-methylarabinose	1.56	(1.89)	0.64	0.96
2,5-Di-*O*-methylarabinose	(1.89)	3.44	0.68	1.02
2,3,4,6-Tetra-*O*-methylgalactose	1.80		1.61	
2,3,4-Tri-*O*-methylgalactose	7.4		2.89	
2,4,6-Tri-*O*-methylgalactose	4.15	4.67	2.08	2.36
2,4-Di-*O*-methylgalactose			3.60	4.26
2,3,4-Tri-*O*-methylglucuronic acid[b]	2.50	3.22	1.76	2.22
2,3-Di-*O*-methyl glucuronic acid[b]	8.32	9.2	2.40	3.10
2,3,4-Tri-*O*-methylglucose[a]	2.59	3.71	1.32	1.79
2,3-Di-*O*-methylglucose[a]			2.40	3.15

[a]Figures in parentheses indicate relative retention-times of components that were incompletely resolved. [b]Detected on cleavage of methylated gums. [c]Detected on cleavage of reduced, methylated gums.

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REFERENCES

1 For references to earlier work, see F. SMITH AND R. MONTGOMERY, *The Chemistry of Plant Gums and Mucilages*, Reinhold Publishing Corp., New York (1959).
2 D. M. W. ANDERSON, SIR EDMUND HIRST, AND J. F. STODDART, *J. Chem. Soc. (C)*, (1967) 1476, and references therein cited.
3 A. M. STEPHEN, *J. Chem. Soc.*, (1951) 646.
4 A. J. CHARLSON, J. R. NUNN, AND A. M. STEPHEN, *J. Chem. Soc.*, (1955) 1428; D. M. W. ANDERSON AND G. M. CREE, *Carbohyd. Res.*, 6 (1968) 214.
5 M. CAPLAN AND A. M. STEPHEN, *Tetrahedron*, 23 (1967) 193.
6 I. J. GOLDSTEIN, G. W. HAY, B. A. LEWIS, AND F. SMITH, *Methods Carbohyd. Chem.*, 5 (1965) 361–370.
7 D. M. W. ANDERSON, SIR EDMUND HIRST, AND J. F. STODDART, *J. Chem. Soc. (C)*, (1966) 1959.
8 G. O. ASPINALL, E. L. HIRST, AND A. NICOLSON, *J. Chem. Soc.*, (1959) 1967.
9 G. O. ASPINALL, A. J. CHARLSON, E. L. HIRST, AND R. YOUNG, *J. Chem. Soc.*, (1963) 1696; G. O. ASPINALL AND R. YOUNG, *ibid.*, (1965) 3003.
10 A. J. CHARLSON, J. R. NUNN, AND A. M. STEPHEN, *J. Chem. Soc.*, (1955) 269.
11 H. NEUKOM, H. DEUEL, W. J. HERI, AND W. KÜNDIG, *Helv. Chim. Acta*, 43 (1960) 64.
12 M. DUBOIS, K. A. GILLES, J. K. HAMILTON, P. A. REBERS, AND F. SMITH, *Anal. Chem.*, 28 (1956) 350.
13 R. U. LEMIEUX AND H. F. BAUER, *Can. J. Chem.*, 31 (1953) 814.
14 E. A. MCCOMB AND R. MCCREADY, *Anal. Chem.*, 24 (1952) 1630.
15 F. SMITH AND A. M. STEPHEN, *J. Chem. Soc.*, (1961) 4892.
16 G. O. ASPINALL AND R. J. FERRIER, *Chem. Ind.* (London), (1957) 1216.

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4-(D-*arabino*-TETRAHYDROXYBUTYL)-4-THIAZOLINE-2-THIONE. SYNTHESIS OF OPTICAL ISOMERS AND ANALOGS

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ABSTRACT

The previously reported[6], unequivocal synthesis of the title compound (**5a**) afforded a general method for the preparation of 4-substituted 4-thiazoline-2-thiones derived from fully acetylated α-halo ketoses. Such use of the α-halo ketoses (**1b**) or (**2b**) (as outlined in Method I of the Scheme) led to the crystalline L enantiomorph (**5b**) that, on cocrystallization with **5a**, gave the DL form (**5c**). By use of the α-bromo ketoses (**1d–1f**), further application of the method yielded amorphous, highly soluble, D-*xylo*, L-*xylo* (crude), and D-*gluco* analogs **5d–5f**, respectively. A *galacto*-1,4-bis-(4-thiazoline-2-thione) analog (**5g**), similarly obtained from the α-bromo ketose (**1g**), was crystalline, high-melting, and highly insoluble. By a modification of the procedure (Method II), treatment of the α-bromo ketoses (**1h–1i**) with the dithiocarbamic acid salts, derived from carbon disulfide and methylamine or 4-aminobutyric acid, led to the crystalline 4-thiazoline-2-thiones (**5h–5i**), respectively, that are also substituted at the *N*-hetero atom. Compounds intermediate in these syntheses are reported.

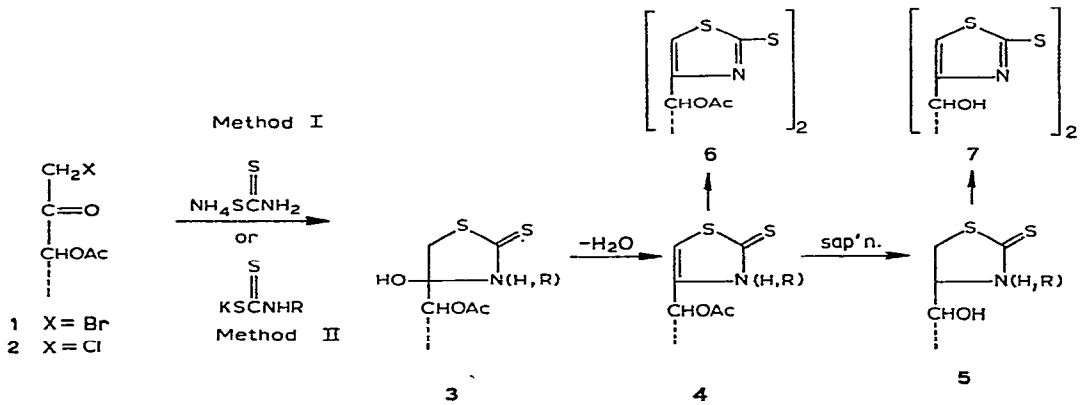
INTRODUCTION

The title compound (**5a**) was first reported by Zemplén *et al.*[1] to be isolable in a trace amount from the reaction of D-fructose with thiocyanic acid in hydrochloric acid solution. The major product obtained was shown to have an oxazolidine-2-thione structure fused to a pyranoid ring. Analogous oxazolidines were isolated[2–4] on similar treatment of L-arabinose, D-galactose, D-glucose, D-lyxose, D-mannose, D-ribose, and D-xylose. A ketose, D-fructose, was the only sugar of the series that was reported to yield a 4-thiazoline-2-thione when treated according to this procedure.

Recently, Jochims *et al.*[4,5] have proposed, on the basis of n.m.r. spectroscopic data, the structure now assigned to the title compound (**5a**). In their study, **5a** was obtained from the reaction of 2-amino-2-deoxy-D-glucose with carbon disulfide, *via* an intermediate 5-hydroxythiazolidine-2-thione that was subsequently dehydrated by refluxing for 16 h in pyridine. The reaction of α-amino carbonyl derivatives with carbon disulfide to form the new class of compounds, 5-hydroxythiazolidine-2-thiones, was reported to be general. Other 4-thiazoline-2-thiones derived from carbohydrates have, however, not yet been reported.

In an independent study, the structure of the title compound (**5a**) was also

determined[6] by degradation to known derivatives and by an unequivocal synthesis. By use of the latter synthesis, as outlined in Method I of the Scheme, **5a** was obtained from the reaction of 3,4,5,6-tetra-*O*-acetyl-1-bromo-1-deoxy-D-*arabino*-hexulose (**1a**) with ammonium dithiocarbamate, *via* a 4-hydroxythiazolidine-2-thione intermediate **3a**. Ready dehydration of **3a** to **4a**, followed by removal of the *O*-acetyl groups, afforded the title compound (**5a**). Earlier literature also reported[7,8] an



a D-*arabino*
b L-*arabino*
c DL-*arabino*
d D-*xylo*
e L-*xylo*
f D-*gluco*
g *galacto*-1,4-bis
h D-*arabino*; R = Me
i L-*arabino*; R = $(CH_2)_3CO_2H$

SCHEME

The series **a**–**e**, **h**, and **i** have a tetrahydroxy(or acetoxy)butyl chain, the series **f** has a pentahydroxy (or acetoxy)pentyl chain, and the series **g** has a 1,2,3,4-tetrahydroxy(or acetoxy)butane-1,4-diyl chain

extension of the synthesis by a modification of the procedure. By the latter route, treatment of an α-halo ketone with a dithiocarbamic acid salt (derived from carbon disulfide and an alkyl- or arylamine, or an amino acid) gave 4-thiazoline-2-thiones substituted at the *N*-hetero atom. It appeared probable that these sequences of reactions (*i.e.*, as outlined in Methods I and II) could be used as a procedure of significant scope for the preparation of 4-thiazoline-2-thiones from carbohydrates. The present article reports application of these methods to preparation of the optical isomers, and certain analogs, of the title compound (**5a**).

The unequivocal synthesis[6] of **5a** largely provided a parallel route to the enantiomorph (**5b**) by known methods of carbohydrate chemistry. The starting point for the preparation of **5b** was L-arabinose. Oxidation with bromine by the method of Fieser *et al.*[9], and isolation of the product from isopropyl alcohol, afforded L-arabinono-1,4-lactone (in 89% yield). Addition of the lactone to methanolic potassium hydroxide[10] gave potassium L-arabinonate (83%); this was acetylated with acetic anhydride and hydrogen chloride to yield tetra-*O*-acetyl-L-arabinonic acid[11] (54%). Thionyl chloride[12] converted the latter into tetra-*O*-acetyl-L-arabinoyl chloride[13] (85%), whereupon treatment with diazomethane yielded 3,4,5,6-tetra-*O*-acetyl-1-

deoxy-1-diazo-L-*arabino*-hexulose[13] (84%). Addition of hydrogen bromide or chloride furnished 3,4,5,6-tetra-*O*-acetyl-1-bromo-1-deoxy-L-*arabino*-hexulose (**1b**) or the 1-chloro derivative (**2b**), respectively. Use of α-halo ketoses **1b** or **2b** permitted synthesis (see Method I) of the intermediate 4-hydroxythiazolidine-2-thione (**3b**) by the ammonium dithiocarbamate method. Acidic dehydration of **3b** gave the acetylated thiazoline structure **4b**, and deacetylation of **4b** yielded 4-(L-*arabino*-tetrahydroxybutyl)-4-thiazoline-2-thione (**5b**). Thiones **4b** and **5b**, on mild oxidation, characteristically yielded the respective disulfides (**6b**) and (**7b**). Cocrystallization of the corresponding enantiomorphs afforded the racemates (**1c**), (**2c**), and (**4c–6c**). All of the products of these reactions were obtained in crystalline form.

4-(D-*xylo*-Tetrahydroxybutyl)-4-thiazoline-2-thione (**5d**), unlike its diastereoisomer (**5a**), is soluble in cold water and in ethanol, and resisted crystallization. Previously, oxidation of tetra-*O*-acetyl-*aldehydo*-D-xylose, obtained from the corresponding diethyl dithioacetal by thiol cleavage[14], was reported by Major and Cook[15] to yield crystalline tetra-*O*-acetyl-D-xylonic acid. The corresponding crystalline acid chloride was obtained by Wolfrom *et al.*[14]. In the present work, the last two intermediates were used satisfactorily as syrups. Treatment of the acid chloride with diazomethane yielded crystalline 3,4,5,6-tetra-*O*-acetyl-1-deoxy-1-diazo-D-*xylo*-hexulose[14]. However, subsequent treatment with hydrogen bromide afforded an α-bromo ketose (**1d**), which crystallized below 0°. Use of **1d** according to Method I gave the product (**5d**) *via* the crystalline, intermediate 4-hydroxythiazolidine and thiazoline structures **3d** and **4d**, respectively. A similar route to the enantiomorph, 4-(L-*xylo*-tetrahydroxybutyl)-4-thiazoline-2-thione (**5e**), required preparation of the new intermediate, L-xylose diethyl dithioacetal (**8**). Other new compounds employed in the preparation were tetra-*O*-acetyl-L-xylose diethyl dithioacetal (**9**), 3,4,5,6-tetra-*O*-acetyl-1-deoxy-1-diazo-L-*xylo*-hexulose (**11**), and their respective racemates, (**10**) and (**12**).

In further use of the method, treatment of 3,4,5,6,7-penta-*O*-acetyl-1-bromo-1-deoxy-D-*gluco*-heptulose (**1f**) according to Method I gave the 4-hydroxythiazolidine (**3f**). Dehydration of **3f** yielded the acetylated thiazoline (**4f**), which gave a disulfide (**6f**). Deacetylation of **4f** afforded 4-(D-*gluco*-pentahydroxypentyl)-4-thiazoline-2-thione (**5f**) as a glass that was soluble in ethanol and in water.

A bis(4-thiazoline-2-thione) compound (**5g**) was prepared from 3,4,5,6-tetra-*O*-acetyl-1,8-dibromo-1,8-dideoxy-*galacto*-2,7-octodiulose (**1g**) *via* the bis(4-hydroxythiazolidine-2-thione) intermediate (**3g**). Simultaneous, rapid dehydration and deacetylation of **3g** in sodium hydroxide solution, and subsequent acidification, gave crystalline 4,4′-(*galacto*-1,2,3,4-tetrahydroxybutane-1,4-diyl)-bis(4-thiazoline-2-thione) (**5g**) directly.

4-Thiazoline-2-thiones substituted at the *N*-hetero atom were obtained from certain carbohydrates by the reactions outlined in Method II. As an example of this approach, treatment of α-bromo ketose **1a** with the dithiocarbamic acid salt derived from methylamine, carbon disulfide, and potassium acetate led to intermediates **3h** and **4h** (syrup), and to the product, 3-methyl-4-(D-*arabino*-tetrahydroxybutyl)-4-

thiazoline-2-thione (**5h**). By the same method, treatment of **1b** with the dithiocarbamic acid salt derived from 4-aminobutyric acid gave, *via* intermediates **3i** and **4i** (syrup), the analogous product, 3-(3-carboxypropyl)-4-(L-*arabino*-tetrahydroxybutyl)-4-thiazoline-2-thione (**5i**).

On the basis of these results, it is expected that chain-lengthened 4-thiazoline-2-thiones may be obtained *via* α-halo ketoses **1** and **2** that are derived from homologous precursors, *e.g.*, 3,4,5,6,7,8-hexa-*O*-acetyl-1-deoxy-1-diazo-D-*glycero*-D-*gulo*-octose[16], 3,4,5,6,7,8,9-hepta-*O*-acetyl-1-deoxy-1-diazo-D-*erythro*-L-*manno*-nonulose[17], etc. The simplest analog of the heterocyclic series, namely, 4-hydroxymethyl-4-thiazoline-2-thione, was reported previously[6].

The heterocyclic forms of the products and their corresponding intermediates described herein were given structural assignments by analogy, and by the similarity of their u.v. or i.r. spectra to those of compounds previously reported. Characteristic data obtained for the new compounds are given in the experimental section.

EXPERIMENTAL

U.v. spectra between 210 and 400 nm were determined with a Perkin–Elmer Model 202 Recording Spectrophotometer, with 1-cm silica cuvettes. Appropriate blank corrections were made for all determinations of optical absorbance. Melting points were determined in open, soft-glass capillaries in an oil bath, and are corrected. Optical rotations were measured with a Rudolph polarimeter by using a 2-dm polarimeter tube.

3,4,5,6-Tetra-O-*acetyl-1-bromo-1-deoxy*-L-arabino-*hexulose* (**1b**). — The following preparation is based on the general method previously reported[18] for the synthesis of the D enantiomorph (**1a**). 3,4,5,6-Tetra-*O*-acetyl-1-deoxy-1-diazo-L-*arabino*-hexulose[13] (13.4 g) was suspended in 400 ml of anhydrous ether, and dry hydrogen bromide was passed into the mixture until the resulting evolution of nitrogen ceased. The solution formed was successively washed with water at 5° and with excess aqueous sodium hydrogen carbonate. The dried (sodium sulfate) organic layer was concentrated *in vacuo*, and the solution diluted with petroleum ether. The crystals which formed on cooling were collected; yield 13.3 g (87%), m.p. 66–67°, unchanged on recrystallization from ether–petroleum ether; $[\alpha]_D^{23}$ −65 ±2° (*c* 4, chloroform).

Anal. Calc. for $C_{14}H_{19}BrO_9$: C, 40.9; H, 4.7; Br, 19.4. Found; C, 41.0; H, 4.8; Br, 19.4.

When the washing steps were omitted from the procedure, the product obtained decomposed on prolonged storage.

3,4,5,6-Tetra-O-*acetyl-1-bromo-1-deoxy*-DL-arabino-*hexulose* (**1c**). — Cocrystallization of **1b** and the D enantiomorph[18] (**1a**) (1.0 g of each) from ether–petroleum ether, and subsequent recrystallization, gave the optically inactive form, yield 1.7 g (85%), m.p. 92°, $[\alpha]_D^{23}$ 0 ±1° (*c* 4, chloroform).

Anal. Calc. for $C_{14}H_{19}BrO_9$: Br, 19.4. Found: Br, 19.2.

3,4,5,6-Tetra-O-*acetyl-1-chloro-1-deoxy*-L-arabino-*hexulose* (**2b**). — 3,4,5,6-Tetra-*O*-acetyl-1-deoxy-1-diazo-L-*arabino*-hexulose[13] (10.0 g) was treated by the

procedure used for the preparation of **1b**, except that hydrogen chloride was employed instead of hydrogen bromide. Recrystallization from ether–petroleum ether gave a pure product, yield 9.1 g (89%), m.p. 76–77°, $[\alpha]_D^{23}$ −68 ±2° (*c* 4, chloroform).

Anal. Calc. for $C_{14}H_{19}ClO_9$: C, 45.9; H, 5.2; Cl, 9.7. Found: C, 45.9; H, 5.6; Cl, 9.4.

*3,4,5,6-Tetra-*O*-acetyl-1-chloro-1-deoxy-*DL-arabino-*hexulose* (**2c**). — Cocrystallization of equal amounts of **2b** and the D enantiomorph[18] from ether–petroleum ether, followed by recrystallization, gave the racemate, m.p. 101°, $[\alpha]_D^{23}$ 0 ±1° (*c* 4, chloroform).

Anal. Calc. for $C_{14}H_{19}ClO_9$: C, 45.9; H, 5.2; Cl, 9.7. Found: C, 45.6; H, 5.5; Cl, 9.7.

*4-Hydroxy-4-(*L-arabino-*tetraacetoxybutyl)thiazolidine-2-thione* (**3b**). — *Treatment* of **2b** (20.2 g) with ammonium dithiocarbamate (7.2 g) by the general method previously described for the preparation of 4-hydroxythiazolidine-2-thiones[19] and for the D enantiomorph[6] (**3a**) in solution in acetone–water gave **3b**, yield 19.8 g (85%). Recrystallized from ether, the product had m.p. 146–147°, λ_{max}^{MeOH} 243, 276 nm (ε 7,500, 15,900).

Anal. Calc. for $C_{15}H_{21}NO_9S_2$: C, 42.5; H, 5.0; N, 3.3; S, 15.1. Found: C, 42.8; H, 5.0; N, 3.2; S, 14.9.

Use of the bromo derivative (**1b**) as the α-halo ketone, instead of the chloro derivative (**2b**), gave similar results.

*4-Hydroxy-4-(*D-xylo-*tetraacetoxybutyl)thiazolidine-2-thione* (**3d**). — Treatment of 3,4,5,6-tetra-*O*-acetyl-1-deoxy-1-diazo-D-*xylo*-hexulose[14] (13.5 g) in ether with hydrogen bromide by the method used for the preparation of **1b** gave a product that crystallized from cold ether, but that was a melt at room temperature. Evaporation of the ether afforded **1d** as a syrup.

Treatment of the latter syrup with ammonium dithiocarbamate (4.6 g) by the procedure used to prepare **3b** yielded **3d**, 12.6 g (80%). Recrystallized from 2 liters of ether by evaporation of the solvent, the product had m.p. 153°, λ_{max}^{MeOH} 243, 277 nm (ε 6,900; 14,400).

Anal. Calc. for $C_{15}H_{21}NO_9S_2$: C, 42.5; H, 5.0; N, 3.3; S, 15.1. Found: 42.5; H, 5.0; N, 3.5; S, 14.7.

*3,4,5,6-Tetra-*O*-acetyl-1-deoxy-1-diazo-*L-xylo-*hexulose* (**11**). — Treatment of tetra-*O*-acetyl-L-xylonyl chloride (26.0 g), in the form of a syrup, with diazomethane according to the procedure described[14] for the preparation of the D enantiomorph afforded **11** as pale-yellow crystals, yield 17.0 g (58%), m.p. 125–126°, unchanged on recrystallization from ether; $[\alpha]_D^{24}$ −47 ±2° (*c* 4, chloroform).

Anal. Calc. for $C_{14}H_{18}N_2O_9$: C, 46.9; H, 5.1; N, 7.8. Found: C, 46.7; H, 5.1; N, 7.9.

*3,4,5,6-Tetra-*O*-acetyl-1-deoxy-1-diazo-*DL-xylo-*hexulose* (**12**). — Cocrystallization of equal amounts of **11** and the D enantiomorph[14] from ether–petroleum ether, and subsequent recrystallization, gave the optically inactive form, m.p. 122°, $[\alpha]_D^{24}$ 0 ±1° (*c* 4, chloroform).

Anal. Calc. for $C_{14}H_{18}N_2O_9$: C, 46.9; H, 5.1; N, 7.8. Found: C, 46.8; H, 5.0; N, 7.5.

4-Hydroxy-4-(L-xylo-*tetraacetoxybutyl)thiazolidine-2-thione* (**3e**). — Treatment of **11** (14.3 g) in ether solution with hydrogen bromide by the procedure used for synthesizing **1b** gave **1e** as a syrup. Treatment of the syrup with ammonium dithiocarbamate (4.9 g) by the procedure used to prepare the D enantiomorph (**3d**) gave **3e**, yield 15.3 g (91%, from **11**). Recrystallized from ether, the product had m.p. 152–153°, λ_{max}^{MeOH} 244, 277 nm (ε 7,200, 15,100).

Anal. Calc. for $C_{15}H_{21}NO_9S_2$: C, 42.5; H, 5.0; N, 3.3; S, 15.1. Found: C, 42,7; H. 5.2; N, 3.7; S, 14.8.

4-Hydroxy-4-(D-gluco-*pentaacetoxypentyl)thiazolidine-2-thione* (**3f**). — The following preparation of **3f** by a previously reported[20] method depends on the interaction of a suspension of the reagents in ether. The procedure used for the preparation of **3b** by interaction of the reagents in solution in acetone–water is, on the basis of comparative reaction times and yields, a preferred method.

A suspension of 3,4,5,6,7-penta-*O*-acetyl-1-bromo-1-deoxy-D-*gluco*-heptulose[18] (25.0 g) and ammonium dithiocarbamate (5.7 g) in ether (300 ml) was stirred for 3 days at reflux temperature. The solid phase was collected, and washed with water. Evaporation of the ether phase afforded a solid residue. The combined solids, precipitated twice from ethanol, gave an amorphous product, yield 9.0 g (35%), λ_{max}^{MeOH} 245, 277 nm (ε 6,500, 13,600),

Anal. Calc. for $C_{18}H_{25}NO_{11}S_2$: C, 43.6; H, 5.1; N, 2.8; S, 12.9. Found: C, 43.7; H, 4.9; N, 2.7; S, 12.9.

4,4'-(galacto-*1,2,3,4-Tetraacetoxybutane-1,4-diyl)-bis(4-hydroxythiazolidine-2-thione*) (**3g**). — Treatment of 3,4,5,6-tetra-*O*-acetyl-1,8-dibromo-1,8-dideoxy-*galacto*-2,7-octodiulose[21] (10.0 g) with ammonium dithiocarbamate (5.5 g) by the procedure used for the preparation of **3b** gave **3g**, separated in three crops, yield 8.8 g (84%). Recrystallized from acetone–water, the product melted at above 300°, λ_{max}^{MeOH} 243, 277 nm (ε 12,900, 25,200).

Anal. Calc. for $C_{18}H_{24}N_2O_{10}S_4$: C, 38.8; H, 4.3; N, 5.0; S, 23.0. Found: C, 39.2; H, 4.0; N, 4.7; S, 22.6.

4-Hydroxy-3-methyl-4-(D-arabino-*tetraacetoxybutyl)thiazolidine-2-thione* (**3h**) — The following preparation is based upon a previously reported[7], general method for the synthesis of 4-hydroxythiazolidine-2-thiones from amines. Carbon disulfide (8.8 ml) was added, dropwise, to a stirred solution (cooled in an ice bath) of potassium acetate (14.2 g) and methylamine (40% in water, 11.2 g) in methanol (100 ml). Stirring and cooling were continued for 3 h. 3,4,5,6-Tetra-*O*-acetyl-1-bromo-1-deoxy-D-*arabino*-hexulose[18] (**1a**) in methanol (100 ml) was added to the stirred, cooled reaction mixture during 30 min. The mixture was kept overnight at room temperature, diluted with water and concentrated *in vacuo*, precipitating **3h**, yield 29.3 g (92%). Two recrystallizations from methanol–water (77% recovery) gave a pure product, m.p. 148–149°, λ_{max}^{MeOH} 253, 273 nm (ε 9,600, 15,800).

Anal. Calc. for $C_{16}H_{23}NO_9S_2$: C, 43.9; H, 5.3; N, 3.2; S, 14.7. Found: C, 43.5; H, 5.3; N, 3.1; S, 14.3.

3-(3-Carboxypropyl)-4-hydroxy-4-(L-arabino-*tetraacetoxybutyl)-thiazolidine-2-thione* (**3i**). — A previously reported[8] modification of the procedure of the foregoing experiment permitted use of an amino acid in this synthesis. Thus, treatment of the dithiocarbamic acid salt, obtained from the reaction of 4-aminobutyric acid (8.7 g) with potassium hydroxide (4.8 g) and carbon disulfide (4.4 ml) in methanol (50 ml), with **1b** (15.0 g) gave **3i** as an amorphous solid, yield 17.0 g (92%). The product was extracted into ether, and the extract was treated with powdered charcoal, filtered, and dried (sodium sulfate). Evaporation of the filtrate *in vacuo* gave a glass, λ_{max}^{MeOH} 255, 276 nm (ε 8,800, 14,400).

Anal. Calc. for $C_{19}H_{27}NO_{11}S_2$: C, 44.8; H, 5.4; N, 2.8; S, 12.6. Found: C, 44.6; H, 5.4; N, 2.6; S, 12.3.

4-(L-arabino-*Tetraacetoxybutyl)-4-thiazoline-2-thione* (**4b**). — Acid dehydration of **3b** (6.0 g) by the procedure reported[6] earlier for the preparation of the D enantiomorph (**4a**) gave the 4-thiazoline-2-thione **4b**, yield 3.9 g (69%). Recrystallized from ether, the product had m.p. 169°, λ_{max}^{MeOH} 317 nm (ε 12,300), $[\alpha]_D^{24}$ +80 ±1° (*c* 2, chloroform).

Anal. Calc. for $C_{15}H_{19}NO_8S_2$: C, 44.4; H, 4.7; N, 3.5; S, 15.8. Found: C, 44.6; H, 4.7; N, 3.2; S, 15.7.

4-(DL-arabino-*Tetraacetoxybutyl)-4-thiazoline-2-thione* (**4c**). — Cocrystallization of equal amounts of **4b** and the D enantiomorph[6] (**4a**) from ether, and subsequent recrystallization, afforded the racemic product, m.p. 173–174°, λ_{max}^{MeOH} 317 nm (ε 12,500), $[\alpha]_D^{24}$ −2 ±2° (*c* 2, chloroform).

Anal. Calc. for $C_{15}H_{19}NO_8S_2$: C, 44.4; H, 4.7; N, 3.5; S, 15.8. Found: C, 44.7; H, 4.5; N, 3.3; S, 15.7.

4-(D-xylo-*Tetraacetoxybutyl)-4-thiazoline-2-thione* (**4d**). — Dehydration of **3d** (9.4 g) by heating it to 185° gave an amorphous product, yield 8.2 g (94%). Reprecipitation, twice, from ether–petroleum ether gave **4d** as a glass, λ_{max}^{MeOH} 317 nm (ε 12,700), $[\alpha]_D^{24}$ +129 ±2° (*c* 4, chloroform).

Anal. Calc. for $C_{15}H_{19}NO_8S_2$: C, 44.4; H, 4.7; N, 3.5; S, 15.8. Found: C, 44.2; H, 4.6; N, 3.4; S, 15.4.

4-(L-xylo-*Tetraacetoxybutyl)-4-thiazoline-2-thione* (**4e**). — Dehydration of **3e** and reprecipitation of the resulting product by the procedure used in the foregoing experiment gave an amorphous product (**4e**), λ_{max}^{MeOH} 317 nm (ε 13,000), $[\alpha]_D^{24}$ −131 ±2° (*c* 4, chloroform).

Anal. Calc. for $C_{15}H_{19}NO_8S_2$: C, 44.4; H, 4.7; N, 3.5; S, 15.8. Found: C, 44.0; H, 4.8; N, 3.2; S, 16.1.

4-(D-gluco-*Pentaacetoxypentyl)-4-thiazoline-2-thione* (**4f**). — Dehydration of **3f** and subsequent reprecipitation of the resultant product by the procedure used for the preparation of **4d** gave the amorphous 4-thiazoline-2-thione (**4f**), λ_{max}^{MeOH} 317 nm (ε 14,000), $[\alpha]_D^{24}$ +133 ±2° (*c* 4, chloroform).

Anal. Calc. for $C_{18}H_{23}NO_{10}S_2$: C, 45.3; H, 4.9; N, 2.9; S, 13.4. Found: 45.6; H, 5.0; N, 2.8; S, 13.8.

4-(L-arabino-*Tetrahydroxybutyl)-4-thiazoline-2-thione* (**5b**). — By repetition of the method used for the preparation of the D enantiomorph[6] (**5a**), a solution of **4b** (8.3 g) and potassium hydroxide (8.3 g) in water (40 ml) was boiled for 2 min, cooled, and made acidic with concentrated hydrochloric acid. The resulting, crystalline precipitate was collected, and rinsed with cold water (10 ml); yield 4.2 g (88%). Recrystallization from water gave pure **5b**, m.p. 218°, λ_{max}^{MeOH} 317 nm (ε 13,800), $[\alpha]_D^{20}$ +80 C, ±2° (*c* 1, *N,N*-dimethylformamide).

Anal. Calc. for $C_7H_{11}NO_4S_2$: C, 35.4; H, 4.7; N, 5.9; S, 27.0. Found: C, 35.6; H, 4.9; N, 5.7; S, 26.6.

4-(DL-arabino-*Tetrahydroxybutyl)-4-thiazoline-2-thione* (**5c**). — Corystallization of equal amounts of **5b** and the D enantiomorph[6] (**5a**) from water, followed by recrystallization, gave the optically inactive form, m.p. 197°, λ_{max}^{MeOH} 317 nm (ε 13,700), $[\alpha]_D^{20}$ 0 ±1° (*c* 1, *N,N*-dimethylformamide).

Anal. Calc. for $C_7H_{11}NO_4S_2$: C, 35.4; H, 4.7; N, 5.9; S, 27.0. Found: C, 35.0; H, 4.8; N, 5.7; S, 26.9.

4-(D-xylo-*Tetrahydroxybutyl)-4-thiazoline-2-thione* (**5d**). — A solution of **4d** (5.4 g) and potassium hydroxide (5.4 g) in water (25 ml) was boiled for 2 min, cooled, acidified with hydrochloric acid, and evaporated *in vacuo* to dryness. The amorphous residue was extracted with ethanol, and the extract was evaporated to dryness *in vacuo*, affording a glass, yield 2.5 g (81%). The product, soluble in water, ethanol, and acetone, had λ_{max}^{MeOH} 317 nm (ε 11,200).

Anal. Calc. for $C_7H_{11}NO_4S_2$: C, 35.4; H, 4.7. Found: C, 35.8; H, 5.0.

Treatment of **4e** by the method used in the foregoing experiment gave a residue that resisted crystallization. The crude L enantiomorph (**5e**) so obtained had λ_{max}^{MeOH} 317 nm (ε 11,800).

4-(D-gluco-*Pentahydroxypentyl)-4-thiazoline-2-thione* (**5f**). — Treatment of **4f** (3.0 g) by the procedure used in the foregoing experiment afforded a quantitative yield of **5f** as a syrup that was soluble in water, λ_{max}^{MeOH} 317 nm (ε 12,100).

Anal. Calc. for $C_8H_{13}NO_5S_2$: C, 35.9; H, 4.9. Found: C, 35.6; H, 5.1.

