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THE DISTRIBUTION OF SUBSTITUENTS IN PARTIALLY ACETYLATED DEXTRAN

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ABSTRACT

A modified procedure for replacing the *O*-acetyl groups in partially acetylated dextran with the stable *O*-methyl group, using methyl vinyl ether as protective reagent, is described. After hydrolysis, the methylated sugars were transformed to the corresponding alditol acetates and analysed by gas–liquid chromatography. The distribution of substituents in two *O*-acetyldextrans prepared by different methods has been studied.

INTRODUCTION

The location of acetyl groups in partially acetylated polysaccharides is attended by several difficulties. Thus, acetyl groups are not only readily hydrolysed under basic conditions and, to a lesser extent, under acidic conditions, but may also migrate[1–5]. This problem has been studied by Bouveng[6–8], who elaborated a method in which the free hydroxyl groups were first protected by reaction with phenyl isocyanate under conditions where the acetyl groups were stable and did not migrate. Subsequent, selective acid hydrolysis of the acetyl groups, methylation, reductive cleavage of the phenylcarbamoyl groups, and, finally, complete hydrolysis of the resulting partially methylated polysaccharide afforded a mixture of sugars and methylated sugars from which the positions of the original acetyl groups could be deduced. The method has recently been applied to partially acetylated cellulose[9]. However, the time-consuming acidic deacetylation[9] (3×72 h) inevitably leads to depolymerisation which, in some cases is, undesirable, and to a loss of phenylcarbamoyl groups[7,9]. The reductive removal of phenylcarbamoyl groups also results in some demethylation.[9]

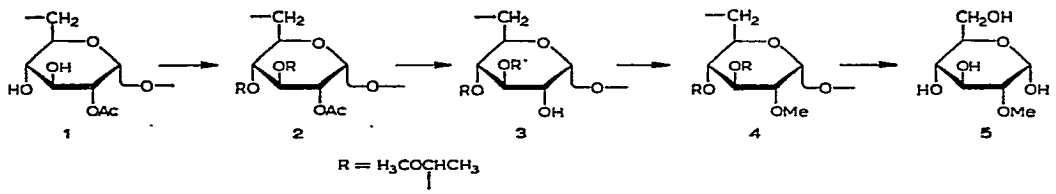
In this paper, the location of *O*-acetyl groups in partially acetylated dextrans, using methyl vinyl ether as protective reagent, is described. This modification simplifies and shortens the method and, at the same time, eliminates the above disadvantages.

RESULTS AND DISCUSSION

The reaction of vinyl ethers with alcohols yields mixed acetals, which, although stable under basic conditions, are very easily hydrolysed by acid. The preparation

of tetrahydropyranyl ethers of starch has earlier been described by Weaver and co-workers[10], and Wolfrom and co-workers[11] have extended this study to the reaction of alkyl vinyl ethers with amylopectin and amylose. In neither of these studies was a completely substituted derivative obtained, even in the presence of a large excess of the reagent.

Attempts to completely substitute an *O*-acetyldextran (**1**), using 2,3-dihydro-4*H*-pyran, methyl vinyl ether, or ethyl vinyl ether in methyl sulphoxide in the presence of *p*-toluenesulphonic acid as catalyst[11], were unsuccessful. It is plausible that, with this procedure, some hydrolysis of the acetal groups occurs during the precipitation in water, despite the precautions to avoid acidity. However, if the reaction product from the *O*-acetyldextran (**1**) and methyl vinyl ether was fractionated directly on a Sephadex LH-20 column, the i.r. spectrum indicated that complete substitution had been attained. Gel filtration thus permitted the quantitative separation of dextran derivative **2** from the reaction products of low molecular weight and from methyl sulphoxide, in the absence of water.



Deacetylation was accomplished in boiling 0.2% methanolic sodium methoxide and was complete after 4 h, giving the derivative **3**. The products of both this stage and the subsequent methylation were isolated most conveniently by gel filtration on Sephadex LH-20.

The *O*-(1-methoxyethyl)*O*-methyl-dextran (**4**) was finally subjected to acid hydrolysis to give a mixture of D-glucose and D-glucose methyl ethers (**5**) which, after reduction and acetylation, was analysed by g.l.c.[12] (Fig. 1.).

The procedure described above was used to investigate the distribution of substituents in two samples of *O*-acetyldextran prepared by using[13] acetic anhydride in aqueous sodium hydroxide (pH 8) and by the action of acetic anhydride in formamide–pyridine[14]. The values for the degree of substitution in each of the two samples, as determined by saponification and from the methoxyl analysis, were in good agreement (Table I). It was necessary to establish that no acetyl migration occurred during the acetalation in methyl sulphoxide. For this purpose, methyl 2-*O*-acetyl-4,6-*O*-benzylidene-α-D-glucopyranoside was treated under exactly the same conditions as for the acetalation. Thin-layer chromatography revealed no trace of the 3-acetate, and thus there was no indication of acetyl migration.

Table II reveals a marked difference in the substitution patterns of two partially acetylated dextrans. Thus, whereas the acetate prepared in aqueous alkali showed almost equal reactivities at C-2, C-3, and C-4, the acetylation in pyridine resulted in a considerable increase in the reactivity at C-2. Differences in the distributions have also been found for the acetylation of monosaccharides with acetic anhydride

under different conditions[15,16]. Williams and Richardson[17] have studied the pattern of reaction of methyl α-D-glucopyranoside with benzoyl chloride in pyridine. They found that the relative reactivities of the secondary hydroxyl groups were in the order

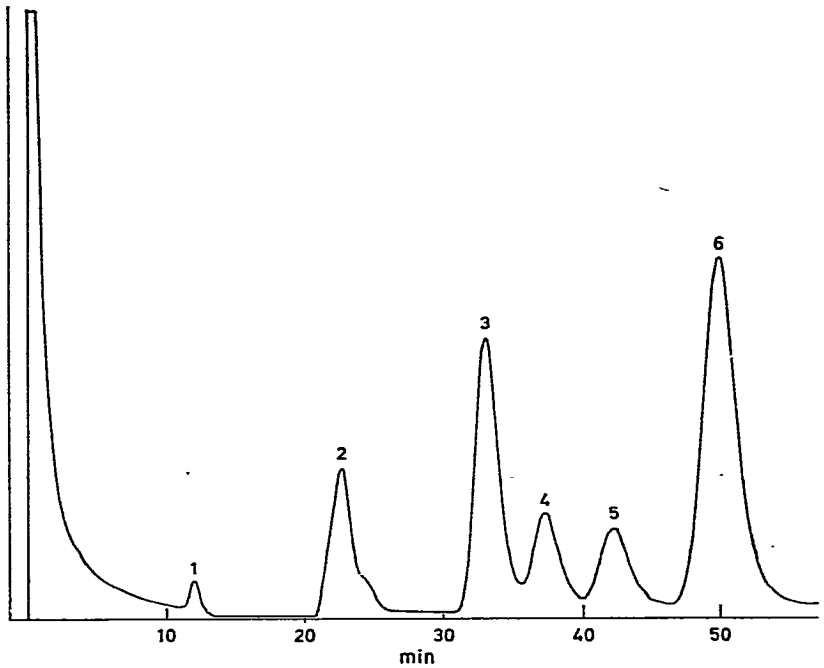


Fig. 1. G.l.c. analysis of an *O*-acetyldextran. (The *O*-acetyl groups are exchanged to *O*-methyl groups.) 1. 1,6-Di-*O*-acetyl-2,3,4-tri-*O*-methyl-D-glucitol (1,6-OAc-2,3,4-OMe-gluc). 2. 1,4,6-OAc-2,3,OMe-gluc + 1,3,6-OAc-2,4-OMe-gluc + 1,2,6-OAc-3,4-OMe-gluc. 3. 1,3,4,6-OAc-2-OMe-gluc. 4. 1,2,4,6-OAc-3-OMe-gluc. 5. 1,2,3,6-OAc-4-OMe-gluc. 6. 1,2,3,4,6-OAc-gluc.

2-OH > 3-OH > 4-OH. Likewise, Chalk and co-workers[18], in a study of the partial mesylation of methyl α-D-glucopyranoside with mesyl chloride in pyridine, established that the 2-OH is more reactive than the 3-OH. It is evident that the substitution pattern obtained for the *O*-acetyldextran, prepared in the presence of pyridine, follows the same general pattern shown by methyl α-D-glucopyranoside.

TABLE I

THE DEGREE OF *O*-ACETYL SUBSTITUTION IN TWO PARTIALLY ACETYLATED DEXTRANS

D. S. determined by saponification	*D. S. determined by OMe-analysis*	*D. S. loss during replacement reaction*
0.62	0.62	0.00
0.73	0.71	0.02

The greater reactivity of the 2-OH in α-D-glucopyranosyl units during acylation in pyridine has been explained by Richardson[19] in terms of hydrogen bonding with

TABLE II

PERCENTAGE MOLAR COMPOSITION OF TWO PARTIALLY ACETYLATED DEXTRANS

O-Acetyl substitution	*D. S.* 0.62[a]	*D. S.* 0.73[b]
2,3,4	2.1	1.0
2,3; 2.4; 3.4	12.2	12.2
2	10.6	22.2
3	11.1	9.3
4	14.2	8.9
0	48.5	46.4

[a]Synthesised by treatment of dextran with acetic anhydride in weakly alkaline sodium hydroxide solution. [b]Synthesised by treatment of dextran with acetic anhydride in pyridine–formamide (1:1).

the oxygen in the axial methoxyl group. A correlation between intramolecular hydrogen bonding and enhanced rates of esterfication had previously been established by Buck *et al.*[20,21]. In the β-D anomers, this hydrogen bonding effect would be less important and an increased reactivity of the 2-OH is not observed[15,18,19]

The similarity in the reactivities of the secondary hydroxyl groups during acetylation in aqueous alkali at pH 8.0 is presumably a result of acetyl migration, the distribution of substituents being thermodynamically rather than kinetically controlled.

The above procedure has also successfully been applied[22] to the location of *O*-acetyl groups in a 10-mg sample of the lipopolysaccharide from *Salmonella typhimurium* 395 MS.

EXPERIMENTAL

All evaporations were conducted under diminished pressure at a bath temperature below 40°. I.r. spectra were obtained with a Unicam SP-200 spectrophotometer. G.l.c. analyses were performed with an F & M Model 810 instrument using a copper column (10 ft × 0.25 in) packed with 3% ECNSS-M (w/w) [copolymer of poly(ethylene glycol succinate) and a nitrile silicone polymer] on Gaschrom Q (100–120 mesh)[22] at 192°; 45 ml of nitrogen per min; injection port at 265°; and flame-ionization detector at 300°.

The *O*-acetyldextrans used in this investigation were prepared by treatment of dextran B-512 ($\overline{M}_w$ 40,000) with acetic anhydride in a weakly basic sodium hydroxide solution[13], and in a mixture[14] of pyridine–formamide (1:1).

Treatment of O-*acetyldextran* **1** *with methyl vinyl ether.* — After being dried in a vacuum (24 h at 100°), the *O*-acetyldextran (5.0 g, D. S. 0.73) was dissolved in anhydrous methyl sulphoxide (100 ml). *p*-Toluenesulphonic acid (0.185 g) was added, and the solution was thermostatted at 15°. With stirring, methyl vinyl ether (15 g), condensed at −10°, was then added in portions. Stirring was continued for 3.5 h at 15°. The clear, red reaction mixture was then placed on a Sephadex LH-20 column

(25 × 7 cm) and eluted with anhydrous acetone (with slight suction). The reaction product was collected and concentrated, giving a red, amorphous substance (**2**, 10.3 g), $[\alpha]_{5760}^{22}$ +133° (*c* 0.86, chloroform). The absence of OH absorption (3400–3600 cm^{-1}) in the i.r. spectrum of this substance indicated that complete substitution had been obtained.

Anal. Calc. for $C_6H_7O_2(OAc)_{0.73}[OCH(CH_3)OCH_3]_{2.27}$: C, 53.1; H, 7.80. Found: C, 53.2; H, 7.73.

Deacetylation of the O-*acetyl*-O-(1-*methoxyethyl*)*dextran* (**2**). — The *O*-acetyl-*O*-(1-methoxyethyl)dextran (5 g) was dissolved in 0.2% methanolic sodium methoxide (100 ml) and refluxed for 4 h. The solution was then placed directly on a Sephadex LH-20 column (80 × 4 cm) and eluted with methanol. The reaction product was collected and concentrated to a red syrup (**3**, 4.5 g), $[\alpha]_{5760}^{22}$ +142° (*c* 0.69, chloroform). The absence of C=O absorption in the i.r. spectrum of this syrup indicated that complete deacetylation had been obtained.

Anal. Calc. for $C_6H_7O_2(OH)_{0.73}[OCH(CH_3)OCH_3]_{2.27}$: C, 52.7; H, 8.10. Found: C, 52.5; H, 8.05.

Complete methylation of the O-(1-*methoxyethyl*)*dextran* (**3**). — The *O*-(1-methoxyethyl)dextran (3.3 g) was dissolved in *N*,*N*-dimethylformamide (DMF) (50 ml), and methylated with methyl iodide (5 ml) and silver oxide (5 g) during 20 h. The reaction mixture was then filtered, and the silver salts were washed with DMF (25 ml). The filtrate and washings were pooled, and the methylation procedure was repeated twice. Benzene (200 ml) was then carefully added to the filtrate, the white precipitate was filtered off, and the benzene was evaporated. The resulting DMF solution was then placed on a Sephadex LH-20 column (80 × 4 cm) and eluted with methanol. The reaction product was collected and concentrated (30°) to a light-yellow syrup (**4**, 3.3 g). The *O*-methyl-*O*-(1-methoxyethyl) derivative (0.5 g) was hydrolyzed in 2N sulphuric acid (50 ml) for 5.5 h at 100° (the compound completely dissolved after 15 min). The solution was then deionised (IR-4B, free base) and concentrated to a syrup. Paper chromatography (butyl alcohol–ethanol–water, 40:11:19) indicated that complete hydrolysis had been obtained.

Anal. Calc. for $C_6H_9O_3(OCH_3)_{0.73}(OH)_{2.27}$; OMe 11.9. Found: OMe 11.6.

Analysis of the hydrolysed O-(1-*methoxyethyl*) O-*methyl-dextran*. — The hydrolysate was reduced with sodium borohydride and acetylated with acetic anhydride[12,19]. The resulting mixture of partially methylated alditol acetates was then analysed by g.l.c.

ACKNOWLEDGMENT

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NUCLEOPHILIC REPLACEMENT REACTIONS OF SULPHONATES

PART I. THE PREPARATION OF DERIVATIVES OF 4,6-DIAMINO-4,6-DIDEOXY-D-GLUCOSE AND -D-GALACTOSE

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ABSTRACT

The nucleophilic displacement reactions of some 4,6-disulphonates of methyl α-D-glucopyranoside and methyl α-D-galactopyranoside have been studied by using sodium azide in *N,N*-dimethylformamide. Displacement occurs at both positions by a bimolecular mechanism, giving the corresponding 4,6-diazides with inversion of configuration at C-4. The azides have been reduced to derivatives of 4,6-diamino-4,6-dideoxy-D-glucose and -D-galactose.

INTRODUCTION

Bimolecular, nucleophilic, displacement reactions of sulphonic esters of carbohydrate derivatives, with the appropriate nucleophiles, offer a ready approach to amino sugars, thio sugars, halogeno sugars, deoxy sugars, etc. Replacement of a terminal sulphonyloxy group is usually accomplished[1] readily in such solvents as acetone, butanone, etc., except in cases where the sulphonyloxymethyl substituent is situated adjacent to either a *cis*-axial group on a six-membered ring, as in the case of galactopyranosides[2], or adjacent to the anomeric carbon, as in ketose 1-sulphonates[3], when replacement is sluggish due to unfavourable steric, polar, and electronic factors. Displacement of secondary sulphonyloxy groups of hexopyranosides with charged nucleophiles is extremely sensitive to steric and electronic factors, which, in many cases, inhibit the reaction altogether, and normally requires the presence of such dipolar aprotic solvents as *N,N*-dimethylformamide, *N*-methyl-2-pyrolidone, or hexamethylphosphoric triamide. The presence of a substituent, other than hydrogen, which is *β-trans*-axial to the departing sulphonate group is the most commonly encountered factor which impedes direct replacement. This is clearly illustrated by 1,2,4,6-tetra-*O*-benzoyl-3-*O*-methanesulphonyl-D-glucopyranoses; the β-anomer is readily converted into β-D-allopyranose pentabenzoate by sodium benzoate in *N,N*-dimethylformamide, whereas the α-anomer, in which the axial anomeric group is *β-trans* to the departing sulphonyloxy group, is resistant to

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replacement[4a]. However, Angyal and his co-workers[4b] have found that methyl 3-*O*-toluene-*p*-sulphonyl-α-D-glucopyranoside 2,4,6-triacetate undergoes a slow displacement reaction with sodium benzoate in boiling *N*,*N*-dimethylformamide, despite a *β-trans*-axial effect, to give the corresponding allopyranoside. The reason for this is probably the stability of the substrate and product under the reaction conditions (66 h in boiling solvent), because, in our experience, most sulphonic esters will not withstand such forcing conditions without extensive decomposition. In the case of certain 4-*O*-methanesulphonyl-mannopyranosides, direct replacement is prevented by the *β-trans*-axial substituent at C-2, but ring contraction is observed as a result of participation of the C-5–O bond in the displacement of the sulphonate group, and furanoside products are formed[5]. Replacement reactions adjacent to the anomeric group are difficult to effect with charged nucleophiles, even if the sulphonate is at a terminal position[3], presumably because of an electron-withdrawing effect from the anomeric group and/or unfavourable polar interactions in the transition state. The fact that examples of replacement at C-2 with neutral nucleophiles, such as hydrazine[6], are known suggests that polar, repulsive forces may be important, because the use of a neutral nucleophile reverses the polarity of the developing carbon-to-nucleophile bond, giving rise to attractive, polar forces in the transition state.

Bimolecular replacement reactions of chlorosulphate groups are extremely sensitive to the "*β-trans*-axial effect", and, furthermore, they resist replacement if sited next to an axial substituent[7]. This effect is due to unfavourable steric (and probably polar) factors in the transition state. However, this factor is probably of less importance in the replacement reactions of sulphonic esters, which are carried out under more-vigorous conditions, because some examples of replacement of a sulphonate group, flanked by an axial substituent, are known[8,9].

Examples of displacement of sulphonate groups by participation of neighbouring, carboxylic-ester groups, although well established in alicyclic chemistry[10], are not so common in the pyranose series[11], although solvolysis of sulphonate groups through the agency of *trans*-acyloxy substituents has been achieved by using sodium fluoride in *N*,*N*-dimethylformamide[12]. In contrast, acylamide groups are excellent participating groups, and many examples of solvolysis of sulphonate groups (situated *trans* to the participating group), *via* oxazoline intermediates, have been cited (*e.g.*, Ref. 13). If such an intermediate underwent rearward nucleophilic attack, it would give rise to a product with overall retention of configuration, but only in two cases has this been observed in the carbohydrate field[14].

In the light of these considerations, a sulphonate group situated at C-4 of a pyranoside, provided the substituent at C-2 is not *trans*-axial, should undergo replacement. Consequently, if reaction occurs in the *C1* (D) [*i.e.*, *1C* (L)] conformation, the 4-sulphonates of xylopyranosides, ribopyranosides, allopyranosides, galactopyranosides, glucopyranosides, and gulopyranosides should undergo nucleophilic displacement. At the outset of this study, only replacement at C-4 of a galactopyranoside had been observed[15], but, since our preliminary report[16], displacement of the 4-sulphonates of xylopyranosides[17], galactopyranosides[18], and glucopyranosides[19] have been

described. In addition, arabinopyranosides[20] and altropyranosides[9] also undergo displacement at C-4 by reacting in the *1C* (D) [*i.e.*, *C1* (L)] conformation.

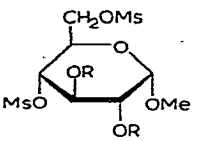
RESULTS AND DISCUSSION

The 2,3-diacetate and the 2,3-dibenzoate of methyl α-D-glucopyranoside 4,6-dimethanesulphonate (**1** and **2**, respectively) were prepared from methyl 4,6-*O*-benzylidene-α-D-glucopyranoside by acylation, followed by mild hydrolysis with acid, and methanesulphonylation of the resulting 4,6-diol. Whereas the dibenzoate **1** was readily obtained pure by this procedure, in an overall yield of 77%, the diacetate was always contaminated with small proportions (~5%) of impurities, from which it could not be freed by repeated recrystallisation. This product was, however, suitable for further reactions. On one occasion, the major impurity was isolated by chromatography, and analysis indicated that it was a tri-*O*-acetyl-mono-*O*-methanesulphonylhexopyranoside. The third acetate group must have been introduced during the hydrolysis of the benzylidene group with aqueous acetic acid. Since it had been reported[21] that D-glucose undergoes selective acetylation at C-6 with 50% aqueous acetic acid, it was thought most likely that the impurity was the 2,3,6-triacetate **3**. Surprisingly, the impurity was not identical with triacetate **3** synthesised from methyl 2,3-di-*O*-acetyl-α-D-glucopyranoside by selective acetylation at C-6 by acetyl chloride, followed by methanesulphonylation. It was shown to be the corresponding 2,3,4-tri-*O*-acetyl-6-*O*-methanesulphonyl derivative[22] (**4**), which was synthesised similarly from the 2,3-diacetate by selective methanesulphonylation, followed by acetylation. The preferential acetylation of the 4-hydroxyl group, rather than the 6-hydroxyl group, during the hydrolysis stage is obscure, and we can offer no satisfactory explanation of this behaviour.

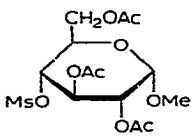
The dimethanesulphonates **1** and **2** reacted smoothly with sodium azide in *N*,*N*-dimethylformamide at 130–155° to give diazido derivatives in 70% and 40% yields, respectively. The lower yield of the diacetate was undoubtedly due to deacetylation during the course of the reaction, and subsequent work has shown that benzoate groups are more satisfactory for blocking purposes in these replacement reactions. The presence of the 3-acyloxy group trans to the departing sulphonate group at C-4 in compounds **1** and **2** offered an alternative pathway to direct bimolecular replacement, and a rigorous structural determination was therefore essential. Direct bimolecular replacement would lead to the 4,6-diazido-galactosides **6** and **7**, whereas involvement of the 3-ester group would lead to the 3,4-acyloxonium ion intermediate **12**, which could undergo ring-opening by azide to give either the 4-azido-glucoside **11** or the 3-azido-guloside **13**. Another possibility is that the 3,4-acyloxonium ion **12** could isomerise to the 2,3-ion **15**; a precedent for this is provided by the work of Paulsen *et al.*[23] on the isomerisation of acetoxonium-pyranose derivatives. The 2,3-ion could then lead to the 3-azido-galactoside **14** or the 2-azido-idoside **16**.

The diazido-diesters were inter-related by their conversion by deacylation, into the same diazidodiol which, upon catalytic reduction, followed by acetylation, gave

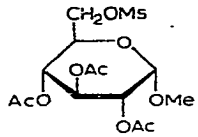
the corresponding diacetamido-di-*O*-acetyl-pyranoside. De-*O*-acetylation gave the diacetamido derivative **9**, which reduced 1.07 moles of periodate per mole. The reduction of one mole of oxidant is consistent only with a 4,6- or 2,6-diazide structure. The n.m.r. spectrum of the diazido-dibenzoate showed, without doubt, that our product was the galactoside **6**, which had been formed by direct bimolecular nucleophilic displacement at C-4. Previous work has shown that the presence of benzoyloxy



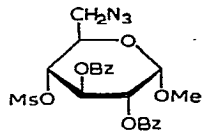
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2 R = Ac



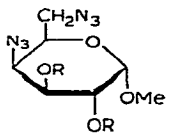
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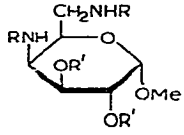
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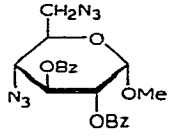
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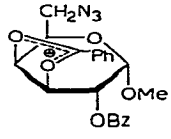
6 R = Bz
7 R = Ac



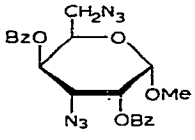
8 R = R' = Ac
9 R = Ac, R' = H
10 R = R' = Bz



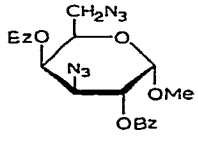
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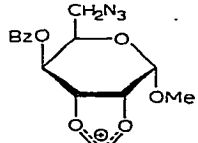
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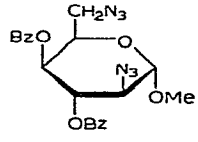
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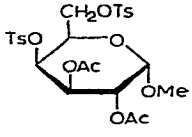
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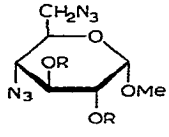
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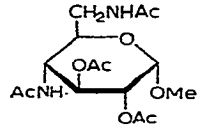
16



17



18, R = Ac
19, R = H



20

groups on a pyranoside ring has a strong deshielding effect on protons attached to the same ring carbon-atom[24,25]. Hence, the signals for these protons are often observed to lower field than those of all other ring protons, including, in many cases, the anomeric proton. The n.m.r. spectrum of the diazido-dibenzoate showed two identical low-field quartets at τ 4.03 and τ 4.41, with splittings of 3.5 and 10.5 Hz, and the doublet due to the anomeric proton was readily recognised at τ 4.82, with a splitting of 3.5 Hz. The low-field quartets must be due to the protons attached to the same carbon atom as the ester groups, and the splittings suggested that these were both axial protons coupled to an axial and an equatorial proton (*i.e.*, *ax–ax*, *ax–eq* coupling). Examination of all possible structures (**6**, **11**, **13**, **14**, and **16**) for the dibenzoate

revealed that only **6** would exhibit two low-field quartets with the observed coupling constants. Furthermore, the asymmetry of the two quartets suggested that they were mutually coupled. The resonance due to H-4 was recognised as a broad doublet at τ 5.74, probably overlapped with that due to H-5. Spin-decoupling experiments using this resonance caused the quartet at τ 4.03 to collapse to a doublet, showing that this was due to H-3. The corresponding diacetate **7** gave a fairly similar, but less well-resolved, n.m.r. spectrum. Further evidence for direct bimolecular replacement of the 4-sulphonate group was provided by the observation that disulphonate **1** was converted into methyl α-D-galactopyranoside tetra-*O*-benzoate by the action of sodium benzoate in *N*,*N*-dimethylformamide.

Application of these azide replacements to methyl 2,3-di-*O*-acetyl-4,6-di-*O*-toluene-*p*-sulphonyl-α-D-galactopyranoside (**17**) gave rise to similar 4,6-diazides having the *gluco*-stereochemistry (**18**). The precursor **17** was synthesised from methyl 4,6-*O*-benzylidene-α-D-galactopyranoside in a manner similar to that described for diacetate **2**. It was noted, however, that toluene-*p*-sulphonylation of the 4-hydroxyl group was exceedingly slow, taking at least twelve days for completion at room temperature, even in the presence of a large excess of the acid chloride. This is in accord with previous observations[24] that, for steric reasons, the 4-hydroxyl group of methyl α-D-galactopyranoside is particularly resistant to acylation. The reaction of the galactoside **17** with sodium azide in *N*,*N*-dimethylformamide proceeded more slowly than with the glucoside **2**, to give a complex mixture from which a syrupy diazido-diacetate **18** was isolated in 21% yield after chromatography. The low yield was attributable to partial deacetylation, because if the crude reaction product was de-*O*-acetylated before isolation of the diazide, the corresponding crystalline diazido-diol **19** could be obtained in 46% yield.

The possibility of neighbouring-group participation does not arise in the case of replacement at C-4 of galactopyranosides, and hence the diazide must arise by a bimolecular displacement reaction and consequently has the *gluco*-stereochemistry. In support of this, it has already been shown that methyl 2,3,6-tri-*O*-benzoyl-4-*O*-methanesulphonyl-α-D-galactopyranoside is converted into methyl α-D-glucopyranoside tetrabenzoate by the action of sodium benzoate in *N*,*N*-dimethylformamide[15]. The n.m.r. spectrum of diazide **18** showed only one completely separated multiplet, but this served to support the *gluco*-configuration. The rather poorly resolved triplet at τ 4.54 ($J \sim 8$ Hz) was assigned to H-3 (*ax–ax*, *ax–ax* coupling), and the complex multiplet centred at τ 5.10 was assigned to H-2 and H-1.

As in the case of the diazido-galactoside **7**, catalytic hydrogenation of diazide **18** gave, after acetylation, variable yields of methyl 4,6-diacetamido-2,3-di-*O*-acetyl-4,6-dideoxy-α-D-glucopyranoside (**20**).

Attempts to obtain reducing sugars from the diazido and diacetamido sugars by acid hydrolysis were unsuccessful, and extensive degradation was observed under the acidic conditions employed. In the case of the acetamido derivatives, this is probably due to nitrogen entering the ring, which may eventually lead to pyrrole derivatives[26].

TABLE I

CHEMICAL SHIFTS (τ-VALUES) AND FIRST-ORDER COUPLING CONSTANTS (Hz) FOR COMPOUNDS 5–7 AND 18

Compound	6	7	18	5
H-1	4.82(d)[a]	5.05(d)	5.1(cm)	4.8(cm)
H-2	4.41(q)	4.85(q)[b]		
H-3	4.03(q)	4.57(q)	4.54(t)[c]	3.92(t)[c]
H-4	5.74(bd)	5.93(bd)	—	5.11(t)
H-5	—	—	—	—
H-6	—	—	—	—
OMe	6.57(s)	6.60(s)	6.60(s)	6.50(s)
OAc	—	7.90(s), 7.93(s)	7.91(s), 7.94(s)	CH_3SO_2 7.13(s)
$J_{1,2}$	3.5	3.0	—	—
$J_{2,3}$	10.5	10.5	~8	~10
$J_{3,4}$	3.5	3.0	~8	9.0
$J_{4,5}$	<1	<1	—	9.0

[a]Key: s, singlet; d, doublet; bd, broad doublet; t, triplet; q, quartet; cm, complex multiplet. [b]An inside limb of this quartet failed to appear. [c]This was observed as a broad triplet, probably virtually coupled to H-1 by *ca* 1.5–2 Hz [*cf.* B. Coxon, *Tetrahedron*, 21 (1965) 3481].

EXPERIMENTAL

All solutions were concentrated under diminished pressure at 45°, and light petroleum (b.p. 60–80°) was used throughout. Optical rotations were determined at 25 ±2° for chloroform solutions, unless otherwise stated. Melting-point determinations were carried out on a Kofler microscope hot-stage and are uncorrected. N.m.r. spectra were measured on a Varian A-60 spectrometer. For column chromatography, silica gel "Davison" grade 950 (60–200 mesh), and, for t.l.c., silica gel G (Merck) were used. The plates were developed by spraying with a solution of 5% sulphuric acid in ethanol and heating them for several min at 120°. Unless otherwise stated, all named products gave single spots on chromatograms, and their i.r. spectra were in accord with the structures assigned to them.

Methyl 2,3-di-O-benzoyl-4,6-di-O-mesyl-α-D-glucopyranoside (1). — Methyl 4,6-*O*-benzylidene-α-D-glucopyranoside (106 g) was dissolved in pyridine (400 ml), and benzoyl chloride (100 ml) was added in portions (3 × 33 ml) over about 5 min. The mixture was kept for 3 h at room temperature, water was then added slowly, the crystals which formed were filtered off and, without further purification or drying, dissolved in acetone (1 litre), and the solution was just acidified with conc. hydrochloric acid (*ca.* 15–30 ml). A further 6 ml of the acid was then added, and the solution was heated under reflux for 1.25 h, when t.l.c. showed the reaction to be complete. An excess of sodium hydrogen carbonate was then added, and the neutral solution was filtered and concentrated, and the resulting syrup was dried *in vacuo*. The product was dissolved in warm pyridine (300 ml), the solution was cooled, and mesyl chloride (70 ml) was added with cooling. After 2.5 h at room temperature, the reaction mixture was diluted carefully with water, and the resulting crystalline product was filtered off

and washed successively with water, ethanol, and ether. Recrystallisation from ethanol (*ca.* 3000 ml) afforded compound **1** (162 g; 77% overall), m.p. 151–152°, $[\alpha]_D$ +146° (*c* 0.5, acetone) (Found: C, 49.35; H, 4.6; S, 11.4. $C_{23}H_{26}O_{12}S_2$ calc.: C, 49.45; H, 4.65; S, 11.5%).

*Methyl 2,3-di-*O*-acetyl-4,6-di-*O*-mesyl-α-*D*-glucopyranoside* (**2**). — Methyl 4,6-*O*-benzylidene-α-D-glucopyranoside (14.8 g) was dissolved in pyridine (40 ml) and acetic anhydride (15 ml) was added. After the mixture had been kept overnight at room temperature, ice (*ca.* 120 g) was added with vigorous stirring, and the crystalline diacetate was collected, washed twice with water, and then suspended in 50% acetic acid (100 ml). The solid dissolved as the temperature was raised, and, after it had been boiled under reflux for 5 min, the solution was cooled slightly, before being concentrated to a mobile syrup, which became more viscous after being washed twice with light petroleum to remove benzaldehyde. The syrup was then dried overnight at 60° *in vacuo*, and dissolved in pyridine (50 ml), and mesyl chloride (10 ml, 3.3 moles) was added in two portions with cooling. After standing overnight at room temperature, the dark-brown solution was slowly poured into ice–water (*ca.* 500 ml) with vigorous stirring. The crystalline product was filtered off, washed with ethanol, and then dried to give 16.0 g (70%) of crude methyl 2,3-di-*O*-acetyl-4,6-di-*O*-mesyl-α-D-glucopyranoside (**2**) (m.p. 162–167°), which was normally used for further reactions without purification. T.l.c. showed a faster moving impurity (*ca.* 5%) and two slower moving, minor impurities (each *ca.* 1%) which co-crystallised with disulphonate **2** from several solvents.

On one occasion, a solution of the impure disulphonate (14.75 g) in chloroform was introduced onto a column of silica gel (400 g), and eluted with chloroform–light petroleum (20:1 v/v) at a rate of *ca.* 30 ml per h, the products being detected by t.l.c. The first product was identified as methyl 2,3,4-tri-*O*-acetyl-6-*O*-mesyl-α-D-glucopyranoside (180 mg, 2%) by comparison with an authentic sample (see below). Recrystallised from ethanol it had m.p. 111–113° and $[\alpha]_D$ +125° (*c* 1.06); lit.[28], m.p. 113°. Methyl 2,3-di-*O*-acetyl-4,6-di-*O*-mesyl-α-D-glucopyranoside (6.0 g, 40%) was then eluted, and had m.p. 174–176° (from ethanol), $[\alpha]_D$ +113° (*c* 1.67) (Found: C, 36.1; H, 5.1; S, 14.65. $C_{13}H_{22}O_{12}S_2$ calc.: C, 35.95; H, 5.1; S, 14.75%).

*Methyl 2,3,6-tri-*O*-acetyl-4-*O*-mesyl-α-*D*-glucopyranoside* (**3**). — A solution of methyl 2,3-di-*O*-acetyl-4,6-*O*-benzylidene-α-D-glucopyranoside (1.00 g) in 50% acetic acid (15 ml) was heated under reflux for 20 min and then concentrated to dryness, the last traces of water being removed by co-distillation with ethanol. The syrup was shaken twice with light petroleum and dried *in vacuo* at 60° overnight, after which it was dissolved in dry pyridine (25 ml) and treated with acetyl chloride (1.1 moles/mole) at −50°. After the mixture had been warmed to room temperature and kept for 2 h, t.l.c. showed a considerable quantity of residual starting material, and more acetyl chloride (0.5 mole/mole) was added, again at −50°. After 2 h, the solution was treated with mesyl chloride (0.8 ml, large excess) and then stored overnight at room temperature. The addition of ice–water (50 ml) gave no precipitate, and the solution was concentrated to dryness. A solution of the resulting dark syrup in

chloroform (200 ml) was washed with water (3 × 150 ml), dried ($CaSO_4$), and, after decolourisation with charcoal, concentrated to a colourless syrup. Crystallisation from ethanol–light petroleum gave a crude (t.l.c.) product (0.82 g; 77%). Four recrystallisations from ethanol gave pure compound **3**, m.p. 130–132°, $[\alpha]_D$ +73° (*c* 1.04) (Found: C, 42.35; H, 5.55; S, 8.2. $C_{13}H_{22}O_{11}S$ calc.: C, 42.2; H, 5.55; S, 8.05%).

*Methyl 2,3,4-tri-*O*-acetyl-6-*O*-mesyl-α-*D*-glucopyranoside* (**4**). — Methyl 2,3-di-*O*-acetyl-α-D-glucopyranoside was prepared, as described above, from the 4,6-benzylidene derivative (1.0 g), and the syrupy product, after removal of benzaldehyde and drying, was treated with mesyl chloride (1.4 moles/mole) at −50°, the reaction being followed by t.l.c. Excess of acetyl chloride was then added, and, after a similar processing, a crystalline product (760 mg, 70%), m.p. 107–110°, was obtained from ethanol. T.l.c. showed the product to be impure, and it could not be purified by recrystallisation. A pure specimen, obtained by chromatography on silica gel with ether–light petroleum (3:1 v/v), had m.p. 111–113°, $[\alpha]_D$ +125° (*c* 1.06); lit.[27], m.p. 113° (Found: C, 42.25; H, 5.55; S, 8.2. $C_{13}H_{22}O_{11}S$ calc.: C, 42.2; H, 5.55; S, 8.05%).

*Methyl 2,3-di-*O*-acetyl-4,6-di-*O*-toluene-*p*-sulphonyl-α-*D*-galactopyranoside* (**17**). — Methyl 2,3-di-*O*-acetyl-4,6-*O*-benzylidene-α-D-galactopyranoside (60 g) was heated for 30 min under reflux with 50% acetic acid (250 ml), and the reaction was shown to be complete by t.l.c. The solution was concentrated to a syrup which was washed with light petroleum to remove benzaldehyde, and dried for 24 h *in vacuo* over phosphorus pentoxide. It was then treated with tosyl chloride (130 g, 4 moles/mole) in pyridine (300 ml); t.l.c. showed that the reaction required 12 days at room temperature to reach completion. After the addition of water (50 ml), the solution was concentrated to a thick syrup which was dissolved in chloroform (600 ml). The chloroform solution was washed successively with 2N hydrochloric acid (2 × 500 ml), saturated aqueous sodium hydrogen carbonate (500 ml), and water (500 ml), and then dried ($CaSO_4$), decolourised with charcoal, and concentrated to a syrup. Crystallisation from ethanol gave disulphonate **17** (68 g, 63%), m.p. 121–123°, $[\alpha]_D$ +106° (*c* 0.82) (Found: C, 51.3; H, 5.1; S, 10.9. $C_{25}H_{30}O_{12}S_2$ calc.: C, 51.2; H, 5.1; S, 10.9%).

Derivatives synthesised via replacement reactions

*Methyl 2,3-di-*O*-acetyl-4,6-diazido-4,6-dideoxy-α-*D*-galactopyranoside* (**7**). — The dimesyl-diacetate **2** (9.5 g) and sodium azide (8.0 g, 5.0 moles/mole) were heated for 18 h at 130° in dry *N*,*N*-dimethylformamide (130 ml). The sodium azide did not dissolve initially, and sodium methanesulphonate was deposited as the reaction proceeded. The cooled mixture was diluted with chloroform (300 ml) and filtered. The solution was then washed with water (3 × 200 ml), dried over calcium sulphate, decolourised with charcoal, and concentrated to a syrup which soon crystallised. Recrystallisation from ethanol gave the diazide **7** (2.40 g, 40%), m.p. 152–153°, $[\alpha]_D$ +100° (*c* 1.24) (Found: C, 40.00; H, 4.65; N, 25.55. $C_{11}H_{16}N_6O_6$ calc.: C, 40.25; H, 4.9; N, 25.6%).

It was found by t.l.c. that the reaction was complete in 3 h if carried out at 150–155°. Excessive de-*O*-acetylation occurred in some preparations, and, in such cases, the yield of the diazido compound was increased by acetylation of the reaction product.

*Methyl 4,6-diazido-2,3-di-*O*-benzoyl-4,6-dideoxy-α-*D*-galactopyranoside* (**6**). — This compound was made in exactly the same way as the 2,3-diacetate, using methyl 2,3-di-*O*-benzoyl-4,6-di-*O*-mesyl-α-D-glucopyranoside (**1**) as starting material. Heating with sodium azide (5 moles/mole) in *N,N*-dimethylformamide for either 3 h at 155° or 18 h at 130° gave yields of 60–70%. Recrystallisation from ethanol gave colourless crystals of the diazide **6**, m.p. 106–108°, $[\alpha]_D$ +40° (*c* 1.00, methanol) (Found: C, 55.75; H, 4.4; N, 18.75. $C_{21}H_{20}N_6O_6$ calc. C, 55.75; H, 4.4; N, 18.6%).

*Methyl 2,3-di-*O*-acetyl-4,6-diazido-4,6-dideoxy-α-*D*-glucopyranoside* (**18**). — Methyl 2,3-di-*O*-acetyl-4,6-di-*O*-toluene-*p*-sulphonyl-α-D-galactopyranoside (20 g) and sodium azide (8 g) were mixed with *N,N*-dimethylformamide (300 ml) and heated for 14 h at 140°. Concentration of the reaction mixture gave a thick, black paste which was fractionated between chloroform and water. The chloroform solution (600 ml) was washed with water (2 × 400 ml), dried ($CaSO_4$), decolourised with charcoal, and concentrated to a syrup. T.l.c. revealed a complex mixture, with at least seven components, three of which preponderated. After being dried *in vacuo* overnight at 60°, the mixture was eluted from silica gel with ether–light petroleum (2:1 v/v). The major component, syrupy compound **18** (1.75 g, 21%), was eluted first, and the low yield was attributable to de-esterification in the reaction mixture (Found: C, 40.7; H, 5.25; N, 23.6. $C_{11}H_{16}N_6O_6$ calc.: C., 40.25; H., 4.9; N., 25.6%).

*Methyl 6-azido-2,3-di-*O*-benzoyl-6-deoxy-4-*O*-mesyl-α-*D*-glucopyranoside* (**5**). — Methyl 2,3-di-*O*-benzoyl-4,6-di-*O*-mesyl-α-D-glucopyranoside (0.83 g) and sodium azide (0.77 g) were heated at 150° in *N,N*-dimethylformamide (10 ml). After 15 min, the cooled mixture was diluted with water to give gelatinous material. The addition of ethanol gave a denser, more easily filterable product. Recrystallisation from ethanol gave the 6-azido derivative **5** (0.54 g, 70%), m.p. 148.5–149°, $[\alpha]_D$ +150° (*c* 1.80) (Found: C, 52.3; H, 4.7; N, 8.2; S, 6.3. $C_{22}H_{23}N_3O_9S$ calc.: C, 52.3; H, 4.55; N 8.3; S 6.35%).

*Methyl 4,6-diazido-4,6-dideoxy-α-*D*-galactopyranoside* (**6**, R=H). — Methyl 2,3-di-*O*-acetyl-4,6-dideoxy-α-D-galactopyranoside (5.00 g) was dissolved in the minimal volume of boiling chloroform, and methanol (50 ml) and N sodium methoxide solution (25 ml) were added. The mixture was kept overnight at room temperature when t.l.c. showed an almost pure product. Concentration gave a colourless syrup which was extracted with boiling chloroform. The filtered extract was concentrated to a syrup which was then dissolved in ether (60 ml) containing a little acetone, and light petroleum was added to give the diazide (**6** R=H) (2.40 g, 65%), m.p. 103–104°, $[\alpha]_D$ +116° (*c* 1.28, ethanol) (Found: C, 34.45; H, 5.0; N, 34.3. $C_7H_{12}N_6O_4$ calc.: C, 34.45; H, 4.90; N, 34.45%). The diazido compound was also prepared from the diazido-dibenzoate **6** in an identical manner.

*Methyl 4,6-diazido-4,6-dideoxy-α-*D*-glucopyranoside.* — (*a*) Methyl 2,3-di-*O*-

acetyl-4,6-diazido-4,6-dideoxy-α-D-glucopyranoside (1.25 g) was dissolved in dry ethanol (50 ml), and 0.84N sodium ethoxide solution (2 ml) was added. T.l.c. showed the reaction to be complete after 2 h at room temperature. A flocculent precipitate was filtered off, and the filtrate was concentrated to yield a syrup which soon crystallised. Recrystallisation was, however, exceedingly difficult, and the product was purified by chromatography on silica gel with ether–light petroleum (2:1 v/v). The diazido-diol (0.43 g, 46%) could then be obtained crystalline by allowing its solution in ether–light petroleum to evaporate in dust-free conditions overnight. It had m.p. 86–88° and $[\alpha]_D$ +110° (*c* 1.18) (Found: C, 34.4; H, 5.0; N, 34.4. $C_7H_{12}N_6O_4$ calc.: C, 34.4; H, 4.9; N, 34.4%).

(*b*) A better method of obtaining the diazido compound **19** was as follows. A mixture of methyl 2,3-di-*O*-acetyl-4,6-di-*O*-toluene-*p*-sulphonyl-α-D-galactopyranoside (22.35 g) and sodium azide (9.0 g) in *N,N*-dimethylformamide (300 ml) was heated for 17 h at 130°. The crude product, obtained as described above, was de-esterified by dissolving it in ethanol (200 ml) and adding 0.92N sodium ethoxide solution (10 ml). T.l.c. showed the reaction to be complete after 18 h, and treatment of the reaction mixture as described in (*a*) gave crude methyl 4,6-diazido-4,6-dideoxy-α-D-glucopyranoside (4.28 g, 46% from the disulphonate). It could then be purified as described in (*a*).

*Methyl 2,3,4,6-tetra-*O*-benzoyl-α-D-galactopyranoside.* — To a solution of 1 g of dimethanesulphonate **1** in *N,N*-dimethylformamide (20 ml) was added sodium benzoate (0.9 g), and the mixture was heated for 20 h at 140°. The solution was then diluted with chloroform and extracted three times with water. The extract was dried ($MgSO_4$) and concentrated to give a syrup, which was crystallised from aqueous methanol to give the galactoside tetrabenzoate, m.p. 117–118°, $[\alpha]_D$ +149° (*c* 1.02), identical with an authentic specimen, for which m.p. 117–119°, $[\alpha]_D$ +151° is recorded[24].

Products arising from reduction of azides

*Methyl 4,6-diacetamido-2,3-di-*O*-acetyl-4,6-dideoxy-α-D-galactopyranoside* (**8**). — Methyl 2,3-di-*O*-acetyl-4,6-diazido-4,6-dideoxy-α-D-galactopyranoside (**7**, 500 mg) in methanol (150 ml) was hydrogenated overnight in the presence of 10% palladium-on-carbon (50 mg). After filtration and concentration, the syrup was dissolved in dry pyridine (40 ml), and acetic anhydride (3 ml, large excess) was added. After standing overnight, concentration of the reaction mixture gave a syrup which crystallised from ethyl acetate to give the 4,6-diacetamido-galactopyranoside **8** (0.33 g, 52%), m.p. 218–219°, $[\alpha]_D$ +123° (*c* 1.00, water) (Found: C, 49.9; H, 6.7; N, 7.65. $C_{15}H_{24}N_2O_8$ calc.: C, 50.0; H, 6.65; N, 7.75%).

The use of platinum oxide (Adams' catalyst) gave similar results, and, with Raney nickel, yields varying from 16–70% were obtained.

The tetra-acetate was also prepared, though in lower yield (16%), by similar reduction and acetylation of methyl 4,6-diazido-4,6-dideoxy-α-D-galactopyranoside.

Methyl 4,6-diacetamido-4,6-dideoxy-α-D-galactopyranoside (**9**). — Methyl 4,6-diacetamido-2,3-di-*O*-acetyl-4,6-dideoxy-α-D-galactopyranoside (**8**, 100 mg) was dissolved in methanol (5 ml), and conc. ammonia (sp.gr. 0.88) (0.5 ml) was added.

After standing for 2 h at room temperature, concentration yielded a syrup, which was crystallised from acetone (10 ml) by adding light petroleum. Filtration gave compound **9** (41 mg; 55%), m.p. 212–213°, $[\alpha]_D$ +151° (*c* 1.0, water) (Found: C, 47.4; H, 7.25; N, 10.0. $C_{11}H_{20}N_2O_6$ calc.: C, 47.1; H, 7.25; N, 10.15%).

Methyl 4,6-dibenzamido-2,3-di-O-benzoyl-4,6-dideoxy-α-D-galactopyranoside (**10**). — Methyl 4,6-diazido-2,3-di-*O*-benzoyl-4,6-dideoxy-α-D-galactopyranoside (**6**, 0.50 g) was dissolved in ethanol (100 ml) and hydrogenated in the presence of Raney nickel for 12 h. Filtration and concentration yielded a syrup which, after being dried *in vacuo*, was dissolved in pyridine (3 ml). The addition of benzoyl chloride (0.5 ml, large excess) produced a white precipitate, the i.r. spectrum of which showed it to be compound **10**. Recrystallisation from chloroform–light petroleum gave a product (51%), m.p. 284–286° (glass-capillary method), $[\alpha]_D$ +52° (*c* 0.35). However, no satisfactory analysis was obtained for this compound, and lack of solubility prevented recrystallisation from the usual solvents.

Methyl 4,6-diacetamido-2,3-di-O-acetyl-4,6-dideoxy-α-D-glucopyranoside (**20**). — Methyl 2,3-di-*O*-acetyl-4,6-diazido-4,6-dideoxy-α-D-glucopyranoside (1.20 g) was dissolved in ethanol (*ca.* 400 ml) and hydrogenated for 23 h in the presence of 10% palladium-on-carbon (0.8 g). T.l.c. showed the absence of starting material, and the solution was filtered, and concentrated to a syrup, which was dried *in vacuo* for 20 h, dissolved in pyridine (25 ml), and then treated with acetic anhydride (5 ml). After 3 h, the solution was concentrated to a brown syrup, shown by t.l.c. to be mainly one component, which was isolated by chromatography on silica gel with chloroform–methanol (18:1 v/v). Crystallisation of the purified material from ethyl acetate gave compound **20** (0.71 g, 54%), m.p. 184–185°, $[\alpha]_D$ +208° (*c* 0.71) (Found: C, 49.9; H, 6.7; N, 7.65. $C_{15}H_{24}N_2O_8$ calc.: C, 50.0; H, 6.53; N, 7.75%).

The yields for this preparation varied widely, and, on one occasion, two unidentified, syrupy compounds were obtained in addition to compound **20**.

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NUCLEOPHILIC REPLACEMENT REACTIONS OF SULPHONATES

PART II. THE SYNTHESIS OF DERIVATIVES OF 4,6-DITHIO-D-GALACTOSE AND -D-GLUCOSE, AND THEIR CONVERSION INTO 4,6-DIDEOXY-D-*xylo*-HEXOSE

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ABSTRACT

The replacement reactions of the 2,3-diacetate and 2,3-dibenzoate of methyl 4,6-di-*O*-mesyl-α-D-glucopyranoside with potassium thiocyanate in *N*,*N*-dimethylformamide have been studied. They proceed mainly *via* an S_N2 mechanism to give the corresponding 4,6-dideoxy-4,6-dithiocyanato-D-galactopyranosides. In the case of the diacetate, however, a small proportion (~1%) of the 4,6-dideoxy-4,6-dithiocyanato-D-glucopyranoside was isolated, which arose by participation of the neighbouring 3-acetoxy substituent. This D-glucopyranoside was also prepared from methyl 2,3-di-*O*-acetyl-4,6-di-*O*-toluene-*p*-sulphonyl-α-D-galactopyranoside by treatment with potassium thiocyanate in *N*,*N*-dimethylformamide.

The 4,6-dideoxy-4,6-dithiocyanato-D-galactoside undergoes base-catalysed hydrolysis to give, presumably, the 4,6-dithio-D-galactopyranoside which spontaneously oxidises with air to give the intramolecular 4,6-disulphide.

Desulphurisation of these 4,6-dithiocyanates and the 4,6-disulphide with Raney nickel, followed by acid hydrolysis, gave 4,6-dideoxy-D-*xylo*-hexopyranose.

INTRODUCTION

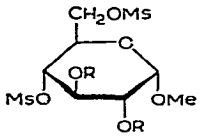
In the preceding paper[1], it was shown that 4,6-disulphonates of methyl α-D-glucopyranoside and methyl α-D-galactopyranoside undergo bimolecular replacement reactions with azide to give, by inversion of configuration at C-4, 4,6-diazido-4,6-dideoxy-D-galactopyranosides and -glucopyranosides, respectively, which are useful intermediates for the synthesis of 4,6-diamino-4,6-dideoxyhexoses. We now wish to report a related study in which the thiocyanate anion is used as the nucleophile for these displacement reactions, affording a method leading to 4,6-dithiopyranosides and, by desulphurisation, to 4,6-dideoxy sugars.

RESULTS AND DISCUSSION

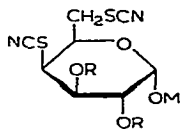
Methyl 2,3-di-*O*-benzoyl-4,6-di-*O*-methanesulphonyl-α-D-glucopyranoside[1] (**1**), on being heated with potassium thiocyanate in *N*,*N*-dimethylformamide, underwent

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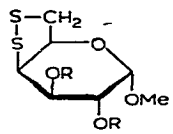
replacement of both sulphonate groups, and a dithiocyanate was readily isolated in 39% yield. By analogy with the azide replacement reaction on the same substrate[1], it was correctly assumed that direct S_N2 replacement had occurred, without participation by the neighbouring 3-benzoate group, to give the 4,6-dithiocyanato-galactoside **3**. The n.m.r. spectrum of the dithiocyanate was closely similar to that of the corresponding diazide[1], suggesting that the two were stereochemically identical. For reasons previously detailed[1], the two low-field quartets at τ 3.96 (J 10.5 and 4.5 Hz) and τ 4.52 (J 10.5 and 4.0 Hz) were assigned to H-3 and H-2, respectively, and the doublet at τ 4.75 (J 4.0 Hz) to H-1. A higher-field quartet at τ 5.60 (J 4.5 and 2.0 Hz) was clearly due to H-4, and the small value (2.0 Hz) for $J_{4,5}$ is a common feature of galactopyranosides[2]. The multiplet at τ 5.30 was due to H-5. These coupling constants afforded unequivocal support for the *galacto* stereochemistry.



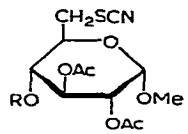
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2, R = Ac



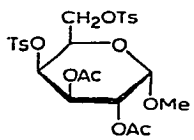
3, R = Bz
4, R = Ac



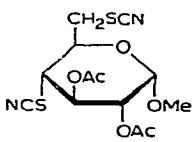
5, R = H
6, R = Ac



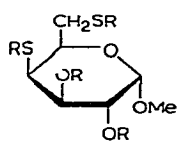
7, R = Ms
8, R = Ac



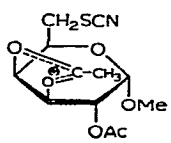
(9)



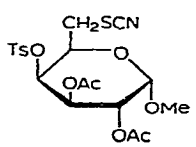
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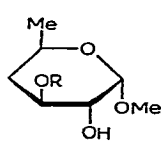
11, R = H
12, R = Ac



13



14



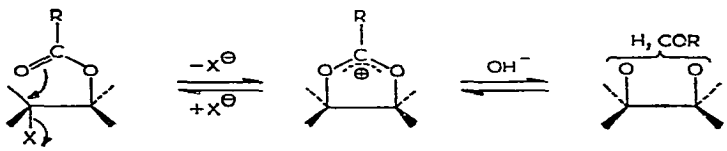
15, R = Me
16, R = H

When the dithiocyanate **3** was hydrolysed with sodium methoxide[3], hydrolysis of the benzoate and thiocyanate groups occurred to give methyl 4,6-dithio-α-D-galactopyranoside (**11**), presumably as a dianion. Before processing, the reaction mixture exhibited a strongly positive nitroprusside-test for thiol, but, immediately after deionisation of the reaction mixture with a cation-exchange resin, a negative thiol test was obtained. A pale-yellow compound was isolated after deionisation, the analytical data of which suggested it to be the intramolecular disulphide **5** derived from the 4,6-dithiogalactopyranoside **11**. This was supported by the fact that it exhibited weak absorption in the ultraviolet at 336 (ε 85) and 226 nm (ε 224); a

λ_{max} at 334 nm (ε 100) is said to be characteristic of a 1,2-dithiacyclopentane system[4]. The corresponding diacetate **6** had λ_{max} 328 (ε 97) and 226 nm (ε 282), and its n.m.r. spectrum showed five well-resolved multiplets due to single ring-protons, the splittings of which were in complete agreement with the structure of the disulphide **5**. The protons immediately adjacent to the ester groups resonated to low field as quartets; that at τ 4.30 (*J* 10.0 and 4.8 Hz) being assigned to H-3 and that at τ 4.74 (*J* 10.0 and 3.5 Hz) to H-2. The H-1 doublet was observed at τ 5.03 (*J* 3.5 Hz), a 1:3:3:1 quartet at τ 5.12 (*J* 3.0 Hz) and a 1:1:1:1 quartet at τ 5.82 (*J* 4.8 and 3.0 Hz) were obviously H-5 and H-4, respectively. The formation of compound **5** represents the first example of an intramolecular disulphide in the carbohydrate series. The ease with which the 4,6-dithiol undergoes oxidation to the disulphide is quite remarkable, and this has prevented its isolation as the free dithiol **11**. The same disulphide was obtained by conversion of the dithiocyanate **3** into the corresponding dithiol **11** by heating with ethanolic sodium sulphide[5]. In this case, the product was isolated by acetylation, and the infrared spectrum of the syrupy acetate indicated that it was probably methyl 4,6-dithio-α-D-galactopyranoside tetra-acetate (**12**), but deacetylation with sodium methoxide gave the disulphide **5**.

The action of potassium thiocyanate in dry *N*,*N*-dimethylformamide on impure methyl 2,3-di-O-acetyl-4,6-di-*O*-methanesulphonyl-α-D-glucopyranoside (**2**), which had previously[1] been shown to contain about 5% of methyl 2,3,4-tri-*O*-acetyl-6-*O*-methanesulphonyl-α-D-glucopyranoside, gave a rather complex mixture from which methyl 2,3-di-*O*-acetyl-4,6-dideoxy-4,6-dithiocyanato-α-D-galactopyranoside (**4**) was isolated in 28% yield by direct crystallisation. T.l.c. showed that the mother liquors contained, in addition to the dithiocyanate, four minor components. Separation of this mixture by column chromatography afforded, in order of elution, methyl 2,3-di-*O*-acetyl-4,6-epidithio-α-D-galactopyranoside (**6**) (3%), the dithiocyanato-galactoside **4** (3.3%), methyl 2,3,4-tri-*O*-acetyl-6-deoxy-6-thiocyanato-α-D-glucopyranoside (**8**), methyl 2,3-di-*O*-acetyl-4,6-dideoxy-4,6-dithiocyanato-α-D-glucopyranoside (**10**) (1%), and methyl 2,3-di-*O*-acetyl-6-deoxy-4-*O*-methanesulphonyl-6-thiocyanato-α-D-glucopyranoside (**7**). It is clear that the disulphide **6** must have arisen by hydrolysis of the corresponding dithiocyanate **4**. This hydrolysis was promoted by traces of water in the reaction mixture; when the replacement was conducted in 95% aqueous *N*,*N*-dimethylformamide, the disulphide **6** was the major product, formed at the expense of the dithiocyanate **4**. The presence of the 6-thiocyanate-2,3,4-triacetate **8** was predictable, since it has already been shown that methyl 2,3,4-tri-*O*-acetyl-6-*O*-methanesulphonyl-α-D-glucopyranoside is the major impurity in the starting material[1]. Its identity was demonstrated by its synthesis from an authentic specimen of methyl 2,3,4-tri-*O*-acetyl-6-*O*-methanesulphonyl-α-D-glucopyranoside by treatment with potassium thiocyanate in hot *N*,*N*-dimethylformamide. It was clearly quite different from methyl 2,3,6-tri-*O*-acetyl-4-deoxy-4-thiocyanato-α-D-galactopyranoside prepared in an analogous way from methyl 2,3,6-tri-*O*-acetyl-4-*O*-methanesulphonyl-α-D-glucopyranoside. The identity of the 4,6-dithiocyanato-glucoside **10** was shown by its synthesis by a different route (see below), and its presence is of considerable note

because it must have arisen as a result of participation by the neighbouring 3-acetoxy group, *via* an acetoxonium ion **13**, followed by attack at C-4 of **13** by thiocyanate (*i.e.*, overall retention of configuration). None of the 3,6-dithiocyanato-gulopyranoside, which would have arisen from the acetoxonium ion **13** by attack at C-3, was detected, although its complete absence was not verified, and several other trace components were detectable by t.l.c. Displacement reactions of sulphonic esters and halogeno derivatives through the agency of neighbouring acyloxy-group participation is well established in carbohydrate chemistry[6], particularly in reactions at the anomeric centre[7]. However, participation of a *trans*-acyloxy group in the displacement of a sulphonyloxy group at C-2, 3, and 4 of a pyranoid ring does not occur so readily[8], particularly when a powerfully nucleophilic anion in a dipolar aprotic solvent is used. Hence, 1,2,4,6-tetra-*O*-benzoyl-3-*O*-toluene-*p*-sulphonyl-β-D-glucopyranoside underwent a bimolecular displacement with sodium benzoate in *N*,*N*-dimethylformamide[9] to give the allopyranoside. When neighbouring-group participation does take place, the intermediary acyloxonium ion usually undergoes solvolysis to give a mono-*O*-acyl derivative of the α,β-*cis*-diol (*i.e.*, inversion of configuration):



This type of solvolysis has been observed by using sodium benzoate[10] or sodium fluoride[11] in *N*,*N*-dimethylformamide. This present example constitutes the first example, in the carbohydrate field, of displacement of a sulphonate group with an anion by neighbouring acetyl-group participation.

The final component obtained from the column, methyl 2,3-di-*O*-acetyl-6-deoxy-4-*O*-methanesulphonyl-6-thiocyanato-α-D-glucopyranoside (**7**) was an intermediate product in the formation of the dithiocyanates **4** and **10**, and could be prepared in good yield from the dimethanesulphonate **2** by treatment with potassium thiocyanate in a mixture of butanone and *N*,*N*-dimethylformamide.

Treatment of methyl 2,3-di-*O*-acetyl-4,6-di-*O*-toluene-*p*-sulphonyl-α-D-galactopyranoside[1] (**9**) with potassium thiocyanate in *N*,*N*-dimethylformamide was much slower than in the glucoside series, although the two series are strictly not comparable, because the sulphonate residues are different in each case. It was difficult to induce the reaction to go beyond 50% completion, since prolonged reaction caused excessive decomposition, and usually a *ca.* 1:1 mixture of the mono- and di-thiocyanates (**14** and **10**, respectively) was obtained, which was fractionated chromatographically. Since there is no possibility of neighbouring-group participation from C-3 in compound **14**, it is reasonable to suppose that the reaction occurred by an S_N2 mechanism to give the 4,6-dithiocyanato-glucoside **10**, and this supposition is established beyond doubt by the first-order coupling constants observed in the n.m.r. spectrum of the dithiocyanate. The n.m.r. spectrum showed a low-field quartet (τ 4.41, *J* 10.5 and

9.0 Hz) due to H-3 and a partly overlapping H-1 doublet (τ 4.97, J 3.0 Hz) and H-2 quartet (τ 5.13, J 3.0 and 9.0 Hz); H-4 was also readily recognised as a triplet (τ 6.83, J 10.5 Hz), and H-5 as a well-resolved septet at τ 5.70. The observed coupling constants (Table I) show that the dithiocyanate exists in the *C1* conformation, in which all of the ring protons, except H-1, are axial, and therefore unequivocally demonstrate the *gluco*-configuration of the product. All attempts to convert the glucoside dithiocyanate **10** into the corresponding intramolecular disulphide, by hydrolysis with sodium methoxide, gave complex mixtures from which no pure product could be isolated. This is in contrast to the *galacto*-analogue which gave the *cis*-fused 4,6-disulphide so readily. The failure of the glucoside to give the disulphide is undoubtedly due to the fact that the product would possess a less-stable, *trans*-fused, bicyclic system.

TABLE I

CHEMICAL SHIFTS (τ-VALUES) AND FIRST-ORDER COUPLING CONSTANTS (Hz) AT 60 MHz FOR COMPOUNDS **3**, **6–8**, AND **10**.

Compound	**3**	**10**	**8**	**7**	**6**
H-1	4.75(d)[a]	4.97(d)	5.01(d)	4.75(cm)	5.03(d)
H-2	4.52(q)	5.16(q)	5.15(q)		4.74(q)
H-3	3.96(q)	4.45(q)	4.50(t)	3.91(t)[b]	4.30(q)
H-4	5.60(q)	6.83(t)	5.10(t)	5.11(t)	5.83(q)
H-5	5.28(cm)	5.70(cm)	5.90(cm)	5.70(cm)	5.10(cm)
H-6	~ 6.65(cm)	~ 6.55(cm)	~6.90(cm)	~ 6.50(cm)	~ 6.65(cm)
OMe	6.45(s)	6.52(s)	6.50(s)	6.50(s)	6.55(s)
OAc		7.90(s)	8.00(s)		7.91(s)
		7.85(s)			
$J_{1,2}$	4.0	3.0	3.5		3.5
$J_{2,3}$	10.5	9.0	9.0	~10	10.0
$J_{3,4}$	4.5	10.5	9.0	9.0	4.8
$J_{4,5}$	2.0	10.5	9.0	9.0	3.0

[a]Key: s, singlet; d, doublet; t, triplet; q, quartet; cm, complex multiplet. [b]Broad triplet with half-peak width of *ca.* 3 Hz.

The dithiocyanate derivatives (**3**, **4**, and **10**) and the disulphides (**5** and **6**) underwent desulphurisation with Raney nickel to give, after de-esterification and acid hydrolysis, crystalline 4,6-dideoxy-α-D-*xylo*-hexopyranose (**16**). At the time of our preliminary communication on this work[12], this was the first description of this dideoxy sugar, which was of some interest because of its occurrence as the 3-methyl ether in the antibiotic chalcomycin[11]. In the meantime, however, a further synthesis from methyl 4-deoxy-α-D-*xylo*-hexopyranoside has been described[14]. The desulphurisation of the dithiocyanate tended to give variable yields of the dideoxy derivative and required a very large excess of Raney nickel. It was therefore better to use the disulphide **5** for the desulphurisation reaction.

EXPERIMENTAL

For notes on general experimental procedure, see the preceding paper[1].

Methyl 2,3-di-O-benzoyl-4,6-dideoxy-4,6-dithiocyanato-α-D-galactopyranoside (**3**). — Methyl 2,3-di-*O*-benzoyl-4,6-di-*O*-mesyl-α-D-glucopyranoside[1] (**1**, 54 g) was heated with potassium thiocyanate (105 g) in *N,N*-dimethylformamide (400 ml) for 46 h at 120°. The precipitated potassium methanesulphonate (25.2 g, 1.94 moles) was filtered off and washed with ethanol and acetone. The combined filtrate and washings were slowly treated with water, causing crystallisation, and the addition of water was continued until an oil began to precipitate. After filtration, the crystalline product was washed with ethanol and then light petroleum to give 25.2 g (54%) of a brown, crystalline product. This was purified by chromatography on silica gel (*ca.* 2 kg) and eluting with chloroform until the dark-coloured band was just about to emerge. The eluate was concentrated to a solid which was recrystallised from acetone–light petroleum to give the dithiocyanate **3** (18.0 g, 39%), m.p. 212–214°, $[\alpha]_D$ +93.5° (*c* 0.32) (Found: C, 56.9; H, 4.2; N, 5.8; S, 13.2. $C_{23}H_{20}N_2O_6S_2$ calc.: C, 57.0; H, 4.15; N, 5.8; S, 13.2%).

Methyl 2,3-di-O-acetyl-4,6-dideoxy-4,6-dithiocyanato-α-D-galactopyranoside (**4**). — Impure methyl 2,3-di-*O*-acetyl-4,6-di-*O*-mesyl-α-D-glucopyranoside (**2**) (10 g) and potassium thiocyanate (20 g) were heated in *N,N*-dimethylformamide (*ca.* 70 ml) for 44 h at 130°. T.l.c. showed a highly complex reaction mixture, which was allowed to cool and then diluted with chloroform (150 ml). The inorganic material was removed by filtration and washed with chloroform and ethanol. The combined filtrate and washings was concentrated to give a black paste which was fractionated between water (100 ml) and chloroform (100 ml), and the organic layer was washed with water (2 × 100 ml), dried ($CaSO_4$), decolourised (charcoal), and concentrated to a syrup which crystallised from ethanol–light petroleum to give compound **4** (2.30 g, 28%), m.p. 183–185°, $[\alpha]_D$ +134° (*c* 1.52) (Found: C, 43.4; H, 4.5; N, 7.6; S, 17.75. $C_{13}H_{16}N_2O_6S_2$ calc.: C, 43.3; H, 4.45; N, 7.75; S, 17.75%).

The mother liquors were concentrated to give a complex, syrupy mixture which was readily fractionated by chromatography [a silica gel to syrup ratio of 85:1 and elution with ether–light petroleum (2:1 v/v)]. The following, crystalline compounds were obtained, in order of elution: methyl 2,3-di-*O*-acetyl-4,6-epidithio-α-D-galactopyranoside (**6**) (200 mg, 2.8%); methyl 2,3-di-*O*-acetyl-4,6-dideoxy-4,6-dithiocyanato-α-D-galactopyranoside (**4**) (275 mg, a further 3.3%); methyl 2,3,4-tri-*O*-acetyl-6-deoxy-6-thiocyanato-α-D-glucopyranoside (**8**) (311 mg, 3.6%); methyl 2,3-di-*O*-acetyl-4,6-dideoxy-4,6-dithiocyanato-α-D-glucopyranoside (**10**) (70 mg, 0.8%); and methyl 2,3-di-*O*-acetyl-6-deoxy-4-*O*-mesyl-6-thiocyanato-α-D-glucopyranoside (**7**) (80 mg, 0.7%). All of these products were identified by comparison with authentic samples prepared elsewhere in this Experimental section.

The reaction was very sensitive towards water; small traces of water changed the composition of the reaction product drastically, so that the disulphide **6** was the major product in 95% aqueous *N,N*-dimethylformamide (see below).

Methyl 2,3-di-O-acetyl-4,6-dideoxy-4,6-dithiocyanato-α-D-glucopyranoside (**10**). — Methyl 2,3-di-*O*-acetyl-4,6-di-*O*-toluene-*p*-sulphonyl-α-D-galactopyranoside (**9**, 14.2 g) and potassium thiocyanate (8.4 g) were heated in dry *N*,*N*-dimethylformamide (200 ml) for 3 h at 160°, when the reaction was judged by t.l.c. to have reached the optimal point. Concentration of the reaction mixture yielded a stiff, black paste which was extracted with chloroform (3 × 150 ml). The combined extracts were washed with water (2 × 150 ml), dried ($CaSO_4$), decolourised (charcoal), and concentrated to give a clear syrup (9.15 g) that was shown by t.l.c. to be a complex mixture. The mixture was partially fractionated by chromatography on silica gel with ether–light petroleum (3:1 v/v).

The dithiocyanate **10**. (1.2 g, 13.8%) was obtained crystalline from ethanol, and had m.p. 144–145°, $[\alpha]_D$ +56° (*c* 1.07) (Found: C, 43.25; H, 4.55; N, 7.8; S, 17.7. $C_{13}H_{16}N_2O_6S_2$ calc.: C, 43.35; H, 4.45; N, 7.75; S, 17.75%).

Eluted before the dithiocyanate was methyl 2,3-di-*O*-acetyl-6-deoxy-6-thiocyanato-4-*O*-toluene-*p*-sulphonyl-α-D-galactopyranoside (**14**) (2.01 g; 18%); m.p. 126–127° (from ethanol), $[\alpha]_D$ +167° (*c* 1.96) (Found: C, 48.15; H, 4.9; N, 2.95; S, 13.45. $C_{19}H_{23}NO_9S_2$ calc.: C, 48.1; H, 4.85; N, 2.95; S, 13.55%).

Methyl 2,3,6-tri-O-acetyl-4-deoxy-4-thiocyanato-α-D-galactopyranoside. — Methyl 2,3,6-tri-*O*-acetyl-4-*O*-mesyl-α-D-glucopyranoside[1] (0.75 g) and potassium thiocyanate (0.9 g) were heated in *N*,*N*-dimethylformamide (10 ml) for 24 h at 135°. The reaction mixture was concentrated to a thick paste and fractionated between chloroform (50 ml) and water (50 ml). The organic layer was washed with water (2 × 50 ml), dried ($CaSO_4$), decolourised (charcoal), and concentrated to an impure syrup which was shown by t.l.c. to contain about 50% of one component. Chromatography on silica gel, with ether–light petroleum (2:1 v/v), gave, after crystallisation from ethanol, the title 4-thiocyanate (90 mg, 13%), m.p. 109–110°, $[\alpha]_D$ +107° (*c* 1.21) (Found: C, 46.75; H, 5.45; N, 3.75; S, 8.9. $C_{14}H_9NO_8S$ calc.: C, 46.55; H, 5.2; N, 3.9; S, 8.8%).

Methyl 2,3,4-tri-O-acetyl-6-deoxy-6-thiocyanato-α-D-glucopyranoside (**8**). — Methyl 2,3,4-tri-*O*-acetyl-6-*O*-mesyl-α-D-glucopyranoside[1] (500 mg) was mixed with potassium thiocyanate (950 mg) and heated in *N*,*N*-dimethylformamide (8 ml) for 2.5 h at 125°. A white precipitate of potassium methanesulphonate was noted after 10 min. The reaction mixture was poured into water (*ca.* 40 ml), and crystals were obtained on storage for 3 days at 0°, giving a crude product (0.276 g, 58%), m.p. 107–110°. The impurities arose from contaminants in the starting material, as revealed by t.l.c., but neither the starting material nor the product could be purified by recrystallisation; the impurities co-crystallised in both cases. A pure specimen of the 6-thiocyanate **8**, obtained by chromatography on silica gel with ether–light petroleum (2:1 v/v), had m.p. 112–114°, $[\alpha]_D$ +200° (*c* 0.87) (Found: C, 46.45; H, 5.25; N, 3.8; S, 9.1. $C_{14}H_{19}NO_8S$ calc.: C, 46.55; H, 5.2; N, 3.9; S, 8.8%).

Methyl 2,3-di-O-acetyl-6-deoxy-4-O-mesyl-6-thiocyanato-α-D-glucopyranoside (**7**). — Methyl 2,3-di-*O*-acetyl-4,6-di-*O*-mesyl-α-D-glucopyranoside (**2**) (1.0 g) and potassium thiocyanate (0.5 g) were heated under reflux for 13 h in 50% *N*,*N*-dimethyl-

formamide–butanone (16 ml). The reaction mixture was concentrated to a solid which was extracted with chloroform (2 × 25 ml), and the combined extracts were washed with water (2 × 25 ml). The chloroform solution was then decolourised with charcoal, dried ($CaSO_4$), and concentrated to give a solid, which was recrystallised from ethanol to give the 6-thiocyanate **7** (0.54 g, 60%), m.p. 171.5–172.5°, $[\alpha]_D$ +150° (*c* 1.06) (Found: C, 39.1; H, 4.65; N, 3.7; S, 16.1. $C_{13}H_{19}NO_9S_2$ calc.: C, 39.3; H, 4.8; N, 3.55; S, 16.1%).

Methyl 2,3-di-O-benzoyl-6-deoxy-4-O-mesyl-6-thiocyanato-α-D-glucopyranoside. — Methyl 2,3-di-*O*-benzoyl-4,6-di-*O*-mesyl-α-D-glucopyranoside (**1**, 0.63 g) was heated for 2 h at 130° with potassium thiocyanate (0.87 g) in *N*,*N*-dimethylformamide (10 ml). The addition of water to the reaction mixture caused the precipitated potassium methanesulphonate to dissolve, and the 6-thiocyanate (0.49 g, 83%) to crystallise. Recrystallisation from ethanol yielded 0.45 g (77%) of the title 6-thiocyanate, m.p. 155–162°, $[\alpha]_D$ +153° (*c* 3.42) (Found: C, 53.2; H, 4.45; N, 2.65; S, 12.25. $C_{23}H_{23}NO_9S_2$ calc.: C, 52.95; H, 4.4; N, 2.7; S, 12.3%).

Methyl 4,6-epidithio-α-D-galactopyranoside (**5**). — (*a*) Methyl 2,3-di-*O*-benzoyl-4,6-dideoxy-4,6-dithiocyanato-α-D-galactopyranoside (**3**, 0.5 g) was treated with a boiling, saturated solution of sodium sulphide in ethanol (10 ml). The material dissolved almost immediately, and the solution was then refluxed for 1 h and concentrated to dryness. The mixture was treated with acetic anhydride (10 ml) and pyridine (5 ml), when a vigorous reaction ensued, which caused the reaction mixture to boil. After storage for 10 min at room temperature, the solution was concentrated to dryness and fractionated between chloroform (50 ml) and water (50 ml). The organic layer was washed with equal volumes of N sodium hydroxide and water, and then dried over sodium sulphate. Concentration yielded a syrup which was shown by t.l.c. to be almost pure. The i.r. spectrum showed peaks due to *O*-ester and *S*-ester, and the product was thus methyl 2,3-di-*O*-acetyl-4,6-di-*S*-acetyl-4,6-dithio-α-D-galactopyranoside (**12**). The syrupy tetra-acetate was then dissolved in methanol (10 ml), and N methanolic sodium methoxide (*ca.* 4 ml) was added to make the solution strongly alkaline. After storage for 1 h at room temperature, the solution was neutralised by cation-exchange resin, and concentrated. Recrystallisation of the residue from ethanol afforded the disulphide **5** as pale-yellow crystals (126 mg, 54%), m.p. 198–200°, $[\alpha]_D$ +210° (*c* 0.17, water), +165° (*c* 1.1). Recrystallisation from acetone–light petroleum (2:1) raised the m.p. to 202–203° [Found: C, 37.35; H, 5.35; S, 28.3; Mol. wt., 227, 228 (osmometer) $C_7H_{12}O_4S_2$ calc.: C, 37.5; H, 5.35; S, 28.6%; Mol.wt., 244].

(*b*) Methyl 2,3-di-*O*-benzoyl-4,6-dideoxy-4,6-dithiocyanato-α-D-galactopyranoside (2.02 g) in N sodium methoxide solution (25 ml) was heated under reflux for 10 min and then kept at room temperature for 30 min. A little water was then added, and the solution was deionised with an excess of cation-exchange resin. After removal of the resin and solvent, the residue was recrystallised from ethanol to give the disulphide **5** (0.47 g, 50%), m.p. 198–199°. Recrystallisation from isopropyl alcohol raised the m.p. to 202–204°.

Methyl 2,3-di-O-acetyl-4,6-epidithio-α-D-galactopyranoside (**6**). — Methyl 2,3-di-*O*-acetyl-4,6-di-*O*-mesyl-α-D-glucopyranoside (**2**) (1.0 g, purified by chromatography) and potassium thiocyanate (2.0 g) in 95% aqueous *N,N*-dimethylformamide (25 ml) were heated for 26 h at 135°. The solution was diluted with water (5 ml) and concentrated to a stiff paste which was fractionated between chloroform (50 ml) and water (50 ml). The organic phase was further washed with water (2 × 50 ml), decolourised (charcoal), dried ($CaSO_4$), and concentrated to a yellow syrup. T.l.c. showed a complex mixture, and the required disulphide **6** was obtained after chromatography on silica gel with ether–light petroleum (2:1 v/v). Recrystallisation from ethanol gave the disulphide diacetate (120 mg, 17%) as yellow crystals, m.p. 108–109°, $[\alpha]_D$ +293° (*c* 0.68) (Found: C, 42.8; H, 5.1; S, 20.5. $C_{11}H_{16}O_6S_2$ calc.: C, 42.8; H, 5.2; S, 20.75%).

*4,6-Dideoxy-*D-xylo-*hexopyranose* (**16**). — (*a*) Methyl 2,3-di-*O*-benzoyl-4,6-dideoxy-4,6-dithiocyanato-α-D-galactopyranoside (**3**) (1.14 g) in dry ethanol (70 ml) was refluxed with a suspension of Raney nickel (*ca.* 15 g) for 2 h. T.l.c. indicated the reaction to be complete and the product to be almost pure. Removal of the catalyst and solvent gave methyl 2,3-di-*O*-benzoyl-4,6-dideoxy-α-D-*xylo*-hexopyranoside, $[\alpha]_D$ +137° (*c* 5.5) (Found: C, 67.5; H, 6.4. $C_{21}H_{22}O_6$ calc.: C, 68.0; H, 6.0%). The product failed to crystallise in the course of 3 days. A solution of this syrup in methanol was treated with a catalytic amount of sodium methoxide, and allowed to stand for 2 h. The solution was diluted with water (10 ml), deionised with cation-exchange resin, and concentrated to give methyl 4,6-dideoxy-α-D-*xylo*-hexopyranoside as a syrup[12] which was dissolved in N sulphuric acid (10 ml) and heated under reflux for 2 h. The hydrolysate was then neutralised with a 5% solution of dioctylmethylamine in chloroform. The aqueous layer was washed with chloroform, and concentrated to give a syrup which soon crystallised. Recrystallisation from acetone afforded 4,6-dideoxy-D-*xylo*-hexopyranose (37 mg, 11% overall), m.p. 137–138°, $[\alpha]_D$ +103 (by extrapolation)→ +33° (2 h, constant value) (*c* 0.75, water) (Found: C, 48.5; H, 8.2. $C_6H_{12}O_4$ calc.: C, 48.7; H, 8.1%); lit.[12], m.p. 143°.

(*b*) Methyl 4,6-epidithio-α-D-galactopyranoside (**5**) (126 mg) was dissolved in dry ethanol (10 ml) and heated under reflux with Raney nickel (*ca.* 3 g) for 2 h. The catalyst and solvent were removed to give a syrup. This product was hydrolysed with N sulphuric acid (4 ml) for 2 h under reflux, and then neutralised with barium carbonate. The syrup obtained after filtration and concentration crystallised, and was recrystallised from acetone to give the dideoxy sugar **16** (6 mg, 7%), m.p. 137–139°, identical with the product from (*a*).

(*c*) In the diacetate series, it was found that the dideoxy sugar could be made by desulphurisation, de-*O*-esterification, and acid hydrolysis of either methyl 2,3-di-*O*-acetyl-4,6-dideoxy-4,6-dithiocyanato-α-D-galactopyranoside (**4**), the *gluco*-epimer **10**, or methyl 2,3-di-*O*-acetyl-4,6-epidithio-α-D-galactopyranoside (**6**), following the methods described in (*a*) or (*b*). In all cases, the yields were low.

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SOME STRUCTURAL FEATURES OF *Citrus limonia* GUM (LEMON GUM)

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ABSTRACT

Lemon gum contains residues of D-galactose, L-arabinose, L-rhamnose, D-glucuronic acid, and 4-*O*-methyl-D-glucuronic acid. Graded, acid hydrolysis affords 3-*O*-β-L-arabinopyranosyl-L-arabinose, 5-*O*-α-L-arabinopyranosyl-L-arabinose, 4-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-L-arabinose, three acidic trisaccharides [including *O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-(1→4)-*O*-α-L-arabinopyranosyl-(1→5)-L-arabinose], and higher acidic oligosaccharides. The methylated gum yields 2,3,4-tri-*O*-methyl-L-rhamnose, 2,3,5- and 2,3,4-tri-, and 2,3-, 2,5-, and 3,5-di-*O*-methyl-L-arabinose, 2,4-di-*O*-methyl-D-galactose, and 2,3,4-tri- and 2,3-di-*O*-methyl-D-glucuronic acid, together with small proportions of 2,3,4,6-tetra-, and 2,3,6-, 2,4,6-, and 2,3,4-tri-*O*-methyl-D-galactose. Partial, acid hydrolysis of the degraded gum, obtained after graded, acid hydrolysis, affords the β-(1→3)- and β-(1→6)-linked D-galactose disaccharides, 4-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-D-galactose, 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose, and 4-*O*-(α-D-glucopyranosyluronic acid)-D-galactose. Its *O*-methyl derivative yields 2,3,4-tri- and 2,3-di-*O*-methyl-L-arabinose, 2,3,4,6-tetra-, 2,3,6-, 2,4,6-, and 2,3,4-tri-, 2,6- and 2,4-di-, and 2-*O*-methyl-D-galactose, and 2,3,4-tri-*O*-methyl-D-glucuronic acid. The degraded gum was subjected to a Smith-degradation, and lemon gum to two successive Smith-degradations, and the products were examined by linkage and methylation analysis. It is concluded that the molecules of lemon gum are based on galactan frameworks of a branch-on-branch type, to which are attached rhamnose-, arabinose-, and uronic acid-containing side-chains.

INTRODUCTION

Earlier investigations[1-8] on the structural features of lemon gum have shown it to contain D-galactose[1], L-arabinose[1], D-glucuronic acid[1], 4-*O*-methyl-D-glucuronic acid[4,6], and rhamnose[4]. Procedures involving graded hydrolysis of the gum with acid have led to the identification of one neutral disaccharide, 3-*O*-β-L-arabinopyranosyl-L-arabinose[5], two acidic disaccharides, 4-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-D-galactose[2,4,6] and 4-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-L-arabinose[4], and an incompletely characterised, acidic trisaccharide[4]. In addition, methyl-

ation analysis[3] has afforded 2,3,5-tri-*O*-methyl-L-arabinose, 2,4-di-*O*-methyl-D-galactose, and 2,3-di-*O*-methyl-D-glucuronic acid, together with some unidentified *O*-methyl sugars, and has prompted the suggestion[6,8] that the polysaccharide has a highly branched structure based on a main chain of D-galactose residues. In view of more recent investigations on the structural features of *Acacia senegal* gum (gum arabic)[9] and *Acacia arabica* gum[10], it is becoming evident that some plant gums of the substituted arabinogalactan type are not based on a main chain of D-galactose residues, but on a branch-on-branch type of structure for the galactan framework. The results reported in this paper show that this structural concept is common to lemon gum, and so may be a general structural feature of many plant gums[8] of the substituted arabinogalactan type.

RESULTS AND DISCUSSION

After purification, the sample[2,4,5] of lemon gum was shown to contain D-galactose (36%), L-arabinose (33%), L-rhamnose (5%), D-glucuronic acid (7%), and 4-*O*-methyl-D-glucuronic acid (16%). Since the sample migrated as a single band on zone electrophoresis on cellulose acetate film in two different buffer systems, it was judged suitable for structural investigations. Graded hydrolysis of the gum with 0.01N sulphuric acid resulted in the release of all of the rhamnose residues, a few of the galactose residues, and most of the arabinose residues, as free arabinose, as two disaccharides, and as a complex acidic fraction, the components of which also contained 4-*O*-methylglucuronic acid residues. The disaccharides were identified as the previously characterised[5] 3-*O*-β-L-arabinopyranosyl-L-arabinose, and as 5-*O*-α-L-arabinopyranosyl-L-arabinose. The latter disaccharide gave only arabinose on acid hydrolysis, and methylation analysis indicated that an arabinose residue was 4-*O*- or 5-*O*- substituted by an arabinopyranose residue. It was identified as the α-(1→5)-linked disaccharide on the basis of its specific rotation value (−9°), which was similar to the value (−14°) reported[11] for this disaccharide isolated from *Virgilia oroboides* gum. The acidic fraction was shown to be composed of the previously characterised[4,7], 4-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-L-arabinose (**1**) and higher, arabinose-containing, homologous, acidic oligosaccharides. The incompletely characterised[4] acidic trisaccharide was shown to be *O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-(1→4)-*O*-α(?)-L-arabinopyranosyl-(1→5)-L-arabinose (**2**).