4,4'-(galacto-*1,2,3,4-Tetrahydroxybutane-1,4-diyl)bis(4-thiazoline-2-thione*) (**5g**). — Simultaneous dehydration and deacetylation of the 4-hydroxythiazolidine **3g** (4.6 g), by the procedure used for the preparation of **5b**, gave the product (**5g**) directly, yield 2.0 g (71%). The product was dissolved in aqueous sodium carbonate, and the solution was treated with powdered charcoal, filtered, and the filtrate acidified with hydrochloric acid. The resulting, lustrous flakes were collected, and had m.p. 253°, $\lambda_{max}^{HCONMe_2}$ 317 nm (ε 24,800), $[\alpha]_D^{23}$ 0 ±1° (*c* 2, *N,N*-dimethylformamide).

Anal. Calc. for $C_{19}H_{12}N_2O_4S_4$: C, 34.5; H, 3.4; N, 7.9; S, 36.4. Found: C, 34.1; H, 3.6; N, 7.7; S, 36.5.

The product was soluble in acetone and *N,N*-dimethylformamide, and insoluble in chloroform, methanol, or water.

3-Methyl-4-(D-arabino-*tetrahydroxybutyl)-4-thiazoline-2-thione* (**5h**). — The

4-hydroxythiazolidine (**3h**) (17.0 g) was suspended in 400 ml of 50 M hydrochloric acid, and the mixture was refluxed for 1 h. Evaporation of the resulting solution yielded a syrup that resisted crystallization. A solution of the syrup and potassium hydroxide (17.0 g) in water (85 ml) was stirred for 4 min at 75°, cooled, and acidified with hydrochloric acid. After a small amount of gum had separated, the solution was treated with powdered charcoal, filtered, and evaporated to dryness. Extraction of the residue with ethanol, and subsequent concentration of the extract, gave a crystalline precipitate, yield 5.0 g, in two crops (51%, from **3h**). Recrystallization from ethanol gave **5h** as white flakes, m.p. 181°, λ_{max}^{MeOH} 313 nm (ε 15,000), $[\alpha]_D^{25}$ −56 ±2° (*c* 2, water).

Anal. Calc. for $C_8H_{13}NO_4S$: C, 38.2; H, 5.2; N, 5.6; S, 25.5. Found: C, 38.5; H, 5.3; N, 5.3; S, 25.9.

The product was soluble in ethanol, methanol, and water, and insoluble in ether.

3-(3-Carboxypropyl)-4-(D-arabino-*tetrahydroyxbutyl-4-thiazoline-2-thione* (**5i**). — The acid dehydration of the 4-hydroxythiazolidine (**3i**) (11.8 g) by the procedure used in the foregoing experiment gave a syrupy product. Treatment of the syrup by the saponification procedure used for the preparation of **5h**, and subsequent acidification, gave a product that crystallized from the aqueous reaction mixture during several weeks at room temperature. Recrystallized from water during several days at room temperature, pure **5i** had m.p. 160°, λ_{max}^{MeOH} 317 nm (ε 13,200), $[\alpha]_D^{25}$ +39 ±2° (*c* 2, water).

Anal. Calc. for $C_{11}H_{17}NO_6S_2$: C, 40.9; H, 5.3; N, 4.3; S, 19.8. Found: C, 41.2; H, 5.2; N, 4.2; S, 19.6.

2,2'-Dithiobis[(4-L-arabino-*tetraacetoxybutyl)thiazole*] (**6b**). — Oxidation of **4b** (3.0 g) in 50% aqueous methanol (250 ml), by the iodine method previously reported[6] [used for the preparation of the D enantiomorph (**6a**)] gave the disulfide, yield 2.3 g (77%). Recrystallized from ether, the product had m.p. 146°, λ_{max}^{MeOH} 266 nm (ε 9,100), $[\alpha]_D^{24}$ +13 ±1° (*c* 4, chloroform).

Anal. Calc. for $C_{39}H_{36}N_2O_{16}S_4$: N, 3.5; S, 15.9. Found: N, 3.2; S, 15.6.

2,2'-Dithiobis[(*4*-DL-arabino-*tetraacetoxybutyl)thiazole*] (**6c**). — A solution of equal amounts of **6b** and the D enantiomorph[6] (**6a**) in ether was evaporated. The resulting racemate resisted crystallization, and had λ_{max}^{MeOH} 266 (ε 9,400), $[\alpha]_D^{24}$ 0 ±1° (*c* 4, chloroform).

Anal. Calc. for $C_{30}H_{36}N_2O_{16}S_4$: C, 44.5; H, 4.5; N, 3.5; S, 15.9. Found: C, 44.5; H, 4.3; N, 3.3; S, 15.7.

2,2'-Dithiobis[(*4*-D-gluco-*pentaacetoxypentyl)thiazole*] (**6f**). — Oxidation of **4f** (4.8 g) by the procedure used for the preparation of **6b** gave **6f**, yield 3.8 g (80%). Recrystallized from ether, the disulfide had mp.. 88°, λ_{max}^{MeOH} 267 nm (ε 9,200).

Anal. Calc. for $C_{36}H_{44}N_2O_{20}S_4$: C, 45.4; H, 4.7; N, 2.9; S, 13.5. Found: C, 45.2; H, 4.6; N, 3.0; S, 13.1.

2,2'-Dithiobis[(*4*-L-arabino-*tetrahydroxybutyl)thiazole*] (**7b**). — Oxidation of **5b** (2.4 g) by the procedure previously reported[6] [used for the preparation of the

D enantiomorph (**7a**)] gave a quantitative yield of the disulfide. Washed with boiling water, the product had m.p. 210°.

Anal. Calc. for $C_{14}H_{29}N_2O_8S_4$: C, 35.6; H, 4.3; N, 5.9; S, 27.1. Found: C, 35.2; H, 4.0; N, 5.6; S, 27.1.

L-*Xylose diethyl dithioacetal* (**8**). — Treatment of a solution of L-xylose (20.0 g) in 24 ml of hydrochloric acid with 20 ml of ethanethiol, according to the procedure previously reported[22] for the preparation of D-xylose diethyl dithioacetal, gave **8**, yield 21.2 g (64%). Recrystallized from ethanol, the product had m.p. 63–64°, $[\alpha]_D^{20}$ $+70 \pm 1°$ (*c* 1, chloroform).

Anal. Calc. for $C_9H_{29}O_4S_2$: C, 42.1; H, 7.9; S, 25.0. Found: C, 41.8; H, 7.9; S, 24.6.

Tetra-O-*acetyl*-L-*xylose diethyl dithioacetal* (**9**). — Acetylation of **8** (14.0 g) in pyridine (50 ml) with acetic anhydride (100 ml), by the procedure previously reported[22] for the preparation of the D enantiomorph, gave **9**, yield 22.0 g (95%). Recrystallized from methanol–water, the product had m.p. 49–50°, $[\alpha]_D^{25}$ $-13 \pm 1°$ H, (*c* 4, chloroform).

Anal. Calc. for $C_{17}H_{28}O_8S_2$: C, 48.1; H, 6.7; S, 15.1. Found: C, 47.8; 6.6; S, 15.6.

Tetra-O-*acetyl*-DL-*xylose diethyl dithioacetal* (**10**). — Cocrystallization of equal amounts of **9** and the D enantiomorph[22] from ethanol–water, and subsequent recrystallization, gave the optically inactive form, m.p. 47°, $[\alpha]_D^{24}0$ $\pm 1°$ (*c* 4, chloroform).

Anal. Calc. for $C_{17}H_{28}O_8S_2$: C, 48.1; H, 6.7; S, 15.1. Found: C, 48.0; H, 6.8; S, 15.0.

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REFERENCES

1 G. Zemplén, A. Gerecs, and E. Illes, *Ber.*, 71 (1938) 590.
2 G. Zemplén, A. Gerecs, and M. Rados, *Ber.*, 69 (1936) 748.
3 W. Bromund and R. M. Herbst, *J. Org. Chem.*, 10 (1945) 267.
4 J. C. Jochims, A. Seeliger, and G. Taigel, *Chem. Ber.*, 100 (1967) 267.
5 J. C. Jochims, *Angew. Chem. Intern. Ed. Engl.*, 5 (1966) 964.
6 W. J. Humphlett, *Carbohyd. Res.*, 6 (1968) 25.
7 R. W. Lamon, W. J. Humphlett, and W. P. Blum, *J. Heterocyclic Chem.*, 4 (1967) 349.
8 R. W. Lamon and W. J. Humphlett, *J. Heterocyclic Chem.*, 0000.
9 M. Fieser, L. F. Fieser, E. Toromanoff, Y. Hirato, H. Heyman, M. Tefft, and S. Bhattacharya, *J. Amer. Chem. Soc.*, 78 (1956) 2825.
10 W. J. Humphlett, *Carbohyd. Res.*, 4 (1967) 157.
11 C. D. Hurd and J. C. Sowden, *J. Amer. Chem. Soc.*, 60 (1938) 235.
12 M. L. Wolfrom and A. Thompson, *Methods Carbohyd. Chem.*, 2 (1963) 24.
13 M. L. Wolfrom and A. Thompson, *J. Amer. Chem. Soc.*, 68 (1946) 791.
14 M. L. Wolfrom, S. M. Olin, and E. F. Evans, *J. Amer. Chem. Soc.*, 66 (1944) 204.

15 R. T. MAJOR AND E. W. COOK, *J. Amer. Chem. Soc.*, 58 (1936) 2474.
16 M. L. WOLFROM AND A. THOMPSON, *J. Amer. Chem. Soc.*, 68 (1946) 1453.
17 M. L. WOLFROM AND H. B. WOOD, JR., *J. Amer. Chem. Soc.*, 77 (1955) 3096.
18 M. L. WOLFROM, S. W. WAISBROT, AND R. L. BROWN, *J. Amer. Chem. Soc.*, 64 (1942) 1701.
19 W. J. HUMPHLETT AND R. W. LAMON, *J. Org. Chem.*, 29 (1963) 2148.
20 T. G. LEVI, *Gazz. Chim. Ital.*, 61 (1931) 719.
21 T. NOGRADY, T. W. DOYLE, AND L. NORRIS, *J. Med. Chem.*, 8 (1965) 656.
22 M. L. WOLFROM, M. R. NEWLIN, AND E. E. STAHLY, *J. Amer. Chem. Soc.*, 53 (1931) 4379.

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LEMON-PEEL PECTIN

PART I. FRACTIONATION AND PARTIAL HYDROLYSIS OF WATER-SOLUBLE PECTIN

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ABSTRACT

The pectin isolated from dried lemon-peel by extraction with cold water has been subjected to partial depolymerisation by acid hydrolysis, acetolysis, and enzymic degradation. Acidic oligosaccharides whose structures were established, or indicated by chromatographic identification of the products of methylation linkage-analysis, include 2-*O*-(α-D-galactopyranosyluronic acid)-L-rhamnose, higher oligosaccharides containing residues of galacturonic acid and rhamnose, 4-*O*-(α-D-galactopyranosyluronic acid)-D-galacturonic acid and the polymer-homologous trisaccharide, 6-*O*-(glucopyranosyluronic acid)galactose and 4-*O*-(glucopyranosyluronic acid)fucose, and 3-*O*-xylopyranosylgalacturonic acid.

INTRODUCTION

Many pectins in which D-galacturonic acid is the principal constituent contain neutral sugars, especially D-galactose, L-arabinose, and L-rhamnose, as integral constituents. Polysaccharides of this type have been examined recently from several sources including sisal flesh[1], lucerne (alfalfa)[2,3], the bark of Amabilis fir[4], apples[5], white-mustard cotyledons[6], sycamore cambial and callus tissues[7], soybean cotyledons[8], and soybean hulls[9]. Investigations in this laboratory on a commercial sample of citrus pectin from oranges and lemons[10] provided further evidence for L-rhamnose as a constituent sugar, and also indicated that chains of (1→4)-linked β-D-galactopyranose residues were present in the acidic polysaccharide, since 4-*O*-β-D-galactopyranosyl-D-galactose and its polymer-homologues were formed as products of partial hydrolysis. However, in view of the mixed origin of the pectin sample and of the uncertain method of extraction, a more detailed examination has been carried out on the pectin isolated from dried lemon-peel by extractions with cold water. We report here the characterisation of acidic oligosaccharides formed on partial depolymerisation of this pectin.

RESULTS AND DISCUSSION

Extraction of dried lemon-peel with cold water furnished a polysaccharide

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preparation constituting some 10% by weight of the raw material. Subsequent experiments (unpublished results) have shown that considerable further quantities of pectin may be extracted under more drastic conditions, and a detailed examination of this material will be undertaken later. Pectin A of constant composition was isolated from the water-soluble fraction after two reprecipitations from water with acetone. Pectin A was apparently homogeneous when examined by boundary electrophoresis. Chromatography on *O*-(2-diethylaminoethyl)cellulose, however, gave two main fractions of acidic polysaccharides, which probably represented pectins differing in degree of esterification, together with a small fraction (*ca.* 5%) of neutral polysaccharide which gave an arabinose* and a galactose* on hydrolysis. This pectin was, however, considered suitable for studies on the acidic oligosaccharides formed on enzymic hydrolysis.

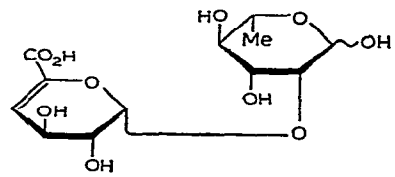
Fractionation of pectin A by precipitation of the insoluble copper salt gave pectin B, but associated neutral polysaccharide was still present. Complete removal of accompanying neutral polysaccharide was achieved by preparative chromatography on *O*-(2-diethylaminoethyl)-Sephadex, which gave pectin C and two minor polysaccharide fractions D and E, to be examined later. Pectin C had a uronic acid content of 76%, and the methoxyl content indicated a degree of esterification of 76%. Hydrolysis gave galacturonic acid, together with arabinose, galactose, rhamnose, and a trace of xylose. The latter polysaccharide fractions gave arabinose and galactose as the main products of hydrolysis; one, as the sole product, and the other, together with acidic sugars.

Partial hydrolysis by acid of pectin B gave a mixture of acidic oligosaccharides which was separated by chromatography on *O*-(2-diethylaminoethyl)-Sephadex, followed where necessary by filter-sheet chromatography, and six oligosaccharides were identified (Scheme I). 2-*O*-(α-D-Galactopyranosyluronic acid)-L-rhamnose (**1**) was characterised by conversion into the crystalline methyl glycoside pentamethyl ether. The nature of the linkages in oligosaccharides **2** and **3** was indicated by the results of methylation. Although no direct evidence was obtained for anomeric and enantiomorphic configurations, the oligosaccharides were chromatographically indistinguishable from 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose and 4-*O*-(β-D-glucopyranosyluronic acid)-L-fucose. The structure of the tetrasaccharide **4** was assigned as an *O*-(galactopyranosyluronic acid)-(1→2)-*O*-rhamnopyranosyl-(1→4)-*O*-(galactopyranosyluronic acid)-(1→2)-rhamnose on the basis of (*a*) partial hydrolysis and (*b*) methylation of the oligosaccharide and the derived alditol. 4-*O*-(α-D-Galactopyranosyluronic acid)-D-galacturonic acid (**5**) and the polymer-homologous trisaccharide (**6**) were each characterised as their crystalline calcium salts and by methylation of the corresponding neutral methyl glycosides obtained by the sequence of reactions involving conversion of the acidic oligosaccharides into the methyl ester glycosides, trimethylsilylation, reduction with lithium aluminium hydride, and removal of protecting groups.

*Omission of configurational prefixes indicates that the sugars or their derivatives were identified by chromatographic methods.

Acetolysis of pectin C resulted in the isolation of acidic oligosaccharides only, and each of these contained residues of galacturonic acid and rhamnose. Oligosaccharides **1** and **4** were characterised as described previously, and oligosaccharide **7** was characterised as *O*-(galactopyranosyluronic acid)-(1→2)-*O*-rhamnopyranosyl-(1→2)-rhamnose by methylation of the trisaccharide and the derived alditol.

1 α-D-Gal*p*A-(1→2)-L-Rha
2 G*p*A-(1→6)-Gal
3 G*p*A-(1→4)-Fuc
4 Gal*p*A-(1→2)-Rha*p*-(1→4)-Gal*p*A-(1→2)-Rha
5 α-D-Gal*p*A-(1→4)-D-Gal*p*A
6 α-D-Gal*p*A-(1→4)-D-Gal*p*A-(1→4)-D-Gal*p*A
7 Gal*p*A-(1→2)-Rha*p*-(1→2)-Rha
8 Xyl*p*-(1→3)-GalA
10 Gal*p*A-(1→2)-Rha*p*-(1→4)-Gal*p*A



9

Scheme I

Degradation of pectin A by a commercial enzyme-preparation afforded three further acidic oligosaccharides. Methylation analysis indicated that oligosaccharide **8** had the structure 3-*O*-xylopyranosylgalacturonic acid.

The tentative structure, 2-*O*-(4-deoxy-β-L-*threo*-hex-4-enopyranosyluronic acid)-L-rhamnose, was assigned to oligosaccharide **9** on the basis of the following observations: (*i*) it had a light-absorption maximum at 231 nm characteristic of α,β-unsaturated carboxylic acids; (*ii*) it gave a red colour (λ_{max} 547 nm) with thiobarbituric acid; (*iii*) hydrolysis gave rhamnose as the sole detectable product; and (*iv*) cleavage of the methylated disaccharide gave the methyl glycoside of 3,4-di-*O*-methylrhamnose as the only recognisable product. The formation of oligosaccharide **9** shows that the enzyme preparation used, like other commercial "pectinases"[12], contains some pectin and/or pectic acid hydro-lyase activity in addition to the hydrolytic activity. Oligosaccharide **10** was assigned the structure, *O*-(galactopyranosyluronic acid)-(1→2)-*O*-rhamnopyranosyl-(1→4)-galacturonic acid, since it contained a single rhamnose and two galacturonic acid residues, one at the reducing position. The site of substitution of the rhamnose residue was apparent from methylation studies, but that of the reducing galacturonic acid residue has been assigned only tentatively.

Additional evidence for the overall structure of the pectin was obtained by a series of reactions involving completion of the esterification of the galacturonic acid residues on treatment of pectin C with ethylene oxide, acetylation, and reduction with lithium borohydride, which resulted in the formation of the carboxyl-reduced polysaccharide. Hydrolysis of this derivative gave galactose as the preponderant product with only traces of other sugars. The presence and nature of other sugar residues were established by gas-chromatographic examination of the methanolysis products from the methylated polysaccharide. The major products were methyl glycosides of 2,3,6-tri-*O*-methylgalactose, but small proportions of methyl glycosides of 2,3,4,6-tetra- and 2,6-di-*O*-methylgalactose, 2,3,5-tri- and 2,3-di-*O*-methylarabinose, and 3,4-di- and 3-*O*-methylrhamnose were also present.

The present results show that many of the structural features which have been established for lucerne[2,3] and soybean[8,9] pectins are also encountered in this lemon-peel pectin. The hexuronic acid content (76%) of the pectin indicates that *ca.* 79% of the sugar residues are D-galacturonic acid. Although glucuronic acid is a trace constituent of this, as of other, pectins, it probably accounts for less than 1% of the sugar residues. Of the neutral sugar constituents, the clearest evidence for a structural role has been obtained in the case of L-rhamnose. It is evident from the characterisation of the acidic oligosaccharides **1**, **4**, **7**, and **10**, which were formed in different ways on partial fragmentation, that residues of this sugar are present in the main chains of the pectin, where they interrupt blocks of 4-*O*-substituted α-D-galacturonic acid residues. The rhamnose residues, however, are unevenly distributed in the galacturono-rhamnan chains and are probably concentrated in certain regions, where they may alternate with galacturonic acid residues, as in oligosaccharide **4**, or occur as adjacent units, as in oligosaccharide **7**.

The other neutral-sugar constituents of lemon-peel pectin probably occur in outer chains. Methylation of the carboxyl-reduced pectin showed that arabinofuranose residues were present together with some non-terminal units. Apart from the partial characterisation of the aldobiouronic acid, namely, 6-*O*-(glucopyranosyluronic acid)galactose (**2**), as a minor product of partial hydrolysis, nothing is yet known of the location of the galactose residues. However, the failure to detect galactose-containing oligosaccharides as acetolysis products under conditions in which they were formed from soybean pectins[8,9] suggests that chains of contiguous galactose residues are largely absent in the lemon pectin. Although xylose is only a trace constituent of the pectin, the isolation of the oligosaccharide 3-*O*-xylopyranosylgalacturonic acid (**8**) on enzymic hydrolysis showed that residues of this sugar are attached to the galacturonan chain, as in soybean-cotyledon pectin[8] and tragacanthic acid[13]. As indicated by the isolation of the two aldobiouronic acids **2** and **3**, this pectin, like those from soybeans[8,9] and lucerne[2,3], contains glucuronic acid and fucose as trace constituents, but there is no evidence for their structural location.

EXPERIMENTAL

General. — Unless otherwise stated, sugars and oligosaccharides were identified by paper chromatography, which was performed on Whatman Nos. 1 and 3 MM papers, with the following solvent systems (v/v): (A) 10:4:3 ethyl acetate–pyridine–water; (B) 18:3:1:4 ethyl acetate–acetic acid–formic acid–water; (C) 18:8:3:9 ethyl acetate–acetic acid–formic acid–water; (D) 4:1:5 butyl alcohol–ethanol–water (upper layer); (E) 200:17:1 butanone–water–ammonia; (F) 9:1:1 butanone–acetic acid–water (saturated with boric acid). The R_G values of methylated sugars refer to rates of movement relative to that of 2,3,4,6-tetra-*O*-methyl-D-glucose (as unity) in solvent D. Paper ionophoresis was conducted in borate buffer at pH 10. Unless otherwise stated, optical rotations were observed for aqueous solutions at *ca.* 18°.

Gas chromatography was performed on columns of acid-washed Celite coated

with (*a*) 10% by weight of butane 1,4-diol succinate polyester (operating temperature, 175°), (*b*) 10% by weight of *m*-bis(*m*-phenoxyphenoxy)benzene (200°), and (*c*) 3% or 5% by weight of neopentylglycol adipate polyester (125 or 150°). All compounds whose identification was by gas chromatography, alone, had the same retention times as those of authentic reference compounds.

Isolation and fractionation of lemon-peel pectin. — Powdered lemon-peel, which was kindly supplied by Mr. W. C. Platt of the Ventura Coastal Lemon Corporation, Ventura, California, had been pre-extracted with isopropyl alcohol to remove lemon oil. Powdered peel (1 kg) was stirred in water (30 liters) at pH 4.8 for 24 h at room temperature. Residual peel was removed in a centrifuge, and the extraction was repeated. The combined, aqueous extracts were poured into acetone (1.5 vol), and the resulting precipitate was washed with acetone–water, dissolved in water, and freeze-dried to give crude pectin (80 g), $[\alpha]_D$ +193° (*c* 0.32). The crude pectin was reprecipitated three times from aqueous solution with acetone, to give pectin A (68 g), $[\alpha]_D$ +218° (*c* 0.31), of constant composition [Found: uronic acid (by decarboxylation), 75; OMe, 10.7%]. A sample of pectin A was chromatographed on *O*-(2-diethylaminoethyl)cellulose (phosphate form)[14] with 0.025M, 0.05M, 0.1M, 0.25M, and 0.5M sodium dihydrogen phosphate buffer at pH 6 as eluant. The fractions were monitored with the phenol–sulphuric acid reagent[15], appropriate fractions were dialysed and concentrated, and polysaccharides were precipitated with acetone, redissolved in water, and isolated by freeze-drying. Hydrolysis of the polysaccharides indicated that a neutral fraction (*ca.* 5%) was eluted with 0.025M buffer, but that all later fractions (largely eluted with 0.1M and 0.25M buffer) were of essentially similar composition.

Aqueous, 7% cupric acetate (200 ml) was added dropwise, with stirring, to pectin A (20 g) in water (4 l). The insoluble copper salt that resulted was removed in a centrifuge, and decomposed by washing with acetone containing 1% of hydrogen chloride, and the precipitate remaining was washed free of acid with ethanol, dissolved in water, and freeze-dried to give pectin B (18.2 g), $[\alpha]_D$ +221° (*c* 0.28) [Found: uronic acid residues, 76; OMe, 10.2%]. Chromatography of a sample of pectin B on *O*-(2-diethylaminoethyl)cellulose as described above showed that the neutral polysaccharide fraction was still present, although in slightly diminished amount.

Pectin A (12 g) in water (600 ml) was adsorbed on a column of *O*-(2-diethylaminoethyl)-Sephadex A-50 (100 g, formate form), and polysaccharide fractions were eluted with water (12 liters), 0.2M formic acid (12 liters), and M formic acid (20 liters). The formic acid solutions were dialysed for 36 h, the pH of the solutions was adjusted to 4.5 by the addition of potassium acetate, and the solutions were concentrated, the pH being kept at 4.5 by the addition of acetic acid. The polysaccharide contents were precipitated with ethanol containing 4% of acetic acid, washed with ethanol, redissolved in water, and freeze-dried. In a typical separation, polysaccharide D (196 mg, eluted with water) had $[\alpha]_D$ +38° (*c* 0.2), and gave, on hydrolysis, arabinose, galactose, and smaller amounts of galacturonic acid, xylose, fucose, and rhamnose [Found: uronic acid residues, 10%]; polysaccharide E (381 mg, eluted with

0.2M formic acid had $[\alpha]_D$ +78° (*c* 0.2), and gave, on hydrolysis, galactose, arabinose, galacturonic acid, and a trace of rhamnose [Found: uronic acid residues, 27%]; and pectin C (7.29 g, eluted with M formic acid) had $[\alpha]_D$ +218° (*c* 0.5), and on hydrolysis gave galacturonic acid, together with arabinose, galactose, and traces of xylose [Found: uronic acid residues, 76; OMe, 10.2%]. Chromatography of pectin C on *O*-(2-diethylaminoethyl)cellulose showed that neutral polysaccharide was absent.

Partial, acid hydrolysis of pectin. — Pectin B (17 g) was heated in 0.5M sulphuric acid (150 ml) for 4 h on a boiling-water bath, during which time degraded polysaccharide separated. Degraded polysaccharide was removed at the centrifuge, and further polysaccharide was precipitated by the addition of acetone (1 vol) and removed by centrifugation. The supernatant liquid was concentrated to remove acetone, rendered neutral with barium hydroxide and barium carbonate, filtered, passed through a column of Amberlite resin IR-120 (H^+) to remove barium ions, and evaporated to a syrup. The barium sulphate precipitate was washed with very dilute sulphuric acid (pH 4), and the washings were rendered neutral by shaking with Amberlite resin LA-1 (5% in chloroform), passed through a column of Amberlite resin IR-120 (H^+), and evaporated to a syrup.

The combined, degraded polysaccharides were rehydrolysed, and the products were isolated in the same way. Residual, degraded polysaccharide (5.94 g) gave, on hydrolysis, galacturonic acid and only traces of neutral sugars. The combined syrups (8.25 g) were adsorbed batchwise on columns of *O*-(2-diethylaminoethyl)-Sephadex A-25 (formate form). Elution of the columns with water removed neutral sugars, and elution with a gradient of water containing 0→0.4M formic acid, and with 0.4M, M, and 3M formic acid gave fractions containing galacturonic acid and acidic oligosaccharides. Further separations by filter-sheet chromatography in solvents B and C gave chromatographically pure samples of oligosaccharides[1-6].

Oligosaccharide **1**. — The sugar (47 mg), R_{Gal}0.83 in solvent B, M_G 0.56, had $[\alpha]_D$ +96° (*c* 0.47), gave galacturonic acid and rhamnose on hydrolysis, and was chromatographically indistinguishable from 2-*O*-(α-D-galactopyranosyluronic acid)-L-rhamnose. The aldobiouronic acid was characterised by conversion into its methyl glycoside pentamethyl ether dihydrate, m.p. and mixed m.p. 69° (hot-stage) and 119° (capillary tube), $[\alpha]_D$ +91° (*c* 0.63, chloroform), and by X-ray powder photography[2].

Oligosaccharide **2**. — The sugar (8 mg). R_{Gal}0.26 in solvent B, M_G 1.04, had $[\alpha]_D$ +6° (*c* 0.4), gave glucuronic acid, glucurono-6,3-lactone, and galactose on hydrolysis, and was chromatographically and ionophoretically indistinguishable from 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose. Gas chromatography of the methanolysis products from the methylated derivative on column *a* showed the presence of methyl glycosides of 2,3,4-tri-*O*-methylglucuronic acid, and 2,3,4- and 2,3,5-tri-*O*-methylgalactose.

Oligosaccharide **3**. — The sugar (15 mg). R_{Gal}0.60 in solvent B, M_G 0.63, had $[\alpha]_D$ −70° (*c* 0.8), gave glucuronic acid, glucurono-6,3-lactone, and fucose on hydrolysis, and was chromatographically and ionophoretically indistinguishable from 4-*O*-(β-D-glucopyranosyluronic acid)-L-fucose[8]. Gas chromatography of the

methanolysis products from the methylated derivative on column *a* showed the presence of methyl glycosides of 2,3,4-tri-*O*-methylglucuronic acid and 2,3-di-*O*-methylfucose.

Oligosaccharide **4**. — The sugar (8 mg). R_{Gal} 0.12 and 0.56 in solvents B and C, M_G 0.67, had $[\alpha]_D$ +92° (*c* 0.4), and gave, on partial hydrolysis, galacturonic acid, rhamnose, and oligosaccharide **1**. Colorimetric determinations of galacturonic acid (carbazole method)[16] and rhamnose (cysteine method)[17] residues indicated their presence in the approximate molar ratio of 1:1 in the oligosaccharide and of 2:1 in the derived alditol (borohydride reduction). Controlled, partial hydrolysis of the derived alditol gave 2-*O*-(galactopyranosyluronic acid)rhamnose and 2-*O*-(galactopyranosyluronic acid)rhamnitol (separated in solvent F). Gas chromatography of the methanolysis products from the methylated oligosaccharide alditol showed the presence of 1,3,4,5-tetra-*O*-methylrhamnitol, and methyl glycosides of 2,3,4-tri- and 2,3-di-*O*-methylgalacturonic acid, and 3,4-di-*O*-methyl-rhamnose.

Oligosaccharide 5. — The sugar (508 mg), R_{Gal} 0.21 and 0.50 in solvents B and C, M_G 0.95, was chromatographically and ionophoretically indistinguishable from 4-*O*-(α-D-galactopyranosyluronic acid)-D-galacturonic acid, gave galacturonic acid, only, on hydrolysis, and furnished a calcium salt, $[\alpha]_D$ +115° (*c* 0.68, 0.5M hydrochloric acid)[2]. The sugar (30 mg) was treated with methanolic 1% hydrogen chloride for 18 h at room temperature, the acid was neutralised with silver carbonate, and the mixture was filtered, and the filtrate evaporated. The resulting methyl ester methyl glycosides (31 mg) were treated with hexamethyldisilazane (0.6 ml) and chlorotrimethylsilane (0.3 ml) in pyridine (3 ml) for 1 h at 25°. The resulting solution was evaporated to dryness. The residue was extracted with ether (2 × 10 ml), and the extract was evaporated to a syrup (52 mg). The trimethylsilyl ethers were reduced by refluxing with lithium aluminium hydride (100 mg) in ether (5 ml) for 2 h, and ethyl acetate was added to decompose the excess of hydride. The precipitate that separated was removed by centrifugation, and shaken with 0.25M sulphuric acid (3 ml) for 1 h. The resulting solution was rendered neutral with barium carbonate, the suspension was filtered, and the filtrate was treated with Amberlite IR-120 (H^+) resin to remove barium ions, and evaporated to a syrup (21 mg). Hydrolysis of a sample of the syrup gave galactose only. The major portion of the neutral disaccharide glycoside was methylated with methyl iodide and silver oxide in *N*,*N*-dimethylformamide, and gas chromatography of the methanolysis products of the methylated derivative on column *c* showed the presence of methyl glycosides of 2,3,4,6-tetra- and 2,3,6-tri-*O*-methylgalactose.