4-*O*-Me-α-D-G*p*A-(1→4)-L-Ara

1

4-*O*-Me-α-D-G*p*A-(1→4)-α-L-Ara*p*-(1→5)-L-Ara

2

The acidic trisaccharide **2** gave disaccharide **1** and arabinose on acid hydrolysis, and methylation evidence indicated that the reducing arabinose residue was 4-*O*- or 5-*O*-substituted by disaccharide **1**. Since the acidic trisaccharide **2** was liberated under very mild conditions of acid hydrolysis, the reducing arabinose residue was assumed to be in the furanose form and, therefore, 5-*O*-substituted. Although

the acidic disaccharide **1**, having the arabinose residue in the pyranose form, was also liberated during mild hydrolysis with acid, degraded gum *A*, isolated after dialysis, was shown to contain appreciable amounts of **1** and no detectable amounts of **2**. The α configuration for the nature of the linkage between the two L-arabinose residues was deduced from the value (+67°) for its specific rotation. Although the characterisation of the α-(1→5)-linked disaccharide would also tend to favour the α configuration, this assignment must be regarded as tentative. Evidence was also

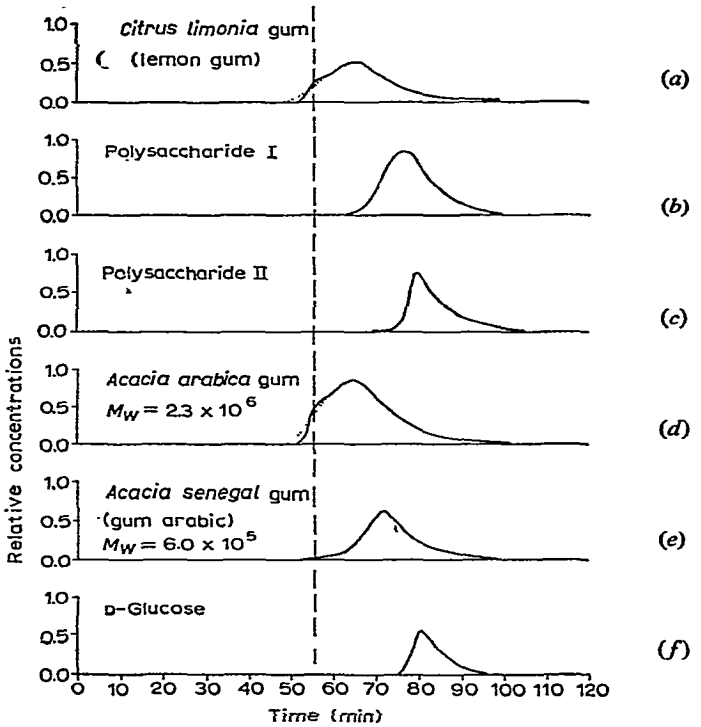


Fig. 1. Elution patterns for lemon gum (*a*), for polysaccharides I and II (*b*) and (*c*), for *A. arabica* gum (*d*), for *A. senegal* gum (*e*), and for D-glucose (*f*) from a "Bio-Glas 500" column (30 × 1.5 cm). Since the column was eluted at a constant flow-rate, the time marked by the broken line corresponds to the void volume of the column.

obtained for the presence of trace amounts of two other acidic trisaccharides, an acidic tetrasaccharide, and an acidic pentasaccharide, all of which yielded arabinose and disaccharide **1** on acid hydrolysis. The last two components proved difficult to investigate on account of autohydrolysis of their syrups. The evidence indicates that fragments of type **3** are structural features of lemon gum.

4-*O*-Me-α-D-Gp*A*-(1→4)-L-Ara*p*-(1→)-(L-Ara*f*)$_n$-(1→

3

where $n = 1$, 2, or 3

Degraded gum *A* contained galactose (66%), arabinose (5%), glucuronic acid (17%), and 4-*O*-methylglucuronic acid (10%). Apart from **1**, partial hydrolysis of degraded gum *A* with acid also yielded another previously characterised[2,4,6] acidic disaccharide, 4-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-D-galactose, together with the β-(1→3)- and β-(1→6) linked D-galactose disaccharides. Paper-chromatographic evidence was also obtained for the presence of 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose and 4-*O*-(α-D-glucopyranosyluronic acid)-D-galactose. Examination of the *O*-methyl derivative of degraded gum *A* yielded 2,3,4-tri- (a trace) and 2,3-di-*O*-methyl-L-arabinose (+/2), 2,3,4,6-tetra-(+), 2,3,6-(+/2), 2,4,6-(+), and 2,3,4-tri-(+ + +), 2,6-(a trace) and 2,4-di-(+ +), and 2-*O*-methyl-D-galactose (a trace), and 2,3,4-tri-*O*-methyl-D-glucuronic acid (+ +). Together with the results from linkage analyses, these *O*-methyl sugars are evidence for terminal, non-reducing L-arabinopyranose, D-galactopyranose, and (4-*O*-methyl)-D-glucopyranosyluronic acid residues, 4-*O*-substituted L-arabinopyranose residues, and 4-*O*-, 3-*O*-, 6-*O*, and 3,6-di-*O*-substituted D-galactopyranose residues. The presence of 2,6-di- and 2-*O*-methyl-D-galactose is ascribed to incomplete methylation[10] of hydroxyl groups at position 4.

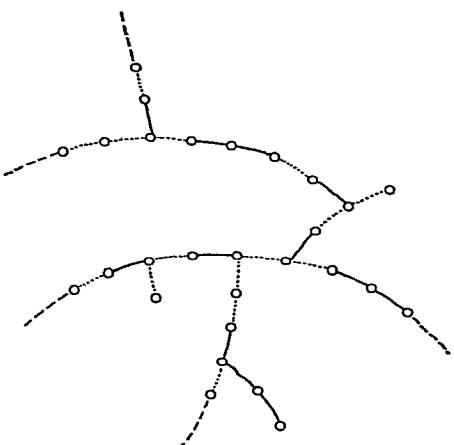


Fig. 2. Diagrammatic representation of part of the galactan framework of a lemon-gum molecule. Uronic acid rhamnose-, and arabinose-containing side-chains are attached to this framework. o = D-galactose residues; — = β-(1→3)-linkages; ··· = β-(1→6)-linkages.

Partial methanolysis of methylated, degraded gum *A* indicated that **4**, **5**, **6**, and **7** are possible structural fragments attached to a highly branched galactan framework.

4-*O*-Me-α-D-Gp*A*-(1→4)-L-Ara*p*-(1→ **4**

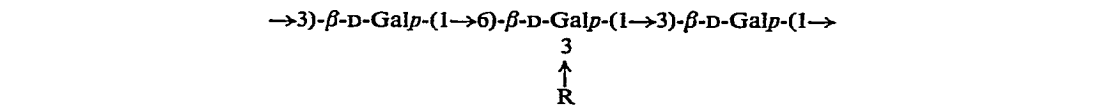
4-*O*-Me-α-D-Gp*A*-(1→4)-D-Gal*p*-(1→ **5**

β-D-Gp*A*-(1→6)-D-Gal*p*-(1→ **6**

α-D-Gp*A*-(1→4)-D-Gal*p*-(1→ **7**

The branched nature of this framework and the mode of distribution of β-(1→3)- and β-(1→6)-linkages was investigated by studying the constitution of a degraded gum *B*,

isolated after a Smith degradation[12] of degraded gum *A*. Partial hydrolysis of degraded gum *B* with acid gave the β-(1→3)- and β-(1→6)-linked D-galactose disaccharides, and examination of its *O*-methyl derivative 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 (a trace), and 2,3,4-tri-*O*-methyl-D-glucuronic acid (a trace). The presence of some 2,6-di- and 2-*O*-methyl-D-galactose is ascribed to under-methylation, and that of 2,3,4-tri-*O*-methyl-D-glucuronic acid to incomplete oxidation[10] of uronic acid residues in degraded gum *A*. The proportions of 2,3,4,6-tetra- and 2,4-di- to 2,4,6- and 2,3,4-tri-*O*-methyl-D-galactose indicate that the galactan framework of degraded gum *B* is a highly branched structure composed of terminal, 3-*O*-, 6-*O*-, and 3,6-di-*O*-substituted D-galactopyranose residues. The presence of 2,3,4-tri-*O*-methyl-D-galactose indicates that **8** is a significant structural feature of degraded gum *A*.



where R = L-Arap-(1→, →4)-L-Arap-(1→, D-Galp-(1→, →4)-D-Galp-(1→, →6)-D-Galp-(1→

8

An examination of methylated lemon gum indicated the presence of 2,3,4-tri-*O*-methyl-L-rhamnose, 2,3,5- and 2,3,4-tri-, and 2,3-, 2,5-, and 3,5-di-*O*-methyl-L-arabinose, 2,4-di-*O*-methyl-D-galactose, and 2,3,4-tri- and 2,3-di-*O*-methyl-D-glucuronic acid, together with a small proportion of 2,3,4-tri-*O*-methyl-D-galactose. Comparison of this methylation evidence with that for degraded gum *A* suggests that the 4-*O*-methyl-D-glucuronic acid- and L-arabinose-containing side chains (**3**) are attached to the C-3 and/or C-6 positions of certain D-galactose residues. The side chains may contain 2-*O*-, 3-*O*-, and/or 5-*O*-substituted L-arabinofuranose residues, and/or 4-*O*-substituted L-arabinopyranose residues, and may be terminated by L-arabinopyranose, L-arabinofuranose, and 4-*O*-methyl-D-glucuronic acid residues. Some D-glucuronic acid residues may be 4-*O*-substituted with L-rhamnopyranose residues as in *A. senegal* gum[13].

A sample of lemon gum was submitted to two successive Smith degradations[12] to give polysaccharides I and II. The low percentage yields of the products, the drastic cleavage of the macromolecules as indicated by molecular-sieve chromatography[14] on porous glass[15], and the results of methylation and linkage analyses demonstrate the preponderance of periodate-vulnerable 6-*O*-substituted D-galactose residues in the galactan framework of the molecules (*cf.* Ref. 10). Molecular-sieve chromatography of lemon gum, polysaccharides I and II, *A. arabica* and *A. senegal* gums, and D-glucose on "Bio-glas 500" gave the elution patterns shown in Fig. 1. The irregularities of the elution patterns for both lemon gum and *A. arabica* gum at comparable elution times suggest that this feature corresponds to the termination of molecular-sieving at the so-called void volume of the column[14]. The gum samples

from *A. arabica* and *A. senegal* have[10] weight-average molecular-weights of 2.3×10^6 and 6.0×10^5, respectively. Comparison of the elution patterns in Fig. 1 indicates that lemon gum has a weight-average molecular-weight of the order of 2×10^6. The fact that lemon gum has a small limiting-viscosity number ($[\eta] = 16.6$ ml/g) suggests that the molecules are compact and approximately spheroidal in shape.

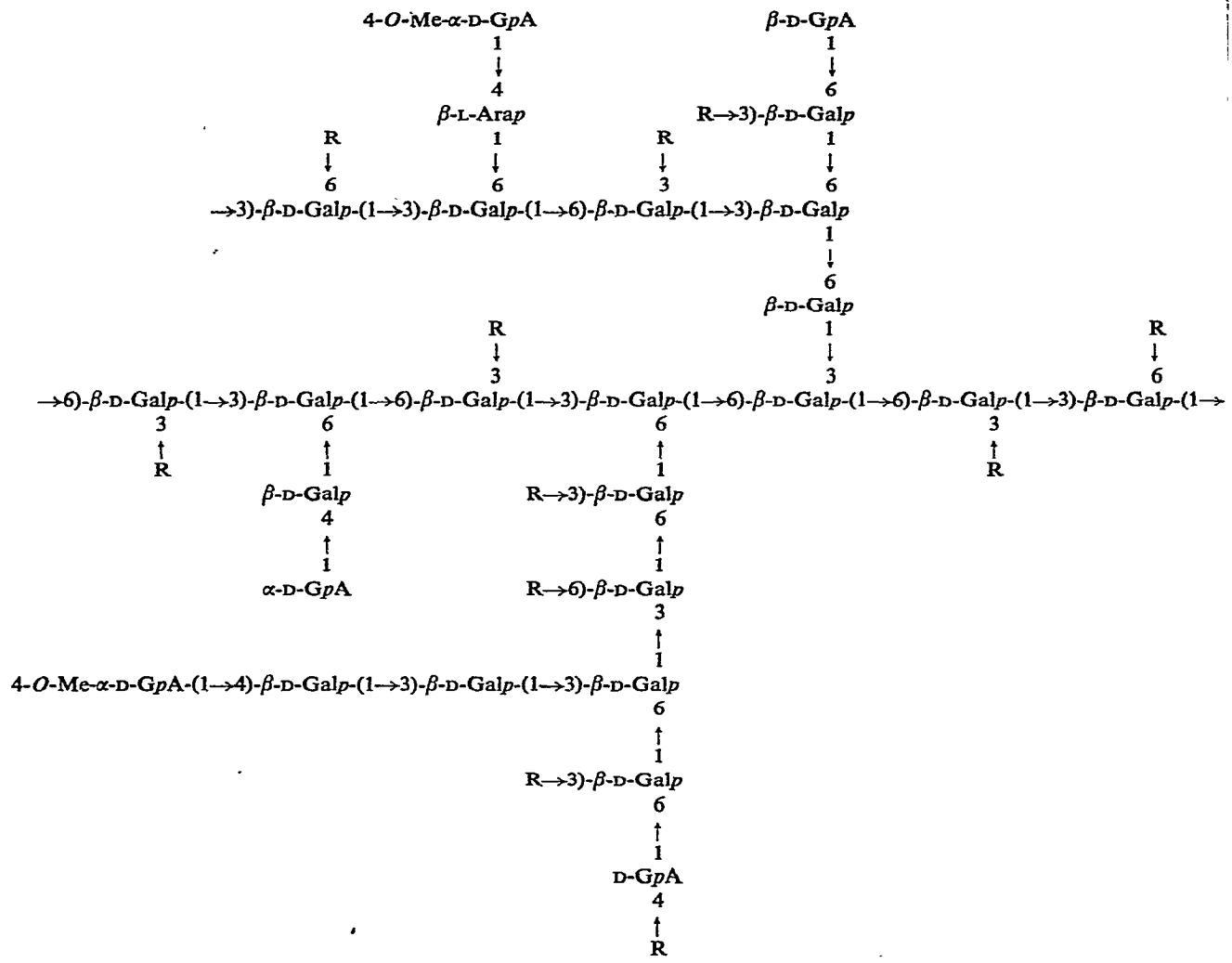


Fig. 3. Possible structural fragment from a lemon-gum molecule. R represents L-Rha*p*-, L-Ara*p*-, L-Ara*f*-, and 4-*O*Me-D-G*p*A-containing side-chains. These chains contain →4)-L-Ara*p*-(→1, →3)-L-Ara*f*-(1→, and →2)-L-Ara*f*-(1→ residues, and may be terminated by L-Rha*p*-(1→, L-Ara*p*-(1→, L-Ara*f*-(1→, or 4-*O*-Me-D-G*p*A-(1→ residues.

Physicochemical behaviour, together with structural evidence, suggests that the gum molecules are based on galactan frameworks of the branch-on-branch type shown diagrammatically in Fig. 2. A possible structural fragment for one of these gum

molecules is represented by the polysaccharide array shown in Fig. 3. When it is considered that lemon gum is probably composed of a spectrum of related molecules (*cf.* Refs. 9, 10, 16), the limitations of this type of structural representation are apparent. It may become possible, as methods of structural elucidation are improved, to interpret structural features of such heteropolysaccharides as lemon gum in terms of probability functions, as has been suggested recently by Painter[17]. Since such conventional techniques, as linkage analysis only serve to yield information regarding structural order, there is clearly a need to develop approaches which will allow the disordered features of certain heteropolysaccharides to be investigated.

EXPERIMENTAL

Melting points were determined with a Fisher–Johns apparatus and are uncorrected. Optical rotations were measured by using a Bendix ETL-NPL automatic polarimeter (type 143A) at 20 $\pm 2°$. Infrared absorption spectra (KBr discs) were recorded on a Beckman model IR5A spectrophotometer. Molecular-sieve chromatography[14] was carried out on a column (30 × 1.5 cm) of "Bio-Glas 500" with 0.25M ammonium formate solution as eluent. Samples (*ca.* 5 mg) were applied to the top of the column, and the effluent was analysed on a Technicon Autoanalyser by using an automated[18] cysteine–sulphuric acid colorimetric method[20]. 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*) butyl alcohol–ethanol–water (3:1:1); (*e*) ethyl acetate–pyridine–water (10:4:3); (*f*) butanone–water–ammonia (*d* 0.88) (200:17:1). R_{Gal} values of sugars 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. Gas–liquid partition chromatography (F and M chromatograph, type 402) of mixtures of methyl glycosides of *O*-methyl sugars[19] 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 60–80 mesh acid-washed Chromosorb W (110 × 0.4 cm) at 175°, and (*ii*) 15% by weight of poly(diethylene glycol adipate) on 60–80 mesh acid-washed Chromosorb W (110 × 0.4 cm) at 175°. Retention times (T) are quoted relative to methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucopyranoside as standard. Neutral sugar percentages were estimated by using the cysteine–sulphuric acid method[20], and uronic acid percentages by the carbazole procedure[21]. 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 6 h with 5% methanolic hydrogen chloride. Acid hydrolyses of oligosaccharides and of methyl glycosides were carried out on a steam bath with N sulphuric acid (for 7 h) and with 0.5N sulphuric acid (for 4 h), respectively.

Purification of lemon gum. — A solution of the gum (115 g) in water (1150 ml) was filtered, dialysed against running tap-water, and freeze-dried to give a product

(81 g), $[\alpha]_D$ +17° (*c* 0.79, water) (Found: N, 0.51; OMe, 2.54; uronic acid, 22; galactose, 36; arabinose, 33; rhamnose, 5%; 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 (17 × 2.5 cm) of cellulose acetate film in both 0.1M ammonium carbonate buffer (pH 8.9) and 0.05M acetate buffer (pH 5.0) at field strengths of 20 volts/cm for 30 min. Polysaccharide bands were located by using the periodate–rosaniline hydrochloride method[22]. Viscosity determinations were carried out in M sodium chloride solutions of lemon gum in a suspended-level dilution viscometer at 24°. The viscometer had a flow time of 193.1 sec for M sodium chloride solution. By using the technique of isoionic dilution, the following values of η_{sp}/c were obtained for known values of *c*, where η_{sp} is the specific viscosity, and *c* the concentration in g/ml: 29.4 ml/g, 0.0290 g/ml; 25.0 ml/g, 0.0193 g/ml; 23.0 ml/g, 0.0145 g/ml; 21.9 ml/g, 0.0116 g/ml. The limiting-viscosity number, $[\eta]$, *i.e.*, $\underset{c\to 0}{\mathrm{Lt}}\ \eta_{sp}/c$, was 16.6 ml/g.

Partial acid hydrolysis of lemon gum and preparation of degraded gum A. — The gum (10 g) was hydrolysed with 0.01N sulphuric acid (250 ml) on a steam bath for 100 h. The cooled solution was neutralised with barium carbonate, filtered, treated with Amberlite resin IR-120 (H^+), and dialysed against water (1.5 l). Dialysis was completed against running tap-water, and freeze-drying yielded degraded gum *A* (4.8 g), $[\alpha]_D$ +23° (*c* 2.14, water) (Found: OMe, 1.74; uronic acid, 26; galactose 66; arabinose, 5%). The diffusate from the dialysis of degraded gum *A* was concentrated to a syrup (4.4 g), a portion (2 g) of which was chromatographed successively in solvents (*a*) and (*b*) on Whatman No. 3MM papers to give three pure monosaccharides, two pure disaccharides, and five fractions containing acidic oligosaccharides.

Fractions 1, 2, and 3 were L-rhamnose monohydrate (22 mg), m.p. 93–94°, $[\alpha]_D$ +6.6° (equil.) (*c* 0.60, water); L-arabinose (301 mg), m.p. 158–159°, $[\alpha]_D$ +103° (equil.) (*c* 1.56, water); and D-galactose (24 mg), m.p. 162–163°, $[\alpha]_D$ +79° (*c* 1.20, water).

Fraction 4 (20 mg) had $[\alpha]_D$ +195° (*c* 0.65, water), gave only arabinose on acid hydrolysis, and had the same paper-chromatographic mobility [R_{Gal} 0.80 in solvent (*a*)] as 3-*O*-β-L-arabinopyranosyl-L-arabinose[5].

Fraction 5 (17 mg) had $[\alpha]_D$ −9° (*c* 0.85, water), gave only arabinose on acid hydrolysis, and had similar paper-chromatographic mobilities [R_{Gal} 1.03 in solvent (*a*), 0.82 in solvent (*b*), and 0.71 in solvent (*c*)] as 5-*O*-α-L-arabinopyranosyl-L-arabinose. A portion of the disaccharide was methylated with methyl iodide and silver oxide in *N*,*N*-dimethylformamide. G.l.c. examination of the methanolysis products revealed peaks having retention times corresponding to the methyl glycosides of 2,3,4-tri- [(*i*) *T* 1.00; (*ii*) *T* 1.02] and 2,3-di-*O*-methyl-L-arabinose [(*i*) *T* 1.46, 1.60, 1.78; (*ii*) *T* 1.51, 1.66, 1.86].

Fraction 6 (118 mg) had $[\alpha]_D$ +129° (*c* 2.36, water), gave 4-*O*-methylglucuronic acid, arabinose, and unchanged starting material on acid hydrolysis, and had the same paper-chromatographic mobility [R_{Gal} 1.12 in solvent (*b*)] as 4-*O*-(4-*O*-methyl-

α-D-glucopyranosyluronic acid)-L-arabinose. Reduction of the derived methyl ester methyl glycosides with lithium aluminium hydride in tetrahydrofuran, followed by acid hydrolysis, gave approximately equal amounts of 4-*O*-methylglucose and arabinose. Methylation of the acidic disaccharide, followed by methanolysis and g.l.c., revealed peaks having retention times corresponding to the methyl glycosides of 2,3,4-tri-*O*-methyl-D-glucuronic acid methyl ester [(*i*) *T* 2.34, 3.03; (*ii*) *T* 2.38, 3.14] and 2,3-di-*O*-methyl-L-arabinose [(*i*) *T* 1.48, 1.66, 1.78; (*ii*) *T* 1.52, 1.67, 1.86].

Fraction 7 (74 mg) had $[\alpha]_D$ +67° (*c* 1.48, water) and a similar paper-chromatographic mobility [R_{Gal} 0.69 in solvent (*b*)] as previously reported[4] for an acidic trisaccharide. On acid hydrolysis, it yielded 4-*O*-methylglucuronic acid, arabinose, and a component that was paper-chromatographically identical [R_{Gal} 1.12 in solvent (*b*)] with Fraction 6. Methylation of the acidic oligosaccharide, followed by methanolysis and g.l.c., revealed peaks having retention times corresponding to the methyl glycosides of 2,3,4-tri-*O*-methyl-D-glucuronic acid methyl ester (+) [(*i*) *T* 2.37, 3.04; (*ii*) *T* 2.40, 3.14] and 2,3-di-*O*-methyl-L-arabinose (++) [(*i*) *T* 1.52, 1.68, 1.84; (*ii*) *T* 1.53, 1.68, 1.87].

Fraction 8 consisted of two components in trace quantities [R_{Gal} 0.62 and 0.50 in solvent (*b*)] and contained approximately 1 molar proportion of uronic acid to 2 of arabinose. On acid hydrolysis, the mixture yielded arabinose and a component that was paper-chromatographically identical [R_{Gal} 1.16 in solvent (*b*)] with Fraction 6.

Fraction 9 (25 mg) had $[\alpha]_D$ +70° (*c* 0.83, water), R_{Gal} 0.41 in solvent (*b*), and contained approximately 1 molar proportion of uronic acid to 3 of arabinose. Acid hydrolysis yielded arabinose and a component that was paper-chromatographically identical [R_{Gal} 1.14 in solvent (*b*)] with Fraction 6.

Fraction 10 (18 mg) had $[\alpha]_D$ +50° (*c* 0.60, water), R_{Gal} 0.25 in solvent (*b*), and contained approximately 1 molar proportion of uronic acid to 4 of arabinose. Acid hydrolysis yielded arabinose and a component that was paper-chromatographically identical [R_{Gal} 1.14 in solvent (*b*)] with Fraction 6.

Partial acid hydrolyses of degraded gum A. — Degraded gum *A* (1 g) was hydrolysed with 0.5N sulphuric acid (250 ml) for 3 h on a steambath. The cooled solution was neutralised with barium carbonate, filtered, treated with Amberlite resin IR-120 (H^+), and concentrated to a syrup, which was chromatographed on Whatman No. 3MM papers in solvents (*b*) and (*a*) successively to give a component identical with Fraction 6 above, and three pure disaccharides.

Fraction 1 (33 mg) had $[\alpha]_D$ + 92° (*c* 1.64, water) and the same paper-chromatographic mobility [R_{Gal} 0.73 in solvent (*b*)] as 4-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-D-galactose. Reduction of the derived methyl ester methyl glycosides with sodium borohydride in aqueous solution, followed by acid hydrolysis, gave approximately equal amounts of 4-*O*-methylglucose and galactose. Methylation of the acidic disaccharide, followed by methanolysis and g.l.c., revealed peaks having retention times corresponding to the methyl glycosides of 2,3,4-tri-*O*-methyl-D-glucuronic

acid methyl ester [(*i*) *T* 2.39, (3.04)] and 2,3,6-tri-*O*-methyl-D-galactose [(*i*) *T* (3.04), 3.91, 4.42].

Fraction 2 (22 mg) had $[\alpha]_D$ +69° (*c* 1.10, water) and the same paper-chromatographic mobility [R_{Gal} 0.49 in solvent (*a*) and 0.53 in solvent (*e*)] as 3-*O*-β-D-galactopyranosyl-D-galactose.

Fraction 3 (18 mg) had $[\alpha]_D$ +34° (*c* 0.90, water) and the same paper-chromatographic mobility [R_{Gal} 0.30 in solvent (*a*) and 0.37 in solvent (*e*)] as 6-*O*-β-D-galactopyranosyl-D-galactose.

Degraded gum *A* (50 mg) was hydrolysed with N sulphuric acid (20 ml) for 5 h on a steambath. Paper chromatography of the hydrolysate in solvent (*b*) showed the presence of 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose (R_{Gal} 0.23) and 4-*O*-(α-D-glucopyranosyluronic acid)-D-galactose (R_{Gal} 0.30), in addition to the other acidic disaccharides and neutral sugars already identified.

Methylation of degraded gum A. — Degraded gum *A* (500 mg) was methylated to give a product (90 mg), $[\alpha]_D$ −7° (*c* 0.40, chloroform) (Found: OMe, 40.2%). Methanolysis of a sample of this product, followed by g.l.c. of the mixture of methyl glycosides, gave the results shown in Table I. Examination of a hydrolysate of the methyl glycosides in solvents (*c*) 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 I).

Partial methanolysis of methylated, degraded gum A. — Methylated, degraded gum *A* (25 mg) was heated under reflux with 2% methanolic hydrogen chloride (10 ml) for 2 h. The cooled methanolic solution was neutralised with silver carbonate, filtered, and concentrated to a syrup, which was hydrolysed with 0.5N sulphuric acid (100 ml) for 4 h on a steambath. The cooled solution was neutralised with barium carbonate, filtered, treated with Amberlite resin IR-120 (H^+), and adsorbed on to a column (8 × 0.5 cm) of DEAE-Sephadex A25 in the formate form[23]. After elution of the neutral *O*-methyl sugars with water (1 litre), the acidic *O*-methyl sugars were eluted with aqueous 3% formic acid (100 ml). After removal of water and formic acid, methanolysis of the syrup and g.l.c. of the products revealed peaks having retention times corresponding to the methyl glycosides of 2,3,4-tri-*O*-methyl-D-glucuronic acid methyl ester (+ + +) [(*i*) *T* 2.36, (3.05)]; 2,3-di-*O*-methyl-L-arabinose (+) [(*i*) *T* 1.48, 1.67, 1.79]; 2,3,6-(+) [(*i*) *T* (3.05), 4.10, 4.47] and 2,3,4-tri- [(*i*) *T* 7.0], and 2,4-di-*O*-methyl-D-galactose (trace) [(*i*) *T* 16.4, 18.4].

Smith degradation of degraded gum A. — Degraded gum *A* (1 g) was dissolved in water (25 ml), and 0.5M sodium metaperiodate solution (25 ml) was added. After 96 h, the amount of formic acid released was 4.85 mmoles/g. The reaction was stopped at this point by addition of ethylene glycol (2 ml), and the solution was dialysed against running tap-water for 48 h. Sodium borohydride (500 mg) was added, and the mixture kept at room temperature for 48 h. After dialysis for a further 48 h, the polyalcohol was hydrolysed with N sulphuric acid for 48 h at 22–24°. After dialysis against running tap-water for 48 h, degraded gum *B* (140 mg, 14%), $[\alpha]_D$ −11° (*c* 1.40 water), was isolated as the freeze-dried product.

TABLE I

EXAMINATION OF METHANOLYSIS AND HYDROLYSIS PRODUCTS FROM METHYLATED, DEGRADED GUM *A*

O-*Methyl sugars*	*Relative retention times* (T) *of methyl glycosides*		R_G *in solvent* (c)	*Approx. relative molar proportions*
	Column (i)	*Column* (ii)		
2,3,4-Tri-*O*-methyl-L-arabinose	1.00	1.03	0.77	Trace
2,3-Di-*O*-methyl-L-arabinose	1.50 (1.76)[a]	1.58 (1.73) (1.87)	0.63	+/2
2,3,4,6-Tetra-*O*-methyl-D-galactose	(1.76)	(1.73) (1.87)	0.89	+
2,3,6-Tri-*O*-methyl-D-galactose	(3.06) (3.94) (4.43)	(3.18) (4.24) (4.72)	0.72	+/2
2,4,6-Tri-*O*-methyl-D-galactose	(3,94) (4.43)	(4.24) (4.72)	0.72	+
2,3,4-Tri-*O*-methyl-D-galactose	7.0	7.8	0.69	+++
2,6-Di-*O*-methyl-D-galactose	—	—	0.51	Trace
2,4-Di-*O*-methyl-D-galactose	16.1, 18.4	18.1, 20.9	0.47	++
2-*O*-Methyl-D-galactose	—	—	0.27	Trace
2,3,4-Tri-*O*-methyl-D-glucuronic acid[b]	2.40 (3.06)	2.48 (3.18)	—	++

[a] Figures in parentheses indicate *T* values of components not completely resolved. [b] As methyl ester methyl glycosides.

Partial acid hydrolysis and methylation of degraded gum B.—Degraded gum *B* (30 mg) was hydrolysed with 0.5N sulphuric acid (20 ml) for 1 h on a steambath. Paper-chromatographic examination of the hydrolysate showed the presence of galactose, a trace quantity of arabinose, two neutral disaccharides having the mobilities of 3-*O*-*β*-D-galactopyranosyl-D-galactose [R_{Gal} 0.49 in solvent (*a*), 0.52 in solvent (*e*)] and 6-*O*-*β*-D-galactopyranosyl-D-galactose [R_{Gal} 0.30 in solvent (*a*), 0.37 in solvent (*e*)], and higher neutral oligosaccharides.

Degraded gum *B* (50 mg) was methylated to give a product, which, on methanolysis followed by g.l.c., revealed peaks having retention times corresponding to the methyl glycosides of 2,3,4,6-tetra-(+) [(*i*) *T* 1.74; (*ii*) *T* 1.72, 1.82], 2,4,6-(+ + +) [(*i*) *T* 3.90, 4.42; (*ii*) *T* 4.06, 4.67], and 2,3,4-tri-(+ +) [(*i*) *T* 7.0; (*ii*) *T* 7.5], and 2,6-(+) [(*i*) *T* 9.6, 11.0, 12.6; (*ii*) *T* 10.1, 12.0, 13.6] and 2,4-di-*O*-methyl-D-galactose (+) [(*i*) *T* 16.3, 18.5), (*ii*) *T* 17.8, 20.5]; and 2,3,4-tri-*O*-methyl-D-glucuronic acid methyl ester (trace) [(*i*) *T* 2.42, 3.03; (*ii*) *T* 2.46, 3.12]. Examination of a hydrolysate of the methyl glycosides by paper chromatography in solvents (*c*) and (*f*) indicated the presence of 2-*O*-methylgalactose, in addition to those *O*-methyl sugars already characterised by g.l.c. of their methyl glycosides.

Methylation of lemon gum. — The gum (500 mg) was methylated to give a product (102 mg), $[\alpha]_D$ −6° (*c* 1.75, chloroform) (Found: OMe, 36.5%). Methanolysis, followed by g.l.c., of the mixture of methyl glycosides indicated the presence of 2,3,4-tri-*O*-methyl-L-rhamnose [(*i*) *T* 0.48; (*ii*) *T* 0.47]; 2,3,5- [(*i*) *T* 0.57, 0.72; (*ii*) *T* 0.56, 0.73] and 2,3,4-tri- [(*i*) *T* (1.04); (*ii*) *T* (1.05)], and 3,5- [(*i*) *T* (1.04), 2.48; (*ii*) *T* (1.05), 2.50], 2,5-[(*i*) *T* (1.83), 3.20; (*ii*) *T* (1.86), 3.40], and 2,3-di-*O*-methyl-L-arabinose [(*i*) *T* 1.50, 1.68, (1.83); (*ii*) *T* 1.55, 1.73, (1.86)]; 2,3,4,6-tetra- [(*i*) *T* (1.83); (*ii*) *T* (1.86)], 2,3,6-[(*i*) *T* (2.97), (3.90), (4.40); (*ii*) *T* (3.15), (4.22), (4.72)], 2,4,6-[(*i*) *T* (3.90), (4.40); (*ii*) *T* (4.22), (4.72)], and 2,3,4-tri- [(*i*), *T* 7.1; (*i*) *T* 7.7], and 2,4-di-*O*-methyl-D-galactose [(*i*) *T* 16.2, 18.5; (*ii*) *T* 17.6, 20.3]; and 2,3,4-tri- [(*i*) *T* 2.30 (2.97); (*ii*) *T* 2.47, (3.15)] and 2,3-di-*O*-methyl-D-glucuronic acid [(*i*) *T* 9.6; (*ii*) *T* 11.7]. Acid hydrolysis of the methyl glycosides, followed by paper-chromatographic examination of the free sugars, confirmed these results.

Preparation, partial hydrolysis with acid, and methylation of polysaccharide I. — Borohydride reduction of periodate-oxidised gum (40.0 g), followed by hydrolysis with N sulphuric acid at 22–24° for 48 h, gave polysaccharide I (10.9 g, 27%), $[\alpha]_D$ −1° (*c* 2.14, water) (Found: uronic acid, 2; galactose, 62; arabinose, 36%). The amount of formic acid released in 48 h was 1.75 mmoles/g. Partial hydrolysis of polysaccharide I (20 mg) with acid, followed by paper-chromatographic examination of the hydrolysate, indicated the presence of galactose, arabinose, two neutral disaccharides having the mobilities of 3-*O*-*β*-D-galactopyranosyl-D-galactose [R_{Gal} 0.49 in solvent (*a*), 0.52 in solvent (*e*)] and 6-*O*-*β*-D-galactopyranosyl-D-galactose [R_{Gal} 0.29 in solvent (*a*), 0.37 in solvent (*e*)], and other oligosaccharides. Polysaccharide I (500 mg) was methylated to give a product (70 mg), $[\alpha]_D$ −32° (*c* 0.68, chloroform) (Found: OMe, 40.1%). Methanolysis, followed by g.l.c. of the mixture of methyl glycosides gave the results shown in Table II. Acid hydrolysis of the methyl glycosides,

followed by paper-chromatographic examination of the free sugars, showed the presence of trace amounts of 2,6-di- and 2-*O*-methylgalactose, in addition to those listed in Table II.

TABLE II

O-METHYL SUGARS IN METHYLATED POLYSACCHARIDES I AND II

O-Methyl sugar	*I*	*II*
2,3,5-Tri-*O*-methyl-L-arabinose	+	++
3,5-Di-*O*-methyl-L-arabinose	++	+++++
2,3,4,6-Tetra-*O*-methyl-D-galactose	++	+
2,4,6-Tri-*O*-methyl-D-galactose	+	+
2,3,4-Tri-*O*-methyl-D-galactose	++	+
2,4-Di-*O*-methyl-D-galactose	++	++
2,3,4-Tri-*O*-methyl-D-glucuronic acid	Trace	—

Preparation, partial hydrolysis with acid, and methylation of polysaccharide II. — Borohydride reduction of periodate-oxidised polysaccharide I (10 g), followed by hydrolysis with N sulphuric acid at 22–24° for 48 h, gave polysaccharide II (1.39 g, 13.9%), $[\alpha]_D$ −27° (*c* 1.70, water) (Found: galactose, 35%; arabinose, 65%). The amount of formic acid released in 48 h was 2.53 mmoles/g. Partial hydrolysis of polysaccharide II (20 mg) with acid, followed by paper-chromatographic examination of the hydrolysate, indicated the presence of galactose, arabinose, two neutral disaccharides having the mobilities of 3-*O*-β-D-galactopyranosyl-D-galactose [R_{Gal} 0.48 in solvent (*a*), 0.52 in solvent (*e*)] and 6-*O*-β-D-galactopyranosyl-D-galactose [R_{Gal} 0.29 in solvent (*a*), 0.36 in solvent (*e*)], and higher neutral oligosaccharides. Polysaccharide II (300 mg) was methylated to give a product (212 mg), $[\alpha]_D$ −60° (*c* 0.54, chloroform) (small i.r. absorption in the range 3400–3600 cm^{-1}). Methanolysis, followed by g.l.c. of the mixture of methyl glycosides, gave the results shown in Table II. Acid hydrolysis of the methyl glycosides, followed by paper-chromatographic examination of the free sugars, showed the presence of trace amounts of 2,6-di- and 2-*O*-methylgalactose, in addition to those listed in Table II.

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STRUCTURAL STUDIES ON THE O-SPECIFIC SIDE-CHAINS OF THE CELL-WALL LIPOPOLYSACCHARIDE FROM *Salmonella typhimurium* 395 MS

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ABSTRACT

The structure of the O-specific side-chains of the cell-wall lipopolysaccharide of *Salmonella typhimurium* 395 MS has been investigated. Methylation analyses of the original lipopolysaccharide, of the material obtained on mild hydrolysis of the lipopolysaccharide with acid, and of a product obtained by acetalation of all of the free hydroxyl groups in the lipopolysaccharide, followed by alkaline deacetylation, have provided the essential information in this study. The mixtures of sugars obtained on acid hydrolysis of the different methylated products were analysed, as alditol acetates, by g.l.c.–mass spectrometry. As a result of these studies, a detailed structure of the repeating unit of these side-chains is presented.

INTRODUCTION

The O-antigenic cell-wall lipopolysaccharides from Gram-negative bacteria have been extensively studied by immunochemical, biochemical, and chemical methods. The field has been summarised in two recent papers[1,2]. The complex structure of these lipopolysaccharides and the difficulty in preparing large amounts of pure material have restricted the use of chemical methods. One of the most important methods in structural polysaccharide chemistry, methylation analysis, seems to have been applied only twice and with ambiguous results. The lipopolysaccharides from smooth forms of *Salmonella* bacteria carry the O-specific side-chains, which constitute the major part of the molecule. These side chains are built up of repeating units, containing up to five sugar residues. For some lipopolysaccharides, the composition and partial structure of these repeating units are known, but the complete structures are not known with certainty. In the present paper, chemical studies of the O-specific side-chains of the lipopolysaccharide from *Salmonella typhimurium* 395 MS are reported. An essential tool in this investigation has

been the use of g.l.c.[3] and mass spectrometry[4] for the qualitative and quantitative analysis of the alditol acetates of methylated sugars.

ISOLATION OF THE LIPOPOLYSACCHARIDE

The lipopolysaccharide was extracted, by the phenol–water method, from cell walls of *Salmonella typhimurium* 395 MS, which contains the O-factors 4, 5, and 12, as established by agglutination tests and phage susceptibility. The isolated lipopolysaccharide was shown to contain the same O-factors by passive hemagglutination and phage inactivation techniques[5]. Acid hydrolysis of the lipopolysaccharide yielded D-glucose, D-galactose, D-mannose, L-rhamnose, and abequose, in agreement with previous results from similar lipopolysaccharides[1,2]. The sugars were not isolated in the present study but were analysed by g.l.c., as their alditol acetates[6,7], and their identity was confirmed by mass spectrometry[8]. A previous analysis[6] of the lipopolysaccharide from the same organism gave the above sugars in the relative molar proportions 30:22:21:18:9.

According to general ideas on the structure of the repeating unit of the lipopolysaccharide of *S. typhimurium*, abequose should occur in the same relative proportion as D-mannose and L-rhamnose. It had always been found, however, in a lower proportion. It therefore seemed probable that part of this sugar had either been lost during the phenol–water extraction or had been destroyed during the acid hydrolysis. The first possibility was eliminated when it was found that no abequose passed through the membrane during the dialysis of the aqueous phase from the phenol–water extraction. In order to minimize destruction during acid hydrolysis, the lipopolysaccharide was hydrolysed in two steps. After the first, mild hydrolysis, during which all terminal abequose residues should be cleaved, the monomeric sugars were separated from the oligo- and poly-meric material by gel filtration. The latter material was then hydrolysed to completion, and the two fractions were combined and analysed. The molar percentages of D-glucose, D-galactose, D-mannose, L-rhamnose, and abequose were 5:26:24:21:24. The last three sugars are known to occur exclusively in the side chains, whereas part of the D-galactose and at least part of the D-glucose originate from the core of the lipopolysaccharide.

O-Factor 5 is known to be associated with the presence of *O*-acetyl groups, which, it is assumed, are linked to C-2 of the D-galactose residues. The presence of *O*-acetyl groups in the lipopolysaccharide was indicated by the infrared band at 1738 cm^{-1}, and further supported by the presence of a singlet at τ 7.95 (typical for acetoxyl) in the n.m.r. spectrum (D_2O).

METHYLATION ANALYSIS

The lipopolysaccharide was methylated by treatment[9] with methylsulphinyl carbanion–methyl iodide in methyl sulphoxide. The fully methylated lipopolysaccharide was hydrolysed, and the mixture of methylated sugars was converted into their

alditol acetates, and analysed by g.l.c.[3] and mass spectrometry[4]. Four main and some minor peaks were obtained on g.l.c. (Fig. 1), and mass spectra were taken at the beginning, maximum, and end of each main peak and at the maxima of the minor peaks.

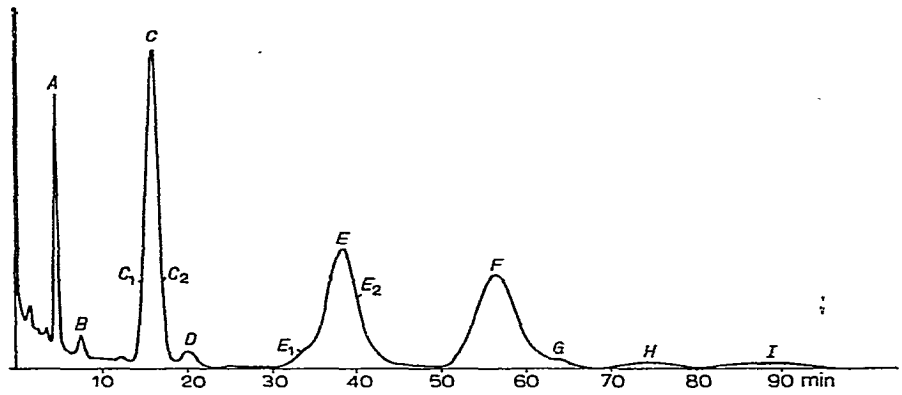


Fig. 1. G.l.c. separation of methylated sugars, as their alditol acetates, obtained from the hydrolysate of the fully methylated lipopolysaccharide.

The component in the first peak (*A*) gave a mass spectrum with the major fragments (at least 10% of the base peak, *m/e* 43) *m/e* 69, 71, 75, 83, 89, 101, 115, 117, 131, 143, and 157. Of these, *m/e* 117, 131, and also 175 (5% of the base peak) are three of the four expected primary fragments from 1,5-di-*O*-acetyl-2,4-di-*O*-methylabequitol (Fig. 2), assuming that fission occurs at a methoxylated carbon atom and that the fragment with the methoxyl carries the positive charge. The fourth primary fragment, *m/e* 189, gave only a peak of low intensity, but the major, secondary fragment, *m/e* 157, is most probably derived from this fragment by loss of methanol (32). Authentic 1,5-di-*O*-acetyl-2,4-di-*O*-methylabequitol, prepared from methyl α,β-abequopyranoside, showed the same retention time and mass spectrum. The component in Fraction *A* is thus derived from 2,4-di-*O*-methylabequose.

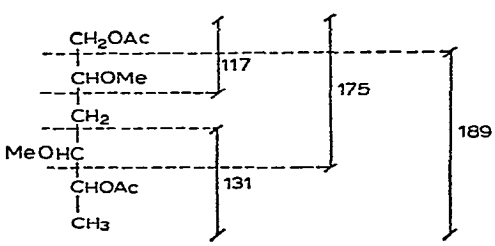


Fig. 2. Primary fragments obtained from 1,5-di-*O*-acetyl-2,4-di-*O*-methylabequitol.

The mass spectrum of the component in peak *B* showed that it was not a partially methylated alditol acetate. It was not further investigated.

The mass spectrum taken at C_1 showed the major fragments, *m/e* 87, 101, 117, 143, and 203. This fragmentation pattern is typical for a 1,4,5-tri-*O*-acetyl-

6-deoxy-2,3-di-*O*-methylhexitol and was also obtained from the acetylated alditol derived from 2,3-di-*O*-methyl-L-fucose. No authentic 2,3-di-*O*-methyl-L-rhamnose was available, but as L-rhamnose is the only 6-deoxyhexose present in the lipopolysaccharide, the main component in *C* must therefore be derived from 2,3-di-*O*-methyl-L-rhamnose.

When the mass spectrum was taken at C_2, additional major fragments at *m/e* 45, 145, and 161 were obtained. These fragments are typical for 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylhexitols, and the fragment *m/e* 145 has not been obtained from any other partially methylated alditol acetate. From the retention time on the ECNSS-M column ($T = 1.0$ relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, $T = 1.00$), one cannot differentiate between the D-glucitol and the D-mannitol derivative. 1,4,5-Tri-*O*-acetyl-2,3-di-*O*-methyl-L-rhamnitol, 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, and 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-mannitol are, however, better resolved on a polyphenyl ether column at 175° ($T = 0.81$, 1.00, and 1.06, respectively), and it could be demonstrated that the minor component in peak *C* was the D-glucitol derivative (from 2,3,4,6-tetra-*O*-methyl-D-glucose).

The component in peak D gave a mass spectrum typical for a 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylhexitol. The retention time ($T = 1.25$) was identical with that of the D-galactitol derivative (from 2,3,4,6-tetra-*O*-methyl-D-galactose).

The mass spectrum of the component in *E*, taken at E_2, is typical for a 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylhexitol. From the shape of the peak, it is obvious that it contains a contaminant having a lower retention time. The mass spectrum taken at E_1 was indistinguishable from that taken at E_2. The *T*-values of the two components are 2.28 and 2.10, respectively, and the corresponding values for the D-glucitol, D-galactitol, and D-mannitol derivatives are 1.95, 2.28, and 2.09, respectively. This establishes that the major component is derived from 2,4,6-tri-*O*-methyl-D-galactose and the minor from 2,4,6-tri-*O*-methyl-D-mannose.

The mass spectrum of the component in peak *F* showed major fragments at *m/e* 45, 85, 87, 99, 101, 127, 129, 161, and 261. Of these, those at *m/e* 45, 161, and 261 are the major primary fragments expected from a 1,2,3,5-tetra-*O*-acetyl-4,6-di-*O*-methylhexitol. The mass spectrum was also indistinguishable, except for small differences in intensities of some of the fragments, from that given by 1,2,3,5-tetra-*O*-acetyl-4,6-di-*O*-methyl-D-glucitol. The retention time ($T = 3.29$) is, however, different from that of the glucitol derivative ($T = 4.02$). Reference materials of the corresponding D-mannitol and D-galactitol derivatives were not available. As D-galactose and D-mannose occur in comparable amounts in the lipopolysaccharide, and most of the D-galactose (but only traces of the D-mannose) has been accounted for in the other fractions, it is reasonable to assume that the component in *F* is derived from 4,6-di-*O*-methyl-D-mannose.

The component in peak *G* afforded the mass spectrum corresponding to a 1,3,4,5-tetra-*O*-acetyl-2,6-di-*O*-methylhexitol. The retention time ($T = 3.65$) is the same as that given by the D-galactitol derivative but differs from that given by the D-glucitol derivative. Since no reference sample of the corresponding D-mannitol derivative

was available, the component is only tentatively identified as the alditol acetate of 2,6-di-*O*-methyl-D-galactose.

The component in peak *H* gave the mass spectrum of a 1,2,4,5-tetra-*O*-acetyl-3,6-di-*O*-methylhexitol. The retention time ($T = 4.30$) differs from that of the D-glucitol derivative ($T = 4.40$). Reference materials of the corresponding D-mannitol and D-galactitol derivatives were not available, and the substance has not therefore been identified.

The component in peak *I* afforded a mass spectrum identical with that of 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methylhexitol. The retention time ($T = 5.10$) was the same as for the D-glucitol derivative, but differred from that of the D-galactitol derivative ($T = 6.35$). The corresponding D-mannose derivative was not available. The substance is therefore only tentatively identified as the alditol acetate of 2,4-di-*O*-methyl-D-glucose.

The results from the methylation analysis are summarised in Table I. The molar percentages of D-glucose, D-galactose, D-mannose, L-rhamnose, and abequose, calculated from the methylation analysis, and assuming that the molar percentage of L-rhamnose is the same as in the original lipopolysaccharide, are 3:24:23:21:7. The agreement between these values and those obtained from the sugar analysis of the lipopolysaccharide, with the exception of abequose, is good. It seems reasonable to assume, however, that a considerable part of the volatile di-*O*-methylabequose and derivatives was lost during the concentrations.

TABLE I

METHYL ETHERS FROM THE HYDROLYSATE OF METHYLATED LIPOPOLYSACCHARIDE

Sugars	T[a]	*Molar proportion*[b] (%)
2,4-Di-*O*-methylabequose	0.32	7.0
2,3-Di-*O*-methyl-L-rhamnose	0.98	21.4
2,3,4,6-Tetra-*O*-methyl-D-glucose	1.00	1.8
2,3,4,6-Tetra-*O*-methyl-D-galactose	1.25	1.3
2,4,6-Tri-*O*-methyl-D-mannose	2.10	2.1
2,4,6-Tri-*O*-methyl-D-galactose	2.28	20.9
4,6-Di-*O*-methyl-D-mannose	3.29	21.2
2,6-Di-*O*-methyl-D-galactose	3.65	1.8
3,6-Di-*O*-methylhexose	4.30	1.1
2,4-Di-*O*-methyl-D-glucose	5.10	1.1

[a]Retention times of the corresponding alditol acetates on the ECNSS-M column relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol. [b]As a considerable proportion of 2,4-di-*O*-methylabequose was lost during the methylation analysis, the % of the methylated sugars are given relative to that of 2,3-di-*O*-methyl-L-rhamnose, assumed to be the same as the % of L-rhamnose in the original lipopolysaccharide.

A sample of the lipopolysaccharide was subjected to mild hydrolysis with acid, during which all of the abequosidic linkages should be broken and most of the other

linkages should be left intact. The oligo- and poly-meric material was recovered, methylated, and hydrolysed, and the partially methylated sugars were reduced and acetylated. The mixture was then analysed by g.l.c. and mass spectrometry, and the main peaks, consisting of the acetylated alditols derived from 2,3-di-*O*-methyl-L-rhamnose, 2,3,4,6-tetra-*O*-methyl-D-galactose, 3,4,6-tri-*O*-methyl-D-mannose, and 2,4,6-tri-*O*-methyl-D-galactose, were identified. The alditol acetate derived from 3,4,6-tri-*O*-methyl-D-mannose showed the expected mass spectrum and the same retention time ($T = 1.95$) as an authentic sample. Reference samples of the corresponding D-glucitol and D-galactitol acetates were not available. None of the components in the other main peaks, however, were derived from D-mannose, and the alditol acetate from 4,6-di-*O*-methyl-D-mannose, which was the main D-mannose derivative present during a similar analysis of the non-degraded lipopolysaccharide, was absent. The evidence is entirely consistent with the designation of the 3,4,6-tri-*O*-methylhexose as the D-mannose derivative. The results are summarised in Table II.

TABLE II

METHYL ETHERS FROM THE HYDROLYSATE OF METHYLATED, DEGRADED LIPOPOLYSACCHARIDE

Sugars	T[a]	*Molar proportion* (%)
2,4-Di-*O*-methylabequose	0.32	1.7
2,3-Di-*O*-methyl-L-rhamnose	0.98	33.4
2,3,4,6-Tetra-*O*-methyl-D-galactose	1.25	18.1
3,4,6-Tri-*O*-methyl-D-mannose	1.95	32.7
2,4,6-Tri-*O*-methyl-D-galactose	2.28	12.7
4,6-Di-*O*-methyl-D-mannose	3.29	1.7

[a]Retention times of the corresponding alditol acetates on the ECNSS-M column relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.

DETERMINATION OF THE POSITION OF THE *O*-ACETYL GROUP

In order to locate the *O*-acetyl groups in the lipopolysaccharide, the hydroxyl groups were first protected, as acetals, by treatment[10] with methyl vinyl ether and a catalytic amount of *p*-toluenesulphonic acid in methyl sulphoxide. The product showed C=O absorption at 1738 cm^{-1} but no absorption in the hydroxyl region. When the *O*-acetyl groups were removed, by treatment with sodium methoxide in methanol, the C=O absorption disappeared, and a very small absorption in the hydroxyl region was observed. The deacetylated product was methylated as above and hydrolysed, and the sugars were reduced with sodium borodeuteride and acetylated. G.l.c.–mass spectrometry of the alditol acetates revealed the presence of D-glucose, D-galactose, D-mannose, and L-rhamnose in the hydrolysate. Abequose was virtually absent, and the only component, not present in the mixture of alditol acetates from the original lipopolysaccharide, had a lower retention time than abequitol acetate.

The mass spectrum of this component showed major fragments at *m/e* 217 (4% of the base peak, *m/e* 43), 157, 118, 115, 102, 83, and 43 (base peak); *m/e* 217 and 118 are the two main primary fragments expected from 2-*O*-methylabequitol acetate, and *m/e* 118 (even) contains the deuterium atom. The major secondary fragments *m/e* 157, 115, and 83 are most probably derived from *m/e* 217 by consecutive losses of acetic acid (60), ketene (42), and methanol (32). The component is therefore derived from 2-*O*-methylabequose. The results demonstrate that only the 2-positions of the abequose residues and no other positions in the side chains of the lipopolysaccharide are acetylated.

ACID HYDROLYSIS OF THE LIPOPOLYSACCHARIDE

A solution of the lipopolysaccharide in 0.05M aqueous sulphuric acid was kept at 80°, and the optical rotation was followed polarimetrically (Fig. 3). A rapid decrease (4 h) of $[\alpha]_{5780}$ from +120 to +87° was observed, followed by a slower decrease to +52° in 21 h. Because of the low solubility of the lipopolysaccharide, these values are not very accurate. From the established properties of glycosides, we can predict that the abequosidic linkages in this lipopolysaccharide should be the most readily hydrolysed, followed by the L-rhamnosidic linkages. This has been confirmed in partial hydrolysis studies on closely related lipopolysaccharides. Together with the knowledge of the specific rotations of α- and β-abequosides[11] and α- and β-L-rhamnosides, respectively, it may be inferred that the abequose residues are α-linked and the L-rhamnose residues are β-linked in the lipopolysaccharide.

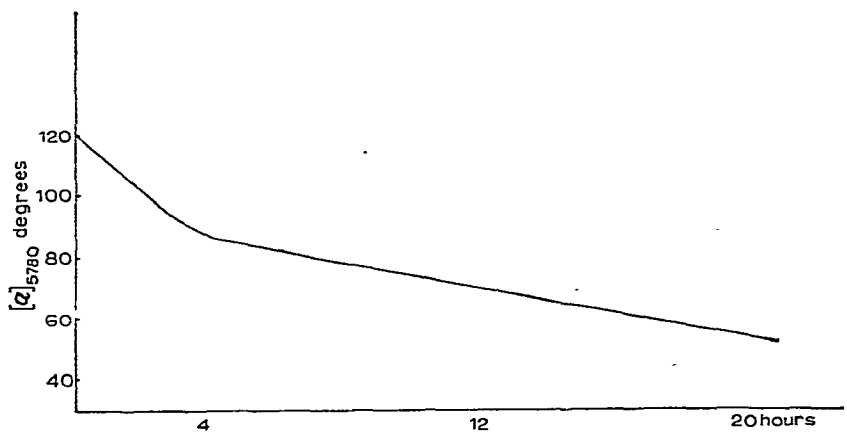


Fig. 3. Optical rotation *versus* time on acid hydrolysis of lipopolysaccharide.

DISCUSSION

From previous studies[1,2] on the O-specific side-chains from group-B *Salmonella*

lipopolysaccharides, the structure of the repeating unit given in Fig. 4 has been suggested.

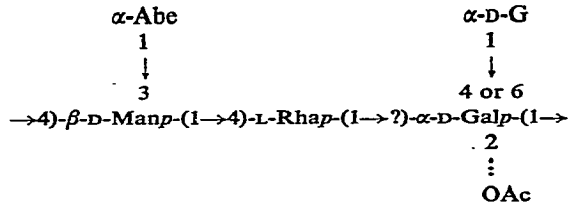


Fig. 4. Previously proposed structure for the repeating unit of the O-specific side-chains of group-B *Salmonella* lipopolysaccharides.

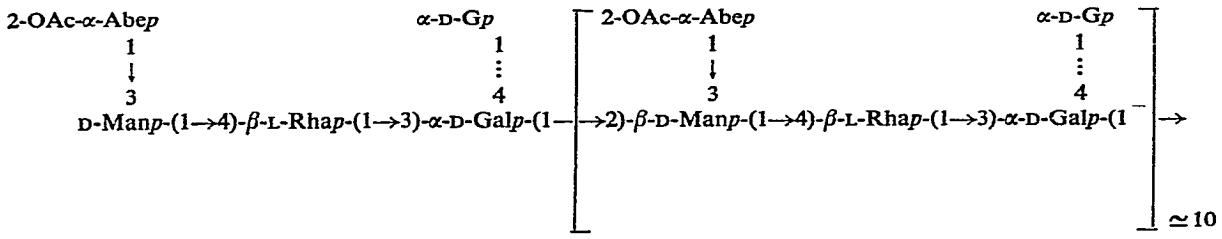


Fig. 5. Proposed structure for the O-specific side-chains of the lipopolysaccharide from *Salmonella typhimurium* 395 MS.

O-Factor 4 is associated with the abequose residue, which is assumed to be linked to the 3-position of D-mannose. The α-linked abequose residue was indicated by inhibition experiments.

O-Factor 5, which is not present in all of the group-B lipopolysaccharides, is associated with an *O*-acetyl group, previously believed to be linked to the 2-position of the D-galactose residue.

O-Factor 1, absent in the present lipopolysaccharide, seems to be associated with an α-D-glucose residue, attached to C-6 of D-galactose.

The chemical nature of the O-factor 12 is not well understood. It has been divided into three subfactors one of which, 12_2, appears to be associated with an α-D-glucose residue, attached to C-4 of D-galactose.

The structural features shown in Fig. 4 have been deduced from partial hydrolysis studies, periodate-oxidation studies, and immunochemical studies. It has further been demonstrated, by biosynthetic studies, that, in the first repeating unit, the D-galactose residue is linked to the core of the lipopolysaccharide.

From the result obtained in the present investigation, the following structure of the repeating unit of the O-specific side-chains in the lipopolysaccharide from *Salmonella typhimurium* 395 MS (4, 5, 12) is proposed (see Fig. 5). This structural scheme is based upon the evidence given below.

The pyranosidic nature of the sugars is evident from the methylation analysis, except for the L-rhamnose. A L-rhamnofuranosidic linkage should, however, be even more readily hydrolysed than the abequopyranosidic linkage, and is therefore excluded.

The sequence of the sugars was demonstrated by the methylation analysis. The occurrence of most of the D-mannose as branched residues, linked through C-2 and C-3 in the nondegraded lipopolysaccharide, and as chain residues, linked through C-2, in the product which had been subjected to mild hydrolysis with acid, demonstrates that the terminal abequose groups are linked to D-mannose in the 3-position. The fact that a considerable proportion of terminal D-galactose residues was found in the hydrolysed product shows that several L-rhamnose residues, originally linked to D-galactose, have been hydrolysed. As most of the D-galactose and all of the L-rhamnose residues occur as chain residues, only the sequence given in Fig. 5 is possible, unless a less-ordered structure or a longer repeating unit is considered.

The nature of the linkages between the sugars is evident from the structure of the main components in the hydrolysate of the fully methylated lipopolysaccharide, namely 4,6-di-*O*-methyl-D-mannose, 2,3-di-*O*-methyl-L-rhamnose, and 2,4,6-tri-*O*-methyl-D-galactose. The observation that the L-rhamnose residues are linked in the 4-position is in agreement with previous results from other group-B lipopolysaccharides. The observation that the D-galactose residues are linked to the 2-position of D-mannose is, however, contrary to previous results from studies on this type of lipopolysaccharide, which had indicated a (1→4)-linkage.

The linkage between L-rhamnose and D-galactose, previously unknown, has now been established as (1→3).

The hydrolysis studies have further shown that the abequose residues are α-linked and that the L-rhamnose residues are β-linked. The configuration of the other linkages is assumed to be the same as in the related lipopolysaccharides studied previously.

Several minor components were obtained in the methylation analysis. The structural significance of these is not clear, especially as some of them have only been tentatively identified. The identification of 2,4,6-tri-*O*-methyl-D-mannose is of special interest, as it most certainly is derived from the 3-*O*-abequosyl-D-mannose residues of the terminal repeating unit of the O-specific side-chains. From the percentage content of this methylated sugar, and of 4,6-di-*O*-methyl-D-mannose, the average number of repeating units in a side chain is estimated to be about 11.

The presence of small but comparable amounts of 2,3,4,6-tetra-*O*-methyl-D-glucose and 2,6-di-*O*-methyl-D-galactose (tentatively identified) may indicate that some of the repeating units carry a D-glucose residue linked to the D-galactose residue in the 4-position, as in lipopolysaccharides having O-factor 12_2. 2,3,4,6-Tetra-*O*-methyl-D-galactose, 2,4-di-*O*-methyl-D-glucose, and the unidentified 3,6-di-*O*-methyl-hexose may be derived from the core, which is known to contain D-glucose and D-galactose residues, part of the latter being terminal.

O-Factor 5 has been ascribed to the presence of *O*-acetyl groups in the side chains of serogroup-B *Salmonella* lipopolysaccharides. As 2-acetamido-2-deoxy-D-galactose was a rather good inhibitor of the 5 anti-5 system, it has been suggested that the *O*-acetyl group is located at the 2-position of the D-galactose residues. The

present results, however, demonstrate, at least for the lipopolysaccharide from *Salmonella typhimurium* 395 MS, that they are located at the 2-positions of the abequose residues, and that none of the other sugar residues in the side chains carry *O*-acetyl groups.

The structure assigned to the O-specific side-chains of the *Salmonella typhimurium* 395 MS lipopolysaccharide has several immunological implications, especially regarding the chemical nature of the O-factors. A discussion of these questions must, however, be postponed until it is known whether the O-specific side-chains of other serogroup-B lipopolysaccharides have the same or different structures.

EXPERIMENTAL

General methods. — Concentrations were carried out at diminished pressure at bath temperatures not exceeding 40°. G.l.c. was carried out on columns (200 × 0.3 cm), containing 3% (w/w) of ECNSS-M on Chromosorb G (80–100 mesh), at 195° (alditol acetates) or at 170° (partially methylated alditol acetates), and, for some separations, on columns containing 15% (w/w) of polyphenyl ether, OS 138, on the same support at 180°. A Perkin–Elmer 881 Gas Chromatograph with flame-ionisation detector was used. It was observed that the response of the detector, on a weight basis, was the same within ±5% for several alditol acetates of different degrees of methylation, and, consequently, differences in response factors have been disregarded. For mass spectrometry, the mixtures of alditol acetates, dissolved in chloroform, were injected into an ECNSS-M column, mounted in an LKB combined gas chromatograph–mass spectrometer. The mass spectra were recorded at an inlet temperature of 250°, ionisation potential of 70 e.v., ionisation current of 60 μamp, and a temperature of the ion source of 290°. Optical rotations were determined with a Perkin–Elmer 141 photoelectric polarimeter. N.m.r. spectra, on a 1% solution of lipopolysaccharide in D_2O, were recorded at 60 MHz on a Varian A-60A spectrometer, supplied with a Varian C-1024 time-averaging computer device.

Characterization of Salmonella typhimurium *395 MS.* — The micro-organism used in the present investigation was characterized by immunochemical methods and by its susceptibility to a set of bacteriophages. For the preparation of the agglutinating antigen, the bacteria were grown in nutrient broth (Difco) for 20 h at 37°. The culture was heated for 2 h at 100°. The undiluted, heat-killed, bacterial suspension was used in the tube agglutination tests. Absorbed, monospecific sera against the O-factors 1, 4, and 5 were used. From each serum, a twofold-dilution series of 0.5 ml was prepared, and 0.5 ml of the antigen was added to each tube. The reaction mixtures were incubated for 18 h at 37°. The last tube showing visible agglutination was recorded. The titers for O-factor 4 were 1:1280 and for O-factor 5 were 1:1280. No agglutinating capacity for O-factor 1 was detected at a serum dilution of 1:20. No monospecific serum against O-factor 12 was available. The presence of this factor in the bacterial strain was demonstrated by slide agglutination, using an anti 1,2,12-serum.

Flagellar antigens were characterised by using the tube agglutination test. Monospecific sera against the factors i, 1, and 2 were used. The titers recorded for all three factors were 1:20480.

According to the above findings, the micro-organism was characterized as *Salmonella typhimurium* according to Kauffmann[12].

Further characterization of the bacterial strain was obtained by phage typing, according to Lilleengen[13]. The strain was lysed by 9 of the 12 phages, and the lysis pattern obtained was recognized as phagetype 3. The micro-organism was also susceptible to phage P22, which only attaches[14] to *Salmonella* strains carrying O-factor 12.

Preparation of the lipopolysaccharide. — For the preparation of lipopolysaccharide, *Salmonella typhimurium* 395 MS bacteria were grown in 5-litre, indented, Erlenmeyer flasks; the culture volume in each flask was 1 litre. The medium had the following composition (g/l): Tryptone (Difco), 10; Yeast extract (Difco), 5; D-glucose, 10; NH_4Cl, 2.5; $Na_2HPO_4 \cdot 2H_2O$, 15; KH_2PO_4, 6; $Na_2SO_4 \cdot 10H_2O$, 0.5; $MgSO_4 \cdot 7H_2O$ 0.2. The flasks were placed on a rotary shaker and incubated for 18 h at 37°. The yield varied from 4 to 6 g of bacteria (dry weight) per litre of culture.

Cell walls were prepared by disintegrating centrifuged bacteria in a Braun disintegrator (B. Braun, Apparatenbau, Melsungen, Germany). Bacteria (7.5 g, wet weight) were suspended in distilled water (30 ml) and shaken together with glass beads (30 g, 0.17–0.18 mm). Tributyl phosphate (0.2 ml) was added as an antifoam. The bacteria were completely disintegrated after 3 min of shaking at maximum speed. The temperature was kept below +5° by a stream of carbon dioxide. After disintegration, the glass beads were removed by filtration through a coarse glass-filter. Initial separation of cell walls from cell debris was effected by centrifugation at 10,000 *g* for 20 min in an IEC-PR2 refrigerated centrifuge at +4°. The cell walls collected as a loosely packed layer on the pellet surface and were washed off by careful rinsing. The cell-wall preparation was then washed, in the cold, twice with 0.1M phosphate buffer (pH 7), once with M sodium chloride, and once with distilled water. The yield of cell-wall preparation was about 10% of the bacteria (dry weights). No intact bacteria could be detected when the cell-wall preparation was examined by phase microscopy. No u.v. absorption (260 nm) was observed, indicating that most of the cytoplasmic material had been removed.

Lipopolysaccharide was extracted from the cell walls by the hot phenol–water procedure of Westphal *et al.*[15]. The aqueous phase was extracted five times with ethyl ether to remove phenol and then dialysed againt distilled water. No sugars could be detected in the dialysis water. The yield of lipopolysaccharide (lyophilised) was about 200 mg per g of cell walls (dry weight).

The lipopolysaccharide preparation was checked by passive hemagglutination[16] and phage inactivation. Alkali-treated lipopolysaccharide, adsorbed to sheep erythrocytes, caused a visible hemagglutination of anti O:4 factor serum diluted 1:1280. No agglutination of anti 0:5 factor serum was observed.

Erythrocytes, to which the lipopolysaccharide had been adsorbed in neutral

solution at 100°, were agglutinated with anti 0:5 factor serum in a dilution of 1:320 and with anti 0:4 factor serum in a dilution of 1:160. Specific 0:12 factor serum was not available. The presence of this factor was confirmed by using the phage-inactivating technique of Lindberg[5]. The concentration of lipopolysaccharide which inactivated 50% of the P22 phage used was 0.03 μg/ml. Several other lipopolysaccharides from bacteria devoid of the 0:12 factor did not inactivate the P22 phage in concentrations of 100 μg/ml.

Sugar analysis of the lipopolysaccharide. — The lipopolysaccharide (5 mg) in 0.5M sulphuric acid (5 ml), was heated for 15 min at 100°, cooled, neutralised with barium carbonate, filtered, and concentrated to 1 ml. This solution was added to a column (40 × 1.5 cm) of Sephadex G-10, which was then irrigated with water. The separation on this column had been previously studied with synthetic mixtures. Oligo- and polysaccharide material was collected between 2–10 ml, and monomeric sugars between 10–30 ml. The former material was further hydrolysed with 0.25M sulphuric acid for 12 h at 100°, neutralised, combined with the monosaccharide fraction, and concentrated. The sugars in the hydrolysate were converted into alditol acetates and analysed by g.l.c.[6,7]. The identities of the sugars were confirmed by mass spectrometry[6,8].

Methylation analysis of the lipopolysaccharide. — The lipopolysaccharide (8 mg), in a 5-ml serum bottle sealed with a rubber cap, was dissolved in dry methyl sulphoxide (1 ml). Nitrogen was flushed through the bottle, and a solution of 2M methylsulphinyl carbanion[9] in methyl sulphoxide (0.8 ml) was added dropwise, using a syringe. The resulting, gelatinous solution was agitated in an ultrasonic bath (40 kc.p.s.) for 30 min, and kept for 8 h at room temperature. Methyl iodide (0.8 ml) was then added dropwise, under external cooling with ice–water, and the resulting turbid solution was agitated for 30 min in the ultrasonic bath, when a clear solution was obtained. This solution was poured into water (50 ml), dialysed, and concentrated to dryness.

A solution of the methylated lipopolysaccharide in 90% formic acid (3 ml) was kept for 2 h at 100° and concentrated to dryness, and the residue was dissolved in 0.13M sulphuric acid (3 ml) and kept for 12 h at 100°. The hydrolysate was neutralised (barium carbonate). The sugars were converted into alditol acetates and analysed by g.l.c.[3]–mass spectrometry[4]. The results are summarised in Fig. 1 and Table I.

Methylation analysis of degraded lipopolysaccharide. — The lipopolysaccharide (10 mg) was hydrolysed with 0.5M sulphuric acid (10 ml) for 15 min at 100°, and the hydrolysate was fractionated into a monosaccharide and an oligomeric–polymeric fraction, as described above. The latter fraction was concentrated to dryness, and dried over phosphorus pentoxide, and the product was methylated and hydrolysed. The analysis of the partially methylated sugars in the hydrolysate was performed as described above. The results are summarised in Table II.

Location of O-acetyl groups in the lipopolysaccharide. — A mixture of lipopolysaccharide (16 mg), methyl vinyl ether (1 ml), *p*-toluenesulphonic acid (9 mg), and methyl sulphoxide (2 ml) was kept[10], under gentle shaking, for 5 h at 15°. The resulting

yellow solution was added to a column (27 × 3 cm) of Sephadex LH-20 (lipophilic), which was then irrigated with dry acetone. The optical rotation of the eluate was determined, and the acetalated lipopolysaccharide (36 mg), which was eluted with the void volume, was obtained uncontaminated with the reagents. It showed carbonyl absorption (1738 cm^{-1}), but no absorption in the hydroxyl region.

This material in 0.25M methanolic sodium methoxide (10 ml) was kept for 24 h at 100°, concentrated to 1 ml and added to a column (15 × 3 cm) of Sephadex LH-20. The column was irrigated with methanol, and the polymeric material was eluted with the void volume (optical rotation), free from the salts. The material (25 mg) showed a faint absorption in the C=O region.

The acetalated, deacetylated lipopolysaccharide (12 mg) was methylated, and the methylated product was hydrolysed as described above. The resulting sugars were reduced to alditols with sodium borodeuteride, acetylated, and analysed by g.l.c.–mass spectrometry.

Acid hydrolysis of the lipopolysaccharide. — A solution of the lipopolysaccharide (1 mg) in water (0.5 ml) was diluted with the same volume of cold 0.1M sulphuric acid, transferred to a 1-cm polarimeter tube, and thermostated at 80°, and the optical rotation was determined at intervals. The results are given in Fig. 3.

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STUDIES WITH RADIOACTIVE SUGARS

PART III[1]. THE MECHANISM OF THE ANOMERISATION OF ETHYL α- AND β-D-XYLOPYRANOSIDE

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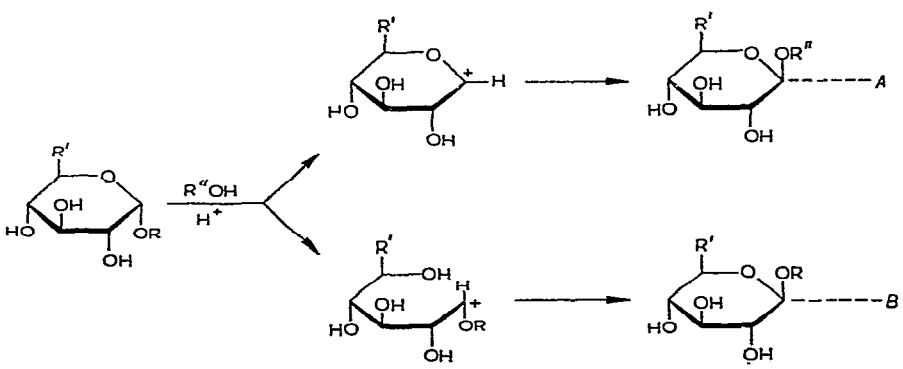
ABSTRACT

Parallel anomerisations of the ethyl D-xylopyranosides in radioactive ethanol and of the radioactive glycosides in unlabelled ethanol have allowed the conclusion to be drawn that the reactions proceed largely by way of a cyclic, ionic mechanism. In principle, these procedures permit a quantitative assessment of the relative significance of this mechanism and a process involving an acyclic ion. The displacement reactions at C-1 occurs predominantly with inversion of configuration, but, particularly with the β-D anomer, significant proportions of isomers having retained anomeric configuration are initially formed.

INTRODUCTION

Although the overall pattern of the alcoholysis of several sugars has been elucidated by the combined application of chromatographic and sensitive analytical procedures[2,3], certain mechanistic details of the contributing reactions have still to be resolved.

Recently, the first direct evidence has been obtained[4] to show that the final step in the methanolysis of D-glucose, *i.e.*, the anomerisation of the D-glucopyranosides, occurs by a cyclic ion mechanism (Scheme 1; Route *A*; R = R″ = Me, R′ = CH_2OH), rather than by a process involving an acyclic ion (Route *B*).



Scheme 1

The elucidation of the mechanism depends upon the recognition of the origin of the aglycon in the initial products of the reaction, and Capon and Thacker[4] performed their experiment by examining the anomerisations of the methyl D-glucopyranosides in fully deuterated methanol, and by using n.m.r. spectroscopy to determine whether the alkyl groups of the first products were deuterated. They showed that, within the limits of their method, the anomerisations occurred exclusively by route *A*, and calculated that solvent attack on the cyclic carbonium ion occurs, for both anomers, very largely from the side opposite to that from which the protonated methoxyl group departs.

RESULTS AND DISCUSSION

Our studies of the ethanolysis of D-xylose[3] have allowed us to examine the pyranoside anomerisations by different methods. Whereas Capon and Thacker[4] followed the overall methyl D-glucopyranoside anomerisations (from the intensities of the resonances of the anomeric protons) and solvent incorporation (from the intensities of the methyl resonances) simultaneously, our procedure involved two parallel experiments. One (E_1), carried out with ethyl D-xylopyranosides radioactively labelled within the sugar rings, afforded information on the overall anomerisations, whereas the other (E_2), with inactive glycosides in labelled ethanol, revealed the extent of solvent exchange. That is, experiment E_1 (Scheme 1; R = R″ = Et, R′ = H, sugar labelled) allows a measurement to be made of the rate of formation of the β-anomer by routes *A* and *B* ($k_A + k_B$); experiment E_2 (Scheme 1; R = Et, R′ = H, R″ = [^{14}C]Et) gives the rate of anomerisation by mechanism *A* (k_A).

For the reaction of ethyl α-D-xylopyranoside, $k_A + k_B$ was found to be 2.6×10^{-5} sec^{-1} under the selected conditions (six determinations; mean deviation 0.4×10^{-5} sec^{-1}; see Table I), and k_A was 2.5×10^{-5} sec^{-1} (six determinations; mean deviation 0.3×10^{-5} sec^{-1}). Similarly, for the reaction of the β-anomer, $k_A + k_B$ was 4.7×10^{-5} sec^{-1} (six determinations; mean deviation 0.5×10^{-5} sec^{-1}), and k_A was 4.6×10^{-5} sec^{-1} (six determinations; mean deviation 0.7×10^{-5} sec^{-1}). It is concluded, therefore, that, in both cases, the ratio k_A:k_B is large [estimated to be at least 10:1; an accurate determination of the ratio was not obtainable with the amounts of radioactivity employed], and consequently that both isomers anomerise predominantly by a cyclic ion mechanism, *i.e.*, by the mechanism operating during the normal acid hydrolysis of pyranosides[5]. As in the work with methyl D-glucopyranoside, a route involving an acyclic acetal, which could lead to solvent incorporation in the products, can be excluded, since furanosides (the first products of alcoholysis of sugar acetals) were not formed in the reactions[4].

Capon and Thacker[4] were unable to measure directly, by the n.m.r. method, the stereospecificity of the displacements, but calculated that a high degree of inversion occurred. In principle, the methods used in this work did allow such direct measurements, since the second experiment (E_2) reveals that both products arise from direct attack on the cyclic carbonium ions. From ethyl α-D-xylopyranoside, the β-anomer

is formed initially in appreciably greater proportions, and from ethyl β-D-xylopyranoside, the initial products are formed with more inversion than retention; in the latter case, the stereospecificity was not so marked. It is concluded, therefore, that shielding of the carbonium ion by the departing ethanol does occur to some extent, but that this is apparently not as extensive (particularly with ethyl β-D-xylopyranoside) as in the anomerisations of the methyl D-glucopyranosides. Likewise, in the methanolyses of phenyl D-glucopyranosides[6], the α-isomer reacts with more inversion than does the β-isomer, presumably because there is more steric interference to attack at C-1 from the "α-side"; with these compounds, inversion also occurred to a high degree (α, 90%; β, 72%).

The high reactivity of ethyl β-D-xylopyranoside, relative to its anomer, is consistent with the findings in the work[4] with methyl D-glucopyranoside, and with the ease of acid hydrolysis of methyl β-D-xylopyranoside and β-D-glucopyranoside, relative to their anomers, which has been explained in terms of dipolar repulsions between the equatorial alkoxyl groups and the unshared electrons on the ring oxygen atom[7]. It is noteworthy, however, that, whereas methyl β-D-galactopyranoside hydrolyses more readily than its anomer, the opposite holds for the ethyl D-galactopyranosides[8].

EXPERIMENTAL

The radiochemical analyses were carried out, as previously described, with an end-window Geiger counter, after resolution of the reaction components on paper chromatograms (Whatman No. 1) developed for 15 h with butanone saturated with water[3]. No corrections were applied for the different distances travelled on the chromatograms by the two glycosides (R_F values: ethyl α-D-xylopyranoside, 0.28; ethyl β-D-xylopyranoside, 0.25), nor for selective adsorption on the resin, since these would not affect the main conclusions drawn from the experiments. Account was taken, however, of activity remaining on an area of paper over which radioactive compounds had passed[3].

Materials. — Radioactive ethyl D-xylopyranosides were prepared by treating D-xylose {1.0 g, containing [^{14}C]-D-xylose (59 μcuries uniformly labelled)} in refluxing ethanol [5 ml, containing hydrogen chloride 1.0%] for 6 h. The acid was neutralised with lead carbonate, and the solids and solvent were removed to leave a syrup (1.18 g) which was shown by paper chromatography and radioautography to contain, almost exclusively, the two pyranosides. Resolution of the syrup, on a column of cellulose developed with butanone saturated with water, gave a fraction (380 mg) which, on recrystallisation from ethyl acetate, afforded the α-D-pyranoside (105 mg., m.p. 109–110°; lit.[3], 111–112°) shown, after chromatographic examination, to contain 1.0% of the β-anomer. A further fraction (80 mg), on recrystallisation from ethyl acetate–light petroleum (b.p. 40–60°), gave the β-anomer (30 mg., m.p. 92–93°; lit.[3], 92–93°) which contained 1.5% of the α-anomer. Corrections for these impurities were applied in the kinetic analyses.

TABLE I

ANOMERISATIONS OF ETHYL [^{14}C]-D-XYLOPYRANOSIDES[a] IN ETHANOL (E_1)

Time (min)	*10*	*15*	*20*	*30*	*45*	*60*	*1,440*
Ethyl α-D-xylopyranoside							
α-Pyranoside	1,370	950	1,225	695	1,320	890	1,250
β-Pyranoside	25	12	35	35	100	75	695
β-Pyranoside, %	1.8	1.3	2.9	4.8	7.6	7.8	36
$k_A + k_B$ ($sec^{-1} \times 10^5$)[b]	3.0	1.5	2.5	2.9	3.2	2.5	
Ethyl β-D-xylopyranoside							
α-Pyranoside	55	75	145	185	240	520	1,130
β-Pyranoside	2,080	2,100	2,220	1,855	2,090	2,620	600
α-Pyranoside, %	2.6	3.5	6.2	9.1	10.3	16.6	65
$k_A + k_B$ ($sec^{-1} \times 10^5$)[b]	4.4	3.9	5.3	5.3	4.2	5.3	

[a]Activities expressed in counts/min. [b]Calculated from the first-order rate equation for equilibration reactions: $k = 2.303 \frac{X_e}{at} \log_{10} \frac{X_e}{X_e - X}$.

TABLE II

ANOMERISATIONS OF ETHYL D-XYLOPYRANOSIDES[a] IN ETHANOL-*1*-^{14}*C* (E_2)

Time (min)	*10*	*15*	*20*	*30*	*45*	*60*	*1,440*
Ethyl α-D-xylopyranoside							
α-Pyranoside	0	10	12	27	70	95	1,180
β-Pyranoside	30	38	60	80	110	205	670
α-Pyranoside (labelled)[b], %	0	0.5	0.6	1.4	3.6	4.8	64
β-Pyranoside (labelled)[b], %							
(*a*) uncorrected	1.5	1.9	3.1	4.1	5.6	10.5	
(*b*) corrected	1.5	1.9	3.1	4.1	5.5	10.1	
k_A ($sec^{-1} \times 10^5$)[e]	2.5	2.2	2.6	2.4	2.2	3.3	
Ethyl β-D-xylopyranoside							
α-Pyranoside	65	70	100	115	285	320	1,190
β-Pyranoside	45	55	90	100	225	255	685
α-Pyranoside (labelled)[c], %							
(*a*) uncorrected	3.4	3.6	5.2	6.0	14.8	16.7	36
(*b*) corrected[d]	3.4	3.5	4.9	5.5	13.7	14.6	
β-Pyranoside (labelled)[c], %	2.4	2.9	4.8	5.2	11.6	13.3	
k_A ($sec^{-1} \times 10^5$)[e]	5.8	4.0	4.3	3.2	5.7	4.6	

[a]Activities expressed in counts/min. [b]Calculated from an average, equilibrium, total count of 1,950 counts/min (see above). [c]Calculated from an average, equilibrium, total count of 1,920 counts/min (see above). [d]The applied corrections compensate for the glycosides formed by anomerisation of the primary products with retained configuration (information from E_1). [e]See footnote, Table I.

The inactive D-xylosides were prepared similarly, as previously described[3]. The specific activities of the D-xylose and ethanol were determined by the courtesy of Dr. I. M. Kerr, National Institute for Medical Research, by scintillation counting.

Anomerisation reactions: radioactive glycosides in inactive ethanol (E_1). — The labelled glycosides (20.0 mg, 1.0 μcurie) were heated under reflux with ethanol (1.0 ml, containing hydrogen chloride, 0.20%) for 24 h. Samples (0.05 ml) were withdrawn, neutralised with Deacidite FF resin (OH^-; 3 mg), and applied to the paper chromatograms without special care being taken to ensure quantitative transfer.

Inactive glycosides in radioactive ethanol (E_2). — The pyranosides (20.0 mg) were heated under reflux in ethanol-*1*-^{14}C [1.0 ml, 153 μcuries (calculated to give glycosides having the same final specific activity as those employed in E_1), containing hydrogen chloride (0.20%)] for 24 h. Samples were withdrawn, and treated as in E_1, but great care was taken to ensure quantitative transfer to the chromatograms.

In the first experiment (E_1), the percentages of anomerised products were readily determined by direct comparison of the activities of the two components of each mixture. In the second (E_2), however, this could not be done, and, instead, the measured activities were compared with the total activities (averages of four determinations) measured in the 24-h (equilibrated) samples, in which it could be assumed that complete incorporation of labelled ethanol had occurred. (This assumption implies that anomerisation has Route *A*, Scheme 1, available, and is clearly valid.)

The results are given in the Tables.

ACKNOWLEDGMENT

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MASS-SPECTROMETRIC INVESTIGATIONS OF PERACETATES OF UNSATURATED CARBOHYDRATES, ANHYDRODEOXYALDITOLS, AND A BRANCHED-CHAIN SUGAR

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ABSTRACT

The mass spectra of a number of peracetates of unsaturated (carbon–carbon double bond) monosaccharides, anhydrodeoxyalditols, and a branched-chain monosaccharide are presented in tabular form. A detailed interpretation of the fragmentation processes undergone by these molecules is presented. The unsaturated carbohydrates are shown to be intermediates in the breakdown of hexose peracetates. The evidence strongly indicates that the stereochemistry of some anhydrodeoxyalditol peracetates can be determined by mass spectrometry. Mass spectrometry is also a useful technique for indicating the position of the branched-chain on a carbohydrate, but not its stereochemistry. The proposed fragmentation processes are supported by the spectra of some isotopically labelled compounds.