Oligosaccharide **6**. — The sugar (923 mg), R_{Gal} 0.24 in solvent C, M_G 0.95, was chromatographically and ionophoretically indistinguishable from the "galacturonotriose" isolated from other pectins, gave galacturonic acid, only, on hydrolysis, and furnished a calcium salt, $[\alpha]_D$ +135° (*c* 0.72, 0.5M hydrochloric acid)[2]. The oligosaccharide (110 mg) was converted into the methyl ester methyl glycosides as described for oligosaccharide **5**, and thence into the trimethylsilyl ethers, which were reduced with lithium aluminium hydride. The resulting methyl galactotriosides

were methylated with methyl sulphate and sodium hydroxide, and methyl iodide and silver oxide, to give the methylated trisaccharide (66 mg). A sample of this derivative was heated with methanolic hydrogen chloride, and gas chromatography of the products on column *c* showed the presence of methyl glycosides of 2,3,4,6-tetra-, 2,3,6-tri-, and (in traces) 2,6-di-*O*-methylgalactose. The major portion (60 mg) of the methylated trisaccharide was hydrolysed in M hydrochloric acid (3 ml) for 8 h on a boiling-water bath, and the cooled solution was rendered neutral with silver carbonate, centrifuged, treated with Amberlite IR-120 (H^+) resin to remove silver ions, and concentrated to a syrup (57 mg). The syrup was adsorbed on a column of 1:1 charcoal–Celite (20 g), and elution with water containing increasing proportions of ethanol gave fractions *i* (4 mg), *ii* (29 mg), and *iii* (17 mg). Fraction *i*, R_G 0.56 and 0.19 in solvents D and E, gave methyl glycosides having the retention times of those of 2,6-di-*O*-methylgalactose. Fraction *ii*, R_G 0.76 and 0.51 in solvents D and E, $[\alpha]_D$ +82° (*c* 0.58, chloroform), gave methyl glycosides having the retention times of those of 2,3,6-tri-*O*-methyl-D-galactose. The sugar was characterised by conversion into 2,3,6-tri-*O*-methyl-D-galactono-1,4-lactone, m.p. and mixed m.p. 97–98°. Fraction *iii*, R_G 0.89 and 0.87 in solvents D and E, $[\alpha]_D$ +97° (*c* 0.34, chloroform), gave methyl glycosides having the retention times of those of 2,3,4,6-tetra-*O*-methyl-D-galactose, and was characterised by conversion into the aniline derivative, m.p. and mixed m.p. 196–197°.

Acetolysis of pectin. — Pectin C (12 g) was acetylated by the method of Carson and Maclay[18], and the acetylated polysaccharide (12.3 g) was dispersed in acetic acid (320 ml). Acetic anhydride (320 ml) was added dropwise to the solution, and concentrated sulphuric acid (32 ml) was added dropwise, with vigorous stirring, during 2 h. The resulting solution was kept for 75 h at room temperature. A precipitate that separated gave galacturonic acid, only, on hydrolysis. The supernatant liquid was poured into ice–water, and brought to pH 3 by the addition of sodium hydrogen carbonate. The precipitated acetates were separated by centrifugation, and dissolved in chloroform, and the aqueous solution was extracted with chloroform (4 × 150 ml). The combined chloroform extracts were evaporated to a syrup (6.5 g), which was deacetylated by treatment of a solution in chloroform (15 ml) and methanol (30 ml) with 0.25M barium methoxide in methanol (30 ml) for 24 h at 0°. The reaction mixture was poured into water, rendered neutral with dilute sulphuric acid, filtered, treated with Amberlite IR-120 (H^+) resin to remove barium ions, and evaporated to a syrup (2.0 g). The syrup was adsorbed onto a column of *O*-(2-diethylaminoethyl)-Sephadex A-25 (formate form) (25 g). Elution with water gave neutral sugars (181 mg), including arabinose, galactose, xylose, and rhamnose, but no oligosaccharides could be detected, either by direct examination or after further separation by chromatography on charcoal–Celite. Elution of the ion-exchange column sequentially with 0.05M, a gradient of 0.05 → 0.4M, 0.4M, and M formic acid gave a series of fractions, from which chromatographically pure samples of oligosaccharides **1** (20 mg), **4** (8 mg), and **7** (11 mg) were isolated after further separation by filter-sheet chromatography. Oligosaccharides **1** and **4** were characterised as described previously, and later fractions,

which contained "galacturonobiose" and "galacturonotriose" and gave galacturonic acid, only, on hydrolysis, were not examined further.

Oligosaccharide **7**. — The sugar, R_{Gal} 0.49 in solvent B, had $[\alpha]_D$ +84° (*c* 0.2), and gave galacturonic acid and rhamnose on hydrolysis. Partial, acid hydrolysis gave 2-*O*-(galactopyranosyluronic acid)rhamnose (oligosaccharide **1**) and rhamnose, and similar treatment of the derived alditol gave the same aldobiouronic acid and rhamnitol. Gas chromatography of the methanolysis products from the methylated oligosaccharide alditol on column *c* showed the presence of 1,3,4,5-tetra-*O*-methylrhamnitol and methyl glycosides of 2,3,4-tri-*O*-methylgalacturonic acid and 3,4-di-*O*-methylrhamnose.

Enzymic degradation of pectin. — Pectin A (20 g) was digested with "Pectinase" (Koch-Light Laboratories) (2 g) in water (10 liters) for 24 h at room temperature, and the enzyme and degraded polysaccharide were precipitated by the addition of acetone (1 vol). The supernatant liquid was concentrated, poured into acetone (4 vol), and kept for 2 days at 4°. The resulting precipitate appeared to contain crystalline galacturonic acid, and was removed by centrifugation. It was dissolved in water, the pH of the solution was adjusted to 7 by the addition of ammonia, and evaporation gave syrup S_1 (9 g). The supernatant liquid was also adjusted to pH 7 by the addition of ammonia, and evaporated to a syrup S_2 (11 g). Although galacturonic acid was the main component of both syrups, the syrups were separately chromatographed on *O*-(2-diethylaminoethyl)-Sephadex A-25 (formate form) (50 g), using water to elute neutral monosaccharides, and 0.05M and a gradient of 0.05 → 0.4M formic acid to elute galacturonic acid and acidic oligosaccharides. Fractions containing three previously unrecognised oligosaccharides were obtained, and chromatographically pure samples of oligosaccharides **8** (12 mg), **9** (19 mg), and **10** (29 mg) were isolated by filter-sheet chromatography.

Oligosaccharide **8**. — The sugar, R_{GalA} 0.53 in solvent B, $[\alpha]_D$ +28° (*c* 0.24), was chromatographically similar to 3-*O*-β-D-xylopyranosyl-D-galacturonic acid, and on hydrolysis gave galacturonic acid and xylose[18]. Hydrolysis of the derived alditol gave xylose as the sole reducing sugar. Gas chromatography of the methanolysis products from the methylated alditol on column *c* showed the presence of 2,3,5,6-tetra-*O*-methylgalactono-1,4-lactone and methyl glycosides of 2,3,4-tri-*O*-methylxylose[8].

Oligosaccharide **9**. — The sugar, R_{GalA} 2.02 in solvent B, had $[\alpha]_D$ +87° (*c* 0.30) and λ_{max} 231 nm. Rhamnose was the only detectable hydrolysis product of the sugar, and rhamnitol, of the derived alditol, but quantitative estimation by the cysteine method[17] indicated the presence of 46% of rhamnose residues in the sugar. The sugar reacted with thiobarbituric acid in a manner characteristic of glycosides of 4-deoxy-L-*threo*-hex-4-enosuronic acid[11] to give a red colour (λ_{max} 547 nm). Gas chromatography of the methanolysis products from the methylated alditol on column *c* showed the presence of the methyl glycoside of 3,4-di-*O*-methylrhamnose and unidentified components.

Oligosaccharide **10**. — The sugar, R_{GalA} 0.12 and 0.50 in solvents B and C, had

$[\alpha]_D$ +85° (*c* 0.4), and gave galacturonic acid and rhamnose on hydrolysis. Colorimetric estimations[16,17] of galacturonic acid and rhamnose indicated that the sugars were present in the ratio of 1.9:1, and, after reduction to the alditol, in the ratio of 1:1. Treatment of the oligosaccharide for 16 h at 100° in 1:1 methanol–water containing 2.5% of triethylamine gave 2-*O*-(galactopyranosyluronic acid)rhamnose as the sole reducing sugar. Gas chromatography of the methanolysis products from the methylated trisaccharide alditol on columns *b* and *c* showed the presence of methyl glycosides of 2,3,4-tri-*O*-methylgalacturonic acid and 3,4-di-*O*-methylrhamnose, and an unidentified component (*T* 3.70 and 9.95 on columns *b* and *c*). This component may have been a derivative of 2,4,5,6-tetra-*O*-methylgalactonic acid, since compounds having the same retention time were observed in the methanolysis products from methylated 3-*O*-(α-D-galactopyranosyluronic acid)-L-galactonic acid ("galacturonobiitol") and methylated 3-*O*-(α-D-galactopyranosyl)-D-galactonic acid (from oxidation of the corresponding galactobiose).

Methylation of carboxyl-reduced pectin. — Pectin C (1 g) in water (100 ml) was passed through Amberlite IR-120 (H^+) resin to remove metal ions, ethylene oxide (25 ml) was added, and the solution was kept for 5 weeks at room temperature, whereupon esterification was complete (pH 6.8). The polysaccharide ester (1 g) was isolated by precipitation with ethanol (3 vol), dissolved in water, and freeze-dried, and was then acetylated by the method of Carson and Maclay[19] to give the acetate (1.1 g). Lithium borohydride (1.1 g) in tetrahydrofuran (25 ml) was added to a suspension of the polysaccharide acetate (1.1 g) in tetrahydrofuran (25 ml), and the mixture was refluxed, with stirring, for 20 h[20]. Excess of hydride was decomposed by the dropwise addition of water, more water (50 ml) was added, and tetrahydrofuran was removed under diminished pressure. Addition of dilute sulphuric acid to the solution (to pH 7) gave a precipitate P, and further polysaccharide precipitates were isolated after dialysis and after addition of ethanol. All precipitates gave galactose as the main sugar on hydrolysis, with only traces of other sugars. Precipitate P was dissolved in 0.5M sodium hydroxide, and precipitation with ethanol gave carboxyl-reduced pectin (322 mg), $[\alpha]_D$ +175° (*c* 0.5, 0.5M sodium hydroxide).

Carboxyl-reduced pectin (220 mg) was treated successively with methyl sulphate and sodium hydroxide, and methyl iodide and silver oxide in *N*,*N*-dimethylformamide, to give methylated, carboxyl-reduced pectin (148 mg), $[\alpha]_D$ +177° (*c* 0.3, chloroform) [Found: OMe, 42.8%]. A sample of the methylated polysaccharide was heated with methanolic hydrogen chloride, and examination of the cleavage products by gas chromatography on columns *a*, *b*, and *c* indicated the presence of methyl glycosides of the following sugars (approximate proportions in parentheses): 2,3,4,6-tetra- (++), 2,3,6-tri- (++++), and 2,6-di-*O*-methylgalactose (++), 2,3,5-tri- (++) and 2,3-di-*O*-methylarabinose (+), and 3,4-di- (+), and 3-*O*-methylrhamnose (+).

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REFERENCES

1 G. O. ASPINALL AND A. CANAS-RODRIGUEZ, *J. Chem. Soc.*, (1958) 4020.
2 G. O. ASPINALL AND R. S. FANSHAWE, *J. Chem. Soc.*, (1961) 4215.
3 G. O. ASPINALL, B. GESTETNER, J. A. MOLLOY, AND M. UDDIN, *J. Chem. Soc.*, submitted for publication.
4 S. S. BHATTACHARJEE AND T. E. TIMELL, *Can. J. Chem.*, 43 (1965) 617.
5 A. J. BARRETT AND D. H. NORTHCOTE, *Biochem. J.*, 94 (1965) 617.
6 S. E. B. GOULD, D. A. REES, N. G. RICHARDSON, AND I. W. STEELE, *Nature*, 208 (1965) 876.
7 R. W. STODDART, A. J. BARRETT, AND D. H. NORTHCOTE, *Biochem. J.*, 102 (1967) 194.
8 G. O. ASPINALL, I. W. COTTRELL, S. V. EGAN, I. M. MORRISON, AND J. N. C. WHYTE, *J. Chem. Soc. (C)*, (1967) 1071.
9 G. O. ASPINALL, K. HUNT, AND I. M. MORRISON, *J. Chem. Soc. (C)*, (1967) 1080.
10 J. L. WHYTE, Ph. D. Thesis, Edinburgh (1964).
11 P. ALBERSHEIM, H. NEUKOM, AND H. DEUEL, *Arch. Biochem. Biophys.*, 60 (1960) 46.
12 P. ALBERSHEIM, H. NEUKOM, AND H. DEUEL, *Helv. Chim. Acta*, 43 (1960) 1422; J. H. MCCLENDON AND J. H. KREISHER, *Anal. Biochem.*, 5 (1963) 295; J. H. MCCLENDON AND J. L. HESS, *J. Food Sci.*, 28 (1963) 289.
13 G. O. ASPINALL AND J. BAILLIE, *J. Chem. Soc.*, (1963) 1702.
14 H. NEUKOM, H. DEUEL, W. J. HERI, AND W. KÜNDIG, *Helv. Chim. Acta*, 43 (1960) 64.
15 M. DUBOIS, K. A. GILLES, J. K. HAMILTON, P. A. REBERS, AND F. SMITH, *Anal. Chem.*, 28 (1956) 350.
16 E. A. MCCOMB AND R. MCCREADY, *Anal. Chem.*, 24 (1952) 1630.
17 Z. DISCHE, *Methods Carbohyd. Chem.*, 5 (1962) 501.
18 G. O. ASPINALL AND J. BAILLIE, *J. Chem. Soc.*, (1963) 1702.
19 J. F. CARSON AND W. D. MACLAY, *J. Amer. Chem. Soc.*, 68 (1946) 1015.
20 D. A. REES AND J. W. B. SAMUEL, *Chem. Ind.* (London), (1965) 2008.

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DEGRADATION OF CARBOHYDRATES

PART IX.* PENTA-*O*-METHYL-*aldehydo*-D-GLUCOSE

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ABSTRACT

The acyclic sugar 2,3,4,5,6-penta-*O*-methyl-D-glucose (**2**) affords an equimolecular mixture of the anhydrous and hydrated aldehyde forms in water at room temperature. In calcium hydroxide solution at 20°, **2** is completely degraded in 20 min to 3-deoxy-tetra-*O*-methyl-*aldehydo*-D-*erythro*-hex-*trans*-2-enose (**3**). The unsaturated aldehyde **3** is converted by dilute acid into 3,4-dideoxy-di-*O*-methyl-*aldehydo*-*keto*-D-*glycero*-hex-*trans*-3-enos-2-ulose.

INTRODUCTION

The degradation of some methylated aldoses by alkali, followed by acid, has been described in previous parts of this series. Pyranose[1,2] and furanose[3] forms of the sugars were studied. The present paper reports on the action of the same reagents on penta-*O*-methyl-*aldehydo*-D-glucose, which can exist in acyclic forms only.

RESULTS AND DISCUSSION

Preparation of the methylated sugars. — Penta-*O*-methyl-*aldehydo*-D-glucose (**2**) was synthesized by Levene and Meyer[4] in 1927, but it was recently claimed[5] that the procedure yields mixtures. The *aldehydo* sugar **2** was therefore prepared in another way, namely, by the methylation of D-glucose dimethyl acetal and hydrolysis of the acetal group. Because the pure (by g.l.c.) acetal **1** and *aldehydo* sugar **2** obtained by the new method had the same physical constants as those reported by Levene and Meyer[4], it is probable that their compounds were pure[6].

D-Glucose dimethyl acetal is known[7]. The preparation starts from D-glucose, and involves the formation of the diethyl dithioacetal, per-*O*-acetylation, demercaptalation to the dimethyl acetal, and, finally, saponification of the acetyl groups[8].

The acyclic sugar **2** was found to be extremely labile to alkali. This is not unexpected, since a large proportion of the sugar exists in the carbonyl form. It is important that it should not be subjected to even weakly basic conditions during the preparation;

*For Part VIII, see Ref. 3. A preliminary account of some of this work has been given[6].

therefore, the acid hydrolyzate of the acetal was not neutralized before being extracted with chloroform.

The i.r. spectrum of **2** given in Fig. 1 shows the complete absence of bands due to unsaturated carbonyl groups, whereas the i.r. spectrum for **2** published by Bayer and Widder[5] appears to be that of a mixture of **2** and **3**.

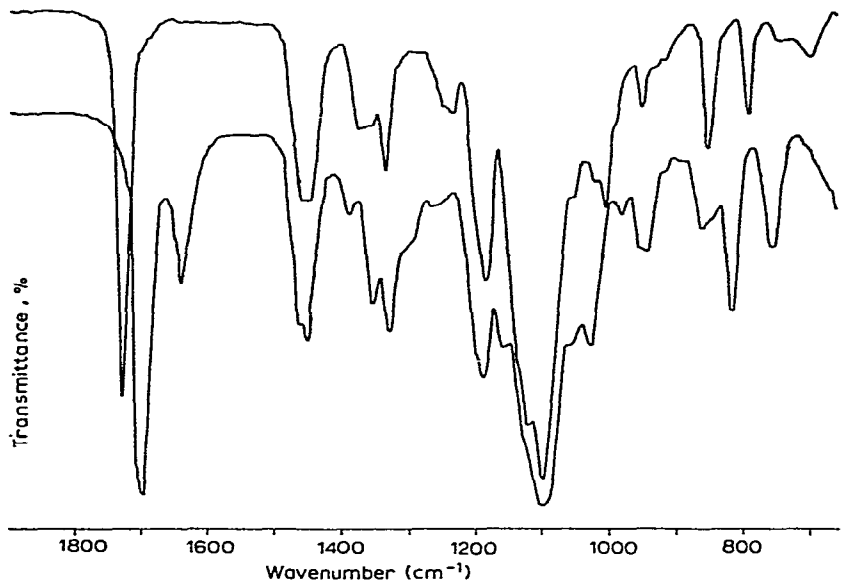
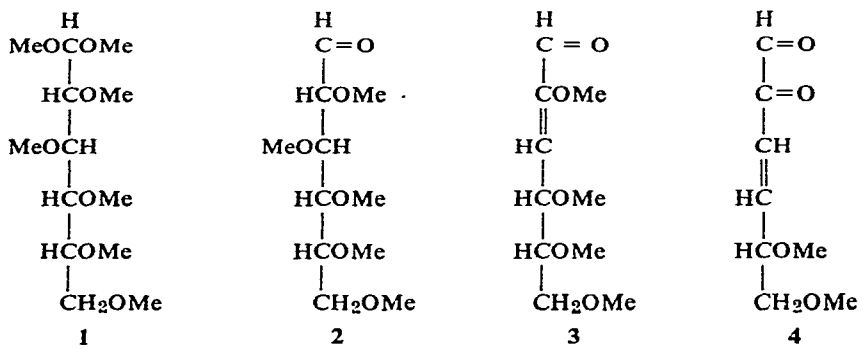


Fig. 1 — Infrared spectra of penta-*O*-methyl-*aldehydo*-D-glucose (**2**), above, and of 3-deoxy-tetra-*O*-methyl-*aldehydo*-D-*erythro*-hex-*trans*-2-enose (**3**), below (*cf.*, Ref. 5).

When the methylated sugar **2** was treated with methanol and an acid catalyst, the dimethyl acetal **1** was re-formed in a few minutes (*cf.*, Ref. 4).



Degradation products. — Sugars having the reducing group free are labile to alkali, the initial reactions of mutarotation and enolization being followed by a β-elimination. For 2-*O*-methylaldo-pyranoses or -furanoses, the group at C-3 is eliminated, to give *cis*-3-deoxyglyc-2-enoses, some weeks at 25° being required for

this reaction to approach completion with saturated calcium hydroxide solution as the reagent[1–3]. Under similar conditions, a rapid elimination occurred from the acyclic sugar **2**, to give, in 20 min, a quantitative yield of the *trans* unsaturated sugar **3**. The formation of *cis*-3-deoxyglyc-2-enoses from aldo-pyranoses and -furanoses was explained by assuming a *cis-trans* equilibrium, and stabilization of the *cis* isomer in a ring form[9]. In the present instance, as a ring form of **3** is impossible, the situation is the same as with other α,β-unsaturated aldehydes where the configuration of the double bond is *trans*.

The unsaturated sugar **3** was degraded by acid, the u.v. peak at 242 nm being replaced by one at 228 nm on heating **3** with dilute hydrochloric acid for a few minutes at 100°. The product was isolated, and shown to be the *trans*-unsaturated aldehyde-ketone **4**, which readily yielded a (2,4-dinitrophenyl)osazone. The same osazone was formed slowly when **2** was treated with (2,4-dinitrophenyl)hydrazine in perchloric acid solution.

Structures. — The structures of the sugar **2** and of its degradation products were ascertained mainly by n.m.r. spectroscopy. The n.m.r. spectrum of **2** in chloroform-*d* solution was that of the aldehyde only (see Fig. 2); it showed a sharp singlet

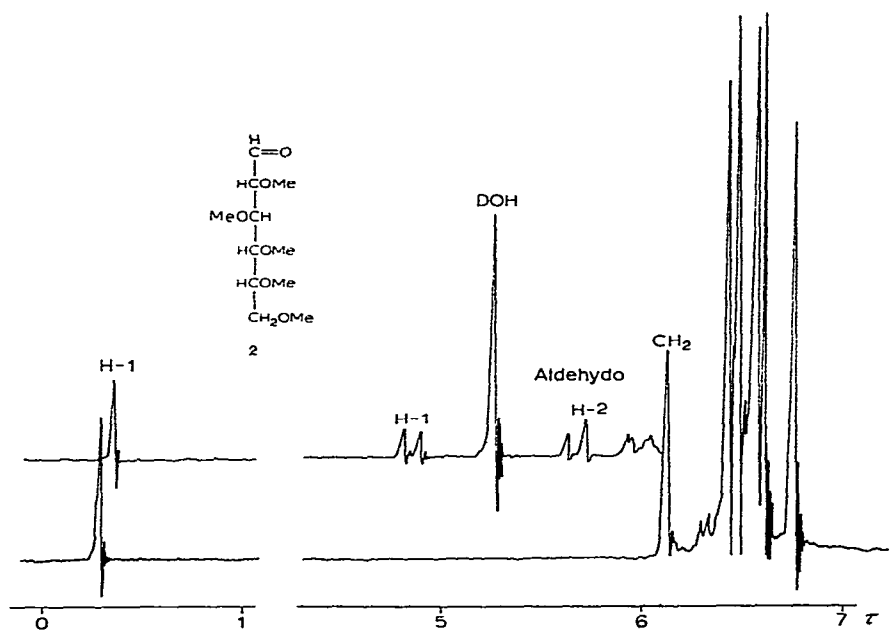


Fig. 2 — N.m.r. spectra of penta-*O*-methyl-D-glucose (**2**) at 60 MHz; in chloroform-*d* solution (below) and in deuterium oxide (above).

($J_{1,2} \sim 0$ Hz) at τ 0.27 (1 proton, HC=O); 5 sharp singlets, τ 6.44, 6.48, 6.58, 6.62, and 6.77 (each approximately 3 protons, 5 OMe); and a peak at τ 6.13 (2 protons, CH_2). The signals of the other four protons were largely obscured by the methoxyl bands. When **2** was dissolved in deuterium oxide, an equilibrium was

established between the aldehyde form and its hydrate. The latter was revealed by a new band, a doublet ($J_{1,2}$ 4.8 Hz) at τ 4.86. At 30°, the two forms were present in equal proportions, as indicated by the intensities of the bands at τ 0.36 (0.5 proton, HC=O) and τ 4.86 [0.5 proton, $HC(OD)_2$]. The 1:1 ratio of the two forms was confirmed by the intensity of a well-resolved signal at high field (τ 6.79, 1.5 proton). This high-field signal was assigned to a methoxyl group of the carbonyl form (*cf.* the signal for the chloroform-*d* solution at τ 6.77, Fig. 2). The freshly distilled sugar **2** showed a strong band in the i.r. at 1728 cm^{-1}, characteristic of the carbonyl group (see Fig. 1).

The n.m.r. spectrum of the unsaturated sugar **3** in either chloroform-*d* or deuterium oxide was clearly that of a single, unsaturated aldehyde (see Fig. 3). If the hydrated form of **3** exists, it can be present in traces only. The spectrum of the

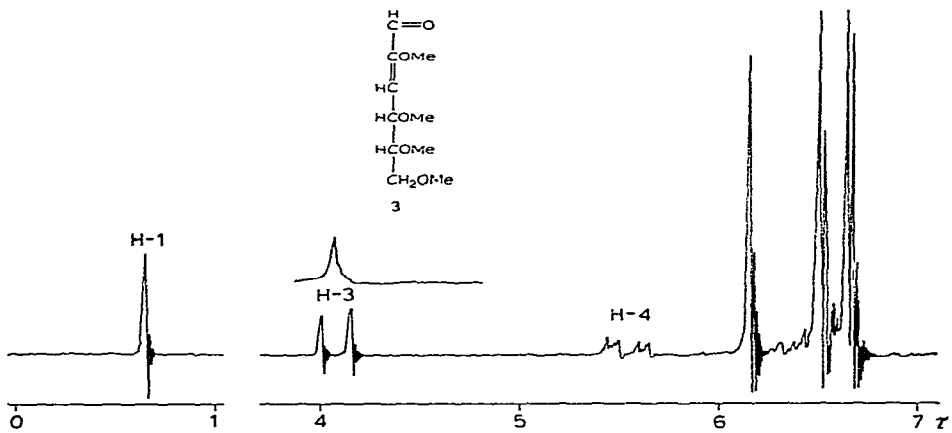


Fig. 3 — N. m. r. spectrum of 3-deoxy-tetra-*O*-methyl-D-*erythro*-hex-*trans*-2-enose (**3**) in chloroform-*d* solution at 60 MHz. Insert shows H-3 decoupled from H-4 (+88 Hz).

chloroform-*d* solution showed a sharp singlet at τ 0.65 (C=C–CH=O), a sharp doublet at τ 4.1 ($J_{3,4}$ 9.0 Hz, H-3), a broad doublet at τ 5.52 ($J_{3,4}$ 9.0 Hz, H-4), sharp singlets at τ 6.17 ($C=C-OCH_3$), 6.50, 6.64, and 6.67 (3 OMe), and a multiplet at τ 6.5 (H-5 and H-6). Each band of the doublet at τ 5.52 was broadened, owing to a small coupling with H-5 and a virtual coupling with H-6, and appeared as a partly resolved doublet of triplets. Two isomers of **3** are possible, the *cis* and the *trans*. The chemical shift of H-3 and H-4 of the two isomers should be different[10]. As only one isomer is present, it is, presumably, the *trans*; evidence supporting this conclusion will be given in a subsequent paper. The i.r. and u.v. spectra of **3** confirmed the α,β-unsaturated aldehyde structure.

The n.m.r. spectrum of **4** in deuterium oxide was essentially first-order. Apart from the signals of two methoxyl groups, the spectrum was closely related to that shown by the *trans* isomer of $CH_2OH–CHOH–CH=CH–CO–CH(OD)_2$, in particular the resonances from H-1 (singlet) and H-3 ($J_{3,4}$ 16.0 Hz) were identical[11].

The presence of the *trans*-α,β-unsaturated ketone group was confirmed by the u.v. and i.r. spectra. Aqueous solutions of **4** were colorless, thus confirming that the aldehyde group was hydrated, whereas the distilled product and its solution in nonhydroxylic solvents showed the typical yellow color of α-dicarbonyl compounds. The n.m.r. spectrum of **4** in chloroform-*d* differed from that in deuterium oxide, in that the signal of H-1 had moved downfield from τ 4.44 to τ 0.63, the characteristic position for resonances of α,β-unsaturated aldehydic protons.

EXPERIMENTAL

General. — The microanalyses were performed by the Australian Microanalytical Service, Melbourne, and the n.m.r. spectra were recorded with a Varian Model A-60 spectrometer by Mr. P. J. Collins of the Division of Coal Research, C.S.I.R.O. The chemical shifts were measured against sodium 4,4-dimethyl-4-silapentane-1-sulfonate (τ = 10.00) when the solvent was deuterium oxide, and, with chloroform-*d*, against tetramethylsilane (τ = 10.00) as internal standard. Optical rotations were determined with a Perkin–Elmer Model 141 polarimeter, the i.r. spectra with a Perkin–Elmer Model 221 spectrometer, and the u.v. spectra with a Beckman Model DK-2A spectrometer.

Gas–liquid chromatography was performed on a Varian Model 200 gas chromatograph fitted with 3-mm columns and a flame ionization detector. All compounds were subjected to g.l.c. on two columns, one packed with the methylsilicone gum, SE30, and the other with Carbowax* 20M, both on Chromosorb G.** Compounds **1** to **4** inclusive gave single peaks on both phases; the retention times are given in Table I.

TABLE I

RETENTION TIMES ON G.L.C.[a]

Column phase	*Compound*			
	1	**2**	**3**	**4**
SE30 at 120°	180	100	48	15
Carbowax 20M at 140°	101	114	63	40

[a]Retention times × 100, relative to methyl tetra-*O*-methyl-α-D-glucofuranoside.

Penta-O-*methyl*-D-*glucose dimethyl acetal* (**1**). — D-Glucose dimethyl acetal[8] (5 g) or the penta-*O*-acetyl derivative thereof (10 g) in acetone (160 ml) was methylated with methyl sulfate (66 ml) and sodium hydroxide solution (80 g in 200 ml of water),

*Union Carbide Corporation.

**Johns Manville Product Corporation.

added simultaneously, in portions, during 100 min. The mixture was stirred vigorously under a nitrogen atmosphere, and was kept at about 56° during the addition; it was then heated for 30 min at 100° to decompose the excess of methyl sulfate, the acetone being allowed to evaporate. The mixture was cooled, and extracted several times with chloroform, and the extracts were combined, dried (sodium sulfate), and evaporated to a syrup (6.5 g). Six such preparations were made; the products were combined, and remethylated with methyl iodide and sodium in liquid ammonia. The product was distilled at 90° (bath temp.) at 0.01 torr, $[\alpha]_D^{25}$ +15.0° (*c* 1, methanol), lit.[4] $[\alpha]_D^{20}$ +15.1° (*c* 4.44, methanol).

Anal. Calc. for $C_{13}H_{28}O_7$: C, 52.7; H, 9.5. Found: C, 52.6; H, 9.5.

The hydrolysis of the acetal **1** (*c* 1) with 20 mM hydrochloric acid at 50° was followed on the recording polarimeter, and found to follow first-order kinetics, half of the compound being hydrolyzed in 3.75 min.

Penta-O-methyl-aldehydo-D-*glucose* (**2**). — The dimethyl acetal **1** (2 ml) was hydrolyzed with 20mM hydrochloric acid (30 ml) for 35 min at 50°. The acid solution was cooled, and extracted with chloroform (3 × 10 ml). The extracts were combined, dried (sodium sulfate), and evaporated to give a quantitative yield of the *aldehydo* sugar as a colorless, mobile oil. The physical data were obtained on freshly prepared and distilled [80° (bath temp.) at 0.01 torr] batches, $[\alpha]_D^{25}$ −35.5° (*c* 1, chloroform), $\lambda_{max}^{H_2O}$ <190 nm, ν_{max} 1728 cm^{-1} (see Fig. 1) (note the absence of an unsaturated carbonyl band, *cf.* Ref. 5). Levene and Meyer[4] gave $[\alpha]_D^{20}$ −35.1° (*c* 4.7, 1,1,2,2-tetrachloroethane). For the n.m.r. spectra, see Fig. 2.

Anal. Calc. for $C_{11}H_{22}O_6$: C, 52.8; H, 8.9. Found: C, 52.9; H, 9.0.

The acetal **1** was re-formed in a few minutes when the aldehyde **2** was dissolved in methanol containing 1% of hydrogen chloride[4]. The mixture was rendered neutral with methanolic sodium methoxide, and evaporated, and the residue was extracted with 2,2,4-trimethylpentane. Examination of the iso-octane extracts by g.l.c. showed that the aldehyde had been converted into the acetal.

3-Deoxy-tetra-O-methyl-aldehydo-D-erythro-*hex*-trans-*2-enose* (**3**). — The dimethyl acetal **1** (2 ml) was hydrolyzed to the aldehyde **2** by the foregoing procedure. The acid solution was rendered neutral with 0.1M sodium hydroxide (6 ml), an equal volume of saturated calcium hydroxide solution (36 ml) was added, and the mixture was kept for 20 min at room temperature for the reaction to proceed to completion. Extraction with chloroform (2 × 40 ml), and removal of the solvent from the dried (Na_2SO_4) extracts, gave a quantitative yield of the unsaturated aldehyde **3** as a colorless oil, $[\alpha]_D^{25}$ +38.8° (*c* 1, water), $\lambda_{max}^{H_2O}$ 242 nm (ε 8200), ν_{max} 1699 and 1642 cm^{-1} (see Fig. 1). For the n.m.r. spectrum, see Fig. 3.

Anal. Calc. for $C_{10}H_{18}O_5$: C, 55.0; H, 8.3. Found: C, 54.7; H, 8.4.

3,4-Dideoxy-5,6-di-O-methyl-aldehydo-keto-D-glycero-*hex*-trans-*3-enos-2-ulose* (**4**). — The glycenose **3** (4.4 g) was heated for 10 min at 100° with 0.1M hydrochloric acid (20 ml), dissolution being complete after 2 min. The reaction mixture was cooled, washed with chloroform (2 ml and 1 ml), rendered neutral with M sodium hydrogen carbonate (2 ml), and extracted with chloroform (12 × 10 ml). The combined

extracts were dried (sodium sulfate), and evaporated to a thick syrup (2.5 g), which distilled at 90° (bath) at 0.05 torr to give **4**, a bright-yellow, viscous oil, $[\alpha]_D^{25}$ +35.3° (*c* 1, water), $\lambda_{max}^{H_2O}$ 228 nm (ε 8700), ν_{max} 1722 (C=O), 1685 and 1629 (C=C−C=O), and 1295 and 975 cm^{-1} (*trans* CH=CH). In hexane solution, **4** also gave λ_{max} 450 nm (ε 17).

Anal. Calc. for $C_8H_{12}O_4$: C, 55.8; H, 7.0. Found: C, 55.6; H, 7.2.

Solutions of **4** in water or alcohols were colorless, probably due to addition of solvent at the aldehyde carbonyl group, with formation of a hydrate or hemiacetal, respectively.

*Formation of an unsaturated osazone from penta-O-methyl-*aldehydo-D-*glucose.* — Freshly distilled **2** (0.5 g) was added to a solution (100 ml) of (2,4-dinitrophenyl)-hydrazine (2 g) in 30% perchloric acid. A precipitate slowly formed: 1 g after 6 h, 0.4 g overnight, and a further 0.1 g, giving a total of 1.5 g of crude osazone. T.l.c.[12] revealed the presence of several compounds. The major product crystallized well from methyl sulfoxide, but still contained traces of impurities that were removed by recrystallization from acetone at 0°, to give red needles, m.p. 206–9°. On t.l.c. plates, the osazone spot turned blue when sprayed with alkali; this color change is the same as that shown by the unmethylated osazone[12].

Anal. Calc. for $C_{20}H_{20}N_8O_{10}$: C, 45.1; H, 3.8; N, 21.0. Found: C, 44.8; H, 3.9; N, 20.9.

The same osazone was precipitated immediately when 3,4-dideoxy-5,6-di-*O*-methyl-*aldehydo-keto*-D-*glycero*-hex-*trans*-3-enos-2-ulose was treated under the above conditions, giving red needles, m.p. and m.m.p. 206–9°, with identical t.l.c behavior.

Anal. Calc. for $C_{20}H_{20}N_8O_{10}$: C, 45.1; H, 3.8; N, 21.0. Found: C, 44.8; H, 3.8; N, 20.8.

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REFERENCES

1 E. F. L. J. ANET, *Carbohyd. Res.*, 1 (1966) 348.
2 E. F. L. J. ANET, *Austral. J. Chem.*, 18 (1965) 837.
3 E. F. L. J. ANET, *Carbohyd. Res.*, 2 (1966) 448.
4 P. A. LEVENE AND G. M. MEYER, *J. Biol. Chem.*, 69 (1926) 175.
5 E. BAYER AND R. WIDDER, *Ann.*, 686 (1965) 197.
6 E. F. L. J. ANET, *Carbohyd. Res.*, 3 (1966) 251.
7 N. K. RICHTMYER, M. ADAMS, AND C. S. HUDSON, *J. Amer. Chem. Soc.*, 61 (1939) 1833.
8 M. L. WOLFROM AND A. THOMPSON, *Methods Carbohyd. Chem.*, 2 (1963) 427.
9 E. F. L. J. ANET, *Austral. J. Chem.*, 16 (1963) 270.
10 G. J. KARABATSOS, G. C. SONNICHSEN, N. HSI, AND D. J. FENOGLIO, *J. Amer. Chem. Soc.*, 89 (1967) 5067.
11 E. F. L. J. ANET, *Austral. J. Chem.*, 15 (1962) 503.
12 E. F. L. J. ANET, *J. Chromatog.*, 9 (1962) 291.

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A REVERSIBLE REACTION BETWEEN REDUCING SUGARS AND A WEAK-BASE ANION-EXCHANGE RESIN

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ABSTRACT

A reaction between D-xylose and Amberlite resin IR-45 (OH^-) was detected during an attempt to remove uronic acids from hemicellulose hydrolysates. Kinetic studies have shown that the reaction is reversible, with the forward reaction being endothermic. The forward reaction is 1st order with respect to D-xylose, and the rate also depends on the proportion of resin. The compound produced is most likely a Schiff's base or a glycosylamine, obtained by the elimination of a molecule of water. This study has shown that caution should be exercised in using a weak-base anion-exchange resin in the presence of reducing sugars.

INTRODUCTION

It is well known that contact between a strong-base anion-exchange resin and reducing sugars leads to epimerisation[1–3] and irreversible sorption, probably associated with alkaline degradation to acidic products[4]. A reaction between reducing sugars and a strong-base resin leading to a reduction in exchange capacity has also been detected[5]. It is usually assumed, however, that the weak-base anion-exchange resins, containing mainly secondary and tertiary amine functional groups, may be used safely in the presence of free sugars. Such uses are frequently very convenient in the neutralisation of solutions resulting from acidic hydrolysis of polysaccharides, or in the separation of uronic acids from neutral sugars.

In attempts to use weak-base anion-exchange resins in such applications, however, we have frequently detected significant losses of neutral sugars and have also found that when sorbed uronic acids are regenerated they are often contaminated by the neutral sugars with which they were previously associated. We have now shown that this effect is caused by the reversible formation of a covalent compound between the resin and the reducing sugar, and we have studied the kinetics of the system, in some detail, for the case of Amberlite resin IR-45 (OH^-) and D-xylose.

RESULTS AND DISCUSSION

The rate of disappearance of D-xylose from aqueous solution in the presence of Amberlite resin IR-45 (OH^-) was determined under various conditions. Fig. 1 shows

a typical rate curve from which the initial rate of reaction could be evaluated. In all cases, the reaction eventually reached a state of apparent equilibrium. A plot of log (initial rate) against log (D-xylose) gives a straight line of slope 1.0 (Fig. 2), indicating that the reaction is first order with respect to D-xylose concentration. Table I shows that the reaction rate, at a constant concentration of D-xylose, is also dependent on the amount of resin present. Examination of the effect of temperature, by the normal Arrhenius plot (Fig. 3), indicates an activation energy of 15 kcal/mole for the process.

TABLE I

VARIATION OF INITIAL RATE WITH RESIN RATIO[a]

Resin ratio (*g of resin/ml of solution*)	*Initial rate* (*mole.litre*$^{-1}$.*sec*$^{-1}$)
0.049	2.183×10^{-6}
0.098	4.67×10^{-6}
0.196	7.33×10^{-6}

[a]Resin batch *A*; temperature, 30.6°; initial concentration of D-xylose, 3.33×10^{-2} moles.litre^{-1}.

The specific nature of the above reaction is indicated by kinetic data obtained on other related systems. With D-glucose and Amberlite IR-45 (OH^-), the rate of disappearance of D-glucose was approximately 10% of that for D-xylose under similar conditions. With D-xylose and Amberlite IR-45 (Cl^-), no uptake of D-xylose occurred over several days.

In a separate series of experiments, resin, which had previously been equilibrated with D-xylose and then separated and quickly washed, was added to a fixed amount of water, and the rate of liberation of D-xylose into the solution was determined for different temperatures (Fig. 4). Once again, a position of apparent equilibrium was eventually reached. The activation energy evaluated for this reverse process (Fig. 5) was 9 kcal/mole.

The above results are consistent with the occurrence of a reversible chemical reaction between free amine groups of the resin and the D-xylose in solution. The kinetic data are in agreement with an overall rate equation of the form,

$$-\frac{\mathrm{d}\,[\text{D-xylose}]}{\mathrm{dt}} = k_1\,[\text{D-xylose}](\theta) - k_2\,(1-\theta),$$

where k_1 and k_2 are the velocity constants of the forward and backward reactions, and θ is the fraction of unreacted amino groups on the resin. The measured activation energies indicate that the forward reaction is endothermic to about 6 kcal/mole. This conclusion was confirmed by the observation that the amount of D-xylose lost from a solution at equilibrium increased with temperature. This means that the forward reaction must occur with an increase in entropy, which precludes any simple addition reaction between the resin and D-xylose. Thus, the reaction is not analogous to the normal process of chemisorption, and, in this respect, it is interesting to note

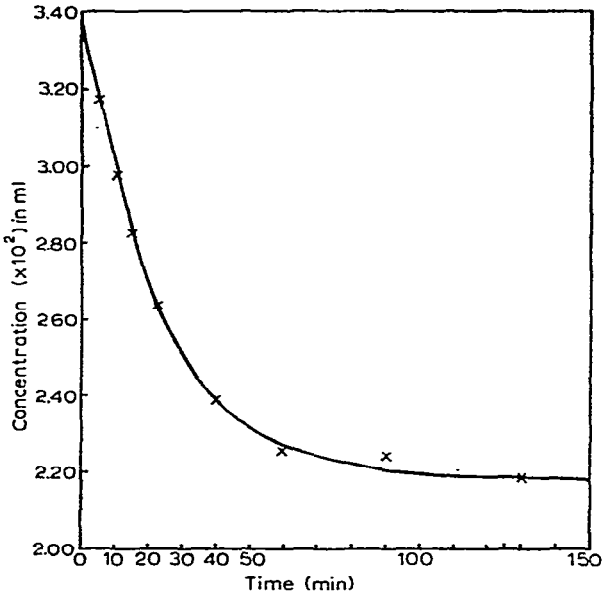


Fig. 1 — Rate curve for the disappearance of D-xylose in the presence of Amberlite resin IR-45 (OH^-). Resin batch *B*. Resin ratio = 0.098 g of resin/ml of solution. Temperature = 41.4°.

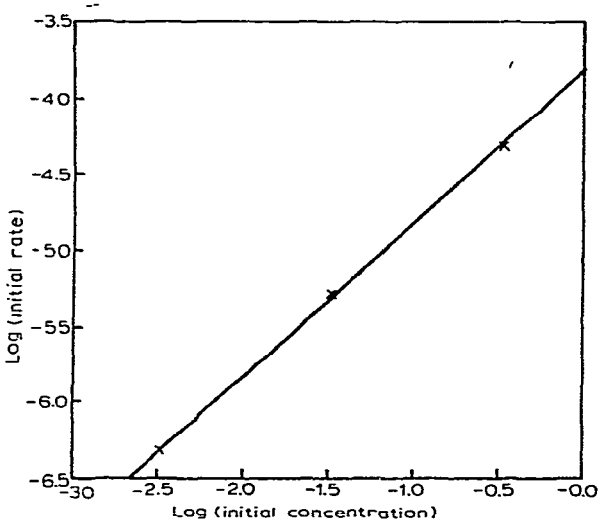


Fig. 2 — Reaction order with respect to D-xylose concentration. Resin batch *A*. Resin ratio, 0.098 g of resin/ml of solution. Temperature = 30.6°.

that equilibrium data obtained from runs with different initial concentrations of D-xylose did not fit the normal Langmuir and Freundlich isotherms.

Quite clearly, the above results indicate formation of a covalent compound between D-xylose and the resin, almost certainly with elimination of another molecule. By analogy with known reactions between amines of low molecular weight and

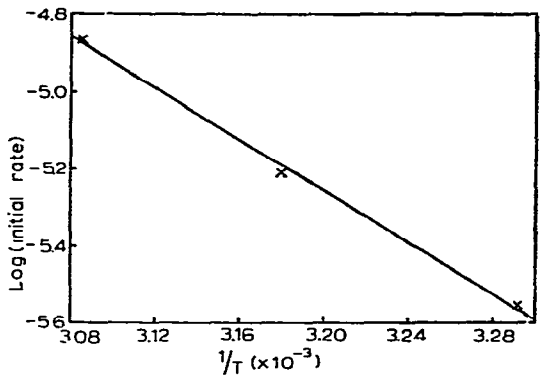


Fig. 3 — Arrhenius plot for the reaction of D-xylose with Amberlite resin IR-45 (OH^-). D-Xylose concentration = 3.33×10^{-2} ml^{-1}. Resin batch *B*. Resin ratio = 0.098 g/ml.

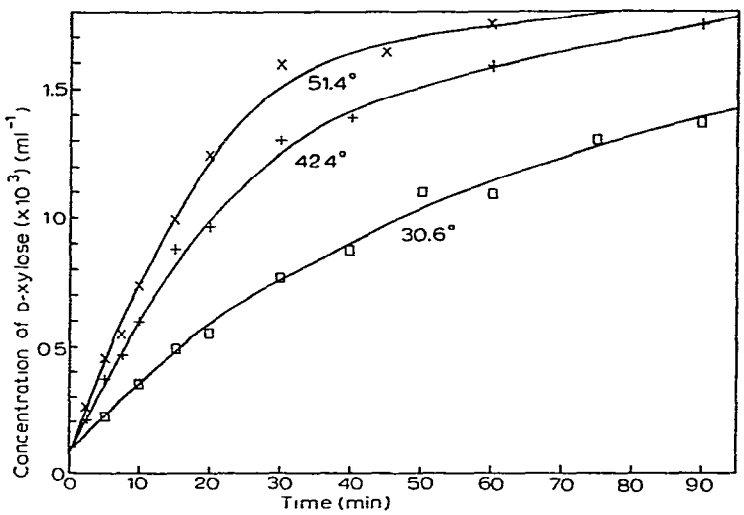
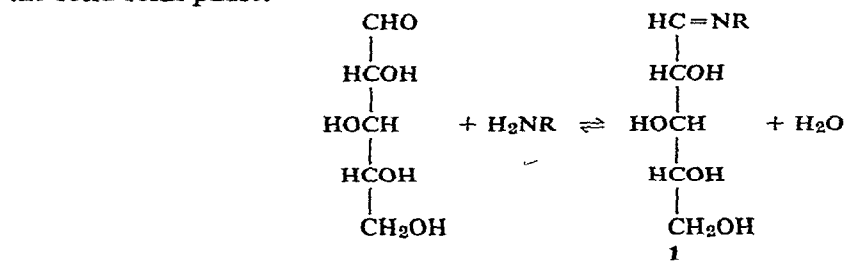


Fig. 4 — Rate curves for the elution of D-xylose from Amberlite resin IR-45 at three different temperatures. Resin batch *B*.

reducing sugars, the most likely explanation is a condensation reaction to form either a Schiff's base (**1**) or a glycosylamine (**2**).

It has not proved possible to obtain any exact details of manufacture of the resin used, but the manufacturers state that its predominant functional groups are of basic strength similar to dipropylamine, although it contains significant amounts of primary, secondary, and tertiary amino groups. On this basis, it seems reasonable to assume that the proportion of primary amino groups may be much lower than that of secondary amino groups, and since Schiff's base can only readily form with primary amines, we conclude that the predominant reaction is the formation of glycosylamine **2**. The rapid reversal of the reaction in neutral, aqueous solution also confirms this conclusion, since the Schiff's base would be expected to be relatively stable under such conditions[6].

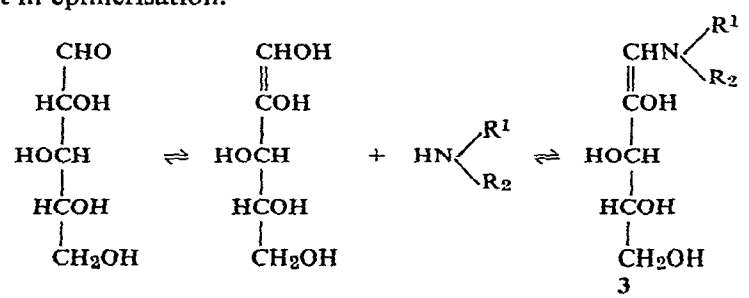
The addition of mineral acid produces a very marked acceleration in the rate of hydrolysis of the resin–D-xylose compound (Fig. 6). This result is rather surprising in view of the observation of Isbell and Frush[6] that the maximum rate of hydrolysis of glycosylamines occurs at *ca.* pH 5 and that the rate at pH 3 is similar to that at pH 7 (the residual acidity in solution in our case was *ca.* 0.1N). We must consider, however, the fact that we have shown that the salt-form of the resin does not react with D-xylose, and it is evident that, in this instance, the reactivity of a glycosylamine in solution must be completely different from a presumed, comparable structure in the solid-resin phase.



1

2

The possibility of formation of an enamine linkage, as shown in structure 3 must be eliminated, since a paper-chromatographic examination of the products of the reverse reaction showed only xylose, whereas the reverse reaction from structure 3 must result in epimerisation.



3

The absence of reaction between D-xylose and the resin in the chloride form indicates that the amine must be in the free base form in order to react. This observation is not in disagreement with the results obtained in preparation of glycosylamines. Such reactions are frequently carried out in the presence of mineral acid, but the molar ratio of acid to base in a preparative reaction is typically[7] 1%.

The fact that D-glucose reacts much less readily than D-xylose with the resin may be associated with the smaller proportion of open-chain form in solutions of the hexose[8].

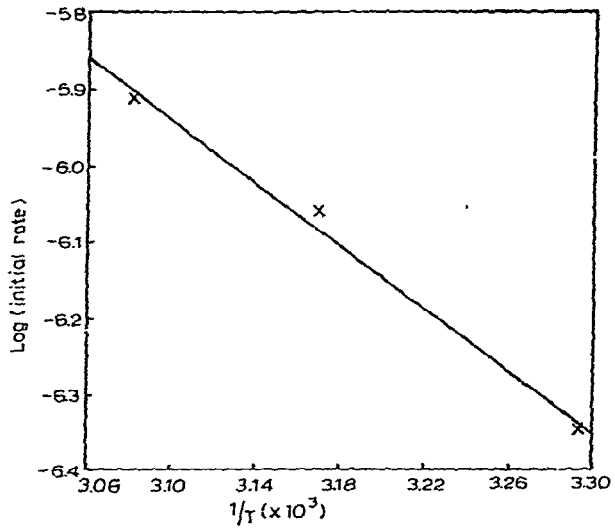


Fig. 5 — Arrhenius plot for elution of D-xylose from Amberlite resin IR-45. Resin batch *B*.

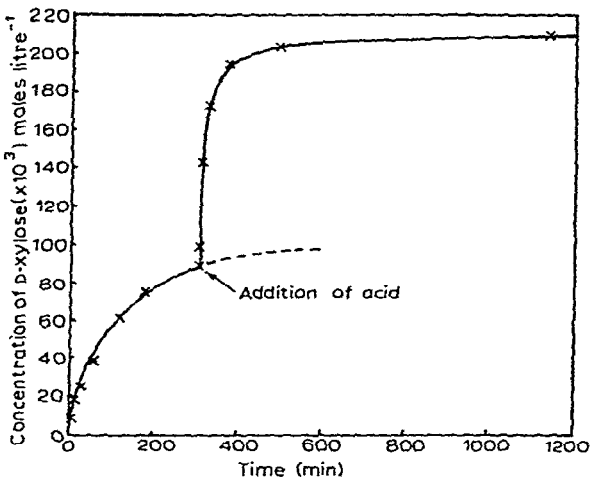


Fig. 6 — Plot of D-xylose concentration against time, showing first the elution with water and then with sulphuric acid.

EXPERIMENTAL

Materials. — The Amberlite resin IR-45 (16–50 mesh) was recycled before use as follows: 2.5 bed-volumes of 1.5N hydrochloric acid was passed through a column of the basic form of the resin at 10 ml/min. This was followed by a water wash and then an excess (2.5 bed-volumes) of N sodium hydroxide at 10 ml/min. The resin was subsequently washed with deionized water until the effluent had the same pH as the wash water. The resin was stored in a moist state. All of the results described were obtained with one or other of two bulked batches of resin (*A* and *B*) derived from two

different commercial samples, and each result is identified as arising from either batch *A* or batch *B*.

Amberlite resin IR-45 (OH^-) was converted into the chloride form by washing with 2.5 bed-volumes of N hydrochloric acid at 10 ml/min and then with water. The resin was stored in a moist state.

α-D-Xylopyranose and α-D-glucopyranose were dried to constant weight at 40°/1 mm, and each gave only a single spot when examined by paper chromatography.

Distilled and subsequently deionised water was used throughout.

Analytical methods. — D-Xylose and D-glucose concentrations were determined by the phenol–sulphuric acid method[9]. Paper chromatography was performed with Whatman No. 1 paper, ethyl acetate–pyridine–water (8:2:1), and silver nitrate–sodium hydroxide[10] and *p*-anisidine[11] spray reagents.

Typical reaction of resin with D-*xylose.* — D-Xylose (*ca.* 1.25 g) was dissolved in water, and the solution was made up to 250 ml and immersed in a thermostatted (±0.05°) water bath. Air was drawn through Amberlite resin IR-45 (OH^-) (62.5 ml, volume measured in a measuring cylinder under water) on a Büchner filter for at least 5 h until it became free-flowing. At this stage, the moisture content of the resin was *ca.* 29% (w/w), and 62.5 ml (in this condition) weighed 35 g.

The dried resin was added to the flask containing the D-xylose solution, which was then stoppered and shaken gently at two cycles/sec. At intervals, samples were withdrawn through a glass sinter, the time at filtration was noted, and samples were subsequently diluted for the phenol–sulphuric acid determination. A plot of D-xylose concentration against time was normally linear for about 20% of the total "uptake", and initial rates were determined from this part of the curve. Resin ratios are expressed as "g/ml", signifying the ratio of resin dry weight to total solution volume.

Typical elution reaction. — Amberlite resin ...-45 (OH^-), which had been equilibrated at 30.6° with a D-xylose solution as described above, was filtered, covered with water (100 ml) for 1 min, and again filtered.

Water (250 ml) was equilibrated in a thermostated bath, the prepared resin (20 ml) was added, and the flask was shaken gently at 2 cycles/sec. Samples were withdrawn at intervals, and D-xylose in the solution was determined as above. A plot of D-xylose concentration in the solution against time was linear for about 20% of the total "desorption", and initial rates were determined from this part of the curve.

In a repetition of the preceding experiment, 6.5 ml of 10N sulphuric acid were added to a system which had almost reached equilibrium at 31°, causing a more rapid and extensive "desorption" of D-xylose (Fig. 6).

Elution of D-*xylose from Amberlite resin IR-45 with* N *sulphuric acid.* — A column containing resin that had reacted with 0.5% D-xylose solution was eluted with N sulphuric acid until the effluent became acidic, and the effluent was made up to 500 ml with water in a standard flask. Some of this solution was used for paper chromatography, after removal of sulphate ion with Amberlite resin IRA-400 (acetate), and showed only xylose. The D-xylose concentration of the solution was found by the

phenol–sulphuric acid method. The optical rotation of the solution was in agreement with the D-xylose concentration so determined.

Attempt to react Amberlite resin IR-45 (Cl^-) with D-xylose. — D-Xylose (*ca.* 1.25 g) was dissolved in water, and the solution was made up to 250 ml and immersed in a thermostated bath at 30.6°. Air was drawn through Amberlite resin IR-45 (Cl^-) (62.5 ml) on a Büchner filter until it became free flowing.

The dried resin was added to the flask containing the D-xylose solution, which was then stoppered and shaken gently at two cycles/sec. At intervals, samples were withdrawn, and the D-xylose concentration was determined as above.

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REFERENCES

1 C. N. TURTON AND E. PACSU, *J. Amer. Chem. Soc.*, 77 (1955) 1059.
2 J. C. SOWDEN, *J. Amer. Chem. Soc.*, 76 (1954) 4487.
3 J. C. SOWDEN AND R. R. THOMPSON, *J. Amer. Chem. Soc.*, 80 (1958) 1435.
4 D. R. BUHLER, R. C. THOMAS, B. E. CHRISTENSEN, AND C. H. WANG, *J. Amer. Chem. Soc.*, 77 (1955) 481.
5 T. SHIMIZU, H. KOZIMA, AND S. YUKIMASA, *Hyogo Noka Daigaku Kenkyu Hokoku, Nogei-kagaku Hen*, 5 (1961) 77.
6 H. S. ISBELL AND H. L. FRUSH, *J. Res. Natl. Bur. Std.*, 46 (1951) 132.
7 E. MITTS AND R. M. HIXON, *J. Amer. Chem. Soc.*, 66 (1944) 483.
8 W. PIGMAN, in W. PIGMAN (Ed.), *The Carbohydrates*, Part 1, Academic Press, New York, 1957, p. 54.
9 L. HOUGH AND J. K. N. JONES, *Methods Carbohyd. Chem.*, 1 (1962) 29.
10 W. E. TREVELYAN, D. P. PROCTER, AND J. S. HARRISON, *Nature*, 166 (1950) 444.
11 L. HOUGH, J. K. N. JONES, AND W. H. WADMAN, *J. Chem. Soc.*, (1950) 1702.

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CARRAGEENANS

PART V. THE MASKED REPEATING STRUCTURES OF λ- AND μ-CARRAGEENANS

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ABSTRACT

For the potassium chloride-soluble fractions of five carrageenans, correlations have been established between 3,6-anhydrogalactose content and the products of methylation and fragmentation. After modification with alkaline borohydride, two subfractions were obtained, and shown to be distinct by methylation analysis and other methods.

Carrageenan is now believed to contain three polysaccharide components: κ-carrageenan, λ-carrageenan, and μ-carrageenan. All have masked repeating structures based on alternately (1→3)- and (1→4)-linked galactose residues, but there are important differences in sulphation pattern and 3,6-anhydride content.

INTRODUCTION

Carrageenan has long been known to contain at least two polysaccharides[1], of which one is selectively precipitated with potassium chloride and named κ-carrageenan. Many features of structure have been characterized for this component[2,3], but there has been less agreement on the nature of the potassium chloride-soluble fraction. In this paper, we show that there are two structurally distinct polysaccharides in the soluble fraction. Both are polymers of alternately β-(1→4)- and α-(1→3)-linked galactose derivatives, but they differ in sulphate ester distribution and certain other respects. On the principle that nomenclature should be related to structure rather than solubility properties[4,5], they are named λ-carrageenan and μ-carrageenan.

RESULTS AND DISCUSSION

Methylation and other methods have shown[4,6] that the potassium chloride-soluble fraction of carrageenan (Sample **1**, Table I) contains both (1→3)- and (1→4)-linked galactose residues. Most of the former type are 2-sulphated, although some are 4-sulphated and others are nonsulphated; the (1→4)-type occurs chiefly as galactose 2,6-disulphate, but also as 3,6-anhydrogalactose (and/or its 2-sulphate) and galactose 6-sulphate. The variation of gross composition from one sample to another[7] might indicate polymer heterogeneity. Several further samples (Table I), kindly supplied

by Dr. E. T. Dewar[7], were therefore examined by methylation, followed by hydrolysis and paper chromatography. The products from each were 3-*O*-methylgalactose, 4,6-di-*O*-methylgalactose, 2,6-di-*O*-methylgalactose, 2,4,6-tri-*O*-methylgalactose, and traces of 2,3,4,6-tetra-*O*-methylgalactose. In addition, 3,6-anhydro-2-*O*-methylgalactose derivatives were detected by gas–liquid chromatography after methanolysis. The relative proportions of 3,6-anhydro-2-*O*-methylgalactose and 2,6-di-*O*-methylgalactose increased from Sample **1** to Sample **5**, whereas 3-*O*-methylgalactose, 4,6-di-*O*-methylgalactose, and 2,4,6-tri-*O*-methylgalactose decreased. With λ-carrageenan defined as a copolymer of 3-linked galactose 2-sulphate, 3-linked galactose, and 4-linked galactose 2,6-disulphate[4], and κ-carrageenan as a copolymer of 3-linked galactose 4-sulphate and 4-linked 3,6-anhydrogalactose with some 4-linked 3,6-anhydrogalactose 2-sulphate[3], the methylation results show that the samples are progressively less "λ-like" from Sample **1** to Sample **5** and progressively more "κ-like". The variations in 3,6-anhydrogalactose content (Table I) point to a similar conclusion. The proportion of 2,3,4,6-tetra-*O*-methylgalactose increased progressively from Sample **1** to Sample **5**, but the amounts were very small indeed and it is not possible to be certain of their significance at present.

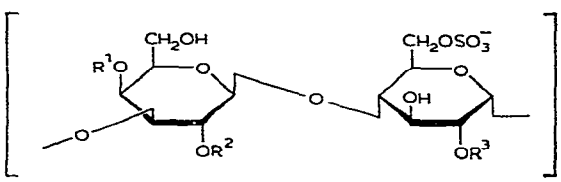
TABLE I

POTASSIUM CHLORIDE-SOLUBLE FRACTIONS OF CARRAGEENAN

Sample number	*Source*	*Time and place of collection*	*Molar ratio, galactose: 3,6-anhydrogalactose: sulphate*
1	*Chondrus crispus*	Prince Edward Island (Nova Scotia), August	1.00:0.01:1.25
2	*Chondrus crispus*	Northumberland Strait (Nova Scotia), May	1.00:0.11:1.22
3	*Chondrus crispus*	Northumberland Strait (Nova Scotia), November	1.00:0.10:1.22
4	*Chondrus crispus*	Ketch Harbor (Nova Scotia), August	1.00:0.29:1.36
5	*Gigartina stellata*	Millport (Scotland), August	1.00:0.43:1.07

The methylation data might therefore suggest that all samples are mixtures of λ-carrageenan and a component which, although not precipitable by potassium chloride, has some structural similarity to κ-carrageenan. This interpretation was confirmed by treatment of Sample **1** with alkaline borohydride to convert any 4-linked galactose 6-sulphate into 3,6-anhydrogalactose residues, followed by addition of potassium chloride. This gave a precipitate *P* (10%) and a soluble polysaccharide *Q* (87%), which were easily distinguished by methylation analysis: *P* gave 2,6-di-*O*-methylgalactose and 3,6-anhydro-2-*O*-methylgalactose as cleavage products with traces of 4,6-di-*O*-methylgalactose and 2,4,6-tri-*O*-methylgalactose, whereas *Q* gave 4,6-di-*O* methylgalactose and 2,4,6-tri-*O*-methylgalactose but no 2,6-di-*O*-methylgalactose

or 3,6-anhydro-2-*O*-methylgalactose. After methanolysis of both *P* and *Q*, carrabiose dimethyl acetal (3,6-anhydro-4-*O*-β-D-galactopyranosyl-D-galactose dimethyl acetal) was isolated as the crystalline hexa-acetate. Quantitative paper chromatography, in experiments published already[8], has shown that the yields of carrabiose dimethyl acetal formed by methanolysis are close to those expected for a polymer built entirely of carrabiose segments. With the evidence from methylation, this shows that the structures of *P* and *Q* are based on alternately arranged 3-linked β-D-galactopyranosyl residues and 4-linked 3,6-anhydrogalactosyl residues, and are therefore of the "masked repeating"[2,3,9] type. The 3,6-anhydrogalactosyl linkages probably have the α-D configuration, by analogy with κ-carrageenan[2,10], and because the α-(1→3)-linked disaccharide has been isolated from the acetolysis products of the potassium chloride-soluble fraction of carrageenan[11]. In *Q* ,the galactose residues are mostly 2-sulphated; the quantitative data[4] suggest that 70% are 2-sulphated and most of the remainder nonsulphated. Virtually all of the 3,6-anhydrogalactose residues in this subfraction were shown to be sulphated, because oxidative hydrolysis of the methylated polysaccharide gave preponderantly 3,6-anhydrogalactonic acid, with little of its 2-methyl ether. The peak at 808 cm^{-1} in the infrared spectrum also indicates[3] a high proportion of 3,6-anhydrogalactose 2-sulphate residues. Before modification with alkaline borohydride, the structure therefore corresponds to **1**, which, by definition[4], is λ-carrageenan.



1 $R^1 = H$; $R^2 = SO_3^-$ (70%) and H (30%); $R^3 = SO_3^-$
2 $R^1 = SO_3^-$; $R^2 = R^3 = H$

The galactose residues in *P* are largely 4-sulphated, since 2,6-di-*O*-methylgalactose was detected as the main, acid-stable hydrolysis product after methylation. The 3,6-anhydride is largely nonsulphated, because oxidative hydrolysis, after methylation, gave 3,6-anhydro-2-*O*-methylgalactonic acid with little 3,6-anhydrogalactonic acid, and because the anhydride 2-sulphate peak[3] in the region of 805 cm^{-1} was virtually absent from the infrared spectrum. Those samples which gave a high yield of 2,6-di-*O*-methylgalactose after methylation and hydrolysis, and which were therefore rich in the precursor of *P*, also had high contents of native 3,6-anhydrogalactose (Table I). This suggests that the 3,6-anhydride, present before modification of the potassium chloride-soluble fraction, occurs in the precursor of *P*. The quantitative data[4] for Sample **1** shows that there is insufficient 3,6-anhydrogalactose to account for more than about 20% of the native 4-linked residues, but the proportion would be higher for Sample **5**, even if λ-carrageenan were totally absent (Table I).

The structure of the precursor of *P* is therefore idealised as **2**, with replacement of galactose 6-sulphate residues by 3,6-anhydrogalactose to an extent which is variable and dependent on source. The further deviations, $R^1 = R^2 = H$; $R^1 = H$, $R^2 = SO_3^-$; and $R^3 = SO_3$, seem to occur to very minor extents and might be artefacts owing to incomplete separation. This polysaccharide represents a new type of carrageenan structure, and we therefore propose the name μ-carrageenan. Its biological relationship to κ-carrageenan has been discussed elsewhere[12].

EXPERIMENTAL

General. — Poly(ethylene glycol adipate) (15% on Gas Chrom P) was the liquid phase for g.l.c. in 4-ft. columns at 175°, with the Pye-Argon Chromatograph fitted with a ^{90}Sr detector. For paper chromatography, the solvents were (*A*) butyl alcohol–ethanol–water (4:1:5, upper phase), (*B*) ammonia (sp. gr. 0.880)–water–ethyl methyl ketone (1:17:200), (*C*) butyl alcohol–ethanol–water (3:1:1), and (*D*) ethyl acetate–acetic acid–formic acid–ethyl methyl ketone–water[2] (17:3:1:15:5). Sprays were *p*-anisidine hydrochloride[13] for reducing sugars and their methyl ethers, and aniline–xylose[14] for aldonic acids and their methyl ethers. Infrared spectra were recorded for polysaccharide films, which were prepared by evaporation of solutions (0.5% w/v) on silver chloride. The analytical methods for sulphate ester and 3,6-anhydrogalactose have been described earlier[15].