INTRODUCTION

During the past few years, the usefulness of mass spectrometry as a technique for the determination of structure of the acetates of pentoses and hexoses has been investigated[1–3]. Fragmentation patterns were suggested that were supported by the spectra of isotopically labelled compounds, mainly esters of acetic-d_3 acid. In addition, the spectra of 1,6-anhydrohexopyranose peracetates (some labelled) have been reported[4]. Although evidence was presented for the possibility of distinguishing furanoses from pyranoses, and of deoxy aldoses from normal aldoses, epimers could not be differentiated. Implicit in the interpretation of the spectra of the peracetates was the assumption that unsaturated carbohydrates (formed by initial expulsion of acetic acid from the parent molecule) constitute important intermediates in the fragmentation pattern. In recent reviews[5,6] dealing with the mass spectra of carbohydrates, the authors have emphasized the importance of the unsaturated carbohydrates in the breakdown of the peracetates. Surprisingly, no investigation of the mass spectra of unsaturated carbohydrates, *per se*, has so far been reported.

In this communication are reported the results of an investigation dealing with the mass spectra of peracetates of unsaturated (containing carbon–carbon double bonds) carbohydrates, anhydrodeoxyalditols (some labelled), and one branched-chain (also labelled) aldose.

The main mass-peaks, and their relative abundance, of the various peracetates are given in Table I.

TABLE I

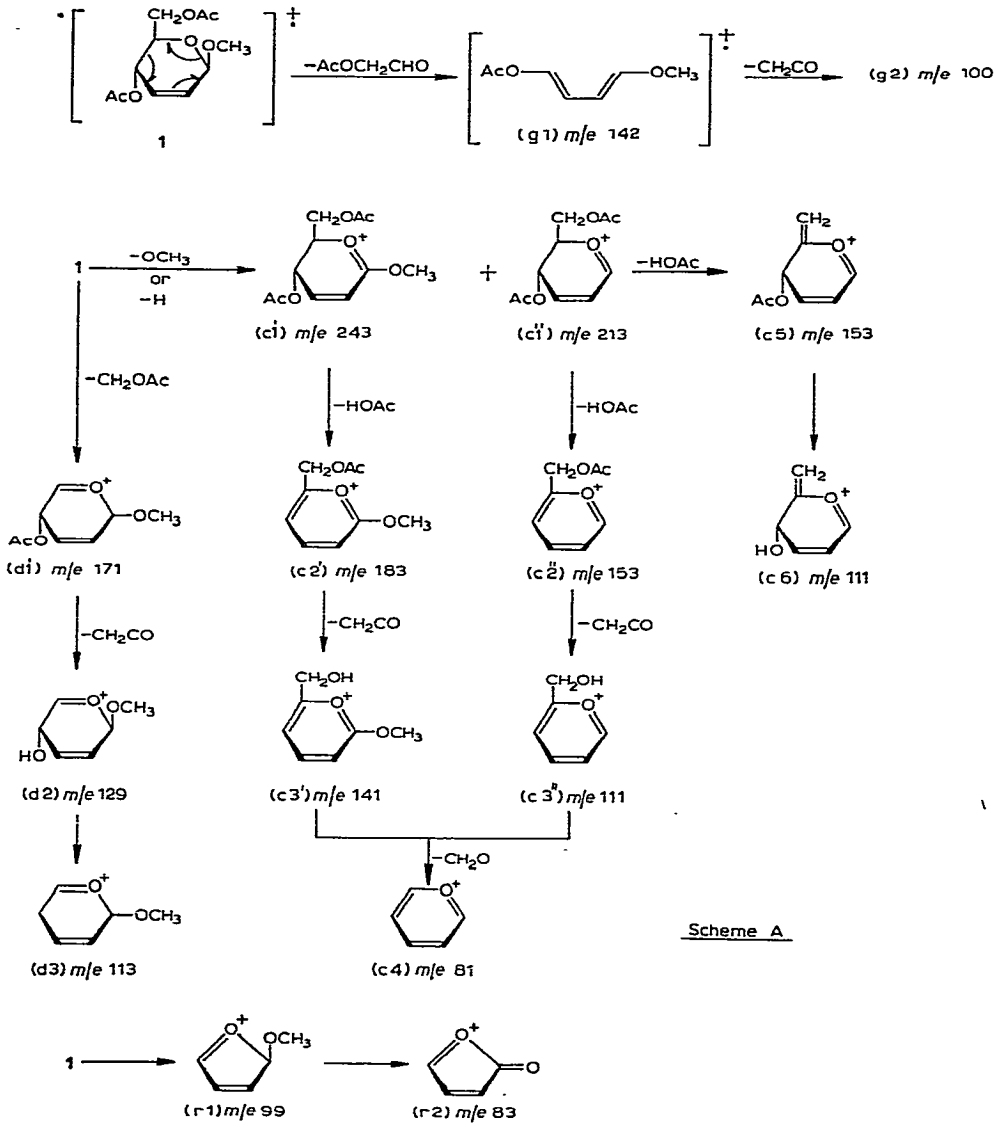
MASS SPECTRA OF SOME UNSATURATED AND DEOXY CARBOHYDRATE PERACETATES: *m/e* VALUES AND RELATIVE INTENSITIES

Compounds									
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
244(0)	330(0)	331(trace)	272	346(trace)	349(trace)	346(0)	404(trace)	404(0)	407(0)
243(1)	288(5)	330(2)	229(3)	286(1)	289(1)	286(1)	344(2)	361(1)	364(2)
213(5)	271(5)	288(3)	212(0)	273(1)	289(0.2)	273(1)	302(2)	345(5)	348(3)
183(2)	228(35)	287(12)	199(2)	244(1)	276(1)	244(0)	284(5)	344(02)	347(1)
171(10)	211(4)	245(10)	170(10)	226(5)	274(5)	226(2)	243(5)	331(1)	334(1)
153(6)	210(10)	228(1)	152(36)	213(10)	247(1)	213(20)	242(30)*	302(1)	305(1)
142(10)	186(12)	227(4)	145(7)	201(8)	229(3)	201(2)	224(110)*	285(1)	288(3)
141(28)	185(3)	197(1)	144(1)	185(10)	228(1)	185(5)	213(5)	273(2)	274(1)
129(22)	169(30)	186(3)	139(90)	184(45)	214(60)	184(25)	211(100)*	271(1)	262(2)
113(4)	168(15)	185(16)	128(22)	171(6)	213(50)	171(8)	206(1)*	259(2)	261(1)
112(5)	155(6)	180(2)*	127(5)	166(10)	204(1)	166(12)	201(3)	242(2)	245(3)
111(34)	145(9)	168(15)	110(100)*	155(2)	187(50)	153(35)	200(13)	225(4)	228(3)
110(7)*	144(25)	167(2)	102(1)	153(15)	186(10)	145(5)	182(100)	211(4)	213(4)
109(5)*	143(5)	157(15)	97(100)*	145(6)	185(6)	142(9)	178(1)*	200(3)	206(2)*
100(100)	139(15)	152(4)*	86(27)	144(3)	174(70)	141(3)	158(8)	199(5)	203(3)
99(4)*	127(12)	145(7)	85(6)	142(13)	173(15)	124(50)	148(3)*	183(9)	202(3)
97(14)*	126(25)	144(25)	81(40)	124(30)	166(2)*	122(1)*	145(60)	182(15)	186(3)
84(2)*	113(10)	143(5)	80(8)*	122(1)*	154(68)	112(20)	141(25)	169(12)	171(6)
83(7)	109(45)	141(2)*	69(8)	112(6)	153(65)	111(100)*	140(40)	158(3)	170(4)
82(6)	102(15)	139(16)	68(10)*	111(40)*	145(55)	110(6)*	139(80)	145(9)	161(3)
81(15)*	100(5)	127(10)	73(33)	110(3)*	144(40)	100(40)	136(2)*	141(3)	160(3)
71(14)	99(20)	126(75)	69(9)	103(13)	143(55)	99(15)	135(1)*	140(15)	154(6)
70(2)*	98(100)	125(6)	60(32)	102(8)	141(18)	95(13)	124(3)	139(25)	145(8)
69(15)	97(80)*	113(25)	54(6)	100(35)	140(15)	93(6)*	112(20)	127(14)*	144(5)
62(2)*	96(13)*	111(3)*		99(12)	127(42)*	83(60)*	111(20)	118(2)*	143(10)
54(3)	81(19)	102(10)*		95(5)	126(29)	81(30)*	102(25)	116(9)	141(6)
40(3)	69(32)	101(3)		93(26)*	125(15)	80(16)*	100(5)	111(26)*	129(8)
39(10)*	60(35)	99(6)		85(2)*	114(94)	72(10)*	97(100)*	110(12)*	118(3)
	54(5)	97(70)*		84(5)*	113(60)	71(40)*	86(15)*	102(8)	114(10)
		94(3)*		83(24)*	112(140)*	70(25)*	84(5)	98(28)	110(8)*
		93(3)*		81(14)*	111(130)*	69(100)	81(45)	97(20)	102(7)
		88(8)*		80(1)*	110(100)*	62(3)*	73(25)*	93(2)*	101(8)
		82(3)*		70(6)*	103(80)	58(10)*	69(61)	86(15)	99(10)*
		81(12)		69(25)	102(48)	54(23)		85(15)*	96(11)*
		75(2)*		67(3)*	101(60)	51(5)*		84(32)	93(3)*
		72(2)*		62(1)*	98(70)			83(60)*	86(10)*
		69(28)		54(8)	97(300)*			82(45)*	84(15)*
		64(2)*		51(1)*	96(50)			81(60)*	83(20)*
		60(16)*			95(64)			80(2)*	82(18)
		54(3)			86(75)*			76(1)*	72(15)
					84(100)			73(27)*	54(5)*
					83(100)			70(35)*	
					82(53)*			58(19)*	
					81(55)*			54(20)*	
					80(30)*			53(20)*	
					73(80)*				
					70(40)*				
					69(54)				
					64(5)*				

(See note, page 63)

DISCUSSION OF RESULTS

*Methyl 4,6-di-O-acetyl-2,3-dideoxy-β-*D-erythro-*hex-2-enoside* (1). — In Scheme A are depicted some of the main fragmentation sequences of compound 1.



1, Methyl 4,6-di-*O*-acetyl-2,3-dideoxy-β-D-*erythro*-hex-2-enoside[7]; 2, tetra-*O*-acetyl-3-deoxy-α-D-*erythro*-hex-2-enopyranose[8]; 3, tetra-*O*-acetyl-1-deoxy-D-*arabino*-hex-1-enopyranose[9]; 4, 3,4,6-tri-*O*-acetyl-D-glucal[10]; 5, 1,4,5,7-tetra-*O*-acetyl-2,6-anhydro-3-deoxy-D-*gluco*-heptitol[11]; 6, 1,4,5,7-tetra-*O*-acetyl-2,6-anhydro-3-deoxy-D-*gluco*-heptitol-*1,1,3-d*$_3$, ref. 11; 7, 1,4,5,7-tetra-*O*-acetyl-2,6-anhydro-3-deoxy-D-*manno*-heptitol[11]; 8, 1,3,4,5,7-penta-*O*-acetyl-2,6-anhydro-D-*glycero*-D-*gulo*-heptitol[12]; 9, 1,2,3′, 4,6 penta-*O*-acetyl-3-deoxy-3-*C*-(hydroxymethyl)-α-D-glucopyranose[13]; 10, 1,2,-3′,4,6-penta-*O*-acetyl-3-deoxy-3-*C*-(hydroxymethyl)-α-D-glucopyranose-*2,3,3′-d*$_3$, ref. 13.

*Metastable peak. The numbers indicate the *m/e* values and relative intensities of the adjoining peaks.

A retro-Diels–Alder reaction of **1**, involving rupture of the C-1–O bond and transfer of an electron to C-5, accompanied by cleavage of the C5–C4 bond and migration of the 2,3-double bond, would lead to the (g1) species. Expulsion of ketene from the latter might be expected to afford the highly abundant (g2) ion having *m/e* 100. The (g) sequence of transformations is supported by the presence of metastable peaks at *m/e* 84 and 70.

Surprisingly, compound **1** gave rise to two series of (c) ions by expulsion of methoxyl or hydrogen from C-1 (the methoxyl was preferentially expelled). Loss of acetic acid, followed by ketene, yielded the relatively abundant (c3) ions. Further loss of formaldehyde from the latter gave the pyronium (c4) ion. Alternatively, the (c1) species lost acetic acid to afford the (c5) ion. The (c) series of ions is clearly supported by metastable peaks at *m/e* 110, 109, and 81.

The presence of the 2,3-double bond led to an enhancement of expulsion of the acetoxymethyl group from C-5, as evidenced by the fact that the (d) series of ions occurred in a higher relative abundance than in D-glucopyranose pentaacetate. A metastable peak found at *m/e* 97 supports the (d1) to (d2) breakdown. Expulsion of oxygen from the (d2) ion (which has no precedent in the literature) to yield the (d3) ion is indicated by the presence of a metastable peak having *m/e* 99. Alternatively, there could be visualized the loss of CH_2O from the (d2) ion to afford an ion having *m/e* 99. Such a transformation is, however, not supported by a metastable peak having the expected value of *m/e* 76.

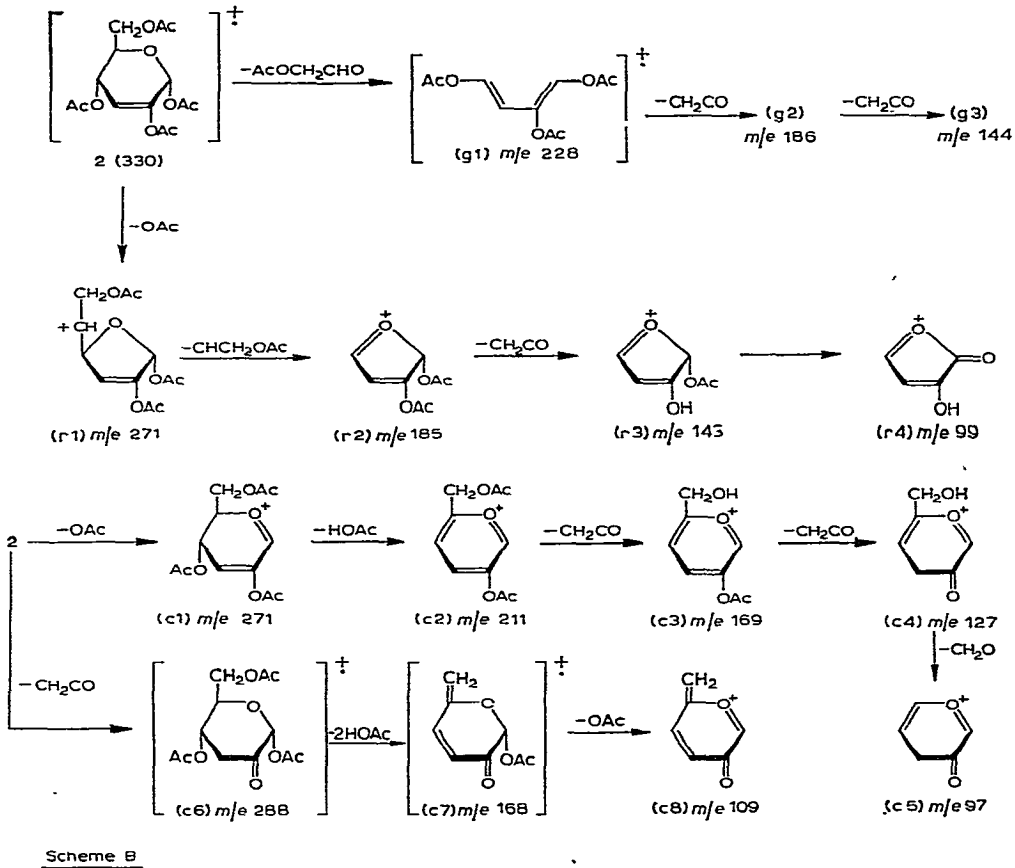
The presence of a peak at *m/e* 99, albeit in relatively low concentration, might indicate that compound **1** undergoes a rearrangement to yield a furan ring. This rearrangement is indicated by the (r) series of ions, and is supported by metastable peaks at *m/e* 39 and 70. An analogous rearrangement has been postulated[1,6] to occur in the fragmentation of the hexose and pentose peracetates.

Although no sequence is shown to account for the presence of the relatively abundant ion at *m/e* 97, the latter might arise by elimination of CH_3OAc and CH_3CO from the (c1″) ion to afford a hydroxypyronium ion having *m/e* 97.

The aforementioned sequences appear to support strongly the contention that 2,3-unsaturated carbohydrates are important intermediates[5,6] in the breakdown of the monosaccharide polyacetates.

Tetra-O-*acetyl-3-deoxy*-α-D-erythro-*hex-2-enopyranose* (**2**). — As expected, compound **2** appears to suffer breakdown (depicted in Scheme B) similar to those outlined for compound **1**. In addition, preliminary expulsion of ketene from **2** affords a keto intermediate (c6) having *m/e* 288, followed by elimination of two molecules of acetic acid and acetate to afford the (c8) ion. Alternatively, prior elimination of acetate, followed by acetic acid, ketene, and then formaldehyde gives the highly abundant pyronium (c5) ion. Surprisingly, there were only two, narrow, metastable peaks in the mass spectrum of compound **2**, found at *m/e* 97 and 96. The latter might offer support for the (c3) to (c4) transformation. Although it is easy to visualize the transformation of the relatively abundant ion having *m/e* 210 (shown in Table I) into ions having *m/e* 168 and 126, by successive loss of two CH_3CO groups, the author

is unable to offer a sequence of steps to account for the presence of the ion having *m/e* 210.



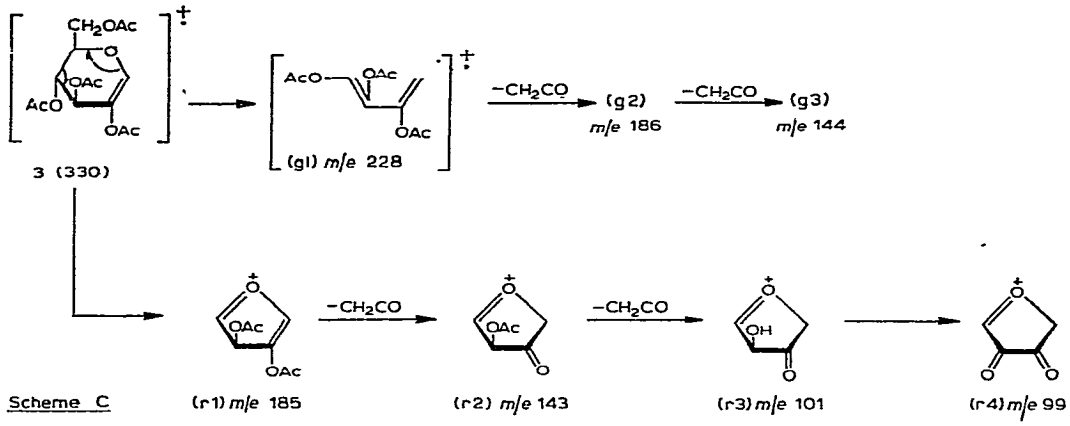
Scheme B

Although compound **1** suffered expulsion of both the allylic anomeric methoxyl group and the hydrogen atom, compound **2** did not expel the anomeric hydrogen atom but only the anomeric acetoxyl group. This might indicate that the C-2 acetoxyl group participated in the expulsion of the anomeric acetoxyl group.

Two routes can be used to account for the ions having *m/e* 185 and 143. The first, shown in Scheme B, envisages a ring contraction of compound **2** to yield the (r) series of ions. Alternatively, these ions could also be formed by loss of CH_3CO from *m/e* 228 followed by subsequent expulsion of ketene. The latter transformation has been observed in other peracetates. Surprisingly, there was no ion at *m/e* 101 [expected on the basis of further loss of ketene from the (r3) ion]. The presence of a relatively abundant ion at *m/e* 99 might indicate the presence of the (4r) ion or a 1,2-diketofuronium ion, but there is no definitive evidence for such a transformation.

*Tetra-*O*-acetyl-1-deoxy-*D*-arabino-hex-1-enopyranose* (**3**). — Although 2,3- and 3,4-unsaturated carbohydrates have been postulated as being intermediates

in the fragmentation of the peracetates[5,6], to the best of the author's knowledge no consideration has been given to the 1,2-unsaturated carbohydrates. The mass spectrum of tetra-*O*-acetyl-1-deoxy-D-*arabino*-hex-1-enopyranose (**3**) (fragmentation sequences are shown in Scheme C) supports the contention that 1,2-unsaturated sugars are also intermediates (albeit to a limited extent) in the fragmentation of glycosyl peracetates. It is interesting that compound **3** gave a molecular ion (*m/e* 330), and, in addition, captured a proton to afford an M + 1 ion (*m/e* 331).

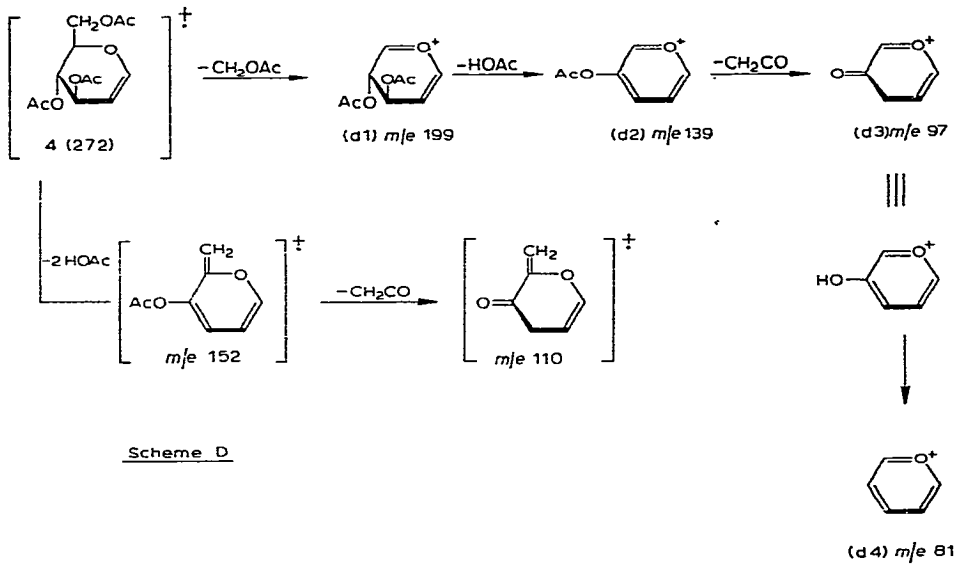


Scheme C

The presence of *m/e* 330 in the mass spectrum of β-D-glucopyranose pentaacetate might, therefore, be attributed to the prior elimination of acetic acid, giving compound **3**. Several of the fragmentation patterns of **3** appear to be similar to those of compound **2**. The (g) series of ions are supported by metastable peaks at *m/e* 152 and 111. Support for the (r) sequence of ions is provided by the fact that metastable peaks occur at 102, 111, 72, and 97. Loss of hydrogen from (r3) affords (r4) [supported by the metastable peak at *m/e* 97]. The presence of metastable peaks at *m/e* 180, 152, and 85 supports the postulation that **3** undergoes loss of acetyl followed by expulsion of acetic acid (330→288→228→186→126).

*3,4,6-Tri-*O*-acetyl-*D*-glucal* (**4**). — The loss of the α-substituent, the CH_2OAc group, with production of the (d) ions, becomes more important in the breakdown of 3,4,6-tri-*O*-acetyl-D-glucal (**4**) (depicted in Scheme D) than in that of the other unsaturated carbohydrates (supported by metastable peaks at *m/e* 97 and 68). The presence of the pyronium ion (*m/e* 81) can be readily accounted for by this fragmentation. In addition, the retro-Diels–Alder fragmentation also takes place to a limited extent, with formation of the (g) ions. The main fragmentation, however, appears to consist of further elimination of acetic and ketene from **4**. The loss of one molecule of acetic acid from **4** yields an ion (*m/e* 212), in very low concentration, which subsequently loses a further molecule of acetic acid to give *m/e* 152, or, alternatively, one molecule of ketene to give *m/e* 170. Apparently, loss of a second molecule of acetic acid from C-6 of compound **4** (supported by a metastable peak at *m/e* 110) occurs more readily than loss of a molecule of ketene from the unsaturated fragment (the transformation

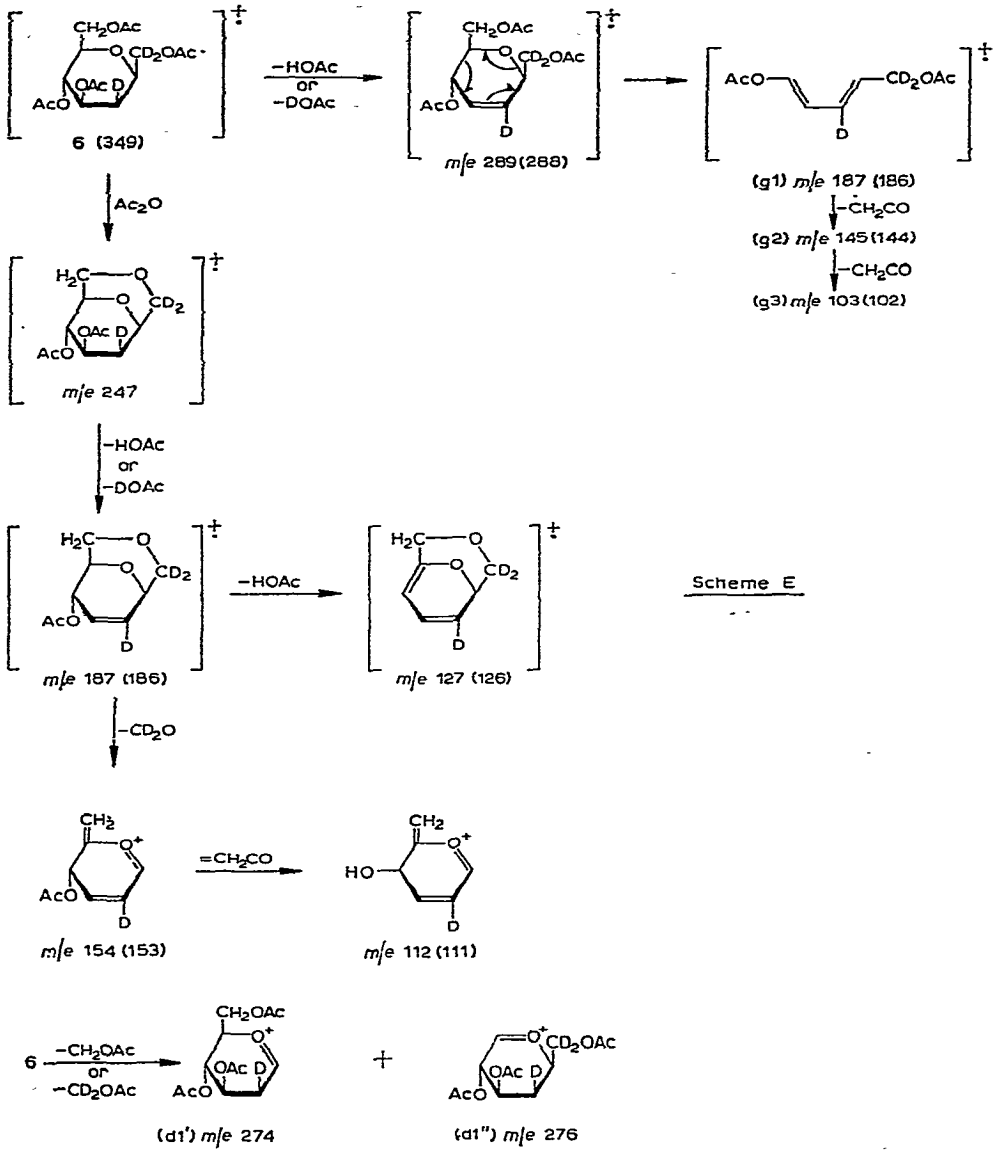
of the ion having *m/e* 152 to *m/e* 110 is supported by a metastable peak at 80) as evidenced by the higher concentration of the former than the latter ion. The presence of the relatively abundant *m/e* 115 is difficult to rationalize. It is possible that this ion might be due to the presence of the ion HOCH = CH—$^{+}$CHOAc, which might arise as a consequence of rupture of C3–C4 and scission of C-5 and the ring-oxygen atom, followed by capture of a hydrogen atom by an oxygen atom. The metastable peak at *m/e* 68 supports the breakdown of the hydroxypyronium ion (by loss of oxygen) to yield the (d4) pyronium ion.



Scheme D

*1,4,5,7-Tetra-*O*-acetyl-2,6-anhydro-3-deoxy-*D*-gluco-heptitol-*1,1,3-d_3 (6). — The mass spectra of 1,4,5,7-tetra-*O*-acetyl-2,6-anhydro-3-deoxy-D-*gluco*-heptitol-*1,1,3-d_3* (6) and of the normal analog showed trace, parent peaks at 349 and 346. The most important fragmentation of **6** (shown in Scheme E) is that involving an elimination of acetic anhydride from the acetoxymethyl groups to give an ion having *m/e* 247. When the normal analog of **6** (namely, **5**) was used, the peak was found at 224. On the other hand, the mass spectrum of 1,4,5,7-tetra-*O*-acetyl-2,6-anhydro-3-deoxy-D-*manno*-heptitol (**7**) (the C-2 epimer of **5**) did not show the presence of any peak at *m/e* 244. Because the presence of two acetoxymethyl groups in the equatorial orientation in **5** or **6** led to an expulsion of acetic anhydride, whereas in the *manno* epimer (having the C-1 acetoxymethyl group in the axial orientation) there was no similar elimination of acetic anhydride, it might be inferred that mass spectrometry can be used in certain cases in deducing the configuration of an unknown asymmetric center of a carbohydrate. To the best of the author's knowledge, this is the first recorded instance of such a claim. After acetic anhydride is eliminated from the parent molecules **5** or **6**, the succeeding fragments that occur (by loss of acetic acid, followed by formaldehyde) in high concentration follow a pathway similar to that envisaged

by Heyns and Scharmann[4] in their mass-spectral study of 2,3,4-tri-*O*-acetyl-1,6-anhydro-D-idose. The presence of metastable peaks at 127, 86, and 81 in the mass spectrum of **6** supports the pathway shown on the left side of Scheme E (247→187→154).

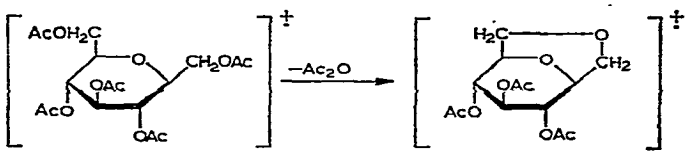


Scheme E

Another fragmentation sequence that occurs in the decomposition of **5** or **6** is the formation of the (g) ions (supported by metastable peaks at *m/e* 112 and 73). This follows the initial loss of acetic acid to form a 2,3-unsaturated fragment having *m/e* 289 (or 288), which subsequently undergoes a retro-Diels–Alder rearrangement to give a fragment *m/e* 187 (or 186). This fragment encompasses C-1 through C-4

after elimination of the ether oxygen atom and C-6 and C-7. Biemann, De Jongh, and Schnoes[1] first proposed such a breakdown in their mass-spectral studies of tetra-*O*-acetyl-2-deoxy-β-D-*arabino*-hexopyranose. It is noteworthy that the presence of the 2-deoxy group results in much greater elimination of C-1 than of the C-6 acetoxymethyl group, to give the (d') and (d") ions (*m/e* 274 and 276, respectively) in the ratio of 6:1. Further breakdown of the (d) ions by elimination of acetic acid and ketene follow sequences already established by other workers[1,3]. The formation of the (d) series of ions is supported by the presence of metastable peaks at *m/e* 166, 111, and 81.

1,3,4,5,7-Penta-O-*acetyl-2,6-anhydro*-D-glycero-D-gulo-*heptitol* (**8**). — The mass breakdown of 1,3,4,5,7-penta-*O*-acetyl-2,6-anhydro-D-*glycero*-D-*gulo*-heptitol (**8**) appears to parallel those of compound **6** and 2,3,4-tri-*O*-acetyl-1,6-anhydro-D-idopyranose[4]. The elimination of 102 mass units from **8** to afford a peak at *m/e* 302 might be due to the elimination of acetic anhydride or of acetic acid, followed by ketene, as envisaged by other workers[1,3]. Although the mass-spectral evidence does not permit an unambiguous assignment of the pathway of breakdown, the fact that there is a close parallel with the peaks encountered in the breakdown of **6** suggests that a prior elimination of acetic anhydride from C-1 and C-7 does, indeed, occur as indicated; if this assumption is made, it may be deduced that the configuration of C-2 can be ascertained from the mass spectrum of **8**.

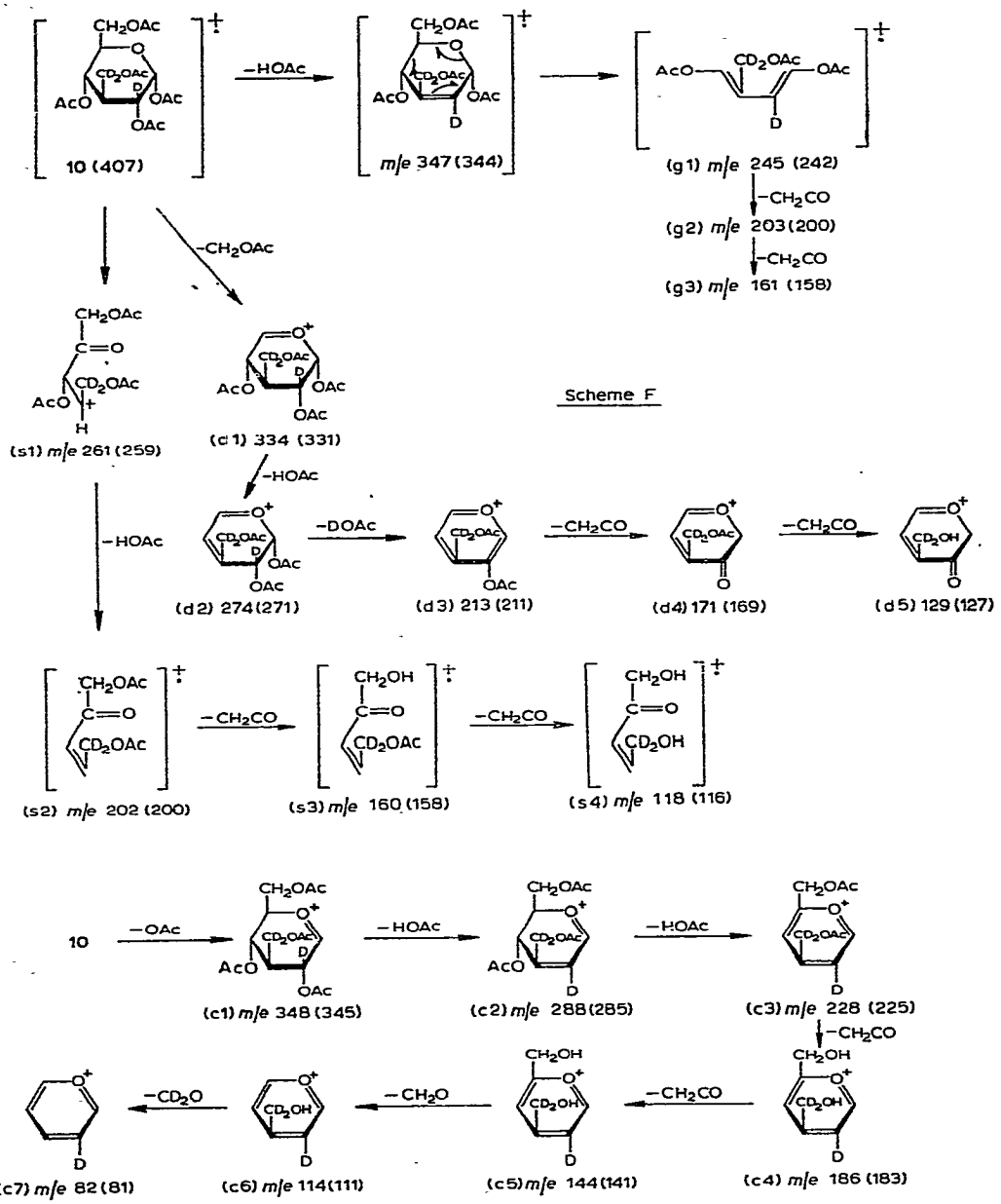


8(*m/e* 404) *m/e* 302

All of the sequences envisaged in the breakdown of 2,3,4-tri-*O*-acetyl-1,6-anhydro-D-idopyranose[4] can be sketched for the fragmentation of **8**.

1,2,3',4,6-Penta-O-*acetyl*-3-*deoxy*-3-C-(*hydroxymethyl*)-α-D-*glucopyranose*-2,3,3'-d_3. — A close examination of the mass spectra of the branched-chain sugars 1,2,3',4,6-penta-*O*-acetyl-3-deoxy-3-*C*-(hydroxymethyl)-α-D-glucopyranose-*2,3,3'-d*$_3$ (**10**) and its nondeuterated analog (**9**) reveals the efficacy of this tool in helping to deduce (or confirm) the structure of a branched-chain sugar. The most important diagnostic fragmentation is that which arises from the cleavage of the C-2–C-3 bond and the ether oxygen–C-1 bond, giving an ion radical fragment (s1) having *m/e* 261 (and 259 for the nondeuterated analog). Such a fragmentation (shown in Scheme F) is not encountered in the breakdown of α-D-glucopyranose pentaacetate. Presumably, this behavior is attributable to the fact that a secondary carbonium ion on C-3 is formed in the breakdown of **10** or **9**. Further breakdown of the fragment *m/e* 261 by elimination of acetic acid and ketene affords clearly visible peaks having the expected values [the (s3) to (s4) transformation is supported by a metastable peak at *m/e* 86].

As expected, the (c) and (d) series of ions are supported by a metastable peak at *m/e* 110 and at 96, respectively.



A fragmentation that might be invoked to deduce the configuration of C-3 of compound **10** is that involving the expulsion of acetic anhydride from **10** to yield a 3′,6-anhydro ring (*m/e* 305), followed by further elimination of 2 molecules of acetic acid and acetate ion to give *m/e* 186. As the mass spectrum of α-D-glucopyranose

pentaacetate also exhibits peaks at 169 and 229 (but not at 288, due to elimination of 102 mass units), it might be argued that this sequence does not establish the configuration at C-3 of compound **10**.

EXPERIMENTAL

Mass Spectra. — The spectra were determined with an A.E.I.-M.S.9 mass spectrometer, equipped with a direct inlet-probe; ionizing potential 70 eV; temperature of the ion source 150°. The sample (≃ 0.5 mg) was sublimed directly from a ceramic rod into the ion chamber.

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STUDIES ON CARBOHYDRATE-METABOLISING ENZYMES

PART XIX*. SWEET-CORN BRANCHING ENZYMES

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ABSTRACT

Extracts of sweet corn contain a branching enzyme which, in contrast to previously known plant branching-enzymes (Q-enzymes), can introduce additional inter-chain linkages into amylopectin, thereby producing a glycogen-type polysaccharide. This new enzyme (amylopectin-branching glycosyltransferase) occurs together with Q-enzyme; the sweet-corn branching enzymes can be separated by gel-filtration.

INTRODUCTION

Although the molecular structures of starch and glycogen are similar, there are significant differences in the enzymes that catalyse their synthesis and degradation[2]. For example, in plants, ADPG [adenosine 5-(α-D-glucopyranosyl pyrophosphate)] is the major glucosyl donor for starch synthesis, whereas uridine 5-(α-D-glucopyranosyl pyrophosphate) is the intermediate in the synthesis of mammalian glycogens[3]. The conversion of linear chains of α-(1→4)-linked D-glucose residues into branched chains with α-(1→6)-D-glucosidic inter-chain linkages is catalysed by branching enzymes. In plant tissues, the branching enzymes are termed Q-enzymes, and can convert amylose into amylopectin, but are not able to introduce additional inter-chain linkages into amylopectin[2]. By contrast, the branching enzymes from glycogen-synthesising tissues, *e.g.*, yeast[4] and muscle[5], can convert amylopectin [containing 4–5% of α-(1→6)-D-glucosidic linkages] into glycogen-type polysaccharides containing 8–10% of inter-chain linkages. To avoid confusion with plant Q-enzymes, these enzymes will be referred to as amylopectin-branching glycosyltransferases.

Since sweet corn (*Zea mays*) differs from most higher plants in synthesising both a two-component starch and a glycogen-type polysaccharide (phytoglycogen)[6], it was of interest to examine extracts of this plant for branching-enzyme activity. This examination has provided evidence for the first-known, plant, amylopectin-branching glycosyltransferase. As reported in a preliminary communication[7], this enzyme is accompanied by debranching-enzyme activity, which hydrolyses α-(1→6)-D-

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glucosidic inter-chain linkages in glycogen and amylopectin; we have recently shown[8] that this latter activity is due to two distinct enzymes named isoamylase and R-enzyme, respectively. Whilst this work was in progress, Lavintman and Krisman[9] independently described the presence of an amylopectin-branching glycosyltransferase in sweet corn, and, recently, Lavintman[10] has shown that this branching enzyme occurs together with a Q-enzyme. We now report details of our experiments, including the separation of sweet-corn Q-enzyme and amylopectin-branching glycosyltransferase.

MATERIALS AND METHODS

Materials. — The following laboratory samples were used: potato amylose, maize amylose, potato amylopectin, rabbit-liver glycogen, and sweet-corn phytoglycogen A. The enzyme extracts were prepared from mature, whole grains of *Zea mays* (var. Golden Bantam), which had been passed through a hammer mill and the resulting flour freed from lipids by extraction with ether at 0°.

Analytical methods. — Total carbohydrate was estimated by using a phenol–sulphuric acid reagent[11]. Reducing sugars were determined on the milligram and microgram scale by the methods of Somogyi[12] and Nelson–Somogyi[13], respectively. The protein content of freeze-dried preparations was measured by the Kjeldahl method or colorimetrically[14], and of solutions by measurement of the absorbance at 280 nm.

The methods for the enzymic analysis of polysaccharides are similar to those used in previous studies of yeast branching-enzyme[4]. β-Amylolysis limits were determined by using Wallerstein barley β-amylase in enzyme digests, at pH 4.6 and 37°, containing 100–150 units of β-amylase per mg of polysaccharide. For isoamylolysis, polysaccharide (about 4–8 mg) was incubated with an equal weight of freeze-dried yeast[15] or sweet-corn isoamylase[8], at pH 6.0 or 6.2, for 24 h at 18°. The enzyme was inactivated by heating, the pH was adjusted to 4.8 with acetate buffer, β-amylase was then added, and the percentage conversion into maltose was measured after a further 24-h incubation at 37°.

Assay of branching enzymes. — These enzymes may be conveniently assayed by measurement of the decrease in iodine staining power of amylose and amylopectin[4]. However, since the relation between this decrease and the number of inter-chain linkages formed is not known, the method is not quantitative, and it is not possible to obtain specific activities calculated in terms of the number of linkages formed. The purity of branching-enzyme preparations has therefore been assessed by the absence of other enzymes (*e.g.*, α-amylase, debranching enzymes) which also affect the iodine staining power of polysaccharides. With enzyme preparations containing both α-amylase and branching enzymes, relative activities were assessed by the method of Krisman[16], in which the decrease in iodine staining power of glycogen is used as a measure of α-amylase activity, and is subtracted from the change observed with amylopectin.

For iodine staining, digests containing polysaccharide (1–4 mg), enzyme

preparation (0.5–2 ml), and buffer, in a total of 3–5 ml, were incubated at 37°. Under these conditions, and with incubation periods of up to 2 h, the decrease in iodine staining power was proportional to time and enzyme concentration. In some digests, fractions from the electrophoresis or gel-filtration studies were analysed directly; in other digests, a solution of freeze-dried preparation in water (0.1–0.2%) was used. Aliquot portions (1 ml) were withdrawn at intervals and added to standard iodine reagent (5 ml), and the absorbances of the solutions were measured at 470, 550, or 630 nm, for glycogen, amylopectin, and amylose, respectively, against iodine–water control solutions. The iodine reagent consisted of 20 ml of 0.2% iodine in 2.0% potassium iodide and 6 ml of 6N hydrochloric acid, diluted with water to 400 ml.

Continuous electrophoresis. — A Beckman Spinco Model CP continuous-electrophoresis cell was used, at a constant voltage (600–900 volts) and a current of 60–70 mamps, for the fractionation of protein material, which was collected in racks of 32 tubes. Electrophoresis was continued for periods of about 42 h. Individual tubes were assayed for protein content (absorbance at 280 nm).

Preparation of sweet-corn extracts. — *Method A.* Air-dried flour (400–500 g) was extracted with 0.05M sodium citrate (pH 6.0 or 7.0) (2 l) for 1–3 h at 18°, with stirring, and the extract was centrifuged first at low speeds (1,000 *g*) to remove insoluble cell-debris and starch, and then at high speeds (using a Sharples centrifuge or an MSE 18 continuous-flow rotor at 15,000 *g*) to give an opalescent solution (about 1,450–1,500 ml).

Method B. Zea mays grains (500 g) were steeped in water for 4 h at 18° and washed in 1% sodium hypochlorite solution followed by distilled water. Subsequent operations at 2° involved homogenisation in 1.5 l of 0.1M sodium citrate buffer (pH 6.3), extraction for a further 2 h, centrifugation at 4,000 *g*, and dialysis for 36 h against running tap-water. Protein was precipitated from the extract by the addition of ammonium sulphate (to 70% saturation), collected by centrifugation at 4,000 *g*, dialysed against running tap-water, adjusted to pH 6.0 by the addition of 0.01M sodium citrate buffer, and freeze-dried. Yield of extract *B*, 2.75 g; protein content, 36.8%.

RESULTS

The branching-enzyme activity of sweet-corn preparations. — Ammonium sulphate fractionation of sweet-corn extract *A* gave four protein fractions; these also contained substantial proportions of polysaccharide, which was identified as phyto-glycogen by iodine staining. The effect of each fraction on the iodine staining power of amylopectin and glycogen is given in Table I, and indicates the presence of various amounts of branching activity and α-amylase.

An attempt to separate these activities in Fraction III by acetone fractionation at low temperatures (−5° to −8°) was not successful. Four protein fractions were obtained, but none were devoid of α-amylase.

Since continuous electrophoresis has been successfully used for the separation

TABLE I

AMMONIUM SULPHATE FRACTIONATION OF SWEET-CORN EXTRACT A^a

Fraction No.	*I*	*II*	*III*	*IV*
Concentration of ammonium sulphate (w/v)	0-22	23-32	33-55	56-70
Yield (g)	6.9	6.1	84.0	1.7
Protein content (%)	3.4	2.2	7.6	1.3
Carbohydrate content (%)	12.0	11.5	71.5	20.0
Amylase + branching activity[b]	0.054	0.060	0.084	0.017
Amylase activity[b]	0.005	0.000	0.005	0.033
Branching activity	0.049	0.060	0.079	—

[a]After precipitation, the fractions were dissolved in water, dialysed for 48 h at 2° against running tap-water, centrifuged, and made 0.1M with respect to citrate buffer at pH 7.0 before freeze-drying. [b]Results are the decrease in absorbance after incubation for 2 h, at 18° for glycogen, and 37° for amylopectin.

of other carbohydrases[17,18], this procedure was applied to a solution of Fraction III [4.0 g dissolved in water (20 ml), and dialysed for 3 h against running tap-water and for 18 h at 0° against 0.01M sodium citrate buffer (pH 6.3)]. The latter buffer was used in the electrophoresis. The distribution of protein in successive racks of 32 tubes followed a similar pattern, with a maximum concentration in Tubes 15–19, although the presence of polysaccharide caused some spreading of the separated components. The activity of selected tubes (numbers 9, 17, and 25) towards amylose, amylopectin, and glycogen was assayed by iodine staining. Tube 9 from seven racks had no significant action on amylose and amylopectin, but caused the iodine staining power of glycogen to increase by about 7–19%. This tube therefore showed debranching activity, but was free from branching activity and α-amylase. Tubes 25 from these racks had little or no action on glycogen (showing the absence of α-amylase), but caused the iodine staining power of both amylose and amylopectin to fall (by 60–65% and 30–40%, respectively, in 2 h), showing the presence of branching activity. Tubes 17 also had little or no action on glycogen, but caused the iodine staining power of amylose to fall only slightly (0–8%), whereas that of amylopectin decreased to a larger extent (10–22%). This suggests that the branching activity in these tubes is different from that in Tubes 25, *i.e.*, Fraction III contained more than one branching enzyme.

Tubes 25 from each rack were pooled and used, in the following experiments, as a source of branching activity.

Action of Electrophoresis-fraction 25 on amylose. — Incubation of Fraction 25 (5 ml) with amylose [20 mg in 20 ml of 0.15M citrate buffer (pH 6.0)] at 37° caused a decrease in the λ_{max} of the iodine complex and in the absorbance thereat. After 0, 1, 3, 6, and 24 h, the λ_{max} was 630, 594, 578, 565, and 556 nm, respectively, whilst the corresponding absorbances fell from 1.22 to 0.71 during the 24 h. This change in iodine staining power was due to the formation of α-(1→6)-D-glucosidic inter-chain

linkages. When maize amylose (about 80 mg) was incubated for 24 h at 37° with Fraction 25 (25 ml) at pH 6.0, the β-amylolysis limit fell from 101 to 77%. Treatment of the "branched-amylose" with yeast isoamylase and β-amylase gave 98% conversion into maltose, showing that the newly formed inter-chain linkages has been hydrolysed by the α-(1→6)-D-glucosidase.

Action of Electrophoresis-fraction 25 on amylopectin. — When Fraction 25 (5 ml) was incubated with a 0.1% solution of potato amylopectin (20 ml) at 37° and pH 6.0, the λ_{max} of the iodine complex fell from 554 to 516 nm during 24 h, and the corresponding absorbance from 0.87 to 0.35. When incubated with 0.3M sodium citrate buffers over the pH range 4 to 8, the greatest decrease in iodine staining power occurred at pH 5.8–6.1 (see Fig. 1). In digests incubated at pH 6.0 and at various temperatures for 30 min, the percentage decreases in iodine staining power, measured at 550 nm, at 20, 30, 40, and 50°, were 6, 4, 19, and 5, respectively.

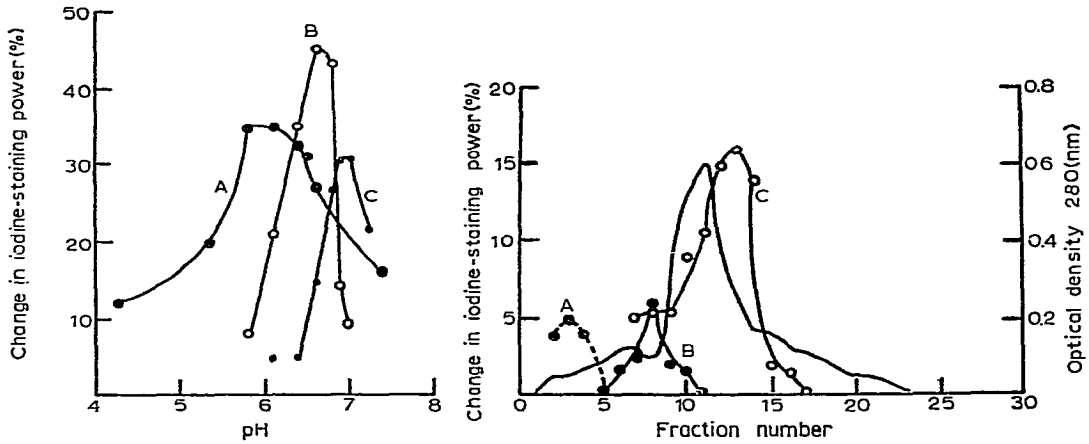


Fig. 1. Effect of pH on the activity of sweet-corn branching enzymes on amylopectin (●—●) and amylose (○—○). Results are expressed as the percentage decreases in iodine staining power at 550 and 630 nm, respectively. Curve A, amylopectin in sodium citrate buffers with Electrophoresis-fraction 25; curves B and C, amylose and amylopectin in sodium citrate–phosphate buffers with freeze-dried branching-enzymes preparation eluted from a column of Sephadex G-100 (see Fig. 2).

Fig. 2. Gel filtration of sweet-corn enzymes on Sephadex G-100. Fractions (5 ml) were collected and assayed against glycogen and amylopectin. Curve *A* represents an *increase* in iodine staining power of glycogen due to debranching-enzyme activity. Curve *B* represents a *decrease* in the iodine staining power of glycogen due to α-amylase. Curve *C* represents a *decrease* in the iodine staining power of amylopectin, which, in Fractions 11–17, is due to branching enzyme. The distribution of protein is shown by the continuous line.

The amylopectin-branching glycosyltransferase was completely inhibited by mercuric chloride at concentrations exceeding 0.05mM. With 0.5, 1.0, and 2.0% ammonium molybdate, the activity fell by 0, 33, and 50%, respectively, during a 15-min incubation period. Sodium borate (1–8mM), which is an inhibitor of potato Q-enzyme[19], had no effect.

The above decreases in iodine staining power of amylopectin were also due to the introduction of new α-(1→6)-D-glucosidic inter-chain linkages. When amylo-

pectin (about 30 mg) was incubated with Fraction 25 (25 ml) at pH 6.0 for 24 h, the α-amylolysis limit fell from 90 to 84%, and the β-amylolysis limit from 59 to 54%. Treatment of the "branched-amylopectin" with a mixture of β-amylase and yeast isoamylase gave 85% conversion into maltose, showing that the outermost inter-chain linkages had been hydrolysed. When phytoglycogen *A* was incubated with β-amylase and yeast isoamylase, the β-amylolysis limit increased from 53 to 76%.

Evidence for the presence of two branching enzymes in sweet-corn extracts. — The above results clearly establish the presence of an amylopectin-branching glycosyltransferase in sweet corn. However, in view of the different activities of Electrophoresis-fractions 17 and 25, the homogeneity of the branching activity was examined.

A sample of freeze-dried extract *B* (1 g, equivalent to 368 mg of protein) was dissolved in 0.01M Tris–hydrochloric acid buffer (50 ml, pH 7.6, and mM with respect to EDTA) and submitted to ultracentrifugation at 105,000 *g* for 2.5 h. The clear, supernatant solution was freeze-dried to give a white powder (0.95 g) having a protein content of 30%. A sample (100 mg) was dissolved in 0.01M tris buffer (3 ml, pH 7.6) and submitted to gel filtration on a column (2 × 30 cm) of Sephadex G-100 at 18°. 0.01M Tris buffer (pH 7.6) was used as eluant, and 5-ml fractions were collected and analysed. The distribution of protein, α-amylase, branching, and debranching activity is shown in Fig. 2. Fractions 11–17 were combined, dialysed against 0.01M citrate–phosphate buffer (pH 6.0) for 24 h at 2°, and freeze-dried to give 0.84 g of a branching-enzyme preparation having a protein content of 2.6%, representing 73% of the total protein applied to the column. As shown in Fig. 1, using phosphate–citrate buffers, activity towards amylose was optimal at pH 6.6, whereas that with amylopectin was greatest at pH 7.0. However, in 0.01M Tris–hydrochloric acid buffer (pH 7.5–8.5), the preparation was active only towards amylopectin, with optimal activity at about pH 8.0. When the preparation was incubated at various temperatures for one hour, activity towards both substrates was optimal at 37°, but the activity towards amylose was selectively inactivated at temperatures above 45° (see Table II).

TABLE II

EFFECT OF TEMPERATURE ON SWEET-CORN BRANCHING ENZYMES

Temperature (degrees)	25	30	37	45	50	60
Substrate[a]						
Amylose	3	30	41	7	0	0
Amylopectin	5	15	22	14	10	0

[a]Results are expressed as the percentage decrease in absorbance of the polysaccharide–iodine complexes measured at 630 and 550 nm, respectively.

In the presence of 0.01mM *p*-chloromercuribenzoate, activity towards both substrates was completely inhibited. With 0.5, 0.05, and 0.005mM mercuric chloride, the activity towards amylose fell by 61, 34, and 0%, whereas, with amylopectin, the percentage inhibition was 100, 75, and 24, respectively.

Since the above results showed the presence of two branching enzymes in the preparation (a Q-enzyme acting only on amylose, and an amylopectin-branching glycosyltransferase acting on both polysaccharides), 0.70 g of the freeze-dried material was again subjected to gel filtration on a column (8 × 70 cm) of Sephadex G-100 fitted to an LKB Model 4900A recycling chromatography equipment. The preparation was passed through the column three times, and samples (3 ml) were then collected and assayed. The results (Fig. 3) show a separation of two branching enzymes, one active towards both polysaccharides, and a Q-enzyme. Appropriate fractions (15–31 and 33–44) were combined and incubated with amylose and amylopectin for 12 h at 37°. The results (Table III) confirm the presence of a separate Q-enzyme and amylopectin-branching glycosyltransferase.

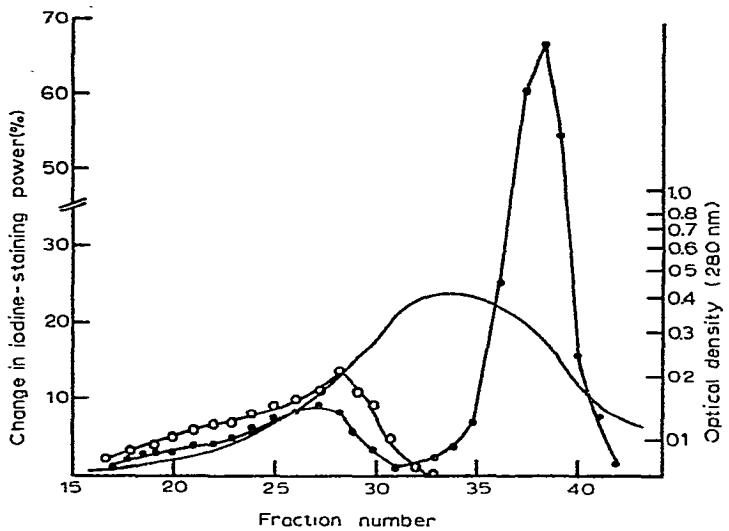


Fig. 3. Separation of Q-enzyme and amylopectin-branching glycosyltransferase by recycling gel-filtration. Changes in the iodine staining power of amylopectin and amylose are shown by ○—○ and ●—●, respectively. The continuous line represents the distribution of protein as measured with an LKB Uvicord.

TABLE III

ACTION OF SEPARATED BRANCHING ENZYMES ON AMYLOSE AND AMYLOPECTIN

	Amylopectin-branching glycosyltransferase	*Q-enzyme*
Fraction number (see Fig. 3)	15-31	33-44
Yield, protein (mg)	5.9	9.8
Action on amylose		
Initial β-amylolysis limit (%)	100	100
Final β-amylolysis limit (%)	63	59
Action on amylopectin		
Initial β-amylolysis limit (%)	61	61
Final β-amylolysis limit (%)	53	61

Characterisation of "synthetic phytoglycogen". — Incubation of the freeze-dried branching enzyme preparation (Sephadex-fractions 11-17; 15 mg) with amylopectin (5 mg) for 24 h at pH 7.0 and 37° caused the λ_{max} of the iodine complex to decrease from 550 to 470 nm and the corresponding absorbance from 1.40 to 0.62. The iodine staining properties of the product were those of a glycogen-type polysaccharide.

Since sweet-corn isoamylase hydrolyses the outermost α-(1→6)-D-glucosidic inter-chain linkages in amylopectin and glycogen[8], the action of this enzyme on a "branched amylopectin" or "synthetic phytoglycogen" was examined. After incubation of amylopectin (15 mg) with branching-enzyme preparation (25 mg) at pH 7.0 for 24 h, the digest was inactivated by heating. Part of the digest (2 ml) was used for measurement of the β-amylolysis limit; sweet-corn isoamylase (20 mg) was added to a second part, and, after incubation, at pH 6.2 for 24 h, the enzyme was inactivated by heating, and the β-amylolysis limit was measured. A third part of the digest was treated with a mixture of sweet-corn isoamylase and β-amylase at pH 4.8, and the apparent percentage conversion into maltose was determined after 24 h. The results (Table IV) show that the amylopectin-branching glycosyltransferase converts amylopectin into a polysaccharide which is very similar to phytoglycogen A with respect to its β-amylolysis limit, and susceptibility to sweet-corn isoamylase.

TABLE IV

ACTION OF SWEET-CORN BRANCHING-ENZYME PREPARATION ON AMYLOPECTIN AND PHYTOGLYCOGENS

	Potato amylopectin	*"Synthetic phytoglycogen"*	*Phytoglycogen* A
Percentage conversion into maltose			
(*a*) β-amylase alone	61	53	52
(*b*) successive action of sweet-corn isoamylase and β-amylase	70	62	64
(*c*) combined action of sweet-corn isoamylase and β-amylase	90	82	87

DISCUSSION

The present investigations have shown that extracts of sweet corn contain a mixture of a Q-enzyme and an amylopectin-branching glycosyltransferase which may be separated from other starch-metabolising enzymes either by continuous electrophoresis or by gel filtration. The two branching enzymes can be separated from each other by recycling gel-filtration.

Lavintman[10] has also obtained evidence for the presence of two branching enzymes in sweet corn. The pronounced effect of different types of buffer on the two activities, including inhibition of Q-enzyme by Tris–hydrochloric acid buffer, and the greater sensitivity of the amylopectin-branching glycosyltransferase to low concen-

trations of mercuric chloride are confirmed. Sweet-corn Q-enzyme differs slightly from other plant Q-enzymes in optimum pH (see Table V) and significantly in optimum temperature.

TABLE V

PROPERTIES OF BRANCHING ENZYMES

Branching enzyme	*Optimum pH*	*Optimum temperature*	*Ref.*
Potato Q-enzyme	7.0	21 ±1°	20
Broad-bean Q-enzyme	*ca.* 7.4	20°	21
Green-gram Q-enzyme	7.0	24°	22
Polytomella coeca Q-enzyme	7.3	25–33°	23
Sweet-corn Q-enzyme	6.6	37°	—
Sweet-corn amylopectin-branching glycosyltransferase	7.0	37°	—
Yeast amylopectin-branching glycosyltransferase	7.0	20°	4

The action of both branching enzymes on amylose results in a loss of iodine staining power which is not due to depolymerisation. Enzyme action introduces new inter-chain linkages into the substrate as shown by a decrease in β-amylolysis limit, which is a measure of the length of the linear outer-chains. With amylopectin, the enzyme preparation causes a decrease in α-amylolysis limit from 90 to 84%, corresponding to an increase in the proportion of inter-chain linkages from 5.4 to 6.6%[7]. Since these inter-chain linkages are hydrolysed by isoamylase preparations from both yeast[15] and sweet corn[8], they are characterised as α-(1→6)-D-glucosidic in type.

The demonstration of a plant branching-enzyme, which differs from Q-enzyme in being able to introduce inter-chain linkages into amylopectin, provides an *in vitro* method for the conversion of the latter into a polysaccharide which resembles phytoglycogen in iodine staining and enzymic degradation properties. Although the localisation of the various starch-metabolising enzymes in the sweet-corn cells has not been investigated, it seems probable that starch and phytoglycogen are metabolised by separate pathways. For starch, this would involve synthesis from sucrose by the insoluble starch-synthetase system, described by Fekete and Cardini[24], and Q-enzyme, whilst phytoglycogen could be formed from ADPG by the combined action of a soluble phytoglycogen synthetase[25] and amylopectin-branching glycosyltransferase. During degradation, the inter-chain linkages in amylopectin may be hydrolysed by R-enzyme, and in phytoglycogen by isoamylase[8].

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NUCLEOPHILIC DISPLACEMENT REACTIONS IN CARBOHYDRATES

PART VII*. THE SOLVOLYSIS OF 3-*O*-BENZYL-1,2-*O*-ISOPROPYLIDENE-α-D-GLUCOFURANOSE 5,6-DISULPHONATES: FURTHER EXAMPLES OF BENZYLOXY-GROUP PARTICIPATION

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(Received March 15th, 1968)

ABSTRACT

Solvolysis of the respective 3-*O*-benzyl-1,2-*O*-isopropylidene-α-D-glucofuranose 5,6-disulphonates in 50% aqueous methanol, in the presence of sodium acetate, afforded 3,6-anhydro-1,2-*O*-isopropylidene-5-*O*-toluene-*p*-sulphonyl-α-D-glucofuranose (**5**) and 3,6-anhydro-1,2-*O*-isopropylidene-5-*O*-methanesulphonyl-α-D-glucofuranose (**6**). 3-*O*-Benzyl-1,2-*O*-isopropylidene-5,6-di-*O*-methanesulphonyl-α-D-allofuranose (**4**) was synthesised and was found to be virtually unaffected in parallel experiments. The reactions leading to the 3,6-anhydro derivatives **5** and **6** are the result of neighbouring-group participation by the benzyloxy group, presumably in the rate-determining ionisation of the primary sulphonic ester groups, with formation and subsequent breakdown of the intermediate oxonium ions.

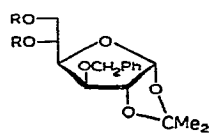
INTRODUCTION AND DISCUSSION

Alkylation and (hemi)acetalation are used extensively to protect carbohydrate hydroxyl and aldehyde groups, respectively, but, in certain instances, the protecting group has been found to participate in nucleophilic displacement reactions. Thus, 1,3,4,6-tetra-*O*-acetyl-2-*O*-methyl-D-glucopyranose was isolated[2], in small yield, from the brominolysis of methyl 3,4,6-tri-*O*-acetyl-2-deoxy-2-iodo-α-D-mannopyranoside in acetic acid, and 6-deoxy-2,3-*O*-isopropylidene-5-*O*-methyl-D-talofuranose was formed[3] on treatment of methyl 5-*O*-bromobenzenesulphonyl-6-deoxy-2,3-*O*-isopropylidene-β-L-allofuranoside with sodium hydroxide in aqueous *p*-dioxane. More recently, a benzoate displacement reaction on 2,3,5-tri-*O*-benzyl-4-*O*-toluene-*p*-sulphonyl-D-ribose dimethyl acetal gave 1-*O*-benzoyl-2,3,5-tri-*O*-benzyl-4-*O*-methyl-L-lyxose methyl hemiacetal as a consequence of methoxy-group participation and migration[4]. The benzyloxy group has been shown[5] to render powerful anchimeric assistance in the ethanolysis of several 2,3,4-tri-*O*-benzylpentitol 1,5-disulphonates (when 1,4-anhydro-sugar derivatives are produced), and the formation of methyl 3,6-anhydro-2-*O*-benzyl-β-D-galactopyranoside on solvolysis of methyl

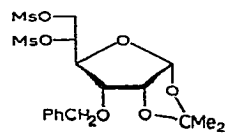
*For Part VI, see Ref. 1.

2,3-di-*O*-benzyl-6-*O*-methanesulphonyl-β-D-galactopyranoside, in aqueous methanol, is clearly the result of a similar participation[1,6].

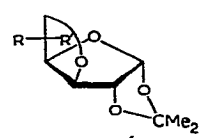
The work of Winstein *et al.*[7] and others[8] on neighbouring methoxy-group participation in solvolytic displacements shows that participation is most favourable by way of five- or six-membered oxonium-ion intermediates. This is also illustrated by the majority of the examples of alkoxy-group participation already reported in the carbohydrate field[1,3-6]. In seeking to extend this work, we examined the solvolysis of 3-*O*-benzyl-1,2-*O*-isopropylidene-5,6-di-*O*-toluene-*p*-sulphonyl-α-D-glucofuranose (**2**) and the related dimethanesulphonate **3**, in 50% aqueous methanol, since solvolysis of the primary sulphonic ester group in these compounds, with neighbouring benzyloxy-group participation, can proceed by way of five-membered, oxonium-ion intermediates. In view of the acid-lability of the acetal group in compounds **2** and **3**, it was necessary to remove (by the addition of sodium acetate) the sulphonic acid liberated, and this precluded the detection of neighbouring-group participation from kinetic data based on a measurement of the acid released. In previous cases of benzyloxy-group participation[1,5,6], the intermediate oxonium-ion was found to react exclusively by $\overset{+}{O}$-benzyl bond-cleavage, and the isolation of anhydro sugars thus provides a criterion for the involvement of intermediates which result from benzyloxy-group participation. It was also of interest to compare qualitatively the solvolyses of disulphonates **2** and **3** with that of 3-*O*-benzyl-1,2-*O*-isopropylidene-5,6-di-*O*-methanesulphonyl-α-D-allofuranose (**4**), which must undergo an unassisted solvolysis.



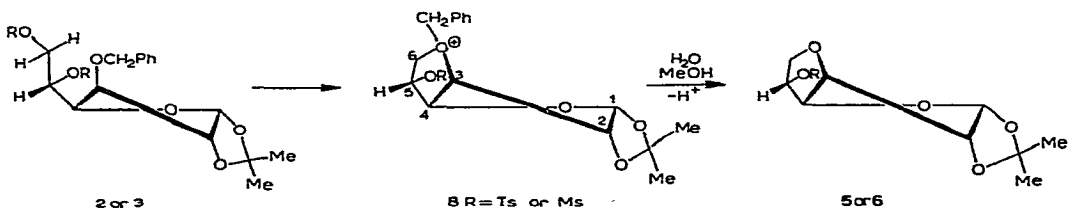
1 R = H
2 R = $\cdot SO_2C_6H_4Me$ = Ts
3 R = $\cdot SO_2Me$ = Ms



4



5 R = OTs, R′ = H
6 R = OMs, R′ = H
7 R = H, R′ = OCOPh



2 or 3 — 8 R = Ts or Ms — 5 or 6

Disulphonate **2** was obtained originally as a syrup by toluene-*p*-sulphonylation of 3-*O*-benzyl-1,2-*O*-isopropylidene-α-D-glucofuranose[9] (**1**). When this procedure was repeated, it was evident from t.l.c. that other products were also formed, but the pure, crystalline disulphonate **2** could be obtained after chromatography on silica gel. Dimethanesulphonate **3** was prepared by methanesulphonylation of the diol **1** and was crystallised directly from the reaction products. The allose disulphonate **4** was

obtained analogously by benzylation of 1,2:5,6-di-*O*-isopropylidene-α-D-allofuranose[10], followed by removal of the 5,6-acetal group and methanesulphonylation.

The solvolyses of disulphonates **2** and **3** were carried out in boiling 50% aqueous methanol, in the presence of sodium acetate, until t.l.c. showed the virtual disappearance of starting material. In the case of ditoluene-*p*-sulphonate **2**, t.l.c. demonstrated the formation of one major product which was indistinguishable in its mobility from that of 3,6-anhydro-1,2-*O*-isopropylidene-5-*O*-toluene-*p*-sulphonyl-α-D-glucofuranose[11] (**5**). The crystalline anhydro-sugar **5** was obtained (~45%) following chromatography on silica gel, and it was identified by comparison with an authentic sample[11]. The solvolysis of dimethanesulphonate **3** afforded a crystalline, major product (62%), together with small proportions of three unidentified products. Elemental analyses for the major product corresponded to those expected for the anhydro sugar **6**, and, in agreement with this structure, the infrared spectrum exhibited no absorptions attributable to aromatic or hydroxyl groups, and n.m.r. spectroscopy demonstrated the presence of an acetal group and a methanesulphonyloxy group. Unequivocal proof for structure **6** was furnished by the isolation of 3,6-anhydro-5-*O*-benzoyl-1,2-*O*-isopropylidene-β-L-idofuranose[12] (**7**), following displacement of the methanesulphonyloxy group by using sodium benzoate in *N*,*N*-dimethylformamide. When the allose disulphonate **4** was solvolysed under conditions comparable to those used with the glucose disulphonates **2** and **3**, it was essentially unaffected (t.l.c.) and could be recovered in approximately 70% yield.

It is clear, from the isolation of anhydro-sugar derivatives **5** and **6** in reasonable yields, that a substantial proportion of the reactions involve a participation by the benzyloxy group. Only rate data can directly implicate benzyloxy-group participation in the rate-determining ionisation of the primary sulphonic ester groups, but the comparative unreactivity of allose disulphonate **4** towards solvolysis indicates that we are probably dealing with anchimerically assisted processes with glucose disulphonates **2** and **3**. A simplified scheme (*cf.* Ref. 7*b*) for the solvolysis of these disulphonates is illustrated on p. 83. The conformations shown are based upon n.m.r. studies[13] of closely related compounds. These indicated that, in 1,2-*O*-isopropylidene-α-D-glucofuranose derivatives, and regardless of the relative sizes of other substituents, the furanoid ring adopts the twist conformation in which C-2 is below and C-3 is above the plane defined by the other ring atoms. With the derived 3,6-anhydrofuranoses, the second five-membered ring adopts the so-called "envelope shape" in which C-4 is displaced above the plane defined by C-3, O, C-6, and C-5. The most likely orientation of the benzyl group in oxonium-ion intermediate **8** is that in which it has an *exo*-configuration with respect to the dioxabicyclo[3.3.0]octane ring system, since non-bonded interactions are least when it is in this position.

EXPERIMENTAL

Thin-layer chromatography (t.l.c.) was performed either on microscope slides or glass plates (3.25 in sq) with Kieselgel G (Merck, 7731) and detection with vanillin–sulphuric acid[14]. N.m.r. spectra were measured in deuteriochloroform (tetramethyl-

silane as internal reference) with a Varian A-60 or a Perkin–Elmer R-10 spectrometer, and infrared spectra were routinely recorded with a Perkin–Elmer 125 spectrometer.

3-O-Benzyl-1,2:5,6-di-O-isopropylidene-α-D-glucofuranose. — This compound was made by the following modified procedure. Sodium hydride (2 g) was added in small portions to a stirred solution of 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose (5.2 g) in *N,N*-dimethylformamide (100 ml), and, after 30 min, a solution of benzyl chloride (5 ml) in *N,N*-dimethylformamide (5 ml) was added dropwise. The reaction mixture was stirred for 2.5 h at room temperature, and the excess of reagents was then decomposed by the careful addition of methanol (10 ml), and the solvents were removed. The residue was extracted with chloroform (150 ml), and this was washed with water (3 × 100 ml) and dried ($MgSO_4$). Removal of the solvent yielded a syrup (7.2 g), $[\alpha]_D$ −19.5° (*c* 1.1, ethanol), which was homogeneous when examined by t.l.c. (ethyl acetate–hexane, 7.5:2.5); lit.[9], $[\alpha]_D$ −28.3° (ethanol). The infrared spectrum of the syrupy material showed negligible absorption at *ca.* 3500 cm^{-1} (OH), and the n.m.r. spectrum revealed the presence of 5 aromatic protons, and 12 protons due to the two acetal groups.

3-O-Benzyl-1,2-O-isopropylidene-α-D-glucofuranose (**1**). — The crude product was obtained by partial hydrolysis of the foregoing compound (1.8 g) in 75% acetic acid (100 ml), essentially as described by Meyer and Reichstein[9]. Chromatography on silica gel (80 g; ethyl acetate–chloroform, 1:1) gave compound **1** (1.2 g), $[\alpha]_D$ −35° (*c* 0.6, chloroform); lit.[9], $[\alpha]_D$ −45° (chloroform). N.m.r. data: τ 2.75 (5 aromatic protons); 4.15 (doublet, $J_{1,2}$ 3.5 Hz, H-1); 5.41 (AB quartet, *J* 12 Hz, benzyl CH_2); 8.53, 8.69 (3-proton singlets, Me_2C).

3-O-Benzyl-1,2-O-isopropylidene-5,6-di-O-toluene-p-sulphonyl-α-D-glucofuranose (**2**). — A solution of the diol **1** (1.2 g) and toluene-*p*-sulphonyl chloride (2 g) in pyridine (70 ml) was stirred for 108 h at room temperature, and the reaction mixture was processed, in the usual manner, to give a material containing at least three components. Chromatography on silica gel (100 g; chloroform–ethyl acetate, 8:1) afforded the disulphonate **2** (0.63 g), m.p. 95–96° (from acetone–light petroleum, b.p. 40–60°), $[\alpha]_D$ −6° (*c* 0.65, chloroform) (Found: C, 57.95; H, 5.5; S, 10.7. $C_{30}H_{34}O_{10}S_2$ calc.: C, 58.2; H, 5.5; S, 10.4%). Meyer and Reichstein[9] recorded $[\alpha]_D$ −6° (chloroform) for a syrupy material. The n.m.r. and infrared spectra were in accord with the structure assigned.

3-O-Benzyl-1,2-O-isopropylidene-5,6-di-O-methanesulphonyl-α-D-glucofuranose (**3**). — To a solution of the diol **1** (0.63 g) in dry pyridine (60 ml) was added methanesulphonyl chloride (0.6 ml) in pyridine (5 ml), and the mixture was kept for 41 h at room temperature. Water (1 ml) was then added, and, after 1 h, the solution was processed, in the usual way, to give a clear syrup. This was crystallised from acetone–light petroleum (b.p. 40–60°) to give the dimethanesulphonate **3** (0.66 g), m.p. 124–125°, $[\alpha]_D$ −18° (*c* 0.8, acetone) (Found: C, 46.95; H, 5.4; S, 13.7. $C_{18}H_{26}O_{10}S_2$ calc.: C, 46.3; H, 5.6; S, 13.7%). N.m.r. data: τ 2.73 (5 aromatic protons); 4.19 (doublet, $J_{1,2}$ 3.5 Hz, H-1); 6.98, 7.05 (3-proton singlets, $MeSO_2$); 8.53, 8.71 (3- proton singlets, Me_2C).

3-O-Benzyl-1,2:5,6-di-O-isopropylidene-α-D-allofuranose. — A 50% suspension of sodium hydride in oil (3 g) was added in small portions to a stirred solution of 1,2:5,6-di-*O*-isopropylidene-α-D-allofuranose[10] (3.6 g) in *N,N*-dimethylformamide (50 ml), and, after 30 min, benzyl chloride (4 ml) in *N,N*-dimethylformamide (4 ml) was added. The mixture was stirred overnight, and the excess of reagents was then decomposed by the addition of methanol (3 ml), and the solvents were removed. The residue was extracted with chloroform (150 ml), and the extract was washed with water (5 × 150 ml) and dried ($MgSO_4$). Removal of the solvent gave a syrup which t.l.c. (chloroform) showed to contain at least four components. Chromatography of a portion (2 g) of the syrup on silica gel (100 g; elution with chloroform) gave material which was crystallised from aqueous methanol to give the product (0.61 g), m.p. 64–65°, $[\alpha]_D$ +110° (*c* 1, methanol) (Found: C, 65.2; H, 7.55. $C_{19}H_{26}O_6$ calc.: C, 65.1; H, 7.5%). N.m.r. data: τ 2.63 (singlet, 5 aromatic protons); 4.25 (doublet, $J_{1,2}$ *ca.* 4 Hz, H-1); 5.33 (AB quartet, *J* 12 Hz, benzyl CH_2); 8.41, 8.64 (singlets, integrated areas 1:3, 2 Me_2C).

3-O-Benzyl-1,2-O-isopropylidene-5,6-di-O-methanesulphonyl-α-D-allofuranose. — A solution of the foregoing compound (0.9 g) in 75% acetic acid (50 ml) was kept for 20 h at room temperature, whereupon t.l.c. (ethyl acetate) indicated that removal of the 5,6-acetal group was essentially complete. Removal of the solvents gave a syrup which was dissolved in ethyl acetate (120 ml). The organic layer was washed with water (100 ml), 2% aqueous sodium hydrogen carbonate (100 ml), and water (2 × 100 ml). Chromatography on silica gel (100 g; ethyl acetate) gave essentially pure 3-*O*-benzyl-1,2-*O*-isopropylidene-α-D-allofuranose (0.64 g), $[\alpha]_D$ +103° (*c* 0.6, methanol), ν_{max} 3400 cm^{-1} (OH) and 1375 cm^{-1} (isopropylidene group), which could not be induced to crystallise.

To a solution of the foregoing diol (0.56 g) in dry pyridine (60 ml) was added methanesulphonyl chloride (0.5 ml) in dry pyridine (5 ml), and the mixture was kept for 2 h at room temperature. Methanol (1 ml) was added, and, after 1 h, the solution was processed in the usual way.

The allose disulphonate **4** (0.63 g), had m.p. 107–108° (from ethyl acetate–light petroleum, b.p. 40–60°), $[\alpha]_D$ +78° (*c* 0.8, chloroform), ν_{max} 1180, 1340 cm^{-1} (sulphonic ester), 1370 cm^{-1} (isopropylidene group) (Found: C, 46.4; H, 5.9; S, 14.4. $C_{18}H_{26}O_{10}S_2$ calc.: C, 46.3; H, 5.6; S, 13.7%).

Solvolysis of 3-O-benzyl-1,2-O-isopropylidene-5,6-di-O-toluene-p-sulphonyl-α-D-glucofuranose (**2**). — The disulphonate **2** (0.3 g) in 50% aqueous methanol (160 ml) containing sodium acetate trihydrate (0.6 g) was heated under gentle reflux for 115 h, whereupon t.l.c. (chloroform–ethyl acetate, 9:1) indicated that the solvolysis was essentially complete. The cooled solution was extracted with chloroform (3 × 80 ml), and the combined extracts were washed with water (50 ml) and dried ($MgSO_4$). The solvent was removed, and the residue was chromatographed on silica gel (100 g; chloroform–ethyl acetate, 9:1) to give anhydro sugar **5** (84 mg, 45%), m.p. 128° (from ethanol), $[\alpha]_D$ +37° (*c* 0.6, chloroform); lit.[11], m.p. 132°, $[\alpha]_D$ +39° (*c* 2.8, chloroform). The infrared spectrum of anhydro sugar **5** was indistinguishable from

that of an authentic sample[11], and no depression of melting point was observed on admixture of the two samples.

Solvolysis of 3-O-benzyl-1,2-O-isopropylidene-5,6-di-O-methanesulphonyl-α-D-glucofuranose (3). — Solvolysis of the dimethanesulphonate 3 (0.41 g) in 50% aqueous methanol (120 ml) containing sodium acetate trihydrate (0.9 g) was carried out as described above. T.l.c. (chloroform–ethyl acetate, 8:1) showed that, after 135 h, a small amount of starting material still remained, and that one major component and at least three minor components were formed. The solution was extracted with chloroform (3 × 80 ml), and the extracts were washed with water (80 ml) and dried ($MgSO_4$). Removal of the solvents gave 3,6-anhydro-1,2-*O*-isopropylidene-5-*O*-methanesulphonyl-α-D-glucofuranose (6; 152 mg, 62%), m.p. 108–109° (from acetone–light petroleum, b.p. 40–60°), $[\alpha]_D$ +62° (*c* 0.6, acetone) (Found: C, 43.0; H, 5.7; S, 11.5. $C_{10}H_{16}O_7S$ calc.: C, 42.85; H, 5.75; S, 11.4%). N.m.r. data: τ 4.15 (doublet, $J_{1,2}$ 3.2 Hz, H-1); 6.93 (3-proton singlet, $MeSO_2$); 8.53, 8.69 (3-proton singlets, Me_2C). Its infrared spectrum contained no absorptions for hydroxyl or aromatic groups.

When compound 6 (0.11 g) was heated under reflux with sodium benzoate (0.3 g) in *N*,*N*-dimethylformamide (6 ml) for 3.5 h, it was converted into 3,6-anhydro-5-*O*-benzoyl-1,2-*O*-isopropylidene-β-L-idofuranose 7 (95 mg), m.p. and mixed m.p. 78–79°, $[\alpha]_D$ +77° (*c* 0.75, chloroform); lit.[12], m.p. 83–84°, $[\alpha]_D$ +79° (*c* 2, chloroform). The infrared spectrum of benzoate 6 was indistinguishable from that of an authentic sample prepared from compound 5, as described by Buchanan and Conn[12].

Solvolysis of 3-O-benzyl-1,2-O-isopropylidene-5,6-di-O-methanesulphonyl-α-D-allofuranose (4). — When this compound was heated under the conditions used in the solvolysis of disulphonates 2 and 3, it was usually recovered in greater than 70% yield, and identified by its mixed m.p. and by infrared spectroscopy. T.l.c. (ethyl acetate) demonstrated that little change had taken place, but traces of two other components were noted after 74 h.

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CARBOHYDRATE–PROTEIN COMPLEX-FORMATION

SOME FACTORS AFFECTING THE INTERACTION OF D-GLUCOSE AND POLYSACCHARIDES WITH CONCANAVALIN A

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ABSTRACT

The effects of temperature, chelating agents, protein denaturants, and *alpha*- and *beta*-amylolysis on concanavalin A–polysaccharide complexes have been studied. Temperatures between 30 and 50° tend to dissociate concanavalin A–glycogen complexes, whereas temperatures above 50° denature concanavalin A solutions. The chelating agent (ethylenedinitrilo)tetraacetic acid slightly inhibits complex-formation between concanavalin A and polysaccharides. Formamide, guanidine hydrochloride, 1,1,3,3-tetramethylurea, and urea competitively inhibit concanavalin A–glycogen complex-formation. Concanavalin A does not protect glycogen from glycogenolysis by *alpha*- or *beta*-amylase, although it does partially protect the ability of the *alpha*-amylolytic end-product to be precipitated with the protein.

An ultraviolet difference-spectrum was generated by the addition of D-glucose to concanavalin A solutions, and it was characterized by inflection points at approximately 256, 264, 278, 287, and 294 nm. In contrast, the optical rotatory properties of concanavalin A solutions were not detectably altered by D-glucose. Moffitt–Yang plots for concanavalin A revealed that the helical content of concanavalin A is approximately 7.5%.

Possible modes of interaction between concanavalin A and neutral sugars or polysaccharides are discussed, and it is suggested that hydrogen bonding plays an important role in their binding.

INTRODUCTION

The reaction between concanavalin A, a phytohemagglutinin isolated from the jack bean, and various polysaccharides, to form insoluble protein–polysaccharide complexes has recently received considerable attention, mainly by Goldstein and associates[1–10]. It has been shown that concanavalin A reacts with neutral polysaccharides through their nonreducing chain-ends[2,7–9]. Several techniques have been employed for characterizing concanavalin A–neutral D-glucan complexes,

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including agar-gel diffusion[4], turbidity of solutions following complex-formation[1,2,4,11–14], immunoelectrophoresis[3], and hapten (monomeric and oligomeric sugar) inhibition[2,8–10].

Other studies have revealed that, in addition to those with neutral D-glucans, concanavalin A forms precipitin-like complexes with a variety of such charged polysaccharides as heparin[12,14], D-glucan phosphates[14], galactan sulfate[13], fucan, RNA, and bacterial lipopolysaccharides[14]. Although many of these polyelectrolytes do not possess the highly branched structures having requisite nonreducing hexose termini, it was shown that, in certain cases, D-glucose could inhibit complex-formation[14]. The latter results suggested that the sugar moiety was involved in the reaction. The present studies have extended these earlier observations with respect to concanavalin A–polysaccharide interactions, and we report on several parameters affecting formation of the complexes.

Such physicochemical methods as optical rotatory dispersion and ultraviolet difference spectroscopy have been utilized for detecting and characterizing concanavalin A–D-glucose (hapten) interactions. In addition, the effects of temperature, chelating agents, protein denaturants, and amylolysis on concanavalin A–polysaccharide complexes have been examined.

MATERIALS AND METHODS

Highly purified concanavalin A was prepared from defatted, jack-bean meal (Sigma, lot 37B-5270) by the method of Agrawal and Goldstein[6]. The lyophilized material was stored in a desiccator until used. Frequently, turbid matter formed by aggregation of protein appeared in solutions of concanavalin A, but it could be separated by centrifugation. The concentration of solutions of concanavalin A (after clarification) was determined by ultraviolet absorption at 280 nm ($E^{1\%}_{1cm} = 11.4$; see ref. 5). Molecular-weight determinations at pH 7.0 (sedimentation equilibrium) gave a value of approximately 70,000 for concanavalin A, in close agreement with the value of 71,000 reported by Olson and Liener[15].