Methylation analysis of potassium chloride-soluble fractions. — The methylation analysis of Sample **1** (Table I) has been described before[4]. Samples **2**–**5** (1 g of each) were separately dissolved in water (100 ml), and each solution was cooled in ice. Methyl sulphate (10 ml) and sodium hydroxide (30% w/v, 30 ml) were added over 6 h with vigorous stirring under nitrogen. After stirring for 16 h, the procedure was repeated five times at room temperature. The solution was dialysed, and concentrated under diminished pressure to 100 ml, and the entire cycle was repeated. The methylated polysaccharides were isolated in yields of 0.8–1.0 g by dialysis and freeze-drying (Found: SO_4, 18.0; OCH_3, 13.6 for Sample **2**; SO_4, 21.5; OCH_3, 13.0 for Sample **3**; SO_4, 20.9; OCH_3, 16.3 for Sample **4**; SO_4, 15.1; OCH_3, 15.3 for Sample **5**). Repeated methylation did not change the pattern of methyl ethers observed after subsequent hydrolysis and paper chromatography.

Each methyl ether, including that of Sample **1**, was heated with aqueous formic acid (45% w/v) for 6 h at 100°. Paper chromatography (solvents *A* and *B* with *p*-anisidine hydrochloride spray) gave the results reported in the Discussion. Samples **3**, **4**, and **5** contained only traces of 2,4,6-tri-*O*-methylgalactose. The relative proportions of the two di-*O*-methylgalactoses were similar to each other in Samples **2** and **3**; in Sample **5**, there was an approximately ten-fold excess of the 2,6 isomer (contrast Sample **1**, for which the 4,6 isomer is present in much the greater amount[4]). Methanolysis (2.3% methanolic hydrogen chloride in a sealed tube for 15 h at 100°, followed by neutralisation with silver carbonate) and g.l.c. confirmed these trends and showed the presence of 3,6-anhydro-2-*O*-methylgalactose derivatives which,

for each sample with our apparatus and conditions, gave a total peak area quite close to the corresponding total area for 2,6-di-*O*-methylgalactose derivatives.

Modification with alkaline borohydride, fractionation, and characterization of the products. — Sample **1** (40 g) was dissolved in water (2 l), and potassium borohydride (2 g) was added. After 20 h at room temperature, 3N sodium hydroxide (1 litre) was added with a further quantity of borohydride (1 g), and the mixture was kept for 24 h at 80°, dialysed against running tap-water, concentrated, and freeze-dried (30.5 g). Part of the product (5 g) was dissolved in water (2 l), and potassium chloride (50 g) was added with stirring. After storage of the mixture overnight, a gelatinous precipitate had separated. This was collected by centrifugation, dissolved in 5% sodium chloride for dialysis against running tap-water, and isolated by freeze-drying (*P*, 0.5 g). The supernatant solution was dialysed against running tap-water and freeze-dried (*Q*, 4.4 g). In the infrared spectrum, *Q* showed strong absorption for ester sulphate at 1220–1260 cm^{-1}, and peaks at 808 and 835 cm^{-1}; there was no peak at 850 cm^{-1}. After purification by reprecipitation with potassium chloride, washing thrice with 5% KCl at the centrifuge, dissolution in sodium chloride, and isolation as before, *P* showed absorption bands at 1240 and 850 cm^{-1}, but little, if any, at 805 and 830 cm^{-1}. Other details of these fractions have been published[8].

Fraction *P* (0.70 g) was refluxed with methanolic hydrogen chloride (0.2% w/v, 500 ml) for 40 min. After being kept over excess of silver carbonate for 20 h, the solution was filtered and concentrated to small volume. Separation on sheets of Whatman 3MM paper with solvent *C* gave syrupy carrabiose dimethyl acetal (0.40 g), which was acetylated by treatment with pyridine (5 ml) and acetic anhydride (3.3 ml) for 1 h at 80°. Evaporation of the reagents, and washing with water by decantation, left hexa-*O*-acetylcarrabiose dimethyl acetal which was crystallised from ethanol as needles (0.22 g, 33% overall), m.p. and mixed m.p. 152–154°, $[\alpha]_D$ −14.0° (*c* 1.1, benzene). Similar treatment of *Q* (0.71 g) also gave syrupy carrabiose dimethyl acetal (0.47 g) and hence the same crystalline hexa-acetate (0.25 g, 34% overall), m.p. and mixed m.p. 153–154.5°, $[\alpha]_D$ −15.5° (*c* 0.9, benzene).

Treatment with methyl sulphate and sodium hydroxide, as already described, converted *Q* purified and *P* (0.25 g of each) into their methyl ethers (0.2 g). Hydrolysis and paper chromatography, and methanolysis and g.l.c., were carried out by methods described above; the results are given in the Discussion. For oxidative hydrolysis, a sample of each (20 mg) was dissolved in water (2 ml), and N sulphuric acid (2 ml) was added with shaking followed by bromine (1–3 drops). The solution was kept at 50° with addition of more bromine as necessary to maintain the concentration. After 24 h, the bromine was removed by aeration, and 6N sulphuric acid (1 volume) was added, followed by heating for 15 h at 100° to hydrolyse the galactosyl bonds and sulphate groups. After neutralisation with barium carbonate, silver carbonate was added to the suspension which was left in the dark with occasional shaking for 72 h, and then filtered and treated with Amberlite resin IR-120 (H^+ form). The solution was concentrated under diminished pressure and compared with 3,6-anhydrogalactonic acid and its 2-methyl, 4-methyl, and 2,4-dimethyl ethers[2], by paper chromatography

in solvent *D* with aniline–xylose spray. The product from *Q* contained 3,6-anhydrogalactonic acid and its 2-methyl ether, in the approximate ratio 20:1. For *P*, the ratio was approximately 1:5.

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REFERENCES

1 D. B. SMITH, W. H. COOK, AND J. L. NEAL, *Arch. Biochem. Biophys.*, 53 (1954) 192.
2 N. S. ANDERSON, T. C. S. DOLAN, AND D. A. REES, *J. Chem. Soc.* (*C*), (1968) 596.
3 N. S. ANDERSON, T. C. S. DOLAN, A. PENMAN, D. A. REES, G. P. MUELLER, D. J. STANCIOFF, AND N. F. STANLEY. *J. Chem. Soc.* (*C*), (1968) 602.
4 T. C. S. DOLAN AND D. A. REES, *J. Chem. Soc.*, (1965) 3534.
5 E. E. PERCIVAL AND R. H. MACDOWELL, *Chemistry and Enzymology of Marine Algal Polysaccharides*, Academic Press, New York, 1967, p. 142.
6 D. A. REES, *J. Chem. Soc.*, (1963) 1821.
7 W. A. P. BLACK, W. R. BLAKEMORE, J. A. COLQUHOUN, AND E. T. DEWAR, *J. Sci. Food. Agr.*, 16 (1965) 573.
8 N. S. ANDERSON AND D. A. REES, in E. G. YOUNG AND J. L. MCLACHLAN (Eds.), *Proceedings of the Fifth International Seaweed Symposium*, Pergamon, Oxford, 1966, p. 329.
9 N. S. ANDERSON AND D. A. REES, *J. Chem. Soc.*, (1965) 5880.
10 J. WEIGL, J. R. TURVEY, AND W. YAPHE, in E. G. YOUNG AND J. L. MCLACHLAN (Eds.), *Proceedings of the Fifth International Seaweed Symposium*, Pergamon, Oxford, 1966, p. 329; see also, J. WEIGL AND W. YAPHE, *Can. J. Microbiol.*, 12 (1966) 939.
11 K. MORGAN AND A. N. O'NEILL, *Can. J. Chem.*, 37 (1959) 1201.
12 D. A. REES, *Ann. Rept. Progr. Chem. (Chem. Soc. London)*, 62 (1965) 469.
13 L. HOUGH, J. K. N. JONES, AND W. H. WADMAN. *J. Chem. Soc.*, (1950) 1702.
14 J. SAARNIO, E. NISKASAARI, AND G. GUSTAFSSON, *Suomen Kemistilehti*, 25B (1952) 25.
15 D. A. REES AND E. CONWAY, *Biochem. J.*, 84 (1962) 411.

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FURTHER OBSERVATIONS ON DERIVATIVES OF 1,6-ANHYDRO-β-D-TALOPYRANOSE; AN EXAMPLE OF ACETAL MIGRATION ACCOMPANYING HYDROLYSIS

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ABSTRACT

2-*O*-Acetyl-1,6-anhydro-3,4-*O*-isopropylidene-β-D-galactopyranose and 1,6-anhydro-3,4-*O*-isopropylidene-2-*O*-methylthiomethyl-β-D-galactopyranose were identified as by-products in the methyl sulphoxide–acetic anhydride oxidation of 1,6-anhydro-3,4-*O*-isopropylidene-β-D-galactopyranose to 1,6-anhydro-3,4-*O*-isopropylidene-β-D-*lyxo*-hexopyranosulose (isolated as the stable 2,2-*gem*-diol derivative). Syntheses of 1,6-anhydro-3,4-*O*-isopropylidene-2-*O*-methyl-β-D-talopyranose and 1,6-anhydro-2-*O*-methyl-β-D-talopyranose from 1,6-anhydro-3,4-*O*-isopropylidene-β-D-talopyranose are described. The former compound exhibited unusual chromatographic behaviour. 1,6-Anhydro-2,3-*O*-isopropylidene-β-D-talopyranose was isolated from an incomplete hydrolysis of 1,6-anhydro-3,4-*O*-isopropylidene-β-D-talopyranose to 1,6-anhydro-β-D-talopyranose, indicating that acetal migration was accompanying hydrolysis. The 2,3-acetal was the major product of reaction of acetone with 1,6-anhydro-β-D-talopyranose in the presence of sulphuric acid.

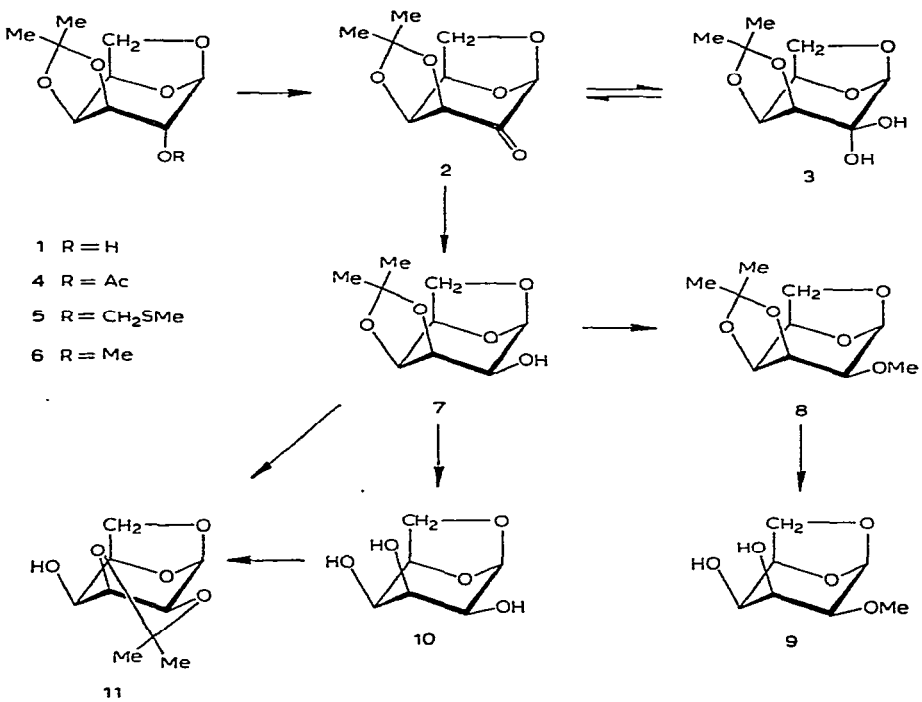
INTRODUCTION

In connection with other work[1], an unambiguous synthesis of 1,6-anhydro-3,4-*O*-isopropylidene-2-*O*-methyl-β-D-talopyranose (**8**) was desirable. An obvious route to the necessary 1,6-anhydro-3,4-*O*-isopropylidene-β-D-talopyranose (**7**) was to invert C-2 of the known[2] 1,6-anhydro-3,4-*O*-isopropylidene-β-D-galactopyranose (**1**) by an oxidation–reduction sequence[3]. When this work was completed, two other groups[4,5] independently reported this inversion; oxidation of compound **1** with ruthenium tetraoxide or methyl sulphoxide–acetic anhydride gave 1,6-anhydro-3,4-*O*-isopropylidene-β-D-*lyxo*-hexopyranosulose (**2**) which was stereospecifically reduced to compound **7** with sodium borohydride. The results obtained in the present work supplement these observations.

DISCUSSION

Oxidation of compound **1** with methyl sulphoxide–acetic anhydride proceeded smoothly, and the product was isolated from ether, not as the ketone **2**, but as the

gem-diol **3**. The previous workers[4,5] have described only the ketonic form **2**. The *gem*-diol **3** could be converted into the ketone **2** by vacuum sublimation, but, on standing in air, the ketone **2** reverted to the *gem*-diol **3**. This behaviour is in keeping with previous work[6], in which it was suggested that formation of *gem*-diols from pyranosuloses was particularly favourable due to an electron-withdrawing effect from C-1. Reduction of the *gem*-diol **3**, or the crude product isolated from the oxidation by removal of solvents *in vacuo*, with sodium borohydride gave the required *talo*-compound **7**. Two by-products were observed in the oxidation. One was the known[2] 2-*O*-acetyl-1,6-anhydro-3,4-*O*-isopropylidene-β-D-galactopyranose (**4**). The second compound was identified as 1,6-anhydro-3,4-*O*-isopropylidene-2-*O*-methylthiomethyl-β-D-galactopyranose (**5**). The n.m.r. spectrum contained a singlet at τ 7.91 (SCH_3), and, on desulphurisation, compound **5** gave a product that was chromatographically indistinguishable from 1,6-anhydro-3,4-*O*-isopropylidene-2-*O*-methyl-β-D-galactopyranose[7] (**6**). Similar thio-ether derivatives have been isolated as by-products in methyl sulphoxide–acetic anhydride oxidations of alkaloids and steroids[8] and, very recently, of carbohydrates[9]. Their mode of formation has been discussed[8].



Methylation of compound **7** with methyl sulphate gave the required methyl ether **8**; the n.m.r. spectrum of ether **8** contained a singlet at τ 6.62 (OCH_3), and the mass spectrum was virtually identical with that of the isomeric *galacto*-compound **6**. Mild hydrolysis with 80% acetic acid removed the acetal group to give 1,6-anhydro-2-*O*-methyl-β-D-talopyranose (**9**). More-vigorous hydrolysis with dilute hydrochloric acid gave a reducing syrup, presumably 2-*O*-methyl-D-talose, since it gave D-*lyxo*-

hexose phenylosazone on treatment with phenylhydrazine–acetic acid. 2-*O*-Methyl sugars are known[10] to give osazones under these conditions.

A curious feature of the methyl ether **8** was its mobility on t.l.c. In one system (alumina–ether), this was barely greater than that of the parent alcohol **7**, and in another (silica gel–ether), it was actually less. The related *galacto*-compounds behaved as expected in these systems, with the methyl ether **6** having approximately twice the mobility of the alcohol **1**. The reason for this anomalous behaviour is not apparent, but it should be noted that the methyl ether **8** also has a relatively high melting point (see Table I for R_F values and melting points).

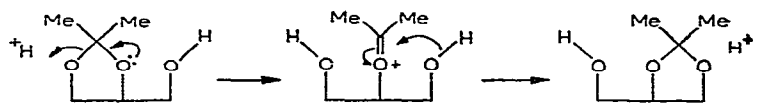
TABLE I

MELTING POINTS AND R_F VALUES FOR SOME DERIVATIVES OF D-GALACTOSE AND D-TALOSE

Compound	*M.p. (degrees)*	R_F[a]	R_F[b]
1,6-Anhydro-3,4-*O*-isopropylidene-β-D-galactopyranose (**1**)	154–156	0.49	0.35
1,6-Anhydro-3,4-*O*-isopropylidene-2-*O*-methyl-β-D-galactopyranose (**6**)	37– 39	0.77	0.87
1,6-Anhydro-3,4-*O*-isopropylidene-β-D-talopyranose (**7**)	120–122	0.57	0.47
1,6-Anhydro-3,4-*O*-isopropylidene-2-*O*-methyl-β-D-talopyranose (**8**)	99–101	0.39	0.62

[a] For silica gel–ether. [b]For alumina–ether.

The use of 80% acetic acid for removing the acetal group from the methyl ether **8** is convenient, since the reagent is without effect on the anhydro structure. In a similar manner, compound **7** was hydrolysed to 1,6-anhydro-β-D-talopyranose (**10**) without any further hydrolysis to D-talose. When the progress of this hydrolysis was followed by t.l.c., the transient appearance of a third compound in addition to **7** and **10** was observed. Its mobility was only slightly less than that of the starting material **7**. It was isolated by preparative t.l.c. and found to be 1,6-anhydro-2,3-*O*-isopropylidene-β-D-talopyranose[4] (**11**), indicating that acetal migration was competing with hydrolysis. Since it is unlikely that acetone would recombine with compound **10** n the aqueous conditions, the reaction is presumably intramolecular:



When the acetal fraction was isolated from an incomplete hydrolysis, the proportion of 2,3-acetal **11** to 3,4-acetal **7** was approximately 3:1 (estimated by g.l.c.). Treatment of 1,6-anhydro-β-D-talopyranose **10** or the 3,4-acetal **7** with acetone containing sulphuric acid gave predominantly the 2,3-acetal **11**, the proportion of

3,4-acetal **7** formed was less than 10%. Evidently the 2,3-acetal **11** is the preferred isomer, possibly because of steric interactions between the C-6 methylene group and the *endo*-methyl group in the 3,4-acetal **7**. A related acetal rearrangement in acidified acetone has been observed recently in the case of 1,2:4,5-di-*O*-isopropylidene-D-psicopyranose, which has a similar arrangement of acetal and hydroxyl groups[9].

It is generally assumed that acetals do not migrate under hydrolysis conditions. Support for this assumption came from experiments with acetals in which hydrolyses were followed spectroscopically when, even in favourable cases, no evidence for migration was obtained[11]. An exception[12] is 2,4-*O*-ethylidene-D-erythrose which rearranges to the 2,3-acetal in dilute sulphuric acid. However, in this case, the acetal rearrangement is accompanied by a cyclisation of the sugar chain to give the favourable[13] bicyclic 2,3-*O*-ethylidene-D-erythrofuranose. The present result appears to be the first example of a simple acetal rearrangement in aqueous solution.

EXPERIMENTAL

General methods. — Unless stated otherwise, R_F values refer to paper chromatography (ascending development) on Whatman No. 1 paper in the system butyl alcohol–water (86:14, v/v). α-Glycols were detected by the periodate–Schiff reagent[14]; reducing sugars were detected with aniline hydrogen phthalate[15]. Silica gel G, Merck, and alumina G, Merck, were used for thin-layer chromatography (t.l.c.); compounds were detected by charring with sulphuric acid. Compounds were converted into their trimethylsilyl derivatives for gas–liquid chromatography on 3% SE-52 silicone rubber gum on 80–100 mesh acid-washed silanised "Celite". N.m.r. spectra were measured for solutions in carbon tetrachloride or deuteriochloroform, with tetramethylsilane as internal standard.

Oxidation of 1,6-anhydro-3,4-O-isopropylidene-β-D-galactopyranose (**1**). — A solution of anhydride **1** (1.0 g) in methyl sulphoxide (10 ml) and acetic anhydride (7 ml) was left for 22 h at room temperature, after which the solvents were removed *in vacuo*. A solution of the residue in ether (10 ml) deposited the *gem*-diol **3** (0.78 g) as needles which, when recrystallised from ether, had m.p. 104–109°; $[\alpha]_D$ −55° (*c* 0.76, water), −87° (*c* 0.64, dichloromethane); ν_{max} 3320, 3480 cm^{-1} (OH) (Found: C, 49.3, H, 6.3. $C_9H_{14}O_6$ calc.: C, 49.5; H, 6.5%). A portion of the hydrate **3** was vacuum-sublimed (100°/0.1 mm) to give the crystalline ketone **2**, m.p. 94–97°, $[\alpha]_D$ −86° (*c* 1.1, dichloromethane) (lit.[4], m.p. 92–93°, $[\alpha]_D$ −89°; lit.[5], m.p. 96–97°, $[\alpha]_D$ −110°), ν_{max} 1755 cm^{-1} (C=O) (Found: C, 54.4; H, 6.2. $C_9H_{12}O_5$ calc.: C, 54.0; H, 6.0%). On standing in the open laboratory overnight, the ketone **2** reverted to the *gem*-diol **3**, m.p. and mixed m.p. 104–109°.

The mother liquors, on standing, gave a small crop of 2-*O*-acetyl-1,6-anhydro-3,4-*O*-isopropylidene-β-D-galactopyranose (**4**), m.p. and mixed m.p. 135–136° after recrystallisation from isopropyl ether.

1,6-Anhydro-3,4-O-isopropylidene-2-O-methylthiomethyl-β-D-galactopyranose (**5**). — Chromatography, on silica gel, of the mother liquors from the preceding experiment and elution with benzene–ether (9:1) gave the thio-ether **5** as a colourless oil

(0.12 g), b.p. 120° (bath)/0.2 mm, $[\alpha]_D$ −146° (*c* 1.4, chloroform), n_D 1.4985; τ 4.92 (1-proton singlet, H-1), 5.48 (2-proton singlet, OCH_2S), 7.91 (3-proton singlet, SCH_3) (Found: C, 50.4; H, 6.7; S, 12.2. $C_{11}H_{18}O_5S$ calc.: C, 50.4; H, 6.9; S, 12.2%).

A sample of the thio-ether **5** (65 mg) was desulphurised by being heated under reflux in ethanol (4 ml) containing Raney nickel (0.5 ml) for 70 min. T.l.c. indicated a single product having the same mobility as 1,6-anhydro-3,4-*O*-isopropylidene-2-*O*-methyl-β-D-galactopyranose (**6**).

1,6-Anhydro-3,4-O-isopropylidene-β-D-talopyranose (**7**). — (*a*) *From the* gem-*diol* **3**. Sodium borohydride (200 mg) was added to a solution of the *gem*-diol **3** (200 mg) in ethanol (2 ml) and water (2 ml). After 45 min, most of the ethanol was removed by evaporation, and the aqueous solution was extracted with dichloromethane. The extract was dried and evaporated, and recrystallisation of the residue from isopropyl ether gave the product **7** (164 mg), m.p. 120–122°, $[\alpha]_D$ −122° (*c* 0.7, chloroform) (lit.[4], m.p. 112–113°, $[\alpha]_D$ −112.5°; lit.[5], m.p. 117–118°, $[\alpha]_D$ −115°); τ 4.74 (1-proton doublet, H-1, $J_{1,2}$ ~3 Hz) (Found: C, 53.4; H, 6.9. $C_9H_{14}O_5$ calc.: C, 53.5; H, 7.0%).

(*b*) *From the crude oxidation product.* The crude oxidation product (from **1**, 1.0 g) was dissolved in ethanol (10 ml) and water (10 ml), and treated with sodium borohydride (1.0 g). After 45 min, work-up as in (*a*) gave the product **7** (0.57 g), m.p. 120–122°.

1,6-Anhydro-3,4-O-isopropylidene-2-O-methyl-β-D-talopyranose (**8**). — Methyl sulphate (0.6 ml) was added to acetone (16 ml) containing the acetal **7** (240 mg) and crushed potassium hydroxide (0.7 g). The mixture was stirred under reflux for 3 h. N Sodium hydroxide (16 ml) was added, and heating was continued for a further hour. The acetone was evaporated off, and the aqueous solution was neutralised with solid carbon dioxide before being extracted with dichloromethane. Evaporation of the dried extract and recrystallisation of the residue from light petroleum gave the methyl ether **8** (220 mg), m.p. 99–101°, $[\alpha]_D$ −53° (*c* 0.6, methanol); τ 4.85 (1-proton doublet, H-1, $J_{1,2}$ ~3 Hz) 6.62 (3-proton singlet, OCH_3) (Found: C, 56.0; H, 7.45. $C_{10}H_{16}O_5$ calc.: C, 55.50; H, 7.45%).

1,6-Anhydro-2-O-methyl-β-D-talopyranose (**9**). — A solution of the acetal **8** (100 mg) in 80% acetic acid (2 ml) was kept for 1 h at 90°. Evaporation of solvents left a crystalline residue. Recrystallised from isopropyl ether, the product **9** (57 mg) had m.p. 90–92°, $[\alpha]_D$ −50° (*c* 0.8, methanol), R_F 0.46 (Found: C, 48.1; H, 7.0. $C_7H_{12}O_5$ calc.: C, 47.7; H, 6.9%).

Hydrolysis of 1,6-anhydro-2-O-methyl-β-D-talopyranose (**9**). — The anhydro compound **9** (90 mg) in N sulphuric acid (2 ml) was kept for 4 h at 100°. The solution was passed through Dowex-1 (OAc^-, 5 ml) and evaporated to dryness. The syrupy residue (100 mg), presumably 2-*O*-methyl-D-talose, had $[\alpha]_D$ −4° (*c* 0.9, water), R_F 0.26.

A solution of the syrup (20 mg) in water (0.3 ml) containing phenylhydrazine (0.04 ml) and acetic acid (0.025 ml) was kept for 2 h at 100°. The yellow crystals, which started to form within 1 h, were filtered off, and washed successively with 10%

acetic acid, water, ethanol, and ether to give D-*lyxo*-hexose phenylosazone (6 mg), m.p. and mixed m.p. 180–185°; the infrared spectrum was identical with that of an authentic sample.

1,6-Anhydro-β-D-talopyranose (**10**). — A solution of the acetal **7** (50 mg) in 80% acetic acid (1 ml) was kept for 30 min at 90°. Solvents were evaporated, and the product **10** (29 mg), recrystallised from acetone, had m.p. 202–205°, $[\alpha]_D$ −82° (*c* 0.7, water) (lit.[4], m.p. 185–186°, $[\alpha]_D$ −80°; lit.[5], m.p. 206–208°, $[\alpha]_D$ −80.5°), R_F 0.34 (Found: C, 44.3; H, 6.2. $C_6H_{10}O_5$ calc.: C, 44.4; H, 6.2%).

Reaction of 1,6-anhydro-β-D-talopyranose (**10**) *with acetone containing sulphuric acid.* — The anhydro compound **8** (80 mg) in acetone (8 ml) containing conc. sulphuric acid (0.08 ml) was kept for 30 min at room temperature. After neutralisation (Na_2CO_3), the solution was evaporated to a crystalline residue. A portion was analysed by g.l.c., and the remainder was recrystallised from isopropyl ether to give the 2,3-acetal **11** (62 mg), m.p. 104–107°, $[\alpha]_D$ −35° (*c* 0.7, chloroform) (lit.[4], m.p. 107–108° $[\alpha]_D$ −34°), τ 4.80 (1-proton doublet, H-1, $J_{1,2}$ ~3 Hz) (Found: C, 53.65; H, 7.1. $C_9H_{14}O_5$ calc.: C, 53.4; H, 7.0%).

Reaction of 1,6-anhydro-3,4-O-isopropylidene-β-D-talopyranose (**7**) *with acetone containing sulphuric acid.* — The acetal **7** (100 mg) was treated with acetone (7 ml) containing sulphuric acid (0.07 ml), as described in the previous experiment, to give the 2,3-acetal **11** (60 mg), m.p. 104–107°.

Partial hydrolysis of acetal 7. — A solution of the acetal **7** (100 mg) was kept in 80% acetic acid (2 ml) for 5 min at 90°. After removal of solvents, the residue was partitioned between dichloromethane and water. Evaporation of the dried dichloromethane extract gave a crystalline residue (57 mg); t.l.c. (silica gel–ether) indicated the two acetals **7**, R_F 0.53, and **11**, R_F 0.46. A portion of the residue was analysed by g.l.c., and a pure sample of the 2,3-acetal **11** (6 mg), m.p. and mixed m.p. 104–107°, was obtained by preparative t.l.c. (silica gel–ether), and recrystallisation from light petroleum.

REFERENCES

1 N. A. HUGHES, *Chem. Commun.*, (1967) 1072.
2 R. M. HANN AND C. S. HUDSON, *J. Amer. Chem. Soc.*, 64 (1942) 2435.
3 W. SOWA AND G. H. S. THOMAS, *Can. J. Chem.*, 44 (1966) 836.
4 D. HORTON AND J. S. JEWELL, *Carbohyd. Res.*, 5 (1967) 149.
5 K. HEYNS, J. WEYER, AND H. PAULSEN, *Ber.*, 100 (1967) 2317.
6 P. M. COLLINS AND W. G. OVEREND, *J. Chem. Soc.*, (1965) 3448.
7 S. P. JAMES, F. SMITH, M. STACEY, AND L. F. WIGGINS, *J. Chem. Soc.*, (1946) 625.
8 J. D. ALBRIGHT AND L. GOLDMAN, *J. Amer. Chem. Soc.*, 89 (1967) 2416.
9 K. JAMES, A. R. TATCHELL, AND P. K. RAY, *J. Chem. Soc.*, (1967) 2681.
10 W. N. HAWORTH, E. L. HIRST, AND E. G. TEECE, *J. Chem. Soc.*, (1931) 2858.
11 N. BAGGETT, K. W. BUCK, A. B. FOSTER, R. JEFFERIS, B. H. REES, AND J. M. WEBBER, *J. Chem. Soc.*, (1965) 3382.
12 C. E. BALLOU, *J. Amer. Chem. Soc.*, 82 (1960) 2585.
13 J. A. MILLS, *Advan. Carbohyd. Chem.*, 10 (1956) 1.
14 J. BADDILEY, J. G. BUCHANAN, R. E. HANDSCHUMACHER, AND J. F. PRESCOTT, *J. Chem. Soc.*, (1956) 2818.
15 S. M. PARTRIDGE, *Nature*, 164 (1949) 443.

Notes

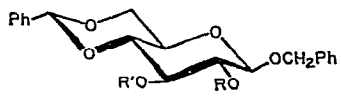
Substitution at the 2- or 3-hydroxyl group of D-glucose

The preparation of compounds having an acid-labile substituent on the 2- or 3-hydroxyl group of D-glucose has at times been hampered by the paucity of suitably protected derivatives. Coxon and Hall[1] have shown that the 3-hydroxyl group of 1,2:4,6-di-*O*-benzylidene-α-D-glucopyranose is axially attached, and may hence be expected to be quite unreactive. Also, although 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose[2] can be alkylated, the action of an acid is required for its deacetalation. The readily available 1,2,4,6-tetra-*O*-acetyl-β-D-glucopyranose[3] is, perhaps, the most useful derivative, but it sometimes displays a diminished reactivity towards potential substituting groups if they are bulky, and this problem is aggravated by changing to the corresponding tetra-*O*-benzoyl compound[4]. In the synthesis of nigerose[5], 4,6-*O*-benzylidene-1,2-*O*-isopropylidene-α-D-glucose was employed, but it is doubtful that an intersaccharidic linkage of greater lability to acids would have survived the removal of the isopropylidene group. Methyl 4,6-*O*-benzylidene-α-D-glucopyranoside seems to become substituted mostly at O-2, as shown in the synthesis of sophorose[6,7], but also requires acidic conditions for removal of the glycosidic methyl group. Methyl 2-*O*-acetyl-4,6-*O*-benzylidene-α-D-glucopyranoside[8], having an equatorially attached 3-hydroxyl group[9], can be used in the synthesis of (1→3)-linked disaccharides[10], but removal of the glycosidic methyl group again requires the rather drastic action of an acid.

Turning now to the D-glucose derivatives that are protected at all of the hydroxyl groups except that at C-2, a somewhat similar situation is encountered. Although 1,3,4,6-tetra-*O*-acetyl-α-D-glucose[11,12] is fairly readily available, albeit in low yield, compounds of this nature have been shown[13,14] to undergo very ready acyl migration from O-1 to O-2. Methyl 3-*O*-acetyl-4,6-*O*-benzylidene-α-D-glucopyranoside[8] cannot be used for the preparation of acid-labile, reducing disaccharides.

In the course of some synthetic work, we prepared the 2-monoacetate (**2**) and 3-monoacetate (**3**) of benzyl 4,6-*O*-benzylidene-β-D-glucopyranoside[15] (**1**) by the method used by Jeanloz and Jeanloz[8] for synthesis of the two monoacetates of the corresponding methyl D-glucoside. With compound **1**, acetylation with *ca.* one molecular equivalent of acetic anhydride led to quite different results, in that the 3-hydroxyl group was preferentially acetylated, instead of that at C-2. Also, the reaction mixture contained a substantial proportion of the known diacetate[16], as well as unreacted starting material. However, both the 3-acetate and the 2-acetate of benzyl 4,6-*O*-benzylidene-β-D-glucopyranoside could be prepared in practicable yield, and isolated in crystalline form. Their structures were confirmed by methylation

according to the procedure of Mastronardi *et al.*[17], followed by hydrolysis, trimethylsilylation, and g.l.c. of the trimethylsilyl ethers[18].