Glycogen, glycogen phosphate, and *Shigella flexneri* lipopolysaccharide (Boivin type) were those used in a previous study[14]. Amylases (*alpha-*, lot AA5703ML, swine pancreas; *beta-*, lot BA5650, sweet potato) were obtained from Worthington Biochemicals, Freehold, New Jersey. Urea, D-glucose, guanidine hydrochloride, formamide, 1,1,3,3-tetramethylurea, and (ethylenedinitrilo)tetraacetic acid (EDTA) were purchased from Eastman Organic Chemicals, Rochester, New York.

Formation of a complex between concanavalin A and polysaccharides was determined turbidimetrically at 450 nm[1,11,13,14] by using 1-cm, rounded cuvettes in a Bausch and Lomb Spectronic 20 colorimeter; some of the measurements were made with a Coleman Jr. spectrophotometer. Details of each variable used in the present procedures are noted in the text. With the exception of the lipopolysaccharide from *S. flexneri*, all solutions of polysaccharides were prepared gravimetrically from dialyzed, lyophilized samples. The anthrone procedure of Mokrasch[16] was used for determining the hexose in polysaccharides.

Ultraviolet difference-spectra were measured in a Cary Model 15 spectrophotometer at room temperature. The four-cuvette method of Herskovits and Laskowski[17] was used; concanavalin A plus D-glucose were in one sample-beam cuvette, and buffer was in the other; in the reference beam, concanavalin A and D-glucose were in separate cuvettes.

Optical rotatory dispersion (o.r.d.) curves were obtained by use of a Jasco-Durrum Model ORD/5 recording spectropolarimeter. These studies were performed at room temperature (approx. 22°). A silica cuvette of 1.0-cm path-length was employed as a sample holder for measurements in the visible region. For ultraviolet measurements, a 9.80-mm silica insert was fitted into the 1.0-cm cuvette, to give an effective path-length of 0.2 mm. The data in the visible region were treated by use of the Moffitt–Yang equation[18,19], namely,

$$[m'] = \frac{a_0 \lambda_0^2}{\lambda^2 - \lambda_0^2} + \frac{b_0 \lambda_0^4}{(\lambda^2 - \lambda_0^2)^2} \quad \text{or} \quad [m'] = \frac{3}{(n^2+2)} \cdot \frac{[\alpha]_\lambda M}{10^2}$$

where $[m']$ is the mean residue rotation, λ is the observed wavelength, a_0 is a constant depending on the conformation of the protein and on the solvent system, b_0 is a constant that depends on the conformation of the protein and that is independent of the solvent system, λ_0 is a constant that has a value, in aqueous systems, of 212 nm, n is the refractive index of the solvent at wavelength λ, M is the molecular weight of concanavalin A, and $[\alpha]_\lambda$ is the specific rotation at wavelength λ. The mean residue weight (MRW) of concanavalin A was calculated from the amino acid data of Olson and Liener[15], and found to be 114. Use of the Moffitt–Yang equation to calculate the helical content of concanavalin A is discussed subsequently.

Amylolytic degradations of glycogen were conducted at 25°, and the final volume of the reaction mixtures for turbidimetric assays of complex-formation was 4.0 ml. For *alpha*-amylolysis, all solutes were suspended in 0.01M phosphate and 0.003M salt at pH 6.9, and the system for *beta*-amylolysis was 0.008M sodium acetate and 0.003M salt at pH 4.8. Turbidimetric assays were made at 450 nm, in 1-cm cuvettes, as a function of time. The degree of hydrolysis of glycogen was determined by subjecting aliquots to 30% potassium hydroxide for 2 h at 100°, cooling, precipitating with 65% ethanol, and determining the nonhydrolyzed glycogen by the anthrone method[16]. In amylolytic studies in the presence of concanavalin A, 3.9-ml suspensions of concanavalin A (4.0 mg) and glycogen (0.4 mg) were incubated for 30 min prior to the addition of 0.1 ml of either *alpha*- or *beta*-amylase (5 μg). In amylolytic studies in the absence of concanavalin A, 2.0-ml suspensions of glycogen (0.4 mg) and the respective enzyme (5 μg) were incubated for the time intervals noted, and were heated for 5 min at 100° in order to inactivate the amylase. The resulting solution of glycogen was brought to 25°, and 2.0 ml of concanavalin A (4.0 mg) in the appropriate buffer was added. After an incubation period of 30 min, the turbidities for complex-formation were determined.

RESULTS AND DISCUSSION

The interactions between carbohydrate-containing macromolecules and proteins are numerous and diverse. Polysaccharides are known to exclude proteins sterically from solution[21] and to precipitate proteins under a variety of conditions, as in formation of *alpha*-amylase–glycogen[22] and muscle phosphorylase–glycogen[23] complexes. Recent studies have shown that, in the presence of gelatin, specific polysaccharides become soluble in solvent systems that normally precipitate the polysaccharides[24,25]. Serum albumin reacts with charged polysaccharides to form insoluble and soluble complexes[26]. In this study, we have examined some of the factors affecting formation of complexes of concanavalin A both with polysaccharides and hexoses.

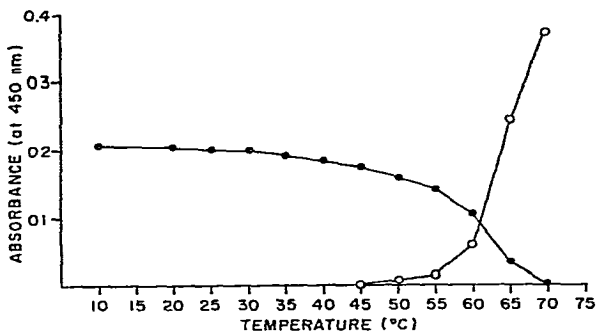


Fig. 1. Effect of temperature on formation of a complex of concanavalin A with glycogen. Concanavalin A (4.0 mg) (—O—), and 4.0 mg of concanavalin A–0.4 mg glycogen (—●—) in 6.0 ml of 0.1M NaCl–0.05M phosphate buffer, pH 7.0, were exposed to the incremental temperature-changes listed, and the turbidities were recorded. Optical absorbances of the concanavalin A–glycogen complexes were corrected for the turbidities of the concanavalin A at the respective temperatures. During incubation, the concanavalin A and glycogen solutions were in separate containers, and were mixed after the desired temperature had been reached.

The data in Fig. 1 show that the precipitin reaction of glycogen–concanavalin A complexes is temperature-dependent. The lack of any temperature dependence on precipitin formation between 10 and 30° parallels the findings of So and Goldstein[3], who reported no significant differences in the solubility of dextran NRRL B-1355-S–concanavalin A complexes between 0 and 25°. At temperatures above 30°, precipitin formation becomes increasingly sensitive to incremental rises in temperature. At temperatures above 50°, a concomitant increase in the turbidities of concanavalin A control-samples, characteristic of many heat-denatured proteins, was evident (see Fig. 1). Consequently, at each temperature, a concanavalin A control determination was made, and its turbidity was subtracted from the turbidity of the respective complex.

The inhibitory effect of relatively high temperatures on glycogen–concanavalin A complexes indicates that the D-glucan binding site(s) on the protein moiety are heat-sensitive. These results suggest that hydrogen bonding is the primary binding mechanism involved in the precipitin reaction. This interpretation is based on the

assumptions that hydrophobic interactions would be enhanced and electrostatic forces would not be greatly affected by the higher temperatures. On the other hand, internal changes in the protein may occur as a consequence of binding to glycogen, and, in turn, tend to stabilize the complex *via* hydrophobic, electrostatic or hydrogen-bonding forces, or both. However, ancillary data, discussed below, support the hydrogen-bonding hypothesis.

Concanavalin A is known to contain 0.029% of manganese (by weight), and removal of the metal results in the elimination of the glycogen-binding ability[5]. Preliminary electron-spin resonance studies confirmed the presence of manganese in our purified, lyophilized preparations of concanavalin A. Thus, if the cation is accessible, a chelating agent such as EDTA should have a strong effect on the interaction properties of concanavalin A. Although this has not been shown in a quantitative way, we have incubated concanavalin A with EDTA, and have then determined the polysaccharide-precipitating ability by adding glycogen, glycogen phosphate, or a lipopolysaccharide of *S. flexneri*. At a molar ratio of EDTA to manganese of 3×10^3, an inhibition of approximately 11 per cent was observed with each of the polysaccharides. Higher ratios (up to 10^5) did not give rise to additional inhibition of the precipitin reaction. These results parallel the findings of others[27], who have reported that calcium ions cannot be removed readily from *alpha*-amylase preparations by chelating agents.

The limited action of EDTA on the precipitin reaction suggests that either the cation is not readily accessible to chelation or a protein–metal–EDTA complex is formed that is still partially functional in the precipitin reaction. It may be possible that EDTA alters the aggregative properties of the complex by some as-yet-unknown mechanism. On the other hand, a favorable equilibrium may exist between metal–protein and metal–EDTA complexes that allows most of the glycogen-binding sites on concanavalin A molecules to remain active. The latter explanation seems implausible, as increasing concentrations of EDTA did not result in corresponding decreases in turbidimetric readings, and molar ratios of EDTA to manganese of 10^5 or greater did not inhibit the precipitin reaction to any greater degree than did much lower concentrations of EDTA.

In earlier studies, it was noted that guanidine hydrochloride and urea competitively inhibit[14] the precipitation of polysaccharides and polyelectrolytes with concanavalin A at pH 5.5. The data in Fig. 2 show that, in addition to guanidine hydrochloride and urea, formamide and 1,1,3,3-tetramethylurea also inhibit the glycogen–concanavalin A reaction at pH 7.0. Methyl sulfoxide and 1,1,3,3-tetramethylguanidine were also tested; however, these potential inhibitors tended to promote protein aggregation, which interfered with the quantitative precipitin reaction. In contrast, a model peptide, namely *N*-methylacetamide, did not inhibit the concanavalin A–glycogen precipitin reaction. The data in Fig. 2 also show that, on a molar basis, guanidine hydrochloride is the most effective inhibitor, whereas a hydrophobic, substituted urea, tetramethylurea, is relatively ineffective as an inhibitor of the precipitin reaction.

The action of the urea–guanidinium class of protein denaturants is not yet completely understood. Several workers believe that these compounds act on proteins by forming hydrogen bonds directly to peptide bonds or to peptide carbonyl groups[28,29]. Others believe that compounds of the urea class change the conformation of proteins

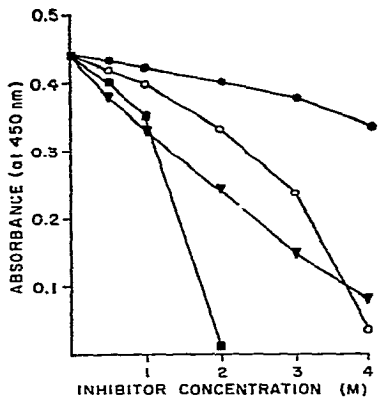


Fig. 2. Effect of protein denaturants on the glycogen–concanavalin A precipitin-reaction. All reaction mixtures contained 0.8 mg of glycogen and 4.0 mg of concanavalin A in 6.0 ml of 0.1M NaCl, 0.05M sodium phosphate buffer, pH 7.0, plus the indicated molarity of the following inhibitors: 1,1,3,3,-tetramethylurea (—●—), urea (—○—), formamide (—▼—), and guanidine hydrochloride, (—■—).

indirectly by changing the structure of the water surrounding the protein[30,31]. In the present study, relatively low concentrations of guanidine hydrochloride, formamide, or urea were needed for initiation of the inhibition of complex-formation (see Fig. 2), whereas much higher concentrations of these reagents are usually required for denaturation of proteins. Agrawal and Goldstein[32] observed that concentrated solutions of urea dissociate concanavalin A–Sephadex complexes..

Concanavalin A dissociates into subunits in the presence of 8.0M urea[15,33] It is likely that the low concentration of inhibitors used in the present study did not elicit a dissociation of concanavalin A into subunits, as 8.0M urea apparently[33]does not completely dissociate the subunit structure of concanavalin A. Assuming that compounds of the urea–guanidinium class are bound directly to proteins *via* hydrogen bonds, it would follow that inhibition of formation of concanavalin A–polysaccharide complex would be a result of rupture of hydrogen bonds between the protein and the polysaccharide. Electrostatic forces are, most probably, not involved in formation of glycogen–concanavalin A complexes. as the precipitin reaction is insensitive to salt over a wide range of concentrations of sodium chloride[3,15]. So and Goldstein[10] and Doyle *et al.*[14] have previously emphasized the importance of hydrogen bonding in concanavalin A–polysaccharide complexes. In contrast, Torii *et al.*[34] have suggested that hydrophobic bonding is important in such antigen–antibody reactions as the dextran–antidextran interaction. The fact that 1,1,3,3-tetramethylurea is not so effective an inhibitor as urea suggests that hydrophobic binding is probably not involved directly in the concanavalin A–glycogen precipitin reaction.

The data in Figs. 3A and 3B show that both *alpha*-amylase (85% glycogenolysis) and *beta*-amylase (30% glycogenolysis) hydrolyze the bound glycogen of glycogen–concanavalin A complexes to the same extent as noted for the respective control samples of glycogen. In contrast, following *alpha*- and *beta*-amylolysis of glycogen in the presence and absence of concanavalin A, the degree of inhibition of the pre-

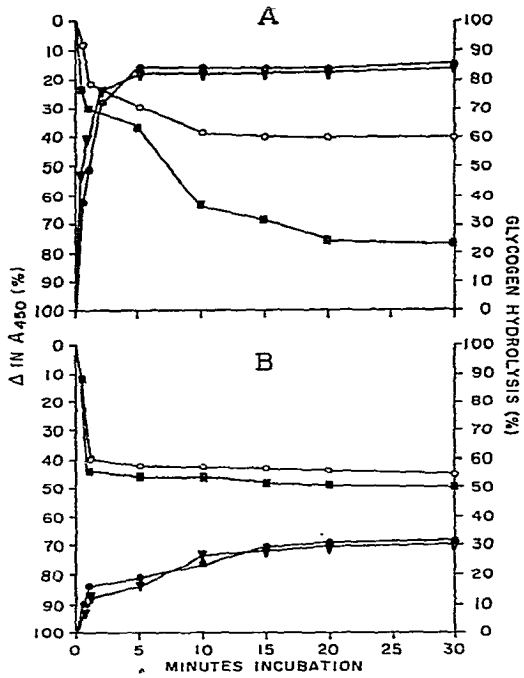


Fig. 3. Effect of amylolysis on glycogen–concanavalin A complexes. A, *alpha*-Amylase hydrolysis, B, *beta*-amylase hydrolysis. Turbidity (—○—) and amylolysis (—●—) of glycogen–concanavalin A complexes during incubation with the respective amylase; turbidity (—■—) and amylolysis (—▼—) of glycogen–concanavalin A complexes following initial exposure of glycogen to the respective amylase.

cipitin reactions was found to be dependent on the specific limit-dextrins that are produced by the respective amylases. After hydrolysis of the glycogen–concanavalin A complex and the glycogen control with *alpha*-amylase for 20 min, the diminution in the turbidities of their concanavalin A complexes were 40 and 75%, respectively (see Fig. 3A). Thus, although concanavalin A does not alter the rate or degree of glycogenolysis by *alpha*-amylase, it does partially protect the ability of the amylolytic end-products to form a precipitate with protein. No similar, differential effects on complex formation were detected for the hydrolytic end-products after *beta*-amylolysis of glycogen in the presence of concanavalin A (see Fig. 3B).

The persistence of glycogen–concanavalin A binding after hydrolysis of glycogen or its complex with concanavalin A by *alpha*- or *beta*-amylase shows that these limit dextrins are capable of eliciting a precipitin reaction with concanavalin A. Previously, Cifonelli *et al.*[12] and Manners and Wright[13] reported relatively high glycogen values

for *beta*-limit dextrins from glycogen. With respect to *alpha*-amylolysis, the results in Fig. 3A are surprising, in that *alpha*-amylase should release from glycogen monomeric and oligomeric sugars that should be capable of inhibiting convanavalin A–D-glucan complex-formation[2]. It is possible that insufficient quantities of the end-products of low molecular weight were released to inhibit the precipitin reaction completely. As *alpha*-amylase acts primarily on the internal structure of glycogen, it seems reasonable to assume that the end-groups of glycogen bound to concanavalin A would remain intact during amylolysis. Based on the fact that limit dextrins elicit precipitin reactions with concanavalin A[12,13], the data in Fig. 3a suggest that the end-products of *alpha*-amylolytic degradation of glycogen–concanavalin A complexes are, most probably, more complex than, and possibly of a different molecular-weight class from, the end-products of glycogenolysis in the absence of the protein.

The action of *beta*-amylase on glycogen involves the nonreducing end-groups, and concanavalin A might be expected to inhibit its action. This was not the case, however, because *beta*-amylolysis of glycogen proceeded at equal rates, and to the same extent, in the presence and absence of concanavalin A. The similar degrees of inhibition of the precipitin reactions (50% decreases in turbidities) after exposure of glycogen, in the presence of concanavalin A, to *beta*-amylase suggest that the *beta*-limit dextrins are identical.

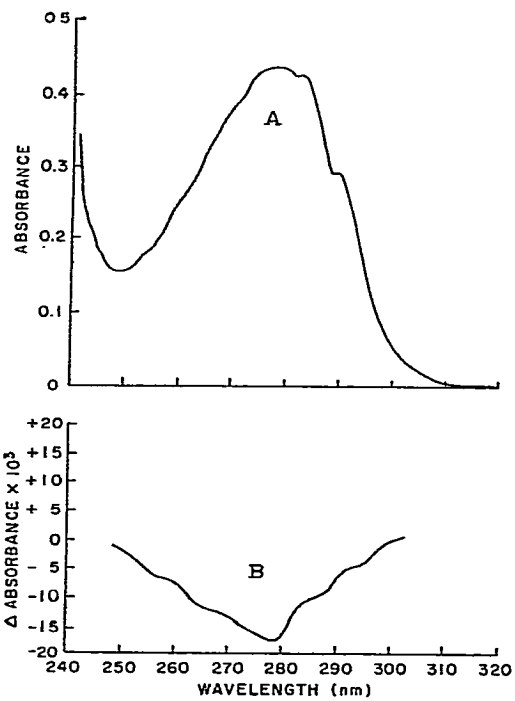


Fig. 4. D-Glucose-induced, ultraviolet difference-spectrum in concanavalin A. Ultraviolet spectrum of 5.3×10^{-6}M concanavalin A in M NaCl (curve A); D-glucose-induced difference spectrum in 2.9×10^{-5}M concanavalin in A in M NaCl (curve B). The concentration of D-glucose was 2.8×10^{-4}M.

Certain small molecules are known to alter the conformations of proteins. For example, cycloamyloses partially prevent the proteolysis of potato phosphorylase[35]. Haptens are thought to change the conformation of antibodies[36], and specific dyes are known to render serum albumin resistant to proteolysis[37]. In the present study, experiments were designed to show whether the binding of D-glucose to concanavalin A could be detected by ultraviolet difference-spectra and by optical rotatory dispersion studies. The data in Fig. 4 show that D-glucose induces an ultraviolet difference-spectrum in concanavalin A. Because of the number of inflection points in the difference spectrum, at approximately 256, 264, 278, 287, and 294 nm, it would appear that the environments of phenyl-L-alanine, L-tyrosine, and L-tryptophan are altered[38]. These environmental changes may be caused by small displacements of the chromophores from their immediate environments, or may reflect an overall, conformational alteration of the protein. In addition, D-glucose may bind directly to these residues. It may also be possible that D-glucose alters the aggregative properties of concanavalin A.

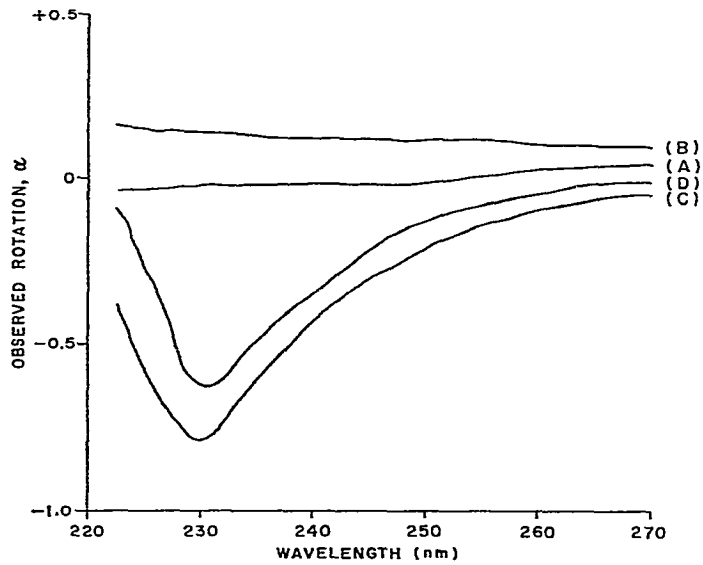


Fig. 5. Effect of D-glucose on the rotatory dispersion properties of concanavalin A. Solvent baseline, M NaCl–0.02M phosphate buffer, pH 7.0 (curve A); 1% D-glucose (curve B); 1% concanavalin A (curve C); 1% D-glucose–1% concanavalin A (curve D).

to give rise to the observed difference-spectrum. So and Goldstein[3] have noted a D-glucose-induced stabilization of concanavalin A solutions. Other studies show that D-glucose alters the susceptibility of L-tyrosyl residues in concanavalin A to nitration with tetranitromethane[39]. Supporting these interpretations are the results of Poretz and Goldstein[40], who have presented data suggesting that hydrophobic groups are in close proximity to the sugar-binding sites on concanavalin A.

The specific rotation of an optically active solute is a constitutive property, and, in the absence of interactions, is additive[18]. However, failure to observe non-

additivity does not exclude the possibility that interactions may have occurred. The data in Fig. 5 show the observed rotations of concanavalin A–D-glucose mixtures over the wavelength range of 270 to 220 nm. The relationship:

$$\frac{\alpha_{(\text{concanavalin A})}}{\alpha_{(\text{concanavalin A + D-glucose})} - \alpha_{(\text{D-glucose})}}$$

should be greater or less than unity if a D-glucose-induced conformational change in concanavalin A occurs (as the solute concentrations were the same, namely, 1%, it is not necessary to convert observed rotations into specific rotations). Results based on nine independent experiments show that the ratio at 233 nm is 0.94 $\pm$0.12. The data indicate that this method is not sensitive enough to detect small, D-glucose-induced, conformational changes in concanavalin A. Results after denaturation with 8.0M urea and 0.1M sodium hydroxide show a ratio of 1.02 $\pm$0.05. Similar results were obtained from the visible region.

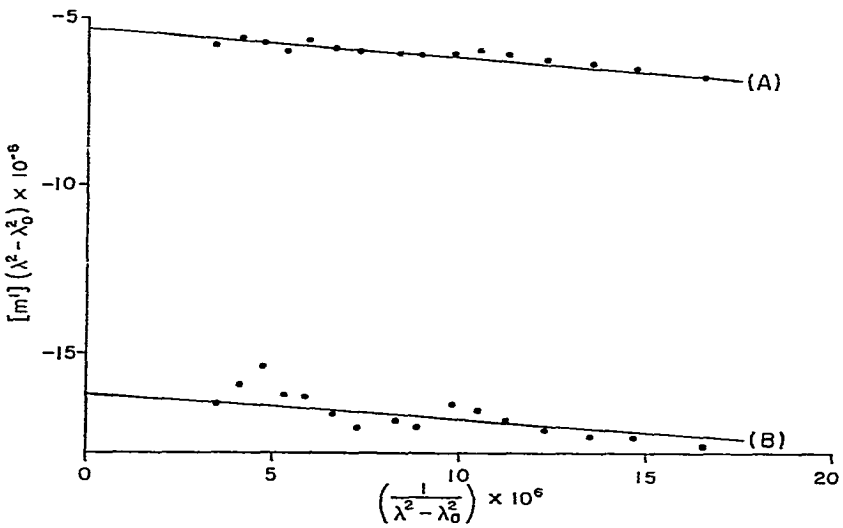


Fig. 6. Moffitt–Yang plots of concanavalin A in buffer and urea. Concanavalin A (1%) in M NaCl–0.02M phosphate buffer, pH 7.0 (curve A); 1% concanavalin A in M NaCl–0.02M phosphate buffer plus 8.0M urea (curve B). Data were taken from the region of 350–650 nm.

In Fig. 6, Moffitt–Yang plots of concanavalin A in buffer and in urea are given. The data were corrected for index of refraction, but not for dispersion. The slope of the curve for concanavalin A (see Fig. 6a) gave a b_0 value of -46.6, which corresponds to a helix content of 7.5%. This calculation is based on a value of -620 for the b_0 value of a completely helical polymer[19]. The a_0 value, which is obtained from the y intercept, was -124. In 8.0M urea, the b_0 value of concanavalin A was -34.0, which corresponds to a helical content of approximately 5.5% and an a_0 value of -350.

The *alpha*-helix content of a protein can be estimated either from b_0 values,

$[R']_{233}$, or $[R']_{198}$, which correspond to the trough and peak mean residue rotations of the peptide bond[19]. Data were obtained for $[R']_{233}$ only, as the $[R']_{198}$ value was inaccessible with the instrumentation available. The value of $[R']_{233}$ for concanavalin A was found to be $-1830 \pm 10°$. This value is low, and is characteristic of proteins having a low helical content (a value of $-11{,}000°$ corresponds to a completely helical polymer)[20]. For the calculation of the helical content, it is necessary to determine $[R']_{233}$ in the completely denatured state, as well as in the native state. However, estimates of $[R']_{233}$ were inconclusive, because of the fact that concanavalin A in 8.0M urea exhibits a negative b_0 value, which indicates only a partial denaturation of concanavalin A. It is possible that the o.r.d. measurements in 8.0M urea were biased by aggregation (*cf.* ref. 33), although Quiocho *et al.*[41] observed a negative b_0 value for carboxypeptidase in 8.0M urea. Thus, it is believed that $[R']_{233}$ data for concanavalin A in 8.0M urea are unreliable. However, from the $[R']_{233}$ data for concanavalin A in buffer, it may be concluded that the protein has a low content of helices. Others[42,43] have recently criticized the use of absolute b_0 values as a criterion for determining helicities, especially when low b_0 values are obtained.

It is well known that, in some proteins, Cotton effects due to aromatic side-chains can affect the shape of the visible and near-ultraviolet o.r.d. curves that are generally believed to result from peptide-bond interactions[44]. Several investigators have studied changes in the rotatory properties of proteins in the presence of small ligands[45,46]. In all cases, there is little or no change in the $[R']_{233}$ value when the ligand interacts with the protein. Fasella and Hammes[46] pointed out the insensitivity of the measurement in determining small conformational changes. The data reported here for concanavalin A–D-glucose support this conclusion. At present, we are examining the circular dichroism band of concanavalin A caused by L-tyrosine and L-tryptophan residues. This method should be more sensitive in detecting concanavalin A–D-glucose interactions if such interactions give rise to asymmetry around the L-tyrosine and L-tryptophan residues. The rotatory dispersion and ultraviolet difference-spectra reported here are strikingly parallel to those for biotin–avidin systems. Although biotin induces an ultraviolet difference-spectrum in avidin[47], there are no changes in its rotatory properties[48].

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1,6-ANHYDRO-4-*O*-BENZYL-β-D-GLUCOPYRANOSE

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ABSTRACT

Benzylation of phenyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranoside (**1**) with benzyl bromide and silver oxide in *N*,*N*-dimethylformamide gave 57% of phenyl 2,3,6-tri-*O*-acetyl-4-*O*-benzyl-β-D-glucopyranoside (**2**). Deacetylation of **2** and treatment with hot alkali converted **2** into crystalline 1,6-anhydro-4-*O*-benzyl-β-D-glucopyranose (**3**) in high yield. Methylation or acetylation of **3** followed in each case by hydrogenation over palladium gave 1,6-anhydro-2,3-di-*O*-methyl- and 2,3-di-*O*-acetyl-β-D-glucopyranose (**4** and **8**), respectively. Hydrolysis of **4** and treatment of the product with aniline gave the known 2,3-di-*O*-methyl-*N*-phenyl-D-glucosylamine. Methylation of **8** with diazomethane followed by deacetylation yielded 1,6-anhydro-4-*O*-methyl-β-D-glucopyranose (**11**).

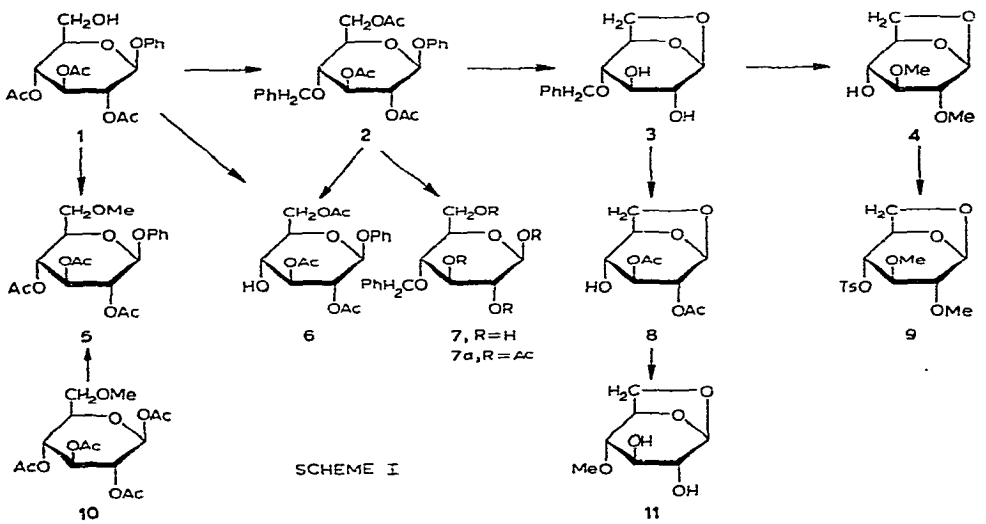
INTRODUCTION

In our investigation of the mechanism of acid-catalyzed polymerization of 1,6-anhydro-β-D-glucopyranose (levoglucosan), we had need for 1,6-anhydrokojibiose and 1,6-anhydrosophorose. For this purpose, we have prepared and characterized 1,6-anhydro-4-*O*-benzyl-β-D-glucopyranose (**3**). Treatment of **3** under Koenigs–Knorr conditions would be expected to produce principally (1→2)-linked 1,6-anhydrodisaccharides, since the hydroxyl group on C-3 of levoglucosan is much less reactive[1–4] than the hydroxyl group on C-2 or C-4. Compound **3** is also of interest, as it could probably be used to prepare D-glucans that are less highly branched than those obtained from levoglucosan.

The sequence of reactions used to synthesize and identify compound **3** is shown in Scheme I.

Treatment of phenyl 2,3,4-tri-*O*-acetyl-6-*O*-trityl-β-D-glucopyranoside with hydrogen bromide in acetic acid gave compound **1**, which crystallized in dimorphic states, one shaped as prisms having m.p. 137–138°, the other as fine needles having m.p. 143–144°. Apparently, neither of these forms corresponds to the one (m.p. 114°) previously reported[5], and furthermore, the measured specific rotation $[\alpha]_D^{25}$ −18.9 ±0.5° (*c* 2.1, chloroform) differed from the reported value $[\alpha]_D^{20}$ −28.3° (*c* 7.2, chloroform). Because of these discrepancies, and because acetyl migration could lead

to one of four different phenyl β-D-glucopyranoside triacetates, the structure of **1** was examined, and firmly established, by n.m.r. studies and by methylation. In methyl sulfoxide-d_6, the n.m.r. signal for the hydroxyl proton of **6** was a triplet at τ 5.20, which is consistent only with the presence of a primary carbinol group[6]. Methylation of **6**, under conditions where acetyl migration does not occur[7], gave phenyl 2,3,4-tri-*O*-acetyl-6-*O*-methyl-β-D-glucopyranoside (**5**). Compound **5** was identical with that prepared by standard methods from the known[7] 1,2,3,4-tetra-*O*-acetyl-6-*O*-methyl-β-D-glucopyranose (**10**).



SCHEME I

Benzylation[8] of **1** with benzyl bromide and silver oxide in *N*,*N*-dimethylformamide gave, in 57% yield, a crystalline product whose n.m.r. spectrum was consistent with a phenyl monobenzylglucoside triacetate (**2**). Preliminary results indicated that **2** was the 4-benzyl ether, as it was readily degraded in alkali to an anhydro sugar and was different from phenyl 2,3,6-tri-*O*-acetyl-3-*O*-benzyl-β-D-glucopyranoside. Hydrogenolysis of **2** over palladium gave phenyl 2,3,6-tri-*O*-acetyl-β-D-glucopyranoside (**6**), whose structure was determined by applying the methods used to determine the constitution of **1**. In this case, the n.m.r. spectrum of **6** in methyl sulfoxide-d_6 showed a doublet at τ 4.31, which confirmed the presence of a secondary hydroxyl group, and methylation of **6** with diazomethane gave[9] phenyl 2,3,6-tri-*O*-acetyl-4-*O*-methyl-β-D-glucopyranoside.

Deacetylation of **2** followed by hydrolysis in acid gave 4-*O*-benzyl-D-glucose (**7**), m.p. 138–139°, $[\alpha]_D^{22}$ +65→+53 ±1° (*c* 0.94, water). Croon and Lindberg[8] reported that the α-D anomer of this derivative has m.p. 178–179°, $[\alpha]_D^{20}$ +96→+52° (*c* 2.0, water). The n.m.r. spectrum of **7** in dry methyl sulfoxide-d_6 shows a poorly resolved doublet of doublets centered at τ 3.82 ($J_{1-OH,H-1}$ 4.5 Hz and $J_{1-OH,H-2}$ ~0.5 Hz) and a doublet at τ 3.44 ($J_{1-OH,H-1}$ 6.4 Hz). These signals were assigned[10] to the anomeric hydroxyl protons of the α- and β-D-pyranose forms, respectively, and, from their

integrated intensities, it was determined that the lower-melting form of 4-*O*-benzyl-D-glucopyranose is a mixture of 47% of the α-D and 53% of the β-D anomer. Compound **7** has infrared absorption bands at 841 and 885 cm^{-1}, which is additional evidence[11] for an anomeric mixture of glycopyranoses. Kim *et al.*[12] recently reported g.l.c. evidence for the presence of two anomeric forms in crystalline 2- and 4-*O*-benzyl-D-glucose*. The n.m.r. signal intensities for the anomeric protons of **7** were used for determining the equilibrium concentration of anomers in water at 60° (72% of the β-D). Assuming that the entropy difference between anomers of 4-*O*-benzyl-D-glucopyranose is equal to the difference of 0.87 cal/mole·degree found[13] for α- and β-D-glucopyranose, the composition of anomers of **7** in equilibrium at 25° was estimated to be 26% α-D and 74% β-D. By using the initial and equilibrium compositions and the corresponding specific rotations of the solutions, and assuming that the rotational contributions of anomers do not change with concentration, the specific rotation of the pure α-D anomer was calculated to be +95°. This value is in good agreement with the reported value, +96°. Acetylation of **7** with acetic anhydride and pyridine yielded 30% of crystalline 1,2,3,6-tetra-*O*-acetyl-4-*O*-benzyl-β-D-glucopyranose (**7a**).

Deacetylation of **2** gave phenyl 4-*O*-benzyl-β-D-glucopyranoside, which was smoothly converted in 82% yield into 1,6-anhydro-4-*O*-benzyl-β-D-glucopyranose (**3**) by heating it for 32 h at 105° in 1:2 (v/v) 2-methoxyethanol–2.6M aqueous potassium hydroxide. Methylation of **3**, followed by hydrogenation over palladium, gave crystalline 1,6-anhydro-2,3-di-*O*-methyl-β-D-glucopyranose (**4**), previously reported[14] as a syrup. Hydrolysis of **4** gave 2,3-di-*O*-methyl-D-glucose, identified by its conversion into 2,3-di-*O*-methyl-*N*-phenyl-D-glucosylamine. Also, compound **4** reacted with *p*-toluenesulfonyl chloride in pyridine to give the known[14] 1,6-anhydro-2,3-di-*O*-methyl 4-*O*-*p*-tolylsulfonyl-β-D-glucopyranose (**9**).

1,6-Anhydro-4-*O*-benzyl-β-D-glucopyranose (**3**) was acetylated and the product hydrogenated, to give 2,3-di-*O*-acetyl-1,6-anhydro-β-D-glucopyranose (**8**), which resisted all attempts at crystallization. G.l.c. and t.l.c. indicated that **8** was homogeneous, and its n.m.r. spectrum showed only two signals for acetyl protons, at τ 7.90 and 7.88. The spectrum of 2,3,4-tri-*O*-acetyl-1,6-anhydro-β-D-glucopyranose shows three signals for acetyl, at τ 7.90, 7.88, and 7.84, and, therefore, the signal at 7.84 probably arises from the acetoxy group on C-4 (and not[15] that on C-2). The diacetate **8** was methylated with diazomethane, and the product deacetylated to give, in good yield, 1,6-anhydro-4-*O*-methyl-β-D-glucopyranose (**11**), which has been prepared in this laboratory by another route and fully characterized[9].

EXPERIMENTAL

General. — Melting points were determined on a Thomas–Hoover, "Unimelt",

*Unfortunately, these authors did not give the physical constants they observed for these benzyl ethers. They also reported that 3-*O*-benzyl-D-glucopyranose consists solely of the β-D form; this was confirmed by the n.m.r. spectrum of 3-*O*-benzyl-D-glucose in methyl sulfoxide-d_6, since only one signal (doublet at τ 3.43, $J_{1-OH,H-1}$ 6.2 Hz) was observed for the proton of the anomeric hydroxyl group.

melting-point apparatus, and are uncorrected. Solutions were evaporated under diminished pressure below 50°. Thin-layer chromatograms were obtained on microscope slides coated with silica gel G (Brinkmann Instruments, Inc., Great Neck, N. Y.) and, after being developed with appropriate solvents, the components were visualized by spraying the plate with a 5% solution of sulfuric acid in methanol and charring the zones on a hot plate. Column chromatography was performed with silica gel (Davison, grade 950, 60–200 mesh) as adsorbent, with 1–2% loading. Fractions were collected at 5-min intervals, at a flow-rate of 1–2 ml/min. I.r. spectra of solids were obtained with a Perkin–Elmer Model 21 recording i.r. spectrophotometer. N.m.r. spectra of protons (^{1}H) were recorded at 25° with a Varian A-60 n.m.r. spectrometer. Chemical shifts are reported as τ values in p.p.m. from the reference signal of either tetramethylsilane (TMS) in chloroform-*d* and methyl sulfoxide-d_6, or of sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) in deuterium oxide. In parentheses after the chemical shift are given the integrated intensities of the signal and an abbreviation for singlet (s), doublet (d), triplet (t), quartet (q), and multiplet (m). Specific optical rotations were determined with a Zeiss–Winkel polarimeter.

Phenyl 2,3,4-tri-O-acetyl-β-D-glucopyranoside (**1**). — Phenyl 2,3,4-tri-*O*-acetyl-6-*O*-trityl-β-D-glucopyranoside (9.8 g, 15.7 mmoles) was dissolved in warm glacial acetic acid (25 ml), and the mixture was cooled to 5° in an ice–water bath. Cold, 32% hydrogen bromide in acetic acid (2.9 ml) was added, the mixture was shaken for 2–3 min, and the insoluble bromotriphenylmethane was removed by filtration through a coarse, sintered-glass funnel. After the precipitate had been washed with cold acetic acid, the filtrate was poured onto ice–water (50 g), and the aqueous phase was extracted three times with chloroform. The chloroform layer was successively washed with water, aqueous sodium hydrogen carbonate solution, and water, and dried (anhydrous sodium sulfate). The chloroform was evaporated, and the residue was crystallized from isopropyl alcohol to give 4.6 g of **1** (77%) having m.p. 135–138°. One recrystallization from the same solvent gave **1** having a constant m.p. (137–138°), $[\alpha]_D^{25}$ −19° (*c* 2.1, chloroform) [lit.[5] m.p. 114°, $[\alpha]_D^{20}$ −28.3° (*c* 7.2, chloroform)]. After melting at 137–138°, **1** frequently resolidified. The higher-melting, dimorphic form was isolated by crystallization of **1** from the melt; it had a sharp melting-point at 143–144°, and m.p. 137–138° mixed with the other form. The form melting at 143–144° was reconverted into the other form by crystallization from isopropyl alcohol. Both forms gave identical n.m.r. spectra in chloroform-*d*: 7.97, 7.95, 7.94 (each 3 protons, s, acetyl); 7.79 (1 proton, t, OH); 6.55–6.09 (3 protons, m); 5.00–4.60 (4 protons, m); 3.13–2.50 (5 protons, m, aromatic).

Anal. Calc. for $C_{18}H_{22}O_9$: C, 56.54; H, 5.80. Found: C, 56.42; H, 5.72.

Phenyl 2,3,4-tri-O-acetyl-6-O-methyl-β-D-glucopyranoside (**5**). — *a.* To 2.0 g of 1,2,3,4-tetra-*O*-acetyl-6-*O*-methyl-β-D-glucopyranose[7] were added 2.0 g of phenol and 0.02 g of *p*-toluenesulfonic acid monohydrate[16]. The mixture was heated under diminished pressure for 1 h on a steam bath, cooled, and dissolved in benzene. The benzene layer was successively washed with water, four times with M aqueous sodium hydroxide, and water, dried (anhydrous sodium sulfate), and evaporated, to give 1.53 g

(70%) of a syrup that contained no starting material but was a mixture of α-D- and β-D-glycosides, as evidenced by t.l.c. with 1:1 petroleum ether (b.p. 60–110°)–isopropyl ether as developer. The β-D-glucoside crystallized at −5° from absolute ethanol to give 1.0 g (46%) of crude material. Two recrystallizations from absolute ethanol gave pure material, m.p. 92–93°.

b. Compound **1** (1.0 g, 2.62 mmoles) was dissolved in dichloromethane (15 ml), and the solution was cooled to −5°. After boron trifluoride etherate (0.04 ml) had been added, a solution of diazomethane in dichloromethane[17], predried with potassium hydroxide pellets and cooled to 0°, was added slowly, with stirring, until a yellow-green color persisted in the reaction mixture. Polymethylene was removed by filtration, and the filtrate was washed with aqueous sodium hydrogen carbonate solution and water, dried (sodium sulfate), and evaporated, and the residue was crystallized from ethanol at −5°. The first crop of crystals weighed 0.83 g (80%) and had m.p. 90–93°. Recrystallization from ethanol gave pure **5**, m.p. 92–93°, mixed m.p. with a sample prepared by procedure *a*, 92–93°; $[\alpha]_D^{23}$ −15° (*c* 0.8, chloroform). The compounds had identical n.m.r. spectra in chloroform-*d*; 7.98 (9 protons, s, acetyl); 6.64 (3 protons, s, methoxyl); 6.53–6.10 (3 protons, m); 5.00–5.40 (4 protons, m); and 6.87–7.50 (5 protons, m, aromatic).

Anal. Calc. for $C_{19}H_{24}O_9$: C, 57.58; H, 6.11. Found: C, 57.34; H, 6.02.

Phenyl 2,3,6-tri-O-acetyl-4-O-benzyl-β-D-glucopyranoside (**2**). — Compound **1** (20 g, 57.5 mmoles) was dissolved in a mixture of dry *N,N*-dimethylformamide (150 ml) and benzyl bromide (60 ml). The mixture was stirred vigorously while silver oxide (60 g) was added portionwise during 1 h, and stirring was continued for an additional 4 h at room temperature. The silver salts were removed by filtration, and the salts were washed with *N,N*-dimethylformamide (50 ml) and chloroform (100 ml). The filtrate was added to 2 liters of 2% aqueous potassium cyanide solution, and this mixture was extracted three times with a total of a liter of chloroform. The extract was washed three times with water, dried (anhydrous sodium sulfate), and evaporated to a thin syrup. Benzyl bromide and benzyl alcohol were removed by evaporation at 70–75°/0.4–0.7 mm. The crystalline residue was dissolved in hot abs. ethanol (100 ml), and the solution was filtered through a bed of charcoal. Examination of the reaction products by t.l.c., with pure isopropyl ether as developer, showed one major (R_F 0.4) and one minor product (R_F 0.6), and no starting-material. The major product crystallized at 5° to give 14.1 g (57%) of crude material, m.p. 106–112°. Two recrystallizations from ethanol gave 11.0 g of pure **2**, m.p. 114–115°, $[\alpha]_D^{26}$ −31° (*c* 2.0, chloroform); n.m.r. data (chloroform-*d*): 8.01 (3 protons, s, acetyl); 7.97 (6 protons,s, acetyl); 6.31–6.15 (2 protons, m); 5.76–5.60 (2 protons, m); 5.38 (2 protons, s,benzylic); 5.00–4.50 (3 protons, m); 3.18–2.55 (10 protons, m, aromatic).

Anal. Calc. for $C_{25}H_{28}O_9$: C, 63.55; H, 5.97. Found: C, 63.50; H, 6.02.

Phenyl 2,4,6-tri-O-acetyl-3-O-benzyl-α- and -β-D-glucopyranoside. — Benzylation[8] of 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose (3.0 g) with benzyl bromide and silver oxide in *N,N*-dimethylformamide, followed by hydrolysis, gave 3-*O*-benzyl-D-glucose, 1.3 g (44%). The product crystallized from acetone at 5° as needles having

a constant m.p. (125–126°), $[\alpha]_D^{25}$ +26→+43 ±1° (*c* 1.2, water) [lit.[18] 127–128°, $[\alpha]_D$ +29.1° (*c* 7.8, water)]. Apparently, 3-*O*-benzyl-β-D-glucopyranose solidifies in different crystalline forms, since other workers[8,19] have found that this derivative has m.p. 137–138°, $[\alpha]_D^{25}$ +16.1→+41.8° (water). 1,2,4,6-Tetra-*O*-acetyl-3-*O*-benzyl-β-D-glucopyranose (530 mg, 1.21 mmoles), prepared[20] by acetylation of 3-*O*-benzyl-β-D-glucopyranose, was fused with 1 g of phenol and 0.02 g of zinc chloride under diminished pressure. The reaction mixture was processed in the usual way, to give 0.28 g of the syrupy phenyl α-D- and β-D-glycoside acetates, which were separated by preparative t.l.c. with isopropyl ether as developer. The faster-moving component was the α-D anomer, which crystallized from absolute ethanol; yield 0.08 g (22%), m.p. 121–122°, $[\alpha]_D^{27}$ +109° (*c* 1.0, chloroform). The slower-moving component was crystallized from ethanol to give 0.134 g (34%) of pure phenyl 2,4,6-tri-*O*-acetyl-3-*O*-benzyl-β-D-glucopyranoside; m.p. 114–115°, mixed m.p. with compound **2**, 82–93°; $[\alpha]_D^{27}$ −31° (*c* 1.9, chloroform).

Anal. Calc. for $C_{25}H_{28}O_9$: C, 63.55; H, 5.97. Found for the α-D anomer: C, 63.49; H, 5.97. Found for the β-D-anomer: C, 63.34; H, 5.97.

*Phenyl 2,3,6-tri-*O*-acetyl-β-D-glucopyranoside* (**6**). — Compound **2** (1 g, 2.12 mmoles) was hydrogenated in 10 ml of glacial acetic acid over 1.0 g of 5% palladium-on-charcoal. The mixture was stirred for 24 h at room temperature under hydrogen (1 atm.), and the catalyst was removed by filtration. The filtrate was freeze-dried, and the residue was crystallized from absolute ethanol at −10°. The total yield (first two crops of crystals) was 0.56 g (69%), m.p. 132–134°. Two recrystallizations from ethanol gave **6** having a constant m.p. of 134.5–135.5°, $[\alpha]_D^{24}$ −56° (*c* 3.7, chloroform); lit.[5] m.p. 130°, $[\alpha]_D^{18}$ −52.2° (*c* 4.0, chloroform).

Anal. Calc. for $C_{18}H_{22}O_9$: C, 56.54; H, 5.80. Found: C, 56.54; H, 5.80.

Compound **6** was identical in all respects with the product obtained by the alkali-catalyzed rearrangement of phenyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranoside (**1**). Methylation of **6** with diazomethane–boron trifluoride gave phenyl 2,3,6-tri-*O*-acetyl-4-*O*-methyl-β-D-glucopyranoside, which was also prepared from the known 1,2,3,6-tetra-*O*-acetyl-4-*O*-methyl-β-D-glucopyranose[9].

*Phenyl 4-*O*-benzyl-β-D-glucopyranoside, 4-*O*-benzyl-D-glucopyranose* (**7**), *and 1,2,3,6-tetra-*O*-acetyl-4-*O*-benzyl-β-D-glucopyranose* (**7a**). — Compound **2** (1.5 g, 31.8 mmoles) was deacetylated in the usual way with sodium methoxide in methanol. The product was crystallized from abs. ethanol at −10° to give 1.05 g (91%) of material having m.p. 117–119°, $[\alpha]_D^{26}$ −16° (*c* 3.2, acetone).

To a solution of phenyl 4-*O*-benzyl-β-D-glucopyranoside (0.73 g, 2.12 mmoles) in *p*-dioxane (20 ml) was added 4.0% aqueous sulfuric acid solution (40 ml), and the mixture was heated for 4 h on a steam bath. The solution was cooled, the acid was neutralized with barium carbonate, and the salts were removed by filtration. The filtrate was evaporated to dryness, the residue was extracted with hot, absolute ethanol, and the extract was evaporated to a syrup (0.77 g), which was purified by column chromatography on 70 g of silica gel, with 15:1 (v/v) ethyl acetate–methanol as eluant. Two fractions were obtained; the first was an oil containing mainly phenol, whereas

the second was a crystalline solid, yield 0.51 g (89%). The crystals were dissolved in hot acetone, and the solution was filtered through a bed of charcoal; on cooling at −10°, 4-*O*-benzyl-D-glucose (7) crystallized as short needles, m.p. 138–139°, $[\alpha]_D^{22}$ +65→+53 ±1° (*c* 0.94, water); n.m.r. data (methyl sulfoxide-d_6): 6.9–6.2 (6 protons, m), 5.80–5.45 (2 protons, m), 5.34, 5.17 (each 1 proton, s, benzylic), 5.30–4.95 (2 protons, m), 3.82 (0.47 proton, doublet of doublets, anomeric hydroxylproton of α-D anomer), 3.44 (0.53 proton, d, anomeric hydroxyl proton of β-D-form), 2.68 (5 protons, s, aromatic); at equilibrium in D_2O at 60°: 6.80–6.00 (6 protons, m), 5.40 (0.72 proton, d, anomeric hydrogen atom of β-D form, $J_{1,2}$ 7.8 Hz), 5.28, 5.22 (each 1 proton,s, benzylic), 4.78 (0.28 proton,d, anomeric hydrogen atom of α-form, $J_{1,2}$ 3.2 Hz), 2.58 (5 protons, s, aromatic).

Anal. Calc. for $C_{13}H_{18}O_6$: C, 57.77; H, 6.71. Found: C, 57.56; H, 6.71.

Compound 7 was acetylated with acetic anhydride and pyridine in the usual way. Crystallization of the product from ethanol gave 30% of 1,2,3,6-tetra-*O*-acetyl-4-*O*-benzyl-β-D-glucopyranose (7a), m.p. 108–109°, $[\alpha]_D^{22}$ 0 ±1° (*c* 0.9, chloroform). The n.m.r. (chloroform-*d*) signal of H-1 was at τ 4.29, $J_{1,2}$ 7.6 Hz, consistent[21] with the assigned β-D anomeric configuration.

Anal. Calc. for $C_{21}H_{26}O_{10}$: C, 57.53; H, 5.98. Found: C, 57.54; H, 5.88.

1,6-Anhydro-4-O-benzyl-β-D-glucopyranose (3). — To 3.2 g (9.25 mmoles) of phenyl 4-*O*-benzyl-β-D-glucopyranoside in a Teflon-lined, bomb calorimeter (Parr Instrument Co.) were added 17 ml of 2-methoxyethanol and 33 ml of 2.6M aqueous potassium hydroxide solution. The mixture was heated for 32 h at 105°, whereupon, the phenyl glycoside (R_F 0.62) was converted into a single new compound (R_F 0.37), as indicated by t.l.c. with 1:1 isopropyl ether–ethyl acetate as irrigant. The cooled solution was rendered neutral with 1.3M aqueous sulfuric acid, and evaporated to dryness, and the residue was extracted three times with hot abs. ethanol. The ethanolic extract was evaporated to dryness, the residue was dissolved in 110 ml of 50% aqueous ethanol, and the solution was passed through 50 ml of Amberlite MB-3 (H^+,OH^-) resin. The resin was washed with 125 ml of 50% aqueous ethanol, and the total effluent was evaporated to a colorless syrup (2.4 g). A small portion of the syrup was distilled at 130–135°/0.15–0.2 mm in a micro-sublimation apparatus. The distillate crystallized. The compound was crystallized from ethyl ether, and gave 1.55 g (65%) of material having m.p. 53–55°. A second crop (0.4 g) brought the total yield of 3 to 1.95 g (82%). One recrystallization from ethyl ether gave pure 3, m.p. 53–54°, $[\alpha]_D^{23}$ −43° (*c* 1.15, ethanol).

Anal. Calc. for $C_{13}H_{16}O_5$: C, 61.89; H, 6.39. Found: C, 62.03; H, 6.33.

1,6-Anhydro-2,3-di-O-methyl-β-D-glucopyranose (4). — A solution of compound 3 (0.47 g, 1.86 mmoles) in *N*,*N*-dimethylformamide (15 ml) and methyl iodide (5 ml) was stirred at room temperature while silver oxide (3.5 g) was added portionwise during 8 h. Stirring was continued for 26 h, and the reaction was processed in the usual way to give 0.48 g of a syrup that was chromatographically pure, as indicated by t.l.c. The product resisted attempts at crystallization, and column chromatography on 25 g of silica gel, with 4:1 chloroform–ethyl acetate as the devel-

oper, again yielded a syrup (0.34 g). This syrup was hydrogenated (1 atm) at room temperature in 3 ml of ethyl acetate over 0.3 g of 10% palladium-on-charcoal. After 24 h, the catalyst was removed by filtration, and the filtrate was evaporated to a syrup, yield 0.23 g (100%), which was purified by column chromatography on silica gel with ethyl acetate as the developer. The eluate containing **4** was evaporated to a syrup that crystallized spontaneously, yield 0.15 g (66%). The crystals were dissolved in ethyl ether, the solution was filtered through a bed of charcoal, and ligroine was added to faint turbidity. The solution was nucleated, and kept at −10°, to give pure **4** (0.10 g), m.p. 43–45°, $[\alpha]_D^{23}$ −87° (*c* 0.9, chloroform).

Anal. Calc. for $C_8H_{14}O_5$: C, 50.52; H, 7.42. Found: C, 50.55; H, 7.49.

Compound **4** (36 mg, 0.19 mmole) was heated for 2 h in a sealed tube at 100° with 4.0 ml of 2M aqueous hydrochloric acid. The mixture was evaporated to dryness at 35°, to give an acid-free residue (41 mg). The hydrolyzate was examined by paper chromatography, with water-saturated butanone as irrigant; the only product detectable with *p*-anisidine–hydrogen chloride spray[22] had R_F 0.2, and was indistinguishable from authentic 2,3-di-*O*-methyl-D-glucose. The hydrolyzate was subjected to preparative paper-chromatography in the same solvent system, and 25 mg (63%) of pure 2,3-di-*O*-methyl-D-glucose was isolated. This D-glucose derivative was refluxed for 5 h with freshly distilled aniline (0.3 ml) in methanol (2.5 ml). After being kept overnight, the solution was concentrated, the last traces of aniline were removed by co-evaporation with methanol and toluene, and the orange-colored syrup (37 mg) was crystallized from methanol–ether–petroleum ether (b.p. 30–60°). One recrystallization gave 13 mg (24%) of pure 2,3-di-*O*-methyl-*N*-phenyl-D-glucosylamine, m.p. and mixed m.p. with an authentic sample, 134°; lit.[23] m.p. 134°.

*1,6-Anhydro-2,3-di-*O*-methyl-4-*O*-p-tolylsulfonyl-β-*D*-glucopyranose* (**9**). — 1,6-Anhydro-2,3-di-*O*-methyl-β-D-glucopyranose (20 mg, 0.10 mmole) was added to 40 mg of *p*-toluenesulfonyl chloride in 0.75 ml of pyridine. The reaction mixture was kept overnight at room temperature, and 0.1 ml of water was added. After 1 h, the product was dissolved in chloroform, and the solution was processed in the usual way. Crystallization at −10° from acetone–ether–petroleum ether (b.p. 30–60°) gave 25 mg of **9**, m.p. 71–73°. One recrystallization gave pure **9**, m.p. 73–75°, $[\alpha]_D^{26}$ −58° (*c* 0.9, chloroform); lit.[14] m.p. 74–77°, $[\alpha]_D^{22}$ −45 ±1° (*c* 0.92, methanol).

*2,3-Di-*O*-acetyl-1,6-anhydro-β-*D*-glucopyranose* (**8**) *and 1,6-anhydro-4-*O*-methyl-β-*D*-glucopyranose* (**11**). — Compound **3** (300 mg, 1.19 mmoles) was acetylated overnight with acetic anhydride and pyridine. The reaction mixture was processed in the usual way, and the product (372 mg) was hydrogenated over 0.4 g of 10% palladium-on-charcoal in 4 ml of ethyl acetate. After 24 h, the mixture was filtered, and the filtrate was evaporated to give **8**, yield 270 mg (91%). Compound **8** could not be induced to crystallize under a variety of conditions. Chromatography on 25 g of silica gel (4:1 ethyl acetate–chloroform as developer) yielded 250 mg of syrupy **8**. The purity of **8** was shown in the following way. T.l.c. with 10:3 isopropyl ether–ethyl acetate as irrigant showed a single component having R_F 0.17 (2,3,4-tri-*O*-acetyl-1,6-anhydro-β-D-glucopyranose had R_F 0.44), and g.l.c. (injection port 200°, column

152°) with 5% SE-30 on DMCS Chromosorb W (5 ft × 1/8 in i.d.) showed one peak. The n.m.r. data for **8** in chloroform-*d* were: 7.90, 7.88 (each 3 protons, s, acetyl), 7.10–6.80 (1 proton, m, hydroxyl), 6.51–5.82 (3 protons, m), 5.50–5.14 (3 protons, m), 4.62–4.52 (1 proton, m, anomeric proton). An analytical sample of **8**, distilled at 90–100°/0.15–0.20 mm, had $[\alpha]_D^{27}$ −45° (*c* 4.9, chloroform).

Anal. Calc. for $C_{10}H_{14}O_7$: C, 48.78; H, 5.73. Found: C, 49.03; H, 5.97.

Syrupy 2,3-di-*O*-acetyl-1,6-anhydro-β-D-glucopyranose (180 mg, 0.73 mmole) was methylated[7] with diazomethane–boron trifluoride reagent. The product, yield 184 mg (90%), was deacetylated in the normal way with sodium methoxide in methanol, to produce 100 mg (78%) of a chromatographically pure (R_F 0.35, t.l.c., 9:1 ethyl acetate–methanol) 4-methyl ether (**11**), which crystallized slowly from a mixture of acetone and petroleum ether (30–60°). A total of two crops of crystals gave 65 mg (51%) of crude material, which was recrystallized once to a constant m.p. of 67–68°; $[\alpha]_D^{25}$ −64° (*c* 3.0, acetone). The mixed m.p. with a sample of **11** prepared by a different procedure[9] was 67–68°.

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Notes

The effect of a purified α-L-arabinofuranosidase on some arabinose-containing polysaccharides

For structural studies on arabinan-containing hemicelluloses and for maceration studies on plant tissues, a purified arabinan-degrading enzyme was required. Arabinan-splitting enzymes are found in many commercial enzyme preparations having high pectinase activity, but for structural studies on complex polysaccharides, only purified enzymes having known substrate specificities should be used. Pectinol R-10 (Rohm & Haas, Philadelphia) was found to contain comparatively high arabinan-splitting activity[1] and was therefore used as the starting material for the isolation of an arabinosidase. In addition to sugar-beet arabinan and phenyl α-L-arabinofuranoside[2], the following substrates were hydrolysed by the crude enzyme: *O*-carboxymethylxylan, *O*-carboxymethylcellulose, starch, methyl α- and β-D-galactopyranoside, phenyl β-D-galactofuranoside, galactomannans (guaran and carubin), pectin, and sodium polygalacturonate.

Arabinan, prepared from sugar-beet chips by extraction with hot lime water[3], and phenyl α-L-arabinofuranoside[2] were used as substrates for the arabinosidase. The hydrolysis was followed by determination of the reducing groups liberated by using the 3,5-dinitrosalicylic acid–phenol reagent[4] (for arabinan) and by measuring (at 270 nm) the phenol liberated (for the phenyl glycosides). One α-L-arabinofuranosidase unit corresponds to the amount of enzyme protein that liberates 1 μmole of phenol per minute under optimal conditions.

Isolation of the α-L-arabinofuranosidase[5] was performed by precipitation of the crude enzyme with methanol, and subsequent chromatography[6] on calcium phosphate gel. The arabinosidase fraction was entirely free from xylanase, α- and β-D-galactopyranosidase, and pectolytic activities, but contained mannanase, amylase, and traces of cellulase and β-D-galactofuranosidase; the latter, however, may be identical with the α-L-arabinofuranosidase[1].

The arabinan-splitting activity of the crude enzyme was shown to be due to the α-L-arabinofuranosidase. This enzyme splits phenyl and methyl α-L-arabinofuranoside, but neither methyl β-L-arabinofuranoside nor methyl α- and β-L-arabinopyranoside are hydrolysed. With arabinan, only monomeric arabinose was liberated from the start of the incubation. The pH optimum was 3.8 to 4.0 in McIlvaine's buffer and the isoelectric point was at pH 3.3. The enzyme was stable for 24 h at room temperature between pH 2 and 8. It was stable at 58° for 2.5 h, but after 2.5 h at 65°, it had lost 60% of its initial activity.

With sugar-beet arabinan, the purified arabinosidase hydrolysed only about

50% of the glycosidic bonds; crude Pectinol R-10, however, completely degraded the arabinan, liberating galactose and rhamnose in addition to arabinose. Even after extended incubation and repeated addition of purified arabinosidase, the hydrolysis did not go beyond 50%. The non-hydrolysed limit-arabinan was still of high molecular weight and could be isolated by chromatography on a Sephadex G-10 column. Acid hydrolysis of this fraction and subsequent paper-chromatography showed ratios of arabinose:galactose:rhamnose of 5:3:1, whereas, in the untreated arabinan, the corresponding ratios were 15:3:1. Most of the arabinose residues in the highly branched sugar-beet arabinan must therefore be situated in the outer regions of the molecule, since the enzyme starts from the non-reducing ends. Further hydrolysis of the limit-arabinan must be blocked by unknown linkages in the arabinan molecules, which can, however, be hydrolysed by other enzymes contained in the crude Pectinol R-10. Galactose or rhamnose residues, or both, might be involved in blocking the arabinosidase. It has been shown earlier[7,8] that chromatographic fractionation of sugar-beet arabinan did not yield any fractions completely free from galactose and rhamnose. The present observations support the view that galactose and rhamnose are constituents of the arabinan molecule and that these residues are concentrated in the inner parts of the molecule[3].

Other arabinose-containing substrates which were hydrolysed by the α-L-arabinofuranosidase include a synthetic arabinan prepared by Kochetkov *et al.*[9], an arabinan from white-mustard cotyledons[10], and an arabino-xylan from wheat flour[5]. The last reaction produced an insoluble xylan which precipitated from solution.

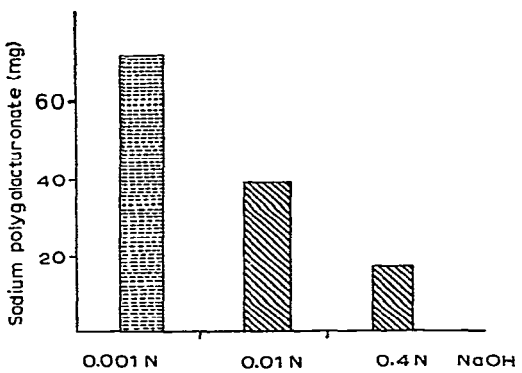


Fig. 1. Chromatography on DEAE-cellulose of a sodium polygalacturonate fraction (70 mg) before ▤ and after treatment ▨ with α-L-arabinofuranosidase (stepwise elution).

A further substrate for the arabinosidase was sugar-beet sodium polygalacturonate having a high content of arabinan. This polysaccharide ("ballast" polysaccharide) was extracted[1] from sugar beet and contained 49% of arabinan. The sodium polygalacturonate was fractionated on a column of DEAE-cellulose[8] into four fractions having different arabinan contents. The third fraction (50% of arabinan), which was eluted by 0.001N NaOH, was rechromatographed, and subsequently

incubated with the purified arabinosidase. This resulted in the splitting off of 24% of the arabinan as arabinose, whereas the polygalacturonate chain remained intact, as shown by a viscosimetric control. The enzymically treated product was rechromatographed on DEAE-cellulose, under the same conditions, and separated into two fractions. The first fraction was eluted with 0.01N NaOH and the second with 0.4N NaOH (see Fig. 1). The two fractions produced by the enzymic treatment, therefore, were more strongly retained by DEAE-cellulose than was the starting fraction. The removal of part of the arabinose from the sodium polygalacturonate makes it more acidic and therefore explains the stronger adsorption on the cellulose exchanger[8]. On the other hand, the investigations strongly support the current thinking of the existence of covalent linkages between arabinans and the polygalacturonic acid chains in pectic substances[8,11].

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Studies on the products obtained by the periodate oxidation of osazones[1]. II

In previous publications[1,2], we have described a number of simple (**1** and **4**) and mixed (**2** and **3**) bishydrazones of mesoxalaldehyde. We have now prepared some substituted compounds of types **3** and **4**, as well as some trishydrazones of types **6**, **9**, and **10**.

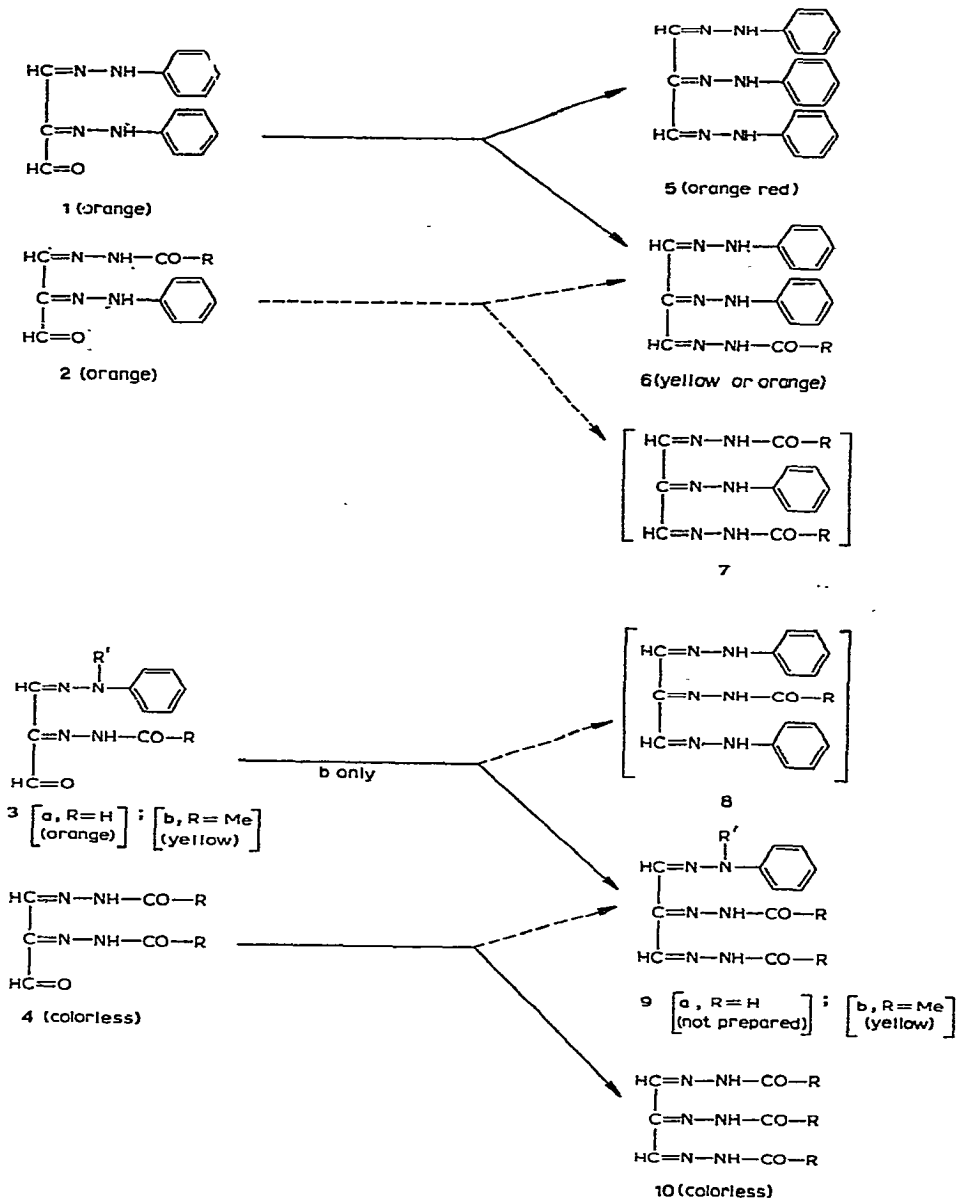
Starting with compound[1] **1** and acylhydrazines, we have prepared a number of mesoxalaldehyde 3-acylhydrazone 1,2-bis(phenylhydrazones) (**6**). These compounds are yellow or orange, and their u.v. spectra are characterized by four peaks, at 204–208, 248–257, 328–346, and 412–416 nm, in addition to shoulders at 219–237 and 277–303 nm. Their i.r. spectra showed a C=N absorption band at 1600–1603 cm^{-1} and the Amide I band at 1638–1660 cm^{-1}.

Theoretically, the trishydrazones of type **6** could also arise from mesoxalaldehyde 1-benzoylhydrazone 2-phenylhydrazone[2] (**2**, R = Ph). However, this trishydrazone (**2**) proved to be very unreactive, and, with arylhydrazines, failed to give trishydrazones of type **6** and did not react with acylhydrazines to give trishydrazones of type **7**.

A number of mesoxalaldehyde bishydrazones of types **3a** and **3b** were also prepared by the periodate oxidation of the corresponding D-*arabino*-hexosulose 2-acylhydrazone 1-phenylhydrazones[2,3] and 2-acylhydrazone 1-(2-methyl-2-phenyl)-hydrazones[3]. The bishydrazones of type **3a** were orange or brown, and their u.v. spectra showed four peaks, at 205–207, 242–244, 312–336, and 387–395 nm. Their i.r. spectra showed three characteristic bands, at 1580–1600 (C=N), 1668–1675 (CONH), and 1700–1720 cm^{-1} (HC=O). They, also, were unreactive, and failed to give trishydrazones of either type **8** or **9a**. The bishydrazones of type **3b**, on the other hand, were yellow, and had two u.v. peaks only, one at 204–206 and the other at 376–384 nm. Their i.r. spectra showed the expected C=N (1600–1615 cm^{-1}), CONH (1690 cm^{-1}), and HC=O (1700–1710 cm^{-1}) bands. Unlike **3a**, compound **3b** gave a color with ferric chloride, and reacted with the appropriate acylhydrazines to give trishydrazones of type **9b**. These were deeper in color than the parent **3b** and had, in addition to the peaks at 204–205 and 396–400 nm of the parent bishydrazone, peaks at 310 and 327 nm.

Bis(acylhydrazones) of type **4**, prepared by periodate oxidation of the parent osazones[4], were colorless, and showed three u.v. peaks, at 206, 257–287, and 322–352 nm. Their i.r. spectra showed the expected bands at 1595–1615 (C=N), 1660–1680 (CONH), and 1690–1710 cm^{-1} (HC=O). They reacted with the corresponding acylhydrazines to give trishydrazones of type **10**. Like the parent bishydrazones (**4**), they were colorless, and gave a green color with ferric chloride. Their u.v. spectra showed peaks at 204–205, 222–245, 278–305, 297–315, and 345–366 nm, and their i.r. spectra had an Amide I band at 1655–1695 cm^{-1}, in addition to the C=N band

at 1600–1608 cm^{-1}. Bishydrazones of type **4** could not be induced to react with arylhydrazines to give trishydrazones of type **9a**.



EXPERIMENTAL

I.r. spectra were recorded on a Unicam SP 200 spectrophotometer, and u.v. spectra on a Unicam SP 800 spectrophotometer.

(*A*) *Mesoxalaldehyde 2-acylhydrazone 1-phenylhydrazones* (**3a**). — The required D-*arabino*-hexosulose 2-acylhydrazone 1-phenylhydrazone[2,3] (1 mmole) was dissolved in 66% ethanol (50–100 ml), and treated at room temperature with metaperiodic acid (4.5 mmoles) in water (10 ml). The aldehyde, which separated at once as an orange or brown precipitate, was filtered off after 15 min, and crystallized from ethanol (see Table I).

(*B*) *Mesoxalaldehyde 2-acylhydrazone 1-(2-methyl-2-phenyl)hydrazones* (**3b**). — The finely powdered D-*arabino*-hexosulose 2-acylhydrazone 1-(2-methyl-2-phenyl)-hydrazone[3] (1 mmole) was suspended in water (20 ml), and treated with metaperiodic acid (4.5 mmoles) in water (10 ml). After the mixture had been kept for 24 h at room temperature, the yellow aldehyde was filtered off, washed, and crystallized from ethanol (see Table I).

Mesoxalaldehyde 1,2-bis[(p-*methoxybenzoyl*)*hydrazone*] (**4**, $R = C_6H_4OMe$-p). — A solution of D-*arabino*-hexosulose bis[(*p*-methoxybenzoyl)hydrazone][4] (1 g) in ethanol (20 ml) was treated with metaperiodic acid (2 g) in water (10 ml), and kept for 2 h at room temperature. The aldehyde that had separated was filtered off, washed, and crystallized from ethanol; needles, m.p. 240–242° (yield 0.4 g).

Anal. Calc. for $C_{19}H_{18}N_4O_5$: C, 59.7; H, 4.8; N, 14.7. Found: C, 60.0; H, 5.1; N, 14.6.

(*C*) *Mesoxalaldehyde 3-acylhydrazone 1,2-bis(phenylhydrazones)* (**6**). — Mesoxalaldehyde 1,2-bis(phenylhydrazone)[1,5] (1 mmole) in ethanol (20 ml) was treated with the required acylhydrazine (1 mmole) and a few drops of glacial acetic acid. The solution was heated on a boiling-water bath for 15 min, and then allowed to cool. The yellow or orange product was filtered off, washed, and crystallized from ethanol (see Table II).

Mesoxalaldehyde 1,2-bis(phenylhydrazone) 3-thiosemicarbazone. — A solution of mesoxalaldehyde 1,2-bis(phenylhydrazone) (1 g) in ethanol (20 ml) was treated with thiosemicarbazide (0.4 g) in 50% ethanol (20 ml), as for the preparation of **6**. The product (0.9 g) crystallized from ethanol in orange, prismatic needles, m.p. 200–202°; ν_{max}^{KBr} 1480 (C=S) and 1605 cm^{-1} (C=N); λ_{max}^{EtOH} 205, 250, 305 (sh), 338, and 400 nm (log ε 4.38, 4.19, 4.28, 4.57, and 4.31); λ_{min}^{EtOH} 222, 275, and 376 nm (log ε 4.06, 3.93, and 4.17).

Anal. Calc. for $C_{16}H_{17}N_7S$: C, 56.6; H, 5.1; N, 28.9. Found: C, 56.1; H, 5.6; N, 28.5.

(*D*) *Mesoxalaldehyde 2,3-bis(acylhydrazone) 1-(2-methyl-2-phenyl)hydrazones* (**9b**). — Mesoxalaldehyde 2-acylhydrazone 1-(2-methyl-2-phenyl)hydrazone (1 mmole) was dissolved in ethanol (10 ml), and treated with the acylhydrazine (1 mmole) and a few drops of glacial acetic acid. The mixture was heated on a steam-bath for 10 min, and cooled, and the yellow product that separated was filtered off, washed, and crystallized from ethanol (see Table II).

(*E*) *Mesoxalaldehyde tris(acylhydrazones)* (**10**). — The mesoxalaldehyde 1,2-bis(acylhydrazone)[2] (1 mmole) in ethanol (100 ml) was treated with the corresponding acylhydrazine (1.5 mmole) and a few drops of glacial acetic acid. The mixture

TABLE I

MESOXALALDEHYDE 1,2-BISHYDRAZONES (3)

Formula	*R*	*M.p., degrees*	*Color*	*Method*	*Yield (%)*	*Found*			*Formula*	*Calc.*		
						C	*H*	*N*		*C*	*H*	*N*
3a	C_6H_4Me-*o*	113–114	orange	A	70	65.8	5.1	18.1	$C_{17}H_{16}N_4O_2$	66.2	5.2	18.2
3a	C_6H_4Me-*m*	154–155	orange	A	80	65.8	4.7	18.2	$C_{17}H_{16}N_4O_2$	66.2	5.2	18.2
3a	C_6H_4OMe-*p*	180–182	orange	A	85	63.0	4.8	17.3	$C_{17}H_{16}N_4O_3$	63.0	5.0	17.3
3a	C_6H_4Cl-*o*	158–159	orange	A	75	58.4	3.7	17.2	$C_{16}H_{13}ClN_4O_2$	58.5	4.0	17.1
3a	C_6H_4Cl-*m*	194–195	orange	A	80	58.5	3.5	17.6	$C_{16}H_{13}ClN_4O_2$	58.5	4.0	17.1
3a	C_6H_4OH-*m*	180–181	orange	A	65	62.4	4.2	17.9	$C_{16}H_{14}N_4O_3$	62.0	4.6	18.1
3a	$C_6H_4NO_2$-*o*	175–176	brown	A	60	56.9	3.4	21.1	$C_{16}H_{13}N_5O_4$	56.6	3.9	20.6
3a	$C_6H_4NO_2$-*m*	158–160	brown	A	70	55.4	3.9	20.0	$C_{16}H_{13}N_5O_4 \cdot 0.5H_2O$	55.2	4.1	20.2
3a	3-pyridyl	200–201	brown	A	80	60.8	4.0	24.1	$C_{15}H_{13}N_5O_2$	61.0	4.4	23.7
3b	CH_3	144–146	yellow	B	90	58.2	5.4	23.1	$C_{12}H_{12}N_4O_2$	58.5	5.7	22.8
3b	C_6H_5	178–180	yellow	B	85	64.7	5.9	17.7	$C_{17}H_{16}N_4O_2 \cdot 0.5H_2O$	64.4	5.4	17.7
3b	C_6H_4Me-*p*	220–222	yellow	B	85	66.6	5.7	17.2	$C_{18}H_{18}N_4O_2$	67.1	5.6	17.4

TABLE II

MESOXALALDEHYDE TRISHYDRAZONES

Formula	*R*	*M.p., degrees*	*Color*	*Method*	*Yield (%)*	*Found*			*Formula*	*Calc.*		
						C	*H*	*N*		*C*	*H*	*N*
6	C_6H_5	169–170	yellow	C	60	62.9	5.7	19.7	$C_{22}H_{20}N_6O \cdot 2H_2O$	62.9	5.7	20.0
6	C_6H_4Me-*m*	217–218	orange	C	60	68.9	5.4	21.0	$C_{23}H_{22}N_6O$	69.3	5.6	21.1
6	C_6H_4Me-*p*	215–216	yellow	C	70	66.6	6.1	20.4	$C_{23}H_{22}N_6O \cdot H_2O$	66.3	5.8	20.2
6	C_6H_4OMe-*p*	192–194	orange	C	75	66.5	5.4	20.1	$C_{23}H_{22}N_6O_2$	66.7	5.4	20.3
6	C_6H_4Cl-*m*	164–165	yellow	C	60	63.1	4.8	20.4	$C_{22}H_{19}ClN_6O$	63.1	4.6	20.1
6	C_6H_4Cl-*p*	193–194	yellow	C	75	62.9	4.5	20.5	$C_{22}H_{19}ClN_6O$	63.1	4.6	20.1
6	$C_6H_4NO_2$-*o*	215–217	orange	C	60	61.6	4.5	23.3	$C_{22}H_{19}N_7O_3$	61.0	4.5	22.8
6	$C_6H_4NO_2$-*m*	150–151	orange	C	65	59.1	4.8	22.1	$C_{22}H_{19}N_7O_3 \cdot H_2O$	59.0	4.7	21.9
6	3-pyridyl	227–228	yellow	C	60	64.3	4.4	24.8	$C_{24}H_{19}N_7O \cdot 0.5H_2O$	63.9	4.9	24.9
6	4-pyridyl	209–210	yellow	C	60	65.9	5.2	24.9	$C_{21}H_{19}N_7O$	65.4	5.0	25.4
9b	C_6H_5	233–235	yellow	D	90	66.8	5.2	18.9	$C_{24}H_{22}N_6O_2 \cdot 0.5H_2O$	66.3	5.3	19.3
9b	C_6H_4Me-*p*	240–241	yellow	D	90	—	—	18.9	$C_{26}H_{26}N_6O_2$	—	—	18.5
10	C_6H_5	243–244	colorless	E	90	64.1	4.5	18.7	$C_{24}H_{20}N_6O_3 \cdot 0.5H_2O$	64.1	4.5	18.7
10	C_6H_4Me-*p*	250–251	colorless	E	85	66.2	5.7	17.3	$C_{27}H_{26}N_6O_3 \cdot 0.5H_2O$	66.0	5.5	17.1
10	C_6H_4OMe-*p*	245–246	colorless	E	90	60.6	4.9	15.7	$C_{27}H_{26}N_6O_6$	61.1	5.0	15.8
10	C_6H_4Cl-*m*	220–222	colorless	E	75	51.4	3.5	15.1	$C_{24}H_{17}Cl_3N_6O_3 \cdot 0.5H_2O$	51.3	3.4	14.7
10	C_6H_4Cl-*p*	259–260	colorless	E	90	52.5	3.3	15.8	$C_{24}H_{17}Cl_3N_6O_3$	53.0	3.2	15.5

TABLE III

INFRARED AND ULTRAVIOLET SPECTRA OF MESOXALALDEHYDE BISHYDRAZONES

Formula	*R*	$\nu_{max}^{KBr}(cm^{-1})$			$\lambda^{EtOH}(nm)$			
		C=N	*CONH*	*HC=O*	λ_{max}	*log ε*	λ_{min}	*log ε*
1	——— (Ref. 1)	1605	——	1665	204, 250, 315, 420	4.30, 4.20, 4.40, 4.30	270, 365	3.90, 3.80
2	C_6H_5 (Ref. 2)	1600	1665	1700	205, 245, 318, 416	4.01, 3.90, 4.01, 3.74	218, 270, 365	3.65, 3.52, 3.45
3a	C_6H_5	1600	1670	1706	205, 242, 322, 395	4.34, 4.26, 4.38, 3.72	217, 275, 363	3.97, 3.83, 3.55
3a	C_6H_4Me-*o*	1590	1673	1715	205, 242, 318, 380	4.37, 4.20, 4.30, 3.62	225, 271, 362	3.90, 3.72, 3.48
3a	C_6H_4Me-*m*	1590	1670	1700	206, 242, 323, 395	4.31, 4.15, 4.11, 3.61	219, 275, 364	3.88, 3.63, 3.45
3a	C_6H_4OMe-*p*	1615	1675	1710	206, 244, 336	4.36, 4.29, 4.19	224, 278	3.80, 3.56
3a	C_6H_4Cl-*o*	1590	1675	1715	207, 243, 314, 376	4.09, 3.91, 4.04, 3.02	224, 271, 358	3.71, 3.42, 3.23
3a	C_6H_4Cl-*m*	1585	1670	1710	206, 242, 321, 394	4.39, 4.24, 4.26, 3.75	222, 274, 360,	4.07, 3.62, 3.52
3a	C_6H_4OH-*m*	1590	1675	1700	207, 240, 320, 384	4.50, 4.31, 4.26, 3.71	222, 277, 366	4.15, 3.83, 3.66
3a	$C_6H_4NO_2$-*o*	1603	1690	1720	207, 244, 312, 372	4.35, 4.29, 4.34, 3.67	222, 275, 347	4.05, 3.98, 3.60
3a	3-pyridyl	1580	1668	1710	205, 242, 318, 390	4.32, 4.24, 4.27, 3.87	218, 279, 352	3.99, 3.79, 3.53
3b	CH_3	1600	1690	1710	204, 249, 270, 376	4.04, 4.21, 4.28, 4.03	225, 256, 318	3.94, 4.19, 3.43
3b	C_6H_5	1600	(1700 broad)		205, 276, 384	4.25, 4.31, 4.06	228, 323	4.04, 3.32
3b	C_6H_4Me-*p*	1615	1690	1710	206, 278, 380	4.25, 4.37, 4.10	233, 325	4.03, 3.38
4	C_6H_5 (Ref. 2)	1603	1680	1695	206, 259, 322	4.45, 4.44, 4.24	218, 298	4.13, 4.06
4	C_6H_4Me-*m* (Ref. 2)	1610	1680	1710	206, 236(sh), 327	3.93, 3.61, 3.64, 3.70	223, 287	3,59, 3.51
4	C_6H_4Me-*p* (Ref. 2)	1615	1680	1690	206, 238, 262(sh), 328	4.05, 3.91, 3.87, 3.94	218, 292	3.81, 3.72
4	C_6H_4OMe-*p*	1610	1660	1690	206, 258, 330	4.43, 4.37, 4.29	222, 300	4.14, 4.21
4	C_6H_4Cl-*m* (Ref. 2)	1600	1670	1690	206, 287, 352	4.45, 4.06, 4.00	245, 324	3.99, 3.97
4	C_6H_4Cl-*p* (Ref. 2)	1595	1680	1700	206, 258, 325	4.42, 4.29, 4.33	218, 292	4.21, 4.17

TABLE IV

INFRARED AND ULTRAVIOLET SPECTRA OF MESOXALALDEHYDE TRISHYDRAZONES

Formula		$\nu_{max}^{KBr}(cm^{-1})$		$\lambda^{EtOH}(nm)$			
	R	*C=N*	*CONH*	λ_{max}	*log ε*	λ_{min}	*log ε*
5	——[1,5]	1605	——	206, 240, 300, 245, 410	4.60, 4.20, 4.40, 4.50, 4.50	275, 315, 365	4.00, 4.30, 4.40
6	C_6H_5	1603	1638	208, 237 (sh), 253, 303 (sh), 338, 414	4.40, 4.17, 4.19, 4.17, 4.50, 4.28	218, 287, 375	4.10, 4.08, 4.14
6	C_6H_4Me-*m*	1605	1658	206, 235 (sh), 250, 303 (sh), 338, 412	4.41, 4.10, 4.11, 4.09, 4.46, 4.23	222, 285, 377	4.03, 4.01, 4.04
6	C_6H_4Me-*p*	1600	1640	205, 233 (sh), 255, 303 (sh), 343, 416	4.47, 4.20, 4.28, 4.32, 4.50, 4.28	221, 285, 376	4.13, 4.16, 4.15
6	C_6H_4OMe-*p*	1610	1655	205, 255, 303 (sh), 338, 414	4.52, 4.27, 4.29, 4.54, 4.29	227, 275, 376	4.18, 4.22, 4.17
6	C_6H_4Cl-*m*	1600	1645	206, 235 (sh), 255, 280 (sh), 342, 410	4.60, 4.28, 4.29, 4.24, 4.58, 4.38	225, 295, 380	4.26, 4.22, 4.24
6	C_6H_4Cl-*p*	1600	1640	206, 248, 277 (sh), 341, 414	4.49, 4.30, 4.20, 4.59, 4.36	221, 292, 378	4.15, 4.16, 4.19
6	$C_6H_4NO_2$-*o*	1605	1645	206, 253, 302 (sh), 328, 412	4.48, 4.31, 4.35, 4.57, 4.36	227, 277, 374	4.21, 4.17, 4.16
6	$C_6H_4NO_2$-*m*	1603	1660	206, 219 (sh), 255, 342, 412	4.44, 4.39, 4.32, 4.51, 4.32	239, 292, 382	4.28, 4.18, 4.19
6	3-pyridyl	1600	1650	204, 255, 342, 412	4.05, 3.86, 4.15, 3.96	230, 285, 380	3.80, 3.80, 3.81
6	4-pyridyl	1608	1658	204, 257, 285, 346, 414	4.35, 4.14, 4.17, 4.46, 4.31	229, 267, 310, 387	4.05, 4.12, 4.09, 4.18
9b	C_6H_5	1605	1670	205, 248, 310, 327, 400	4.39, 4.23, 4.46, 4.43, 4.13	230, 263, 365	4.16, 4.19, 4.05
9b	C_6H_4Me-*p*	1615	1670	204, 225 (sh), 260 (sh), 310, 327, 396	4.43, 4.22, 4.20, 4.48, 4.48, 4.12	238, 263, 365	4.16, 4.19, 4.05
10	C_6H_5	1603	1655, 1693	204, 222, 287, 297 (sh), 358	4.38, 4.25, 4.42, 4.41, 4.33, 4.34	216, 240, 323	4.24, 4.18, 4.24
10	C_6H_4Me-*p*	1608	1660, 1690	205, 230, 290, 300, 360	4.50, 4.25, 4.44, 4.44, 4.29	218, 247, 295, 335	4.19, 4.13, 4.44, 4.29
10	C_6H_4OMe-*p*	1608	1650, 1680	206, 245, 305, 315, 366	4.46, 4.24, 4.44, 4.34, 4.30	222, 262, 310, 342	4.08, 4.18, 4.43, 4.25
10	C_6H_4Cl-*m*	1600	1680, 1690	208, 295, 348	4.63, 4.32, 4.17	245, 334	3.11, 4.16
10	C_6H_4Cl-*p*	1600	1660, 1680	207, 234, 278, 300 (sh), 317, 345	4.25, 4.22, 4.13, 4.04, 4.01, 4.00	217, 258, 300, 310	4.08, 4.11, 4.07, 4.04

was heated on a steam-bath for 30 min, and then allowed to cool. The colorless trishydrazone was filtered off, washed, and crystallized from ethanol (see Table II).

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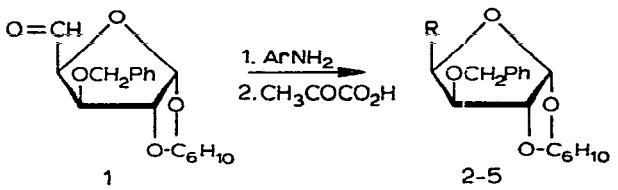
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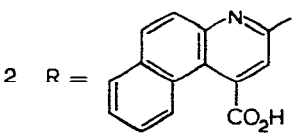
Preliminary communication

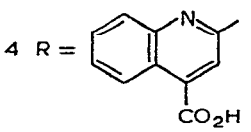
The Doebner synthesis in the carbohydrate series

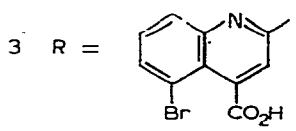
We have examined the behaviour of 3-*O*-benzyl-1,2-*O*-cyclohexylidene-α-D-*xylo*-pentodialdo-1,4-furanose[1] (1) with various arylamines in the Doebner synthesis. The condensation proceeds in the usual way, giving the corresponding substituted cinchoninic acids (2–5).

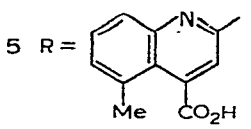


1 2-5









This synthesis was accomplished by preliminary storage of a mixture of compound 1 and the arylamine in ether for 24 h to form the Schiff base, with subsequent addition of freshly distilled pyruvic acid and storage of this mixture for 10 days; all operations were performed at room temperature. Preparative fractionation of the final mixture on alumina gave the following products:

3-*O*-Benzyl-4-(4-carboxybenzo[*f*]quinol-2-yl)-1,2-*O*-cyclohexylidene-α-D-*xylo*-tetrofuranose (2) (27.5%), white needles from ethyl acetate, m.p. 198° (decomp.), $[\alpha]_D^{15}$ −7° (*c* 0.6, chloroform); λ_{max} 243 (inflection) (ε 33,400) and 256 nm (ε 36,000); ν_{max} 1710 (medium), 1670 (weak), and 1599 cm^{-1} (medium) (Found: C, 72.45; H, 5.59; N, 2.40. $C_{31}H_{29}NO_6$ calc.: C, 72.79; H, 5.67; N, 2.74%).

3-*O*-Benzyl-4-(5-bromo-4-carboxyquinol-2-yl)-1,2-*O*-cyclohexylidene-α-D-*xylo*-tetrofuranose (3) (10.9%), an amorphous powder, $[\alpha]_D^{15}$ −125° (*c* 1.2, chloroform); λ_{max} 252 (ε 9900) and 304 nm (ε 3900); ν_{max} 1709 (medium) and 1592 cm^{-1} (medium) (Found: C, 59.85; H, 4.76; Br, 14.26. $C_{27}H_{26}BrNO_6$ calc.: C, 60.0; H, 4.81; Br, 14.81%).

3-*O*-Benzyl-4-(4-carboxyquinol-2-yl)-1,2-*O*-cyclohexylidene-α-D-*xylo*-tetrofuranose (4) (13.9%), an amorphous powder, $[\alpha]_D^{15}$ −102° (*c* 1, chloroform); λ_{max} 235 (ε 9700) and 308 nm (ε 3800); ν_{max} 1710 (medium), 1594 (medium), and 1510 cm^{-1} (medium) (Found: C, 69.71; H, 6.18. $C_{27}H_{27}NO_6$ calc.: C, 70.28; H, 5.85%).

3-*O*-Benzyl-4-(4-carboxy-5-methylquinol-2-yl)-1,2-*O*-cyclohexylidene-α-D-*xylo*-tetrofuranose (5) (24.4%), white needles from ethyl acetate, m.p. 158° (decomp.), $[\alpha]_D^{15}$ −143° (*c* 1.4, chloroform); λ_{max} 236 (ε 13,000) and 322 nm (ε 5300); ν_{max} 1709 (medium), 1601 (medium), and 1512 cm^{-1} (medium) (Found: C, 70.58; H, 6.14. $C_{28}H_{29}NO_6$ calc.: C, 70.73; H, 6.10%).

Removal of the cyclohexylidene residue was accomplished by methanolysis of compound 2 in the presence of dry hydrogen chloride.

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Book review

Khimia Uhlewodov (Chemistry of the Carbohydrates) N. K. KOCHETKOV, A. F. BOCHKOV, B. A. DMITRIEV, A. J. USOV, O. S. CHIZHOV and W. N. SHIBAEV edited by M. N. PASTUSHENKO and L. A. PANTELEEVA. Published by "Khimia", Moscow, 1967, 672 pp., 19 Tables, 17 × 25.5 cm, cloth-back, 7000 copies printed, Rubles 3.75 (approximately $4.00).

This book is an exceedingly valuable presentation of the chemistry and biochemistry of carbohydrates from the modern point of view. It is a well organized and carefully documented monograph that consists of three parts (22 chapters).

Part I describes the chemistry of monosaccharides in 14 chapters which cover: Structure and Stereochemistry (32 pages, 32 references); Physical Properties and Methods of Analysis (optical rotatory dispersion; u.v., i.r., n.m.r., and mass spectra of methylated sugars; conformational analysis; 27 pages, 81 references); Redox Reactions and Action of Acids and Bases (34 pages, 126 references); Reactions and Derivatives of the Free Carbonyl (*aldehydo* and *keto*) Group (oximes, phenylhydrazones, osazones, formazans, and dithioacetals, 17 pages, 117 references); Reactions and Derivatives of the Hydroxyl Groups (esters, ethers, acetals, elimination reactions; 54 pages, 209 references); Reactions and Derivatives of the Anomeric Hydroxyl Group (nucleophilic displacements, halides, azides, ortho-esters, glycosides, anhydro sugars, glycosylamines, glycals; 51 pages, 267 references; Ketoses (14 pages, 85 references); Deoxy Sugars (15 pages, 90 references); Amino Sugars (properties, structure, amino sugars having nitrogen in the ring; 35 pages, 128 references); Uronic Acids (17 pages, 99 references); Higher Sugars (occurrence in Nature, synthesis; 14 pages, 94 references); Other Monosaccharides (sialic acids, branched-chain sugars, thio sugars, 1-thioglycosides; 31 pages, 152 references); Basic Biochemistry of Monosaccharides (fermentation reactions, metabolism of sugars in plants and animals, biosynthesis, and photosynthesis; 45 pages, 183 references); and Analysis and Identification (7 pages, 90 references).

Part II is concerned with the chemistry of oligosaccharides; the 3 chapters describe: Natural Sources, Isolation, and Properties (10 pages, 47 references); Structure Determination (molecular weight, methods of degradation; 28 pages, 75 references); and Synthetic Methods (17 pages, 65 references). Part III presents a sound introduction to the chemistry of polysaccharides and biopolymers containing carbohydrate moieties. Five chapters discuss: Natural Sources and Methods of Isolation (12 pages, 82 references); Chemical and Physical Methods for the Determination of Structure (27 pages, 181 references); Characteristic Members of the Polysaccharide Group (animal, bacterial, cell, plant, and synthetic; 33 pages,

425 references); Mixed Biopolymers Containing Carbohydrate Moieties (glycoproteins, glycolipids; 38 pages, 175 references); Biochemistry of Polysaccharides and Biopolymers (biological function, biosynthesis, and metabolic degradation; 23 pages, 146 references). An appendix (10 pages, called "conclusion") summarizes various physicochemical aspects associated with the chemistry of mono-, oligo-, and polysaccharides. The book concludes with a Subject Index and a Table of Contents. Unfortunately, an Author Index is not provided. An insert at the end of the monograph gives 16 corrigenda. The book covers the literature through the year 1965, and even contains a few references for 1966 and 1967. The nomenclature in at least one chapter (Deoxy Sugars, pp. 250–265) strictly adheres to the Rules of Carbohydrate Nomenclature [*J. Org. Chem.*, 28 (1963) 281].

In general, the monograph is up to date and scientifically sound, and the formulas are adequate. The book contains an abundance of synthetic methods, well presented and well described; some of the newest synthetic procedures recently developed by Kochetkov and coworkers, which may have escaped the attention of the western reader, are also included, and these add considerably to the value of the book. Examples are: a method for lengthening the carbon chain of a sugar by two carbon atoms (p. 325), and the use of the reagent $(PhO)_3P \cdot CH_3I$ for replacement of a hydroxyl group by iodine (pp. 152–155, 174, 180, and 260).

The monograph discusses only those reaction mechanisms that, by cross-checking by different methods, appear to be valid. The book uses the term "inversion of configuration" instead of the customary "Walden inversion" (pp. 152–155, 188–192, 196, 199, 260, and 271). A formula supposedly representing a quasi-aromatic structure of a phenylosazone (p. 117) is out of date; some of the print is illegible (p. 235); and a somewhat more extensive presentation of the chemistry of dicarbonyl sugars (p. 116) would have been welcome. In discussions of conformations, the book uses the Reeves system exclusively (pp. 38–46). Because, in this connection, the chirality is immaterial and irrelevant, it is unfortunate that the Isbell–Tipson system for naming conformers is not even mentioned, although this system is useful when the conformations of members of the D-series and the L-series are considered simultaneously.

But these are quite minor criticisms; all in all, this book is an extremely valuable contribution, and an excellent textbook for graduate study, a personal copy of which should be acquired by all carbohydrate chemists who can read Russian; certainly, its extremely modest price should be no bar to its purchase. The English translation is eagerly awaited. It should be valuable to all carbohydrate chemists, biochemists, and medicinal chemists.

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P.M R EVIDENCE FOR THE CONFORMATIONS OF 1,2-*O*-BENZYLIDENE-α-D-GLUCOFURANOSE DERIVATIVES

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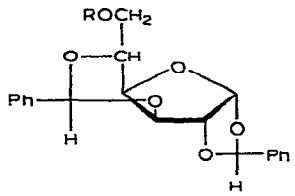
ABSTRACT

Proton coupling constants and chemical shifts of two 1,2-*O*-benzylidene- and four 1,2 3,5-di-*O*-benzylidene-α-D-glucofuranose derivatives have been measured by p.m r spectroscopy at 100 MHz From these parameters, it is deduced that the furanoid rings of the mono- and di-benzylidene acetals exist mainly in symmetric and non-symmetric twist conformations, respectively The *m*-dioxane ring of the dibenzylidene acetals adopts that chair conformation in which C-6 of the sugar is axially attached

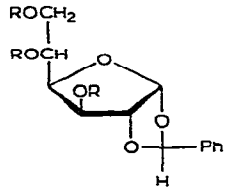
INTRODUCTION

The conformational influence of cyclic acetal substituents fused to furanose and pyranose rings is important to an understanding of the factors that determine the conformations of carbohydrate molecules As part of a continuing study of steric and dipolar interactions in such systems, the conformations, in solution, of a number of bicyclic and tricyclic 1,2-*O*-benzylidene-α-D-glucofuranoses have been investigated by p m r spectroscopy at 100 MHz. The compounds[1] examined were 1,2 3,5-di-*O*-benzylidene-α-D-glucofuranose (**1**), and its 6-*O*-methyl, 6-*O*-acetyl, and 6-*O*-benzoyl derivatives (**2**, **3**, and **4**, respectively), and 1,2-*O*-benzylidene-α-D-glucofuranose (**5**) and its 3,5,6-tribenzoate (**6**) Compounds **1** and **5** are products of the reaction of D-glucose with benzaldehyde in the presence of zinc chloride and acetic acid[1].

Compounds **5** and **6** afforded the opportunity for comparison of the conformational effect of the fusion of a 1,2-*O*-benzylidene group (to a furanose ring) with that of fusion of a 1,2-*O*-isopropylidene group, reported in an investigation of 1,2-*O*-isopropylidene-α-D-*xylo*-hexofuranose derivatives[2] The results obtained from compounds **1**–**4** allow assessment of the effect of fusing a six-membered ring to a system containing two *cis*-fused, five-membered rings As is well known, reactions of carbohydrates with aldehydes and ketones yield different types of sugar and acetal ring-systems according to the particular catalysts and carbonyl compounds that are employed[1,3], and the conformational stability of the derivatives now examined may be expected to have some bearing on this problem.



1 R = H
2 R = Me
3 R = Ac
4 R = Bz



5 R = H
6 R = Bz

DISCUSSION

The spectra of solutions of compounds **1–6** in chloroform-*d* or pyridine all displayed the proton signals of H-1, H-2, and H-3 as doublets, that at lowest field being assigned to H-1. Those of H-4 H-6, and H-6′ usually appeared as quartets, and H-5 either as a broadened triplet, or as a complex multiplet The spectrum (see Fig. 1) of the 6-methyl ether **2** is a good example of the dibenzylidene series, and, for the two monobenzylidene acetals examined, the tribenzoate (**6**) provided the better spectrum (see Fig 2) Since, in some of the spectra, the multiplets overlapped one another in a complex way, solvent shifts and double-resonance experiments were used for confirming assignments For the dibenzylidene acetals (**1–4**), the benzylic protons in all cases resonated as singlets at different chemical shifts For a given solvent, the chemical shift (see Table I) of the singlet at lower field showed the smaller change on variation of the substituent on C-6 This singlet was, therefore, assigned as the benzylic proton remote from C-6, namely, that in the 1,2-benzylidene group, and the singlet at higher field, as that of the 3,5-benzylidene group Support for these assignments came from the chemical shifts of the benzylic protons in the monobenzylidene acetals **5** and **6**, which were closer to the range of shifts for the low-field, benzylic resonances of **1–4** than to the range of those at higher field For compound **1** in pyridine, the chemical-shift difference between its benzylic resonances was appreciably less than that for any other compound and solvent combination studied, and it seems likely that hydrogen bonding between the hydroxyl group on C-6 and the nitrogen atom of pyridine causes the 3,5-benzylic proton to lie in a deshielding zone of the solvent molecule In contrast, for random orientations of a molecule of an aromatic solvent, it has been suggested that a neighboring solute molecule more commonly lies in the shielding zone of the ring current[4] In both chloroform-*d* and pyridine solutions, the chemical shift between H-6 and H-6′ is larger in the 6-esters **3**, **4**, and **6** than in the nonacylated compounds, apparently as a result of the greater diamagnetic anisotropy of the acyl groups There are many rotamers about the C-6–O-6 bond in which H-6 and H-6′ have a different orientation with respect to the acyl group, but the available physical evidence is not such as to indicate which of these is likely to be favored

The coupling constants exhibited by the benzylidene derivatives are shown in Table II, and it may be seen that the absolute value of $J_{2,3}$ in **1–4** and **6** is, in all instances, less than the resolving power of the spectrometer, that is, less than 0 4 Hz

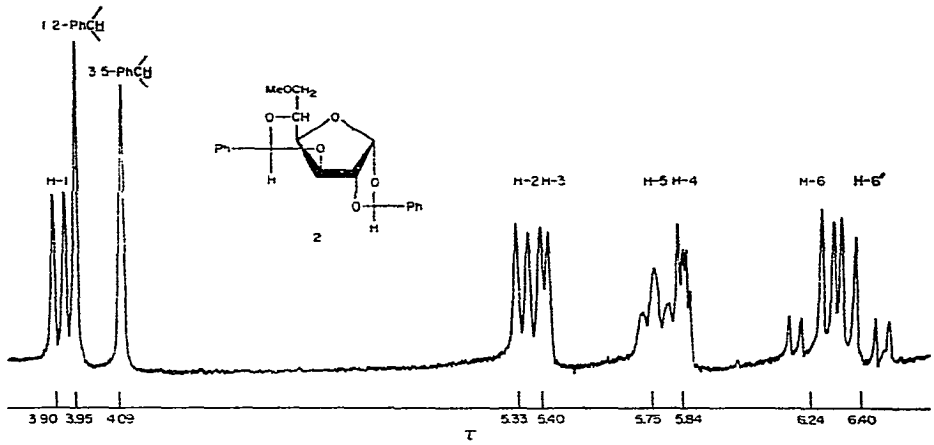


Fig 1. P m r spectrum of 1,2 3,5-di-*O*-benzylidene-6-*O*-methyl-α-D-glucofuranose (2) in chloroform-*d* at 100 MHz The signals of the methyl and phenyl groups are not shown.

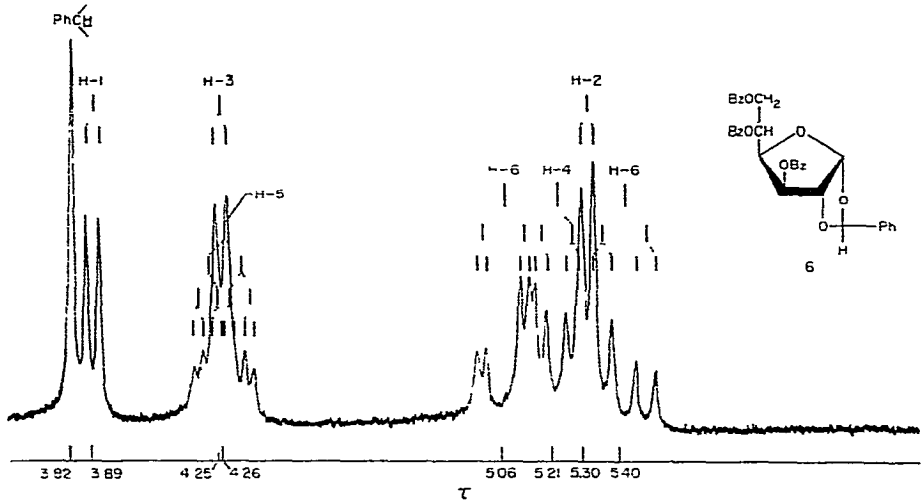


Fig 2 P m r spectrum of 3,5,6-tri-*O*-benzoyl-1,2-*O*-benzylidene-α-D-glucofuranose (6) in chloroform-*d* at 100 MHz The phenyl resonances are omitted

If the explicit form (*1*) of the Karplus equation[5], namely,

$$\phi = \cos^{-1}\left\{\frac{0\,5 \pm [0\,25 + 36(J + 0\,28)]^{\frac{1}{2}}}{18}\right\} \qquad (1)$$

is used to provide an approximate theoretical interpretation, a positive coupling $J_{2\,3}$ in the range of 0 to 0 4 Hz implies a proton dihedral angle $\phi_{2\,3}$ of about 72–78° or 99–104°, whereas a negative $J_{2\,3}$ value in the range of 0 to −0 2869 Hz suggests that $\phi_{2,3}$ is about 78–88°, or 88–99° The figure −0 2869 represents the minimum value of the coupling constant that will allow equation (*1*) to have a real solution, and it corresponds to $\phi \approx 88°$ These values may also be obtained by differentiation

TABLE I

CHEMICAL SHIFTS (τ VALUES) OF 1,2-*O*-BENZYLIDENE-α-D-GLUCOFURANOSE DERIVATIVES

	Derivative	*Solvent*	*H-1*	*H-2*	*H-3*	*H-4*	*H-5*	*H-6*[a]	*H-6'*[a]	*Ph*	*1,2-PhCH*	*3,5-PhCH*	*R-6*[b]
1	3,5-*O*-Benzylidene	$CDCl_3$	3.87d[c]	5.28d	5.45d	5.85[d]	5.81[d]	6.08q	6.14q	2.65m	3.96	4.19	8.15[e,f]
	R-6 = H	C_5H_5N	3.65d	5.12d[d]	5.09d[d]	5.43[d]	5.44[d]	5.75q[b]	5.88q[d]	[g]	3.71	3.77	3.52[e]
2	3,5-*O*-Benzylidene	$CDCl_3$	3.90d	5.33d	5.40d	5.84q	5.75t[e]	6.24q	6.40q	2.70m	3.95	4.09	6.70
	R-6 = Me	C_5H_5N	3.68d	5.16d	5.22d	5.62q	5.53t[de]	6.24q[b]	6.36q[d]	[g]	3.72	3.90	6.82
3	3,5-*O*-Benzylidene	$CDCl_3$	3.89d	5.32d	5.50d	5.98q	5.64	5.47q[b]	5.84q	2.70m	3.97	4.28	7.99
	R-6 = Ac	C_5H_5N	3.66d	5.13d	5.24d	5.68q[d]	5.38m	5.20q[b]	5.69q[d]	[g]	3.73	3.99	8.09
4	3,5-*O*-Benzylidene	$CDCl_3$	3.86d	5.27d	5.41d	5.84q	≈5.48m[d]	5.24q[b]	5.52q[d]	2.68m 2.00m	3.96	4.16	2.68m 2.00m
	R-6 = Bz	C_5H_5N	3.61d	5.07d	5.13d	5.54q[d]	≈5.16m[d]	4.92q	5.40q	[g] 1.92m	3.67	3.85	[g] 1.92m
5	R-3 = R-5 = R-6 = H[b]	C_5H_5N	3.67d	5.08d[d]	5.08d[d]	5.39m[d]	5.42m[d]	5.73m[b]	5.87q[d]	[g]	3.75		3.58
6	R-3 = R-5 = R-6 = Bz	$CDCl_3$	3.89d	5.30d	4.25d	5.21q[d]	4.26m[d]	5.06q	5.40q[d]	2.69m 2.13m	3.82		2.69m 2.13m
		C_5H_5N	3.57d	5.00d	3.91d	4.82q[d]	3.91m[d]	4.81q[b]	5.21q	[g]	3.47		[g]

[a]H-6 is the methylene proton at lower field, H-6' is that at higher field [b]R-3, R-5, and R-6 refer to the *O* substituents at C-3, C-5, and C-6, respectively [c]Signal multiplicities are indicated by d (doublet), t (triplet), q (quartet), or m (multiplet) [d]Incompletely resolved multiplet [e]Broad band. [f]Assignment confirmed by deuterium exchange [g]Obscured by solvent signals

TABLE II

FIRST-ORDER, PROTON COUPLING-CONSTANTS (Hz) OF 1,2-*O*-BENZYLIDENE-α-D-GLUCOFURANOSE DERIVATIVES AT 100 MHz

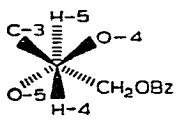
	Derivative	*Solvent*	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6}$[a]	$J_{5,6'}$[a]	$J_{6,6'}$
1	3,5-*O*-Benzylidene	$CDCl_3$	3.6	<0.4	2.3	≈	5.2 (5.32)[b]	5.1 (5.055)[b]	11.4
	R-6 = H[c]	C_5H_5N	3.6	<0.4			≈4.9	≈5.7	≈11.5
2	3,5-*O*-Benzylidene	$CDCl_3$	3.7	<0.4	2.5	1.5	3.7	4.3	10.3
	R-6 = Me	C_5H_5N	3.7	<0.4	2.4	1.2	4.3	4.8	10.3
3	3,5-*O*-Benzylidene	$CDCl_3$	3.6	<0.4	2.4	1.35	6.1	3.5	9.7
	R-6 = Ac	C_5H_5N	3.6	<0.4	2.4	1.2	≈7.7	3.1	9.9
4	3,5-*O*-Benzylidene	$CDCl_3$	3.6	<0.4	2.4	1.5	8.1	4.3	13.1
	R-6 = Bz	C_5H_5N	3.6	<0.4	2.2	1.2	7.4	4.0	10.7
5	R-3 = R-5 = R-6 = H[c]	C_5H_5N	3.4		≈3.3		2.9	≈4.9	≈11.7
6	R-3 = R-5 = R-6 = Bz	$CDCl_3$	3.6	<0.4	3.4	8.9	2.5	5.7	12.3
		C_5H_5N	3.6	<0.4	3.3	≈9.0	2.5	6.4	12.2

[a]H-6 is the methylene proton at lower field, H-6′ is that at higher field [b]The figures in parentheses were obtained from an ABX analysis of the H-6 (A) and H-6′ (B) signals, with H-5 as the X nucleus [c]R-3, R-5, and R-6 refer to the *O*-substituents at C-3, C-5, and C-6, respectively

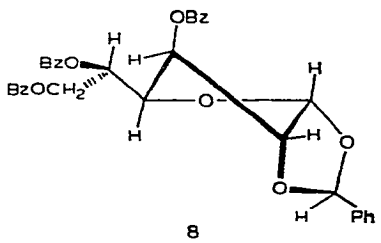
of the Karplus equation to give the derivative of J with respect to ϕ. However, as $J_{2,3}$ was not resolved in **1**–**4** and **6**, its sign cannot be determined, and, therefore, from equation (*1*), it can only be speculated that $\phi_{2,3}$ lies in the approximate range of 72–104°. In order that H-2 and H-3 may achieve such a dihedral angle, it is necessary that the furanoid ring exist in one or more of those nonplanar conformations in which C-3 is above a plane containing C-1, C-2, and C-5 or the ring-oxygen atom (O-4) or both (assuming that the ring is viewed in the usual orientation with O-4 at the back, and C-1 on the right). For compounds **5** and **6**, the values $J_{1,2}$ 3.4–3.6 and $J_{3,4}$ 3.3–3.4 are very nearly equal, so that* $\phi_{1,2} \approx \phi_{3,4}$. As pointed out in a previous study[2] of the conformations of 1,2-*O*-isopropylidene-α-D-*xylo*-hexofuranose derivatives, this observation implies that C-2 and C-3 are placed symmetrically on opposite sides of a plane through C-1, C-4, and O-4, and hence, that C-3 lies above this plane and C-2 below it (T^3_2). For a coupling constant of 3.3–3.6 Hz, equation (*1*) implies a dihedral angle of about 47–49° (neglecting the alternative value of 127–129°, which is too large for *cis*-vicinal protons on the carbon atoms of a five-membered ring). Thus, it appears that the furanoid ring of **5** and **6** has approximately the same twist conformation (T^3_2) as that in the 1,2-*O*-isopropylidene derivatives[2], in which the C_2 symmetry axis of the pentagon formed by the ring bonds passes through O-4 and through the mid-point of the C-2–C-3 bond. Previous calculations[6] of the geometry

*Substituent electronegativity effects at C-1 may cause some slight deviations in the values of these angles

of C_2 twist conformations of five-membered rings indicate that, in such a conformation, angles $\phi_{1,2} \approx \phi_{3,4} \approx 48°$ should be accompanied by $\phi_{2,3} \approx 120°$ minus $57° \approx 63°$ This value is 9° less than the minimum value of $\phi_{2,3}$ estimated from equation (*1*) The angles suggest that the furanose ring is slightly less twisted than the "maximally puckered half-chair model" of cyclopentane described by Brutcher and Bauer[7] The large value (8 9–9 0 Hz) of $J_{4,5}$ for **6** indicates that the favored rotamer about the C-4–C-5 bond is **7**, in which $\phi_{4,5}$ is 180°, and in which C-3 and C-6, and O-4 and O-5 are also in a true *trans* relationship The conformation of **6** may, therefore, be depicted as in **8**



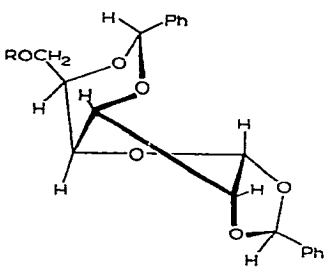
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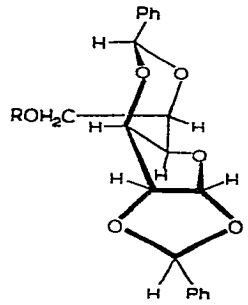
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In the dibenzylidene acetals **1–4**, the values of $J_{3,4}$ (2 2–2 5 Hz) are significantly smaller than those of $J_{1,2}$ (3 6–3 7 Hz), and also smaller than the general range of values of $J_{3,4}$ observed for the 1,2-*O*-isopropylidene-α-D-*xylo*-hexofuranoses[2] This result implies that $\phi_{3,4}$ is larger than $\phi_{1,2}$, and, therefore, it cannot be inferred that the furanoid rings of **1–4** exist in symmetric, twist conformations The fact that $J_{2,3}$ is also less than 0 4 Hz for **1–4** shows that the shapes of their furanose rings are not markedly different from those in **5** and **6** However, it appears that fusion of the 3,5-benzylidene ring to the 1,2-*O*-benzylidene-α-D-glucofuranose system has the effect of distorting the symmetric twist conformation (as in **9**) towards the envelope conformation (V^3 as in **10**) in which C-3 is above the plane through C-4 ,O-4, C-1, and C-2 For the V^3 conformation, $\phi_{1,2}$ is always smaller than $\phi_{3,4}$, irrespective of the angle of buckle of the ring[6] In contrast to the large value of $J_{4,5}$ for **6**, the values of $J_{4,5}$ (1 2–1 5 Hz) for compounds **2–4** are small, indicating that the angle $\phi_{4,5}$ is, in these two cases, quite different From equation (*1*), these values correspond to an angle $\phi_{4,5} \approx 62–64°$, or $\approx 112–115°$. Because the *m*-dioxane ring in **1–4** is *cis*-fused to C-3 and C-4 of the furanoid ring, it can exist in either of two chair conformations, as in **9** (or **10**), and **11** Measurements made with Barton molecular models of these compounds indicate that $\phi_{4,5}$ is $\approx 70°$ for conformations **9** and **10**, but $\approx 160°$ for the alternative chair conformation (**11**) If it can be assumed that the *m*-dioxane ring exists in that chair conformation having its phenyl substituent equatorial, the conformations of the dibenzylidene acetals must be most closely related to **9** and **10**, in which H-4 and H-5 are both equatorially oriented with respect to the *m*-dioxane ring The small values of $J_{4e\,5e}$ are similar to values of $J_{e\,e}$ previously reported for pyranoid rings[8–11] Because a large value of $J_{4,5}$ would be anticipated for the alternative chair conformation (**11**), its contribution to the conformational equilibrium can only be small Moreover, this conformation of the *m*-dioxane ring requires that

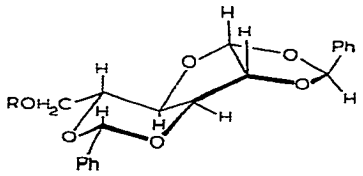
the furanose ring exist in a different envelope or twist conformation, in which C-3 lies below the plane of C-4, O-4, C-1, and C-2, or C-4, O-4, and C-1, respectively Measurements of Barton models of these conformations gave $\phi_{2,3} \approx 160°$, and the observed values of $J_{2\ 3}$ are not consistent with such an angle Conformation **11** may, therefore, be disregarded on the basis of coupling-constant evidence from both the *m*-dioxane ring and the furanoid ring



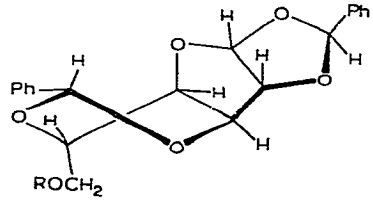
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10



11



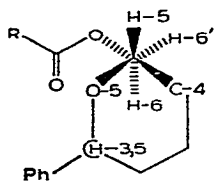
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A possible alternative to the chair forms **9** and **10** of the *m*-dioxane ring is the skew form (**12**), in which H-4 and H-5 are also both equatorial, and, in Barton models, have a dihedral angle* $\phi_{4,5} \approx 50°$ This possibility may, however, be discounted by a consideration of the change in angle $\phi_{3,4}$ when **5** is converted into its 3,5-benzylidene acetal. Introduction of the 3,5-benzylidene group causes $J_{3\ 4}$ to decrease from 3 3–3 4 to 2 2–2 5 Hz From equation (*1*), it may be inferred that $\phi_{3\ 4}$ has increased from $\approx 49°$ to $\approx 55°$ The value of $J_{1,2}$, and, presumably, of $\phi_{1,2}$, is changed little in this process In the skew conformation **12**, $\phi_{3\ 4}$ is $\approx 33°$, whereas, in idealized versions of the chairs **9** and **10**, it would be 60°. It must be concluded, therefore, that the decreased value of $J_{3\ 4}$ corresponds to fusion of a chair form to the furanoid ring, and not of a skew form, the increased value of $\phi_{3\ 4}$ being a consequence of the 60° angle required in a perfect chair It seems unlikely that the decreased value of $J_{3,4}$ could be due to an electronegativity effect, since the first atoms of the substituents at C-3 and C-4 are, in all cases, the same.

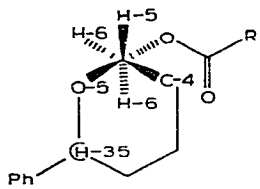
In the esters **3** and **4**, there is a larger difference between $J_{5\ 6}$ and $J_{5\ 6}$, than

*The configuration at the 3,5-benzylic carbon atom of **11** and **12** is depicted as the opposite of that in **9** and **10**, so as to maintain its phenyl substituent in an equatorial orientation

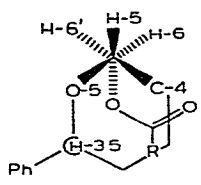
there is for the hydroxy and methoxy analogs **1** and **2** The increased values of $J_{5,6}$ (6 1–8 1 Hz) for **3** and **4** must reflect a greater population of the rotamer about the C-5–C-6 bond, in which H-5 is *trans* to H-6. As the H-6 and H-6′ resonances cannot be assigned individually, this rotamer could be either **13** or **14**, in which the acyloxy group at C-6 is *trans* to either C-4 or O-5, respectively, and is also directed away from the *syn*-axial 3,5-benzylic hydrogen atom (H-3,5) In the less-favored rotamer (**15**), in which $\phi_{5,6} = \phi_{5,6}$, the acyloxy group has a *gauche* relationship with both



13



14



15

C-4 and O-5, and is directed toward the *syn*-axial H-3,5 These observations support the common assumption that an axially oriented acyloxymethyl group tends to adopt a position in which the acyloxy group is directed away from *syn*-axial substituents, so as to minimize 1 3-diaxial interactions The favored conformation (related to **9** and **10**) of the dibenzylidene acetals has C-6 in axial orientation on the *m*-dioxane ring, whereas, in the unfavored chair conformation **11**, this substituent is attached equatorially, but is associated with a different conformation of the furanoid ring This is surprising, since (hydroxymethyl), (methoxymethyl), and (acyloxymethyl) substituents at C-5 of a pyranoid ring normally show a strong tendency to assume the equatorial orientation[10–14]. Inspection of molecular models of **9** and **10** shows that the slight flattening of the *m*-dioxane chair that results from the fusion thereto of a furanoid ring causes some separation of the *syn*-axial H-3 atom and the 5-$ROCH_2$ group Bridging of the O-3 and O-5 atoms of **5** by a benzylidene group causes a small distortion of the shape of its furanoid ring, but this is not sufficient to convert it into **11** It may be noted that, in the favored conformations **8**, **9**, and **10**, the dihedral angle of the C-2–O-2 and C-3–O-3 bonds (and their dipoles) is large ($\approx$ 170–180°), whereas, in the unfavored conformation **11**, it is considerably smaller ($\approx$ 85°) Thus, **11** may be destabilized by the dipolar interactions of O-2 and O-3. However, **11** also has some of the characteristics of an "H-inside" conformation[15], since H-2, H-5, and H-3,5 occupy *endo* positions with respect to the fused furanoid and *m*-dioxane rings. Inspection of Dreiding molecular models of this conformation suggested that the steric interactions H-3,5 H-2, H-3,5 C-2, and C-1 C-5, and of the 3,5-benzylic carbon atom (C-3,5) with H-2 and C-2 might be significant, since the internuclear distances (1 9, 2 5, 3 1, 2 6, and 3 0 Å, respectively, measured from the models are, in each case, somewhat less than the sum of the van der Waals radii for each atom pair (2 4, 2.9, 3 4, 2 9, and 3.4 Å, respectively)

Conversely, **9** and **10** resemble a stable "O-inside" conformation[15], because O-3, O-4, and O-5 are *endo* with respect to the C-3,C-4 ring junction These confor-

mations do not contain the interactions that appear to be present in **11**, as C-1 relative to C-5, and C-2 relative to C-3,5, are almost true *trans* The results may also explain the observation that reactions of D-glucose with acetone in the presence of zinc chloride and phosphoric acid, or other condensing agents[16], do not give an appreciable yield of 1,2 3,5-di-*O*-isopropylidene-α-D-glucofuranose, because, if this compound existed in the same conformation (resembling **9** or **10**) as the 1,2·3,5-dibenzylidene acetals, one of the methyl groups in its 3,5-isopropylidene moiety would be in *syn*-axial relationship with the $ROCH_2$ substituent on C-5 Such an interaction would be similar in magnitude to that between *syn*-diaxial methyl groups[17], but, because the C–O bond-length is less than that of C–C, the interaction energy might well be greater than the 3 7 kcal $mole^{-1}$ measured previously for *syn*-diaxial methyl substituents on a cyclohexane ring[18] By the same token, it appears improbable that the dibenzylidene acetals **1–4** would contain the phenyl ring of the 3,5-benzylidene group in *syn*-axial relationship with the $ROCH_2$ substituent, and, in this sense, therefore, conformations **9** and **10** define the absolute configuration of the 3,5-benzylic carbon atom The configuration of the 1,2-benzylic carbon atom does not appear to be known, but, from steric considerations, its phenyl substituent might be expected to favor assumption of the *exo* orientation with respect to the fused five-membered rings However, **1** and **5** were obtained in only low yield from the reaction of D-glucose with benzaldehyde[1], and hence, these compounds may not be the thermodynamically favored products

EXPERIMENTAL

General — The p m r spectra were recorded under frequency-swept conditions at 100 MHz on a Varian Associates Model HA-100 n m r spectrometer*. Tetramethylsilane was used to provide an internal standard (τ 10 00) Sweep widths were verified with an electronic counter, and a modulating frequency for double-resonance experiments was supplied from an auxiliary oscillator. Spectra were obtained for solutions containing 80–212 mg of a compound in 0 35–0 8 ml of chloroform-*d* or pyridine Coupling constants and chemical shifts were measured from spectra recorded at sweep widths of 250 Hz and 1000 Hz, respectively The spectra were analyzed by first-order methods, unless stated otherwise

Spectral assignments — Assignments of multiplets that were made by matching their spacings were confirmed by observation of the solvent shifts produced on changing from chloroform-*d* to pyridine, and by frequency-swept, spin-decoupling experiments performed on the solutions in chloroform-*d* For compounds **2**, **3**, **4**, and **6**, strong irradiation at the frequency of the doublet at low field (H-1) caused the doublet at $\tau \approx 5$ 3 to collapse to a singlet, thereby confirming its assignment as H-2 Conversely, irradiation of **2** and **6** at τ 5 3 produced collapse of the low-field doublet to a singlet.

*Mention of a commercial instrument in this paper does not imply recommendation or endorsement by the National Bureau of Standards, nor does it imply that the equipment identified is necessarily the best available for the purpose

Irradiation of **2** at $\tau \approx 5.8$ (H-4 and H-5) resulted in the simplification of the doublet at τ 5.40 to a singlet (H-3), and also removed the smaller couplings from the quartets at τ 6.24 and 6.40, thus giving rise to one AB quartet (H-6 and H-6′) having a large, geminal coupling.

For compound **4**, irradiation at τ 5.84 (H-4) led to collapse of the doublet at τ 5.41 to a singlet (H-3), and also to sharpening of the one H-5 peak that could be clearly observed

Irradiation of **6** at τ 5.2 (H-4) caused collapse of the doublet at τ 4.25 (H-3) to a singlet, and simplified the complex multiplet at τ 4.26 (H-5)

ACKNOWLEDGMENTS

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BROMINE OXIDATION OF INOSITOLS FOR PREPARATION OF INOSOSE PHENYLHYDRAZONES AND PHENYLOSAZONES

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ABSTRACT

The application of bromine oxidation of inositols to give inososes, followed by conversion of the latter into phenylhydrazones or phenylosazones, is described. The diketone from *myo*-inositol gives a phenylosazone in 22–30% yield, and L-inositol gives a monoketone phenylhydrazone (12% yield) and a diketone phenylosazone (28% yield), the corresponding enantiomorphs were obtained in 8% and 29% yield, respectively The diketone from quebrachitol yields a new phenylosazone (29% yield) and no monoketone was isolated Pinitol gave a new diketone phenylosazone (10–15% yield) which showed rapid mutarotation in 1 1 (v/v) ethanol–*p*-dioxane In addition, a new phenylosazone has been obtained from DL-*epi*-inosose-2 phenylhydrazone (+)-*proto*-Quercitol (from acorns) has been converted into a phenylosazone (20% yield) *myo*-Inositol has been converted by bromine oxidation into DL-*xylo*-pentahydroxy-2-cyclohexen-1-one in low yield

INTRODUCTION

Methods for the preparation of "osones" and other dicarbonyl derivatives of sugars have received extensive study[1,2] For dicarbonyl derivatives of inositols, oxidation by *Acetobacter suboxydans* has been used most[3,4], alternatively, oxidation of inososes or their phenylhydrazones in the presence of phenylhydrazine and acetic acid has been employed[5]

The application of sodium hypobromite for oxidation of cyclitols has been reported[6–8]. With sugars, bromine in buffered solution at low temperature oxidizes aldoses quantitatively to their corresponding lactones[9], whereas ketoses are essentially unaffected under these conditions Glycosides are known to undergo oxidation under more vigorous conditions[10].

Except for a general statement by Posternak[11], in which he claims that, on hypobromite oxidation of inositols, yields of inososes are not satisfactory, we are

*Part III Methods in Inositol Chemistry For Part II, see *Carbohyd Res*, 6 (1968) 489

unaware of any published report on oxidation of inositols with bromine to give monoketoinositols (inososes), although D-quinic acid (D-1,3-dideoxy-*epi*-inositol-2-carboxylic acid) gives[12] a monoketo derivative in 60% yield, and oxidation of 1,2,3-*cis*-cyclohexanetriol with hypobromite gives[13] a monoketone in a yield of about 2%

A re-examination of the usefulness of bromine for oxidation of cyclitols is the subject of this paper.

In the previous applications of sodium hypobromite as an oxidant in the cyclitol field, preparation of cyclitol diketones was described, and these products were isolated as phenylosazones; for example, on a 1-gram scale, *myo*-inositol was oxidized with bromine in the presence of sodium carbonate buffer, and addition of phenylhydrazine gave[8] DL-*myo*-inosose-1 phenylosazone* in a yield of about 7%. Similarly, phenylosazones have been obtained from oxidation of (+)-1-deoxy-*muco*-inositol [(+)-*proto*-quercitol][6] with aqueous bromine, and from oxidation of (−)-1-deoxy-*myo*-inositol [(−)-viburnitol] with sodium hypobromite[7], however, the yields were not stated

RESULTS AND DISCUSSION

Initially, the oxidation of *myo*-inositol and of L-inositol by bromine in sodium carbonate and in sodium acetate was examined Preliminary evidence (t l c) indicated that both mono- and di-ketones were formed in significant proportions in the oxidation mixtures The yield of each product appeared to be greater in the oxidations employing the acetate buffer Consequently, acetate-buffered oxidations were then studied exclusively

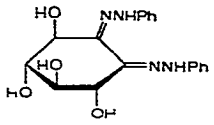
The ice-cold solution of the cyclitol was oxidized with a *ca* 35% molar excess of bromine (calculated for mono-oxidation) in the presence of sufficient sodium acetate to buffer all of the hydrobromic acid that might be formed The oxidation products were isolated by conventional methods, either as phenylhydrazones or as phenylosazones For the latter, the concentration of the oxidized cyclitol was adjusted by dilution with water to 3–6% (pH 5–6) to obtain the maximal yield of the osazone Over-oxidation was observed if the cyclitol–bromine mixture was kept for 48 h or longer; the phenylosazones from over-oxidized mixtures (96 h) were found to contain considerable proportions (3–5%) of a red pigment that was difficult to remove

Addition of phenylhydrazine to the oxidation product of *myo*-inositol gave DL-*myo*-inosose-1 phenylosazone (**1** and **2**) only, in 22–30% yield; none of the monoketone was isolated Starting with L-inositol, however, D-*myo*-inosose-1 phenylhydrazone (**3**) was obtained in 12% yield, and, alternatively, D-*myo*-inosose-1 phenylosazone (**2**) was isolated in 28% yield D-Inositol gave the corresponding enantiomorphs **4** and **1**, in 8 and 29% yields, respectively.

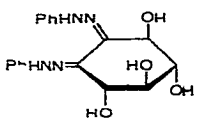
T l c showed evidence of both the mono- and the di-keto products from quebrachitol [1-*O*-methyl-*levo*-inositol] and pinitol [5-*O*-methyl-*dextro*-inositol],

*The nomenclature of this and other inositol derivatives used in this paper is based on the Fletcher–Anderson–Lardy system of H G Fletcher, Jr , L Anderson, and H A Lardy, *J Org Chem* , 12 (1951) 1238, see also ref 14

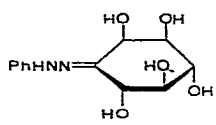
but only the diketo derivatives of each were actually isolated (as phenylosazones, compounds **5** and **6**, respectively)



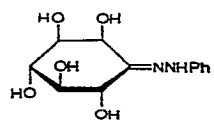
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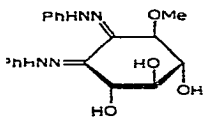
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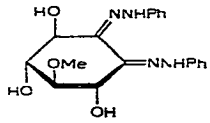
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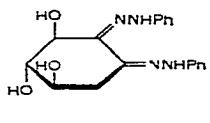
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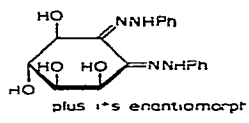
5



6



7



plus its enantiomorph

8

Efforts to obtain either monoketo derivative as a phenylhydrazone were unsuccessful, mainly because of their high solubility. Although the yield of phenylosazone **5** was 29%, the yield of **6** was only 10–15%

The crude phenylosazone **6** contained a considerable proportion of an unknown, yellow component having a higher R_F value (see Experimental Section), in addition to several red-orange components, the mixture also contained a small proportion (2%) of L-*myo*-inosose-1 phenylosazone. this can arise from the parent compound by demethylation to D-inositol, followed by oxidation of the latter to mono- and di-keto derivatives

The mutarotation of phenylosazone **6**, exhibiting a rapid change from $[\alpha]_D$ -507 to 0.00°, followed by a slow change to $+130°$, indicates occurrence of at least two reactions The mutarotation of **6** must be associated with the presence of a remote 5-methoxyl group, as phenylosazone **5**, having a 3-methoxyl group, shows no mutarotation*

Under similar conditions of reaction, quebrachitol proved to be more stable toward bromine oxidation, and the crude phenylosazone **5** isolated contained only a red component; practically no demethylation was observed

Compound **5** and **6** have not been reported previously They were identical in all physical properties with the phenylosazones which we have prepared in two steps by (*1*) catalytic oxidation of quebrachitol and pinitol to the corresponding inososes as described by Post and Anderson[14], and (*2*) further conversion into their osazones Column chromatography of the crude phenylosazone **6** obtained by catalytic oxidation of pinitol (through pinitol inosose followed by treatment with phenylhydrazine) showed the presence of the same yellow and red impurities as were observed in the product from bromine oxidation On the other hand, quebrachitol was found to be more stable toward oxidation, and the phenylosazone **5** isolated contained less of the decomposition products than phenylosazone **6**

By bromine oxidation in acetate buffer, (+)-*proto*-quercitol gave the diketone

*In general the specific rotations of inosose phenylosazones in 1 1 (v/v) ethanol–*p*-dioxane are somewhat higher than those in 1·1 (v/v) ethanol–pyridine

phenylosazone (7) in approximately 20% yield (based on the average concentration of (+)-*proto*-quercitol present in dried, ground acorns)

In the presence of sodium pivalate or propionate and their corresponding acids (instead of sodium acetate–acetic acid) as buffers in oxidations with phenylhydrazine of the inososes to their phenylosazones, the products initially isolated were less colored, and were therefore considered to be of higher quality, than those obtained in the acetate buffer. Substitution of hydrochloric, sulfuric, or formic acid for the buffer gave no phenylosazone

Excess bromine may conveniently be removed by addition of acrylamide, acrylonitrile, diethylamine, methyl methacrylate, or sodium hydrogen sulfite, or by bubbling of ethylene–air mixture, sodium hydrosulfite or sodium thiosulfate interfere in the redox cycle that is apparently necessary for the formation of the phenylosazone, and therefore cannot be used The red pigment present in crude inosose phenylosazones can conveniently be removed by treatment of the crude product with a warm, aqueous ethanolic solution of sodium hydrosulfite ($Na_2S_2O_4$)

It is recommended that final solutions containing D- or[14] L-*myo*-inosose-1 intended for conversion into the corresponding phenylosazones be diluted with methanol (or ethanol) up to 30%, prior to treatment with phenylhydrazine, in order to keep the sparingly soluble D- or L-*myo*-inosose-1 phenylhydrazones in solution, otherwise, the inosose phenylosazone obtained may contain a considerable proportion of the phenylhydrazone. In cases where a mixture of inosose phenylhydrazone and phenylosazone is suspected, the quick, resin test[15] reveals the presence of the reducing component (inosose), if it is present, the mixture is dissolved in warm methanol and re-treated with phenylhydrazine in aqueous acetic acid; the conversion of a solid inosose phenylhydrazone into the phenylosazone under the conditions described is incomplete

It may be noted, however, that inososes or their phenylhydrazones may differ in their rates of conversion into the corresponding phenylosazones under the conditions described For example[16], DL-*epi*-inosose-2 or[17] L-*epi*-inosose-2 or their phenylhydrazones are more difficult to convert into the corresponding phenylosazones under the conditions given; this indicates thermodynamic stability of L-*epi*-, or D-*epi*-, or DL-*epi*-inosose-2 phenylhydrazones, as compared to the readily convertible *myo*-inosose-2 or D- or L-*myo*-inosose-1 phenylhydrazones The Fiesers[18] attributed the slow formation of DL-*epi*-inosose-2 phenylosazone (8) from its phenylhydrazone to its having a chair conformation, which restricts the plane of the C=N bond from becoming parallel to a HCOH group and thus retards the Amadori hydrogen shift, the possible mechanisms for the formation of the phenylosazones from sugars[19,20] or other compounds[21], including the Amadori-type rearrangement, have recently been discussed The difficulty in preparation of DL-*epi*-inosose-2 phenylosazone (8), under the conditions described, could also be ascribed to the moderate solubility of DL-*epi*-inosose-2 phenylhydrazone; this compound requires a considerable proportion of methanol (or other suitable solvent) to keep it in solution. A small quantity of the

previously unreported DL-*epi*-inosose-2 phenylosazone (**8**) has now been prepared by a laborious procedure

The action of bromine on inositols is stereospecific and can be compared with catalytic or bacterial oxidants which, as is known, show a tendency to attack axial hydroxyl groups

Oxidation of *myo*-inositol with potassium hypobromite for 36 h, followed by neutralization with potassium carbonate and cooling, gave, in low yield, the crystalline potassium salt of DL-*xylo*-pentahydroxy-2-cyclohexen-1-one, an enolic form of a diketoinositol prepared by the oxidation of *myo*-inositol with nitric acid[22]. It is interesting that both oxidants (bromine and nitric acid) produce the same diketoinositol intermediate

EXPERIMENTAL

Materials — *myo*-Inositol was a purified, commercial* preparation (Corn Products Refining Co., Argo, Illinois) Crude quebrachitol* (Plant Division, U S Rubber Co., Rockefeller Center, New York, N Y) was purified by recrystallization (carbon) from aqueous ethanol containing 10% of acetic acid (solvent *A*), m p 191–193°, $[\alpha]_D^{22}$ −80 5° (*c* 2 37, water) Crude pinitol* (Western Pine Association Research Laboratory, Portland, Oregon) was purified by recrystallization from solvent *A*, m p 185–186°, $[\alpha]_D^{22}$ +66° (*c* 2 5, water) L-Inositol was prepared by demethylation of purified quebrachitol A mixture of quebrachitol (25 g) and 47% hydriodic acid (50 ml) was refluxed for 30 min, the warm solution was added portionwise to warm absolute ethanol (400 ml) with stirring, yield of crude L-inositol, 20–22 g; after recrystallization from warm aqueous ethanol, it had m p 243–244°, $[\alpha]_D^{22}$ −64 9° (*c* 2 0, water) D-Inositol was prepared by demethylation of pinitol by the same procedure, after recrystallization from warm aqueous ethanol, it had m p 246–248° $[\alpha]_D^{22}$ +65.1° (*c* 2 2, water)

I r spectra were recorded with a Perkin–Elmer 257 grating spectrophotometer*, the absorption bands (see Table I), particularly those found for N-H (bending) or Ph-N groups of inosose phenylhydrazones or phenylosazones, were in good agreement with those found for other mono-, bis-, and tris-(phenylhydrazones)[23] U v and visible spectra were recorded with a Beckman DK-2 spectrophotometer*, all inosose phenylosazones showed absorption bands at 254–259, 308–321, and 389–399 nm, as does D-*myo*-inosose-1 phenylosazone[24] However, many inosose phenylosazones show, in addition to the three bands, a broad band or a shoulder in the region at 227–230 nm The n m r spectra of the inosose phenylosazones (recorded with a Varian A-60 spectrometer, for solutions in methyl sulfoxide, TMS internal standard, δ scale) showed a low-field signal at 12 5–13 p p m (chelate ring-structure), similar to that for D-*myo*-inosose-1 phenylosazone[24] *p*-Dioxane was distilled from a small proportion of lithium aluminum hydride, and was stored in the frozen state until used

*Mention in this article of certain commercial instruments or chemical compounds does not constitute endorsement by the National Bureau of Standards

TABLE I

INFRARED ABSORPTION BANDS (cm^{-1}) OF INOSOSE PHENYLHYDRAZONES AND PHENYLOSAZONES[a]

Assignment	*Compound number*[b]							
	1	2	3	4	5	6	7	8
OH, NH								3575 m
		3480 m						3485 s
		3420 s	3440 sh	3450 s				
	3380 s		3380 s		3390 w	3370 w	3390 s	
		3340 s	3320 s	3300 s	3300 m			3300 s
	3250 s					3280 s	3290 s	3240 s
							3115 m	
C=N	1640 m	1638 m	1657 m	1652 w	1654 w	1680 sh	1688 sh	1690 sh
Phenyl ring	1600 s	1600 s	1605 s	1600 s	1600 s	1600 s	1600 s	1600 s
	1585 w		1588 w					1530 s
		1500 sh		1510 m	1509 s	1510 s	1509 s	1510 m
		1494 m		1490 w	1490 w	1488 m	1490 m	1490 s
N–H (bending)								1575 s
				1562 sh	1560 w	1560 s		1560 s
	1550 s			1555 s	1555 w	1555 sh	1550 s	
		1518 m			1500 s			
Ph–N	1170 s	1170 s	1170 s	1170 s	1170 s	1170 s	1170 s	1170 m
			1149 m	1155 w	1158 sh	1155 w		1150 w
	1130 m	1135 m	1130 s			1130 s	1130 w	1140 m
	1120 m	1120 m		1122 s	1125 s		1121 s	
Fingerprint		1390 w						
region	1340 m		1332 m	1350 w	1340 sh	1340 sh	1340 sh	1330 m
		1312 m	1320 m	1320 m			1325 w	
	1309 m		1310 m	1310 sh		1308 w		1310 sh
		1300 sh		1300 sh	1300 w		1300 m	1300 sh
	1290 w	1270 w	1282 sh					1289 m
	1260 s	1250 s	1258 s	1255 s	1250 s	1250 s	1255 s	1260 s
			1248 sh				1248 sh	
			1230 m			1230 sh	1238 sh	1235 m
	1220 w	1220 w		1215 sh			1228 sh	1218 sh
	1210 w		1200 m		1210 sh	1205 m	1210 w	
	1180 w	1180 w		1190 w			1190 w	1190 w
	1170 s	1170 w	1170 s	1170 s	1170 m	1170 s	1170 s	1170 m
	1150 w	1150 sh	1149 m	1155 w	1158 sh	1155 w	1153 sh	1150 w
	1130 m	1135 m	1130 s			1130 s		1140 m
	1120 m	1120 m		1122 s	1125 m		1121 s	
	1115 m			1109 w		1110 sh		1110 w
	1095 m	1095 s	1100 s		1090 w	1100 s	1105 s	1090 w
		1080 w	1080 m	1079 w		1075 w	1080 sh	1076 s
	1070 s	1070 s			1070 w		1070 s	
		1050 m	1058 s	1050 s	1050 m	1050 w	1050 w	1050 w
						1035 s	1038 s	1040 m
								1030 m
		1020 sh						1025 w
	1010 m		1010 s					1015 m

TABLE I (CONTINUED)

	1000 m						1000 w
990 s	988 w		990 s	995 m	995 w	990 w	990 w
				960 w	970 w	955 s	970 sh
930 m	924 w	935 s		935 w	935 sh	925 m	950 m
	920 m		920 sh	915 m	912 s		
			900 s				900 m
	890 m			890 m	898 w		890 w
880 m	880 sh	880 m			880 w	885 m	885 w
				870 sh	875 w	865 w	870 m
	860 sh		860 w				860 sh
		852 s	850 s				850 w
				830 m	830 m		840 sh
			820 w			825 s	820 sh
		785 s		790 w		780 m	790 m
				772 w	775 m		
750 s	752 s	750 s	750 s	750 s	750 s	750 s	750 s
690 s	695 s	690 s	690 s	690 s	698 s	690 s	695 sh
					680 w		690 s
675 m	672 w	670 w	680 w	665 sh	665 sh	670 w	678 w
							660 sh

[a]Spectra were measured for Nujol mulls [b]Key to compounds 1, *myo*-inosose-2 phenylhydrazone, 2, L-*myo*-inosose-1 phenylhydrazone, 3, DL-*epi*-inosose-2 phenylhydrazone, 4, DL-*myo*-inosose-1 phenylosazone, 5, DL-*epi*-inosose-2 phenylosazone, 6, quebrachitol inosose phenylosazone, 7, pinitol inosose phenylosazone 8 (+)-*proto*-quercitol phenylosazone

Purification of inosose phenylosazones was performed by column chromatography on Florisil* (100–200 mesh, Floridan Co., Philadelphia, Pennsylvania) Generally, a solution of the crude phenylosazone in ethyl acetate was filtered, placed on a column of suitable size, and eluted with 1 l (v/v) 95% ethanol–ethyl acetate or with 95% ethanol More difficultly soluble phenylosazones were dissolved in *p*-dioxane, and eluted with ethanol or 19 l (v/v) ethanol–*p*-dioxane T l c was performed on silica gel G (5 × 20 cm plates, 250 μm thick) for 60 min Separations on columns were monitored by t l c on microscope slides

Preparation of DL-myo-*inosose-1 phenylosazone* (1 and 2) *from* myo-*inositol* — *Procedure A* A precooled solution (3°) of *myo*-inositol (25 g, 139 mmole) and sodium acetate trihydrate (52 g) in 200 ml of water was placed in an ice-bath, and treated with bromine (30 g, 187 mmole). The vessel was stoppered loosely, and the reaction mixture was stirred for 24 h at room temperature (It is important that the temperature of the reaction mixture be kept below 5° for 6–8 h from the beginning of the oxidation) Glacial acetic acid (60 ml) was added, and the excess of bromine was removed by bubbling through the solution a mixture of ethylene and air for 10 to 15 min T l c. of the clear solution [on microcrystalline cellulose[25], 1 mm thick, 5 × 20 cm glass plate, for 60 min with 3·1 l (v/v) butanol–acetic acid–water and the inosose spray[26] (*A*) or the alkaline silver nitrate spray[27] (*B*)] then revealed the presence of two reducing components The faster-moving compound (lilac spot, spray *A*, or dark spot, spray *B*) was found to be *myo*-inosose-2 (major component), the slower-

moving, minor component (yellow spot, spray *B*) is believed to be derived from a diketoinositol To the solution was then added sufficient sodium acetate trihydrate (15 g) to give a negative test with Congo Red paper, the solution was diluted to about 2 5 l with water, and cooled to 10° to 15°, and phenylhydrazine (35 g, 320 mmole) was added. The mixture was stirred for 1 h, and kept for 48 h at room temperature with occasional stirring The orange-red crystals of **1** and **2** that separated were filtered off, washed successively with water, cold 1 1 (v/v) ethanol–water, and cold 95% ethanol, and dried at 50°, weight of crude product 8 3 g Dilution of the filtrate with water (1 liter), and further storage for 36 h, produced an additional crop (4 2 g), total yield 12 5 g (25%), range in several experiments, 11–15 g (22 to 30%)

The crude phenylosazone (**1** and **2**) contained a red impurity*†, R_F 0 96 [2·1 1 (v/v) benzene–acetic acid–butanone], 0 87 (solvent *A*), the osazone had R_F 0 30 The colored material was removed by suspending the crude phenylosazone (10 g) in 100 ml of a freshly prepared, 12% aqueous solution of sodium hydrosulfite ($Na_2S_2O_4$) containing 40 ml of ethanol, and stirring the suspension for 30 min at 80–85° By this time, the red color has disappeared; the suspension was then diluted with water, and cooled The light-yellow phenylosazone was filtered off, washed successively with water and aqueous ethanol, and dried, yield 8 5 g (85%), the product was about 96% pure (i r. spectrum)

The product can be further purified by dissolving it in methanol containing potassium hydroxide (5%) and reprecipitating it by dilution with water, or by recrystallization from aqueous *N*,*N*-dimethylformamide, aqueous 2-methoxyethanol–pyridine, or aqueous ethanol–*p*-dioxane A sample purified by column chromatography (*p*-dioxane–95% ethanol) showed a homogeneous, yellow spot on a thin-layer chromatogram [3 2 1 (v/v) butanone–benzene–acetic acid], R_F 0 41 ±0 01 (solvent *B*), m p 199–201°, (lit [5] m p 199–200°), λ_{max}^{MeOH} 229 (sh) (ε ~15,800), 257 (ε ~23,900), 318 (ε ~11,000), and 398 nm (ε ~20,800)

Procedure B A precooled solution (3°) of *myo*-inositol (100 g, 556 mmole) and sodium acetate trihydrate (208 g) in 800 ml of water was oxidized with bromine (120 g, 750 mmole) for 24 h according to Procedure A The reaction mixture was then treated with acetic acid (100 ml), and excess bromine was removed by addition of aqueous sodium hydrogen sulfite (20% solution, 38 ml). Methanol (500 ml) was then added, and the mixture was diluted to *ca* 4 l with ice-cold water, and treated with phenylhydrazine (100 g), and the mixture was kept for 92 h at room temperature, with occasional stirring The yield of crude, somewhat dark-red **1** and **2** was 31 g (20%), range 29–32 g (18–21%)

In another, otherwise identical, preparation, the excess of bromine was removed by bubbling ethylene–air through for about 55 min; the yield of crude **1** and **2** isolated after 96 h was 41 g (28%)

*The nature of the red impurity present in this and other compounds will be discussed in a subsequent article †Treatment of an ice-cold solution (0 to 5°) with phenylhydrazine sometimes produces a colorless, crystalline compound of unknown structure Recrystallized from ethanol, the compound melted at 226–227°, λ_{max}^{MeOH} 227 and 274 nm Found C, 60 5, H, 5 80

Oxidation of myo-*inositol with hot bromine water* — To a solution of *myo*-inositol (50 g, 278 mmole) in water (200 ml) was added bromine (30 g, 187 mmole) The solution was refluxed (hood) for 90 min, and cooled, and excess bromine was removed by means of sodium hydrogen sulfite. The solution was successively diluted with water (1.8 l) and glacial acetic acid (50 ml), rendered neutral to Congo Red paper with sodium acetate, and treated with phenylhydrazine (35 ml) DL-*myo*-Inosose-1 phenylosazone (**1** and **2**) was obtained in two crops the first crop (3 g) after 36 h, and the second crop (5 g) after an additional 48 h; total yield, 8 g (8.1%).

Preparation of D-myo-*inosose-1 phenylosazone* (**2**) *from* L-*inositol* — A precooled solution (5°) of L-inositol (12 g, 70 mmole) and sodium acetate trihydrate (26 g) in 100 ml of water was treated with bromine (15 g, 93 mmole) by the foregoing procedure, and the mixture was kept for 24 h at room temperature. Glacial acetic acid (30 ml) was added, and the excess of bromine was removed by means of ethylene–air at room temperature Sodium acetate (8 g) was added, and dilution of the solution with methanol (300 ml) and water (700 ml), followed by treatment with phenylhydrazine (20 ml) and occasional stirring, gave a product that was collected in two crops, first crop (3 8 g) after 36 h, and second crop (3 2 g) after dilution with water (1 liter) and further storage (48 h), the total yield of **2** was 7 g (28%) The purified product showed essentially the same R_F value as DL-*myo*-inosose-1 phenylosazone (**1** and **2**) (solvent *B*), m p 206–208° (lit.[3] m p 217°, 210°, lit[24] m p 207–209°), $[\alpha]_D^{23}$ +268 ±10° (24 h), +243 ±5° [*c* 0 01, 1 1 (v/v) ethanol–pyridine], $[\alpha]_D^{23}$ +329 ±20° [*c* 0 01, 1 1 (v/v) ethanol–*p*-dioxane, no mutarotation) [lit[3] $[\alpha]_D^{25}$ +240→214° (1 1 (v/v) ethanol–pyridine)] The product was identical with an authentic sample of D-*myo*-inosose-1 phenylosazone[3]

Preparation of D-myo-*inosose*-1 *phenylhydrazone* (**3**) *from* L-*inositol* — A precooled solution (5°) of L-inositol (12 g, 70 mmole) and sodium acetate trihydrate (26 g) in 100 ml of water was treated with bromine (15 g, 93 mmole) by the foregoing procedure, and the mixture was kept for 24 h at room temperature. The solution was cooled to 5°, and the excess of bromine was removed by means of ethylene–air Sodium acetate (8 g) was added, and the solution was diluted with ice–water (1 liter at 3°), phenylhydrazine (20 ml) was added, and the mixture was stirred vigorously (ice-bath) for 30–45 min The product was separated by filtration, and successively washed with water, ice-cold 50% aqueous ethanol, and ethanol, to give colorless to pale-pink crystals of **3**, yield 5 g (12%) A sample recrystallized from 2-methoxyethanol and then from methanol gave colorless needles, m p 195–197° (lit[14] m p 196–197°); λ_{max}^{MeOH} 278 (ε ~23,200), 302 nm (sh) (ε ~10,350) The product was identical with an authentic sample of D-*myo*-inosose-1 phenylhydrazone[14] The filtrate and washings were combined, diluted with methanol (300 ml) and water to about 700 ml, and kept for 60 h at room temperature, the orange product (6 g, 25 2%) was identical with **2**

Preparation of L-myo-*inosose-1 phenylosazone* (**1**) *from* D-*inositol.* — A precooled (5°) solution of D-inositol (12 g, 70 mmole) and sodium acetate trihydrate (26 g) in 100 ml of water was treated with bromine (15 g, 93 mmole) by the procedure

for oxidation of L-inositol. The yield of **1** was 7.3 g (29%); the product was identical with an authentic sample[3].

Preparation of L-myo-*inosose-1 phenylhydrazone* (**4**) *from* D-*inositol.* — Oxidation of D-inositol (12 g, 80 mmole) with bromine (15 g, 93 mmole) by the procedure described for L-inositol gave **4** (5 g, 12%) and **1** (5 8 g, 24 4%), each product was identical with an authentic sample[3]

*Preparation of 3-*O*-methyl-*myo-*inosose-1 phenylosazone* (**5**) *from quebrachitol* — A precooled solution (5°) of quebrachitol (1-*O*-methyl-*levo*-inositol) (25 g, 120 mmole) and sodium acetate trihydrate (52 g) in 200 ml of water was treated with bromine (30 g, 187 mmole) by the foregoing procedure Acetic acid (80 ml) and sodium acetate (20 g) were added, the solution was cooled to 10°, and the excess of bromine was removed by adding, with stirring, diethylamine (75 ml) Dilution with methanol (300 ml) and water (2 2 1), followed by treatment with phenylhydrazine (35 ml), gave a product that was collected in two crops. first crop (8 8 g) after 36 h, and the second crop (6 2 g) after further dilution with water (1 liter) and storage (48 h), the total yield of crude **5** was 15 g (29%) The crude product contained red impurities, R_F 0 93, 0 87 (solvent *A*), the osazone had R_F 0.66 A product of about 98% purity (i r spectrum) could be obtained as follows the crude phenylosazone **5** was treated with sodium hydrosulfite as described for DL-*myo*-inosose-1 phenylosazone (**1** and **2**) The resulting product (3 g, dry wt) was stirred with warm ethanol (15 ml), and cooled, and the suspension was filtered, to give 2 2 g (73%) of light-yellow needles, m p 191–193° A sample purified by column chromatography (95% ethanol) and recrystallized from aqueous ethanol gave golden-yellow needles, m p 195–197°, $[\alpha]_D^{23}$ −485 ±15° [*c* 0 05, 1 1 (v/v) ethanol–*p*-dioxane], no appreciable mutarotation was observed after 60 min A sample for analysis was dried for 2 h at 140°/0 01 torr.*

Anal Calc for $C_{19}H_{22}N_4O_4$. C, 61 60, H, 5.98, N, 15 12 Found C, 61.80, H, 6.10; N, 15 20

The product showed a single yellow spot on a thin-layer chromatogram, R_F 0 60 ±0 02 (solvent *B*), λ_{max}^{MeOH} 228 (sh) (ε ~16,000), 258 (ε ~24,300), 318 (ε ~11,500), 397 nm (ε ~20,500)

Consumption of periodic acid[3] — Quebrachitol phenylosazone (**5**, 37 mg, 10 mmole) in 60% aqueous ethanol (97 ml) was treated with aqueous periodic acid (3 ml, 536 6mM) at room temperature An aliquot (10 ml) was withdrawn and analyzed every 15 min (6 determinations) The excess of periodic acid was determined with sodium arsenite; 2.80 ml of the titrant (100.3mM) was consumed, the average consumption of periodic acid per mole of osazone **1** was 2 06 moles After 1 week, overoxidation had occurred, indicating that the methoxyl group had undergone scission

*Preparation of 5-*O*-methyl-*myo-*inosose-1 phenylosazone* (**6**) *from pinitol* — A precooled solution (5°) of pinitol (5-*O*-methyl-*dextro*-inositol) (12 5 g, 60 mmole) and sodium acetate trihydrate (2 6 g) in 100 ml of water was treated with bromine

*Dried at 25°/0 1 torr, a hemihydrate was obtained

(15 g, 935 mmole) by the foregoing procedure. Glacial acetic acid (35 ml) and sodium acetate (8 g) were added, the solution was cooled to 10°, and the excess of bromine was removed by bubbling ethylene–air through, with stirring, for 10 min. The clear solution was diluted with water (700 ml) and methanol (300 ml), and treated with phenylhydrazine (20 ml) The solution was kept for 96 h at room temperature, and the product was collected in two crops, first crop (7–8 g) after 48 h, and the second crop (1.6 g) after dilution with water (1 l), the total yield of crude **6** was 9.4 g (31%). The crude product contained a considerable proportion of decomposition products; R_F 0 96, 0 93 (red), 0 84 (orange-yellow) (solvent *A*) The osazone had R_F 0.68. The crude product (7 g) was treated with sodium hydrosulfite (10% solution, 70 ml) at 90° to give the phenylosazone **6**, 4.5–5.5 g (64–78%). This product was further purified by column chromatography. a solution of **6** (1 g) in ethanol (100 ml) was passed through a column of Florisil (4 × 35 ml) and eluted with 95% ethanol The first zone (orange-yellow) was discarded; the second zone (light yellow) was collected and concentrated to dryness, and the resulting solid was recrystallized from aqueous ethanol to give **6** as light-yellow crystals; yield 0 2 g (20%), m p. 206–207° (with darkening at 204°), $[\alpha]_D^{23}$ −507 ±25° (3 min) (rapid mutarotation) → 0 00° (63 min) → +88 ±5° (123 min) → +130 ±15° [final, 24 h, *c* 0 01, 1·1 (v/v) ethanol–*p*-dioxane] A sample* for analysis was dried for 2 h at 140°/0 1 torr

Anal Calc. for $C_{19}H_{22}N_4O_4$ C, 61 60, H, 5.98; N, 15 12 Found C, 61 84; H, 6 12, N, 15.15.

The product showed a single, yellow spot on a thin-layer chromatogram, R_F 0 58 ±0 02 (solvent *B*), λ_{max}^{MeOH} 230 (sh) (ε ~15,300), 258 (ε ~24,700), 321 (ε~11,300), 396 nm (ε ~20,000) One mole of **6** consumed 0 97 mole of periodic acid

Preparation of DL-epi-*inosose-2 phenylosazone* (**8**) *from* DL-epi-*inosose-2 phenylhydrazone* — A solution of DL-*epi*-inosose-2 phenylhydrazone[16] (colorless needles from 2-methoxyethanol, m p 188–190°) (0 5 g, 6 mmole) in methanol (200 ml) was diluted with water (50 ml) and treated with phenylhydrazine (2 ml) and acetic acid (10 ml) The reaction mixture (in a loosely stoppered bottle) was kept for 24 h at room temperature, water (30 ml) was then added and the solution was kept for 24 h The mixture was then transferred to a beaker, and allowed to evaporate slowly (hood) for 36–48 h An orange-red solid (0 2 g) was collected, treated with aqueous sodium hydrosulfite (10 ml), and purified by column chromatography (*p*-dioxane–95% ethanol), orange-yellow fractions [λ_{max}^{MeOH} 230 (sh), 305, 328, and 396 nm] were discarded; a slow-moving, yellow zone was collected, and evaporated to dryness, and the resulting solid was recrystallized from aqueous ethanol–*p*-dioxane to give **8** as yellow crystals; yield 0 05 g (7 6%), m p. 202–204°, R_F 0 38 ±0 01 (solvent *B*); λ_{max}^{MeOH} 229 (sh) (ε ~15,800), 258 (ε ~23,900), 318 (ε ~11,900), 398 nm (ε ~21,200). A sample for analysis was dried for 2 h at 140°/0 1 torr

*A sample dried at 25°/0 1 torr contained 0 5 molecule of water of crystallization per molecule Calc for $C_{19}H_{22}N_4O_4$ 0 5 H_2O C, 60 14, H, 6 10, N, 14 80 Found C, 60 20, H, 5 95, N, 14 90

Anal Calc. for $C_{18}H_{20}N_4O_4$: C, 60.65, H, 5.65, N, 15 72 Found C, 60 80, H, 5.75, N, 15.60.

One mole of **8** consumed 3 12 moles of periodic acid

Preparation of (+)-proto-*quercitol** *phenylosazone* (**7**) *from acorns* — Acorn flour (1 kg), from dried acorns crushed in a ball mill, was soaked in distilled water (2 l) for 48 h at room temperature, with occasional stirring The suspension was filtered, and the solid was washed with water (600 ml) The dark-brown filtrate and washings were combined, lead(II) acetate (35 g) in 20% acetic acid (120 ml), and Celite (50 g) were added, and the suspension was stirred for 30 min and filtered The filtrate and washings were combined (about 3 l), and treated with hydrogen sulfide, and the suspension was filtered through a layer of Celite The solid was washed with water, and the filtrate and washings were combined (about 3 2 l), treated with sodium acetate trihydrate (200 g) and glacial acetic acid (150 ml), and cooled to 10° Bromine (90 g) was added, and the mixture was stirred for 48 h at room temperature To the mixture was added sodium acetate (100 g), the solution was cooled to 10°, and the excess of bromine was removed with diethylamine (50 ml). Phenylhydrazine (100 ml) was added, and the suspension was stirred for 2 h, and kept for 96 h at room temperature The product was collected in three crops. the first crop (29 g) after 48 h, the second crop (25 g), following dilution with water (1 liter), after an additional 24 h; and the third crop (3 g) after an additional 24 h The total yield of light-yellow **7** was 57 g (20%)

A sample recrystallized from aqueous ethanol–*p*-dioxane had m p 201–203° (dec.) (lit [28] m p 199–200°), $[\alpha]_D^{23}$ +102 ±5° [*c* 0 01, 1.1 (v/v) ethanol–*p*-dioxane] [lit [28] $[\alpha]_D^{25}$ +65° (1 1 (v/v) ethanol–pyridine)], R_F 0 28 ±0 01 (solvent *B*), λ_{max}^{MeOH} 229 (sh) (ε ~15,100); 255 (ε ~21,300), 308 (ε ~11,800), and 393 nm (ε ~18,600) The product was identical with an authentic sample[6 28] of **7**

Potassium hypobromite oxidation of myo-*inositol, isolation of* DL-xylo-*pentahydroxy-2-cyclohexen-1-one* — A precooled solution (5°) of *myo*-inositol (25 g, 139 mmole) and potassium acetate (37 g) in 200 ml of water was treated with bromine (30 g, 187 mmole), and stirred for 36 h at room temperature The excess of bromine was removed by means of air–ethylene, and the solution was treated with solid potassium carbonate, and stirred for about 20 min at 90° until it remained permanently basic (pH 9–10) The dark solution was then concentrated until crystallization started, and the suspension was cooled and filtered. The filtrate was concentrated to about 20 ml, cooled, nucleated, and kept for ten days in a refrigerator, with occasional shaking and scratching After separation of a small quantity of blue crystals of potassium rhodizonate, the filtrate deposited light-yellow crystals, yield 1 2 g (4%) After recrystallization from aqueous acetone, the product exhibited the properties of an authentic sample of the potassium salt of DL-*xylo*-pentahydroxy-2-cyclohexen-1-one[19]

*(+)-*proto*-Quercitol is named (+)-1-deoxy-*muco*-inositol by the Fletcher–Anderson–Lardy system

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THE SYNTHESIS OF METHYL 3-*O*-ETHYL-α-D-GLUCOPYRANOSIDE

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ABSTRACT

Two syntheses of methyl 3-*O*-ethyl-α-D-glucopyranoside (**6**) are described A comparison of the n m r spectra of a series of substituted methyl α-D-glucopyranosides in methyl sulfoxide-d_6 allows the assignment of chemical shifts and coupling constants to the hydroxyl protons in substituted methyl α-D-glucopyranosides

INTRODUCTION

During an investigation of the products obtained by the vinylation of methyl α-D-glucopyranoside[2], methyl 3-*O*-ethyl-α-D-glucopyranoside was required Several syntheses have been described for the homologous methyl 3-*O*-methyl-α-D-glucopyranoside[3–6] and the parent sugar, 3-*O*-ethyl-D-glucose[7,8]

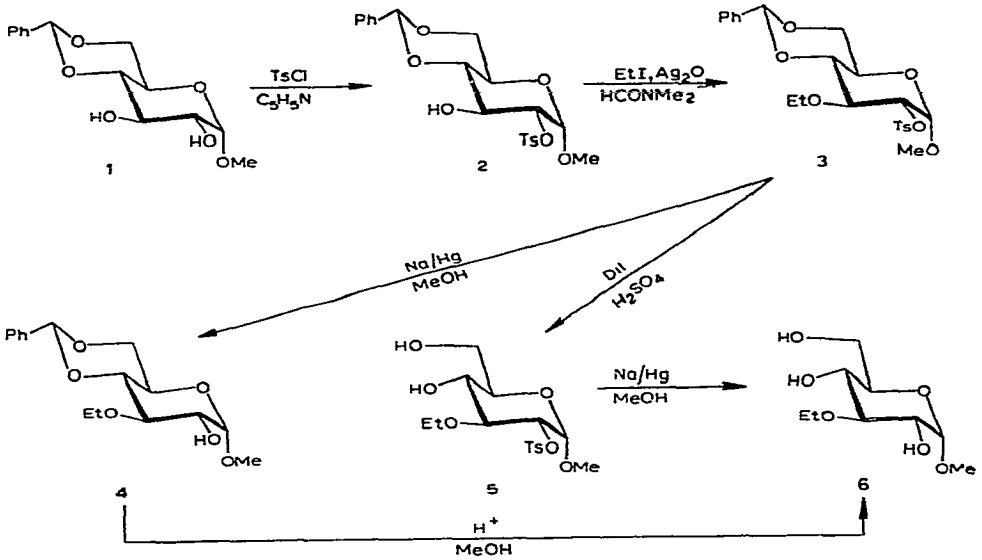
The first of two synthetic methods used in this study was selected because of interest in the relation of n m r spectra and structures of the intermediate methyl D-glucopyranosides The second confirmatory method was chosen for its simplicity

DISCUSSION

Synthesis — In the first approach (see Scheme 1) methyl 4,6-*O*-benzylidene-α-D-glucopyranoside (**1**) was *p*-toluenesulfonylated[3 6 9] to yield methyl 4,6-*O*-benzylidene-2-*O*-*p*-tolylsulfonyl-α-D-glucopyranoside (**2**) The n m r spectrum of ester **2** in chloroform-*d* shows the H-2 signal as a multiplet, apparently overlapped by the signals of H-3 and H-5 Confirmation of the structure is provided by the spectrum in methyl sulfoxide-d_6 (see Table I) Ethylation of compound **2** gave methyl 4,6-*O*-benzylidene-3-*O*-ethyl-2-*O*-*p*-tolylsulfonyl-α-D-glucopyranoside (**3**) Ethylation of the 3-hydroxyl group causes the H-3 signal to be moved upfield[10], exposing the quartet due to H-2 These compounds show only two of the three characteristic[11] α-glucopyranoside bands in their i r spectra (see Table II)

Desulfonylation, or removal of the benzylidene group, from **3** was accomplished by established methods[3]. When removal of the benzylidene group was performed before desulfonylation, the intermediate methyl 3-*O*-ethyl-2-*O*-*p*-tolylsulfonyl-α-D-glucopyranoside (**5**) was obtained as a glass that has not yet been crystallized

Other workers have encountered similar difficulties[6]. The specific rotation of the crude product 5, $[\alpha]_D^{22}$ +70° (*c* 0.99, chloroform), is congruous with that reported for its



Scheme 1

TABLE I

N M R SPECTRA OF SUBSTITUTED METHYL α-D-GLUCOPYRANOSIDES IN[a] METHYL SULFOXIDE-d_6

Compound	*Aromatic protons*		*PhCH H-1*		*Ring protons*	*OMe*	*2-OR*	*3-OR′*	*4-OR″*	*6-OR‴*
	Ts	*Ph*								
1[b]		2 54	4 42	5 34d (3 2)	5 57–6 75	6 68	4 86d (4 8)	5 12d (6 6)		
2[c]	2.10d (8 6) 2 52d	2 58	4 45	5 33d (3 5)	5 57–6 92	6 82	CH_3Ar 7 65	4 37d (6 5)		
3[c]	2 13d (8 1) 2 53d	2 62	4 42	5 26d (3 4)	5 57–6 90	6 95	CH_3Ar 7 68	CH_3CH_2- 9 28t (7 0)		
4[b]		2 55	4 36	5 35d (2 2)	5 74–6 74	6 69	4 98d (4 6)	CH_3-CH_2- 8 98t (7 0)		
6[b]				5 46d (2 2)	6 10–6 90	6 71	5 11d (4 8)	CH_3-CH_2 8 98t (7 0)	5 38d (5 5)	5 58t (5 8)
(methyl α-D-glucopyranoside)[b]				5 43d (3 4)	6 15–6 90	6 67	5 18d (4 7)	5 29d (5 2)	5 38d (5 9)	5 56t (5 8)

[a]Chemical shifts given as τ values, *J* values are given in Hz and are enclosed in parentheses, external standard Me_4Si at τ = 10 0 checked before and after each measurement, d, doublet, m, multiplet, q, quartet, t, triplet [b]Spectrum obtained at 100 MHz [c]Spectrum obtained at 60 MHz.