2. R = $COCH_3$; R' = H
3. R = H; R' = $COCH_3$

Since all of the masking groups in these two intermediates can be removed under the mild conditions of (*a*) catalytic transesterification and (*b*) hydrogenation over palladium black, they appear promising for the synthesis of acid-labile 2-*O*- and 3-*O*-substituted derivatives of D-glucose.

EXPERIMENTAL

Benzyl 3-O- and 2-O-acetyl-4,6-O-benzylidene-β-D-glucopyranoside. — Benzyl 4,6-*O*-benzylidene-β-D-glucopyranoside[15] (**1**) (7.0 g) was dissolved in pyridine (60 ml) containing anhydrous pyridinium hydrochloride prepared from 3 ml of pyridine. Drierite was added to ensure an anhydrous reaction medium, and the mixture was cooled to 0°. Acetic anhydride (2.0 ml) was added slowly, and the mixture was allowed to warm slowly to room temperature. After several days, water was added, and the mixture was extracted with dichloromethane. The extract was successively washed with cold, aqueous 1.5M sulfuric acid and saturated, aqueous sodium hydrogen carbonate solution, dried (sodium sulfate), and evaporated to a syrup (7.5 g). T.l.c. [silica gel, 3:1 (v/v) benzene–ether] showed unreacted starting material, two spots for the monoacetates, and a spot for the known diacetate[16]. Column chromatography (silica gel), with the same eluant, was somewhat hampered by the fact that the two monoacetates have very similar R_F values. but they could be separated, although losses occurred owing to overlapping of bands.

The material, eluted after the diacetate, was recrystallized from 1:1 (v/v) ether–cyclohexane to yield compound **3** (1.120 g, 14.4%), m.p. 145–146°, $[\alpha]_D^{20}$ −80.6° (*c* 1.6, chloroform).

Anal. Calc. for $C_{22}H_{24}O_7$: C, 65.99; H, 6.04. Found: C, 66.00; H, 6.20.

The third compound to be eluted from the column was recrystallized from ether–cyclohexane to yield pure **2** (390 mg, 5%), m.p. 167–168°, $[\alpha]_D^{20}$ −98.4° (*c* 1.14, chloroform).

Anal. Calc. for $C_{22}H_{24}O_7$: C, 65.99; H, 6.04. Found: C, 66.25; H, 6.15.

Methylation experiments. — A sample (*ca.* 15 mg) of **3** was dissolved in ether, and methylated with diazomethane in the presence of boron trifluoride as described by Mastronardi *et al.*[17]. After 0.5 h in acetone–Dry Ice, t.l.c. [3:1 (v/v) benzene–ether] revealed that the methylation was almost complete. The reaction mixture was successively washed with aqueous sodium hydrogen carbonate and water, dried

(sodium sulfate), and evaporated to a crystalline residue which was recrystallized from ether–cyclohexane; m.p. 167–169°. A solution of the methyl ether in M hydrogen chloride in methanol was heated in a steam bath for 5 h, and evaporated to dryness; the product was dissolved in 2M hydrochloric acid, and the solution was reheated in the same way. Evaporation and drying gave a syrup that was converted into its tetrakis(trimethylsilyl) derivative. G.l.c. on a column (0.25 in × 6 ft) of 3% SE 52 on Gas-Chrom A showed peaks indistinguishable from those shown by the tetrakis-(trimethylsilyl) derivative of authentic 2-*O*-methyl-D-glucose. Under the conditions used, the 2- and 3-methyl ethers give completely resolved peaks.

A sample (*ca.* 15 mg) of **2** was methylated as for **3**. T.l.c. showed some residual **2**, but the sample consisted mostly of methylated product. Methanolysis and hydrolysis as before, followed by g.l.c. of the tetrakis(trimethylsilyl) derivative, showed that 3-*O*-methyl-D-glucose was present, as well as a minor proportion of D-glucose. There was a minor peak (unidentified) for a compound more volatile than the per(trimethylsilyl) derivative of either 2-*O*- or 3-*O*-methyl-D-glucose.

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REFERENCES

1 B. COXON AND L. D. HALL, *Tetrahedron*, 20 (1964) 1685.
2 E. RECONDO AND H. RINDERKNECHT, *Helv. Chim. Acta*, 43 (1960) 1653.
3 K. FREUDENBERG AND E. PLANKENHORN, *Ann.*, 536 (1938) 257.
4 C. P. J. GLAUDEMANS, unpublished results.
5 A. M. GAKHOKIDZE, *Zh. Obshch. Khim.*, 16 (1946) 1923; *Chem. Abstr.*, 41 (1947) 6210.
6 K. FREUDENBERG AND K. SOFF, *Ber.*, 69 (1936) 1245.
7 B. COXON AND H. G. FLETCHER, JR., *J. Org. Chem.*, 26 (1961) 2892.
8 R. W. JEANLOZ AND D. A. JEANLOZ, *J. Amer. Chem. Soc.*, 79 (1957) 2579.
9 B. COXON, *Tetrahedron*, 21 (1965) 3481.
10 N. K. KOCHETKOV, A. J. KHORLIN, AND A. F. BOCHKOV, *Tetrahedron*, 23 (1967) 693.
11 R. U. LEMIEUX AND G. HUBER, *Can. J. Chem.*, 31 (1955) 1040.
12 W. M. CORBETT, J. KIDD, AND A. M. LIDDLE, *J. Chem. Soc.*, (1960) 616.
13 C. P. J. GLAUDEMANS AND H. G. FLETCHER, JR., *J. Org. Chem.*, 29 (1964) 3286.
14 W. A. BONNER, *J. Org. Chem.*, 24 (1959) 1388.
15 A. KLEMER, *Chem. Ber.*, 92 (1959) 218.
16 A. L. RAYMOND, R. S. TIPSON, AND P. A. LEVENE, *J. Biol. Chem.*, 130 (1939) 47.
17 I. O. MASTRONARDI, S. M. FLEMMATTI, J. O. DEFERRARI, AND E. G. GROS, *Carbohyd. Res.*, 3 (1966) 177.
18 C. C. SWEELEY, R. BENTLEY, M. MAKITA, AND W. W. WELLS, *J. Amer. Chem. Soc.*, 85 (1963) 2497.

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Preparation of carboxymethylchitin

The hydrolysis of chitin by lysozyme[1] suggests that derivatives of chitin might also be hydrolyzed by this enzyme[2]. Chitin derivatives, however, are difficult to prepare due to the limited alkali-swelling properties of chitin. This communication reports the preparation of a carboxymethylchitin whose properties are sufficiently defined and reproducible to allow the polymer to be used as a potential lysozyme substrate. The carboxymethylchitin was prepared by a new procedure which is more rapid than earlier methods[3,4].

EXPERIMENTAL

Chitin (4 g) was mixed with dimethyl sulfoxide (20 ml) for 24 h. The mixture was crushed to a pulpy consistency, and the excess solvent was removed by filtration. The chitin filter-cake was then washed with ethyl alcohol, dried, and ground to a powder. Chitin was added, in small portions, to 65% NaOH (50 ml), and the slurry was stirred for 1 h. The mixture was filtered, and the cake pressed to a wet weight of approximately 14 g.

This material was added to 2-propanol (50 ml), containing monochloroacetic acid (6 g), and the mixture was stirred for 1 h. The material isolated on filtration was added to water (200 ml), and the pH adjusted to neutrality with conc. HCl. The mixture was stirred for 1 h. The resulting viscous solution was passed through glass wool, and then added slowly to one liter of acetone. The sodium salt of carboxymethylchitin thus obtained was washed with absolute ethyl alcohol and dried (3.5 g).

The carboxymethylchitin was again dissolved in water (120 ml), and the solution stirred for 1 h. The brown, viscous solution was centrifuged at 4000 rpm for 15 min and the supernatant was slowly added to 1 liter of acetone. The fibrous precipitate was washed with ethanol.

The carboxymethylchitin was crushed to a fine powder and extracted with 95% ethyl alcohol for 2 h in a Soxhlet extractor. The product was dried (1.8 g) and stored in a desiccator over phosphorus pentoxide. The degree of substitution was determined as 0.99 with an acid wash-titration technique[5].

Anal. Calc. for $(C_{10}H_{14}O_7NNa)_n$: C, 42.5; H, 4.95; N, 4.94; acetyl, 15.2. Found: C, 41.44; H, 4.85; N, 4.63; acetyl, 14.5*. The analysis was calculated assuming a degree of substitution of one.

A degree of polymerization of 200 was determined by measuring the reducing-sugar value[6] before and after complete acid hydrolysis of the polymer. The infrared spectra of carboxymethylchitin films were determined on the free acid and sodium

*Analysis by Midwest Microlab, Indianapolis, Indiana.

salt forms of the polysaccharide and are presented in Fig. 1. The ratio of the band intensities at 1736 and 1560 cm^{-1} may be considered proportional to the carboxylic acid and hexosamine content of the polysaccharide[7], and was found to be 1.04. This indicates that one *N*-acetyl group corresponds to approximately one carboxymethyl group per repeating unit of the carboxymethylchitin polymer.

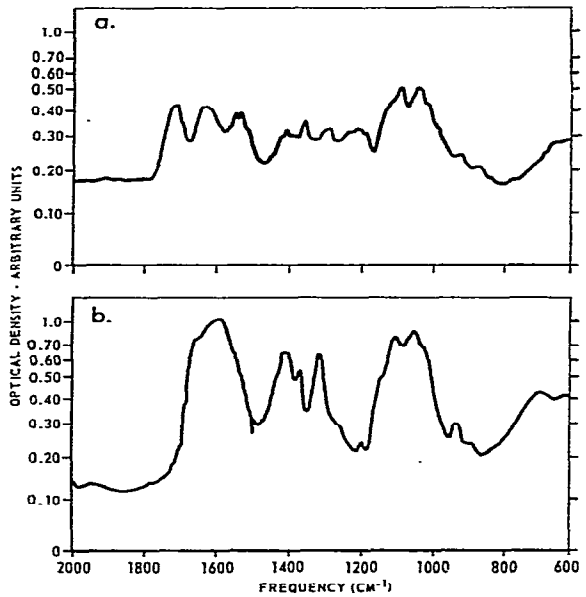

Fig. 1. Infrared spectra of carboxymethylchitin; a. free acid; b. salt form.

The hydrolysis of this chitin derivative by lysozyme has been studied by determination of the viscosity and of the change in reducing power. The compound is a substrate for lysozyme, and the results of this study will be reported at a later date.

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REFERENCES

1 L. R. BERGER AND R. S. WEISER, *Biochim. Biophys. Acta*, 26 (1957) 517.
2 K. HAMAGUCHI AND M. FUNATSU, *J. Biochem.* (Tokyo), 46 (1959) 1659.
3 S. OKIMASU, *Nippon Nogeikagaku Kaishi*, 32 (1958) 303: *Chem., Abstr.*, 54 (1960) 10458.
4 S. N. DANILOV AND E. A. PLISKO, *Zh. Obshch. Khim.*, 31 (1961) 469: *Chem., Abstr.*, 55 (1961) 23356.
5 R. W. EYLER, E. D. KLUG, AND F. DIEPHUIS, *Anal. Chem.*, 19 (1947) 24.
6 S. DYGERT, L. H. LI, D. FLORIDA, AND J. A. THOMA, *Anal. Biochem.*, 13 (1965) 367.
7 S. F. D. ORR, *Biochim. Biophys. Acta*, 14 (1954) 173.

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Some partially benzylated pentose derivatives

Over the course of the past few years, a number of partially benzylated derivatives of the pentoses have been prepared in this laboratory for a variety of purposes. Inasmuch as some of them may prove to be of utility for further syntheses at the hands of other researchers, we describe them briefly here.

Both of the anomeric methyl D-xylopyranosides (**1**) readily gave crystalline 2,3,4-tribenzyl ethers (**2**) and, on hydrolysis, these afforded a splendidly crystalline 2,3,4-tri-*O*-benzyl-D-xylopyranose. This ether showed a small levomutarotation in both of two solvent systems and is, therefore, like its D-glucopyranose analog[1], the α-D anomer (**3**). Its structure was confirmed through its reduction to 2,3,4-tri-*O*-benzylxylitol, a *meso* compound isolated as the bis(*p*-nitrobenzoate) (**4**). Benzoylation of **3** gave the two anomeric 1-*O*-benzoyl-2,3,4-tri-*O*-benzyl-D-xylopyranoses (**5**), but only the β-D anomer could be obtained in crystalline form.

Next, benzyl β-D-ribopyranoside[2] was benzylated to give its crystalline 2,3,4-tribenzyl ether (**6**). The cleavage of the glycosidic linkage in this compound was accomplished with hot, aqueous acetic acid—surprisingly mild conditions in comparison with those employed for the hydrolysis of benzyl 2,3,4,6-tetra-*O*-benzyl-α-D-glucopyranoside[3] and methyl 2,3,5-tri-*O*-benzyl-L-arabinofuranoside[4]. The product, 2,3,4-tri-*O*-benzyl-D-ribopyranose (**7**), was obtained as a chromatographically homogeneous syrup; it yielded a crystalline *p*-nitrobenzoate which was levorotatory and thus, probably, was the β-D anomer (β **8**).

Finally, we report some brief, but unusual, results obtained in the L-arabinofuranose series. On shaking a methanolic suspension of 2,3,5-tri-*O*-benzyl-1-*O*-*p*-nitrobenzoyl-β-L-arabinofuranose[4] and palladium black with hydrogen, it was noted that approximately three molecular equivalents of the gas were absorbed rapidly, and that the rate of absorption then slowed markedly. During the fast phase of the reduction, the mixture became thick because of the precipitation of a pale, yellow-orange compound. On purification, this was found to have the elemental composition of a tri-*O*-benzyl-*O*-*p*-nitrosobenzoylpentose; on hydrolysis, it gave 2,3,5-tri-*O*-benzyl-β-L-arabinofuranose[5] and a compound having properties corresponding to those of

REFERENCES

1 L. R. BERGER AND R. S. WEISER, *Biochim. Biophys. Acta*, 26 (1957) 517.
2 K. HAMAGUCHI AND M. FUNATSU, *J. Biochem.* (Tokyo), 46 (1959) 1659.
3 S. OKIMASU, *Nippon Nogeikagaku Kaishi*, 32 (1958) 303: *Chem., Abstr.*, 54 (1960) 10458.
4 S. N. DANILOV AND E. A. PLISKO, *Zh. Obshch. Khim.*, 31 (1961) 469: *Chem., Abstr.*, 55 (1961) 23356.
5 R. W. EYLER, E. D. KLUG, AND F. DIEPHUIS, *Anal. Chem.*, 19 (1947) 24.
6 S. DYGERT, L. H. LI, D. FLORIDA, AND J. A. THOMA, *Anal. Biochem.*, 13 (1965) 367.
7 S. F. D. ORR, *Biochim. Biophys. Acta*, 14 (1954) 173.

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Some partially benzylated pentose derivatives

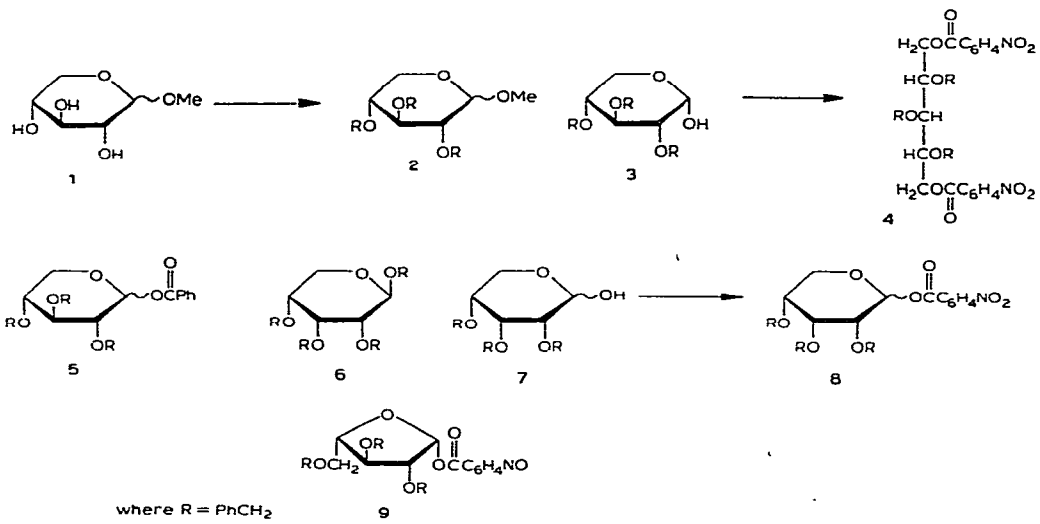
Over the course of the past few years, a number of partially benzylated derivatives of the pentoses have been prepared in this laboratory for a variety of purposes. Inasmuch as some of them may prove to be of utility for further syntheses at the hands of other researchers, we describe them briefly here.

Both of the anomeric methyl D-xylopyranosides (**1**) readily gave crystalline 2,3,4-tribenzyl ethers (**2**) and, on hydrolysis, these afforded a splendidly crystalline 2,3,4-tri-*O*-benzyl-D-xylopyranose. This ether showed a small levomutarotation in both of two solvent systems and is, therefore, like its D-glucopyranose analog[1], the α-D anomer (**3**). Its structure was confirmed through its reduction to 2,3,4-tri-*O*-benzylxylitol, a *meso* compound isolated as the bis(*p*-nitrobenzoate) (**4**). Benzoylation of **3** gave the two anomeric 1-*O*-benzoyl-2,3,4-tri-*O*-benzyl-D-xylopyranoses (**5**), but only the β-D anomer could be obtained in crystalline form.

Next, benzyl β-D-ribopyranoside[2] was benzylated to give its crystalline 2,3,4-tribenzyl ether (**6**). The cleavage of the glycosidic linkage in this compound was accomplished with hot, aqueous acetic acid—surprisingly mild conditions in comparison with those employed for the hydrolysis of benzyl 2,3,4,6-tetra-*O*-benzyl-α-D-glucopyranoside[3] and methyl 2,3,5-tri-*O*-benzyl-L-arabinofuranoside[4]. The product, 2,3,4-tri-*O*-benzyl-D-ribopyranose (**7**), was obtained as a chromatographically homogeneous syrup; it yielded a crystalline *p*-nitrobenzoate which was levorotatory and thus, probably, was the β-D anomer (β **8**).

Finally, we report some brief, but unusual, results obtained in the L-arabinofuranose series. On shaking a methanolic suspension of 2,3,5-tri-*O*-benzyl-1-*O*-*p*-nitrobenzoyl-β-L-arabinofuranose[4] and palladium black with hydrogen, it was noted that approximately three molecular equivalents of the gas were absorbed rapidly, and that the rate of absorption then slowed markedly. During the fast phase of the reduction, the mixture became thick because of the precipitation of a pale, yellow-orange compound. On purification, this was found to have the elemental composition of a tri-*O*-benzyl-*O*-*p*-nitrosobenzoylpentose; on hydrolysis, it gave 2,3,5-tri-*O*-benzyl-β-L-arabinofuranose[5] and a compound having properties corresponding to those of

p-nitrosobenzoic acid[6]. The product from the reduction is, presumably, 2,3,5-tri-*O*-benzyl-1-*O*-*p*-nitrosobenzoyl-β-L-arabinose (**9**); it was obtained in 18% yield. The facile isolation of this compound may be attributed to the fortuitous circumstance of its very low solubility in methanol. On treatment of 2,3,5-tri-*O*-benzyl-1-*O*-*p*-nitrobenzoyl-α-L-arabinofuranose[4] in methanol solution with hydrogen in the presence of palladium black, there was also found a sharp change in the rate of reduction when about 3.5 molar equivalents of the gas had been absorbed. In this case, however, the solution was clear and colorless. On concentration at 40°, the



solution yielded a strongly red syrup, suggesting the formation of an azo derivative through the condensation of a *p*-nitrosobenzoyl residue with a *p*-aminobenzoyl residue. It is possible that the somewhat abrupt decrease observed in the rate of reduction of these *p*-nitrobenzoates is occasioned by the formation of *p*-aminobenzoyl groups whose basicity would be expected to diminish the catalytic activity of palladium.

EXPERIMENTAL

Melting points correspond to corrected values.

*The anomeric methyl 2,3,4-tri-O-benzyl-D-xylopyranosides (α- and β-**2**).* — To a stirred solution of methyl α-D-xylopyranoside[7] (α-**1**, 7 g) in redistilled tetrahydrofuran (100 ml) was added powdered potassium hydroxide* (35 g), and then α-bromotoluene (43 ml) was added dropwise. The mixture was boiled for 15 h, under reflux with stirring and cooled, and the insoluble material was removed by filtration

*Hooker Chemical Corp., Niagara Falls, New York.

on a layer of Celite, the latter being washed thoroughly with dichloromethane. The combined filtrate and washings were concentrated *in vacuo*, finally at 250° (bath temp.) at <1 torr. On standing at room temperature for several hours, the pale-yellow syrup crystallized; two recrystallizations from isopropyl ether (5 ml/g) gave pure α-**2**, 15 g (81%), m.p. 61°, $[\alpha]_D^{20}$ +50.5° (*c* 5.22, dichloromethane).

Anal. Calc. for $C_{27}H_{30}O_5$: C, 74.63; H, 6.96. Found: C, 74.72; H, 7.16.

Methyl β-D-xylopyranoside[7] (β-**1**, 8 g) was treated in a similar way, to give β-**2** which was crystallized from methanol (10 ml/g); yield 20 g (94%), m.p. 70–72°, $[\alpha]_D^{20}$ +1.57° (*c* 2.64, dichloromethane).

Anal. Calc. for $C_{27}H_{30}O_5$: C, 74.63; H, 6.96. Found: C, 74.86; H, 7.18.

*2,3,4-Tri-*O*-benzyl-*α*-D-xylopyranose* (**3**). — A mixture of α-**2** (6 g), glacial acetic acid (120 ml), and 2M hydrochloric acid (48 ml) was heated for 15 h at 80°. After being cooled, the mixture was poured slowly into stirred ice-water (1 l), and the resulting crystals were removed by filtration. After treatment with decolorizing carbon, recrystallization from methanol (10 ml/g) gave **3** (3 g, 52%), m.p. 130–131°, $[\alpha]_D^{20}$ +25.1° (*c* 3.06, dichloromethane, 15 min after dissolution). In 9:1 (v/v) *p*-dioxane–water (*c* 2.48), it showed $[\alpha]_D^{20}$ +28.1 (15 min)→+24.6° (23 h), and in 9:1 (v/w) pyridine–phenol (*c* 6.1), it showed $[\alpha]_D^{20}$ +29.9 (extrapolated to zero time)→+23.1° (9 h).

Anal. Calc. for $C_{26}H_{28}O_5$: C, 74.26; H, 6.71. Found: C, 74.43; H, 6.77.

The compound was also obtained by similar hydrolysis of β-**2**.

*2,3,4-Tri-*O*-benzyl-1,5-di-*O*-p-nitrobenzoylxylitol* (**4**). — To a stirred solution of **3** (1 g) in methanol (25 ml), a solution of sodium borohydride (0.2 g) in methanol (5 ml) was added dropwise. Stirring was continued for 30 min after the addition of the sodium borohydride was complete, and the mixture was then kept overnight at room temperature. After acidification with 2M hydrochloric acid, the solution was deionized by successive passage through columns of Amberlite IR-120 (H^+) and Amberlite IR-45 (OH^-) ion-exchange resins. The solution and washings were combined and evaporated *in vacuo* to a syrup having $[\alpha]_D^{20}$ ±0° (*c* 2.88, dichloromethane). The syrupy 2,3,4-tri-*O*-benzylxylitol (1 g) was *p*-nitrobenzoylated in the usual way, to give **4**, as pale-yellow, short needles (from methanol), m.p. 98°.

Anal. Calc. for $C_{40}H_{36}N_2O_{11}$: C, 66.66; H, 5.03; N, 3.89. Found: C, 66.77; H, 4.28; N, 3.93.

*The anomeric 1-*O*-benzoyl-2,3,4-tri-*O*-benzyl-*D*-xylopyranoses* (α- and β-**5**). — A mixture of pyridine (45 ml) and benzoyl chloride (5 ml) was cooled to −15°, and stirred while 2,3,4-tri-*O*-benzyl-α-D-xylopyranose (**3**, 9 g) was added in small portions. After the addition was complete, stirring was continued for 0.5 h at −15°, and the mixture was kept overnight at room temperature. The excess of benzoyl chloride was decomposed by addition of water (5 ml) and, 0.5 h later, the mixture was diluted with dichloromethane. The dichloromethane layer was successively washed with water, cold 1.5M sulfuric acid, and cold, half-saturated, aqueous sodium hydrogen carbonate solution, dried (sodium sulfate), and evaporated *in vacuo* to a syrup; yield 10 g, $[\alpha]_D^{20}$ +43.0° (*c* 2.30, dichloromethane). The syrup was dissolved

in benzene (50 ml), and the solution was applied to a column of silica gel* (60 g) packed in benzene. Benzene was used for elution at a flow rate of 1 ml/min, and 100-ml portions of eluate were collected. After 600 ml of benzene had been used, the eluant was changed to 4:1 (v/v) benzene–ether. Fractions 2 to 6 were individually concentrated, and the material in them was found to show $[\alpha]_D^{20}$ +59.9 to 57.4° (dichloromethane); these fractions were pooled, and dried *in vacuo* at 56°. The 1-*O*-benzoyl-2,3,4-tri-*O*-benzyl-α-D-xylopyranose (α-**5**) (4.2 g, 38%) could not be induced to crystallize.

Anal. Calc. for $C_{33}H_{32}O_6$: C, 75.55; H, 6.15. Found: C, 74.46; H, 6.42.

Evaporation of fractions 7 and 8 afforded semicrystalline solids. The material from fraction 8 was dissolved in ethyl acetate (8 ml), the solution was filtered, and pentane (80 ml) was added to the filtrate to give 1 g (9%) of crystalline product. Two recrystallizations from isopropyl ether afforded pure 1-*O*-benzoyl-2,3,4-tri-*O*-benzyl-β-D-xylopyranose (β-**5**), m.p. 89–90°, $[\alpha]_D^{20}$ −44.1° (*c* 2.06, dichloromethane).

Anal. Calc. for $C_{33}H_{32}O_6$: C, 75.55; H, 6.15. Found: C, 75.45; H, 6.31.

An alternative procedure gave β-**5** in higher yield. 2,3,4-Tri-*O*-benzyl-α-D-xylopyranose (6 g) was dissolved in 60 ml of 9:1 (v/w) pyridine–phenol, and the solution was kept at 20° until mutarotation had ceased (20 h). The solvent was removed *in vacuo*, and the resulting colorless syrup was dissolved in dichloromethane (50 ml). The solution was washed successively with cold aqueous sodium hydroxide (2%), 1.5M sulfuric acid, and saturated aqueous sodium hydrogen carbonate, dried (sodium sulfate), and evaporated *in vacuo* (bath temp., 40°) to a syrup (5.8 g) which was benzoylated with benzoyl chloride (3 ml) and pyridine (30 ml). The reaction mixture was processed in the usual way, and the product was crystallized from isopropyl ether; yield 1.5 g (20%), m.p. 89–90°, $[\alpha]_D^{20}$ −44.6° (*c* 2.13, dichloromethane).

*Benzyl 2,3,4-tri-*O*-benzyl-β-*D*-ribopyranoside* (**6**). — To a solution of benzyl β-D-ribopyranoside[2] (5.00 g) in *p*-dioxane (13 ml) was added powdered potassium hydroxide (18 g), and the resulting suspension was stirred and boiled under reflux while α-chlorotoluene (18 ml) was added dropwise. Two hours after the addition was complete, the stirring was halted, and the mixture was cooled and subjected to steam distillation to remove *p*-dioxane, benzyl alcohol, and the excess of α-chlorotoluene. The mixture of crude product and water was twice extracted with ether, and the combined extracts were evaporated *in vacuo* to a syrup that was freed of benzyl ether at 110° (bath temp.) at 0.005 torr. On storage at −5°, the residue (8.75 g) crystallized, m.p. 42–45°; successive recrystallizations from 1:2 (v/v) ether–pentane (twice), absolute ethanol, isopropyl ether–pentane, and, finally, ether–pentane, gave **6**; m.p. 46–48° and $[\alpha]_D^{20}$ −30.1° (*c* 4.31, chloroform).

Anal. Calc. for $C_{33}H_{34}O_5$ C, 77.62; H, 6.71. Found: C, 77.80; H, 6.79.

*2,3,4-Tri-*O*-benzyl-*D*-ribopyranose* (**7**). — A solution of **6** (1.02 g) in a mixture of glacial acetic acid (6 ml) and water (1 ml) was heated for 32.5 h at 95°, water being

*Silica gel, Grade 980 (60-200 mesh), from Davison Chemical Co., Baltimore, Md., was used without pretreatment.

added in 0.5-ml portions at 3 min, 5 h, 7 h, 26 h, and 32 h. The progress of the hydrolysis was followed by t.l.c. on Silica Gel G (E. Merck) with 99:1 (v/v) dichloromethane–methanol. After being cooled, the mixture was poured into a large volume of water, and the crude product was extracted with dichloromethane. The extract was washed with saturated, aqueous sodium hydrogen carbonate solution, dried (magnesium sulfate), and evaporated *in vacuo* to a syrup that was chromatographed on a column of silica gel (200 g, E. Merck No. 7734), with 9:1 (v/v) benzene–ether, and collection of 20-ml portions of eluate. Fraction 35 contained benzyl alcohol; fraction 40 yielded material having chromatographic properties similar to those of **6**. Fractions 62 to 79 gave a positive test when t.l.c. samples were sprayed with *p*-anisidine hydrochloride and heated; they were pooled, and concentrated to a syrup that reduced Benedict solution; yield 0.177 g, $[\alpha]_D^{20}$ −1.5° (*c* 1.77, dry dichloromethane), strong i.r. absorption at 3500 cm^{-1} (OH).

Anal. Calc. for $C_{26}H_{28}O_5$: C, 74.26; H, 6.71. Found: C, 74.52; H, 6.49.

p-Nitrobenzoylation of **7** gave, from absolute ethanol, crystalline 2,3,4-tri-*O*-benzyl-1-*O*-*p*-nitrobenzoyl-D-ribopyranose (**8**); m.p. 110–111°, $[\alpha]_D^{20}$ −46° (*c* 1.09, chloroform); recrystallization from ethanol–pentane failed to change its m.p.

Anal. Calc. for $C_{33}H_{31}NO_8$: C, 69.58; H, 5.49. Found: C, 69.72; H, 5.41.

2,3,5-Tri-O-benzyl-1-O-p-nitrosobenzoyl-β-L-arabinofuranose (**9**). — A suspension of 2,3,5-tri-*O*-benzyl-1-*O*-*p*-nitrobenzoyl-β-D-arabinofuranose[4] {4.6 g, m.p. 76°, $[\alpha]_D^{20}$ +43.1° (*c* 3.56, dichloromethane)} in methanol (100 ml) was treated with palladium black [prepared from palladium chloride (0.303 g) by the method of Schmidt and Staab[8]] and shaken under an atmosphere of hydrogen. The absorption of hydrogen was initially rapid, 585 ml (corresponding to 2.9 molar equivalents) being taken up in 1 h; thereafter, it became much slower. By the time that this rate change was evident, the mixture had become a pale yellow-orange slurry. Catalyst and solid product were removed on a fluted filter and the solid product was lixiviated from the catalyst with dichloromethane. Evaporation of the dichloromethane solution *in vacuo* (bath temp. 40°) afforded a pale-orange residue which was recrystallized from ethyl acetate–methanol and then twice from boiling methanol (200 ml); yield 0.8 g (18%), m.p. 123–124°, $[\alpha]_D^{20}$ +74.1° (*c* 1.16, dichloromethane).

Anal. Calc. for $C_{33}H_{31}NO_7$: C, 71.59; H, 5.64; N, 2.53. Found: C, 71.52; H, 5.71; N, 2.69.

A mixture of **9** (1.2 g), glacial acetic acid (24 ml), and 2M hydrochloric acid (9.6 ml) was heated for 1 h at 90°. A flocculent, yellow-orange precipitate was formed and, after being cooled, the suspension was filtered (filtrate *A*), to give 0.35 g (107%) of a solidproduct. This material did not melt or change its appearance by 300°; Alway[6] has reported that *p*-nitrosobenzoic acid darkens when heated above 250°. The substance was virtually insoluble in boiling methanol, but readily soluble in aqueous ammonia and in sodium hydrogen carbonate solution; from its solution in the latter, it could be precipitated by the addition of hydrochloric acid.

Evaporation of filtrate *A* gave a syrup (0.8 g) which was crystallized from cyclohexane solution; m.p. 85–87°, $[\alpha]_D^{20}$ +6.13° (*c* 2.83, dichloromethane). The

melting point of a mixture with authentic 2,3,5-tri-*O*-benzyl-β-L-arabinofuranose[5] was undepressed.