3-*O*-methyl homolog, $[\alpha]_D^{20}$ +79 5° (*c* 1 11, chloroform)[6] Compound **5** was converted into methyl 3-*O*-ethyl-α-D-glucopyranoside (**6**) by treatment with sodium amalgam in methanol

TABLE II

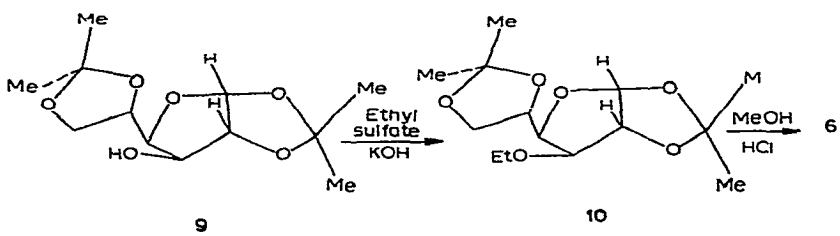
TYPES 1 AND 2a BANDS IN THE INFRARED SPECTRA OF CERTAIN METHYL α-D-GLUCOPYRANOSIDES

Compound	*Solvent or dispersant*	*Type 1* (cm^{-1})	*Type 2a* (cm^{-1})
1	$CHCl_3$	918[a]	845
2	$CHCl_3$	918	838
3	$CHCl_3$	918	840
4	$CHCl_3$	918	837
5	CCl_4	915	835
6	KBr	910	840
8	CCl_4	905	937

[a]Polystyrene showed a band at 1603 cm^{-1} in all cases

When desulfonylation was effected prior to removal of the benzylidene group, crystalline methyl 4,6-*O*-benzylidene-3-*O*-ethyl-α-D-glucopyranoside (**4**) was obtained The i r. spectrum of **4** is in accord with the replacement of a *p*-tolylsulfonyloxy substituent by a hydroxyl group, and the n m r spectrum confirms the substitution pattern (see Table I) Hydrolysis of **4** gave a product (**6**) identical with that obtained from **5** The identity of **6** was confirmed by its hydrolysis[7,8] to 3-*O*-ethyl-D-glucose (**7**)

In the second synthetic method (see Scheme 2), 3-*O*-ethyl-1,2 5,6-di-*O*-isopropylidene-α-D-glucofuranose (**10**), a known compound[7,8], was treated with methanolic hydrochloric acid to yield **6**, identical with methyl 3-*O*-ethyl-α-D-glucopyranoside prepared by the first method

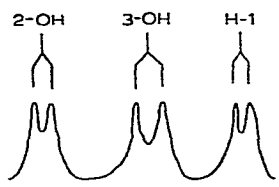


9 10

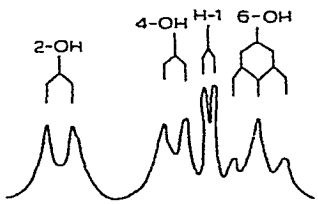
Scheme 2

The n m r. spectra in methyl sulfoxide-d_6 — The usefulness of highly purified and neutral solvents for the detection of vicinal H-O-C-H splittings has been recorded in a number of publications[12–22] Chapman and King have shown that primary, secondary, and tertiary hydroxyl protons can be easily and conclusively differentiated by n m.r. when methyl sulfoxide-d_6 is used as the solvent[16] Variance in the coupling constants of different alcohols has been recorded[16–22], and it is likely that the Karplus

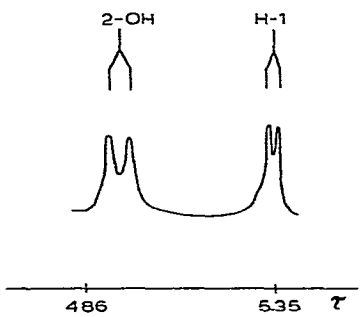
equation[23] should hold qualitatively for the H-O-C-H system[18,21] The advantage of this solvent for n m r. studies on saccharides has been utilized by Casu *et al.*[17,19,21], who have been able to assign, conclusively, the 1-OH, H-1, and 6-OH protons for α-D-glucopyranose, and assign tentatively, the 2-OH, 3-OH, and 4-OH protons for methyl α-D-glucopyranoside The 2-OH proton was assigned the largest coupling constant on the assumption[21] that "this hydroxyl would be most affected by the changes in the stereochemistry at C-1" The 3-OH and 4-OH protons have been assigned on the basis of Perlin's work[22] Perlin reported that double-resonance experiments confirmed these assignments for α-D-glucopyranose, that is, the signals of the 1-OH, C-1, 4-OH, 3-OH, 2-OH, and 6-OH protons appear at progressively higher field Based on these assignments, the results of the present investigation suggest that, proceeding from α-D-glucose to the α-D-glucopyranoside, a change in the order of the 2-OH and 4-OH signals occurs (see Table I) Perlin and Casu and co-workers have shown that analysis of the hydroxyl region of n m r spectra of saccharides gives valuable information concerning the substitution patterns of the sugars The present investigation extends this approach



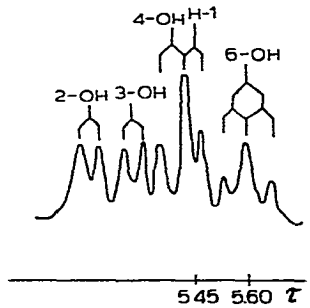
Methyl 4,6-*O*-benzylidene-α-D-glucopyranoside (1)



Methyl 3-*O*-ethyl-α-D-glucopyranoside



Methyl 4,6-*O*-benzylidene-3-*O*-ethyl-α-D-glucopyranoside (4)



Methyl α-D-glucopyranoside

Fig 1a N m r spectra of methyl 4,6-*O*-benzylidene-α-D-glucopyranoside (1) and methyl 4,6-*O*-benzylidene-3-*O*-ethyl-α-D-glucopyranoside (4) in methyl sulfoxide-d_6 at 100 HMz

Fig 1b N m r spectra of methyl 3-*O*-ethyl-α-D-glucopyranoside (6) and methyl α-D-glucopyranoside in methyl sulfoxide-d_6 at 100 MHz

A comparison of the n.m r spectra of compounds **1**, **4**, and **6** (Figs 1a and 1b) leads to the assignments indicated for the hydroxyl protons Although these assignments are not unequivocal, it seems unlikely that both the line-position and coupling constant of the 2-OH would shift in compounds **4** and **6** Inspection of Corey–Pauling and Buchi–Dreiding models revealed that the environment of the 2-OH is very similar in these two compounds. Although the precise spatial position of the 1-OMe group cannot be thus determined, the regular effect of the axial O-1 atom on the H-3 and H-5 axial protons in α-D-glucopyranosides[10] indicates that an electron pair on this oxygen atom is so oriented as to be approximately equidistant between the two, and, therefore, the methoxyl group may also have a favored orientation The effect of an oriented 1-OMe group would be to limit the environment of the 2-OH group Positioning of a 3-OEt group as in compounds **4** and **6** would further limit the environment of the 2-OH group, and a very obvious decrease in the H-1–H-2 coupling constant for compounds **4** and **6** can be seen The assignments for the hydroxyl groups of methyl α-D-glucopyranoside (see Fig 1b) are consistent with the above pattern The i r spectra of these compounds have the same characteristics in the 3600–3400 cm^{-1} region as have been reported previously[17 19 21]

EXPERIMENTAL

General — Melting points are uncorrected T l c was performed on silica gel G (E Merck, Germany) with benzene–ethanol or benzene–butyl alcohol mixtures The plates were developed by spraying the dried chromatogram with a 10% (w/v) solution of phosphomolybdic acid, followed by heating for 5 min at *ca* 100°. I r spectra were recorded with a Perkin–Elmer Infracord spectrometer, model 137, and optical rotations were measured with a Bendix Automatic polarimeter or a Rudolph polarimeter Model 80 N m r spectra were recorded with Varian Associates A-60 and HA-100 spectrometers (60 and 100 MHz), with tetramethylsilane (τ = 10 00) as the internal or external standard Methyl sulfoxide-d_6 and chloroform-*d* were obtained from Merck, Sharp and Dohme, Ltd , Canada, and were used without purification Most of the samples for n m r studies were degassed The assignment of all hydroxyl peaks was confirmed by deuterium oxide exchange Elemental analyses were performed by Schwarzkopf Microanalytical Laboratory, Woodside, New York

Methyl 4,6-O-benzylidene-α-D-glucopyranoside (**1**) — The preparation of this compound has been described[24], m p 161°, $[\alpha]_D^{25}$ +95° (*c* 1, ethanol)

Methyl 4,6-O-benzylidene-2-O-p-tolylsulfonyl-α-D-glucopyranoside (**2**) — This compound was prepared by the method of Bolliger and Prins[3], m p 153–154°, $[\alpha]_D^{15}$ +61° (*c* 2 75, chloroform) Its i r spectrum showed $\nu_{max}^{CHCl_3}$ 3600 (OH), 1370, 1145 cm^{-1} (sulfonic ester), and other characteristic bands given in Table II

Methyl 4,6-O-benzylidene-3-O-ethyl-2-O-p-tolylsulfonyl-α-D-glucopyranoside (**3**) — A solution containing 50 ml of *N*,*N*-dimethylformamide, 2 0 g (4 6 mmoles) of **2**, and 2.0 ml (25 mmoles) of ethyl iodide was stirred with 4 2 g (18 mmoles) of silver oxide for 28 h at room temperature The mixture was filtered and the filtrate evaporated to dryness The crude product, yield 1 4 g (66%), was recrystallized to

TABLE III

N.M R SPECTRA OF SUBSTITUTED METHYL α D-GLUCOPYRANOSIDES IN CHLOROFORM-*d* OR PYRIDINE[a]

Compound	*Aromatic protons*		*Benzylic proton*	*H-1*	*H-2*	*H-3*	*H 4, H-5, H 6e, H-6a*	*1-OMe*	*2-OR*	*2-OR'*
	Ts	*Ph*								
2[b]	1 98d (J = 8 4) 2 51d	2 46	4 38	5 03d ($J_{1,2}$ = 3 4)	5 52m	5 61–6 60	5 61–6 60	6 57	CH_3Ar, 7 50	7 37
3[b]	1.98d (J = 8 5) 2 53d	2 50	4 41	5 00d ($J_{2,3}$ = 3 9)	5 57q ($J_{2,3}$ = 8 8)	5,80–6 70	5,80–6 70	6 56	CH_3Ar, 7 54	CH_3CH_2, 9 03t (J = 7)
8[b]	2,03d (J = 8 4) 2 56d 2.24d (J = 8 2) 2 95d	2 58	4 62	4 88d ($J_{1,2}$ = 3 9)	5 55q ($J_{2,3}$ = 9 6)	4 77t ($J_{3,4}$ ~ 9 8) ($J_{2,3}$ ~ 9 8)	5 60–6 50	6 56	CH_3Ar, 7 52 or 7 71	7 52 or 7 71
1[c]		3 03	4 88	5 59d ($J_{1,2}$ = 3 5)	6 10–6 45	6 10–6 45	6 50–6 70	7 27	3 68	3 68

[a]All spectra obtained at 60 MHz, chemical shifts given as τ values, J values are given in Hz and are enclosed in parentheses, d, doublet, m, multiplet, q, quartet; t, triplet [b]Spectrum obtained in chloroform *d* [c]Spectrum obtained in pyridine, external standard Me_4Si at τ = 10 0 checked before and after each measurement

constant rotation from ether containing a few drops of petroleum ether (b p 40–60°), m p 108–109°, $[\alpha]_D^{22}$ +45° (*c* 1, chloroform) On t l c. with 49.1 butyl alcohol-benzene, the product showed one spot having an R_F value much greater than that of **2** The i r. spectrum showed $\nu_{max}^{CHCl_3}$ 1370 and 1160 cm^{-1} (sulfonic ester), but no OH absorption at 3600 cm^{-1} (see Table II)

Anal Calc for $C_{23}H_{28}O_8S$ C, 59 48; H, 6 03, S, 6 90 Found C, 59 79; H, 6 14; S, 6 92

Methyl 4,6-O-benzylidene-3-O-ethyl-α-D-glucopyranoside (**4**) — To a stirred solution of 25 ml of methanol containing 500 mg (1 1 mmoles) of **3** was added, in five equal portions, 20 g of 2 5% sodium amalgam during 4 h. The solution was decanted from the mercury, mixed with water, and rendered neutral with carbon dioxide After evaporation of the aqueous methanol, the residue was extracted with chloroform The extract was washed with water, dried (sodium sulfate), and evaporated Addition of ether to the resulting syrup caused formation of a precipitate which was recrystallized from chloroform–ether to yield 281 mg (84%) of crystals, m p 168–169°, $[\alpha]_D^{22}$ +111 1° (*c* 0 99, chloroform) It showed one spot having an R_F value much smaller than that of **3** on t l c with 1 49 butyl alcohol–benzene Its i r spectrum showed $\nu_{max}^{CHCl_3}$ 3600 cm^{-1} (OH), and other characteristic bands given in Table II

Anal Calc for $C_{16}H_{22}O_6$ C, 61 94, H, 7 97 Found C, 61 86, H, 8 08

Methyl 3-O-ethyl-2-O-p-tolylsulfonyl-α-D-glucopyranoside (**5**) — A mixture of 1 0 g (2 2 mmoles) of **3** with 8.5 ml of 0 005M sulfuric acid in 8 5 ml of methanol was stirred for 1 h at room temperature After neutralization of the acid with barium carbonate, and filtration, the solution was evaporated, starting material (0 2 g) was recovered. The filtrate was evaporated to dryness under diminished pressure to yield 0 45 g of product (69%, based on starting material recovered) as a glass, $[\alpha]_D^{22}$ +70° (*c* 0 99, chloroform) Its i r spectrum showed $\nu_{max}^{CCl_4}$ 3550 (OH), 1380, 1182 cm^{-1} (sulfonic ester), and other characteristic bands given in Table II The product showed only one spot on t l c with 1 19 butyl alcohol–benzene

Anal Calc for $C_{16}H_{24}O_8S$ C, 51 05; H, 6 43, S, 8 52 Found C, 51 31, H, 6 25, S, 8 63

Methyl 3-O-ethyl-α-D-glucopyranoside (**6**) *from methyl 4,6-O-benzylidene-3-O-ethyl-α-D-glucopyranoside* (**4**) — A mixture of 0 75 g (3 4 mmoles) of **4** in 15 ml of 0 05M sulfuric acid in methanol was stirred for 1 h at room temperature After neutralization of the acid with barium carbonate, and filtration, concentration of the filtrate gave some unchanged starting material The filtrate was evaporated under diminished pressure to yield 0 35 g of product (70%, based on starting material recovered), m p 139–140°, $[\alpha]_D^{25}$ +152° (*c* 1, ethanol) The i r. spectrum showed ν_{max}^{KBr} 3450 (OH), 1000 cm^{-1}, and other characteristic bands given in Table II The product showed only one spot on t l c with 1 4 ethanol–benzene, and g l c of the *O*-(trimethylsilyl) derivative gave a single peak from each of several different columns[2]

Anal. Calc for $C_9H_{18}O_6$ C, 48 64, H, 8 16 Found. C, 48 55, H, 7 95.

Methyl 3-O-ethyl-α-D-glucopyranoside (**6**) *from methyl 3-O-ethyl-2-O-p-*

tolylsulfonyl-α-D-glucopyranoside (**5**) — Desulfonylation of **5** was accomplished by use of sodium amalgam as described for the preparation of **4** From 0 5 g (1.3 mmoles) of **5**, the syrup that resulted after evaporation of the methanol was crystallized from methanol–ether to yield 0 25 g (85%) of crystalline product, m p 137–140°, $[\alpha]_D^{25}$ +152° (*c* 1, ethanol) The products obtained from **4** and **5** were identical with each other, as shown by m p , mixed m p , i r and n m r spectra, and t l c and g l c

3-O-Ethyl-D-glucose (**7**) — A mixture of 0 1 g (0 5 mmole) of **6** and 15 ml of M sulfuric acid was stirred for two days at room temperature After neutralization of the acid with barium hydroxide, evaporation of the filtrate gave crude 3-*O*-ethyl-D-glucose, 0 06 g (67%) Recrystallization from ethanol yielded a crystalline product, m.p 156–157° (lit.[8] 155 5–157 5°), $[\alpha]_D^{25}$ +61° (equil , *c* 1, ethanol)

Methyl 4,6-O-benzylidene-2,3-di-O-p-tolylsulfonyl-α-D-glucopyranoside (**8**) — The preparation of this compound has been described[25], m p 147–148°, $\nu_{max}^{CHCl_3}$ 1370 and 1160 cm^{-1} (sulfonic ester), and other characteristic bands as given in Table II

1,2 5,6-Di-O-isopropylidene-α-D-glucofuranose (**9**) — The preparation of this compound has been described[26], m p 109–110°

3-O-Ethyl-1,2 5,6-di-O-isopropylidene-α-D-glucofuranose (**10**) — To a solution of 26 g (0 1 mole) of **9** in 300 ml of tetrahydrofuran was added 30 ml of ethyl sulfate and 25 g of powdered sodium hydroxide The mixture was boiled for 3 h under reflux with rapid stirring, and then cooled A second addition of 30 ml of ethyl sulfate and 25 g of powdered sodium hydroxide was made, followed by boiling for 3 h under reflux, with stirring After filtration, and the removal of tetrahydrofuran and ethyl sulfate by distillation, the residue was passed through an alumina column to yield 26 g (86%) of a liquid, $\nu_{max}^{CHCl_3}$ 2990 s, 2940 sh, 1475 m, 1440 m, 1405 s, 1380 s, 1365 s, 1340 m, 1285 m, 1250 s, 1215 s, 1195 s, 1165 s, 1122 s, 1080 vs, 1020 vs, 920 vs, 890 m, and 850 ms cm^{-1}

Methyl 3-O-ethyl-α-D-glucopyranoside (**6**) *from 3-O-ethyl-1,2 5,6-di-O-isopropylidene-α-D-glucofuranose* (**10**) — A solution of 26 g (0 1 mole) of **10** in 9 ml of conc hydrochloric acid and 300 ml of methanol was refluxed overnight After neutralization of the acid with sodium hydrogen carbonate, the suspension was filtered, and the filtrate was dried (magnesium sulfate), and evaporated to yield 19 g (95%) of a solid Repeated recrystallization from methanol gave a crystalline product, 14 g (70%), which was identical in all respects with **6** prepared as described previously

ACKNOWLEDGMENTS

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SYNTHESIS OF NEW SUGAR DERIVATIVES HAVING POTENTIAL ANTITUMOUR ACTIVITY

PART XI* 2,5 3,6-DIANHYDRO-D-GLUCITOL

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ABSTRACT

The action of sodium methoxide on 1,6-dichloro-1,6-dideoxy-D-mannitol and 1,6-di-*O*-methanesulphonyl-D-mannitol was studied The "*β*-mannide" that was isolated from the resulting mixture was shown to be 2,5 3,6-dianhydro-D-glucitol.

INTRODUCTION

In prior publications, the synthesis and reactions of some 1,2 5,6-dianhydro-3,4-*O*-isopropylidene derivatives of D-mannitol, D-glucitol, and L-iditol have been described We were also interested in such diepoxides from the biological point of view, since they belong to the class of biological alkylating agents some of which possess cytostatic activity Moreover, it can be assumed that some of the cytostatic hexitol derivatives, *e g*, 1,6-di-*O*-methanesulphonyl-D-mannitol[3], and 1,6-dibromo-1,6-dideoxy-D-mannitol[4] and -galactitol[5], owe their activity to the formation of diepoxides as metabolic intermediates To study this question, synthesis of the hypothetical diepoxide intermediate, 1,2 5,6-dianhydro-D-mannitol, was attempted**

RESULTS AND DISCUSSION

1,6-Dichloro-1,6-dideoxy- and 1,6-di-*O*-methanesulphonyl-D-mannitol were treated with methanolic sodium methoxide, and the reaction was monitored by thin-layer chromatography The starting materials disappeared rapidly, with the appearance of components having R_F 0 82 (*A*), 0.66 (*B*), and 0 58 (*C*) This picture was almost independent of the reaction conditions Substance *C* (minor component) was indistinguishable from 1,4 3,6-dianhydro-D-mannitol

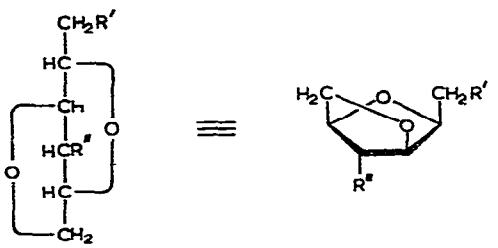
Distillation of the syrupy product yielded substances *B* and *C*, substance *A* could no longer be detected by t l c It is possible that, during the distillation, substance *A* underwent conversion into *C*, since the spot of the latter compound became more intense.

*Part X. *Carbohyd Res*, 3 (1966) 38 Designated erroneously as Part IX
**Whilst this paper was being prepared, the synthesis of 1,2 5,6-dianhydro-D-mannitol was described [M Jarman and W C J Ross, *Chem Ind* (London), (1967) 1789]

Crystalline substance *B* (major component) was isolated and, from its physical constants and those of its dimethanesulphonate, appeared to be identical with a dianhydrohexitol ("*β*-mannide") prepared[6,7] by treating 1,6-dichloro-1,6-dideoxy-D-mannitol with sodium amalgam

Seven dianhydrohexitols arising from D-mannitol or D-mannitol derivatives are described in the literature[8-13], but there are only three whose structures are known 1,4 3,6-dianhydro-D-mannitol[9] (isomannide), 1,5 3,6-dianhydro-D-mannitol[10] (neomannide), and 1,2 5,6-dianhydro-D-mannitol[13]. We have now identified compound *B* (*β*-mannide) as 2,5 3,6-dianhydro-D-glucitol (**1**), on the basis of the following facts

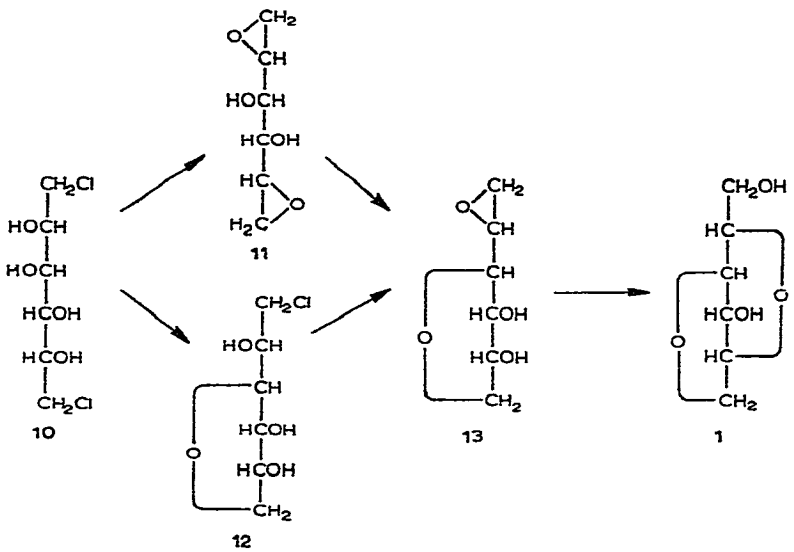
Substance *B* remained unchanged on treatment with hot, dilute acids and bases, thereby excluding the presence of three- or four-membered anhydro-rings The presence of a primary hydroxyl group was indicated by the conversion of compound *B* into a crystalline monoacetyl-monotrityl derivative (**2**) This supposition was confirmed when the di-*O*-methanesulphonyl- and di-*O*-toluene-*p*-sulphonyl derivatives (**3** and **4**) yielded crystalline monoiodomonosulphonyl derivatives (**5** and **6**, respectively) on treatment with sodium iodide in acetone The iodine of compounds **5** and **6** was removed by catalytic hydrogenolysis, and the resulting compounds (**7** and **8**) were desulphonylated by sodium amalgam The resulting dianhydromonodeoxyhexitol (**9**) contained a *C*-methyl group, which was indicated by the analytical results, and by the n m r. spectrum ($CDCl_3$ solution) which contained a 3-proton doublet at δ 1 35 (J 7 Hz)



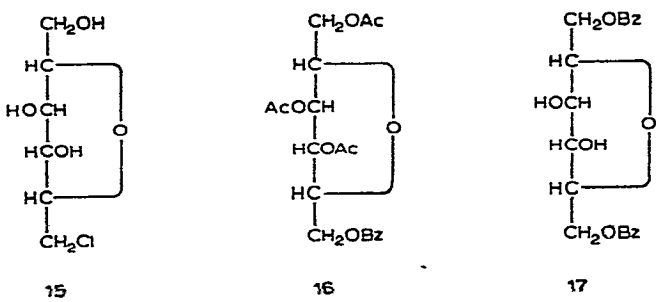
1	R′ = R″	= OH	
2	R′ = OTr ,	R″	= OAc
3	R′ = R″	= OMs	
4	R′ = R″	= OTs	
5	R′ = I ,	R″	= OMs
6	R′ = I ,	R″	= OTs
7	R′ = H ;	R″	= OMs
8	R = H	R″	= OTs
9	R = H ,	R″	= OH

Consistent with the absence of vicinal hydroxyl groups, the dianhydro compound **1** fails to consume periodate The assignment of the D-*gluco* configuration to the "*β*-mannide" is based on the absence of small anhydro rings and the presence of a primary hydroxyl group, as demonstrated above, which precludes an unchanged *manno* configuration The probable reaction mechanism is consistent only with the D-*gluco* configuration It is known that D-mannitol and some of its derivatives can easily form 1,4(3,6)-anhydro-D-mannitol The anhydro ring in this compound could be formed either directly or in two steps *via* an intermediate epoxide Thus, from 1,6-dichloro-1,6-dideoxy-D-mannitol (**10**) or 1,6-di-*O*-mesyl-D-mannitol, 1,2 5,6-dianhydro-D-mannitol (**11**), or 1,4(3,6)-anhydro-6(1)-chloro-6(1)-deoxy-D-mannitol (**12**) and 1,4(3,6)-anhydro-6(1)-*O*-mesyl-D-mannitol, respectively, should be formed first under alkaline conditions In a second step, the diepoxide might undergo

rearrangement by an intramolecular SN2 attack of the 3(4)-hydroxyl group, producing the monoepoxide **13**, which would then undergo a similar rear-side attack of the 5(2)-hydroxyl group, with simultaneous Walden inversion at C-2(5), to give product **1**. The monoanhydromonochloro- (**12**) and the analogous monoanhydromonomesyl derivative could form the "*β*-mannide" in a similar way. Thus, the prerequisite to the establishment of a 2,5-anhydro ring is the intermediate formation of at least one epoxide group



The D-*gluco* configuration of "*β*-mannide" could be verified experimentally Treatment of compound **1** with fuming hydrochloric acid at 100° results in cleavage of the 3,6-anhydro ring, with formation of the monochloromonodeoxyanhydrohexitol **15** The chlorine atom of acetylated compound **15** was displaced by a benzoyloxy group upon treating it with sodium benzoate in boiling *N*,*N*-dimethylformamide The resulting monobenzoate **16** was deacylated (sodium methoxide), and the resulting anhydrohexitol was benzoylated with two equivalents of benzoyl chloride to give a crystalline di-*O*-benzoyl derivative that is identical with 2,5-anhydro-1,6-di-*O*-benzoyl-D-glucitol[11] (**17**)



The structure of the substances *A* and *C* could not be elucidated, because attempts to separate them were unsuccessful. The chromatographic behaviour of substance *C* was indistinguishable from that of 1,4:3,6-dianhydro-D-mannitol. However, the compound could also be the dianhydrohexitol described by Wiggins[7], who gave no experimental details of the preparation and derivatives of his compound. Compound *A* might be one of the supposed intermediates **11**, **12**, and **13**, since it can be detected on the chromatogram by 4-*p*-nitrobenzylpyridine[12], which gives a positive reaction, exclusively, with alkylating agents.

EXPERIMENTAL

Melting points are uncorrected. Thin-layer chromatography (t.l.c.) was performed on Kieselgel with ethyl acetate–propan-2-ol–water (3:5:2:7:1), and detection by a mixture of 0.1M potassium permanganate and 5N sulphuric acid (1:1). All evaporations were carried out in a rotary evaporator under diminished pressure. N.m.r. spectra were obtained by using a JEOL J.N.M.-C-60 spectrometer.

2,5:3,6-Dianhydro-D-glucitol (**1**). — A suspension of 1,6-dichloro-1,6-dideoxy-D-mannitol (**10**, 22 g) or 1,6-di-*O*-methanesulphonyl-D-mannitol (33.8 g) in 2N sodium methoxide (100 ml) was stirred for 8 h and then kept overnight at room temperature. Examination by t.l.c. revealed components *A*, *B*, and *C*, having approximate R_F values of 0.82, 0.66, and 0.58, respectively. The solid precipitate was filtered off, the filtrate was evaporated, and the syrupy residue was dissolved in ethanol. The filtered solution was evaporated, and distillation of the residue gave a product (8.7 g), b.p.$_{0.15}$ 125–140°, which gave only two spots on t.l.c., corresponding to components *B* and *C*. After dissolution in ethanol (10 ml), the mixture was treated with ethyl acetate (20 ml) and stored for 20 h at 0°. The crystals of compound **1** were filtered off, and washed with ethyl acetate; yield, 3.2 g, m.p. 117–119°, $[\alpha]_D^{20}$ +94.4° (*c* 1, water) (Found: C, 49.23; H, 7.10. $C_6H_{10}O_4$ calc.: C, 49.38; H, 6.84%).

The mother liquor contained compound **1** and substance *C* (t.l.c.). The residue (5 g), remaining after distillation, consisted of sodium salts and a polymeric material.

4-O-Acetyl-2,5:3,6-dianhydro-1-O-trityl-D-glucitol (**2**). — The dianhydro compound **1** (0.5 g) was dissolved in pyridine (5 ml) and treated with trityl chloride (1 g). The solution was kept for 24 h at room temperature, acetic anhydride (1 ml) was added, and the reaction mixture was evaporated next day. The residue was dissolved in a chloroform–ice–water mixture, and the organic layer was washed, dried (Na_2SO_4), and evaporated. The resulting syrup was dissolved in ethanol and treated with water, whereupon compound **2** was precipitated as a crystalline powder (1 g), m.p. 143–145° (from ethanol), $[\alpha]_D^{20}$ +30.7° (*c* 1, chloroform) (Found: C, 75.52; H, 6.29. $C_{27}H_{26}O_4$ calc.: C, 75.34; H, 6.0%).

2,5:3,6-Dianhydro-1,4-di-O-methanesulphonyl-D-glucitol (**3**). — A solution of compound **1** (5.5 g) in pyridine (20 ml) was treated at 0° with methanesulphonyl chloride (7.3 ml). The reaction mixture was kept at room temperature overnight and then poured onto ice. The precipitated oil became solid after treatment with fresh

water The product was recrystallised from acetone (40 ml) to give compound **3** (7 5 g), m p. 123–125°, $[\alpha]_D^{20}$ +50 16° (*c* 1, chloroform), Wiggins[7] recorded m p 121° (Found· C, 31 86; H, 4 80; S, 20 92 $C_8H_{14}O_8S_2$ calc C, 31 78, H, 4 67, S, 21 21%)

*2,5 3,6-Dianhydro-1,4-di-*O*-toluene-*p*-sulphonyl-*D*-glucitol* (**4**) — A solution of the dianhydro compound (**1**, 2 g) in pyridine (10 ml) was treated with toluene-*p*-sulphonyl chloride (5.1 g) as described above Recrystallization from ethanol (60 ml) gave compound **4** (3 5 g), m p 123–124°, $[\alpha]_D^{20}$ +37 7° (*c* 1, chloroform); lit[7], m p 115 5° (Found C, 52 68, H, 4 81, S, 14 35 $C_{20}H_{22}O_8S_2$ calc. C, 52 85; H, 4 88, S, 14 11%).

*2,5:3,6-Dianhydro-1-deoxy-1-iodo-4-*O*-methanesulphonyl-*D*-glucitol* (**5**) — A solution of compound **3** (7 g) and sodium iodide (21 g) in acetone (70 ml) was heated in a sealed tube for 8 h at 120° After cooling, the sodium methanesulphonate was filtered off, and the filtrate was evaporated The syrupy residue was dissolved in chloroform, the solution was washed with water and N sodium thiosulphate, dried, and evaporated The residue was dissolved in acetone (50 ml) and treated again with sodium iodide (3 g), as described above The resulting product gave compound **5** (7 5 g), after recrystallization from methanol (20 ml), m p 111–113°, $[\alpha]_D^{20}$ +61 06° (*c* 1, chloroform) (Found C, 25 61, H, 3 40, I, 38 55, S, 10 05 $C_7H_{11}IO_5S$ calc· C, 25 18, H, 3 32; I, 38 02, S, 9 60%)

*2,5.3,6-Dianhydro-1-deoxy-1-iodo-4-*O*-toluene-*p*-sulphonyl-*D*-glucitol* (**6**) — A solution of compound **4** (10 g) and sodium iodide (10 g) in acetone (100 ml) was treated, as described above, to give compound **6** (8 2 g), m p 113–114°, $[\alpha]_D^{20}$ +46 7° (*c* 1, chloroform) (Found C, 37 95, H, 3 91, I, 30 3, S, 7 94 $C_{13}H_{15}IO_5S$ calc C, 38 00, H, 3 68, I, 30 95, S, 7 82%)

*2,5 3,6-Dianhydro-1-deoxy-4-*O*-methanesulphonyl-*D*-glucitol* (**7**) — A solution of compound **5** (5 g) in methanol (100 ml) was hydrogenated at room temperature and normal pressure in the presence of Raney nickel (5 g) The calculated amount of hydrogen was consumed in 1 h The catalyst was removed, the solution was evaporated, and the residue was dissolved in chloroform The solution was washed with water and aqueous sodium thiosulphate, dried (Na_2SO_4), and evaporated The residue was crystallized from ether–light petroleum (b p 40-60°) to give compound **7** (1 7 g), m p 58–60°, as needles containing ether as solvent of crystallization After drying under diminished pressure over paraffin, the needles became opaque and had m p 92–93°, $[\alpha]_D^{20}$ +69 3° (*c* 1, chloroform) (Found C, 40 02, H, 5 83, S, 15 42 $C_7H_{12}O_5S$ calc C, 40 37, H, 5 81, S, 15 40%)

*2,5 3,6-Dianhydro-1-deoxy-4-*O*-toluene-*p*-sulphonyl-*D*-glucitol* (**8**) — Compound **6** (20 5 g) was hydrogenated, as for the methanesulphonate **5**, to give, after recrystallization from ether–light petroleum, compound **8** (10.7 g), m.p. 85–86°, $[\alpha]_D^{20}$ +60 1° (*c* 1, chloroform) (Found. C, 54 92, H, 5 63; S, 11.20 $C_{13}H_{16}O_5S$ calc C, 54 91, H, 5.67, S, 11 28%)

2,5.3,6-Dianhydro-1-deoxy-D-glucitol (**9**) — The toluene-*p*-sulphonate **10** (5 g) was dissolved in 80% methanol (400 ml) and shaken with freshly prepared 4% sodium amalgam (120 g) for 20 h The decanted solution was neutralised with carbon dioxide,

filtered, and evaporated The residue was dissolved in ethyl acetate, and the solution was filtered and evaporated This treatment was repeated three times The final residue was crystallized from ethyl acetate–light petroleum to give compound **9** (1 8 g), m p 81–82°, $[\alpha]_D^{20}$ +107 9° (*c* 1, water) (Found C 55 16; H, 7 99, *C*-CH_3, 11 5 $C_6H_{10}O_3$ calc · C, 55 37; H, 7 74, *C*-CH_3, 11 2%)

1,3,4-Tri-O-*acetyl-2,5-anhydro-6-chloro-6-deoxy*-D-*glucitol* — A solution of compound **1** (10 g) in conc hydrochloric acid (100 ml) was heated in a sealed tube for 8 h at 100°. The solution was evaporated, and water and benzene were each twice distilled from the residue, which was then dissolved in pyridine (50 ml) and treated with acetic anhydride (20 ml). Next day. the reaction mixture was processed in the usual way to give the product (2 4 g) as a colourless syrup. b $p_{0\ 25}$ 115–120°, $[\alpha]_D^{20}$ −4 4° (*c* 1, chloroform) (Found C, 46 54, H, 5 78, Cl, 11 90 $C_{12}H_{17}ClO_7$ calc C, 46 67; H, 5 55, Cl, 11 48%)

2,5-Anhydro-1,6-di-O-*benzoyl*-D-*glucitol* (**17**) from *1,3,4-tri*-O-*acetyl-2,5-anhydro-6-chloro-6-deoxy*-D-*glucitol* — The foregoing syrup (1 6 g) was dissolved in *N,N*-dimethylformamide (50 ml) and stirred with sodium benzoate (5 g) for 8 h under reflux The suspension was concentrated to a syrup that was dissolved in chloroform–water. The organic layer was washed with aqueous sodium hydrogen carbonate and water, dried (Na_2SO_4), and, after treatment with charcoal, evaporated to a glass Traces of the solvent were removed at 10^{-2} torr over paraffin The colourless residue (compound **16**) contained only traces of chlorine

The syrupy product **16** was dissolved in methanol (10 ml) and kept overnight at room temperature after addition of N sodium methoxide (0 1 ml). The solution was neutralized with a cation-exchange resin, and evaporated after filtration The residue was treated with water, methyl benzoate was removed by extraction with ether, and the aqueous solution was treated with charcoal filtered, and evaporated Ethanol was twice evaporated from the residue to give syrupy 2,5-anhydro-D-glucitol which was freed from traces of solvents at 60° and 10^{-2} torr

The crude anhydro compound (0 4 g) was dissolved in pyridine (5 ml) and treated with benzoyl chloride (2 equivalents, 0 6 ml) at 0°. The reaction mixture was kept overnight at room temperature and then poured onto ice The precipitated oil was extracted with chloroform, and the extract was washed, dried (Na_2SO_4), and evaporated The residue was crystallized from benzene (2 ml), by addition of light petroleum (b p 60–80°), to give compound **7**, m p 135–137°, $[\alpha]_D$ +1 2° (*c* 2, ethanol), lit [11], m p 137–138°, $[\alpha]_D$ +3 2° (*c* 1 8, ethanol) (Found C, 64 75, H, 5 57 $C_{20}H_{20}O_7$ calc C, 64 51, H, 5 41%)

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DEGRADATION OF CARBOHYDRATES

PART X* 2,3,4,5-TETRA-*O*-METHYL-D-GLUCOSE

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ABSTRACT

2,3,4,5-Tetra-*O*-methyl-D-glucose (**6**) has been found to occur as a mixture of the β-D-septanose form and the acyclic form In water, the hydrate of the acyclic aldehyde form is present, as well as the anhydrous aldehyde Glucosidation with methanol in the presence of acid gave first the dimethyl acetal, and then a mixture of the methyl β-D-septanoside and this acetal The absence, for the α-D-septanose anomer, of conformations having low free-energy explains the exclusive appearance of the β-D anomer. The aldose **6** was degraded, in 2 min at 20°, by a solution of calcium hydroxide to a mixture of the *cis*- and *trans*-3-deoxy-D-*erythro*-hex-2-enoses with two 3,6-anhydroaldoses The structures of the compounds were ascertained largely by n.m r spectroscopy

INTRODUCTION

The degradation of some methylated aldoses by alkali, followed by acid, was described in previous parts of this series Pyranose[1,2], furanose[3], and acyclic[4] forms of the sugars were studied The present paper reports the action of the same reagents on 2,3,4,5-tetra-*O*-methyl-D-glucose, which can exist in a septanose form and the acyclic form

RESULTS AND DISCUSSION

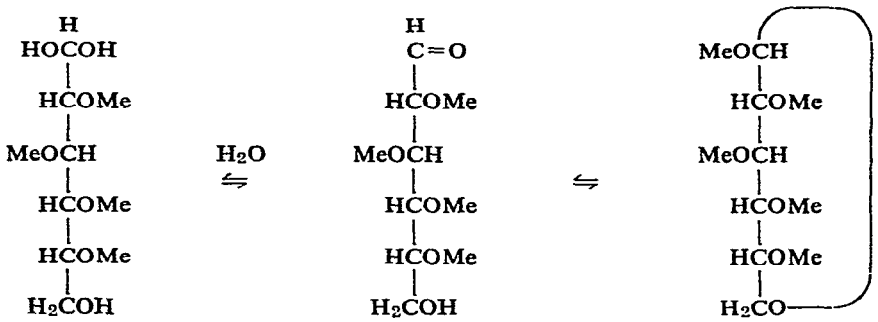
*Preparation of 2,3,4,5-tetra-*O*-methyl-*D*-glucose* The preparation started from 6-*O*-benzyl-D-glucose, and involved the formation of the diethyl dithioacetal (**1**), per-*O*-acetylation to give **2**, demercaptalation of acetate **2** to the dimethyl acetal, methanolysis of the acetyl groups to give compound **3**, methylation to the tetramethyl ether (**4**), and hydrogenolysis of the benzyl groups to give **5**; finally, hydrolysis of the acetal gave the desired aldose, 2,3,4,5-tetra-*O*-methyl-D-glucose (**6**)

*For Part IX, see Ref 4

H R'CR'		R	R'	R''
HCOR	1	H	SEt	CH_2Ph
ROCH	2	Ac	SEt	CH_2Ph
HCOR	3	H	OMe	CH_2Ph
HCOR	4	Me	OMe	CH_2Ph
H_2COR''	5	Me	OMe	H

The starting material for the synthesis, 6-*O*-benzyl-D-glucose, has been reported once[5], but the preparation was on a small scale A more convenient source of 6-*O*-benzyl-D-glucose is now available, namely, from the hydrolysis of 6-*O*-benzyl-3,5-*O*-benzylidene-1,2-*O*-isopropylidene-D-glucose[6], which is readily prepared in large quantity

When the methylated sugar **6** was treated with methanol containing an acid catalyst, the dimethyl acetal **5** was re-formed in about one minute The acetal **5** was then largely converted into the β-D-septanoside **7** within about 24 h This is one instance in which acetal formation definitely precedes glycosidation, with most sugars, the acetals are not important as precursors of the glycosides[7]. The methyl β-D-septanoside **7** was isolated in the pure state, it appears to be the only D-glucoseptanose derivative thus far known.



β-D-septanose (see **11a** and **11b**)

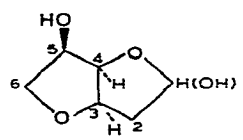
The methylated sugar **6** was extremely labile to alkali, no doubt because a large proportion exists in the carbonyl form It is, therefore, important that it should not be subjected to even weakly basic conditions during the preparation Samples of **6** that had been stored at 0° for some weeks became resistant to alkali, although, on g l c, the normal peak appeared. Apparently, some kind of heat-reversible polymerization had taken place.

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

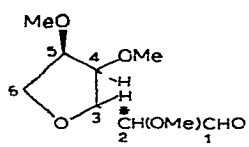
For 2-*O*-methylaldo-pyranoses or -furanoses, the group at C-3 is eliminated to give *cis*-3-deoxyglyc-2-enoses, some weeks at 25° being required for this reaction to approach completion with saturated calcium hydroxide solution as the reagent[1—3]. Under similar conditions, the elimination from 2,3,4,5,6-penta-*O*-methyl-D-glucose occurs rapidly (20 min) and quantitatively, but the configuration of the unsaturated sugar[4] is *trans*, as with ordinary α,β-unsaturated aldehydes. The formation of *cis*-3-deoxyglyc-2-enoses from aldo-pyranoses and -furanoses may be explained by assuming a *cis–trans* equilibrium and stabilization of the *cis* isomer by adoption of a ring form[8] The unsaturated sugar **8**, derived from **6**, presented an intermediate case, approximately equal proportions of the *cis* and *trans* forms being present, although stabilization of the *cis* form by formation of a septanose ring is possible The stabilization is not fully effective, because the *cis* isomer occurs largely in the acyclic form.

The sugar **6** is degraded by alkali even more quickly than penta-*O*-methyl-D-*aldehydo*-glucose, 2 min instead of 20 being sufficient for the disappearance of the starting material The greater reactivity of **6** may be due to assistance by the hydroxyl group at C-6 of the enolization or the β-elimination The second possibility is implicated, because 3,6-anhydroaldoses (**9a** and **9b**) are formed, as well as the unsaturated sugar, and the two products appear to be in equilibrium Anhydroaldoses have not been detected among the products of alkaline degradation of other 2-*O*-methyl-aldoses

In some instances, the hydroxyl group at C-6 was protected by substitution, and, in the others, it must be assumed that the unsaturated sugars were stabilized by the formation of pyranose or furanose rings When 2-deoxy-D-*arabino*-hexose ("2-deoxy-D-glucose") was treated with alkali, the product isolated was[9] the 3,6-anhydride, "3,6-anhydro-2-deoxy-D-glucose" In this latter example, because the hydroxyl group at C-4 is not substituted, two five-membered rings can form, thus stabilizing the anhydroaldose in the D-*arabino* configuration, in contrast to **9a** and **9b**, which probably have the opposite configuration at C-3 (D-*allo* or D-*altro*)



3,6-Anhydro-2-deoxy-D-*arabino*-hexofuranose



9a and **9b**

These two types of degradation product of **6** were readily separated from each other because the anhydroaldoses, unlike the unsaturated sugars, are not readily extracted from aqueous solution by chloroform The reaction mixtures were also analyzed by g l c. (see Table II) Two minor peaks, **9a** and **9b**, were assigned to 3,6-anhydroaldoses, two (**8a** and **8b**) to unsaturated sugars, and one, present in the early stages, to the unchanged aldose. Treatment of the products with bromine in carbon

tetrachloride caused the replacement of the two unsaturated-sugar peaks by two new ones, probably the dibromo compounds; 3,6-anhydroaldoses are stable to bromine[9] The n m r spectrum of **6** that had been degraded by alkali showed, as well as the unsaturated-sugar signals, 2 signals, typical of saturated *aldehydo*-aldoses[4] (δ ~9.7), which were absent from the spectrum of the purified, unsaturated aldose Four 3,6-anhydroaldoses are possible, but apparently only the two forms, **9a** and **9b** having the more-stable ring, namely, the D-allose and D-altrose derivatives, were formed (*cf.* Ref 10)

The unsaturated sugars **8** were degraded by acid, the u v absorption peak at 246 nm being replaced by one at 228 nm when **8** was treated for few minutes at 100° with dilute hydrochloric acid. It is assumed, by analogy with the 6-*O*-methyl derivative[4] of **8**, that an unsaturated ketoaldehyde was formed, but the compound was not isolated

Structures. The structures of 2,3,4,5-tetra-*O*-methyl-D-glucose (**6**) and of derived compounds were largely determined by n m r spectroscopy. The sugar **6** gave complex n m r spectra (see Fig 1) In chloroform-*d* solution, two forms were present, the carbonyl or *aldehydo* form (40%) and the β-D-septanose (60%). The spectrum of the *aldehydo* form was characterized by a sharp singlet at τ 0 25 (HC=O)

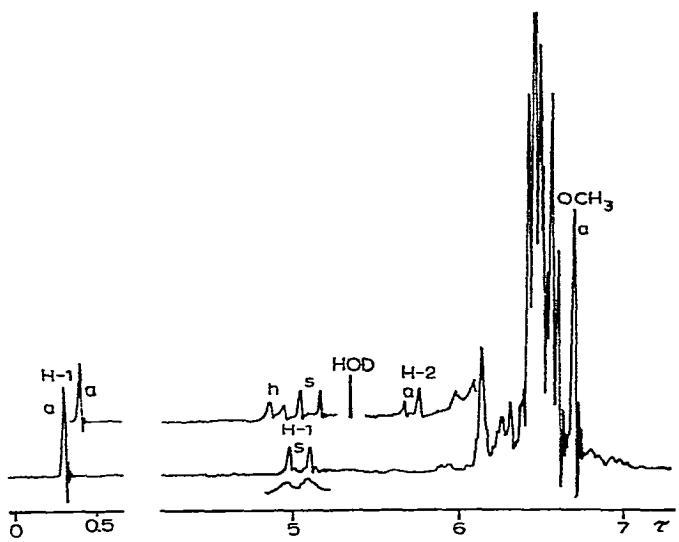


Fig 1 The n m r. spectrum, at 60 MHz, of 2,3,4,5-tetra-*O*-methyl-D-glucose (**6**) + 1 drop of deuterium oxide in chloroform-*d* solution (below), and in deuterium oxide (above) Assignment of peaks a, *aldehydo* form, h, hydrated *aldehydo* form, s, β-D-septanose Bottom insert, part of the spectrum for the chloroform-*d* solution without added deuterium oxide

and by the signal of a methoxyl group at high field[4]. The β-D-septanose form was indicated by a broad doublet ($J_{1,2}$ 7 5 Hz) at τ 5 03 that was sharpened by shaking the sample with deuterium oxide, thus confirming that the signal was due to H-1, since the coupling with the anomeric hydroxyl proton was removed by the addition

of deuterium oxide. That this doublet at τ 5 03 was from the β-D-septanose form of **6** was deduced from a comparison with the spectrum of **7** (see Fig. 2)

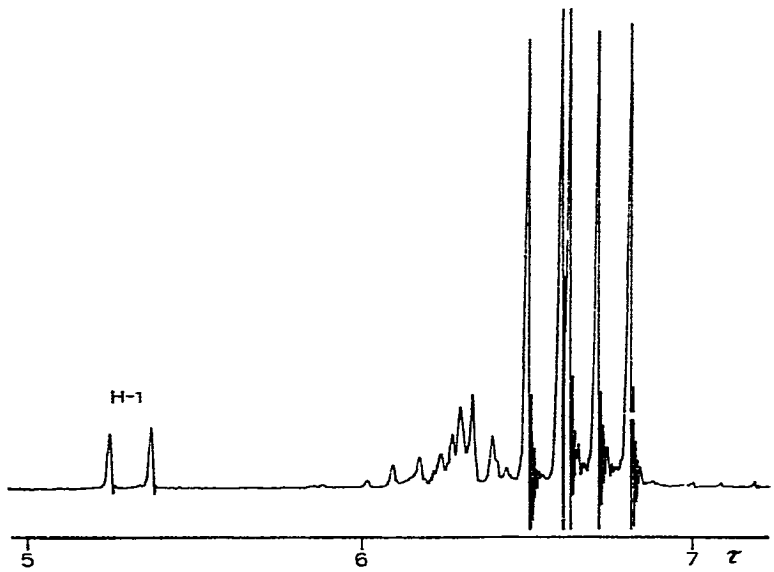


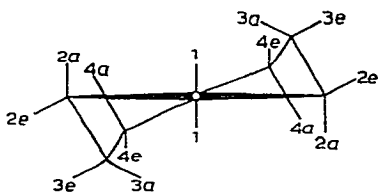
Fig 2 The n m r spectrum, at 60 MHz, of methyl tetra-*O*-methyl-β-D-glucoseptanoside (**7**) in benzene solution showing clearly resolved signals of H-1 and of five *O*-methyl groups

In deuterium oxide solution, **6** gave an n m r. spectrum showing three forms, the β-D-septanose, the *aldehydo* form, and the *aldehydo* hydrate Integration of the signals from H-1 of each form showed that they were present in approximately equal proportions at 30°, whereas, at 90°, the anhydrous carbonyl form amounted to 50%. The three forms were characterized by their H-1 signals· the anhydrous *aldehydo* form gives a sharp singlet at τ 0 37, the hydrate, a doublet ($J_{1,2}$ 4 8 Hz) at τ 4.88, and the β-D-septanose, a doublet ($J_{1\ 2}$ 7.6 Hz) at τ 5 12. One of the methoxyl signals is at high field and originates from the anhydrous carbonyl form[4] The doublet at τ 4 88 was assigned to the hydrate, because it had the same splitting and chemical shift as that of H-1 of the hydrated form of 2,3,4,5,6-penta-*O*-methyl-D-glucose and because, like the latter, it was not present when the sugar was dissolved[4] in chloroform-*d* That the doublet at τ 5 12 was due to the β-D-septanose was deduced from its similarity to the doublet at τ 5 03 given when the compound was in chloroform-*d* solution Since the anomeric effect, which favors the α-D anomer[10], is smaller for the free sugar **6** (0 55 kcal/mole in water) than for the methyl glycosides (1 4 kcal/mole in methanol)[10], and since the α-D-septanoside was not detected in the glycosidation reaction, **6** presumably does not exist as the α-D anomer to any appreciable extent in aqueous solution.

The n m.r spectra obtained from solutions of methyl β-D-septanoside **7** in benzene, pyridine, chloroform-*d*, and deuterium oxide all showed the signal of the

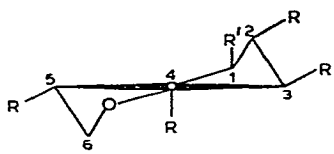
anomeric proton as a sharp doublet ($J_{1,2}$ 7 35 Hz), with no indication of the presence of the α-D anomer. The resonances of the five *O*-methyl groups of **7** were completely resolved when benzene was used as the solvent (see Fig 2) The absence of the α-D anomer was also indicated by g l c. on several liquid phases The β-D configuration of **7** was deduced from its large, negative, optical rotation, and from the splitting of the n m r signal of H-1 (7 35 Hz), which is similar to that observed for β-D-glucopyranose[10], where the dihedral angles of H-1 and H-2 are similar and close to 180° The true coupling-constants are probably larger in both cases, because of virtual coupling[11]

The energies of the various conformations of cycloheptane have been computed by Hendrickson[12] The form having the lowest energy is the skew-chair **10** When his results are applied to the septanosides, the situation becomes more complicated, because of ring substitution and of the asymmetry of the sugar The number of possible skew-chair forms increases to 14 for compound **7**, as compared with one for cycloheptane Substituted cycloheptanes are in their forms of lowest energy when the bonds to substituents are equatorial, bonds 1 of **10** being intermediate between equatorial and axial bonds[12]. Many of the 14 skew-chair conformations of **7** involve impossible overlap of axial substituents; however, two forms, **11a** and **11b**, have no axially



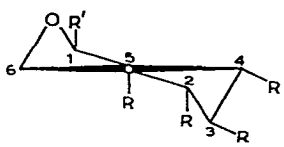
10

Geometry of cycloheptane[12]
(a, axial, e, equatorial)



11a

R = OMe R' = OH for 6
R = R' = OMe for 7



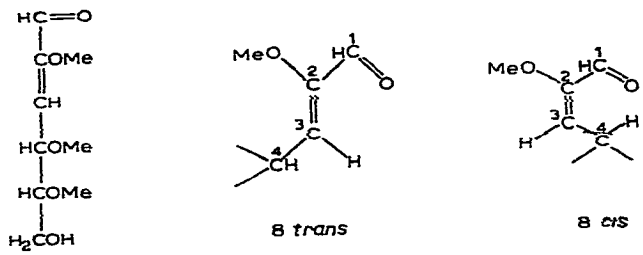
11b

Favored conformations of β-D-glucoseptanose

attached *O*-methyl groups, and a further four forms have only one axially attached substituent On the other hand, the α-D anomer of **7** has no skew-chair forms lacking an axial substituent, and the four forms having one axial substituent (all in the **4a** position), unlike those of the β-D anomer, have the axial group in each instance on the same side of the ring as the methoxyl group at C-1 in compound **10**. The α-D anomer would thus have a very much higher free-energy than the β-D anomer.

Although the anomeric effect favors the α-D anomer, the β-D anomer should preponderate when this effect is relatively small, as with methyl glycosides[10], and this was found experimentally.

The unsaturated sugar **8** gave two partially resolved peaks on g l.c, and therefore consists of at least two forms, probably *cis* and *trans* (**8a** and **8b** in Table II).



8

Similarly, the n m r spectrum showed two major sets of peaks (see Fig. 3) The signals for H-1, H-3, and H-4 in one set were the sharper, and coincided with those of 3-deoxytetra-*O*-methyl-*aldehydo*-D-*erythro*-hex-2-enose[4], they are assigned to

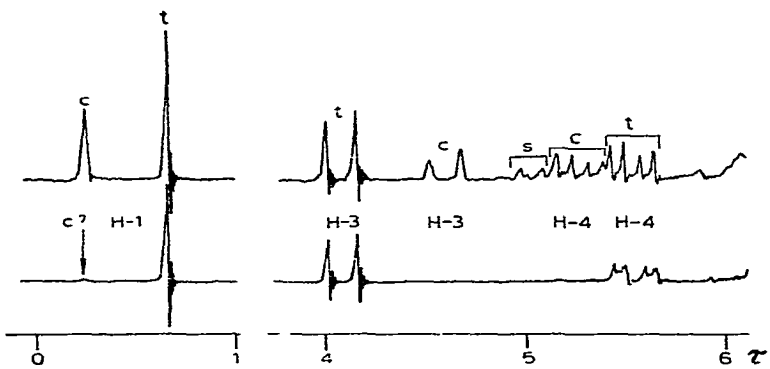


Fig 3 The n m r spectrum, at 60 MHz, of the unsaturated aldoses (**8**) in chloroform-*d* solution, upper trace, lower trace, the spectrum of 3-deoxytetra-*O*-methyl-*aldehydo*-D-*erythro*-hex-*trans*-2-enose[4] under the same conditions Assignments of peaks c, *cis* isomer, s, *cis*-septanose(?), t, *trans* isomer

the *trans* isomers The second set of peaks originated from the *cis* form, and differed in being broader and having the signals of H-1 and H-4 downfield, and that of H-3 upfield, from the corresponding signals for the *trans* form (see Fig 3) Weak signals at $\tau \sim 5.0$ were present in the spectrum, and may be due to cyclic hemiacetal forms of the *cis* isomer

The assignment of *cis* and *trans* configurations depends on the fact that the chemical shifts of H-3 and H-4 are not the same for the two isomers, because of different spatial arrangements about the anisotropic, carbonyl group[13] The proton at C-3 of the *trans* isomer, and that at C-4 of the *cis* isomer, can approach the carbonyl group and be deshielded, so that their signals appear at lower fields[13]

EXPERIMENTAL

General. — The microanalyses were performed by the Australian Microanalytical Service, Melbourne, and the n.m.r. spectra were obtained on a Varian model A-60 n.m.r. 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 when the solvent was deuterium oxide, and, with the other solvents, against tetramethylsilane (τ 10.00) as the internal standard. Optical rotations were determined with a Perkin–Elmer Model 141 polarimeter, the i.r. spectra on a Perkin–Elmer Model 221 i.r. spectrometer, and the u.v. spectra with a Beckman Model DK-2A u.v. spectrometer.

Gas–liquid chromatography was performed on a Varian Model 200 gas-chromatograph fitted with 3-mm columns and a flame-ionization detector. All volatile compounds were subjected to g.l.c. on columns packed with the methylsilicone gum, SE30, on Chromosorb G*, and all except **4** were run on two other phases, either QF1** or Carbowax*** 20M, both on Chromosorb G. Most of the retention times are given in Tables I and II.

TABLE I

RELATIVE RETENTION TIMES IN G.L.C. OF METHYLATED METHYL D-GLUCOSIDES[a]

Column phase	*Column temp (degrees)*	β-p	α-p	12	β-f	α-f
SE30	120	65	80	87	90	100
QF1	145	57	74	83	88	100
Carbowax 20M	140	52	80	81	88	100

[a]Fully methylated methyl D-glucosides, *p*, pyranoside, *f*, furanoside; **12**, β-D-septanoside.

TABLE II

RELATIVE RETENTION TIMES IN G.L.C.

Column phase	*Column temp (degrees)*	**5**	**6**	**7**	**8a**	**8b**	**9a**	**9b**
SE30	140	195	115	100	68	63	50	47
QF1	145	262	262	100	174	174	138	112

6-O-Benzyl-D-glucose — A solution of 6-O-benzyl-3,5-*O*-benzylidene-1,2-*O*-isopropylidene-α-D-glucose[6] (100 g) in *p*-dioxane (400 ml) and M hydrochloric acid

*Johns-Manville Product Corporation
**Dow-Corning Corporation
***Union Carbide Corporation

(100 ml) was heated for 30 min on a boiling-water bath, cooled, and rendered almost neutral with M sodium hydroxide The reaction mixture was then washed with hexane (2 × 400 ml), and evaporated to a syrup, to which water (400 ml) was added The aqueous solution was washed with ether (30 ml), and evaporated to a syrup that was mixed with acetone After the addition of some carbon (Norit), the suspension was filtered, and the filtrate was evaporated to a syrup which slowly crystallized when exposed to air Paper chromatography [4.1 1 (v/v) butanol–acetic acid–water[14]] showed only one spot, no glucose being present The compound had m p 93°, and $[\alpha]_D^{25}$ +40 3° (*c* 1, water) (lit.[5] m p. 92–93 5°, $[\alpha]_D^{20}$ +70 2→+39.3°).

6-O-Benzyl-D-glucose diethyl dithioacetal (**1**) — Finely powdered 6-*O*-benzyl-D-glucose (50 g), hydrochloric acid (d 1 19, 42 ml) and ethanethiol (50 ml) (all precooled to 0°) were mixed, and the mixture was vigorously shaken for 10 min at 0° The acid was then neutralized with a concentrated solution of sodium hydroxide, and the mixture was extracted with ethyl acetate On removal of the solvent, the extracts yielded the dithioacetal (70 g). The crude material was used for the acetylation Two recrystallization from ether at 0° gave colorless needles, m.p. 63 5–64°, $[\alpha]_D^{25}$ −6.7° (*c* 1.04, chloroform)

Anal Calc for $C_{17}H_{28}O_5S_2$, C, 54 2; H, 7 5, S, 17 0 Found· C, 54 3, H, 7 6; S, 17 0

Tetra-O-acetyl-6-O-benzyl-D-glucose diethyl dithioacetal (**2**) — Compound **1** (112 g), pyridine (360 ml), and acetic anhydride (720 ml) were mixed at 0°, and the mixture was kept overnight at room temperature, and poured, with stirring, into ice–water (5 liters) After 30 min, the syrup that had separated was dissolved in chloroform, and the extract was washed several times with water, dried (sodium sulfate), and evaporated to dryness The product did not crystallize, but was apparently pure, since, on t l c. on silica gel with 1 1 (v/v) water-saturated ethyl acetate–toluene, only a single spot was visible

6-O-Benzyl-D-glucose dimethyl acetal (**3**) — The diethyl dithioacetal **7** (40 g) in dry methanol (400 ml) was vigorously stirred with cadmium carbonate (48 g) and mercuric chloride (130 g) for 2 h, in an all-glass apparatus, under reflux 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 did not crystallize The products from 5 batches (160 g) were combined, and deacetylated with sodium methoxide in methanol (900 ml + 0 5 g of sodium) The reaction mixture was kept overnight, and then concentrated to about 200 ml, and dry ether was added to precipitate a large amount of a dark syrup The clear, supernatant liquor was decanted as quickly as possible, before the product crystallized out The solution yielded the product **3** (35 g) Recrystallization from acetone, and then from methanol, gave stout needles, m.p 93°, $[\alpha]_D^{25}$ +12.7° (*c* 1, water)

Anal Calc for $C_{15}H_{24}O_7$, C, 57.0, H, 7.7. Found C, 57.1, H, 7.7

The hydrolysis of the acetal **3** (*c* 1) with 20 mM hydrochloric acid at 50° was followed on a recording polarimeter, and was found to follow first-order kinetics,

half being hydrolyzed in 5 min (*cf* the rate for 2,3,4,5,6-penta-*O*-methyl-D-glucose[4]). That the product was 6-*O*-benzyl-D-glucose was confirmed by its optical rotation of $[\alpha]_D^{25}$ +40° (*c* 1, water) and by paper chromatography [4 1 1 (v/v) butanol–acetic acid–water[14]]

6-O-Benzyltetra-O-methyl-D-glucose dimethyl acetal (**4**). — The acetal **3** (5 g) in *N*,*N*-dimethylformamide (200 ml) was methylated with methyl iodide (33 ml) and silver oxide (50 g) The mixture was shaken for 2 days, the silver oxide being added in small portions during this time The solid was filtered off and washed with more solvent and with chloroform. The filtrate and washings were combined and evaporated, the residue was dissolved in chloroform, the solution was washed with an aqueous solution of potassium cyanide, and the aqueous layer was re-extracted several times with chloroform. The extracts were combined, dried (sodium sulfate), and evaporated to a syrup (6 g), which was distilled at 170° (bath)/0 01 torr, $[\alpha]_D^{25}$ +3 5° (*c* 0 9, methanol) On g l c with the SE30 column at 200°, the acetal **4** gave a single peak having a retention time 12 times that of compound **8**

Anal. Calc. for $C_{19}H_{32}O_7$; C, 61 3, H, 8.7. Found C, 61.3, H, 8 4.

2,3,4,5-Tetra-O-methyl-D-glucose dimethyl acetal (**5**). — The 6-benzyl ether **4** was hydrogenolyzed in methanol solution with palladium (10%) on calcium carbonate as the catalyst The product showed only one peak by g l c , and distillation gave the analytically pure compound as a syrup, $[\alpha]_D^{25}$ +20 3° (*c* 1 08, water)

Anal. Calc for $C_{12}H_{26}O_7$, C, 51 0, H, 9 3 Found· C, 49 7, H, 9 0

The hydrolysis of the acetal **5** (*c* 1) with 20 mM hydrochloric acid at 50° was followed on a recording polarimeter. About half of the acetal was hydrolyzed in 5 min

2,3,4,5-Tetra-O-methyl-D-glucose (**6**) — The acetal **5** (1 g) was treated with 20 mM hydrochloric acid for 40 min at 50° The reaction mixture was cooled, and extracted with chloroform, and the extract was dried (sodium sulfate) and evaporated to give **6** as a syrup (see Fig 1 for n m r. spectra).

Methyl tetra-O-methyl-β-D-glucoseptanoside (**7**) — To a refluxing solution of 2,3,4,5-tetra-*O*-methyl-D-glucose (**6**, 5 g) in absolute methanol (75 ml) was added an ion-exchange resin of the sulfonic acid type (2 ml) in dry methanol, and the suspension was refluxed, with stirring, for 22 h. Samples of the supernatant liquor were withdrawn at intervals and analyzed by direct injection into the gas chromatograph The first sample, taken after 1 min, already consisted essentially of the acetal **5** with some free sugar **6**, but no glycoside. After 22 h, less than 5% of **6** remained as the free sugar, the rest having being converted into the methyl β-D-septanoside (70%) and the dimethyl acetal (30%) The resin was filtered off, the filtrate was concentrated, and the residue was chromatographed on an alumina column, with hexane as the eluant. Because the septanoside was the only non-hydroxylic form, it emerged first from the column That portion of the eluate which contained the pure septanoside (by g l c) was evaporated, and the residue was distilled, $[\alpha]_D^{25}$ −75° (*c* 1.25, water) and −37 6° (*c* 1 05, chloroform).

Anal. Calc for $C_{11}H_{22}O_6$ C, 52 8, H, 8 9. Found C, 53.0, H, 8 9.

To follow the above reaction by g.l.c., a special column was made with 2.1% (v/v) SE30–QF1 as the stationary phase. The retention times of **5**, **6**, and **7** were in the ratios of 5.3.2, giving a clean separation (*cf.* Table II).

Reaction of **6** *with alkali.* — A solution of **6** (350 mg) in 20 mM calcium hydroxide (40 ml) was kept for 2 min at room temperature, rendered neutral with 8 ml of 100 mM hydrochloric acid, and extracted with chloroform (10 × 40 ml). The extracts were combined, dried (sodium sulfate), and concentrated. G.l.c. showed that no unchanged sugar remained, and that 4 new components were present: two major ones (the unsaturated sugars **8a** and **8b**) and two minor ones (the 3,6-anhydroaldoses **9a** and **9b**) (see Table II). When a sample of the product was treated with a slight excess of bromine in carbon tetrachloride, and the solution was subjected to g.l.c., the peaks **8a** and **8b** were absent, and two new peaks having longer retention times, probably the dibromo compounds, appeared. The peaks of the anhydroaldoses were unaffected, but were removed when a solution of the original mixture in chloroform was washed with water, leaving the pure unsaturated sugars in the chloroform. That the material not extracted by water consisted of the unsaturated sugars was confirmed by the strong u.v. absorption at 246 nm, by i.r. bands at 1699 and 1632 cm^{-1}, and by the n.m.r. spectra (see Results and Discussion, and Fig. 3). This degradation of **6** was monitored by g.l.c.; shorter reaction times or lower temperatures resulted in incomplete reactions, and longer times gave a higher proportion of the 3,6-anhydroaldoses.

The unsaturated sugars **8** were degraded by acid (*cf.* Ref. 4). The u.v. absorption at 246 nm disappeared, and was replaced by a peak at 229 nm, which reached a maximum after treatment with 20 mM hydrochloric acid during 5 min at 100°. The product could not be gas-chromatographed, and was not isolated.

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ISOLEMENT À PARTIR DE L'URINE HUMAINE NORMALE D'UN FUCOHÉTÉROPOLYSACCHARIDE À ACTIVITÉ DE GROUPE SANGUIN O(H)

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(Reçu le 19 janvier 1968, sous forme revisée, le 6 mai 1968)

ABSTRACT

Starting from the components of urine soluble in 50% ethanol, a heteropolysaccharide containing L-fucose having O(H) blood-group activity has been isolated from normal human urine with the use of DEAE-cellulose and CM-cellulose chromatography Its homogeneity was established by ultracentrifugation, electrophoresis, and gel filtration L-Fucose forms 20% of the molecule The results of mild acid and enzymic hydrolysis suggest that the L-fucose residue is located at the non-reducing end of the chain and is linked glycosidically. The other components are galactose and 2-amino-2-deoxyglucose, and in smaller proportions, mannose, glucose, and 2-amino-2-deoxygalactose The fucoheteropolysaccharide inhibits the hemagglutination of O(H) erythrocytes with eel antiserum and *Ulex europaeus* lectin

SOMMAIRE

Un fucohétéropolysaccharide possèdant une activité de groupe sanguin O(H) a ete isolé de l'urine humaine normale à partir des constituants urinaires solubles dans l'éthanol à 50%, par chromatographie sur DEAE-cellulose et CM-cellulose. Son homogénéité est fondee sur les résultats de l'ultracentrifugation, de l'electrophorèse et de la gel filtration Le L-fucose represente 20% de la molecule et les résultats des hydrolyses acide ménagée et enzymatique, et de l'oxydation periodique suggèrent que le L-fucose occupe une position à l'extremité non réductrice de la chaîne, étant relié au reste du polysaccharide par son groupe réducteur Les autres constituants sont essentiellement le galactose et la glucosamine, et en plus faibles proportions le mannose, le glucose et la galactosamine. Le fucohétéropolysaccharide inhibe l'hémagglutination des érythrocytes O(H) par le sérum d'anguille et par la lectine *Ulex europaeus*

INTRODUCTION

La presence dans l'urine de substances à activité de groupe sanguin a eté démontrée et diverses préparations actives, mais de nature chimique imprécise ont été obtenues[1].

Plus récemment Masamune *et al*[2] rapportèrent la présence de mannose, de fucose, de galactose, d'acide sialique et d'osamine dans les préparations urinaires brutes, mais sérologiquement actives. A partir des substances non dialysables de l'urine, King *et al.* caractérisèrent deux fractions, l'une ultrafiltrable[3], l'autre non ultrafiltrable[4] mais contenant toutes les deux du galactose, du fucose, du mannose, des hexosamines et des amino-acides, et présentant une activite A et B, sans activité O(H).

Un pourcentage éleve de fucose est une des caracteristiques des substances non dialysables de l'urine[5,6], et des fractions riches en fucose ont eté obtenues par chromatographie sur DEAE-cellulose[7] et par electrophorese de zone[8].

Les travaux de Morgan et Watkins[9,10] ont montre le rôle du fucose dans l'activité de groupe sanguin du système ABO, et Price Evans *et al*[11] ont revélé que l'urine des sécréteurs contient des quantités significativement plus élevées de fucose que les non sécréteurs, fait confirmé ensuite par Lundblad[12]

Il apparaissait alors possible que les activites de groupes sanguins urinaires, ou une partie de ces activités soient localisées dans les fractions riches en fucose Une telle fraction douée d'activité O(H) a été obtenue[13] tandis que des glycopeptides de composition voisine, mais sérologiquement inactifs ont été décrits par Lundblad[14]

Ce mémoire rapporte l'isolement d'un fucohéteropolysaccharide à activité O(H), présent dans l'urine humaine normale et dont la composition en glucides est très semblable à celle des substances des groupes ABO

PARTIE EXPÉRIMENTALE

MÉTHODES

Gel filtration — Les gels de dextran Sephadex G-25 et G-50 (50-100 mesh), ont été suspendus dans 10–20 volumes de NaCl à 1%, lavés à l'eau, puis équilibrés dans le tampon acétate de pyridine 0 1M, pH 5 3. La suspension fut dégazée, puis versée dans une colonne, le gel sédimentant à la pression atmosphérique

Le produit à filtrer a été solubilise dans un volume minimal de tampon (1–5 ml selon les dimensions de la colonne) L'élution a été effectuée par le tampon ci-dessus, des fractions de 4–5 ml étant recueillies sur lesquelles furent déterminés les hexoses[15]

Chromatographie sur DEAE-cellulose — La DEAE-cellulose a éte équilibrée dans le tampon collidine–pyridine–acide acétique 5 mM, pH 8.7 La suspension de DEAE-cellulose dans ce tampon a été dégazée et introduite dans une colonne de 4 cm de diamètre Après sédimentation, la hauteur de l'échangeur etait de 30 cm La préparation à fractionner a été introduite dans la colonne et l'elution effectuee de façon discontinue à pH et molarité variables de ce tampon Des fractions de 20 ml ont été recueillies sur lesquelles furent effectués les dosages des hexoses

Chromatographie sur CM-cellulose — La CM-cellulose a été traitee avec l'hydroxyde de sodium 0.5M, puis lavée à l'eau et enfin équilibrée par le tampon acétate de pyridine 0 1M, pH 5 3. La suspension de l'échangeur dans le tampon

a été dégazée, puis introduite dans une colonne de 3 cm de diamètre. Après sédimentation, la hauteur de l'échangeur était de 30 cm Un litre d'eau distillée a été ensuite passé à travers la colonne, de telle sorte que la CM-cellulose soit finalement équilibrée avec ce dernier solvant La préparation à étudier, dissoute dans l'eau, a été introduite dans la colonne L'élution a été effectuée de façon discontinue avec successivement l'eau distillée, les tampons acétate de pyridine 0 01M, pH 5.3; 0 025M, pH 5 3, acétate de pyridine–triméthylamine M, pH 7.7. Des fractions de 10 ml ont été recueillies sur lesquelles furent déterminés les hexoses[15] et les groupements α-aminés[16]

Électrophorèse sur papier. — La technique d'électrophorèse de Machebœuf *et al*[17] a été utilisee (appareil Leres) avec les tampons acide formique 0 5% (pH 2 3) et acetate de pyridine (0 6M en pyridine, pH 6 4) La migration a été poursuivie pendant 2–3 h sous 400 volts, les révélations furent effectuées par la ninhydrine (groupes α-NH_2) et par le réactif de Schiff après oxydation périodique.

Ultracentrifugation — Les déterminations de la constante de sédimentation ont été effectuées à la Station Centrale du C N R S (Directeur Madame Wurmser) dans la centrifugeuse SPINCO Modèle E à 59 780 tours/min En raison du faible poids moleculaire du produit, ces opérations furent réalisées après la formation d'une frontière synthetique Le solvant utilisé était un tampon phosphate–chlorure de sodium (2 8, 0.1M, pH 6.8), les concentrations de produits employés étant de 2–10 mg/ml

PREPARATION ET PROPRIÉTÉS DU FUCOPOLYSACCHARIDE

Isolement — Un extrait aqueux de 100 litres d'urine humaine dialysée et lyophilisée, a été additionné[7] d'un volume d'éthanol à −5° Le précipité forme a été écarté, et la solution hydroalcoolique surnageante concentrée sous vide à faible volume (Fraction S-50) Cette fraction fut d'abord dessalée par filtration sur Séphadex G-25, puis passée sur une colonne de DEAE-cellulose (pH 8 7, 5 mM)

Le produit sérologiquement actif et riche en fucose n'a pas été retenu sur l'échangeur de cellulose et fut trouvé en totalité dans le 1er pic (Fraction I) Cette fraction a été lyophilisee, elle représente 40 mg/litre et sa composition est rapportée dans le Tableau I

Une seconde chromatographie, réalisée dans les mêmes conditions (pH 8 7, 5 mM) a été effectuée sur la fraction I On a observé une résolution de cette fraction en un pic non retenu sur DEAE-cellulose (Fraction IA) et un pic elué par ce même tampon, mais avec un certain retard Enfin un 3e pic a éte elué à pH 5 3, M (Fig 1)

La fraction IA, représentant 12 mg/litre d'urine, a été dissoute dans 50 ml d'eau et passée sur une colonne de CM-cellulose équilibrée dans l'eau distillée La plus grande partie du produit sérologiquement actif n'a pas éte retenue sur l'échangeur (Fraction IB), tandis qu'une 2e fraction a été éluée par l'acétate de pyridine 0 025M à pH 5 3 (Fig 2)

La fraction IB presente une très petite impureté polysaccharidique en électrophorèse sur papier à pH 2 4 dans l'acétate de pyridine 0 1M La fraction a été

passée sur une colonne de Séphadex G-50 (100 × 2 cm) équilibrée avec le tampon acétate de pyridine 0.1M, pH 5.3. La courbe d'élution (Fig. 3) s'est révélée unique et symétrique La fraction IC a été lyophilisée Elle représente 9 mg/litre d'urine

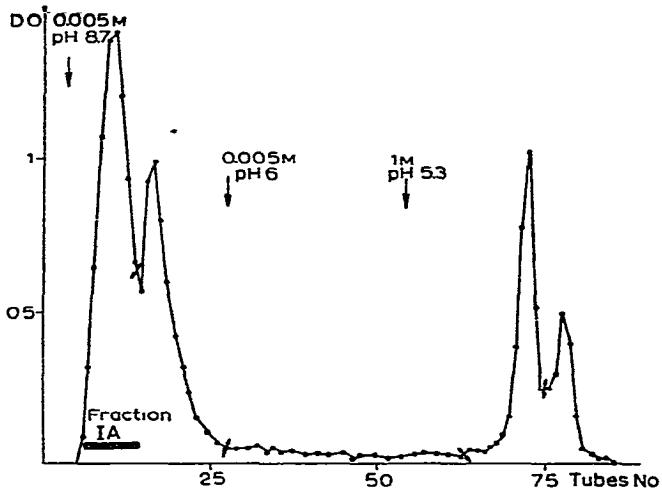


Fig 1 Deuxième chromatographie sur DEAE-cellulose de la fraction non retenue sur DEAE-cellulose des constituants urinaires non dialysables, solubles dans l'éthanol a 50% L'élution discontinue est effectuée avec les tampons acétate de collidine–pyridine 0 005M, pH 5 3, acétate de pyridine 0 05M, pH 6, puis M, pH 5 3 La courbe représente le dosage des hexoses[15]

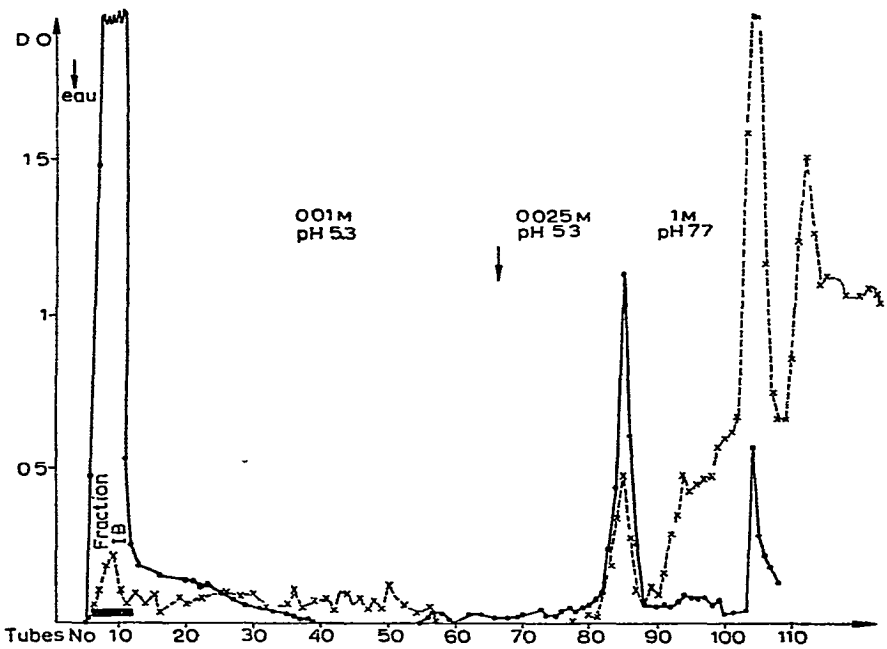


Fig 2 Chromatographie sur CM-cellulose de la fraction IA (Fig 1) Colonne équilibrée d'abord avec le tampon acétate de pyridine 0 1M, pH 5 3, puis avec l'eau distillée Elution discontinue d'abord avec l'eau distillée, puis avec le tampon acétate de pyridine 0 025M, pH 5 3 •———• hexoses, ×– – – × groupes α-aminés

Composition du fucohétéropolysaccharide. — En électrophorèse aux pH 2 4 et 6 4, ce produit s'est révélé homogène, il se situe au niveau des oses neutres témoins, sous forme d'une bande unique bien délimitée, révélable par le réactif de Schiff après oxydation periodique, mais non révélable par la ninhydrine ou l'Amidoschwarz

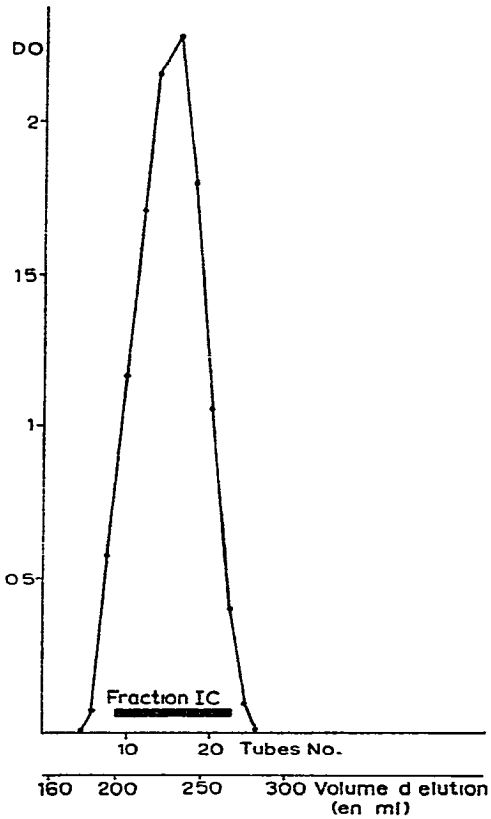


Fig 3 Gel filtration sur Séphadex G-50 (100 × 2 cm) équilibre par le tampon acétate de pyridine 0 1M, pH 5 3 de la fraction IB obtenue par chromatographie sur CM-cellulose L'élution a été effectuée par le tampon acétate de pyridine 0 1M, pH 5 3 et la courbe représente le dosage des hexoses[15]

En ultracentrifugation, le fucopolysaccharide a montré un pic unique et symétrique dont la constante de sédimentation, S^0_{20} 0 83 S, est indépendante de la concentration en produit de 0 2 à 1%.

La filtration sur Séphadex G-50 (1 × 50 cm) de ce polysaccharide dans le tampon acétate de pyridine 0 2M à pH 5 3 s'est traduite par un pic unique et symétrique dont le volume d'exclusion (V_1) est supérieur au volume vide (V_0) de la colonne, déterminé avec le bleu dextran $V_0 = 18$ ml et $V_1 = 27$ ml Dans les mêmes conditions, le polysaccharide a été exclu d'une colonne de Séphadex G-25 (1 × 40 cm) ($V_0 = V_1 = 21$ ml).

Ces trois techniques, électrophorèse, ultracentrifugation et gel filtration permettent de vérifier l'homogénéité du polysaccharide étudié

Le poids moléculaire n'a pu être déterminé par les techniques physiques

impliquant la constante de sédimentation et la constante de diffusion ou la viscosité; en effet la viscosité du produit est trop faible et la diffusion trop considérable pour obtenir une valeur précise, ce qui suggère une faible valeur pour le poids moléculaire; l'étude du poids moléculaire par osmométrie à tension de vapeur (Mechrolab Hewlett Packard) n'a pas donné de résultats satisfaisants Cependant la constante de sédimentation, le comportement sur Séphadex G-25 et G-50, la détermination de la galactosamine et le pouvoir réducteur suggèrent un poids moléculaire voisin de 4 000

Les résultats des dosages des constituants glucidiques, effectués selon les méthodes décrites précédemment[15], sont rapportés dans le Tableau I Les oses neutres représentent 70% du poids total dont 20% du fucose; les hexoses ont été

TABLEAU I

COMPOSITION EN GLUCIDES DES FRACTIONS S-50, I ET IC (FUCOHÉTÉROPOLYSACCHARIDE) ISOLÉES DE L'URINE HUMAINE NORMALE

	S-50[a]	I[b]	IC[c] (Fucohétéropolysaccharide)
	%	%	%
Hexoses totaux	26	36	48 5
Galactose			38 7 (9)
Mannose			7 4 (2)
Glucose			3 2 (1)
Fucose	4 5	11	19 4 (5)
2-Acetamido-2-désoxyhexoses	25	21 5	28 3
Glucosamine			23 5 (5)
Galactosamine			5 2 (1)
Acide sialique	18	7 7	< 0 2
Protéines	29	24	< 0 1

[a]S-50 : constituants urinaires non dialysables, solubles dans l'éthanol a 50% [b]I 1er pic apparu au cours de la première chromatographie sur DEAE-cellulose [c]Entre parenthèses, rapports molaires

déterminés par rapport à une courbe étalon de galactose–mannose (4 1), selon les résultats de la chromatographie sur papier d'un hydrolysat acide du polysaccharide Bien que les groupes acétyles n'aient pas été dosés, il est admis que les osamines sont acétylées, en effet, après hydrolyse ménagée du polysaccharide, une importante partie des osamines a été libérée sous forme de 2-acetamido-2-désoxyglucose L'acide sialique est absent.

La chromatographie sur papier Schleicher et Schull 2043 b d'un hydrolysat de ce polysaccharide par l'acide chlorhydrique M pendant 4 heures selon la méthode décrite précédemment[18] a révélé la présence de trois constituants principaux : le fucose, le galactose et la glucosamine, en outre, le mannose, le glucose et la galactosamine sont présents en petites quantités

Les amino-acides ont été déterminés avec l'auto-analyseur Technicon sur 2 mg (0 5 μmole) de produit, hydrolysés par l'acide chlorhydrique 6M pendant 6 et 22 h à 105°, à la concentration de 0 5 mg/ml. Les hydrolysats furent rapidement évaporés

sous vide en présence d'hydroxyde de potassium et d'anhydride phosphorique Des valeurs non significatives ont été obtenues glycine, 0.1 μmole, proline, 0 08 μmole et les autres amino-acides en proportions inférieures à 0 03 μmole sur la base d'un poids moléculaire de 4 000

La faible proportion d'amino-acides, jointe à la présence d'un pouvoir reducteur correspondant à un poids moléculaire de 4 000, suggère que le produit étudié est un hétéropolysaccharide, et non un glycopeptide qui impliquerait au moins une molécule d'un amino-acide par molécule de produit.

Inhibition de l'hémagglutination par le fucohétéropolysaccharide — Le test d'inhibition de l'hémagglutination est utilisé Les réactifs anti H(O) furent le sérum d'anguille* et un extrait de graines d'*Ulex europaeus* les graines (1 g) ont eté broyees et extraites avec la solution tampon chlorure de sodium 0 1M–phosphate de sodium 0 05M, pH 6 8 (5 ml) Après 24 h d'agitation mecanique, l'extrait a ete sépare des substances insolubles par centrifugation et l'activité O déterminée Les érythrocytes humains O (Centre de Transfusion Sanguine, Paris), provenant de sang citrate, ont éte lavés avec une solution de chlorure de sodium 0.85% dans le tampon phosphate 0.025M, pH 7 2 Une suspension à 4% a été utilisée; la solution d'agglutinine (4 doses d'agglutination 0.1 ml) a été incubée avec différentes dilutions (ordre 2, 0 1 ml) de la substance Après 30 min, la suspension d'érythrocytes du groupe O (0 1 ml) a été ajoutée Dans tous les essais, le L-fucose a éte inclus comme témoin Les produits qui n'inhibaient pas à la dose de 5 mg/ml ont été considérés comme inactifs

Avec la technique décrite ci-dessus, la quantite minimale de L-fucose donnant une inhibition complète était de 0 5 mg/ml avec le sérum d'anguille et de 1 mg/ml avec l'extrait d'*Ulex europaeus* Dans les mêmes conditions, la quantité minimale active de fucopolysaccharide etait de 0 5 mg/ml avec le sérum d'anguille et de 0 25 mg/ml avec l'extrait d'*Ulex europaeus* Ainsi sur le plan pondéral, l'activité du fucopolysaccharide est du même ordre de grandeur que celle de L-fucose vis-à-vis du sérum d'anguille, sur la base molaire, le polysaccharide décrit est 10–20 fois plus actif que le L-fucose

Hydrolyse acide ménagée — Le produit (20 mg) dissous dans l'acide chlorhydrique 0 1M (5 ml) a été hydrolysé pendant des temps de 0, 5, 10, 15, 20, 30, 40 et 60 min à 100°. A chacun de ces temps d'hydrolyse, un prélèvement (0 1 ml) a été effectué sur lequel fut déterminé le pouvoir réducteur selon Somogyi[19]

Après 1 heure d'hydrolyse, l'équivalent de 0.12 mg de glucose/mg de produit a été libéré. L'hydrolysat a été concentré et fractionné sur Séphadex G-25 dans l'acide acétique 0.1M Le 1er pic ne contenait pas de glucides libres et, après hydrolyse par l'acide chlorhydrique M pendant 4 h, la chromatographie a révélé essentiellement deux constituants, le galactose et la glucosamine, avec de faibles quantités de mannose, de glucose, de fucose et de galactosamine, le 2e pic était constitué presque exclusivement de fucose libre, représentant plus de 50% du fucose total du polysaccharide

*Le sérum d'anguille nous a été aimablement donné par le Docteur Fontaine du Muséum d'Histoire Naturelle, Paris, que nous remercions très vivement

Ainsi plus de la moitié des molécules de fucose est facilement libérable avec apparition d'activité réductrice, ce qui suggère que les molécules de fucose sont situées aux extrémités non réductrices de la chaîne et liées glycosidiquement au reste du polysaccharide.