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REFERENCES

1 O. T. SCHMIDT, T. AUER, AND H. SCHMADEL, *Chem. Ber.*, 93 (1960) 556.
2 H. G. FLETCHER, JR., AND R. K. NESS, *J. Amer. Chem. Soc.*, 76 (1954) 760.
3 M. E. TATE AND C. T. BISHOP, *Can. J. Chem.*, 41 (1963) 1801.
4 R. BARKER AND H. G. FLETCHER, JR., *J. Org. Chem.* 26 (1961) 4605.
5 S. TEJIMA AND H. G. FLETCHER, JR., *J. Org. Chem.*, 28 (1963) 2999.
6 F. J. ALWAY, *Ber.*, 37 (1904) 333.
7 C. S. HUDSON, *J. Amer. Chem. Soc.*, 47 (1925) 265; C. T. BISHOP AND F. P. COOPER, *Can. J. Chem.*, 40 (1962) 224.
8 O. T. SCHMIDT AND W. STAAB, *Chem. Ber.*, 87 (1954) 393.

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Chromatographic identification of some monosaccharides by thin-layer chromatography of their methylated methyl glycosides

In some cases, identification of monosaccharides by paper and thin-layer chromatography (t.l.c.) is unreliable because of similarity in the R_F values. For example, rhamnose, glucose, and xylose were tentatively identified[1] by paper chromatography in the hydrolysate of goloturine (a glycoside isolated from the sea cucumber), but a more-detailed study[2] showed that the sugars are glucose, 6-deoxyglucose, xylose, and 3-*O*-methylglucose. It is known[3] that apiose cannot be distinguished from rhamnose by paper chromatography. The methods for gas–liquid chromatographic identification of monosaccharides are rather complicated and not sufficiently reliable[4], and preparation of crystalline derivatives requires considerable amounts of material. Thus, additional information for the identification of sugars is required.

The present paper describes a simple and convenient procedure for the identification of monosaccharides by t.l.c. of their methylated methyl glycosides. Methods involving t.l.c. have been described previously[5,6]. Monosaccharides form mixtures of methyl glycosides after partial methylation. T.l.c. of these mixtures on silica gel

melting point of a mixture with authentic 2,3,5-tri-*O*-benzyl-β-L-arabinofuranose[5] was undepressed.

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7 C. S. HUDSON, *J. Amer. Chem. Soc.*, 47 (1925) 265; C. T. BISHOP AND F. P. COOPER, *Can. J. Chem.*, 40 (1962) 224.
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The present paper describes a simple and convenient procedure for the identification of monosaccharides by t.l.c. of their methylated methyl glycosides. Methods involving t.l.c. have been described previously[5,6]. Monosaccharides form mixtures of methyl glycosides after partial methylation. T.l.c. of these mixtures on silica gel

or on natural adsorbents[7] gives characteristic patterns for glucose, galactose, mannose, arabinose, xylose, fucose, rhamnose, apiose, and chalcose (Fig. 1). The standard

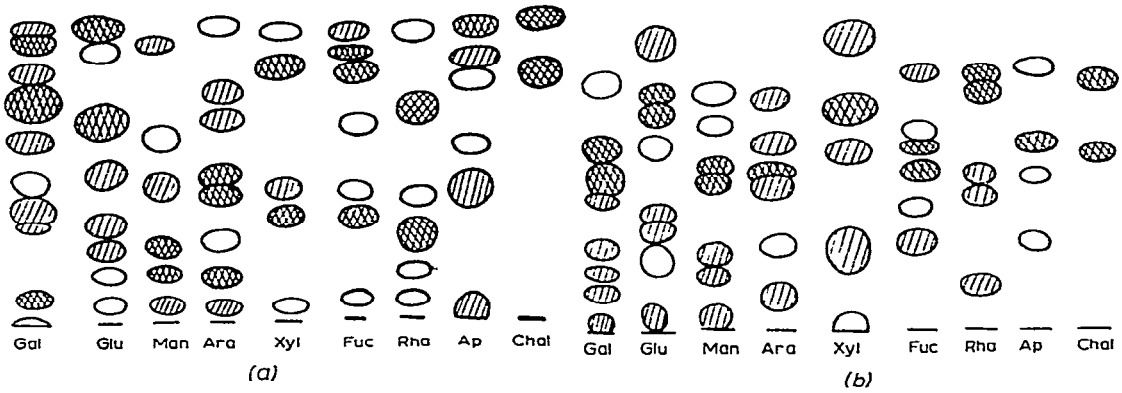

Fig. 1 — T.l.c. (solvent *A*) of products of partial methylation of monosaccharides. (*a*) Silica gel; (*b*) natural adsorbents[7]. Intensity of spots: ,(≠) strong; (//), medium; ◯, weak.

procedure adopted for partial methylation of monosaccharides (a modification of the method in Ref. 8) is reproducible over a wide range of sugar concentrations, and enables ready identification when 1–2 mg of the parent monosaccharide are available. After partial methylation, the monosaccharide is subjected to t.l.c., and the resulting pattern is compared with that of an authentic sample. The chromatographic procedure[7,9] requires approximately 100 μg of a mixture of methylated methyl glycosides. On detection, the glycosides gave spots having characteristic colours as follows (on natural adsorbents): glucose and galactose, black; mannose, deep-violet; arabinose, green; xylose, violet; fucose and rhamnose, yellow; and chalcose, brown. It must be noted that the chromatograms on natural adsorbents may be kept without any change during more than 6 months. The application of the technique to the analysis of the monosaccharide composition of polysaccharides from *Zosteraceae* plants has been described elsewhere[10].

EXPERIMENTAL

Methylation. — The sample of pure monosaccharide (1–10 mg) was dissolved in 2 drops of water and treated with methyl sulphate (2 drops) in the presence of 20% alkali (1 drop) during 0.5 h at 20°. A drop of alkali was then added, and the reaction mixture was heated for 0.5 h at 50°. Finally, 2 more drops of alkali were added, and the mixture was heated for 0.5 h at 70°. The cooled mixture was neutralized with 3% sulphuric acid, the products of methylation were extracted with chloroform–butyl alcohol (15:1 v/v) (0.5 ml), and the aqueous layer was removed by absorption into filter paper. The extract was used for t.l.c.

Chromatography. — The preparation of plates was carried out as previously described[7,9]. Aliquots containing approximately 100 μg of the methylation products

mixture were applied to the plates, and the following solvent systems were employed: *A*, benzene–acetone (2:1, v/v); *B*, chloroform–methanol (20:1, v/v). The detection reagent was 2% potassium dichromate in conc. sulphuric acid (110°/15 min).

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REFERENCES

1 R. F. NIGRELLI, J. D. CHANLEY, S. K. KOHN, AND H. SOBOTKA, *Zoologica*, 40 (1955) 47.
2 J. D. CHANLEY, R. LEDEEN, J. WASE, R. F. NIGRELLI, AND H. SOBOTKA, *J. Amer. Chem. Soc.*, 81 (1959) 5180.
3 D. J. BELL, F. A. ISHERWOOD, AND N. E. HARDWICK, *J. Chem. Soc.*, (1954) 3702.
4 W. W. WELLS, C. C. SWEELEY, AND R. BENTLEY, *Biomedical Application of Gas Chromatography*, Academic Press, New York, 1964, p. 169.
5 M. GEE, *Anal. Chem.*, 35 (1963) 350.
6 G. W. HAY, B. A. LEWIS, AND F. SMITH, *J. Chromatog.*, 11 (1963) 479.
7 V. T. BYKOV, A. A. VASKOVSKAYA, AND V. E. VASKOVSKY, *J. Chromatog.*, 30 (1967) 643.
8 E. L. HIRST AND E. PERCIVAL, *Methods Carbohyd. Chem.*, 2 (1963) 146.
9 YU. S. OVODOV, E. V. EVTUSHENKO, V. E. VASKOVSKY, R. G. OVODOVA, AND T. F. SOLOV'EVA, *J. Chromatog.*, 26 (1967) 111.
10 R. G. OVODOVA, V. E. VASKOVSKY, AND YU. S. OVODOV, *Carbohyd. Res.*, 6 (1968) 328.

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Rates of attack of some α-amylases upon various substrates

The α-amylase enzymes which are obtained from a wide variety of sources (animal, plant, and micro-organisms) may be expected to differ more from each other than do the β-amylases which are only obtained from higher plants. The initial action of α-amylases on amylose has generally been considered to be random, such that the long chain is attacked indiscriminately throughout its length with the production of shorter chains[1,2]. In contrast to the β-amylases, the α-amylases are able to cleave branched substrate molecules between the branches.

The present note indicates a simple method for the comparison of the activities of α-amylases from various sources.

EXPERIMENTAL

The α-amylases were isolated from soya beans, barley, and malted barley by A. W. MacGregor[3]. Amylose and amylopectin were prepared by standard techniques (*e.g.*, Greenwood[4]) from the potato plant (*Solanum tuberosum*). Glycogen was isolated from rabbit liver by extraction with trichloroacetic acid[5]. The β-limit dextrins were prepared by exhaustive digestion of the amylopectin or glycogen with α-amylase-free β-amylase[6]. All of these substrates were dissolved in distilled water, and the enzyme–substrate ratios were adjusted to between 300–400 units of specific α-amylase activity per mg of polysaccharide substrate. The substrate concentration was 1.5 mg/ml, and the digest volume was 40 ml. Activity was measured by a previously reported[3] procedure. Before light-scattering measurements were taken, the solutions were filtered until dust-free by means of Millipore filters (pore diameter 1.2 μ for amylopectin, 50 nm for glycogen and amylose). The measurements were made at constant temperature (20 ±1°) with the mercury green line (546 nm) and the angles 45°, 90°, and 135°.

Samples of the enzymes were added directly to the light-scattering cells from an Agla micrometer syringe. The enzyme preparations were so active that only very

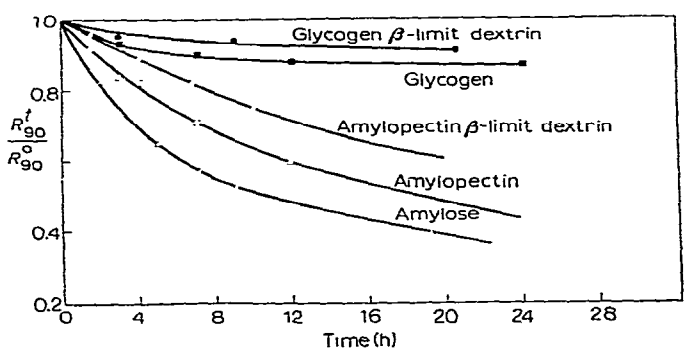

Fig. 1 — The action of soya-bean α-amylase upon various substrates as indicated by the relative decrease in light-scattering at 90° (some points have been omitted for clarity).

small volumes, of the order of 0.01 ml, were required. This eliminated the problem of filtering the enzyme solution to make it dustfree, with the consequent danger of denaturation. The decrease in the scattered light was followed over a period of hours from the addition of the enzyme.

Initially, results were treated simply as a measure of the relative decrease of 90°-scatter at any one time, compared to the 90°-scatter of the original substrate. This resulted in plots similar to those of Banks *et al.*[6] (See Fig. 1). However, the curvature of this type of plot made any estimate of the relative ratio extremely unreliable.

An alternative method of plotting the data was found to be far more satisfactory. The scattering ratio at 90° is defined by

$$\frac{Kc}{R_{90}} = \frac{1}{P_\theta \overline{M}_1} + Bc, \qquad (1)$$

where R_{90} = measured scattering ratio at 90°, K = optically defined constant, c = total concentration of polymer, P_θ = particle scattering factor, $\overline{M}_1$ = apparent weight-average molecular weight, and B = second virial coefficient.

However, since B is effectively zero for the present polymer substrates[7],

$$R_{90} = K(P_\theta M_1).c = K.\overline{M}_w.c, \qquad (2)$$

where $\overline{M}_w$ = true weight-average molecular weight.

From the viewpoint of enzyme kinetics, the actual concentration of substrate is the number of bonds available for hydrolysis (N). Then, since $N = M_w.c.n$, where n = number of hydrolysable bonds per unit molecular weight,

$$R_{90} = K^1 N. \qquad (3)$$

K^1 is not a true constant, since P_θ will vary with $\overline{M}_1$ and particle shape. However, the variation of P_θ with $\overline{M}_1$ is not considerable, and it is considered unlikely that the molecules will undergo conformational changes (*e.g.*, helix→coil), at least during the early stages of hydrolysis. Therefore K^1 is assumed to be appreciably constant. (P_θ corrections were made by using the data of Beattie and Booth[8].) Amylose was assumed to be basically a helix[9], and the other substrates were assumed to be in random-coil configurations.

From equation *3*,

$$\frac{R^0_{90}}{R^t_{90}} = \frac{N}{N - N^1},$$

where superscripts refer to the time (t) of measurement and N^1 = number of hydrolysed bonds.

Since log (R^0_{90}/R^t_{90}) is linearly related to time (Fig. 2), it is clear that, in the initial stages, the enzymic hydrolysis is first order. Further, it will be indicated below that the relative rates of hydrolysis can be derived theoretically.

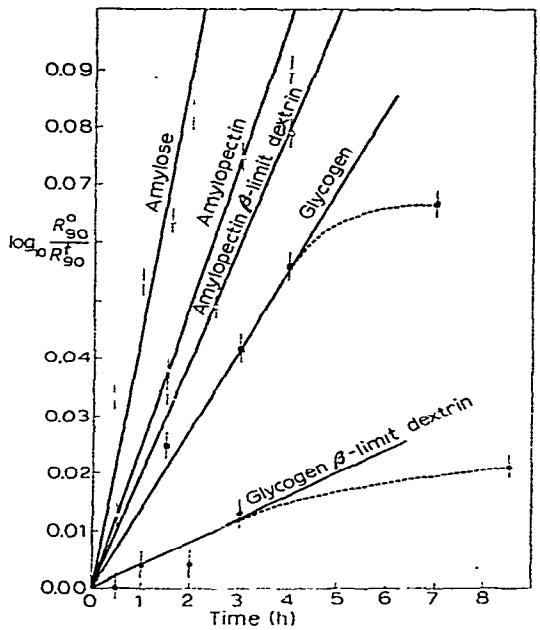

Fig. 2 — A first-order reaction rate plot for the action of soya-bean α-amylase upon various substrates, using the data in Fig. 1.

DISCUSSION

The results shown in Table I indicate the rates expressed relative to amylose. From these results, it seems clear that the modes of action of the three enzymes are essentially similar. Soya-bean α-amylase appears to be able to penetrate the highly branched molecules of glycogen and glycogen β-limit dextrin with greater ease than the other two enzymes. This suggests that it was being less sterically hindered, probably because of a smaller molecular size.

The relative rates of attack of the enzyme can be predicted from the number of sites on the molecule available for enzymic attack. For stereochemical reasons, sites at, and immediately adjacent to, (1→6)-linked groups are unlikely to be attacked, *i.e.*, 3 sites per branch point. It is also likely that, in highly branched structures, exterior chains will be preferentially attacked, and hence the relative rate will be dependent on the amount of material in the external chains. The amount of this material has been calculated statistically by Erlander[10] from mathematical models. The relative first-order rates will be proportional to the amount of material available, and these theoretical rates and their derivation are shown in Table II.

It can be seen that they agree relatively well with the measured experimental values. Again it appears that the barley and malted-barley α-amylases are unable to penetrate denser structures as well as the soya-bean α-amylase, suggesting that the latter is a smaller molecule. Further, the agreement between the theoretical and experimental rate values confirms that the reaction is initially of the first order.

TABLE I

EXPERIMENTAL RATES OF ENZYME ATTACK ON VARIOUS SUBSTRATES (RELATIVE TO AMYLOSE)

Substrate	*α-Amylase*		
	Soya bean	*Malted barley*	*Barley*
Amylose	1.00	1.00	1.00
Amylopectin	0.58	0.68	0.67
Amylopectin, β-limit dextrin	0.46	0.41	0.40
Glycogen	0.32	0.23[a]	0.04
Glycogen, β-limit dextrin	0.10	0.09[a]	0.02

[a]Activity of enzyme increased sevenfold.

TABLE II

THEORETICAL RATES OF ENZYME ATTACK ON VARIOUS SUBSTRATES (RELATIVE TO AMYLOSE)

Substrate	*Branching* (%)	*No. of sites remaining available*[a] (%)	*Material in external chains*[b] (%)	*Relative rates*
Amylose	0	100	100	1.00
Amylopectin	4	88	60	0.53
Amylopectin, β-limit dextrin	9	73	48	0.35
Glycogen	10	70	40	0.28
Glycogen, β-limit dextrin	20	40	25	0.10

[a]Calculated assuming that 3 sites per branch point are unavailable from steric considerations. [b]Calculated by Erlander[10].

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REFERENCES

1 K. MYRBACK, *Advan. Carbohyd. Chem.*, 3 (1948) 251.
2 C. T. GREENWOOD, A. W. MACGREGOR, AND E. A. MILNE, *Carbohyd. Res.*, 1 (1965) 303.
3 C. T. GREENWOOD AND A. W. MACGREGOR, *J. Inst. Brewing*, 71 (1965) 405.

4 W. BANKS, C. T. GREENWOOD, AND J. THOMSON, *Makromol. Chem.*, 31 (1959) 197.
5 M. R. STETTEN, H. M. KATZEN, AND D. STETTEN, *J. Biol. Chem.*, 222 (1956) 587.
6 W. BANKS, C. T. GREENWOOD, AND I. G. JONES. *J. Chem. Soc.*, (1960) 150.
7 R. GEDDES, PH. D. Thesis, University of Edinburgh, (1965).
8 W. H. BEATTIE AND C. BOOTH, *J. Phys. Chem.*, 64 (1960) 696.
9 V. S. R. RAO AND J. F. FOSTER, *Biopolymers*, 1 (1963) 527.
10 S. R. ERLANDER, *J. Polymer Sci.*, 37 (1959) 91.

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Studies on carbohydrate-metabolising enzymes
Part XVIII.* The α-L-arabinosidase activity of almond emulsin

Although almond emulsin is usually regarded as a β-glucosidase, it shows a range of glycosidase activities towards aryl β-D-glucosides and β-D-galactosides, and towards various disaccharides. There is now convincing evidence that the activity towards *p*-nitrophenyl β-D-glucopyranoside and -galactopyranoside is due to the same enzyme[2,3], and we have recently shown that this enzyme also hydrolyses *p*-nitrophenyl β-D-xylopyranoside[4]. Since these results imply a lack of specificity towards C-4 and C-5 of the substrate, the activity towards *p*-nitrophenyl α-L-arabinopyranoside has now been examined.

The substrate was synthesised from 2,3,4-tri-*O*-acetyl-β-L-arabinopyranosyl bromide by the method of Snyder and Link[5], and had m.p. 201–202°, $[\alpha]_D^{20}$ −21.9° (*c* 0.25, water) {lit.,[5] m.p. 201–202°, $[\alpha]_D^{20}$ −22.4°.} Enzymic hydrolysis was measured by the rate of release of *p*-nitrophenol[3]. When a 0.016% solution of substrate was incubated at pH 5.5 with a 0.02% solution of almond emulsin (protein content 95%; prepared by the method of Tauber[6]) at 37° for 10 min, there was a linear relation between activity and enzyme concentration.

Over the pH range 4.1–6.6, the pH-activity curves with *p*-nitrophenyl β-D-glucopyranoside and α-L-arabinopyranoside were similar, with optima at 5.5; this is identical to the β-D-xylosidase activity[4]. The proportions of the three activities were about 40:8:1, respectively. As shown in Tables I and II, there was no selective inactivation of one glycosidase activity on heating the emulsin to various temperatures, or on the addition of the inhibitors D-glucono-1,5-lactone[7] or erythritol[3] to the digests. The ratio of β-D-glucosidase/α-L-arabinosidase activities varied only slightly (from about 5.6 to about 4.4) with these treatments. All of these results suggest that the β-D-glucosidase and α-L-arabinosidase activities are due to the same enzyme, and unambiguous evidence was therefore sought by protein fractionation experiments.

Since continuous curtain electrophoresis provides a valuable method for the separation of carbohydrases[8,9], 20 ml of a 2.5% solution of emulsin (equivalent

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Since continuous curtain electrophoresis provides a valuable method for the separation of carbohydrases[8,9], 20 ml of a 2.5% solution of emulsin (equivalent

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TABLE I

EFFECT OF PARTIAL INACTIVATION BY HEAT ON β-D-GLUCOSIDASE AND α-L-ARABINOSIDASE ACTIVITIES

Temperature[a] (degrees)	50	55	60	65	70
Inactivation (%)					
Arabinosidase	6	13	25	55	90
Glucosidase	3	13	30	60	92

[a]Enzyme solutions were heated to the stated temperatures for 15 min, cooled rapidly, and then assayed at 37°.

TABLE II

EFFECT OF INHIBITORS ON β-D-GLUCOSIDASE AND α-L-ARABINOSIDASE ACTIVITIES[a]

D-*Glucono-1,5-lactone* (mM)	0.8	1.6	2.4	3.2	4.0
Arabinosidase	54	67	74	78	82
Glucosidase	61	75	80	82	86
Erythritol (mM)	2	4	20	40	200
Arabinosidase	2	7	19	35	78
Glucosidase	7	11	30	47	83

[a]Results are expressed as percentage inhibition.

to 475 mg of protein) in 0.05M phosphate buffer (pH 8.0) was subjected to electrophoresis in a Beckman–Spinco model CP apparatus, using 0.005M phosphate buffer (pH 8.0), at 700 volts and 25 mamp. Samples were collected, and assayed for protein content (by extinction measurements at 280 nm) and arabinosidase activity, and were combined to give three fractions. Fraction 1 (47 mg of protein) showed the greatest β-D-glucosidase and α-L-arabinosidase activities (ratio, *ca.* 5:1), which were similarly decreased by varying concentrations of D-glucono-1,5-lactone and erythritol.

Fraction 1 (8 mg of protein) was chromatographed on a column (18.5 × 1.4 cm) of DEAE-cellulose (OH^- form), using a gradient of sodium chloride (0–0.4M) for elution. The rate of elution of the β-D-glucosidase and α-L-arabinosidase activity (see Fig. 1) showed clearly that no separation of activities had occurred. In a second experiment, the fraction showing maximum α-L-arabinosidase activity also hydrolysed *p*-nitrophenyl β-D-galactopyranoside and -xylopyranoside.

Acrylamide-gel electrophoresis has been successfully used for the separation of carbohydrases[10], and has now been applied to an emulsin fraction (2 mg) from the continuous electrophoresis experiment. Using a Shandon disc-electrophoresis apparatus, with 75 volts and 9 mamp, and a 7.5% polyacrylamide gel in 0.01M phosphate buffer (pH 7.0), one diffuse band of protein was detected. The gel was sectioned, and each section assayed for enzymic activity. The β-D-glucosidase and α-L-arabinosidase activities occurred in identical sections, and the peaks of activity coincided. The electrophoresis was repeated in the presence of 6M urea[9]. The distribution of activities is shown in Fig. 2(*a*).

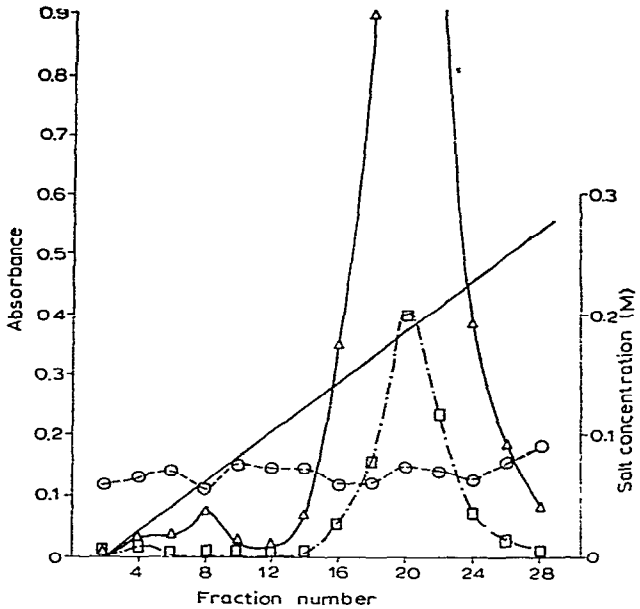

Fig. 1. Pattern of elution of emulsin fraction 1 from DEAE-cellulose, showing the distribution of protein (0 — — —0), β-D-glucosidase (—△—), and α-L-arabinosidase (—⊡—) activities. Fractions of 8 ml were collected.

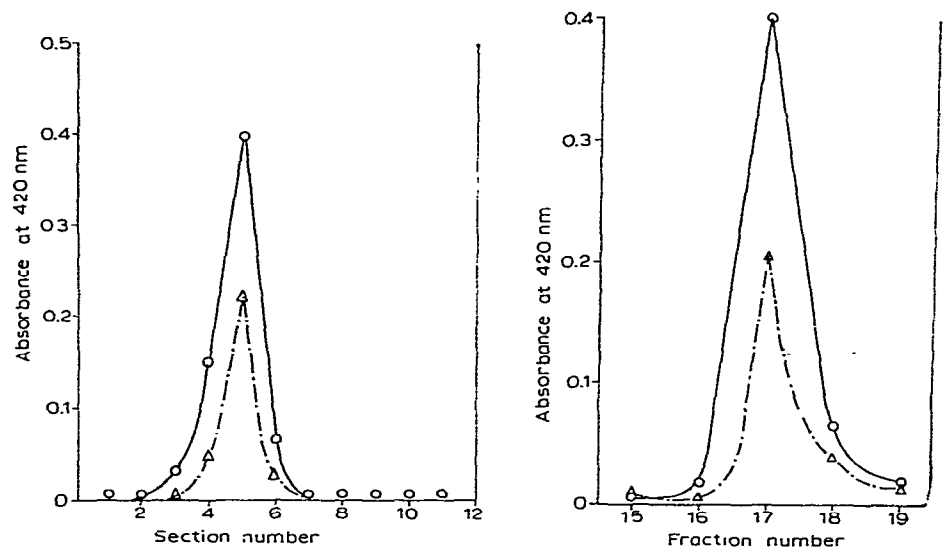

Fig. 2. Distribution of almond glycosidases on (*a*) electrophoresis on polyacrylamide gel in 6M urea, (*b*) on elution from a column of pachyman. β-D-Glucosidase and α-L-arabinosidase activities are represented by (—○—) and (—△—), respectively. Samples for β-D-glucosidase activity were diluted, as appropriate, before assay. After electrophoresis, 5-mm sections of the gel were assayed; the protein migrated from the point of application (section 0) towards the anode. In the chromatographic separation, 3-ml fractions were collected.

Mixtures of carbohydrases have been separated by chromatography on columns of insoluble polysaccharides[11]. Fraction 1 (2 mg of protein) was therefore dissolved in 0.005M phosphate buffer (pH 5.2) and chromatographed on a column (8 × 0.8 cm) of the β-glucan pachyman[12], using phosphate buffers (0.005M, pH 5.2; and 0.05M pH 7.2) as eluants. Samples were collected and assayed for activity. A single, symmetrical peak, showing both β-D-glucosidase and α-L-arabinosidase activity, was obtained, as shown in Fig. 2(*b*), which also hydrolysed *p*-nitrophenyl β-D-galactopyranoside and -xylopyranoside.

On the basis of these three different techniques of separation, we conclude that the *p*-nitrophenyl α-L-arabinosidase activity of sweet almonds is due to the same enzyme as that which hydrolyses the corresponding β-D-glucopyranoside, β-D-galactopyranoside, and β-D-xylopyranoside. Since this work was completed, Conchie and coworkers[13] have reached the same conclusion on the basis of enzyme inhibition studies. It is now evident that, in certain tissues, *e.g.*, pig kidney[14] and limpet[13], the α-L-arabinosidase activity is due to an enzyme with a similarly wide specificity, whereas, in other tissues, *e.g.*, apricot emulsin[9] and marine algae[4], a mixture of specific glycosidases is present.

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REFERENCES

1 D. J. MANNERS, I. R. PENNIE, AND J. R. STARK, *Carbohyd. Res.*, 6 (1968) 20
2 R. HEYWORTH AND P. G. WALKER, *Biochem. J.*, 83 (1962) 331.
3 M. V. KELEMEN AND W. J. WHELAN, *Arch. Biochem. Biophys.*, 117 (1966) 423.
4 D. J. MANNERS AND J. P. MITCHELL, *Biochem. J.*, 103 (1967) 43P.
5 J. A. SNYDER AND K. P. LINK, *J. Amer. Chem. Soc.*, 74 (1952) 1883.
6 H. TAUBER, *J. Biol. Chem.*, 99 (1932) 257.
7 J. CONCHIE AND G. A. LEVVY, *Biochem. J.*, 65 (1957) 389.
8 D. H. HUTSON AND D. J. MANNERS, *Biochem. J.*, 94 (1965) 783.
9 Y. SHIBATA AND K. NISIZAWA, *Arch. Biochem. Biophys.*, 109 (1965) 516.
10 R. SIEPMANN AND H. STEGEMANN, *Z. Naturforsch*, 22 (1967) 949.
11 Y. HASHIMOTO AND K. NISIZAWA, in E. T. REESE (Ed.), *Advances in Enzymic Hydrolysis of Cellulose and Related Materials*, Pergamon, Oxford, 1963, p. 93; I. A. CHERKASOV, N. A. KRAVCHENKO, AND E. D. KAVERZNEVA, *Dokl. Akad. Nauk SSSR*, 170 (1966) 213; *Chem. Abstr.*, 66 (1967) 43914.
12 S. A. WARSI AND W. J. WHELAN, *Chem. Ind.* (London), (1957) 1573.
13 J. CONCHIE, A. L. GELMAN, AND G. A. LEVVY, *Biochem. J.*, 106 (1968) 135.
14 D. ROBINSON AND H. E. ABRAHAMS, *Abstr. Fourth F.E.B.S. Meeting, Oslo*, 1967, p. 40.

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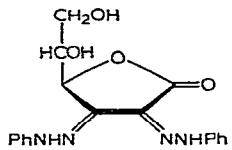
Preliminary communications

The structure of dehydro-L-ascorbic acid phenylosazone

The 2,3-bis(phenylhydrazone) of L-*threo*-2,3-hexodiulosonolactone (dehydro-L-ascorbic acid phenylosazone) has been formulated[1] as the 1,4-lactone **1**. We now present evidence that the structure of the osazone is that of the 1,5-lactone (**2**).



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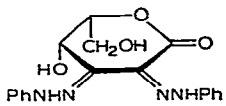

2

(*a*) Even on prolonged treatment, the osazone does not react with periodic acid, whereas structure **1** would be expected to react. This behavior agrees with structure **2**, which lacks two adjacent hydroxyl groups. (*b*) The infrared spectrum of dehydro-L-ascorbic acid phenylosazone shows a band for carbonyl absorption at 1720 cm^{-1}, as would be expected[2] for a 1,5-lactone, but not for a 1,4-lactone, which would absorb at a higher frequency. (*c*) Finally, in the n.m.r. spectrum of the diacetate of dehydro-L-ascorbic acid phenylosazone (obtained by treating the osazone with boiling acetic anhydride) the H-6 signal appears as a doublet at τ 5.55, that of H-4 as a doublet at τ 4.69, and that of H-5 as a multiplet at τ 4.47. In other words, the shielding of the sugar-chain protons decreases in the order H-6, H-4, H-5, which is more in agreement with structure **2** than with structure **1**. For **1**, H-4 would be expected to resonate at a lower field than H-5, and the order of the τ values of the sugar-chain protons would be H-4, H-5, H-6. The signals of the two imino protons appeared at τ −0.87 and τ −1.83 and those of the two acetyl methyl groups at τ 8.0 and 7.9.

We therefore conclude that, during osazone formation, the 1,4-lactone ring of dehydro-L-ascorbic acid rearranges to the 1,5-lactone form, and propose structure **2** for the phenylosazone, namely, L-*threo*-2,3-hexodiulosono-1,5-lactone 2,3-bis-(phenylhydrazone).

The preparation of **2** was achieved by the interaction of phenylhydrazine with dehydro-L-ascorbic acid (obtained by air oxidation of L-ascorbic acid). It was crystallized twice to remove traces of 1-phenyl-4-phenylazo-3-(L-*threo*-1,2,3-trihydroxypropyl)pyrazolin-5-one[3] (**3**). The pure product (**2**) had m.p. 218–220°; λ_{max}^{EtOH} 261, 274(sh), 352, and 444 nm (log ε 4.34, 4.29, 3.91, and 4.31); λ_{min}^{EtOH} 220, 319, and 383 nm (log ε 3.97, 3.80, and 3.91); ν_{max}^{KBr} 1720 (C=O) and 3400 cm^{-1} (OH).

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REFERENCES

1 R. W. HERBERT, E. L. HIRST, E. G. V. PERCIVAL, R. J. W. REYNOLDS, AND F. SMITH, *J. Chem. Soc.*, (1933) 1270.
2 S. A. BARKER, E. J. BOURNE, R. M. PINKARD, AND D. H. WHIFFEN, *Chem. Ind.* (London), (1958) 658.
3 H. HASSELQUIST, *Arkiv Kemi*, 4 (1952) 375.