Hydrolyse enzymatique — Le produit (10 mg) a été incubé à 37° en présence de β-galactosidase (Sigma) à pH 7, de suc d'*Helix pomatia* à pH 5 4, ou d'un extrait de *Trichomonas foetus** à pH 7 5 Apres 48 h, le pouvoir réducteur a été déterminé[19] La β-galactosidase est inactive, l'extrait d'*Helix pomatia* a libéré seulement de petites quantités de substances réductrices, tandis que l'extrait de *T foetus* s'est révélé très actif (0 1 mg d'ose libéré exprimé en glucose/mg de polysaccharide) Cet hydrolysat a été fractionné sur Séphadex G-25 Le 1er pic était dépourvu de glucides libres, contenait essentiellement du galactose et de la glucosamine, et peu de fucose, révélables seulement en chromatographie après hydrolyse par l'acide chlorhydrique M Le 2e pic etait constitué essentiellement de fucose libre, et d'une petite quantite de galactose libre

Ces résultats corroborent les conclusions avancées, d'après les résultats de l'hydrolyse acide ménagée

TABLEAU II

EFFET DE L'HYDROLYSE ACIDE MENAGEE (A), DE L'HYDROLYSE ENZYMATIQUE (B) ET DE L'OXYDATION PERIODIQUE (C) SUR LES CONSTITUANTS GLUCIDIQUES DU FUCOHETÉROPOLYSACCHARIDE[a]

	A	*B*	*C*
	%	%	%
Hexoses	97	81	61
Fucose	52	41	0
Hexosamines	98	100	75

[a]Constituants glucidiques restants

Oxydation periodique — L'oxydation a été effectuée avec le métapériodate de sodium 0 05M dans un tampon acétate 0 1M, pH 4 5, à +4° et à l'obscurité La concentration en produit était de 1 mg/ml et le rapport molaire périodate/polysaccharide égal à 250. Après 24 h, l'oxydation a été arrêtée par addition de 1,2-éthanediol et la consommation de périodate déterminée.

La solution concentrée a été filtrée sur Séphadex G-25, après dilution avec l'acide acétique 0 1M, et la fraction polysaccharidique oxydée ainsi obtenue, est traitée par le borohydrure de sodium pendant 16 h à 4° Après ce temps, l'excès de borohydrure fut décomposé par l'acide acétique et la solution passée sur Dowex-50 X 4 (H^+, 100–200 mesh), puis concentrée à sec Le résidu a été repris 5 fois par le méthanol suivi d'évaporation pour éliminer les dernières traces de boro-

*L'extrait de *Trichomonas foetus* nous a été aimablement donné par le Dr Watkins que nous remercions très vivement

hydrure Enfin, la fraction obtenue est hydrolysée par l'acide chlorhydrique 0 1M, pendant une nuit à 22°, ce qui libère les liaisons acétals présentes dans la fraction[20] L'hydrolysat a été concentré et passé sur Séphadex G-25 dans l'acide acétique 0.1M pour séparer le polysaccharide oxydé et les polyols éventuellement formés

La consommation de periodate après 24 h d'oxydation fut de 14 moles/mole de polysaccharide sur la base d'un poids moléculaire égal à 4 000

L'analyse chimique et chromatographique des constituants glucidiques du polysaccharide oxydé puis réduit, a été effectuee en comparaison avec celle du polysaccharide natif On a observé la disparition totale du fucose, la destruction de 40% des hexoses (essentiellement du galactose) et de 25–30% des osamines

La présence et la resolution des polyols présents dans le 2e pic de la filtration sur Séphadex ont été déterminées par chromatographie sur papier avec les solvants alcool butylique–acide acétique–eau (4 1 5) et pyridine–acetate d'ethyle–acide acétique–eau (5 5 3 1) en présence des témoins suivants glycerol, érythritol et glycolaldehyde Les polyols ont éte révélés par le nitrate d'argent et par un réactif plus spécifique, le métaperiodate de sodium–benzidine Une tache très importante de glycérol apparaît et une tache plus faible au niveau de l'erythritol

DISCUSSION

La composition chimique de l'héteropolysaccharide décrit dans ce travail est semblable à celle des substances à activite de groupe sanguin ABO Toutefois ce fucopolysaccharide urinaire se distingue de ces dernières par un poids moléculaire et une activite nettement plus faibles, et par l'absence d'amino-acides, il se rapproche plutôt des oligosaccharides (tri- à hexa-saccharides) obtenus après dégradation des substances à activité de groupe sanguin Pusztai et Morgan[21] avaient effectivement montré la perte considérable d'activité qui résultait de la proteolyse des substances de groupe sanguin, la partie polysaccharide non modifiée conservant seulement une activité sérologique résiduelle minimale

Le rôle du fucose dans la spécificité de groupe sanguin O(H) a été bien démontre par Watkins et Morgan[9,10] le fucose inhibe l'agglutination des érythrocytes de groupe sanguin humain O par les agglutinines anti H(O) du sérum d'anguille ou de certaines légumineuses (lectines ou phytohémagglutinines)

Des préparations riches en fucose de type glycopeptidique ont été obtenues à partir de l'urine, mais les unes présentaient une activité A et B et pas d'activité $O^{3\,4}$, et les autres étaient sérologiquement inactives[12], seul l'heteropolysaccharide décrit ici possède une spécifité O(H).

L'activité des fucosides se revèle dépendre de la série L, de la forme pyranose et de la liaison α-L du fucose[22], la structure α-L-fucosyl-(1→2)-β-D-galactosyl étant impliquée dans les groupes déterminants des substances H(O)[23 24]. Les résultats partiels obtenus avec l'hétéropolysaccharide urinaire s'accordent bien avec le rôle et la position du fucose dans les molécules actives, en effet l'oxydation periodique libère la totalité du fucose et abolit l'activité sérologique, tandis que l'hydrolyse

enzymatique clive essentiellement le fucose avec de petites quantités de galactose et apparition d'une activité réductrice Le fucose est donc en position externe, et lié glycosidiquement au reste de la molécule, il est en outre libéré par une préparation de *T. foetus* qui hydrolyse les liaisons α-L fucosides[25]

Des différences dépendant de la nature des agglutinines ont été signalées par Springer *et al*[26] dans l'activité inhibitrice des oligosaccharides à fucose, il en est de même du fucopolysaccharide urinaire dont l'activité inhibitrice de l'hémagglutination est plus importante vis-à-vis des lectines que du sérum d'anguille

L'origine du polysaccharide est encore inconnue et il n'est pas certain qu'il provienne des substances de groupes sanguins cellulaires ou solubles, car il contient de petites quantités de mannose et de glucose, de même que les autres fractions riches en fucose déjà mentionnees[3 4 12] Or ces deux hexoses ne paraissent pas entrer dans la constitution des substances solubles des groupes sanguins ABO.

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A METHOD FOR ANALYSIS OF AMINO SUGARS: SPECIFICITY AND MECHANISM OF THE REACTION

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ABSTRACT

Two methods for the analysis of amino sugars are presented. Ratios of the colorimetric values obtained with these two methods permit the preliminary characterization of several amino sugars and direct determination when they are in admixture with 2-amino-2-deoxy-D-glucose.

The main features of the mechanism of the color reaction are outlined. Factors affecting the reproducibility of the Elson and Morgan analysis and the role of 2-methylpyrrole in the production of color are discussed.

INTRODUCTION

A valuable method for the analysis of amino sugars is based on their reaction with an alkaline solution of 2,4-pentanedione; the products of this reaction yield, with an acid solution of *p*-dimethylaminobenzaldehyde, a colored solution (Elson and Morgan reaction).

This report describes a modification of a method based on the Elson and Morgan reaction[1]. It considers the possible variables involved in its reproducibility and discusses the mechanism of the color reaction.

RESULTS AND DISCUSSION

Analysis. — The "Hot-Reaction" method*, when the chromogens are formed at 100°, and the "Cold-Reaction" method, when the chromogens are formed at 0° (see Experimental), were used for establishing colorimetric ratios ("Hot-Reaction" to "Cold-Reaction" ratio). These ratios, useful for the preliminary identification of a single amino sugar, are also useful in the direct quantitative analysis of a mixture. As shown in Table I, of all the amino sugars analyzed, a high color intensity ratio was obtained only with 2-amino-2-deoxy-D-glucose. In the "Cold-Reaction" method

*The "Hot-Reaction" and "Cold-Reaction" methods are modifications of previously published "Total-Hexosamine" and "Hexosamine" methods, respectively[1]. The new procedures of analysis are at least twice as sensitive.

this amino sugar gave about 3% of the color intensity produced by 2-amino-2-deoxy-D-galactose

TABLE I

PRODUCTION OF COLOR BY AMINO SUGARS

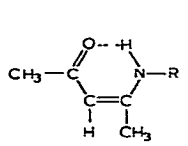
Amino sugar (*0 22 μmole*)	*Absorbance*				
	Cold-Reaction (*525 nm*)		*Hot-Reaction* (*535 nm*)		
		ε^a		ε^a	*Ratio*[b]
2-Amino-2-deoxy-D-glucose	0 020	524	0 598	15700	30 0
2-Amino-2-deoxy-D-allose[c]	0 121	3170	0 616	16350	5 2
2-Amino-2-deoxy-D-galactose	0 600	15750	0 596	15630	1 0
2-Amino-2-deoxy-D-altrose	0 975	25500	0 584	15300	0 60
2-Amino-2-deoxy-D-mannose	0 981	25700	0 492	12900	0 50
2-Amino-2-deoxy-D-gulose	1 073	28200	0 452	11850	0 42
2,6-Diamino-2,6-dideoxy-D-allose	0 152	3980	0 238	6230	1 57
2,6-Diamino-2,6-dideoxy-D-mannose	1 160	30400	0 242	6340	0 21
2,6-Diamino-2,6-dideoxy-D-gulose	1 170	30600	0 334	8750	0 29
2-Amino-2-deoxy-D-glucuronic acid	0 077	2020	0 534	13800	6 83
2-Amino-2-deoxy-D-xylose	0 403	10580	0 664	17400	1 65

[a]Molar absorbance coefficient, based on the concentration of amino sugar (chromogens transformed), in the colored solution [b]"Hot-Reaction" to "Cold-Reaction" [c]Amorphous prepared from a crystalline derivative [P H Gross, K Brendel, and H K Zimmerman Jr *Ann Chem* 683 (1965) 175]

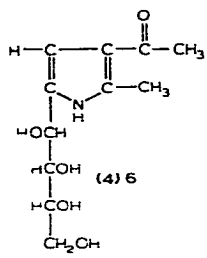
Since amino acids and neutral carbohydrates are known to interfere with the Elson and Morgan reaction[2], colorimetric ratios may be obtained which could, mistakenly, be attributed to an amino sugar. This can be avoided by purification of the hydrolyzed sample[3] When an ion-exchange column is used for the separation and analysis, artifacts of this nature are usually found in elution volumes just following the void volume of the column[4] The hydrolysis and analysis (by the ion-exchange procedure of Gardell[4]) of a protein–polysaccharide (15% protein) from the *nucleus pulposus* of whale intervertebral discs is given as example After this sample was hydrolyzed with 4M hydrochloric acid in a nitrogen atmosphere for 14 h at 100°, three elution peaks were obtained; the second and third peaks corresponded to 2-amino-2-deoxy-D-glucose and 2-amino-2-deoxy-D-galactose, respectively The first elution peak was a complex mixture in which several amino acids and galactose were identified by paper chromatography The "Hot-Reaction" to "Cold-Reaction" ratio of this elution volume was 10 However, the color of the solution was anomalous and similar to the color produced by mixtures of amino acids and neutral carbohydrates[5]. The same sample, when hydrolyzed with 6M hydrochloric acid in evacuated, sealed tubes for 21 h at 110°, gave only two peaks, corresponding to 2-amino-2-deoxy-D-glucose and 2-amino-2-deoxy-D-galactose

Reaction intermediates — 2-Methylpyrrole[6,7], 3-acetyl-2-methylpyrrole[7],

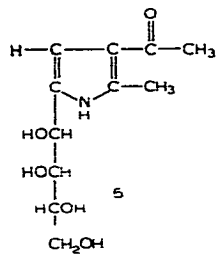
3-acetyl-5-(D-*arabino*-tetrahydroxybutyl)-2-methylpyrrole[8–10] (**4**) and 2-deoxy-2-[2-(4-oxo-2-pentenyl)amino]-D-glucose[11] (**1**) have been isolated from the reaction product of 2-amino-2-deoxy-D-glucose with 2,4-pentanedione The enamine derivatives **2** and **3**, products obtained from the condensation of 2-amino-2-deoxy-D-galactose and



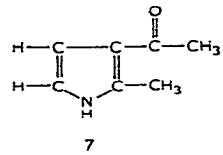
1 R = 2-deoxy-D-glucose
2 R = 2-deoxy-D-galactose
3 R = 2-deoxy-D-mannose



(4) 6



5



7

2-amino-2-deoxy-D-mannose, respectively, with 2,4-pentanedione and 3-acetyl-5-(D-*lyxo*-tetrahydroxybutyl)-2-methylpyrrole (**5**), obtained from 2-amino-2-deoxy-D-galactose, have not been reported before

Role of 2-methylpyrrole — A high color-yield derivative, isolated from the reaction product of a hexosamine with an alkaline solution of 2,4-pentanedione, has been identified as 2-methylpyrrole, this was suggested to be the most important chromogen of the Elson and Morgan reaction[7] This view was challenged by García González *et al*[10], who pointed out that the color produced in the Elson and Morgan reaction and several of its modifications showed an absorption curve with maximum at 530 nm, whereas 2-methylpyrrole gives a maximum at 548 nm with the Ehrlich reagent

Two possible mechanisms have been suggested for the formation of 2-methylpyrrole[7 12 13] Although both appear valid and seem to operate, the role of 2-methylpyrrole in the color produced by the reaction is not clear as yet

The absorption spectra, observed by using the "Hot-Reaction" and "Cold-Reaction" methods, suggest that smaller amounts of 2-methylpyrrole are formed when the chromogens are produced at a lower temperature (Fig 1) The formation of 2-methylpyrrole was demonstrated by lyophilization of the reaction mixtures prior to the development of color, and by analysis of the water of lyophilization with the Ehrlich reagent. Whereas only a trace amount of a volatile chromogen (2-methylpyrrole)[7] was found when the conditions of incubation of the "Cold-Reaction" method were used, considerably higher amounts of 2-methylpyrrole were obtained when the conditions of the "Hot-Reaction" method were used. Thus, when the "Hot-Reaction" method was applied to 2-amino-2-deoxy-D-galactose (one of the amino sugars giving the higher yields of 2-methylpyrrole), the color intensity developed in the water of lyophilization by addition of the Ehrlich reagent was about two-thirds of the total color intensity of the non-lyophilized solution When the "Cold-Reaction" method was applied to 2-amino-2-deoxy-D-galactose, the contribution to the color of the solution by 2-methylpyrrole was found to be negligible (less than 1%) From these results, it is clear that, whatever mechanisms are postulated for the formation of

chromogens by the "Cold-Reaction" method, the formation of 2-methylpyrrole cannot be considered important. However, in the "Hot-Reaction" method, 2-methylpyrrole seems to be one of the predominant factors responsible for the color of the solution.

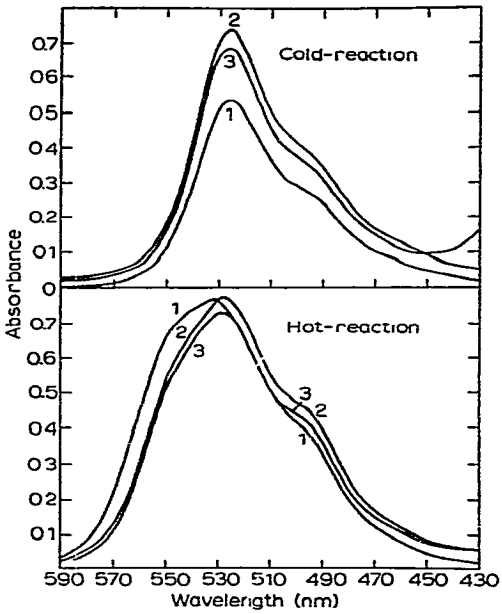


Fig 1 Absorption spectra produced by hexosamines 1 2-amino-2-deoxy-D-galactose, 2 2-amino-2-deoxy-D-mannose, and 3 2-amino-2-deoxy-D-glucose

Transformations of 2-methylpyrrole catalyzed by oxygen and light were observed A fresh alkaline solution of 2-methylpyrrole gave a higher color intensity with the Ehrlich reagent than an equivalent solution that had been previously heated in a glass stoppered tube at 100° However, if a solution containing 2-methylpyrrole is heated in the dark and in the absence of oxygen (in presence of nitrogen), the color intensity of the solution produced with the Ehrlich reagent remains essentially the same In fact, if the "Hot-Reaction" method is used for the analysis of 2-amino-2-deoxy-D-galactose, the color yield increases by 20% when the reaction is carried out in the dark and in the presence of nitrogen gas In a previous study, 2-methylpyrrole could not be identified by paper chromatography[7]. A possible explanation for this was that the compound may have been lost by evaporation from the paper before it was sprayed with the Ehrlich reagent, or partly lost during the heating of analytical samples with an alkaline solution of 2,4-pentanedione[7]. Both evaporation and decomposition of 2-methylpyrrole are variables which may affect the reproducibility of the "Hot-Reaction" method

On the basis of qualitative observations, a possible mechanism of color formation by the "Cold-Reaction" method was proposed[1] (see footnote on Page 1) which would involve the formation of different amounts of enamine derivatives *vs*

chromogens. We have found, however, by quantitative paper-chromatographic analysis, that 2-amino-2-deoxy-D-glucose produced about 20% of **1** and 10% of 3-acetyl-2-methylpyrrole (7), 2-amino-2-deoxy-D-galactose produced about 5% **2** and 20% of 3-acetyl-2-methylpyrrole (7); and 2-amino-2-deoxy-D-mannose produced trace amounts of **3** and about 25% of 3-acetyl-2 methylpyrrole (7). The remaining product(s) of the reaction from 2-amino-2-deoxy-D-glucose was a component having a lower paper chromatographic mobility (R_F 0.12) than the original hexosamines, which gave no color with the Ehrlich reagent and a pink color with the Elson and Morgan reagents An analogous paper-chromatographic component was also formed, but in smaller amount, from 2-amino-2-deoxy-D-galactose, none of this product could be detected when 2-amino-2-deoxy-D-mannose was used. The compound(s) with R_F 0 12 was presumably produced by degradation, since the hexosamines incubated for 18 h at 0° in an alkaline solution (pH 9.5) containing no 2,4-pentanedione produced a similar paper, chromatographic component Apparently 2-amino-2-deoxy-D-glucose is less reactive with 2,4-pentanedione and, as a free hexosamine, is subjected to the deteriorating conditions of an alkaline incubation for a longer period of time than other amino sugars

The low reactivity of 2-amino-2-deoxy-D-glucose with the reagent and the formation of predominantly low color-yield chromogens derived from its enamine derivative **1** seems to be, then, the cause for the negligible color produced by this hexosamine in the "Cold-Reaction" method

In addition to the enamine derivatives and the 3-acetyl-2-methylpyrrole which were produced by the hexosamines, strong color-yield chromogens were also observed on paper chromatograms when 2-amino-2-deoxy-D-galactose and 2-amino-2-deoxy-D-mannose were used in the reaction mixture ("Cold Reaction" method). It was, however, difficult to detect these chromogens (presumably because of their low concentrations in this reaction mixture) when 2-amino-2-deoxy-D-glucose was used as the reactant

EXPERIMENTAL

Ehrlich reagent — A solution of *p*-dimethylaminobenzaldehyde (1 g) in 3 8M perchloric acid (50 ml) was diluted to 100 ml with 95% ethanol

Alkaline 2,4-pentanedione reagent — 2,4-Pentanedione (3 ml) was diluted to 100 ml with 0 7M sodium carbonate The pH of this solution was 9 8 A solution of this reagent (0 5 ml) with 0 3M hydrochloric acid (0 25 ml) had a pH 9 5 When the solution was heated for 20 min at 100°, the pH was 9 4

Cold-Reaction method — Samples (0 25 ml) containing less than 80 μg of amino sugar hydrochloride in 0 3M hydrochloric acid were introduced into tubes refrigerated with ice; alkaline 2,4-pentanedione reagent (0.5 ml) was added to each tube The tubes were glass stoppered, submerged in crushed ice, and incubated in a refrigerator at 0° After 18 h, 90% ethanol (4 ml) was added while the tubes were under refrigeration This was followed by the addition of the Ehrlich reagent (1 ml) The mixtures were shaken after each addition and finally incubated for 1 h at 70°.

After the incubation period, the solutions were cooled for 1 h in a water bath (20–25°), and absorbance measurements were made at 525 nm

Hot-Reaction method — To samples (0 25 ml) containing less than 80 μg of amino sugar hydrochloride in 0 3M hydrochloric acid was added alkaline 2,4-pentanedione reagent (0 5 ml). The solutions were heated in glass stoppered tubes for 20 min at 100°. After cooling, first 90% ethanol (4 ml) and then the Ehrlich reagent (1 ml) were added. The mixtures were shaken after each addition The solutions were then cooled for 1 h in a water bath (20–25°) and absorbance measurements were made at 535 nm

All absorbance readings were made in rectangular cuvettes of 1-cm light-path length with a Gilford spectrophotometer, model 2000

Color intensity — The "Cold-Reaction" method showed a range of linearity of the color up to 3 mg for 2-amino-2-deoxy-D-glucose and 2-amino-2-deoxy-D-glucuronic acid For the remaining amino sugars, the color-linearity range extended to about 80 μg When the "Hot-Reaction" method was used, all the amino sugars analyzed showed a color linearity range up to about 80 μg Intercepts at the absorbance axis were from 0 01 to about 0 04 for both methods of analysis Relative standard deviations (σ/mean) × 100 of the "Hot-Reaction" to "Cold-Reaction" ratios were from 3 to 6%.

Identification of chromogens and intermediates — This identification was made by descending paper chromatography on Whatman Paper No. 1 with propyl alcohol–water (89 28) for 16 h at 5°. Reaction mixtures containing hexosamine (500 μg) in alkaline 2,4-pentanedione reagent (0 25 ml) were incubated by the "Hot-Reaction" and "Cold-Reaction" methods described above Purple spots of the same chromatographic mobility—(R_F 0 71) corresponding to **1**, **2**, and **3**—appeared immediately on the paper after it was sprayed with a solution of 5% $FeCl_3 \cdot 5\,H_2O$ in 50% ethanol–water (v/v) The 5-tetrahydroxybutylpyrrole derivatives **4** and **5** were identified by spraying the paper with the Ehrlich reagent. Both compounds **4** and **5** showed the same chromatographic mobility (R_F 0 65), **4**, however, showed initially a yellow spot which turned blue after 24 h, whereas **5** showed initially a yellow spot, which turned purple after 24 h, and 3-acetyl-2-methylpyrrole (R_F 0 90), initially a pink spot, which turned violet after 24 h. The relative color intensity of the spots produced by the enamine intermediates was estimated with the aid of a paper-scanner densitometer 30 min after spraying The color intensity of the substituted pyrrole derivatives was estimated after 24 h The Ehrlich reagent and the Elson and Morgan reagents for paper spraying were prepared according to Partridge and Westall[14]

Analysis of 2-methylpyrrole — To a solution of an amino sugar (800 μg) in 0 3M hydrochloric acid (0.5 ml) was added alkaline 2,4-pentanedione reagent (1 ml) The mixture was incubated according to the "Cold-Reaction" and "Hot-Reaction" methods At the end of the incubation period the reaction mixture was frozen and lyophilized. From the water of lyophilization, collected in a tube refrigerated with a solid carbon dioxide and acetone bath, an aliquot (0 25 ml) was assayed for 2-methylpyrrole with the Ehrlich reagent. Recovery of 2-methylpyrrole was higher than 95%

The enamine **1** and the (tetrahydroxybutyl)pyrrole derivative of 2-amino-2-deoxy-D-glucose (**5**) were prepared by the methods of García González *et al.*[10], and 3-acetyl-2-methylpyrrole and 2-methylpyrrole by the method of Cornforth and Firth[7]. Several attempts were made to prepare **6** from 2-amino-2-deoxy-D-mannose, none of them succeeded It is of interest to note, however, that cyclization of **1** and **3** should give the same (tetrahydroxybutyl)pyrrole derivative **4**. Compounds **4** and **6** are, therefore, identical

Compounds **2** and **3** showed properties in common with those exhibited[10,11] by **1**, and in neutral or alkaline solutions (pH 9 5) underwent ring closure reactions to produce 3-acetyl-2-methylpyrrole and the corresponding (tetrahydroxybutyl)pyrrole derivatives **5** and **4** (**6**) respectively With the exception of 2-methylpyrrole, the remaining intermediates gave, under analytical conditions, colored solutions of lower intensity than those produced by the corresponding hexosamines[10,11]

2-Deoxy-[2-(4-oxo-2-pentenyl)amino]-D-galactose (**2**) — A mixture of triethylamine (25 ml), 2,4-pentanedione (25 ml), and pyridine (0 5 ml) in abs methanol (50 ml) at 0° was added to solid 2-amino-2-deoxy-D-galactose hydrochloride (1 g) The reaction mixture was stirred for 2 h at 0° under nitrogen and concentrated in a flash evaporator below 15° to about 25 ml To this solution was added 100 ml of 50% (v/v) petroleum ether in ethyl ether, an amorphous precipitate separated, which was dried in vacuum, dissolved in abs methanol, and precipitated again by the addition of a large excess of ethyl acetate Compound **2** separated first as an oil which crystallized after being kept for four months at 5°, 0 15 g, m p 115° with softening at 100°; $[\alpha]_D^{20}$ −133 5° (*c* 0 12, water); $\lambda_{max}^{H_2O}$ 312 nm (ε 18,000), λ_{max}^{KBr} 6 16 μm (> C=O), weak band at 3 16 μm for intramolecularly bonded > NH[15,16]

Anal Calc for $C_{11}H_{19}NO_6$· C, 50 56, H, 7 33, N, 5.36 Found C, 49.91, H, 7.32, N, 5 80

2-Deoxy-2-[2-(4-oxo-2-pentenyl)amino]-D-mannose (**3**) — To a solution of 2-amino-2-deoxy-D-mannose hydrochloride (1 g) in 0.8M sodium carbonate (3 ml) at 0° was added 1 ml of 45% (v/v) of 2,4-pentanedione in acetone The reaction mixture (pH 7 6) was stirred for 3 days at 0° and lyophilized The residue of lyophilization was extracted with two portions of 10 ml each of abs methanol and filtered Addition to the methanol solution of an excess of ethyl acetate (120 ml) precipitated a yellow hygroscopic material which was rapidly removed by filtration Compound **3** crystallized out (0 25 g) from the filtrate after being kept overnight at 5°; m p 127–29°; $[\alpha]_D^{20}$ −197° (*c* 0 3, water), $\lambda_{max}^{H_2O}$ 312 nm (ε 21,400), λ_{max}^{KBr} 6 18 μm (> C=O) weak band at 3.16 μm for intramolecularly bonded > N-H[15,16]

Anal Calc for $C_{11}H_{19}NO_6$ C, 50.56; H, 7 33, N, 5 36 Found C, 50.63, H, 7.53, N, 5.30

The yellow hygroscopic material was shown by paper chromatography to consist of several compounds, among them **3** and a second major component having the same paper-chromatographic mobility and color behavior (with the Ehrlich reagent) as **4**

3-Acetyl-5-(D-lyxo-tetrahydroxybutyl)-2-methylpyrrole (**5**). — A solution of

0 8M sodium carbonate (3 ml) at 0° was added to 2-amino-2-deoxy-D-galactose hydrochloride (1 0 g), 1 ml of 45% (v/v) 2,4-pentanedione in acetone was then added (pH of final mixture 7 6) and the mixture was kept for 3 days at 0° This solution was then lyophilized and extracted with two portions of 10 ml each of abs methanol. After filtration an excess of ethyl acetate (120 ml) was added, the mixture was filtered again, and the solution was kept overnight in the refrigerator, compound **5** crystallized out (0 1 g); m p 134–35°, $\lambda_{max}^{H_2O}$ 212, 247, 290 nm (ε 18,400, 7460, 625), λ_{max}^{KBr} 3 08 μm (> NH), 6 12 μm (>C=O); $[\alpha]_D^{20}$ +20° (*c* 1.0, water)

Anal Calc for $C_{11}H_{17}NO_5 \cdot H_2O$ C, 50 56; H, 7 33; N, 5 36 Found C, 50 80, H, 7 12, N, 5 34

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SYNTHESIS AND PROPERTIES OF 3,6-DIDEOXYHEXULOSONIC ACIDS AND RELATED COMPOUNDS

A CONVENIENT PREPARATION OF 3-DEOXY-D-*erythro*-HEXULOSONIC ACID (3-DEOXY-2-KETO-D-GLUCONIC ACID)* STEREOSELECTIVITY OF NUCLEOPHILIC ADDITION TO TRIOSE CARBONYL

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ABSTRACT

Synthesis and characterization of the epimeric 3,6-dideoxy-D(and L)-hexulosonic acids are described The configuration of each epimer has been established by measurements of rate of periodate oxidation, and each assignment agrees with that determined by enzyme-specificity studies The n.m.r. spectra of the epimeric 3,6-dideoxyhexulosonic acids and that of 3-deoxy-DL-*glycero*-pentulosonic acid show that each compound exists in solution mainly as the 2,5-hemiacetal. A convenient preparation of the bacterial metabolite 3-deoxy-D-*erythro*-hexulosonic acid ("3-deoxy-2-keto-D-gluconic" acid) is reported The stereospecificity of the aldol reactions employed in these syntheses is discussed

INTRODUCTION

In the course of studies on the stereochemistry of the enzymic conversion of 3-deoxy-L-*glycero*-pentulosonate into 2,5-dioxopentanoate[1], it was found necessary to have on hand all four stereoisomers of 3,6-dideoxyhexulosonic acid Syntheses of the related compounds, *N*-acetylneuraminic acid and 3-deoxy-D-octulosonic acids, have already been performed by Cornforth *et al*[2] and Heath[3], respectively Their method, which involves aldol reaction of oxalacetic acid with the appropriate aldehyde (see Scheme I), has been extended and modified for the preparation of potassium 3-deoxy-DL-*glycero*-pentulosonate[4] at neutral pH The successful application of this modified procedure to the synthesis of the 3,6-dideoxyhexulosonic acids is now described. At the same time, it was noted that no simple method is at present available for the preparation of 3-deoxy-D-*erythro*-hexulosonic acid ("3-deoxy-2-keto-D-gluconic" acid), a well known, bacterial metabolite[5]. A convenient synthesis of this compound, by a similar method, is therefore reported here

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It was also of general interest to determine the ratios of epimers arising in these reactions, and to attempt rationalization of the results by consideration of the relevant steric and polar effects

RESULTS AND DISCUSSION

Oxalacetic acid was condensed with D-, L-, or DL-lactaldehyde under neutral, aqueous conditions[4], and the resultant mixture, containing pyruvate (arising from the decarboxylation of oxalacetate) and a pair of epimeric 3,6-dideoxyhexulosonates, was fractionated on Dowex-1 (formate) resin Under the conditions used, pyruvic acid remained adsorbed on the resin, whereas the epimeric 3,6-dideoxyhexulosonates were eluted as clearly separated peaks The pure epimers **1** and **2** were isolated as the potassium salts, and were shown to be chromatographically homogenous in four solvent systems (see Table I) Specific rotations for both pairs of enantiomorphs are recorded

TABLE I

CHROMATOGRAPHIC DATA FOR POTASSIUM 3,6-DIDEOXYHEXULOSONATES

	R_F[a]	R_F[b]	R_F[c]	R_F[d]
Isomer **1** (*erythro*)	0 25	0 53	0 72	0 78
Isomer **2** (*threo*)	0 30	0 55	0 72	0 78

[a]6 4 3 (v/v) butyl alcohol–pyridine–water [b]3 1 (v/v) propyl alcohol–0 2M NH_4OH [c]70 15 1 (v/v) *tert*-butyl alcohol–formic acid–water [d]6 3 1 (v/v) propyl alcohol–formic acid–water

TABLE II

OPTICAL ROTATORY DATA[a] FOR POTASSIUM 3,6-DIDEOXYHEXULOSONATES

Sample	*Concentration (g/100 ml)*	$[\alpha]^{22}_{437}$, *degrees*	$[\alpha]^{22}_{589}$, *degrees*
1a (*erythro*)	7 32	−21 4	−11 5
1b	5 42	+22 0	+11 3
2a (*threo*)	5 00	+41,4	+18 2
2b	4 10	+39 2	−17 1

[a]Length = 1 dm, solvent, H_2O; pH, 7 5

in Table II Solutions of either isomer in 2M hydrochloric acid showed a slow development of a pH-dependent, u v absorption At low pH (< 7.5), the spectra showed a broad band at λ_{max} 232 nm ($\varepsilon \sim 4000$), which, at high pH (> 7.5), shifted to λ_{max} = 265 nm ($\varepsilon \sim 3600$). This behavior is attributed to the presence of the chromophore C=C(OH)C=O arising by lactonization of the original free acid, and is similar to that of 4-hydroxy-2-oxobutyro-1,4-lactone, which absorbs at 226 nm

(enol, ε 4000) and 261 nm (enolate anion, ε 2600)[6] It seems likely that the 1,4- (not the 1,5) lactone is formed, as only in the former would the presence of a 1,2-dicarbonyl grouping in the ring favor the formation at equilibrium of the conjugated enol[7]

The compounds were characterized further by preparation of the crystalline semicarbazones and, in the case of isomer **2**, of the (2,4-dinitrophenyl)hydrazone All of these derivatives gave correct analyses for a dehydrated species, assumed to be the 1,4- or 1,5-lactone that would be expected to be formed under the acidic conditions of the preparation Isomers **1** and **2** were both shown to exist in equilibrium with the 2,5-hemiacetal forms, as evidenced by their observed mutarotation and their i r. spectra (weak C=O absorption at 1705 cm^{-1}); the n m r spectra in deuterium oxide supported this conclusion In order to obtain unequivocal peak-assignments, the spectrum of 3-deoxy-DL-*glycero*-pentulosonate **3a** + **3b** (K^+ salt) was first examined The spectrum [see Fig 1(a)] showed peaks centered at τ 5 5 (1 proton), τ 6 0 (2 protons),

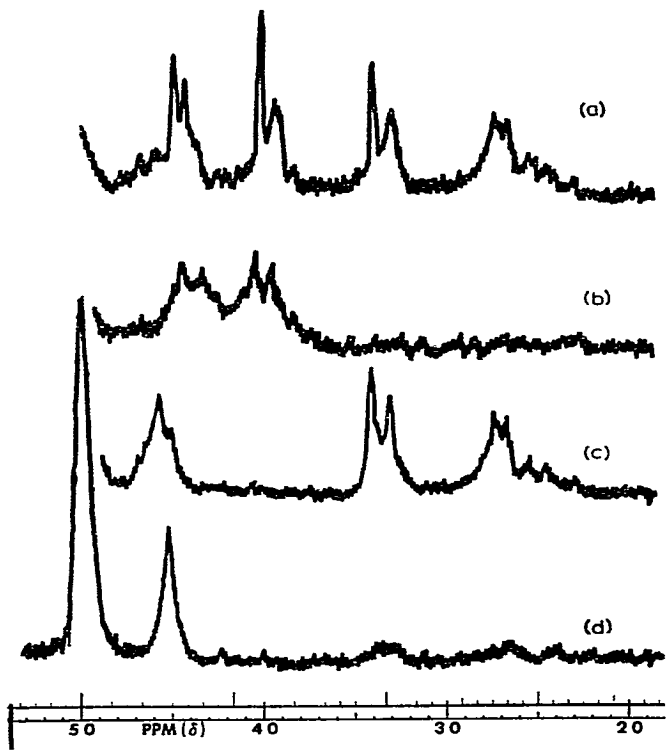


Fig 1 N m r spectra of 3-deoxy-DL-*glycero*-pentulosonate and deuterated analogs See text for explanation

τ 6 6 (1 proton), and τ 7 3 (1 0 proton) The peaks at τ 6 6 and τ 7 3 gradually diminished in intensity ($t_{1/2} \sim 3$ days at 25°), but the ratio of their intensities remained constant [see Fig 1 (b)] This loss of absorption was reversible, by equilibration of the sample with water, the peaks reappeared. Therefore, these peaks were assigned to the exchangeable protons at C-3, and the spectrum observed was rationalized in terms of an equilibrium

between the acyclic and the 2,5-hemiacetal forms On the basis of the chemical shifts observed[8], the band at τ 6 6 was assigned to H-3 of the acyclic form, and that at τ 7.3 was assigned to those of the cyclic hemiacetal As expected, the splitting patterns of both peaks are not simple The ratio of absorption intensities at τ 7 3 and τ 6 6 indicated a *ca* 1 1 ratio for the cyclic–acyclic equilibrium The peaks centered at τ 5 5 and τ 6 0 were assigned to H-4 and H-5, respectively, on the basis of their expected chemical shifts (τ secondary > τ primary) and the assumption that the chemical shift of each proton is not greatly affected by change from the acyclic to the cyclic form These assignments were confirmed by examination of the spectrum of 3-deoxy-DL-*glycero*-pentulosonate-*5-d*$_2$ [see Fig 1(c)], which showed absorption bands at τ 5 5, τ 6 6, and τ 7 3, but no absorption at τ 6 0 As noted previously, the peaks at τ 7 3 and τ 6 6 disappeared slowly [see Fig 1(d)], thus confirming the assignment of the remaining peak (at τ 5 5) to H-4.

The n m r spectrum of 3,6-dideoxy-DL-*erythro*-hexulosonate is shown in Fig 2; the spectrum of the *threo* isomer was very similar. By analogy with the spectrum of 3-deoxy-DL-*glycero*-pentulosonate, the following assignments were made τ 5 5, H-4 and H-5, τ 6 7, H-3 (acyclic, apparent doublet), τ 7 4, H-3 (cyclic form, apparent ABX system), τ 8 4, terminal methyl group. The peaks at τ 6 7 and τ 7 4 both diminished slowly by exchange with solvent deuterium, confirming their assignment to the α-ketonic protons. From the ratio of intensities of the H-3 peaks, it was estimated that the 3,6-dideoxyhexulosonic acids exist to about 85% as the cyclic hemiacetals

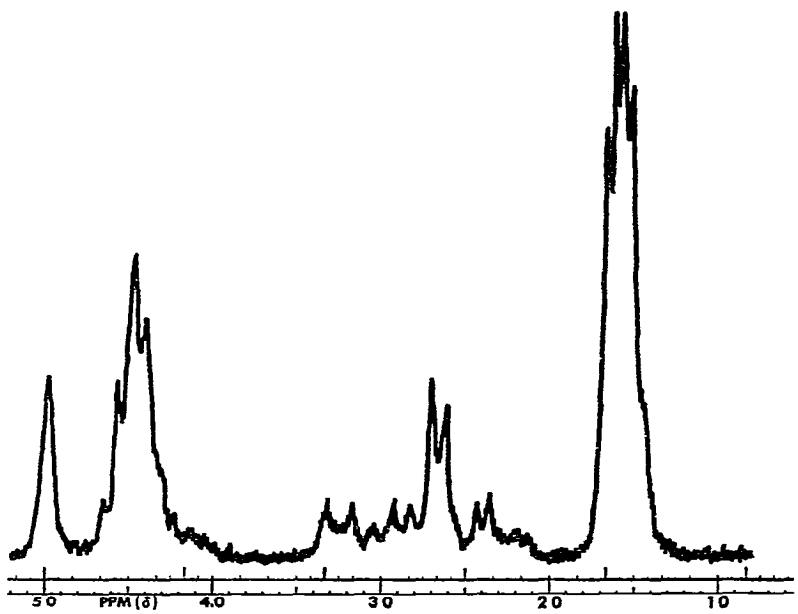


Fig 2 N m r spectrum of 3,6-dideoxy-DL-*erythro*-hexulosonate

The configurations of isomers **1** and **2** were determined from the results of periodate-oxidation studies The relative rates of periodate cleavage of a number of

pairs of diastereoisomeric 1,2-diols have been measured, and it has been clearly shown that the *threo* isomer is oxidized at a rate greater than that of the corresponding *erythro* isomer[9]. This method was therefore applied in the present study. In order to avoid any complication due to the existence of 3,6-dideoxyhexulosonic acids as cyclic hemiacetals, both isomers were oxidatively decarboxylated with hydrogen peroxide to the corresponding, acyclic 3,4-dihydroxypentanoic acids The relative rates of periodate oxidation of these diols were measured spectrophotometrically by the loss of absorption at 223 nm (periodate ion), the results are shown in Table III

TABLE III

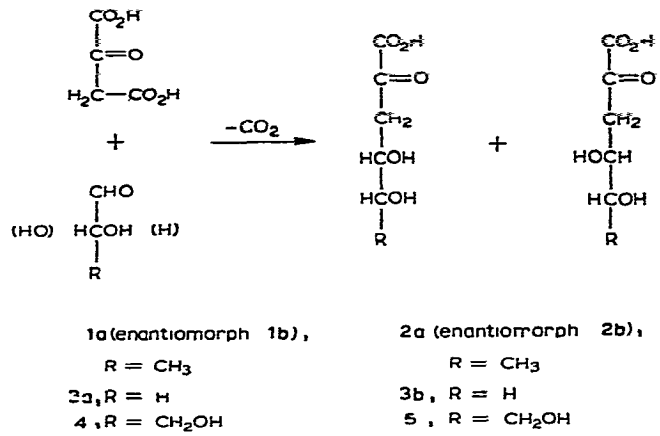
PERIODATE OXIDATION OF 3,6-DIDEOXYHEXULOSONATES

Isomer	*Molarity*	*pH*	*Initial rate**	*Initial rate* **2** / *Initial rate* **1**
1	0.417×10^{-4}	4.0	0.231	1.9
2		4.0	0.445	
1	2.085×10^{-4}	4.0	0.123	2.6
2		4.0	0.325	
1	4.170×10^{-4}	4.0	0.488	2.4
2		4.0	1.150	
1	0.417×10^{-4}	5.5	0.249	2.1
2		5.5	0.532	
1	2.085×10^{-4}	6.5	0.194	2.4
2		6.5	0.466	

*Units are arbitrary, being dependent, in each pair of measurements, on the speed of the recorder chart

It may be seen that the diol derived from isomer **2** was oxidized 1.9–3.2 times faster than the diol derived from isomer **1** under several conditions of pH and concentration of substrate It was concluded, therefore, that isomer **1** has the *erythro* configuration, whereas isomer **2** has the *threo* configuration This assignment of configuration is in agreement with enzyme-specificity studies Of the four 3,6-dideoxyhexulosonates, only isomer **1b** (derived from L-lactaldehyde) is active towards 3-deoxy-L-*glycero*-pentulosonate dehydrase[1]. Because this enzyme is specific for the "C-4-L" configuration[4], the reactive isomer **1b** must have the L-*glycero* configuration at C-4, and must therefore be the L-*erythro* isomer. The corresponding isomer **2b** must possess the *threo* configuration.

Synthesis of the 3-deoxy-D-hexulosonic acids **4** and **5** was effected by condensation of oxalacetic acid with D-glyceraldehyde by the procedure used for the 3,6-dideoxyhexulosonic acids Paper electrophoresis showed the product mixture to contain, in addition to pyruvate and unchanged D-glyceraldehyde, two other ketonic acids, **4** and **5**, in the approximate ratio of 10:1 By fractionation on Dowex-1 (formate) resin, it was possible to isolate approximately half of the total yield of α-keto acid as isomer **4**, but it was not practicable to isolate appreciable quantities of isomer **5**



SCHEME I

Isomer **4** was isolated as the syrupy potassium salt, which was chromatographically and electrophoretically homogenous, having R_F values in agreement with those previously reported for 3-deoxy-D-*erythro*-hexulosonic acid (see Table IV) (Isomer **5** has about the same R_F values as isomer **4**, as expected from their very similar structures) It was thus concluded that compounds **4** and **5** are the epimeric 3-deoxy-D-hexulosonic acids, and their configurations were assigned by comparison of the observed and previously reported optical rotations Isomer **4** had $[\alpha]_D^{22}$ −31.6°, which compares well with the value of $[\alpha]_D^{22}$ −29.2° observed by Merrick and Roseman for the D-*erythro* isomer[10] A roughly equimolar mixture of isomers **4** and **5** (as determined by paper electrophoresis) had $[\alpha]_D^{22}$ −7 8°, in agreement with the value of −7 1° calculated by using the rotation at $[\alpha]_D^{22}$ reported by Kuhn *et al*[11] for the D-*threo* epimer Thus, it was concluded that isomer **4** is 3-deoxy-D-*erythro*-hexulosonate, and isomer **5** is the *threo* epimer. Compound **4** was further characterized by its (2,4-dinitrophenyl)hydrazone, apparently the first crystalline derivative to be reported, except for the calcium salt[10]

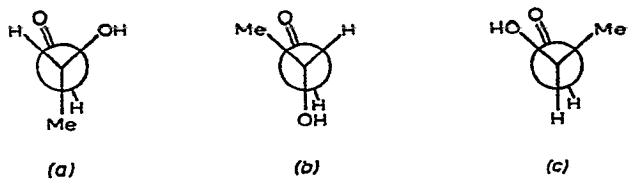
TABLE IV

CHROMATOGRAPHIC AND ELECTROPHORETIC DATA FOR POTASSIUM 3-DEOXY-D-HEXULOSONATES

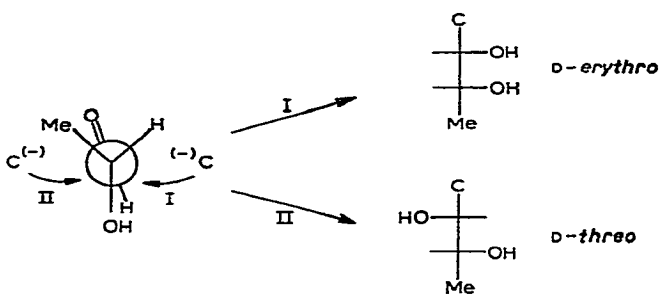
	R_F[a]	R_F[b]	R_F[c]	R_F[d]	M_G[e]
Isomer **4** (*erythro*-)	0 73 (0 89[f])	0 63 (0 68[f])	0 51 (0 59[f])	0 20 (0 35[g])	1 4
Isomer **5** (*threo*-)	0 73	0 68	0 51		1 6

[a] 6 1 3 methanol–0 88 NH_4OH–water [b] 80 15 5 methanol–formic acid–water [c] 6 3 1 propyl alcohol–formic acid–water [d] 4 1 1 butyl alcohol–acetic acid–water. [e] $M_G = \frac{\text{mobility of compound}}{\text{mobility of D-glyceraldehyde}}$ [f] See ref 10 [g] See ref 14

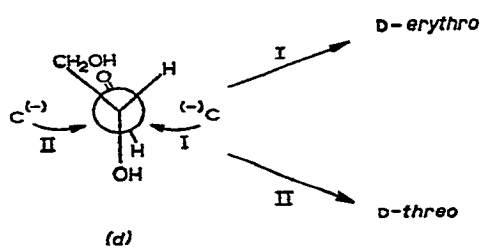
The stereoselectivity of the above condensations is significant, and may be rationalized by the following considerations Free D-lactaldehyde may exist in solution as three rotamers.



Conformer (*c*) is probably least favored, and, although there is little steric favoring for either (*a*) or (*b*), the latter is probably favored because of a minimization of dipolar interactions Nucleophilic addition to the carbonyl group of conformer (*b*) of lactaldehyde is likely to occur by mode I



that is, by the least sterically hindered approach (Cram's rule[16]) Thus, the resultant diol is more likely to be the *erythro* isomer than the *threo* Actually, when $C^{(-)}$ is the pyruvate carbanion, the *erythro–threo* ratio is about 2 1 For addition of the same carbanion to D-glyceraldehyde, whose most favored rotamer is probably (*d*)



(*d*)

the preference for mode I of attack should be greater, since the hydroxymethyl group should hinder attack by mode II more effectively than would a methyl group; this conclusion is borne out, since the *erythro–threo* ratio for this reaction was found to be greater than 10 1.

According to the above discussion, it should be possible to predict the steric outcome of the cyanohydrin synthesis, which involves a similar carbonyl-addition

reaction However, previous results[15] show that the ratio of epimers produced by the addition of cyanide ion to most of the reducing monosaccharides changes greatly according to the reaction conditions, especially the pH. Three factors may account for this Firstly, the attacking nucleophile, namely, cyanide, is much smaller than the pyruvate carbanion, and its favoring of a particular mode of attack may not be critically dependent on the steric factors just discussed Secondly, most additions of cyanide to reducing monosaccharides previously reported involve substrates that are more complex than either lactaldehyde or glyceraldehyde, and the conformation of their acyclic form is not yet known with certainty Finally, the ratio of epimeric adducts may be thermodynamically, not kinetically, controlled, since the establishing of equilibrium may be rapid under certain conditions In such cases, Cram's rule is not applicable[16] For this reason, the ratios of epimers produced in the synthesis of neuraminic acid and 3-deoxy-D-*manno*-octulosonic acid, already mentioned, could not have been predicted by the simplified considerations discussed earlier, because these reactions were conducted at pH 11, where the rate of reverse aldolization is probably significant

Thus, an essential factor for successful prediction of the stereospecificity of nucleophilic additions to carbonyl groups of reducing sugars is the choice of conditions for kinetic control of the products, this is exemplified by the syntheses here described

EXPERIMENTAL

General. — N m r. spectra were recorded with a Varian A-60A n m r spectrometer Chemical shifts in deuterium oxide were referred to the residual hydroxyl (HOD) proton arbitrarily assigned to τ 5 0 on the Varian chart scale* Hydroxylic protons of samples were pre-exchanged with deuterium by freeze-drying with 99.7% deuterium oxide at least twice. Paper chromatography (ascending) was performed with Whatman No 1 paper and alkaline silver nitrate or semicarbazide sprays for detection I r. (KBr disc) and u v. spectra (in ethanol) were obtained with a Perkin–Elmer SP-621 and a Unicam SM-800 spectrometer, respectively 3-Deoxy-DL-*glycero*-pentulosonate and 3-deoxy-DL-*glycero*-pentulosonate-*5*-d_2 were prepared as previously described[4], but with the simplified purification procedure now described for the 3,6-dideoxyhexulosonates D- and L-Lactaldehyde were prepared from L- and D-threonine by oxidation with ninhydrin[12] DL-Lactaldehyde was a gift from Dr Perry A Frey

3,6-Dideoxyhexulosonic acids. — A typical procedure is as follows Oxalacetic acid (2 4 g, 18 mmoles) was added slowly to a solution of L-lactaldehyde (9 mmoles) in 90 ml of 0.05M potassium phosphate buffer (pH 7 5), the pH being kept between 6 and 8 by addition of 40% potassium hydroxide After 15 h at room temperature, the pH was adjusted to 4, and the solution was degassed The resultant solution was

*Under the conditions employed, the HOD proton signal appears at τ = 4 7 relative to the methyl protons of sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS)

admitted to a column (3 8 × 50 cm) of Dowex-1 (formate), 200–400 mesh, and eluted with 0 23M formic acid (2 liters), followed by 0 46M formic acid Fractions (22 ml) were collected, and assayed quantitatively for α-keto acid by the semicarbazide method[13] Fractions 5–15 contained unreacted L-lactaldehyde (*ca* 5 mmoles), which was retained for further use. Fractions 112–128 and 135–154, containing isomers **1** and **2**, respectively, were pooled individually, intermediate fractions were discarded The purified solutions of isomers **1** and **2** so obtained were freed of formic acid by several evaporations under diminished pressure, care being taken that the flask temperature did not rise above 15° Each isomer was obtained as a colorless syrup, which was dissolved in a small volume (5–10 ml) of water and brought to pH 7 5 with 40% potassium hydroxide The compounds could be stored indefinitely in solution at pH 7 5 and at 5° without significant deterioration, or could be isolated as the partly crystalline potassium salts by freeze-drying The yield of isomers **1** and **2** thus obtained was 1.9 and 0 9 mmoles, respectively, as estimated by semicarbazide assay The D and DL compounds were prepared similarly from D- and DL-lactaldehyde, respectively All compounds prepared by the above method were homogenous by paper chromatography, and had the R_F values recorded in Table I Specific rotations are given in Table II The optically pure isomers exhibited rapid mutarotation towards zero The i r spectra of isomers **1** and **2**, obtained with amorphous, freeze-dried potassium salts, were grossly similar, but showed clear differences in the region of 9–10 μm Bands due to hydroxyl groups (2–9 μm), carboxylate anion (6 2 μm), and ketonic carbonyl (5 9 μm, weak), were present

Semicarbazones. — *A Isomer* **1** (DL) A solution of semicarbazide hydrochloride (133 mg, 1 14 mmoles) and sodium acetate (140 mg, 1 71 mmoles) in 1 ml of water was added to a solution of isomer **1** (0 57 mmole) in 1 ml of water, and the mixture was kept overnight After acidification of the mixture to pH 1 7 with conc hydrochloric acid, yellowish crystals (109 mg, m p 234–236° dec) were formed, these were removed by filtration after 24 h After two recrystallizations from water, the pale-yellow prisms had m p 231–233° (dec , changed in crystalline form at ~180°), λ_{max} 250 nm (ε 11,000) (Found C, 41 82; H, 5 63, N, 20 48 $C_7H_{11}N_3O_4$ (lactone) calc C, 41.88, H, 5 5, N, 20 8%) The i r spectrum was consistent with the expected structure

B Isomer **2b**. A solution of semicarbazide hydrochloride (56 mg, 0 5 mmole) and sodium acetate (62 mg, 0 75 mmole) in 1 ml of water was added to a solution of isomer **2b** (0 5 mmole) in 1 ml of water, and the mixture was kept overnight at room temperature After acidification of the solution to pH 1.5 with conc hydrochloric acid, white crystals were formed (20 mg, m p 225–230°, dec.), and these were removed by filtration After recrystallization from water, the white needles had m p 229–230°, dec ; λ_{max} 250 nm (ε 11,000) (Found C, 41 91, H, 5 52, N, 21 00 $C_7H_{11}N_3O_4$ (lactone) calc C, 41 88, H, 5 5; N, 20 8%) The i r spectrum was grossly similar to, but not identical with, that of the previous semicarbazone, its major bands were in the same regions

(2,4-Dinitrophenyl)hydrazones — *A Isomer* **1** (DL) (2,4-Dinitrophenyl)-

hydrazine (100 mg, 0 5 mmole) was dissolved in 3 ml of conc. hydrochloric acid by warming, and the solution was diluted to 20 ml with water, and added to a warm solution of isomer **1** (0 5 mmole) in 10 ml of water The mixture was kept for 5 min at 50°, and kept overnight at room temperature The resulting, yellow precipitate (105 mg, m p. 205–208°, dec) was removed by filtration, washed with a little ethanol, and dried. Two recrystallizations from methanol gave the pure derivative, m p 217–218°, dec (Found C, 44 59; H, 3 84, N, 17 11; $C_{12}H_{12}N_4O_7$ (lactone) calc C, 45 0, H, 3 7; N, 17 28%) The i r spectrum was consistent with the expected structure

B Isomer **2** (DL) The (2,4-dinitrophenyl)hydrazone was prepared in a manner similar to the preceding preparation, but attempts to recrystallize the crude material failed. The i r spectrum was, however, grossly similar to that of the previous derivative

Periodate-oxidation studies — A. Oxidative decarboxylation of isomers **1** *and* **2**, Solutions I and II, each containing 50 μmoles of isomer **1a** or isomer **2a** and 500 μmoles of hydrogen peroxide in a total volume of 1 2 ml of water, were prepared, and kept for 15 h at 5° (pH 5 5) Semicarbazide assays[13] at this stage showed the reaction to be complete, since there was no remaining absorption for α-keto acid semicarbazone at 250 nm Excess of hydrogen peroxide was decomposed by treatment with 1 mg of catalase at room temperature for 24 h (A negative peroxide test by starch–KI was obtained after less than 1 h) The solutions of the resultant diols were used without further purification in the experiments next described.

b Measurements of rate of periodate oxidation Each of the above solutions (1, 5, or 10 μl), derived from isomer **1a** or isomer **2b**, was added to 1-cm, quartz cuvettes containing 1 ml of 0 1mM sodium periodate in 10mM sodium formate buffer (pH 4 0) Under these conditions, oxidation was complete in less than 1 h, and the rate of loss of periodate-ion absorption at 223 nm was followed spectrophotometrically with a Unicam SP-800 spectrometer The measurements were repeated at pH 5 5 and pH 6 5, the results are recorded in Table III The initial rates of oxidation shown are expressed in arbitrary units based on the initial slope of the curve of absorbance at 223 nm against time Since the initial concentration of periodate ion and diol are the same in each pair of measurements, the ratio of the arbitrary, initial rates gives the ratio of the rate constants for oxidation of the pair of diols

Small samples of solutions containing, separately, the diol derived from isomer **1a** and isomer **2a**, at pH 1, were heated for 15 min at 90° to effect lactonization Aliquots of each of the resulting solutions were added to the buffered periodate solution as previously described No loss of periodate-ion absorption at 223 nm occurred, supporting the conclusion that the loss of periodate absorption previously observed was caused by diol cleavage and not by oxidation of impurities

*3-Deoxy-*D*-hexulosonic acids* — Oxalacetic acid (2 64 g, 20 mmoles) was slowly added to a solution of D-glyceraldehyde (276 mg, 8 mmoles) in 50 ml of 50 mM potassium phosphate buffer pH 7 5, while the pH was kept between 6 and 8 by addition of 40% potassium hydroxide solution. After 15 h at room temperature, the pH was adjusted to 4, and the solution was degassed. The composition of the product mixture

was determined by paper electrophoresis of aliquots removed at this stage Electrophoretograms (on Whatman No 3 paper) in 50mM borate buffer showed two, clearly separated, keto acid spots, **4** and **5**, in addition to pyruvate and unchanged D-glyceraldehyde The compounds **4** and **5** were shown to be present in the ratio of *ca* 10 1, as determined by elution of the spots with water, and assay by the semicarbazide method (The u v. spectra of the resultant semicarbazone solutions were typical for α-keto acids, showing a broad maximum at 250 nm.) The resultant solution was admitted to a column (3 8 × 75 cm) of Dowex-1 (formate), 200–400 mesh, and eluted with 230 to 460 mM formic acid (linear gradient, 2 5-liter reservoirs). Fractions (20–25 ml) were collected, and assayed for α-keto acid with semicarbazide Fractions eluted after the void volume contained unreacted D-glyceraldehyde, and fractions 110–140 contained the 3-deoxyhexulosonic acids as partly overlapping zones Fractions 110–121, containing electrophoretically pure isomer **4**, and fractions 122–140, containing isomers **4** and **5**, were pooled individually After removal of formic acid by several evaporations under diminished pressure, the solutions were brought to pH 7 with 40% potassium hydroxide solution The potassium salt of isomer **4** was obtained as a syrup by freeze-drying From a total yield of 1 5 mmole of α-keto acid obtained in this way, it was possible to isolate up to 1 mmole of pure isomer **4** Isomer **4** had $[\alpha]_{437}^{22}$ −55.8°, $[\alpha]_{589}^{22}$ −31 6° (*c*, 1 67, water at pH 6) A mixture of **4** and **5**, shown by electrophoresis to be about equimolar in both isomers, had $[\alpha]_{437}^{22}$ −24 2°, $[\alpha]_{589}^{22}$ −7 8° (*c*, 0 97, water at pH 6) The i r spectrum of isomer **4** (freeze-dried potassium salt) showed bands at 2 9 (OH) and 6 2 μm (CO_2^-), but no ketonic carbonyl (5 9 μm) The (2,4-dinitrophenyl)hydrazone of isomer **4** was prepared by a method similar to that described for the hydrazones of the 3,6-dideoxyhexulosonic acids After recrystallization from ethyl acetate–heptane, the (2,4-dinitrophenyl)-hydrazone of **4** had m p 189–191° (dec) (Found C, 42 32, H, 3 67, N, 16 25 $C_{12}H_{12}N_4O_8$ (lactone) calc C, 42 37, H, 3 56, N, 16 47%)

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OPTICAL ROTATORY DISPERSION OF SUGARS*

IV. CONFIGURATION AND CONFORMATION OF URONIC ACIDS AND ACETAMIDO SUGARS

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ABSTRACT

The optical rotatory dispersion properties of uronic acids and acetamido sugars have been investigated For both categories of sugars, two optically active transitions have been implicated as the foremost factors determining the shapes of the rotatory dispersion curves in the spectral region below 230 nm By a study of difference curves, the rotatory properties associated with the individual amide and carboxyl chromophores were isolated The curves for the uronic acids were characterized by positive Cotton-effect peaks near 220 nm and negative Cotton effects shown below 190 nm These features were ascribed to the influence of the equatorial carboxyl group on the pyranoid ring The acetamido sugars were found to exhibit curves having a Cotton-effect minimum near 220 nm and a Cotton-effect peak at 200 nm The present data have also been related to the results obtained previously for sugars lacking acetamido or carboxyl substituents, and these conform with the established views relating far-ultraviolet rotations to the configuration and conformation of the cyclic forms of sugars

INTRODUCTION

Optical rotatory dispersion (o r d) studies have recently been reported for a large number of cyclic sugars in solution[1-4], and empirical relationships between the shapes of the o r.d curves and known features of configuration and conformation of these sugars have been derived[3]. Rotational properties in the spectral region below 210 nm were shown to be particularly significant, since many of the curves were no longer plain in this spectral region, and, in some instances, abrupt changes in the direction of rotation were observed These inflections resulted from the combined

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influence of the partial rotatory contributions of the individual asymmetric centers in the spectral region approaching the optically active absorption bands, rather than from extrema of Cotton effects Circular dichroism (c d) spectra indicated[5] that optically active transitions for simple sugars are located at wavelengths well below 200 nm

Such sugar derivatives as lactones[6], *C*-nitro alcohols[7,8], sugar nitrates[9–11], and sugar–molybdate complexes[12] exhibit Cotton effects in the spectral regions of their respective absorption bands, and the o r.d. data were related to structural features of these molecules Chondroitin sulfates A, B, and C exhibit Cotton-effect peaks near[13] 220 nm and the *C1*(L) conformation of the L-idosyluronic moiety in chondroitin sulfate B was proposed on the basis of the o r.d results Disulfated disaccharides obtained from chondroitin sulfate by digestion with chondroitinase have also been studied by o r d methods[14]. O r d and c.d measurements have recently been applied to a study of the conformation of oligosaccharides (such as blood-group substances[14] and milk oligosaccharides[15]) that contain acetamido sugar residues

The present study is a report on the o r d. properties of some representative acetamido sugars and uronic acids. The results have been compared to the data obtained previously with simple sugars[1,3,4], and attempts have been made to isolate and evaluate the rotatory contribution associated with the amide and carboxylate chromophores

RESULTS AND DISCUSSION

In our previous studies, the suggestion was made that the electronic transitions associated with the ring-oxygen atom of the furanoid or pyranoid forms of sugars govern the shapes of the o r d. curves in the spectral region[3] between 210 and 185 nm. General rules were therefore proposed that related the direction of rotation in the far-ultraviolet to the spatial disposition of the ring substituents relative to the ring-oxygen atom[3] The introduction of acetamido or carboxyl substituents on the ring should induce Cotton effects that are directly related to the position of these chromophores, and, as a first approximation, may be studied apart from the remainder of the molecule In addition, the effect of an acetamido or carboxyl substituent on the optically active absorption bands of the ring-oxygen atom may be evaluated

Acetamido sugars — O r.d curves for some sugars having equatorial acetamido substituents* at C-2 are shown in Fig 1A The Cotton effect curves in the 230–190 nm spectral region exhibit small troughs or minima near 220 nm and distinct peaks near 200 nm The molar rotations of the α-D-glycosidic forms are more positive than those of the β-D forms, indicating that the rotations are highly dependent on the configuration at C-1. C d spectra for some acetamido sugars are shown in Fig. 1B *N*-Acetyl-D-mannosamine (2-acetamido-2-deoxy-D-mannose) (having an axial acetamido substituent on C-2) has a positive ellipticity band near 210 nm, as opposed to the negative c d bands for *N*-acetyl-D-glucosamine (2-acetamido-2-deoxy-D-

*To simplify the discussion, the sugars of the D-series are considered to be in the *C1* conformation.

glucose) and *N*-acetyl-D-galactosamine (2-acetamido-2-deoxy-D-galactose). The sign of the c.d bands for the reducing sugars should not be affected by the configuration at the reducing position of the molecule[5] (C-1), unless there is a direct interaction between the substituents resulting in configurational changes of the amide itself. On the other hand, the o r d properties in this spectral region are greatly influenced by the relative amounts of each anomeric form present (Fig 1A) Therefore, in order to isolate and assess the rotatory contribution of an acetamido substituent and to avoid consideration of equilibrium mixtures of the two anomeric forms, the ensuing o.r.d studies were made only on methyl glycosides of the 2-acetamido-2-deoxy sugars

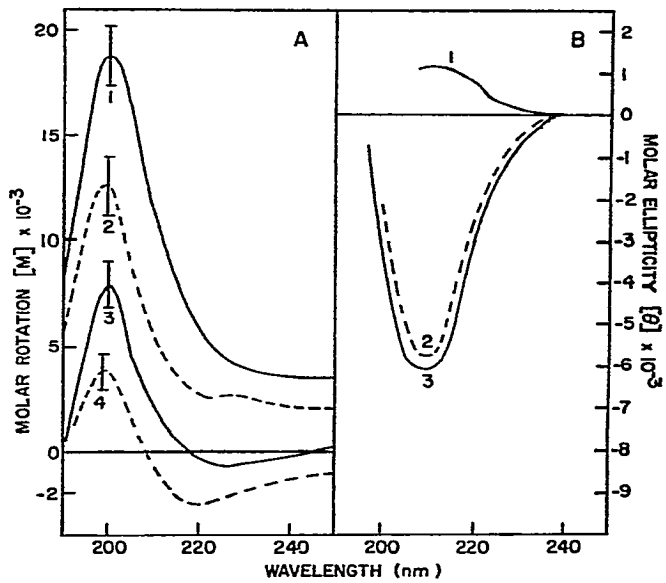


Fig 1A Optical rotatory dispersion curves for methyl glycosides of acetamido sugars Curve 1, methyl 2-acetamido-2-deoxy-α-D-galactopyranoside, curve 2, methyl 2-acetamido-2-deoxy-α-D-glucopyranoside, curve 3, methyl 2-acetamido-2-deoxy-β-D-galactopyranoside, curve 4, methyl 2-acetamido-2-deoxy-β-D-glucopyranoside

B Circular dichroism spectra for acetamido sugars Curve 1, *N*-acetyl-D-mannosamine, curve 2, *N*-acetyl-D-glucosamine, curve 3, *N*-acetyl-D-galactosamine

In the spectral region below 225 nm, the superposition of multiple Cotton effects and the background rotations of the entire sugar molecule hamper the interpretation of o r.d. data for the individual acetamido sugars O.r d difference-curves were therefore employed The partial rotatory contribution of an equatorial acetamido substituent at C-2, as compared to a hydroxyl group, may be approximated by subtracting the o.r d. curves of methyl α- and β-D-galacto- and -D-gluco-pyranosides from the corresponding curves for methyl 2-acetamido-2-deoxy-α- and β-D-galacto- and -D-gluco-pyranoside (see Fig. 2). The difference curves in Fig 2 show a small trough or minimum near 220 nm and a substantial peak at 200 nm, and they all have similar shapes. Probably, two optically active amide transitions are responsible for the shape of the difference curves; a weak n–π^* transition at the longer wavelengths with a

π–π^* transition below 200 nm A negative ellipticity band has been described previously for sugars containing equatorial acetamido groups on C-2[15], and the o r.d difference data presented here imply that a more intense, positive, optically active, absorption band is responsible for the 200-nm Cotton-effect peak

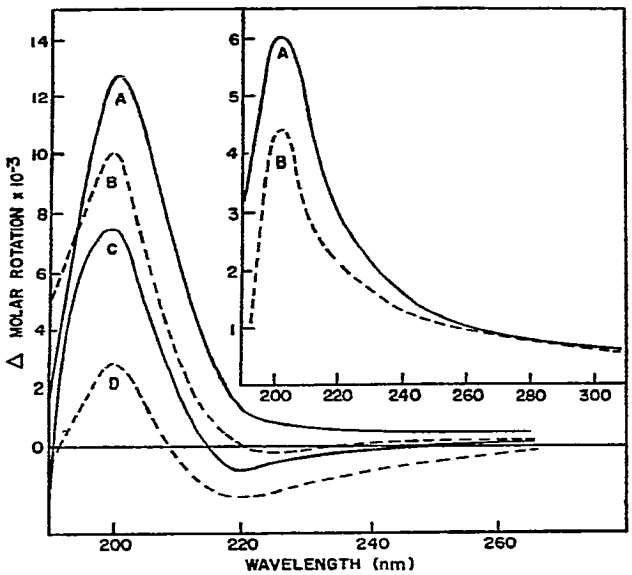


Fig 2 Optical rotatory dispersion difference-curves Each curve represents the difference in molar rotation for the two sugars indicated Curve A, methyl 2-acetamido-2-deoxy-α-D-galactopyranoside minus methyl α-D-galactopyranoside, curve B, methyl 2-acetamido-2-deoxy-β-D-galactopyranoside minus methyl β-D-galactopyranoside, curve C, methyl 2-acetamido-2-deoxy-α-D-glucopyranoside minus methyl α-D-glucopyranoside curve D methyl 2-acetamido-2-deoxy-β-D-glucopyranoside minus methyl β-D-glucopyranoside
Insert Contribution of the configuration at C-4 Curve A, methyl 2-acetamido-2-deoxy-α-D-galactopyranoside minus metnyl 2-acetamido-2-deoxy-α-D-glucopyranoside, curve B, methyl 2-acetamido-2-deoxy-β-D-galactopyranoside minus methyl 2-acetamido-2-deoxy-β-D-glucopyranoside

Although the profiles of the difference curves shown in Fig. 2 are alike, the divergence in rotational magnitudes may be ascribed to asymmetric interactions of the 2-acetamido substituent with the other components In particular, the partial rotations of the acetamido substituents at C-2 are highly dependent upon the configuration at C-1 Rotational differences between the methyl 2-acetamido-2-deoxy-α- and -β-D-glucopyranosides do not coincide with the corresponding differences between the methyl α- and β-D-glucopyranosides (Hudson's 2A values) (see Table I), at wavelengths below 220 nm This discrepancy is also observed for the D-galactose derivatives The differences between the *galacto* and *gluco* derivatives in the 2-acetamido-2-deoxy sugar series are almost identical, however, with the corresponding differences between the methyl glucopyranosides and methyl galactopyranosides (see Table I), even at the lower wavelengths.

It has previously been shown that the configuration at C-4 of the pyranoid ring has a considerable influence on the far-ultraviolet o.r d properties of sugars[3]. These

TABLE I

MOLAR ROTATIONAL DIFFERENCES FOR METHYL GLYCOSIDES OF D-GLUCOSE AND D-GALACTOSE AND THEIR 2-ACETAMIDO-2-DEOXY DERIVATIVES

Compounds[a]	*Molar rotation*					
	Wavelength (nm) 500	300	250	220	210	200
α-D-GNAc − β-D-GNAc	560	940	3550	5100	6700	9100
α-D-GalNAc − β-D-GalNAc	550	2070	3720	5260	8400	10,900
α-D-G − β-D-G	530	1770	2980	5040	5040	6200
α-D-Gal − β-D-Gal	560	1930	3300	5200	6440	8200
(α-D-GalNAc − β-D-GalNAc) − (α-D-GNAc − β-D-GNAc)	−10	130	150	160	1700	1800
(α-D-Gal − β-D-Gal) − (α-D-G − β-D-G)	30	160	320	160	1400	2000

[a]GNAc and GalNAc represent the methyl 2-acetamido-2-deoxy-D-gluco- and -galacto-pyranosides G and Gal are the methyl D-gluco- and -galacto-pyranosides

effects have been attributed[3] to the additional axis of polarizability difference for a pyranoid ring having an axial substituent on C-4 and to the restrictive influence of the configuration at C-4 on the free rotation of the hydroxymethyl group on C-5 In addition, the c d band below 200 nm is negative for D-galactose derivatives and positive for D-glucose derivatives[5]

Difference curves obtained from the values for the 2-acetamido-2-deoxy-D-galactose derivatives minus those for the 2-acetamido-2-deoxy-D-glucose derivatives (see Fig 2, insert) exhibit an inflection point and a change in direction of rotation (from positive to negative) near 205 nm, and follow the same pattern as those reported earlier[1 3 4] for their counterparts lacking the acetamido substituents For these sugars, it appears that the acetamido substituent on C-2 does not substantially affect the specific o r d properties associated with the configuration at C-4

Uronic acids — O r d curves in the u v spectral region for some representative glycosides of uronic acids are shown in Fig 3 The curves for D-glucuronic acid and D-galacturonic acid (not shown) also have peaks at 218 ($[M]_{218}$ 1550) and 223 nm ($[M]_{223}$ 2100), respectively The positive Cotton-effect extrema may be ascribed to the influence of the equatorial carboxyl group on C-5 of the pyranoid ring The positions of the peaks are not exclusively dependent on the nature and position of the optically active transition of the carboxyl group, since the partial rotatory contributions of the other asymmetric centers of the molecule also become prominent in this spectral region O r d difference curves were therefore utilized for further isolating the rotatory characteristics of the carboxyl group

Rotational values for the glycosides of D-galactose, D-glucose, and D-mannose[3] were subtracted from those of the glycosides of the corresponding uronic acids (see Fig 4), to approximate the rotatory contribution of a carboxylic acid group compared to that of a hydroxymethyl group on C-5 The general shapes of all of the difference curves are similar In addition, the difference rotations are all highly

negative below 210 nm, suggesting that an intense, negative, Cotton effect is present at wavelengths below those accessible with the present instrumentation Carboxylic acids have[18] absorption maxima at 205 nm and below 170 nm, and the energy separation between the two transitions of the carboxyl chromophore is much greater than that for the amide transitions already discussed The peaks near 220 nm are, therefore,

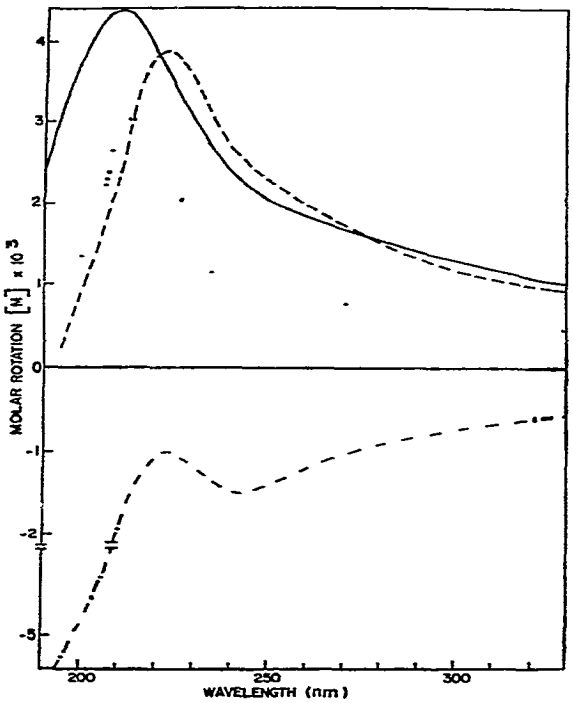


Fig 3 Optical rotatory dispersion curves for some glycosides of uronic acids ———, methyl α-D-glucopyranosiduronic acid, — — —, methyl α-D-galactopyranosiduronic acid, , methyl α-D-mannopyranosiduronic acid, — — — —, propyl β-D-glucopyranosiduronic acid

always manifest in the o r d. curves of the uronic acids, and do not overlap with other peaks, whereas the trough in this spectral region is not always clearly defined in the o r d curves for the acetamido sugars The stronger negative transition below 170 nm is responsible for the lower, or more negative, rotations above 230 nm in the difference curves (see Fig 4), and accounts for the more negative specific rotations at the sodium D line for uronic acids, as compared to those of the corresponding sugars lacking a carboxyl group

It should be noted that the three difference-curves for sugars having an equatorial hydroxyl group on C-4 (obtained by subtracting the data for the α- and β-D-glucopyranosides and α-D-mannopyranoside from those of the corresponding glycosides of the uronic acids) show a distinct trough near 240 nm (see Fig 4) that is absent from the curves for the D-galactose derivatives This effect may result from the intramolecular interaction of the carboxyl group with the equatorial hydroxyl group on

C-4 and with the ring-oxygen atom. The occurrence of such an interaction is currently being investigated by application of c.d. measurements, and will be described in a forthcoming publication.

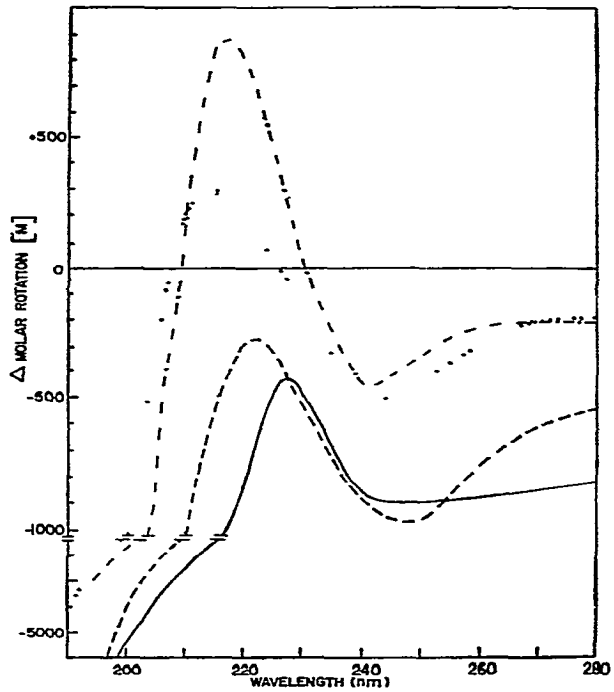


Fig 4 Optical rotatory dispersion difference-curves Each curve represents the molar rotational difference between the uronic acids indicated and the corresponding sugars having a hydroxymethyl group instead of a carboxyl group at C-5. — — —, methyl α-D-mannopyranosiduronic acid minus methyl α-D-mannopyranoside, , methyl α-D-glucopyranosiduronic acid minus methyl α-D-glucopyranoside, — — —, propyl β-D-glucopyranosiduronic acid minus methyl β-D-glucopyranoside, ——— methyl α-D-galactopyranosiduronic acid minus methyl α-D-galactopyranoside

In Fig 5, the effects of the disposition of the hydroxyl substituents on C-2 and C-4 on the rotatory properties of the uronic acids are examined The curve obtained by subtracting the rotations of methyl α-D-glucopyranosiduronic acid from those of methyl α-D-galactopyranosiduronic acid exhibits a characteristic inflection at 230 nm and a change in direction of rotation from positive to negative The curve for methyl α-D-mannopyranosiduronic acid minus methyl α-D-glucopyranosiduronic acid has a shoulder near 230 nm, and a change in direction of rotation from negative to positive below 210 nm Analogous types of inflections are characteristic of the difference curves obtained with the corresponding sugars lacking carboxylic acid moieties[3,4]

There is a "blue shift" in the position of the Cotton-effect peak for the sodium and the potassium salts of the uronic acids, but the shapes of the curves and the values of the molar rotations at the peaks correspond closely to those of the free acids Thus,

peaks near 205 nm were observed for the (methyl α-D-mannopyranosid)uronates ($[M]_{205}$ 2900) and (methyl α-D-glucopyranosid)uronates ($[M]_{205}$ 4400), and near 215 nm for (methyl α-D-galactopyranosid)uronates ($[M]_{215}$ 3760) The rotational values at the peaks for the corresponding free acids are $[M]_{215}$ 2950, $[M]_{213}$ 4400, and $[M]_{225}$ 3800, respectively (see Fig 3) The difference curve obtained by subtracting the value for the D-glucosiduronate from that for the D-galactosiduronate is similar to that

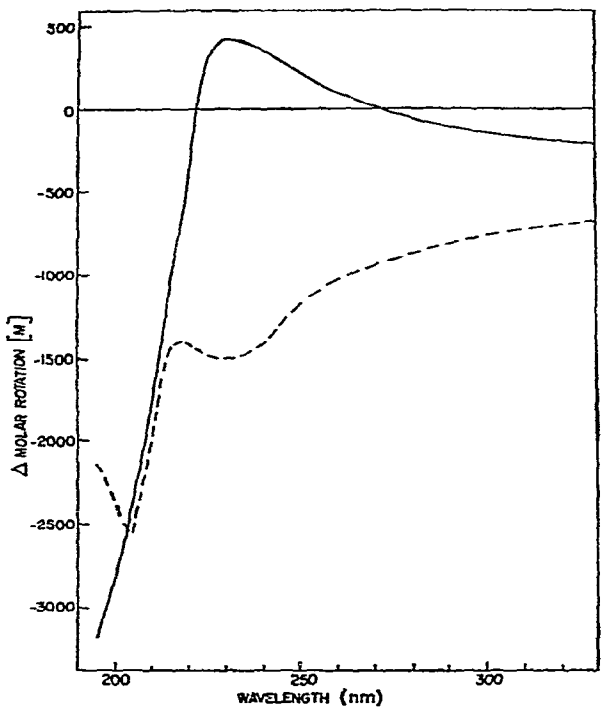


Fig 5 Optical rotatory dispersion difference-curves for the uronic acids ——— methyl α-D-galactopyranosiduronic acid minus methyl α-D-glucopyranosiduronic acid, – – – methyl α-D-mannopyranosiduronic acid minus methyl α-D-glucopyranosiduronic acid

obtained with the corresponding free acids, and shows the characteristic inflection and change in direction from positive to negative near 220 nm Also, the curve for the D-mannopyranosiduronate minus the D-glucopyranosiduronate exhibits the same change in direction of rotation from negative to positive near 200 nm as that obtained with the corresponding free acids A "blue shift" is observed in the Cotton-effect peak for the sodium salt of poly(α-D-galactosiduronic acid) (pH 9 0) as compared to the free acid (pH 2 8) The rotational magnitudes at the peaks are comparable, however, indicating that the conformation of the macromolecule is independent of the state of ionization of the carboxyl groups

EXPERIMENTAL

Materials — Most of the sugar derivatives employed for this study were

obtained from commercial sources, or were synthesized by using commercially available starting-materials Each compound was checked for optical purity by measurement of the specific rotation at 589 nm, and the values agreed with those reported in the literature We are grateful to Dr R Ledeen for samples of methyl 2-acetamido-2-deoxy-α- and -β-D-galactopyranoside, to Drs E Kabat and K. Lloyd for methyl 2-acetamido-2-deoxy-α- and -β-D-glucopyranoside, to Dr T E Timell for propyl β-D-glucopyranosiduronate, to Dr E E Dickey for the (methyl α-D-glucopyranosid)uronates, to Dr O Samuelson for the (methyl α-D-mannopyranosid)-uronates, and to Dr C A Marsh for the (methyl α-D-galactopyranosid)uronates

All solutions were prepared in distilled water, and solutions of the free uronic acids were obtained by treatment of their salts with Dowex-50(H^+) ion-exchange resin The pHs of the uronic acid solutions were near 3, and their salts were studied at pH 7–9 O r d data for the mutarotating sugars were recorded after constant values of the specific rotation had been attained

Spectropolarimetric studies — The o r d measurements were made with a Cary Model 60 spectropolarimeter. Cylindrical, quartz cells having path lengths of 0 05, 0 2, 1 0, and 10 mm were used Absorption values of 2 0 were never exceeded, and 1.0 per cent sugar solutions were employed for the visible and near-ultraviolet rotations, appropriate dilutions were prepared for the far-ultraviolet o r d measurements The slit-width of the polarimeter was programmed for half band-widths of less than 1 5 nm through the entire spectral range For increased intensity of light and for precision of the measurements in the far-ultraviolet, a Xenon arc, $XBO\text{-}450_W/4$ lamp was used C d measurements were made by use of the c d accessory for the Cary Model 60 spectropolarimeter. The experimental conditions were similar to those employed for the spectropolarimetric measurements

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THE SPECIFIC SUBSTANCE FROM *Diplococcus pneumoniae* TYPE 34 (U S. TYPE 41) : THE LOCATION OF THE *O*-ACETYL GROUPS

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ABSTRACT

Methyl 6-*O*-acetyl-β-D-galactofuranoside has been prepared It strongly inhibits the antigen–antibody precipitation in the homologous *Diplococcus pneumoniae* Type 34 immune system The parent methyl β-D-galactofuranoside does not inhibit this precipitation This result suggests very strongly that the acetyl groups in the capsular polysaccharide of *D. pneumoniae* Type 34 are located on O-6 of the internal D-galactofuranosyl residues

INTRODUCTION

The structure of the capsular polysaccharide (S-34) of *Diplococcus pneumoniae* Type 34 has recently been elucidated by Baddiley and coworkers[1,2]. The antigen contains *O*-acetyl groups, and, by treatment with a weak base, can be *O*-deacetylated[2] to give a modified polysaccharide showing a very much diminished interaction[3] with homologous rabbit antibody This diminished interaction of S-34 with serum is due to *O*-de acetylation, rather than to extensive molecular degradation** of the antigen[2]

Since half of the D-galactofuranose residues that are linked to D-galactose in S-34 are resistant to periodate oxidation[3], it appears that they carry the acetyl groups at either O-5 or O-6, probably at O-6. Each internal residue is linked by a β-D-glycosidic linkage to the next sugar residue in the polymer chain. It was therefore of interest to synthesize methyl 6-*O*-acetyl-β-D-galactofuranoside and test its inhibitory power on the S-34/rabbit anti-*D. pn* 34 system

RESULTS AND DISCUSSION

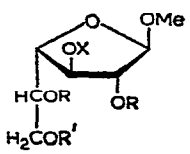
Methyl β-D-galactofuranoside (**1**) was prepared according to the procedure of Augustad and Berner[5] The per(trimethylsilyl) ether[6] of compound **1** gave a single

*Chemical Foundation Fellow, 1967–1968

**To confirm this supposition, a 0 5% solution of S-34 in aqueous ammonia was deacetylated, and the product was dialyzed against ten times its volume of physiological saline The outer and inner solutions were tested by double diffusion on agar by the technique of Ouchterlony[4] Only the non-dialyzable fraction showed immunochemical interaction with antibody to S-34

peak on g l.c. (column A) The n m r spectrum in methyl sulfoxide showed a singlet at τ 6 75 for the glycosidic methoxyl hydrogen atoms The two C-6 hydrogen atoms resonated at τ 6 53. Compound **1** was dissolved in pyridine, and acetylated at $-30°$ to $-40°$ for 2 5 h with 1 2 molar equivalents of acetic anhydride T.l c. indicated that about 50% of the material was present as a monoacetate Isolation of this fraction by silica-gel column chromatography gave in 44% yield, a syrup from which pure methyl 6-*O*-acetyl-β-D-galactofuranoside (**2**) was obtained in crystalline form in an overall yield of 17 4% The n m r spectrum of **2** has a broad singlet for H-1 at τ 5 33, a singlet for the glycosidic methyl proton at τ 6.7, and a singlet for the acetyl protons at τ 7.95 The signal for the two H-6 protons has shifted from τ 6 53 in the spectrum of **1** to τ 6–5 9 in the spectrum of **2**, owing to the presence of the deshielding acetate group at C-6.