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Proof of structure, stereochemistry, and synthesis of perosamine (4-amino-4,6-dideoxy-D-mannose) derivatives

Recently, numerous examples of 4-amino-4,6-dideoxy hexoses have been isolated from natural sources[1], including antibiotics and bacterial cell-walls. As part of the investigation of the chemistry of this new class of amino sugars, derivatives of 4-amino-4,6-dideoxy-D-mannose were synthesized. As this work was nearing completion, Schaffner and Lee reported the isolation of a basic carbohydrate moiety, perosamine, from the acid hydrolyzate of the heptaenic antibiotic perimycin[2]. Available evidence led to the tentative assignment of the 4-amino-4,6-dideoxy-D-mannose structure for perosamine. This preliminary communication describes the total synthesis and structure proof of derivatives of 4-amino-4,6-dideoxy-D-mannose, and establishes the conclusive identity of this sugar with natural perosamine.

Methyl 3,4-anhydro-6-deoxy-α-D-talopyranoside (**1**), m.p. 65–66°, was prepared from the known methyl 6-deoxy-2,3-*O*-isopropylidene-4-*O*-methylsulfonyl-α-D-mannopyranoside[3] by procedures previously used with success in the L-rhamnose series[4]. Treatment of **1** with lithium azide and ammonium chloride in 2-methoxyethanol[5] for 6 h at reflux afforded a mixture of azides **3** and **9**. Methyl 4-azido-4,6-dideoxy-α-D-mannopyranoside **3**, m.p. 81.5–82.5°, $[\alpha]_D^{27}$ +127° (*c* 1.0, methanol) and methyl 3-azido-3,6-dideoxy-α-D-idopyranoside **9**, m.p. 52–53°, $[\alpha]_D^{26}$ +111° (*c* 1.05, methanol), were obtained in 17% and 45% yields, respectively, after preparative thin-layer chromatography. Since the primary goal was to obtain the product resulting from opening at C-4 by azide ion, methyl 3,4-anhydro-2-*O*-benzoyl-6-deoxy-α-D-talopyranoside (**2**) m.p. 72–73° was prepared in the hope of increasing the ratio of products obtained from attack at C-4 relative to those obtained from opening at C-3. The reaction of **2** with lithium azide and ammonium chloride for 18 h resulted in a mixture of five azides which were separated by preparative t.l.c.: **3**, m.p. 81.5–82.5° (20%); **4**, as an oil (4%); **5**, m.p. 116–117° (8%); **9**, m.p. 52–53° (3%); **10**, m.p. 106–107° (11%). When the reaction was carried out in a sealed tube for 18 h at 110°, only azides **3** (43%), **9** (9%), and **10** (2%) could be isolated by preparative t.l.c.

REFERENCES

1 R. W. HERBERT, E. L. HIRST, E. G. V. PERCIVAL, R. J. W. REYNOLDS, AND F. SMITH, *J. Chem. Soc.*, (1933) 1270.
2 S. A. BARKER, E. J. BOURNE, R. M. PINKARD, AND D. H. WHIFFEN, *Chem. Ind.* (London), (1958) 658.
3 H. HASSELQUIST, *Arkiv Kemi*, 4 (1952) 375.

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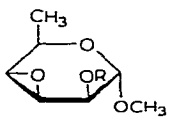
Proof of structure, stereochemistry, and synthesis of perosamine (4-amino-4,6-dideoxy-D-mannose) derivatives

Recently, numerous examples of 4-amino-4,6-dideoxy hexoses have been isolated from natural sources[1], including antibiotics and bacterial cell-walls. As part of the investigation of the chemistry of this new class of amino sugars, derivatives of 4-amino-4,6-dideoxy-D-mannose were synthesized. As this work was nearing completion, Schaffner and Lee reported the isolation of a basic carbohydrate moiety, perosamine, from the acid hydrolyzate of the heptaenic antibiotic perimycin[2]. Available evidence led to the tentative assignment of the 4-amino-4,6-dideoxy-D-mannose structure for perosamine. This preliminary communication describes the total synthesis and structure proof of derivatives of 4-amino-4,6-dideoxy-D-mannose, and establishes the conclusive identity of this sugar with natural perosamine.

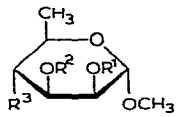
Methyl 3,4-anhydro-6-deoxy-α-D-talopyranoside (**1**), m.p. 65–66°, was prepared from the known methyl 6-deoxy-2,3-*O*-isopropylidene-4-*O*-methylsulfonyl-α-D-mannopyranoside[3] by procedures previously used with success in the L-rhamnose series[4]. Treatment of **1** with lithium azide and ammonium chloride in 2-methoxyethanol[5] for 6 h at reflux afforded a mixture of azides **3** and **9**. Methyl 4-azido-4,6-dideoxy-α-D-mannopyranoside **3**, m.p. 81.5–82.5°, $[\alpha]_D^{27}$ +127° (*c* 1.0, methanol) and methyl 3-azido-3,6-dideoxy-α-D-idopyranoside **9**, m.p. 52–53°, $[\alpha]_D^{26}$ +111° (*c* 1.05, methanol), were obtained in 17% and 45% yields, respectively, after preparative thin-layer chromatography. Since the primary goal was to obtain the product resulting from opening at C-4 by azide ion, methyl 3,4-anhydro-2-*O*-benzoyl-6-deoxy-α-D-talopyranoside (**2**) m.p. 72–73° was prepared in the hope of increasing the ratio of products obtained from attack at C-4 relative to those obtained from opening at C-3. The reaction of **2** with lithium azide and ammonium chloride for 18 h resulted in a mixture of five azides which were separated by preparative t.l.c.: **3**, m.p. 81.5–82.5° (20%); **4**, as an oil (4%); **5**, m.p. 116–117° (8%); **9**, m.p. 52–53° (3%); **10**, m.p. 106–107° (11%). When the reaction was carried out in a sealed tube for 18 h at 110°, only azides **3** (43%), **9** (9%), and **10** (2%) could be isolated by preparative t.l.c.

Catalytic hydrogenation of **9** gave methyl 3-amino-3,6-dideoxy-α-D-idopyranoside (**11**), m.p. 166–167°. The physical constants of **11** agreed with those of its L-enantiomorph, and thus established its structure. Compound **11** was prepared also in 80% yield by treatment[6] of **1** with ethanol–ammonia at 130°.

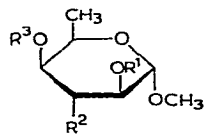
Catalytic hydrogenation of **3** afforded methyl 4-amino-4,6-dideoxy-α-D-mannopyranoside (**6**) m.p. 152–153°, $[\alpha]_D^{27}$ +82.5° (*c* 1.0, methanol). The physical constants of **6** agreed with those of methyl perosaminide prepared by methanolysis of perosamine[2]. Acetylation of **6** with acetic anhydride in methanol at 0° gave its *N*-acetyl derivative **7**, m.p. 184–186°. The m.p. of the mixture of **7** with methyl *N*-acetylperosaminide (m.p. 184–185.5°) was undepressed. The infrared spectra (KBr) of the two compounds were identical. Acetylation of **6** with excess acetic anhydride in pyridine afforded the triacetyl derivative **8**, m.p. 160–161°, $[\alpha]_D^{23}$ +95.6° (*c* 0.98, methanol).



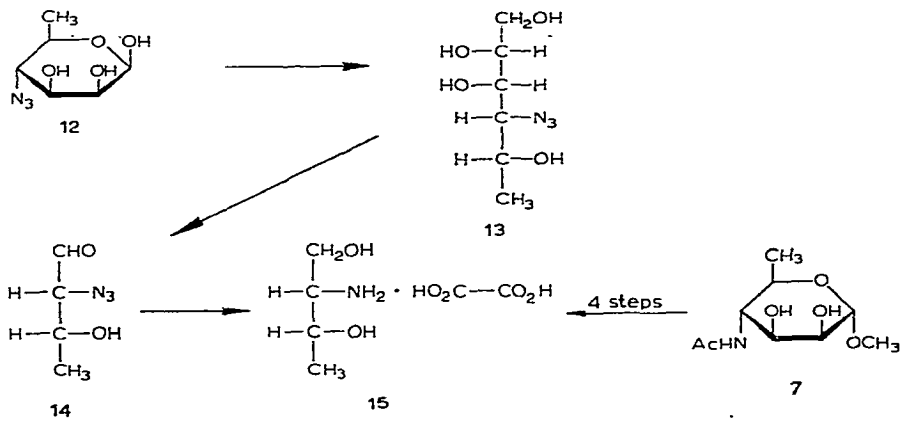
1 R = H
2 R = Bz



3 $R^1 = R^2 = H$; $R^3 = N_3$
4 $R^1 = H$; $R^2 = Bz$; $R^3 = N_3$
5 $R^1 = Bz$; $R^2 = H$; $R^3 = N_3$
6 $R^1 = R^2 = H$; $R^3 = NH_2$
7 $R^1 = R^2 = H$; $R^3 = NHAc$
8 $R^1 = R^2 = Ac$; $R^3 = NHAc$



9 $R^1 = R^3 = H$; $R^2 = N_3$
10 $R^1 = Bz$; $R^2 = N_3$ $R^3 = H$
11 $R^1 = R^3 = H$; $R^2 = NH_2$



The structure and absolute configuration of the manno azide **3**, and consequently the structure of the amino sugar glycoside **6**, was proven by degradation to crystalline 2-amino-2,4-dideoxy-D-erythritol oxalate. Acid hydrolysis of **3** gave 4-azido-4,6-dideoxy-β-D-mannose, **12**, (50%), m.p. 118–118.5°, $[\alpha]_D^{27}$ +21.6°→57.4° (0.5 h, *c* 1.0, methanol). Selective sodium borohydride reduction of **12** in water at 0° afforded the

corresponding alditol **13**, m.p. 72–81°, $[\alpha]_D^{27}$ +5° (*c* 0.9, methanol). Sodium metaperiodate oxidation of either free sugar **12** or alditol **13** gave 2-azido-2,4-dideoxy-D-erythrose **14**. Sodium borohydride reduction and hydrogen oxalate salt formation gave crystalline 2-amino-2,4-dideoxy-D-erythritol hydrogen oxalate (**15**), m.p. 174–175° (dec.), $[\alpha]_D^{26}$ −28° (*c* 0.93, water), identical in all respects with authentic hydrogen oxalate, m.p. 174–175° (dec.), $[\alpha]_D^{25}$ −27° (*c* 0.6, water)[7]. Also, synthetic *N*-acetyl derivative **7** was converted into 2-amino-2,4-dideoxy-D-erythritol hydrogen oxalate (**15**) by sequential sodium metaperiodate cleavage, sodium borohydride reduction, acid hydrolysis, and hydrogen oxalate salt formation[7].

All new compounds reported herein had acceptable elemental analyses.

ACKNOWLEDGMENTS

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REFERENCES

1 C. L. STEVENS, K. NAGARAJAN, AND T. H. HASKELL, *J. Org. Chem.*, 27 (1962) 2991; C. L. STEVENS, F. A. DANIHER, *J. Am. Chem. Soc.*, 85 (1963) 1552; R. W. WHEAT, E. L. ROLLINS, AND J. M. LEATHERWOOD, *Biochem. Biophys. Res. Commun.*, 9 (1962) 120; C. L. STEVENS, P. BLUMBERGS, F. A. DANIHER, R. W. WHEAT, A. KIYOMOTO, AND E. L. ROLLINS, *J. Am. Chem. Soc.*, 85 (1963) 3061; C. L. STEVENS, P. BLUMBERGS, D. H. OTTERBACH, J. L. STROMINGER, M. MATSUHASHI, AND D. N. DIETZLER, *ibid.*, 86 (1964) 2937; C. L. STEVENS, P. BLUMBERGS, F. A. DANIHER, J. L. STROMINGER, M. MATSUHASHI, AND D. N. DIETZLER, *ibid.*, 86 (1964) 2939; C. L. STEVENS, G. E. GUTOWSKI, K. G. TAYLOR, AND C. P. BRYANT, *Tetrahedron Letters*, (1967) 649; B. JANN AND K. JANN, *Europ. J. Biochem.*, 2 (1967) 26.
2 C. H. LEE AND C. P. SCHAFFNER, *Tetrahedron Letters*, (1966) 5837.
3 C. L. STEVENS, R. P. GLINSKI, K. G. TAYLOR, P. BLUMBERGS, AND F. SIROKMAN, *J. Am. Chem. Soc.*, 88 (1966) 2073.
4 G. CHARALAMBOUS AND E. PERCIVAL, *J. Chem. Soc.*, (1954) 2443.
5 R. D. GUTHRIE AND D. MURPHY, *Chem. Ind.* (London), (1962) 1473.
6 J. JARÝ, K. ČAPEK AND J. KOVÁŘ, *Collection Czech. Chem. Commun.*, 28 (1963) 2171.
7 C. L. STEVENS, S. K. GUPTA, R. P. GLINSKI, G. E. GUTOWSKI, AND C. P. BRYANT, *Tetrahedron Letters*, (1968) 1817.

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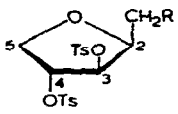
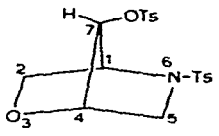
The synthesis of functionally substituted 3-oxa-6-azabicyclo[2.2.1]heptanes

As a part of our continuing interest in the synthesis and reactions of bicyclic systems, containing two heteroatoms, we now report on the synthesis of functionally substituted 3-oxa-6-azabicyclo[2.2.1]heptanes (4, 7). Previously, there has been no report of the synthesis of such chiral derivatives in the literature.

When the trisulphonate[1] 1 was heated with sodium azide in *N,N*-dimethylformamide for 1 h at 120°, thin-layer chromatography indicated the presence of a major product and a small proportion of unchanged starting material. A 65% yield of the crystalline azide 2, m.p. 108–110°, $[\alpha]_D$ + 32.3° (*c* 1, chloroform), was obtained. If either the temperature or the reaction time was increased, additional products were formed. Hydrogenation of the azide 2 with Adam's catalyst, followed by toluene-*p*-sulphonylation, gave compound 3, m.p. 155–157°, $[\alpha]_D$ +15° (*c* 0.7, chloroform).



1 R = OTs
2 R = N_3
3 R = NH–Ts



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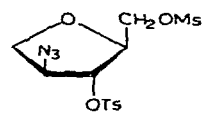

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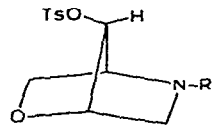
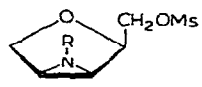
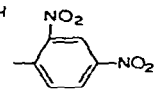


6 R = H
7 R = Ts



8 R = H



9 R =

Treatment of compound 3 with refluxing methanol containing 1 molecular proportion of sodium methoxide for 3 h afforded 93% of a crystalline product, m.p. 138–139°, $[\alpha]_D$ –4° (*c* 1.15, chloroform). It had a molecular weight of 423 (mass spectrometry), and its n.m.r. and mass spectral data were fully consistent with its being (1*S*,4*S*,7*R*)-7-(toluene-*p*-sulphonyloxy)-3-oxa-6-azabicyclo[2.2.1] heptane (4). Thus, base removal of the amide proton in compound 3 was accompanied by an intramolecular nucleophilic displacement at C-4, resulting in the formation of the oxa-aza-norbornane derivative 4.

The diastereoisomer 7 was obtained from the readily available (2*S*,3*S*,4*S*)-4-azido-2-methanesulphonyloxymethyl-3-(toluene-*p*-sulphonyloxy)tetrahydrofuran[2] (5). The azido group in compound 5 is *trans* to the C-3 toluene-*p*-sulphonyloxy group and thus suitably arranged, after reduction, for rearward attack leading to the epimine 8; it is *cis* to the C-2 mesyloxymethyl group, rendering possible a cyclization to (1*S*,4*S*,7*S*)-7-(toluene-*p*-sulphonyloxy)-3-oxa-6-azabicyclo[2.2.1]heptane (6).

When the azide 5 was refluxed for 3 h in isopropyl alcohol containing sodium borohydride, cyclization occurred, and the bicyclic system 6 formed was converted *in situ* into its *N*-(toluene-*p*-sulphonyl) derivative 7, m.p. 103–104°, $[\alpha]_D$ −14° (*c* 1.05, chloroform), in a 90% yield. The analytical and spectral data of the isolated product are compatible only with structure 7, and its n.m.r. and mass spectra display strong similarities with those of its diastereoisomer 4.

A small amount of the epimine 8 was also present in the reaction mixture. It was characterized as its *N*-dinitrophenyl derivative, which was shown to be identical with the authentic compound[2] 9.

The synthetic approach described in this note appears to be generally applicable for the synthesis of functionally substituted, chiral, norbornane systems, containing heteroatoms at positions 3 and 6.

Satisfactory elemental analyses have been obtained for all of the compounds described.

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REFERENCES

1 J. Cléophax, J. Hildesheim, and S.D. Géro, *Compt. Rend., Ser.C,* 265 (1967) 257.
2 J. Cléophax, J. Hildesheim, A.M. Sépulchre, and S.D. Géro, *Compt. Rend., Ser.C,* 266 (1968) 720.

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Oxidation of dehydro-L-ascorbic acid phenylosazone

The red dehydro-L-ascorbic acid phenylosazone was recently shown[1] to exist in the 1,5-lactone bis(phenylhydrazone) form (**1a**). We have found that mild oxidizing agents, such as cupric chloride and iodine, convert this osazone into a yellow dehydro-osazone (**2a**). The u.v. spectrum of **2a** shows maxima at 205, 219, 239, 280, and 366 nm, and its i.r. spectrum has a lactone band at 1720 cm^{-1} in exactly the same position as that of the red osazone **1a**, suggesting that the lactone ring remains unaffected during the oxidation. Compound **2a** gave, on acetylation, a yellow mono-*O*-acetyl derivative (**2b**) which showed a broad, unresolved band at 1740 cm^{-1} (ester and lactone). The n.m.r. spectrum of the yellow acetate **2b** (see Fig.1) was quite similar to that of the

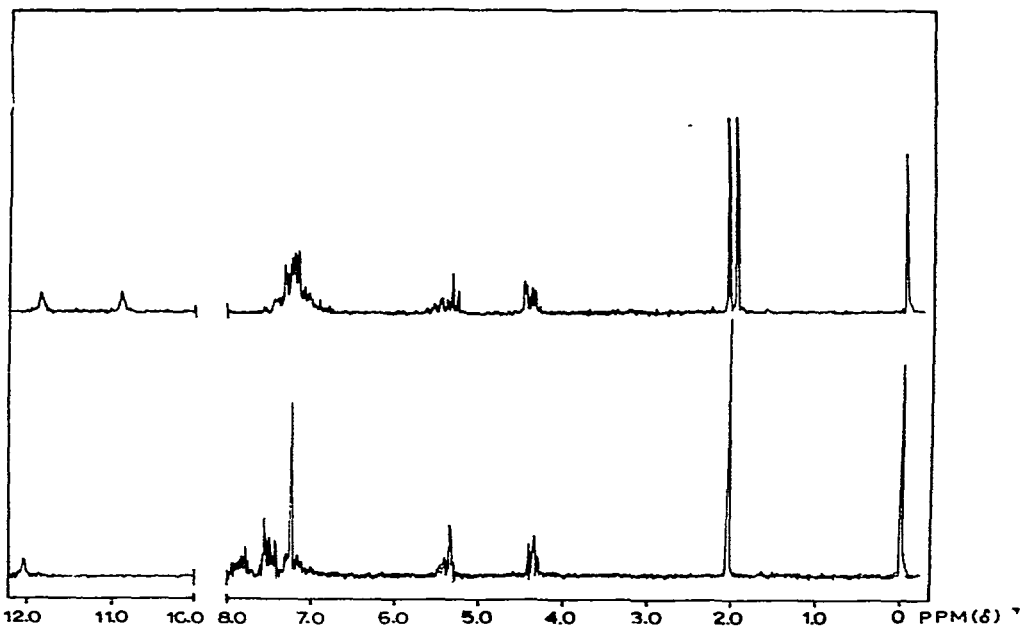


Fig.1. N.m.r. spectrum in chloroform-*d*. Upper graph (**1b**); lower graph (**2b**).

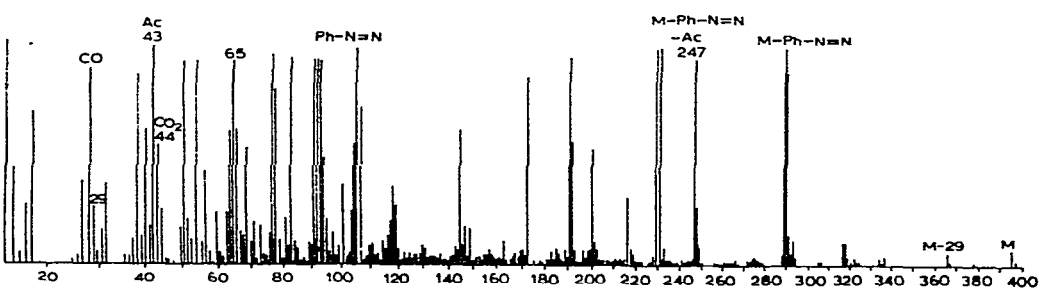
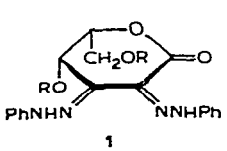
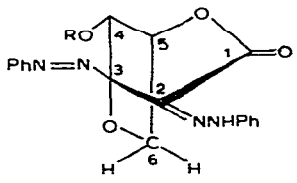


Fig.2. Mass spectrum of **2b**.

red 4,6-di-*O*-acetyl-dehydro-L-ascorbic acid phenylosazone (**1b**), except for the fact that it showed one acetyl group instead of two, and one imino proton instead of two. The methylene group of C-6 was at 5.64 followed at τ 4.67 and τ 4.56 by the C-4 and C-5 protons. The mass spectrum (see Fig.2) of **2b** agreed with a molecular weight of 394.5; it showed major peaks at 105 and M-105 due to the splitting of the Ph-N=N group, and a peak at 43 and M-105-43 due to the further splitting of the acetyl group. In addition, it showed the 65 peak of *N*-substituted anilines, and the 44 (CO_2) and 28 (CO) peaks, all of which agree with the assigned structure (**2b**), namely, 4-*O*-acetyl-3,6-anhydro-3-phenylazo-L-*xylo*-hexulosono-1,5-lactone phenylhydrazone, in which the primary hydroxyl on C-6 of the osazone precursor has cyclized with C-3, with subsequent oxidation to phenylazo of the hydrazo group formed.



1



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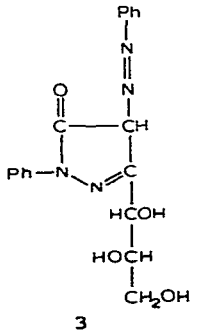

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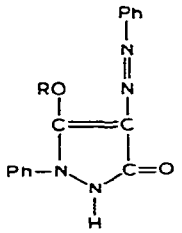

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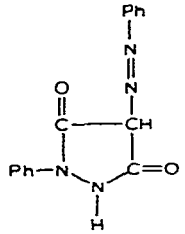

5

(a , R=H ; b, R=Ac)

That the cyclization of the primary hydroxyl group involved C-3, not C-2, was apparent when the dehydro-osazone (**2a**) was treated with alkali, and the mixture was acidified. Under these conditions, the parent, red osazone (**1a**) yields 1-phenyl-4-phenylazo-3-(L-*threo*-1,2,3-trihydroxypropyl)pyrazolin-5-one[2] (**3a**); the dehydroderivative (**2a**), on the other hand, cyclized to a yellow compound (**4a**), which proved to be the enol form of the known 1-phenyl-4-phenylazopyrazolidin-3,5-dione (**5**). The presence of the carbonyl oxygen atom on C-3 of **4a** is strongly indicative of the involvement of the C-3 hydrazone residue of **2a** in anhydro-ring formation, especially since, sugar under similar treatment, phenylosazones yield[3] glyoxal bis(phenylhydrazone) with no oxygen atom on C-2.

The yellow cyclization product **4a** showed one carbonyl band at 1685 cm^{-1}, and, on acetylation, it yielded a mono-acetate (**4b**) having a carbonyl band at 1710 (CONH) and at 1740 cm^{-1} (OAc); its n.m.r. spectrum revealed the *O*-acetyl protons at τ 7.33, deshielded by the heterocyclic ring; the phenyl protons appeared at τ 2.62, and

the deuterable, imino proton showed at τ −2.50. The mass spectrum of the acetate agreed with a molecular weight of 322, and showed many of the peaks of the parent **2b**, such as the Ph-N=N and M-Ph-N=N, the Ac at 43, etc., all of which agree with structure **4b**. Final proof of this structure was reached by acetylation of the known 1-phenyl-4-phenylazopyrazolidine-3,5-dione[4] (**5a**); this tautomer of **4a**, the i.r. spectrum of which possesses two carbonyl bands (at 1745 and 1720 cm^{-1}), gave, on acetylation, the same acetyl derivative **4b** as the one obtained from **4a**.

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REFERENCES

1 H. El Khadem and S.H. El Ashry, *Carbohyd. Res.*, (1968) in press.
2 H. Ohle, *Ber.*, 67 (1934) 1750.
3 O. Diels, R. Meyer, and O. Onnen, *Ann.*, 525 (1936) 94.
4 A. Michaelis and R. Burmeister, *Ber.*, 25 (1892) 1510.

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A mild method for the hydrolysis of acetal groups attached to sugars and nucleosides

Acetal groups are among the most useful protecting groups in sugar and nucleoside chemistry. Their removal, usually by aqueous acid, is, however, often difficult and is sometimes incompatible with other groups present. For removing these groups, we now report a new technique that is unusually mild, very rapid, and compatible with most of the common functional groups that are used in carbohydrate chemistry.

As part of an unambiguous synthesis of 1,6-dichloro-1,6-dideoxygalactitol[1], it was necessary to remove the acetal groups from 1,6-dichloro-1,6-dideoxy-2,4:3,5-di-*O*-isopropylidenegalactitol[2]. Aqueous acetic acid was ineffective, and use of a strongly acidic, ion-exchange resin in aqueous ethanol resulted in incomplete removal of the acetal groups, accompanied by solvolysis of the primary halide groups. When the protected sugar was dissolved in 90% (v/v) aqueous trifluoroacetic acid at room temperature, a high yield of deacetalated material of analytical purity was obtained within 10 min. The method has been extended to a variety of sugars, and the results are presented in Table I. It is clear from this summary that the method is applicable to benzylidene, as well as isopropylidene, acetals, and that the reagent does not noticeably affect halogen, benzoyl, sulfonyl, amino, or azido groups. We have noted no cleavage of the glycosyl–base linkage in the deacetalation of the one purine nucleoside derivative thus far studied. The applicability to a hexos-3-ulose derivative is also noteworthy. The trifluoroacetic acid reagent has excellent solvent properties and high volatility, thus simplifying processing procedures.

In a typical procedure, the protected compound (1 g) was dissolved in 10 ml of 9:1 (v/v) trifluoroacetic acid–water. After the compound had dissolved, the mixture was kept for 5–10 min at room temperature; the solvent was then evaporated off *in vacuo* (bath temperature <50°). Ordinarily, trituration of the residue with ether left a crystalline solid that was either analytically pure or could be recrystallized readily. For ether-soluble compounds, trituration of the residue with hot hexane usually removed traces of trifluoroacetic acid and, for example, benzaldehyde. In a number of cases, the product crystallized from the trifluoroacetic acid solution.

Further extension of the method to nucleoside chemistry is in progress, as is a study of the conditions necessary for hydrolysis of methylene acetals. Extension of the method to the hydrolysis of trityl ethers is also under investigation.

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TABLE I[a]

HYDROLYSES WITH AQUEOUS TRIFLUOROACETIC ACID

Starting material	*Product*	*Yield (%)*[b]	$[\alpha]_D$, *degrees*[c]	m.p., *degrees*[d]
1,6-Dichloro-1,6-dideoxy-2,4:3,5-di-*O*-isopropylidene-galactitol[2]	1,6-Dichloro-1,6-dideoxy-galactitol[1]	83–96		181–182
2,4:3,5-Di-*O*-isopropylidene-1,6-di-*O*-(methylsulfonyl)-galactitol[3]	1,6-Di-*O*-(methylsulfonyl)-galactitol[4]	83–84		140–141
2,4:2,5-Di-*O*-isopropylidene-1,6-di-*O*-*p*-tolylsulfonyl-galactitol[5]	1,6-Di-*O*-*p*-tolylsulfonyl-galactitol	76		127–129
1,6-Di-*O*-benzoyl-2,4:3,5-di-*O*-isopropylidene-galactitol[1]	1,6-Di-*O*-benzoyl-galactitol[1]	90		210–215
1,2:5,6-Di-*O*-isopropylidene-D-glucofuranose	D-Glucose	64	+52.3	144–147.5
1,2:5,6-Di-*O*-isopropylidene-3-*O*-*p*-tolylsulfonyl-D-allofuranose[6]	3-*O*-*p*-Tolylsulfonyl-D-allose	82–84	+ 3.5	140–143[e]
6-Azido-6-deoxy-1,2-*O*-isopropylidene-D-glucofuranose[7]	6-Azido-6-deoxy-D-glucose	68	+58.2	133.5–138
1,2:5,6-Di-*O*-isopropylidene-D-*ribo*-hexos-3-ulose[8]	D-*ribo*-Hexos-3-ulose[9]		+55.6[f]	119–125
3,5-*O*-Benzylidene-1,2-*O*-isopropylidene-6-*O*-*p*-tolylsulfonyl-D-glucofuranose[10]	6-*O*-*p*-Tolylsulfonyl-D-glucose[11]	44	+37.6	109–112[g]
Methyl 4,6-*O*-benzylidene-2-*O*-*p*-tolylsulfonyl-α-D-glucopyranoside[12]	Methyl 2-*O*-*p*-tolylsulfonyl-α-D-glucopyranoside[13]	60	+87.5 ($CHCl_3$)	137–138
Methyl 4,6-*O*-benzylidene-3-*O*-*p*-nitrobenzoyl-2-*O*-*p*-tolylsulfonyl-α-D-glucopyranoside	Methyl 3-*O*-(*p*-nitrobenzoyl)-2-*O*-*p*-tolylsulfonyl-α-D-glucopyranoside	65		180–182
5′-*O*-Acetyl-8-bromo-2′,3′-*O*-isopropylideneadenosine[14]	5′-*O*-Acetyl-8-bromoadenosine trifluoroacetate	51		145–149

[a] Correct elemental analyses were obtained for all new compounds, and their n.m.r. and i.r. spectra were consistent with the structures assigned. All other compounds had been reported in the literature, and their physical constants compared well with the literature values, except where otherwise noted. [b] Yield of analytical material. [c] End values in water (*c*, 1.0) unless otherwise noted. [d] Uncorrected. [e] Prior to recrystallization from ethanol, the product had m.p. 123.5–125°. [f] Reported[9] as the hydrate isolated from hydrolysis of "3-ketosucrose" The reported $[\alpha]_D^{26}$ value of +14.8° may indicate incomplete anomeric equilibration. [g] The lit.[11] values are $[\alpha]_D^{26}$ +39° and m.p. 132–133°. We attribute the difference in m.p. to anomeric differences, as the elemental analysis was correct.

of the authors, and not necessarily those of the Cancer Chemotherapy National Service Center.

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REFERENCES

1 K. Butler and W.A.W. Cummings, *J. Chem. Soc.*, (1956) 636.
2 T.P. Bird, W.A.P. Black, E.T. Dewar and J.B. Hare, *Chem. Ind.* (London), (1961) 1077.
3 S.S. Brown and G.M. Timmis, *J. Chem. Soc.*, (1961) 3656.
4 L. Vargha, J. Kuszmann and B. Dumbovich, Austrian Patent 214,417; *Chem. Abstr.*, 55 (1961) 15365.
5 R.M. Hann, W.D. Maclay and C.S. Hudson, *J. Amer. Chem. Soc.*, 61 (1939) 2432.
6 D.T. Williams and J.K.N. Jones, *Can. J. Chem.*, 45 (1967) 7.
7 F. Cramer, H. Otterbach, and H. Springmann, *Ber.*, 92 (1959) 384.
8 O. Theander, *Acta Chem. Scand.*, 18 (1964) 2209.
9 S. Fukui and R.M. Hochster, *J. Amer. Chem. Soc.*, 85 (1963) 1697.
10 A.S. Meyer and T. Reichstein, *Helv. Chim. Acta*, 29 (1946) 139.
11 J. Compton, *J. Amer. Chem. Soc.*, 60 (1938) 395.
12 R.W. Jeanloz and D.A. Jeanloz, *J. Amer. Chem. Soc.*, 79 (1957) 2579.
13 H.B. Wood, Jr., R. Allerton, H.W. Diehl, and H.G. Fletcher, Jr., *J. Org. Chem.*, 20 (1955) 875.
14 M. Ikehara and H. Tada, *Chem. Pharm. Bull.*, (Tokyo), 15 (1967) 94.

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