In order to confirm the structure of the acetate **2** thus obtained, an alternative synthetic route was undertaken Methyl β-D-galactofuranoside (**1**) was tritylated in pyridine solution to give methyl 6-*O*-trityl-β-D-galactofuranoside (**3**) Unlike Siddiqui and Urbas[7], we were not able to crystallize this compound, but the n.m r. spectrum showed it to be the monotrityl derivative **3** Benzylation, followed by detritylation, afforded methyl 2,3,5-tri-*O*-benzyl-β-D-galactofuranoside (**5**) The n.m.r. spectrum of **5** confirmed the presence of three benzyl groups Acetylation of **5** gave methyl 6-*O*-acetyl-2-3,5-tri-*O*-benzyl-β-D-galactofuranoside (**6**), whose n m r. spectrum confirmed



1 R = H, R' = H
2 R = H, R' = Ac
3 R = H, R = Tr
4 R = PhCH$_2$, R' = Tr
5 R = PhCH$_2$, R' = H
6 R = PhCH$_2$, R' = Ac

the presence of three benzyl groups, one *O*-methyl group, and one acetyl group, and showed a sharp singlet at τ 4.98 for H-1 Reductive debenzylation of **6** with hydrogen over palladium black yielded methyl 6-*O*-acetyl-β-D-galactofuranoside (**2**), which was purified by chromatography, and crystallized from ether The two samples of **2** were identical in melting point, specific rotation, n.m r. spectrum, and retention time in g l c of their trimethylsilyl ethers (columns A and B) Furthermore, methylation of **3** with silver oxide and methyl iodide, followed by removal of the trityl group, yielded a methyl tri-*O*-methyl-β-D-galactofuranoside which was identical by g l c (column B) with the product obtained by the methylation of **2** by the method of Mastronardi *et al*[8], followed by *O*-deacetylation

Rabbit serum to *D pneumoniae* Type 34*, diluted with 9 volumes of physiological saline, was brought to pH 6 with dilute hydrochloric acid, and set up with increasing volumes of a 0 01% solution of S-34 in physiological saline After one week at $+5°$, the precipitates were removed by centrifuging, and the clear, supernatant liquor was examined for antibody excess by the Ouchterlony double-diffusion method[4].

*Capsular titer 256, year of production 1941 Staten Seruminstitut, Copenhagen, Denmark

In this way, the equivalence zone was found for the diluted serum and the antigen solution Tubes were then set up with and without potential inhibitors at +5°, with the equivalence ratio of antigen and serum as indicated in Table I From the results listed in this Table, it is clear that the 6-*O*-acetylglycoside **2** inhibits the precipitation of the immune system rather markedly. This effect is good evidence that the acetyl groups in S-34 are situated at O-6 of the internal D-galactofuranoside residues.

EXPERIMENTAL

General — Melting points are corrected. Gas–liquid chromatography was conducted with an F & M Model 5750 Research Chromatograph, with the following colums (A) 3% SE 52 on Gas Chrom A, 6 ft, (B) 10% NPGS on Chromosorb W, 6 ft, and (C) 5% EGS on Chromosorb P, 6 ft The n m r spectra were recorded with a Varian A-60 n m r. spectrometer

Methyl 6-O-acetyl-β-D-galactofuranoside (**2**) — Methyl β-D-galactofuranoside[5] (**1**, 3 2 g) in pyridine (40 ml) was cooled to about −40° (Dry Ice), and mixed with acetic anhydride (2 g; 1 2 molar equivalents), and the mixture was kept cold for 2 5 h After 16 h at room temperature, the mixture was evaporated to dryness Since about 50% of the mixture appeared to be monoacetate [by t l c. on silica gel with 1 19 (v/v) methanol–ether], it was added to the top of a column of silica gel and eluted with 1 19 (v/v) methanol–ether The monoacetate (1 4 g) was recrystallized three times from ether, to yield pure methyl 6-*O*-acetyl-β-D-galactofuranoside (**2**) (650 mg, 17 4%), m p 86–87°, $[\alpha]_D^{20}$ −117° (*c* 0 5, ethyl alcohol)

Anal Calc for $C_9H_{16}O_7$ C, 45 74; H, 6 82 Found C, 45 67, H, 6 95

Methyl 6-O-trityl-β-D-galactofuranoside (**3**) — Methyl β-D-galactofuranoside[5] (**1**, 2 5 g) was tritylated with 1 3 molar equivalents of trityl chloride in pyridine solution The reaction was monitored by t l c (silica gel, ethyl acetate) After purification by column chromatography on silica gel, pure methyl 6-*O*-trityl-β-D-galactofuranoside (**3**) (3 8 g) was obtained as an amorphous substance having $[\alpha]_D^{20}$ −49 4° (chloroform), lit [7]. −46 3°.

Anal. Calc for $C_{26}H_{28}O_6$ C, 71 56, H, 6 47 Found C, 71 34, H, 6 34

Methyl 6-O-acetyl-2,3,5-tri-O-benzyl-β-D-galactofuranoside (**6**) — The trityl ether **3** (3 6 g), dissolved in purified *p*-dioxane (30 ml), was mixed with powdered potassium hydroxide (18 g). Benzyl chloride (20 ml) was added dropwise during 2 h, while the mixture was stirred and heated on a steam bath, after the addition, heating and stirring were continued for 3 h The mixture was cooled (ice bath), diluted with water, and extracted with three 200-ml portions of dichloromethane The extracts were combined, washed with water, dried (magnesium sulfate), and evaporated to a syrup from which benzyl alcohol was removed by azeotropic distillation with water. Purification of the residue by column chromatography on silica gel (benzene) gave amorphous methyl 2,3,5-tri-*O*-benzyl-6-*O*-trityl-β-D-galactofuranoside (**4**) (5 2 g), $[\alpha]_D^{20}$ −46 5° (*c* 0.7, chloroform)

Detritylation of **4** was achieved by brief (30-sec) treatment with one molar

equivalent of hydrogen bromide[9], and the product was purified by column chromatography on silica gel [1 9 (v/v) ether–benzene] to yield methyl 2,3,5-tri-*O*-benzyl-β-D-galactofuranoside (**5**) (3.15 g), $[\alpha]_D^{20}$ −41° (*c* 1.8, chloroform) The detritylated material **5** (2.75 g) was acetylated with acetic anhydride (10 ml) in pyridine (50 ml), the reaction was monitored by t l c. [1 9 (v/v) ether–benzene] The mixture was evaporated with water, toluene, and ethyl alcohol The pure, amorphous product, methyl 6-*O*-acetyl-2,3,5-tri-*O*-benzyl-β-D-galactofuranoside (**6**), was thus obtained in almost 100% yield, $[\alpha]_D^{20}$ −47° (*c* 1 0, chloroform)

Anal Calc. for $C_{30}H_{34}O_7$ C, 71 13, H, 6 76 Found. C, 71 33, H, 6.55

Methyl 6-O-acetyl-β-D-galactofuranoside (**2**) — Compound **6** (2 85 g) in ethyl alcohol was hydrogenated for 20 h in the presence of palladium black (prepared from 3 g of $PdCl_2$ by hydrogenation, followed by very thorough washing with methanol and ethanol) T l c on silica gel [10 2 1 (v/v) ethyl acetate–propyl alcohol–water] then showed the absence of **6**, and a single spot for the product Purification by column chromatography on silica gel [1 19 (v/v) methanol–ether] gave a syrup that crystallized from ether to yield methyl 6-*O*-acetyl-β-D-galactofuranoside (**2**) (840 mg, 64%), m p 87–88°, $[\alpha]_D^{20}$ −117° (*c* 0 13, methanol). The n m r spectrum and g l c of **2** thus prepared were identical with those of **2** prepared by direct acetylation

Compound **2** from the direct acetylation of **1** was methylated in ether by the method of Mastronardi *et al*[8]. The product was deacetylated, and the product was identical (g l c , columns A and B) with the product from the methylation (methyl iodide–silver oxide) of **3** followed by detritylation

Inhibition of immune precipitation — The rabbit serum to *D pneumoniae* Type 34 showed a single precipitin line against S-34 by the Ouchterlony agar double-diffusion technique, and no precipitin line against C-substance with either 0 01% (from Pn II) or 0 05% solutions of C-substance from *D pneumoniae* Type IV* Under the conditions used, a clear precipitin line was obtained with those concentrations of C-substance and a pneumococcal horse-serum (Type II) containing 98 μg of antibody nitrogen of anti-C antibody per ml*, but not with a rabbit serum

TABLE I

EFFECT[a] OF COMPOUNDS **1** AND **2** ON PRECIPITATION OF THE IMMUNE SERUM

Diluted rabbit serum (μl)	*0 01% S-34 in 0 85% saline* (μl)	*Compound* **1** (%)	*Compound* **2** (%)	*Antibody N precipitated* (μg)	*Inhibition* (%)
200	40	—	—	24 5	—
200	40	0 75–1 1	—	25 8	0
200	40	—	0 8	12 1	50 5
200	—	—	—	0	—

[a]All measurements in triplicate

*Gifts from Professor M Heidelberger

(to Pn II) containing 32 μg of AN per ml of anti-C antibody The Type 34 rabbit serum was carefully brought to pH 6 0 with dilute hydrochloric acid, diluted with physiological saline (9 volumes), and clarified by prolonged centrifugation It was next established that all homologous antibodies in 200 μl of the diluted serum were precipitated by 40 μl of a 0 01% solution of the capsular polysaccharide (S-34) from *D pneumoniae* Type 34 [The preparation of S-34 used contained *ca.* 20% of C-substance It was not further purified, since the serum used showed no antibody to this antigen. 3 ml of diluted serum was absorbed with 50 μl of 0 1% C-substance (Pn IV). Repetition of the inhibition experiment as in Table I gave virtually identical results] Precipitin experiments were then set up with this amount of antigen and serum, with and without compounds **1** and **2** (see Table I). After 10 days at+5°, the tubes were centrifuged, the supernatant liquor was decanted, and the residue was washed three times with cold, physiological saline on a centrifuge The contents of each tube were dissolved in 100 Msodium hydroxide, the solutions were transferred to micro-Kjeldahl flasks, and the amount of precipitated antibody-nitrogen was measured (see Table I).

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SYNTHESIS OF THE *N*-CARBOXY-α-AMINO ACID ANHYDRIDES OF SEVERAL *O*-ACETYLATED SERINE GLYCOSIDES

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ABSTRACT

The *O*-acetylated *O*-(β-glycopyranosyl)-L-serine derivatives of D-galactose, lactose, cellobiose, 2-acetamido-2-deoxy- and 2-deoxy-2-dodecamido-D-glucose, and *O*-acetylated *O*-(α-L-rhamnopyranosyl)-L-serine were synthesized by Koenigs–Knorr condensation of the corresponding acetobromo sugars with *N*-benzyloxycarbonyl-L-serine benzyl ester, followed by hydrogenolysis of the benzyloxycarbonyl and benzyl ester groups Treatment of the *O*-acetylated serine glycosides with phosgene gave the corresponding *N*-carboxy-α-amino acid anhydrides or Leuchs' anhydrides which are useful reagents to link sugars to linear and branched synthetic polypeptide antigens The free serine glycosides were obtained by treatment of the *O*-acetylated derivatives with methanolic ammonia

INTRODUCTION

Synthetic polypeptides or poly-α-amino acids have been used extensively during the past few years as model antigens in the study of the various physical and chemical parameters that are necessary to endow a macromolecule with the capacity to elicit antibodies in experimental animals[1] Since sugars are of great importance as determinants in many natural antigens we began to investigate their influence on the immunological properties of such synthetic antigens Of particular interest was the question whether sugar molecules when attached to a non-antigenic amino acid polymer would be able to convert it into an immunogen

For this study, a method was developed to link D-glucose to a branched synthetic polypeptide *via* the β-D-glucoside of serine[2,3] *O*-(2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl)-L-serine was synthesized from 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide and *N*-benzyloxycarbonyl-L-serine benzyl ester by the Koenigs–Knorr procedure, followed by hydrogenolysis of the benzyloxycarbonyl and benzyl ester groups Treatment of the serine derivative with phosgene gave the *N*-carboxy anhydride (or Leuchs' anhydride) of *O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-L-serine. The polymerization reaction of this anhydride in aqueous medium, using as initiators the free α-amino terminals of the side chains of a branched synthetic polypeptide, led to elongation of the side chains with short peptides of *O*-acetylated

glucopyranosylserine units After removal of the *O*-acetyl groups, the resulting synthetic glycoprotein analog was used for immunological studies

It soon appeared to be of interest to extend these experiments to other monosaccharides and, if possible, to disaccharides In addition we wanted to determine if introduction of an internal adjuvant in the form of long-chain fatty acids attached to the amino group of D-glucosamine might increase the immunogenicity of sugar–polypeptide conjugates. Similar structures are present in the so-called lipid A fraction of bacterial endotoxins, and are possibly related to their adjuvant activity[4]

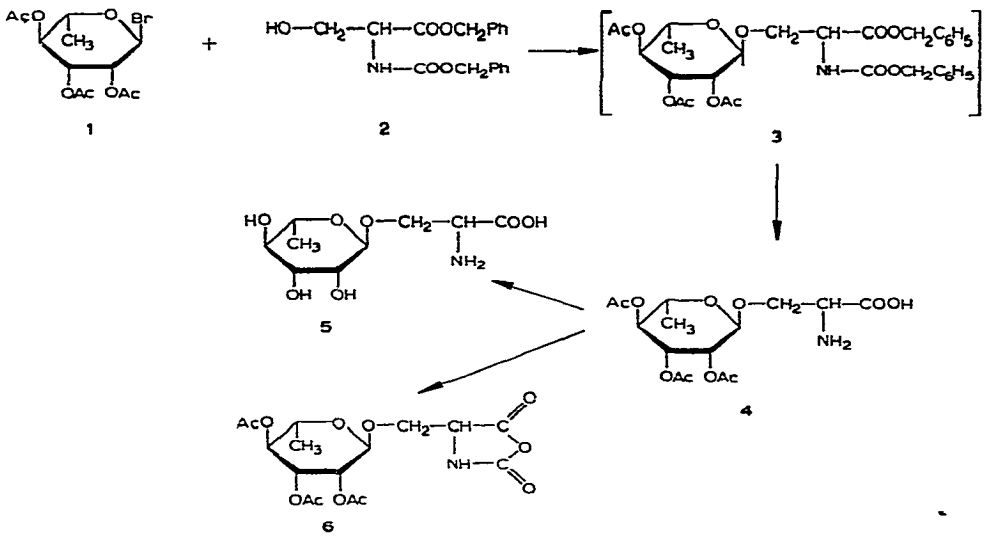
Few reports had appeared on the synthesis of serine glycosides[5,6] before interest in this type of compound was stimulated by the finding that glycosides of serine or threonine form the covalent linkage between peptide chains and carbohydrate moieties of several glycoproteins[7] In a preliminary communication Lindberg and Silvander[8] described the synthesis of β-D-xylopyranosylserine The relatively low yield of the product obtained was probably due to the difficulty of removal of the *N*-tosyl and methyl ester protecting groups of serine During the course of the present work, Brendel and Davidson[9] published the synthesis of α- and β-D-xylopyranosylserine, while Kum and Roseman[10] synthesized β-D-xylopyranosyl-, β-D-glucopyranosyl-, and β-D-galactopyranosyl-serine by essentially the same procedure as outlined above for the synthesis of the glucose derivative *O*-(2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-*N*-(2,4-dinitrophenyl)-L-serine methyl ester was prepared by Vercellotti and Luetzow[11] as a model compound to study the alkali lability of serine glycosides More recently Derevitskaya *et al*[12] also described the synthesis of β-D-glucopyranosyl-and β-D-galactopyranosyl-serine by the Koenigs–Knorr procedure using *N*-benzyloxycarbonyl-L-serine methyl ester as starting material The same authors prepared β-D-galactofuranosylserine by a new method using *O*-acetylated 1,2-*O*-alkyl orthoesters of sugars as glycosylating agents

The present report deals with the synthesis of several *O*-acetylated and unesterified glycosides of serine by a procedure analogous to the one used previously for the preparation of the corresponding glucose derivative[3] The *O*-acetylated serine glycosides were treated with phosgene in order to prepare the corresponding *N*-carboxy anhydrides, which are useful reagents for the binding of sugars to synthetic polypeptides

RESULTS AND DISCUSSION

Serine glycosides of mono- and di-saccharides — The serine glycosides of L-rhamnose, D-galactose, lactose, and cellobiose were synthesized as exemplified for the rhamnose derivative in Scheme 1. The respective acetylglycosyl bromide derivative **1** was condensed with *N*-benzyloxycarbonyl-L-serine benzyl ester (**2**) in the presence of mercuric cyanide in nitromethane as solvent[13] Only the *O*-(hepta-*O*-acetyl-β-cellobiopyranosyl)-*N*-benzyloxycarbonyl-L-serine benzyl ester (analog of **3**) crystallized directly from the crude reaction product and was obtained in pure form Generally the reaction mixtures were complex, as shown by thin-layer chromatography, and the isolation of the fully substituted glycosides (**3** and analogs) would have been possible

only by chromatography on silica gel This operation, which is very inconvenient for large-scale preparations, was avoided by the following procedure: The crude reaction product was hydrogenated to remove the benzyloxycarbonyl and benzyl ester protecting groups The *O*-acetylated serine glycosides (**4** and analogs) were precipitated with ethyl acetate or ethyl acetate–ether, and most of the side products resulting from the decomposition or hydrogenation of unreacted acetylglycosyl bromides remained



Scheme 1

in the supernatant. The main impurities still present were serine and small amounts of unidentified ninhydrin-positive substances which on paper chromatograms migrated between serine and the *O*-acetylated serine glycosides (**4** and analogs) In the case of lactose, all these impurities could be removed by crystallization from water, but the L-rhamnose and D-galactose derivatives **4** were too soluble for application of the same procedure The latter derivatives were conveniently purified in relatively large amounts by partition chromatography on a column of moist Celite as stationary phase and water-saturated butyl alcohol as mobile phase[14]. The *O*-acetylated serine glycosides (**4** and analogs) were eluted first. They crystallized readily and were chromatographically pure A very low and broad second peak, which also contained a serine glycoside, appeared sometimes It was clearly separated from the first peak. This compound, possibly the anomer glycoside, had a slightly lower paper-chromatographic mobility than that of the main product. It could not be isolated in crystalline form or in sufficient amount for further characterization.

The serine glycosides (**5** and analogs) were prepared from the *O*-acetylated derivatives (**4** and analogs, **12** and **13**) by treatment with methanolic ammonia at 4°.

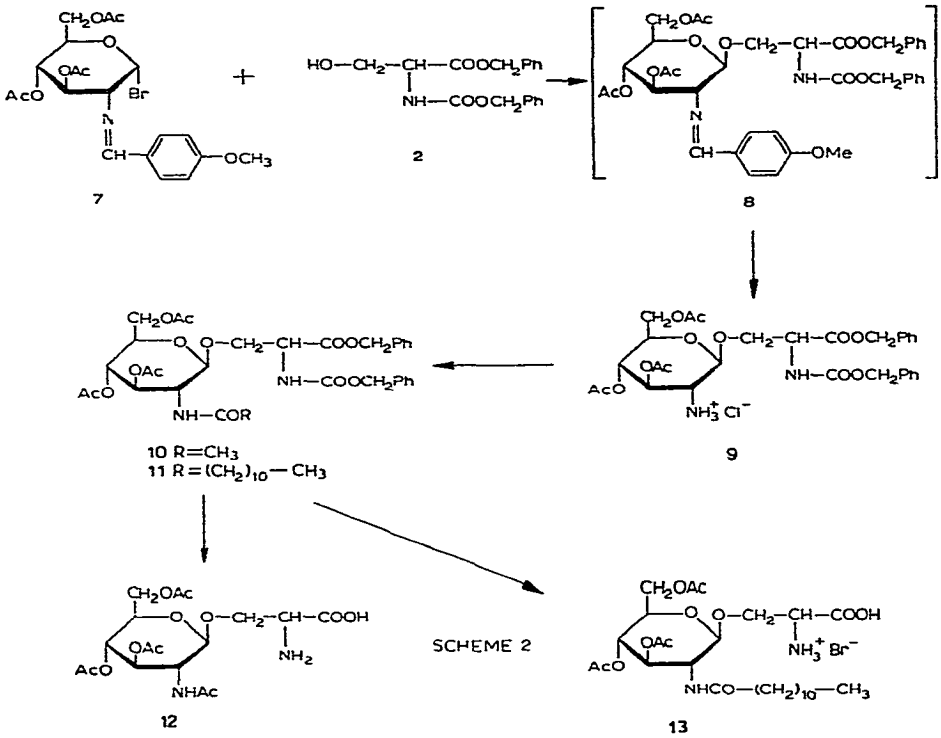
Several reports in the literature and our own experience in the synthesis of serine glucoside indicated that both mercuric cyanide and silver carbonate, when used as acid acceptors in the Koenigs–Knorr reaction, favor the formation of the same

anomeric glycoside[13,15]. This is the β-D-glycoside for most of the common sugars such as D-glucose, D-galactose, D-xylose, and lactose There are some cases, however, in which α-D-(1→2)- and α-D-(1→3)-linked disaccharides have been isolated from reactions with the 2,3,4,6-tetra-*O*-acetyl-α-D-glycosyl bromide derivatives of D-galactose, D-xylose, and L-fucose in the presence of mercuric cyanide[16–18]. On the other hand, 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl bromide and 2,3,4-tri-*O*-acetyl-α-L-rhamnopyranosyl bromide usually give α-D- and α-L-glycosides, respectively, under conditions similar to those of our condensation procedure[19] In these cases the configuration at the anomeric center is retained, probably owing to participation of the C-2 acetoxy group of D-mannose and L-rhamnose in the glycosidation reaction As shown in Table I, a comparison of the optical rotation of the synthetic serine glycosides with that of the corresponding α-D- and β-D-methyl glycosides indicates that the β-glycosides of D-galactose, lactose, cellobiose, and the α-glycoside of L-rhamnose have been obtained as the major products

Serine glycosides of 2-acylamido-2-deoxy-D-glucose — For the reasons just mentioned, we planned to link, in addition to L-rhamnose, D-galactose, lactose, and cellobiose, various 2-acylamido-2-deoxy-D-glucoses (acyl = acetyl, lauroyl, and eventually higher fatty acids) to synthetic polypeptides It was obvious to use also for this purpose the "serine glycoside" method

Several glycosyl halide derivatives of 2-amino-2-deoxy-D-glucose are known as glycosylating agents[20] They differ mainly in the manner in which the amino group is protected For our purpose, an amino-blocking group, which could be easily removed after glycoside synthesis and replaced by an acetyl or lauroyl group appeared to be advantageous We selected 3,4,6-tri-*O*-acetyl-2-deoxy-2-(*p*-methoxybenzylidene)amino-α-D-glucopyranosyl bromide (**7**) because the *p*-methoxybenzylidene group could be removed under mild acidic conditions[21], whereas blocking groups such as dinitrophenyl[22] or dichloroacetyl[20] require alkaline hydrolysis which might cause β-elimination of the serine glycoside Condensation of 3,4,6-tri-*O*-acetyl-2-deoxy-2-(*p*-methoxybenzylidene)imino-α-D-glucopyranosyl bromide (**7**) with *N*-benzyloxycarbonyl-L-serine benzyl ester (**2**) was performed in the presence of silver carbonate (Scheme 2) In contrast to the experiments described in the preceding part, mercuric cyanide gave completely negative results A crystalline side product was separated from the reaction mixture in considerable quantity. The nature of this compound, probably a nonreducing disaccharide, is currently under investigation Apparently the same side product has been isolated by Hardy *et al*[23], but it was not studied in detail The crude reaction product containing the fully protected serine 2-amino-2-deoxy-D-glucopyranoside (**8**) was then treated with dilute acid and *O*-(3,4,6-tri-*O*-acetyl-2-amino-2-deoxy-β-D-glucopyranosyl)-*N*-benzyloxycarbonyl-L-serine benzyl ester hydrochloride (**9**) was easily obtained in pure form The low yield of 15% was probably due in part to the use of impure starting material (**7**) (because of its instability it was not recrystallized before use), and to the formation of the side product just mentioned After acylation of the free amino group of **9** with acetic anhydride or dodecanoyl (lauroyl chloride), the benzyloxycarbonyl and benzyl ester groups of **10**

and **11** were removed by hydrogenolysis to give the *O*-(3,4,6-tri-*O*-acetyl-2-acylamido-2-deoxy-β-D-glucopyranosyl)-L-serines (**12** and **13**).



SCHEME 2

Scheme 2

N-Carboxy-α-amino acid anhydrides are usually prepared by treatment of amino acids with phosgene in inert solvents (Fuchs–Farthing method)[24] Using this procedure it was possible to synthesize the *N*-carboxy anhydrides of the *O*-acetylated serine glycosides (**6**) shown in Table IV All the anhydrides, except the lactose derivative, are crystalline solids which in the case of 2-acetamido-2-deoxy-D-glucose and L-rhamnose contain 1 molecule of dioxane Their equivalent weights were determined by titration with sodium methoxide in inert solvents[25] For the synthesis of long-chain, linear poly-α-amino acids by polymerization in organic solvents, *N*-carboxy-α-amino acid anhydrides of very high purity are required[24] If only short peptide chains are to be attached to the amino groups of a macromolecule by polymerization in aqueous solution, such highly purified anhydrides are not essential Nevertheless, the anhydrides of the serine glycosides were carefully purified by repeated crystallization or precipitation, and several of these anhydrides were used to attach serine glycosides to branched synthetic polypeptides

EXPERIMENTAL

General — Melting points were determined with a Tottoli melting-point

apparatus (Buchi, Flawil, Switzerland) and are uncorrected Optical rotations were measured with a Perkin–Elmer Model 141 polarimeter Descending paper chromatography was performed on Whatman No 1 paper with the following solvents (v/v) butyl alcohol–pyridine–water (6:4:3) (solvent A) and (6 5 5) (solvent B) The serine glycosides were detected by spraying with a 1% solution of ninhydrin in acetone and heating to 90° Thin-layer chromatography was performed on glass plates coated with Kieselgel G (E. Merck, Darmstadt, Germany). Depending on the type of com-

TABLE I

OPTICAL ROTATIONS OF SERINE GLYCOPYRANOSIDES AND OF THE CORRESPONDING METHYL α- AND β-D-GLYCOPYRANOSIDES

Compound	$[\alpha]_D$	*Solvent*	*Temp* (°C)	$[M]_D$
O-(α-L-Rhamnosyl)-L-serine[a]	− 41 3	water	22	−10377
Methyl α-L-rhamnoside[26]	− 62 5	water	20	−11137
Methyl β-L-rhamnoside[26]	+ 95 4	water	20	+17000
O-(β-D-Galactosyl)-L-serine[a]	− 1 6	water	22	−428
O-(β-D-Galactosyl)-L-serine[10]	− 2 1	water	24	−561
Methyl β-D-galactoside[26]	− 0 4	water	20	−78
Methyl α-D-galactoside[26]	+196 6	water	20	+38177
O-(β-Lactosyl)-L-serine[a]	− 0 6	water	22	−258
Methyl β-lactoside[27]	+ 5 6	water	26	+1995
Phenyl α-lactoside[28]	+ 88 7	water		+37113
O-(β-Cellobiosyl)-L-serine[a]	− 17 4	water	22	−7472
Methyl β-cellobioside[28]	− 19 1	water	20	−6806
Methyl α-cellobioside[28]	+ 96 8	water	20	+34493
O-(2-Acetamido-2-deoxy-β-D-glucosyl)-L-serine[a]	− 32 1	water	22	− 9896
O-(2-Acetamido-2-deoxy-β-D-glucosyl)-L-serine[6]	− 31 0	water	24	− 9557
O-(2-Deoxy-2-dodecanamido-β-D-glucosyl)-L-serine[a]	− 29 2	MeOH	22	−13098
Methyl 2-acetamido-2-deoxy-β-D-glucoside[29]	− 43 0	water		−10116
Methyl 2-acetamido-2-deoxy-α-D-glucoside[29]	+105 0	water		+24701

[a]This publication

pound the following solvents were used. chloroform–ethyl acetate (70 30, v/v), benzene–ethyl acetate (50 50, v/v), and ethyl acetate–methanol (20 80 v/v) The spots were detected by spraying with a 10% (v/v) solution of sulfuric acid in ethanol and subsequent heating, or with ninhydrin All compounds, except the Leuchs anhydrides, were routinely tested by thin-layer or paper chromatography, and after purification gave single spots Evaporations were performed with a flash evaporator at 40° bath-temperature and 12 mm Hg Solvents were purified and dried according to known procedures[30] Celite 535 was washed with conc. hydrochloric acid, then with distilled water until neutral, and dried The *N*-benzyloxycarbonyl-L-serine benzyl ester was prepared according to Baer and Maurukas from *N*-benzyloxycarbonyl-L-serine[31] Deacetylation of the *O*-acetylated serine glycosides (4 and analogs) was performed in all cases by treatment with methanolic ammonia as described below in detail for the rhamnose derivative The solvents used for recrystallization of the free serine

TABLE II

PROPERTIES AND ANALYSES OF SERINE GLYCOSIDES

O-*Glycopyranosyl*-L-*serine*	*Solvent of crystallization*	*Yield*[a] %	*M p* (°C) (*dec*)	$[\alpha]_D^{22}$ (*deg*)	*Solvent* (c, *l*)	R_{Ser}[b]	*Formula*	*Calc*			*Found*		
								C	*H*	*N*	*C*	*H*	*N*
α-L-Rhamnosyl-	H_2O-MeOH	61	173–175	−41.3	water	0 81	$C_9H_{17}NO_7\ H_2O$	40 15	7 11	5 20	40 32	7 24	5 11
β-D Galactosyl-	H_2O-MeOH	84	140–142	− 1.6	water	0 56	$C_9H_{17}NO_8$	40 45	6 41	5 24	40 59	6 62	5 37
β-Lactosyl-	H_2O-MeOH	70	200–206	− 0 6	water	0 34	$C_{15}H_{27}NO_{13}$ H_2O	40 27	6 53	3 13	40 40	6.68	3.10
β-Cellobiosyl-	H_2O-MeOH	73	211–228	−17.4	water	0 42	$C_{15}H_{27}NO_{13}$	41 96	6 34	3 26	42 00	6 28	3 17
2-Acetamido-2-deoxy-β-D-glucosyl-	H_2O-MeOH	77	200–216	−32 1	water	0 88	$C_{11}H_{20}N_2O_8$	42 85	6.54	9 09	42 79	6 60	8 96
2-Deoxy-2-dodecamido-β-D glucosyl-	MeOH-H_2O	85	174–178	−29 2	MeOH	4 3	$C_{21}H_{40}N_2O_8$	56 23	8 99	6 25	56 10	9 12	6 21

[a]The yields given in this Table refer to the deacetylation reaction

[b]Mobility on paper chromatograms relative to serine in solvent B

glycosides (**5** and analogs) and the yields are given in Table II The *N*-carboxy-α-amino acid anhydrides of the various serine glycosides (**6** and analogs) were prepared by treatment of the *O*-acetylated serine glycosides (**5** and analogs) with phosgene, essentially according to the procedure given for the rhamnose derivative The properties of these compounds are listed in Table IV Their equivalent weights were determined as described by Berger *et al*[25]. The anhydride (4-10 mg) was dissolved in anhydrous dioxane (about 0 7 ml) and titrated in the presence of thymol blue as indicator with 0 1M sodium methoxide delivered from an Agla micrometer syringe (Burroughs Wellcome and Co , London)

O-(*2,3,4-Tri*-O-*acetyl*-α-L-*rhamnopyranosyl*)-L-*serine* (**4**) — 2,3,4-Tri-*O*-acetyl-α-L-rhamnopyranosyl bromide[32] (**1**) (3 53 g, 10 mmoles) and *N*-benzyloxycarbonyl-L-serine benzyl ester (**2**) (3 3 g, 10 mmoles) were dissolved in anhydrous nitromethane and powdered mercuric cyanide (2 8 g, 11 mmoles) was added After being stirred for 48 h at 40° with exclusion of moisture, the mixture was diluted with benzene, and the mercuric salts were filtered off The filtrate was extracted 3 times with M aqueous potassium bromide, 3 times with a saturated sodium bicarbonate solution, and finally with water The organic phase was dried with anhydrous sodium sulfate and evaporated to a colorless syrup which was dissolved in ethanol Water was added dropwise until a point was reached just before a slight uniform turbidity appeared The solution was hydrogenated at room temperature and atmospheric pressure in the presence of 5% palladium-on-charcoal The uptake of hydrogen was essentially complete after 3 h. After filtering off the catalyst, the solution was evaporated, and the remaining syrup was repeatedly dissolved in ethyl acetate and evaporated again to remove water Finally the syrup was dissolved in ethyl acetate, and ether was slowly added until precipitation was complete After centrifugation the supernatant was discarded, and the sediment was dissolved in a minimal amount of methanol. Part of the product crystallized Crystallization was completed by addition of ethyl acetate and ether The yield of crude acetylated *O*-(α-L-rhamnopyranosyl)-L-serine (**4**) was 2 5 g (66%)

The main impurity still present, L-serine, was removed by partition chromatography[14]. Dry Celite (165 g) was thoroughly mixed with water saturated with butyl alcohol (150 ml of lower phase) and suspended in butyl alcohol saturated with water (upper phase) This mixture was poured onto a column (3.5 × 71 cm) The solution of the crude glycoside (**4**, 2 5 g) in butyl alcohol (8 ml) was applied to the column, and elution was performed at a rate of about 60 ml/h with butyl alcolol saturated with water. Fractions of 15 ml were collected Samples of each fraction were deposited on paper and stained with ninhydrin. Most of compound **4** was eluted in fractions 50–84, however, an additional small amount was eluted in fractions 85–100 Fractions 50–84 were pooled and evaporated. The chromatographed material was completely free from serine, and contained only trace amounts of a substance of slightly lower chromatographic mobility. This substance was removed by two recrystallizations from methanol–ether (yield 1 9 g)

Fractions 85–100 together with the combined mother-liquors were rechromato-

graphed on a column prepared from 60 g of Celite, and 5-ml fractions were collected Fractions 55–88 again contained 0 34 g of pure **4**, and fractions 128–190 contained a small amount of another rhamnosylserine which could not be crystallized The paper chromatographic mobility of this compound was slightly lower than that of the main product **4**. The total yield of pure **4** was 2 2 g (58%) (Table III)

O-(α-L-*Rhamnopyranosyl*)-L-*serine* (**5**) — *O*-(2,3,4-Tri-*O*-acetyl-α-L-rhamnopyranosyl)-L-serine (**4**, 0 46 g), dried *in vacuo*, was dissolved in anhydrous methanol (30 ml) The solution was cooled with ice, and then saturated with dry ammonia with exclusion of moisture The mixture was kept for 3 days at 4° and was then evaporated. The residue, after removal of acetamide by sublimation for 2 h at 60° and 0 2 mm Hg, was recrystallized twice from water–methanol The yield of *O*-(α-L-rhamnopyranosyl)-L-serine (**5**) was 0 20 g (61%) (Tables I and II)

O-(*2,3,4-Tri*-O-*acetyl*-α-L-*rhamnopyranosyl*)-N-*carboxy*-L-*serine anhydride* (**6**) — *O*-(2,3,4-Tri-*O*-acetyl-α-L-rhamnopyranosyl)-L-serine (**4**) (0 51 g), dried *in vacuo* over phosphorus pentaoxide, was suspended in anhydrous dioxane (40 ml) Dry phosgene was passed through the suspension for 1 h at 50° Within a few minutes the glycoside dissolved completely. The solution was evaporated to remove the excess phosgene The residue could not be crystallized from ethyl acetate–petroleum ether, but crystallized from dioxane–petroleum ether For recrystallization a small amount of dry charcoal was added, this effectively adsorbed trace amounts of chlorine-containing impurities As far as possible all operations were performed with exclusion of moisture and with anhydrous solvents The 3 times recrystallized anhydride **6** (0 56 g, 85%) began to melt at 65° and decomposed at 76° It contained one molecule of dioxane which was slowly released at 0 2 mm Hg by gradually raising the temperature from 60 to 80° (loss of weight 16 8%, calc for 1 molecule of dioxane 17 9%) The dioxane-free anhydride slowly decomposed from 80° to 115° (Table IV)

O-(*2,3,4,6-Tetra*-O-*acetyl*-β-D-*galactopyranosyl*)-L-*serine* — This compound was prepared essentially according to Kum and Roseman[10], but isolation and purification were performed as described for the rhamnose derivative The chromatographically pure β-D-galactoside was obtained in 25% yield

O-(*Hepta*-O-*acetyl*-β-*lactopyranosyl*)-L-*serine* — The condensation reaction of hepta-*O*-acetyl-α-lactopyranosyl bromide[33] (15 4 g, 22 mmoles) with *N*-benzyloxycarbonyl-L-serine benzyl ester (7 3 g, 22 mmoles), followed by hydrogenation was performed as described The crude reaction product was dissolved in a minimal amount of hot water The lactoside crystallized upon cooling with ice It was washed with ice water, redissolved in a small amount of ethanol, precipitated with ethyl acetate and ether, and again recrystallized from water The yield of chromatographically pure product, which contained 3 molecules of water, was 6.7 g (39%) (Table III)

O-(*Hepta*-O-*acetyl*-β-*cellobiopyranosyl*)-N-*benzyloxycarbonyl*-L-*serine benzyl ester* — Hepta-*O*-acetyl-α-cellobiopyranosyl bromide[33] (7 0 g, 10 mmoles) was treated with *N*-benzyloxycarbonyl-L-serine benzyl ester (3 3 g, 10 mmoles) in the presence of mercuric cyanide (2 8 g, 11 mmoles) as described for the rhamnose derivative The crude reaction product crystallized from methanol After several recrystal-

TABLE III

PROPERTIES AND ANALYSES OF *O* ACETYLATED SERINE GLYCOSIDES

O-(*Per*-O-*acetyl-glyco-pyranosyl*)-L-*serine*	*M p* (°C)	$[\alpha]_D^{23}$ (*deg*)	*Solvent* (*c*, *l*)	R^a_{Ser}	R^a_F	*Formula*	*Calc*				*Found*			
							C	*H*	*N*	*Br*	*C*	*H*	*N*	*Br*
2,3,4-Tri-*O*-acetyl-α-L-rhamnosyl	169–171 (dec)	−65 2	water	4.3	0 56	$C_{15}H_{23}NO_{10}$	47 75	6 14	3 71		47 46	6 25	3 84	
2,3,4,6-Tetra-*O*-acetyl-β-D-galactosyl-	141–145 (dec)	−10 0	water	4 4	0 57	$C_{17}H_{25}NO_{12}$	46 89	5 79	3 22		46 98	5.87	3.26	
Hepta-*O*-acetyl-β-lactosyl-	168–170	−16.2	water	4 8	0 62	$C_{29}H_{41}NO_{20}$ $3H_2O$	44 79	6 09	1 80		44.93	6.24	1 90	
Hepta-*O*-acetyl-β-cellobiosyl-	165–177 (dec.)	−13 5	acetic acid	4 5	0 58	$C_{29}H_{41}NO_{20}$	48 13	5 71	1 94		48.07	5 66	1 85	
2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucosyl- (12)	160–162 (dec.)	−47 3	methanol	3 5	0 48	$C_{17}H_{26}N_2O_{11}$	47.00	6.03	6 45		46 99	6 01	6.48	
3,4,6 Tri-*O*-acetyl-2-deoxy-2-dodecamido β-D glucosyl-hydrobromide (13)	147–151 (dec)	−11 3	methanol	6 0	0 83	$C_{27}H_{47}N_2O_{11}Br$	49 47	7 23	4.27	12 19	49 00	7.41	4 11	12.08

[a]Mobility on paper chromatograms relative to serine (R_{Ser}) and to the solvent (R_F) in solvent A

TABLE IV

PROPERTIES AND ANALYSES OF THE *N*-CARBOXY ANHYDRIDES OF *O*-ACETYLATED SERINE GLYCOSIDES

O-(*Per*-O-*acetyl-glycopyranosyl*)-N-*carboxy*-L-*serine anhydride*	*Solvent of crystallization*	*Yield* %	*M p* (°C) (*dec*)	*Equiv wt*		*Formula*	*Calc*			*Found*		
				Calc	*Found*		*C*	*H*	*N*	*C*	*H*	*N*
2,3,4-Tri-*O* acetyl α-L-rhamnosyl-	dioxane–petroleum ether	85	65– 76	491 5	494	$C_{16}H_{21}NO_{11}$ $C_4H_8O_2$[a]	48 88	5 95	2 85	48 98	5 93	2 96
2,3,4,6-Tetra-*O*-acetyl-β-D-galactosyl-	ethyl acetate–petroleum ether	52	90– 98	461 4	469	$C_{18}H_{23}NO_{13}$	46 86	5 02	3 04	46 67	5 21	2 99
Hepta-*O*-acetyl-β-lactosyl-	precipit from dioxane-petroleum ether, lyophilized from dioxane	90		749 7	753	$C_{30}H_{39}NO_{21}$	48 07	5 24	1 87	47 62	5 38	1 78
Hepta-*O*-acetyl-β-cellobiosyl-	ethyl acetate–petroleum ether	81	199–204	749 7	750	$C_{30}H_{39}NO_{21}$	48 07	5 24	1 87	47 78	5 37	1 75
2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucosyl-	dioxane–petroleum ether	63	192–193	548 5	558	$C_{18}H_{24}N_2O_{12}$ $C_4H_8O_2$[b]	48 17	5 88	5 11	47.83	5 89	5 18
				460 4		$C_{18}H_{24}N_2O_{12}$	46 96	5 25	6 09	46 73	5.45	5.89
3,4,6-Tri-*O*-acetyl-2-deoxy-2-dodecamido-β D-glucosyl-	ethyl acetate–petroleum ether	66	169–173	600 7	590	$C_{28}H_{44}N_2O_{12}$	55 99	7 38	4 66	56 03	7 65	4 59

[a]The crystalline compound contains 1 molecule of dioxane which is slowly released by gradually raising the temperature from 60 to 80° at 0 2 mm Hg Loss of weight 16 8%, calc 17 8%

[b]The crystalline compound contains 1 molecule of dioxane which is released at 70° and 0 2 mm Hg Loss of weight 16 19%, calc. 16 06%

lizations from methanol, the yield was 4 5 g (47.5%), m p 145–148°, $[\alpha]_D^{23}$ −16 4° (*c* 1, ethyl acetate)

Anal Calc for $C_{44}H_{53}NO_{22}$· C, 55 75; H, 5.64, N, 1 48 Found C, 55.66, H, 5.61, N, 1.64.

O-(*Hepta-O-acetyl-β-cellobiopyranosyl*)-L-*serine* — *O*-(Hepta-*O*-acetyl-β-cellobiopyranosyl)-*N*-benzyloxycarbonyl-L-serine benzyl ester (9 48 g, 10 mmoles) was dissolved in methanol–ethanol (1:1, 350 ml) and a small amount of water was added. The solution was hydrogenated in the presence of (5%) palladium-on-charcoal (0 5 g) at atmospheric pressure, and the reaction product was crystallized from water–ethanol to give 6 67 g (92%) (Table III)

O-(*3,4,6-Tri-O-acetyl-2-amino-2-deoxy-β-D-glucopyranosyl*)-N-*benzyloxycarbonyl-L-serine benzyl ester hydrochloride* (**9**) — 3,4,6-Tri-*O*-acetyl-2-deoxy-2-(*p*-methoxybenzylidene)-amino-α-D-glucopyranosyl bromide (24 3 g, 50 mmoles), prepared according to Zervas and Konstas[21] and used without recrystallization, and *N*-benzyloxycarbonyl-L-serine benzyl ester (**2**, 16.5 g, 50 mmoles) were dissolved in anhydrous 1,2-dichloromethane (100 ml) Silver carbonate (13 8 g, 50 mmoles) and anhydrous calcium sulfate (40 g) were added, and the mixture was shaken for 2 days in the dark Calcium sulfate and silver salts were filtered off, washed with 1,2-dichloromethane, and the filtrate was evaporated Upon redissolving the oily residue in acetone (90 ml) and cooling, a crystalline compound (2 4 g, m p 243–245°) was obtained, probably a nonreducing disaccharide

Conc hydrochloric acid (3 ml) was added to the mother liquor, and the solution was boiled for 1 min and evaporated *in vacuo* The residue was dissolved in ether (100 ml) and water (35 ml) Upon shaking or stirring for a few minutes, compound 9 crystallized, mainly at the interphase of ether and water It was filtered off and recrystallized from methanol–water and from methanol–ether to give 4 9 g (15%) of pure compound **9**, m p 213–218° (dec), $[\alpha]_D^{25}$ −4° (*c* 1 234, methanol)

Anal. Calc. for $C_{30}H_{37}ClN_2O_{12}$ C, 55 17, H. 5 71, Cl, 5 43; N, 4 29 Found C, 55 04, H, 5 62, Cl, 5 56, N, 4 33

O-(*2-Acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl*)-N-*benzyloxycarbonyl-L-serine benzyl ester* (**10**) —Compound **9** (3 27 g, 5 mmoles) was dissolved in anhydrous pyridine (25 ml), and acetic anhydride (2 ml) was added with shaking The solution was kept in a stoppered flask for 1 day at room temperature, and was then poured into ice water A precipitate which formed was filtered off, washed with ice water, dried and recrystallized several times from ethyl acetate–petroleum ether and from methanol–water, yield 3 12 g (94 5%), m p 160–161°, $[\alpha]_D^{23}$ −13 9° (*c* 1, acetic acid).

Anal Calc for $C_{32}H_{38}N_2O_{13}$ C, 58 35, H, 5 81, N, 4 25 Found C, 58.46, H, 5 90, N, 4.18

O-(*2-Acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl*)-L-*serine* (**12**) — Compound **10** (3 3 g, 5 mmoles) dissolved in ethanol (250 ml) was hydrogenated in the presence of palladium-on-charcoal in the usual way Crystallization from ethanol gave 2 0 g (92%) (Table III)

O-(3,4,6-Tri-O-acetyl-2-deoxy-2-dodecamido-β-D-glucopyranosyl)-N-benzyloxycarbonyl-L-serine benzyl ester (**11**) — Compound **9** (6 53 g, 10 mmoles) was partially dissolved in anhydrous pyridine (15 ml) and dodecanoyl chloride (lauroyl chloride) (2 18 g, 10 mmoles) was added with shaking After being kept for 1 day at room temperature, the solution was poured into ice water The precipitate which formed was washed with ice water, dried, and recrystallized 3 times from ethyl acetate–petroleum ether to give 6 55 g (82%), m p. 146–148°, $[\alpha]_D^{23}$ −5.8° (*c* 1, ethyl acetate)

Anal Calc for $C_{42}H_{58}N_2O_{13}$ C, 63 14; H, 7 32, N, 3 51 Found: C, 63 04, H, 7 40, N, 3 53

O-(3,4,6-Tri-O-acetyl-2-deoxy-2-dodecamido-β-D-glucopyranosyl)-L-serine (**13**) — Compound **11** (4 0 g, 5 mmoles) dissolved in 1:1 ethyl acetate–methanol (270 ml) was hydrogenated in the usual way Crystallization from methanol–water was very slow and a gel formed easily. Therefore, the hydrobromide was prepared by adding a few drops of 48% (w/w) aqueous hydrobromic acid until the solution of **13** in a small amount of methanol became acidic. Upon addition of ether, fine needles were obtained which were recrystallized from methanol–ether (2 7 g, 82%) (Table III)

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REACTION OF HEPARITIN SULFATE WITH NITROUS ACID*

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ABSTRACT

Degradation of heparitin sulfate with nitrous acid at −20° has been shown to be a useful technique for the study of structural features of this mucopolysaccharide. Products obtained from this reaction indicate that heparitin sulfate is composed of multiple, alternating sections containing 2-acetamido-2-deoxy-*O*-(glycuronosyl)-D-glucose or 2-deoxy-*O*-glycosyluronic-2-sulfamido-D-glucose repeating units, each separate section containing only *N*-acetylated or *N*-sulfated 2-amino-2-deoxy-D-glucose residues. Variation in size of the individual sections is indicated by the data.

INTRODUCTION

Heparitin sulfate is a polymer consisting of repeating units of uronic acid and 2-amino-2-deoxy-D-glucose, the latter being either *N*-sulfated or *N*-acetylated. It has been suggested that heparitin sulfate is a hybrid molecule with the hexosamine units alternately *N*-sulfated and *N*-acetylated[1,2]. Enzymic studies by Linker and Sampson[3], however, indicated that this polysaccharide contains a region with repeating units of uronic acid and *N*-acetylhexosamine and a separate region with repeating units of uronic acid and *N*-sulfated hexosamine.

Application of nitrous acid to structural studies of heparitin sulfate[4,5] is made possible by the presence of both *N*-acetyl and *N*-sulfated hexosamine residues. Since nitrous acid at −20° reacts only with the *N*-sulfated units, this reaction was used for the study of certain structural features of heparitin sulfate. This report is concerned with the characterization of certain fractions obtained as a result of this reaction. The data indicate that heparitin sulfate is composed of fragments which contain 2-acetamido-2-deoxy-D-glucose and uronic acid repeating units and other fragments containing uronic acid and 2-deoxy-2-sulfamido-D-glucose. The heterogeneity of the products isolated demonstrates the occurrence of multiple regions of varying lengths interspersed throughout the molecule.

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EXPERIMENTAL

Methods. — Analytical methods for the quantitative determination of uronic acid and hexosamine components, reducing groups, total carbohydrate content, total sulfate and *N*-sulfate residues have been reported previously[6]. The orcinol reaction for uronic acid of Hoffman *et al.*[7] was used for determination of carbazole to orcinol ratios. Galactose was determined by the cysteine–sulfuric acid method of Dische[8].

Paper chromatography of hydrolyzates obtained from heparitin sulfate fractions was performed by development with 2-methyl-2-butanol–2-propanol–water (8:2:3). The paper chromatograms were sprayed with an aniline oxalate or a silver reagent. The hydrolyzates were obtained by heating samples with M hydrochloric acid for 3 h and neutralizing with Dowex-3 (CO_3^{2-}). Amino acids were visualized by spraying chromatograms with ninhydrin.

Purification of heparitin sulfate. — By-products of the preparation of heparin, obtained from lung tissue, were gifts from Dr. L. L. Coleman of the Upjohn Company, Kalamazoo, and Dr. H. H. R. Weber of Wilson Laboratories, Chicago. The crude products were fractionated on Dowex-1 columns as described earlier[9,10]. Fractions eluted with 1.25M sodium chloride were used for the present work. Analyses of a typical preparation gave the following molar ratios for hexosamine to uronic acid to total sulfate to *N*-sulfate to nitrogen: 1.00:1.51:1.03:0.52:1.06. Amino acids, as determined by the Technicon autoanalyzer, were present only in trace amounts, the serine to hexosamine ratio reaching generally a maximum of 1:100. Less than 0.1% of the total hexosamine was 2-amino-2-deoxy-galactose. Determination of the molecular weight from osmotic pressure data, kindly performed by Dr. M. B. Mathews, gave a value of 12.5×10^3 (*cf.* ref. 5).

Application of the purified heparitin sulfate to a 200-cm Sephadex G-25 column (beaded, fine grade) gave a single peak emerging with the void volume. After digestion with testicular hyaluronidase, gel filtration on Sephadex G-25 did not show the presence of retarded material, indicating the absence of hyaluronic acid or chondroitin 4- or 6-sulfates.

Preparation of nitrous acid reagent. — Nitrous acid solutions were prepared as described by Scanley[11] with the following modifications: Peroxide-free 1,2-dimethoxyethane was obtained by shaking with Dowex-1 (CO_3^{2-}, 20–50 mesh) and then by passage through a column of this resin. The peroxide-free solvent, stored in a brown bottle at −20°, gave a negative starch–iodide test for at least 2 months. Removal of peroxides from the solvent was necessary because of interference with the indole colorimetric method[12] used for following the reaction of heparitin sulfate with nitrous acid.

An approximately 0.1M solution of the nitrous acid reagent was prepared in the following way: a 3.5% solution of sodium nitrite (10 ml) was mixed with 1,2-dimethoxyethane (20 ml). After being cooled to −20° the solution was passed through a column of Dowex-50 (X-8, H^+, 50–100 mesh, 2 × 8 cm) which had previously been equilibrated at −20° with 60% 1,2-dimethoxyethane. The column was finally washed

with 60% 1,2-dimethoxyethane (20 ml) and the washings were pooled with the effluent to give a nitrous acid solution approximately 0.1M. This solution has generally been used within a few hours of preparation, although, when stored in a dry-ice chest for 1 or 2 weeks, the solution remained active.

Treatment of heparitin sulfate with nitrous acid. — Since the salt of heparitin sulfate does not react with nitrous acid, the preparation was converted to the free acid form by passage at 2° over Dowex-50.

The heparitin sulfate preparation (250 mg) was dissolved in water (15 ml), and the solution passed at 2° through a column of Dowex-50 (X-12, H^+, 200–400 mesh, 2.2 × 5 cm). The absorbed material was eluted from the column with 3 separate 5-ml portions of water. The effluents were pooled and peroxide-free 1,2-dimethoxyethane (60 ml) was added. The mixture was cooled to −20°, and a freshly prepared solution of 0.1M nitrous acid in 60% 1,2-dimethoxyethane (40 ml) was added.

2,5-Anhydro-D-mannose formed during the reaction at −20° was estimated by removal of 0.1-ml aliquots at various time intervals and immediate transfer to tubes, kept at 2°, containing 12% ammonium sulfamate (0.5 ml) and water (1.4 ml). The tubes were shaken occasionally during a period of 1 h. The 2,5-anhydro-D-mannose content was then determined by the indole colorimetric method[11]. In this determination, the anhydro-mannose values obtained for heparitin sulfate are generally 60% higher than those given by a comparable amount of 2-amino-2-deoxy-D-glucose after treatment with nitrous acid at room temperature. A correction of this value was applied to all analyses.

Treatment of substances containing free and N-*substituted amino groups with nitrous acid.* — The substances were tested at a concentration of 1 mg per ml of 60% dimethoxyethane. The solutions were mixed with one-half volume of 0.1M nitrous acid at −20°, and the reaction mixtures were kept for 24 h, except for the solutions containing amino acids which were treated for 4 h. The following compounds having free amino groups were tested: 2-amino-2-deoxy-D-glucose hydrochloride, 2-amino-2-deoxy-D-galactose hydrochloride, chondrosine [2-amino-2-deoxy-3-*O*-(β-D-glucopyranosyluronic acid)-D-glucose], heparosin [4-*O*-(2-amino-2-deoxy-α-D-glucopyranosyl)-D-glucopyranosyluronic acid], glycine, proline, and de-*N*-sulfated heparin (prepared by heating heparin with 0.04M hydrochloric acid for 100 min). The substances having *N*-substituted groups which were tested were the *N*-(2,4-dinitrophenyl) derivatives of aspartic acid, serine, lysine, and de-*N*-sulfated heparitin sulfate (prepared by the conditions described for de-*N*-sulfated heparin).

The reaction of hexosamine-containing compounds was determined by the indole reaction as described above. The reaction of the *N*-(2,4-dinitrophenyl) derivatives and of the free amino acids was determined on the basis of recovery of the original compound by chromatography.

Gel filtration of products obtained from treatment of heparitin sulfate with nitrous acid. — After the treatment of heparitin sulfate with nitrous acid had been completed (8–12 h), an equal volume of methanol, pre-cooled to 0°, was added, and the mixture was concentrated *in vacuo* to half its volume in a bath maintained at 0–5°.

This procedure was repeated once, and the pH of the mixture was finally adjusted to 6–7 with dilute sodium hydroxide. After concentration to 3–4 ml, the solution was applied to a column of Sephadex G-25 (bead form, 30-80 μm, 2.2 × 200 cm). Elution was performed with 0.2M sodium acetate in 10% ethanol. Fractions of 5 ml were collected each 40 min and analyzed for uronic acid, hexosamine, carbohydrate, and 2,5-anhydro-D-mannose content.

Reduction of gel filtration products. — The reduction was accomplished as follows: To the sample (2 ml) was added a freshly prepared solution of 1% sodium borohydride (0.15 ml). After incubation for 1 h at room temperature with occasional mixing, 6M acetic acid (0.15 ml) was added. In control mixtures the order of reagent additions was reversed, the borohydride being degraded by acetic acid prior to addition of the sample.

In order to be sure that disappearance of reducing activity did not result from the action of alkaline borate (produced from borohydride), a second control utilizing a borate solution at pH 10.5 was performed. No change in 2,5-anhydro-D-mannose content was observed.

RESULTS

The reaction with nitrous acid of the *N*-sulfate groups present in heparitin sulfate and heparin is complete in approximately 1 h at room temperature under the conditions described by Dische and Borenfreund[12]. In the present study it was found that a similar reaction occurred at −20° with nitrous acid in 60% 1,2-dimethoxyethane. Fig. 1 shows that the rates of reaction are appreciably lower than at room temperature; complete reaction at −20° requires approximately 8 h for heparitin sulfate and 2 h for heparin. Under these conditions hexosamine derivatives containing free or *N*-(2,4-dinitrophenyl)-substituted amino groups were unreactive, even after 24 h. These compounds, however, did react with nitrous acid at room temperature. The disparity in rates of reaction between heparin and heparitin sulfate is not clear at present. However, the generally larger concentration of sulfate and especially of *N*-sulfate groups in heparin as compared to heparitin sulfate suggests that this property influences the increased rate of reaction of heparin with nitrous acid.

Gel filtration of heparitin sulfate fractions after nitrous acid treatment. — For study of the products formed from heparitin sulfate, the nitrous acid treatment was performed for 12 h at −20°. Completion of the reaction was confirmed by the lack of further anhydromannose formation[13] after nitrous acid treatment at 25°. The neutralized, concentrated solution was applied to a Sephadex G-25 column (fine, beaded), and the fractions were eluted with 0.2M sodium acetate in 10% ethanol. The elution pattern from a typical reaction mixture is shown in Fig. 2.

Fraction I was slightly, if at all, retarded on the column, suggesting a molecular size for this material of approximately 3000 or higher. This peak contained approximately 20% of the hexosamine present in the original polysaccharide.

Hydrolysis of this fraction with M hydrochloric acid for 2 h gave oligosaccharide fractions in which the reducing end was almost entirely composed of uronic acid, indicating a similarity to the hydrolysis pattern of heparin[9]. The position of the next discrete peak (II) coincided approximately with the elution position of a tetrasaccharide[14] from Sephadex G-25, and peak (II) contained approximately 15% of the initial hexosamine content. The third peak (III) contained no hexosamine and emerged from the Sephadex column as expected for a disaccharide. In addition to its presence in the 2 major fractions, the remaining hexosamine was distributed in the less distinct fractions found in intermediate positions (Ia, Ib).

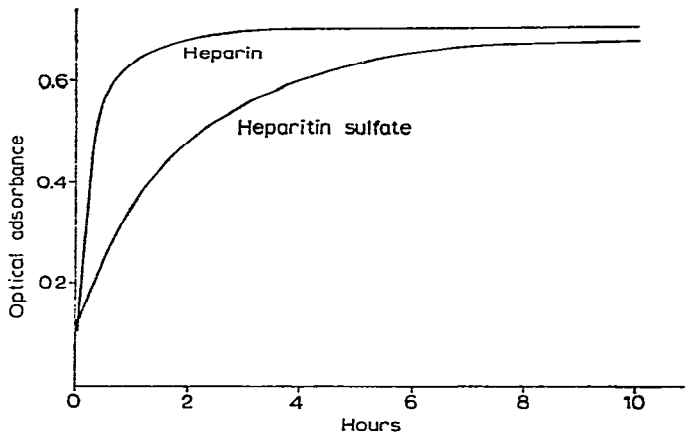


Fig 1. Reaction rates of heparin and heparitin sulfate with nitrous acid at −20°. Optical absorbance (492–520 nm) was based on the indole reaction. The heparin used contained 98% of *N*-sulfated 2-amino-2-deoxy-D-glucose units, and the heparitin sulfate preparation had approximately 50% of this constituent as determined by recovery of hexosamine after treatment with nitrous acid.

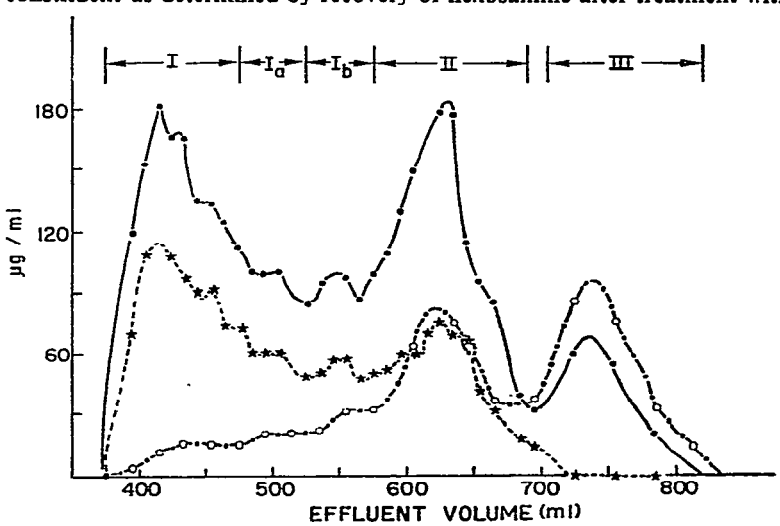


Fig. 2. Gel filtration pattern from Sephadex G-25 of products obtained after reaction of heparitin sulfate with nitrous acid. Fractions were analyzed for uronic acid, •—•—•; hexosamine •—•—•; 2,5-anhydro-D-mannose, ○—○—○. Methods used are given in the Experimental Section.

Composition of gel filtration fractions. — Analyses of the fractions obtained by gel filtration are reported in Table I. As the molecular size increases, the ratio of 2,5-anhydro-D-mannose to uronic acid decreases and concomitantly a larger amount of hexosamine is found. Osmotic pressure measurements of fraction I indicated a mole-

TABLE I

COMPOSITION OF GEL FILTRATION FRACTIONS OBTAINED FROM REACTION OF HEPARITIN SULFATE WITH NITROUS ACID[a]

Peak	*Uronic acid*	$[\alpha]_D^{25}$	*2,5-Anhydro-D-mannose*[b]	*Ester sulfate*	*Carbazole / orcinol*	*Hexosamine recovery*[c], %
I	1.48	+80°	0.05	0.19	1.66	42
I_a	1.68		0.14		1.62	18
I_b	1.66		0.24			13
II	2.38	+46°	1.20	0.74	1.01	27
III	1.00	+ 6°	1.46		0.38	

[a]Analytical results expressed as ratios to hexosamine taken as 1.00, except for III in which uronic acid is expressed as 1.00. The methods used are described in the Experimental Section. [b]Results corrected as indicated in the Experimental Section. [c]Recovery based on total hexosamine obtained from the Sephadex column.

cular weight of approximately 3000. The high positive optical rotation of fraction I suggests a preponderance of α-D-glycosidic linkages. Paper chromatography of hydrolyzates of fraction I showed the presence of xylose and galactose. The molar ratio of sulfate to hexosamine of 0.19 agrees with the findings of Linker and Sampson[3] that most of the ester sulfate of heparitin sulfate resides on the *N*-sulfated 2-amino-2-deoxy-D-glucose residues. The high carbazole to orcinol ratio is similar to that of original heparitin sulfate. These data are consistent with the hypothesis that fraction I is derived from a region of the heparitin sulfate molecule that contains an average of 6–7 disaccharide units of 2-acetamido-2-deoxy-D-glucose and uronic acid. Associated with this portion of the molecule is the galactosylgalactosylxylosyl linkage region. The results in Table II indicating a molar ratio of galactose to hexosamine of 1:3 support the chain size just given.

Fractions Ia and Ib are similar to fraction I except for decreasing molar ratios of hexosamine to anhydromannose. Like fraction I, they also contain galactose and xylose as demonstrated by paper chromatography of hydrolyzates. The data are in agreement with the suggestion that these fractions are derived from segments of the heparitin sulfate molecule similar to those giving rise to fraction I, but containing somewhat fewer successive 2-acetamido-2-deoxy-D-glucopyranosyluronic acid repeating-disaccharide units.

In contrast to the above fractions, fraction II contains nearly identical amounts of anhydromannose and hexosamine. Reduction with borohydride (Table II) resulted in a loss of anhydromannose only, indicating the reducing terminal position of the anhydromannose in this fraction. In contrast to fraction I, no galactose or xylose was

found on chromatography of hydrolyzates of this fraction. The sulfate to hexosamine ratio of fraction II was 0.75 indicating that some of the disaccharide repeating units lacked sulfate. The region of emergence from the Sephadex column, considered together with the analyses, suggests that this fraction consists primarily of a tetrasaccharide having two uronic acid residues, one hexosamine, and a reducing terminal anhydromannose residue. The sulfate is present in ester linkage, presumably to the anhydromannose moiety. Such tetrasaccharide units are expected to arise from interior fragments containing alternating *N*-sulfated and *N*-acetylated hexosamine units linked to a uronic acid unit. The absence of xylose and galactose suggests that these tetrasaccharides are not adjacent to the linkage region, since otherwise the presence of glucosyluronicgalactosylgalactosylxylose units would be expected in fraction II.

TABLE II

COMPOSITION OF REDUCED GEL-FILTRATION FRACTIONS[a]

Fraction	*Uronic acid*	*Hexosamine*	*2,5-Anhydro-D-mannose*	*Carbohydrate, as galactose*[b]
I Untreated	1.00	0.65	0.05	0.23
Reduced	0.95	0.61	0	0.20
II Untreated	1.00	0.45	0.56	
Reduced	0.93	0.38	0	
III Untreated	1.00	0	1.5	
Reduced	0.81	0	0	

[a]Analytical results are expressed as molar ratios to uronic acid of untreated samples. [b]Galactose determinations by the cysteine–sulfuric acid method of Dische[7].

Fraction III contains no hexosamine and a maximal amount of anhydromannose. Reduction with borohydride (Table II) resulted in a loss of anhydromannose, indicating the reducing position of this residue. No galactose or xylose was found in hydrolyzates of this fraction. Sulfate analyses were unsatisfactory because of difficulty in completely removing inorganic sulfate. The analytical data together with the pattern of elution from Sephadex G-25 suggests that this fraction is predominantly composed of a disaccharide. Such disaccharide units would be expected to derive from repeating disaccharide units of uronic acid and *N*-sulfated 2-amino-2-deoxy-D-glucose. Similar disaccharide fractions, though in increased amounts, are obtained from heparin preparations after reaction with nitrous acid.

The carbazole to orcinol ratio becomes progressively lower in the successive fractions and reflects an increasing 2,5-anhydro-D-mannose content, rather than a variation in L-iduronic acid content, since this component appears to be present only in minor proportion in all fractions.

DISCUSSION

Heparitin sulfate appears to consist of a family of compounds having varying

ratios of *N*-sulfated to *N*-acetylated 2-amino-2-deoxy-D-glucose as well as a varying content of ester sulfate[9]. The preparations used in this study were obtained by fractionation on Dowex-1 of the by-products obtained in the commercial preparation of heparin. For convenience in interpretation of the results, a heparitin sulfate fraction was selected that contained equimolar ratios of *N*-acetylated and *N*-sulfated glucosamine.

The present study of the structure of heparitin sulfate is based on the observation that nitrous acid reacts with the *N*-sulfated but not the *N*-acetylated hexosamine units of the mucopolysaccharide. During the reaction, 2,5-anhydro-D-mannose is formed from the *N*-sulfated hexosamine residues, and concurrent cleavage of the adjacent glycoside bond occurs, producing fragments containing 2,5-anhydro-D-mannose at the reducing end.

Various structures that may be postulated for heparitin sulfate are shown in Fig. 3. These are shown in linear form with glycopeptide residues (GP) at the reducing ends in conformity with previous evidence[5,15] suggesting that galactose and xylose form a "linkage region" between heparitin sulfate and protein similar to that present in heparin[16] and chondroitin 4-sulfate[17].

(A) $[\text{HexA-GNAc-HexA-GNSO}_3\text{H}]_n$— GP → n(HexA-GNAc-HexA-AMan) + GP

(B) $[\text{HexA-GNSO}_3\text{H}]_n$—$[\text{HexA-GNAc}]_n$—GP → n(HexA-AMan) + $(\text{HexA-GNAc})_n$— GP

(C) $[\text{HexA-GNSO}_3\text{H}]_d$—$[\text{HexA-GNAc}]_c$—$[\text{HexA-GNSO}_3\text{H}]_b$—$[\text{HexA-GNAc}]_a$— GP

↓

$[\text{HexA-GNAc}]_c$—HexA-AMan + (b + d − 1) (HexA-AMan) + $[\text{HexA-GNAc}]_a$— GP

Fig. 3. Illustration of structural formulations possible for linear arrangements of heparitin sulfate. Abbreviations: HexA, hexuronic acid; AMan, 2,5-anhydro-D-mannose; GNAc, 2-acetamido-2-deoxy-D-glucose; $GNSO_3H$, 2-deoxy-2-sulfamido-D-glucose; GP, linkage region residue. Use of the letter subscripts has no implication as to size of individual sections and they are used only to express in quantitative terms the products obtained after reaction.

The first structure (A) indicates a hybrid molecule having disaccharide units with alternating *N*-acetylated and *N*-sulfated hexosamine residues. Treatment with nitrous acid would produce a glycopeptide residue along with tetrasaccharides containing 2 units of uronic acid, 1 unit of 2-acetamido-2-deoxy-D-glucose and 1 unit of 2,5-anhydro-D-mannose at the reducing end. Structure B should yield anhydromannose-containing disaccharides in a proportion related to the N-sulfated hexosamine content and oligosaccharides composed of 2-acetamido-2-deoxy-*O*-(glycosyluronic)-D-glucose repeating units attached to the glycopeptide residues initially present in the mucopolysaccharide. A more complex pattern results from structure C. It would include not only the two types of product encountered from structure B, but also fractions arising from the internal part of the carbohydrate chain and containing both hexosamine and anhydromannose residues.

The complexity of products formed after treatment of heparitin sulfate with nitrous acid, as indicated by the gel filtration pattern obtained from Sephadex G-25 (Fig. 2), can be explained only by structure C. Analytical results (Table I) indicate that approximately 40% of the total recovered 2-acetamido-2-deoxy-D-glucose residues are present in fraction I. Since this fraction contains most of the galactose and xylose residues originally present in the mucopolysaccharide and has a molecular weight of approximately 3000, it is apparent that the linkage part of the carbohydrate chain attached to the protein moiety is linked in major part to chains containing 6 or 7 repeating units of 2-acetamido-2-deoxy-*O*-(glucosyluronic)-D-glucose. The isolation, in a yield of approximately 25%, of tetrasaccharides containing uronic acid, 2-acetamido-2-deoxy-D-glucose, and 2,5-anhydro-D-mannose in the ratio 2:1:1 (fraction II) indicates that single 2-acetamido-2-deoxy-*O*-(glucosyluronic)-D-glucose units are distributed in the interior of the molecule or at the non-reducing terminus. The intermediate fractions (Ia, Ib) having various proportions of 2-acetamido-2-deoxy-D-glucose and 2,5-anhydro-D-mannose, as well as D-galactose and D-xylose residues, further demonstrate the variations in chain lengths of the residues containing 2- acetamido-2-deoxy-D-glucose in heparitin sulfate.

From the preceding results heparitin sulfate may be depicted as a molecule having a variable distribution of 2-acetamido-2-deoxy-*O*-(glucosyluronic)-D-glucose repeating units, the sections of longest size being attached mostly to the galactosyl-xylosyl-protein part, and those of shortest size predominating further away from the carbohydrate-protein linkage. Sections containing 2-deoxy-2-sulfamido-*O*-(glycosyluronic)-D-glucose residues similarly show random distribution, with the exception that such residues do not occur in detectable amounts near the glycopeptide linkage area. The ester sulfate content of the 2-acetamido-2-deoxy-D-glucose residues is minor as compared with that of the *N*-sulfated hexosamine-containing units.

ACKNOWLEDGMENTS

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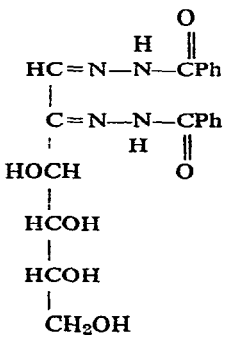
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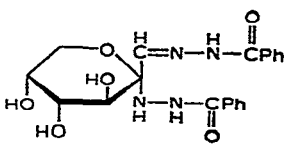
Note

Cyclic forms of monosaccharide benzoylosazones

In a previous communication[1], we showed that D-*arabino*-hexosulose bis-(benzoylhydrazone) (**1**) could be prepared from the Amadori compounds[2] 1-deoxy-1-*p*-toluidino-D-fructose and 1-*p*-anisidino-1-deoxy-D-fructose by refluxing for 8 hours with benzoylhydrazine, or from D-glucose benzoylhydrazone and *p*-toluidine or aniline by refluxing with benzoylhydrazine for 18 hours When the time of refluxing in the above experiments was decreased to 4 hours, an isomeric benzoylosazone (**2**) was obtained, it was found that compound **2** is also formed when D-glucose or D-fructose (D-*arabino*-hexulose) is refluxed with benzoylhydrazine in the presence of aniline or *p*-toluidine



1



2

A comparison of the properties of the two isomers is given in Table I Compound **2** is dextrorotatory, whereas the previously prepared[3] **1** is levorotatory, and, on acetylation, **1** gives a crystalline acetate, whereas **2** yields an amorphous product The Amide I band of **2** is at 1660 cm^{-1}, whereas **1** shows two amide bands, at 1665 and 1675 cm^{-1}, the ultraviolet absorption spectra were similar, but **2** showed a slight hypsochromic shift (see Fig 1)

TABLE I

COMPARISON OF COMPOUNDS **1** AND **2**

Compound	*M p , degrees*	$[\alpha]_D$, *degrees*	*M p of acetate, degrees*	λ_{max}^{EtOH}, *nm*	ν_{max}^{KBr}, cm^{-1}
1	186–188	−89 8	232–233	204, 254, 330	3450, 1665, 1675
2	179–180	+88 6	—	204, 238, 318	3150, 1660

Most of the properties of **1** and **2** are quite similar, thus, they both give brown colorations with ferric chloride, and fail to give the formazan reaction Like **1**, when boiled with aqueous cupric sulfate, compound **2** gives the glycosulose (osone), identified by conversion into D-*arabino*-hexosulose 1-(2-methyl-2-phenyl)hydrazone[4]. Similarly, when treated with phenylhydrazine in ethanolic acetic acid, **1** and **2** give D-*arabino*-hexulose phenylosazone by transhydrazonation (exchange of hydrazone residues), showing that the two compounds possess the same configuration on C-3, C-4, and C-5 (*i e*, no inversion had taken place)

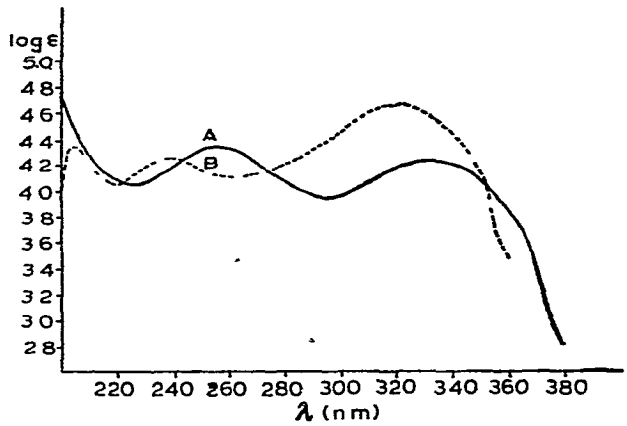
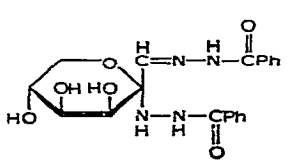


Fig 1 — Ultraviolet spectra of (A) D-*arabino*-hexosulose bis(benzoylhydrazone) (**1**), and (B) D-*ar a bino*-hexulopyranose benzoylosazone (**2**)

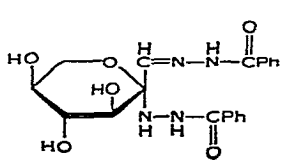
Periodate oxidation of **1** resulted in the consumption of three moles of oxidant per mole, and yielded one mole of formaldehyde and two moles of formic acid, in addition to crystalline mesoxalaldehyde bis(benzoylhydrazone)[5] On periodate oxidation, the isomeric compound consumed only two moles of oxidant per mole, and yielded an amorphous aldehyde and one mole of formic acid, no formaldehyde was produced The isomeric compound was therefore tentatively assigned the pyranoid structure **2**, which agrees with these findings.

The cyclic D-*lyxo*-hexulopyranose benzoylosazone (**3**) was also obtained, by refluxing 1-deoxy-1-*p*-toluidino-D-tagatose[2] with benzoylhydrazine, or by refluxing D-galactose benzoylhydrazone with benzoylhydrazine and some *p*-toluidine. Its u v spectrum was identical with that of **2**, and on periodate oxidation, it consumed two moles of oxidant per mole of the benzoylosazone

Similarly, the cyclic L-*xylo*-hexulopyranose benzoylosazone (**4**) was obtained by refluxing L-sorbose with benzoylhydrazine in the presence of aniline or *p*-toluidine



3



4

Cyclic pentopyranose benzoylosazones from D-arabinose and D-xylose were prepared by refluxing the sugar or its benzoylhydrazone with benzoylhydrazine and aniline or *p*-toluidine They had similar u v spectra, and their oxidation with periodic acid resulted in the consumption of one mole of periodic acid per mole of the pentose benzoylosazone The detailed structures of these compounds, which have been arbitrarily depicted as the α-D anomers, are at present under investigation

The oxime of 1-*p*-anisidino-1-deoxy-D-fructose, obtained by heating this Amadori compound with hydroxylamine in ethanolic acetic acid, is also described

EXPERIMENTAL

I r spectra were recorded on a Unicam SP 200 spectrophotometer, and u v spectra on a Unicam SP 800 spectrophotometer

Cyclic D-arabino-*hexulose benzoylosazone* (2) — *Method a* A solution of 1-deoxy-1-*p*-toluidino-D-fructose[2] (2 g) and benzoylhydrazine (2 g) in ethanol (30 ml) containing 5 ml of 2M acetic acid was refluxed for 2 h The product, which separated after 24 h, was filtered off, washed with ethanol, and dried (yield 0 9 g) It crystallized from ethanol in needles, m p 179–180° (dec), $[\alpha]_D^{20}$ +88 6° (*c* 0 53, pyridine), ν_{max}^{KBr} 1600 (C=N), 1660 (CONH), and 3450 cm^{-1} (OH), λ_{max}^{EtOH} 204, 238, and 318 nm (log ε 4 39, 4 24, and 4 67), λ_{max}^{EtOH} 220 and 260 nm (log ε 4 12 and 4 12), it gave a characteristic, brown color with ferric chloride

Anal Calc for $C_{20}H_{22}N_4O_6$· C, 58 0, H, 5 3, N, 13 5 Found C, 57 7, H, 5 7, N, 13 8

Method b A solution of D-glucose (2.5 g), benzoylhydrazine (3 2 g), and *p*-toluidine (1 5 g) or aniline (1 5 ml) in ethanol (35 ml) containing 5 ml of 2M acetic acid was refluxed for 4 h. The product, which separated after 24 h, was filtered off, washed, and dried (yield 1.2 g and 1 3 g, respectively); it crystallized from ethanol in needles, m p and mixed m p. 180°. It had the same optical rotation as, and its u v and i r spectra were identical with those of, the product obtained in *a*

Method c A solution of D-glucose benzoylhydrazone (2 5 g), benzoylhydrazine (1 2 g), and *p*-toluidine (1 5 g) or aniline (1.5 ml) in ethanol (35 ml) containing 5 ml of 2M acetic acid was refluxed for 4 h The product, which separated after 24 h, was filtered off, washed, and dried (yield 1 3 g, and 1 35 g, respectively), it had the same m p , optical rotation, and u v. and i r spectra as the product obtained in *a*

Method d. A solution of D-fructose (2 5 g), benzoylhydrazine (3 2 g), and *p*-toluidine (1 5 g) or aniline (1.5 ml) in ethanol (35 ml) containing 5 ml of 2M acetic acid was refluxed for 4 h, and afforded a product (yield 0 8 or 0 9 g, respectively) identical with that obtained in *a*.

Properties of the cyclic D-arabino-*hexulose benzoylosazone — 1 Periodate oxidation* A suspension of the cyclic D-*arabino*-hexulose benzoylosazone (0 036 g) in a solution of metaperiodic acid (HIO_4 $2H_2O$) (0 07 g) in water (5 ml) was shaken for 72 h at room temperature. The suspension was filtered, and the filtrate was diluted with water to 50 ml, treated with excess arsenite, and then back-titrated against iodine [consumption of periodic acid per mole (414 42), 1 96 mole]

Another sample (0.1 g) was treated with metaperiodic acid (0.7 g) in water (5 ml) for 72 h, and the filtrate was treated with 1% dimedon solution; no formaldehyde dimedon compound separated

A third sample (0.1 g) was suspended in 50 ml of water containing sodium metaperiodate (0.7 g), and the mixture was shaken for 24 h. The excess of periodate was decomposed by addition of ethylene glycol (1 ml), and the formic acid formed was titrated against barium hydroxide, with Methyl Red as the indicator, 0.7 mole of formic acid was produced per mole.

The acyclic bis(benzoylhydrazone) (**1**) consumed three moles of periodic acid per mole, and yielded two moles of formic acid and one mole of formaldehyde (isolated as its dimedon derivative[6], m.p. and mixed m.p. 180°).

2 Action of cupric sulfate A suspension of cyclic D-*arabino*-hexulose benzoylosazone (1 g) in water (25 ml) was heated for 3 h under reflux with cupric sulfate (2 g). The filtrate was de-ionized with ion-exchange resin, and evaporated under diminished pressure. The product was identified as D-*arabino*-hexosulose (D-glucosone) by conversion at room temperature into D-*arabino*-hexosulose 1-(2-methyl-2-phenyl)-hydrazone[4], m.p. and mixed m.p. 171°.

3 Transhydrazonation A mixture of cyclic D-*arabino*-hexulose benzoylosazone (0.5 g) with a solution of phenylhydrazine (0.3 ml) in ethanol (15 ml) and acetic acid (1 ml) was refluxed for 1.5 h, on cooling, D-*arabino*-hexulose phenylosazone separated. It was filtered off (yield 0.1 g), and recrystallized from ethanol, giving needles, m.p. and mixed m.p. 210° (dec.).

D-lyxo-*Hexulose benzoylosazone* (**3**). — *Method a.* A solution of 1-deoxy-1-*p*-toluidino-D-tagatose (2 g) and benzoylhydrazine (2 g) in ethanol (30 ml) containing 5 ml of 2M acetic acid was refluxed for 6 h. The product, which separated after 24 h at room temperature, was filtered off, washed, and dried (yield 0.9 g). It crystallized from ethanol in needles, m.p. 214–215° (dec.), $[\alpha]_D^{20}$ −52° (*c* 0.89, pyridine); ν_{max}^{KBr} 1600 (C=N), 1660 (CONH), and 3445 cm^{-1} (OH), λ_{max}^{EtOH} 204, 238, and 318 nm (log ε 4.11, 3.99, and 4.41); λ_{min}^{EtOH} 220 and 260 nm (log ε 3.89 and 3.86), it gave a characteristic, brown color with ferric chloride solution. Periodate consumption, 2 moles per mole.

Anal. Calc. for $C_{20}H_{22}N_4O_6 \cdot H_2O$: C, 56.0; H, 5.6; N, 13.0. Found: C, 56.4; H, 5.8; N, 13.0.

Method b. A solution of D-galactose (2.5 g), benzoylhydrazine (3.2 g), and *p*-toluidine (1.5 g) in ethanol (30 ml) containing 5 ml of 2M acetic acid was refluxed for 7 h. The product, which separated after 24 h at 25°, was filtered off, washed, and dried (yield 1.2 g), it crystallized from ethanol in needles, m.p. and mixed m.p. 214°, and was identical with the product obtained by method *a*.

Method c. A solution of D-galactose benzoylhydrazone (2.5 g), benzoylhydrazine (1.2 g), and *p*-toluidine (1.5 g) in ethanol (35 ml) containing 5 ml of 2M acetic acid was refluxed for 7 h. The product (1.1 g) obtained was identical with that obtained by method *a*.

Cyclic L-xylo-*hexulose benzoylosazone* (**4**). — A solution of L-sorbose (1 g),

benzoylhydrazine (1 6 g), and aniline (1 ml) or *p*-toluidine (0 7 g) in ethanol (20 ml) containing 3 ml of 2M acetic acid was refluxed for 6 h The product, which separated after 24 h at 25°, was filtered off, washed with ethanol, and dried (yield 0 4 g, 0 45 g, respectively) It crystallized from ethanol in needles, m p 206–209° (dec); $[\alpha]_D^{20}$ +64 2° (*c* 0 43, pyridine), ν_{max}^{KBr} 1600 (C=N), 1670 (CONH), and 3500 cm^{-1} (OH), λ_{max}^{EtOH} 204, 238, and 318 nm (log ε 4.47, 4 34, and 4.80); λ_{min}^{EtOH} 220 and 260 nm (log ε 4 25 and 4 22); it gave a characteristic, brown color with ferric chloride solution

Anal Calc for $C_{20}H_{22}N_4O_6 \cdot H_2O$ C, 56 0; H, 5 6, N, 13 0 Found C, 55 5, H, 6 0, N, 13 1

Cyclic D-erythro-*pentulose benzoylosazone* — *Method a* A solution of D-arabinose (2 5 g), benzoylhydrazine (3 2 g), and *p*-toluidine (1.5 g) in ethanol (35 ml) containing 5 ml of 2M acetic acid, was refluxed for 5 h. The product (1 3 g), which separated after 24 h at 25°, was filtered off, washed, dried, and crystallized from ethanol in needles, m.p. 234–235° (dec); $[\alpha]_D^{20}$ −21° (*c* 0 48, pyridine), ν_{max}^{KBr} 1600 (C=N), 1670 (CONH), and 3450 cm^{-1} (OH), λ_{max}^{EtOH} 204, 239, and 319 nm (log ε 4 5, 4 4, and 4 8); λ_{min}^{EtOH} 220 and 261 nm (log ε 4 2 and 4 2), it gave a characteristic, brown coloration with ferric chloride solution

Anal Calc. for $C_{19}H_{20}N_4O_5$ C, 59 4, H 5 2; N, 14 6 Found C, 59 9; H, 5.6, N, 15 1.

Method b A solution of D-arabinose benzoylhydrazone (2 5 g), benzoylhydrazine (1 2 g), and *p*-toluidine (1.5 g) in ethanol (35 ml) containing 5 ml of 2M acetic acid was refluxed for 5 h The product (1 3 g), which separated after 24 h at 25°, was filtered off, washed, and dried, it was identical with the product obtained by method *a*

Cyclic D-threo-*pentulose benzoylosazone* — A solution of D-xylose (1 g) benzoylhydrazine (1 2 g), and *p*-toluidine (0 7 g) in ethanol (20 ml) containing 3 ml of 2M acetic acid was refluxed for 5 h The product, which separated after 24 h at 25°, was filtered off, washed with ethanol, and dried (yield 0 35 g) It crystallized from ethanol in needles, m p 230–232° (dec), $[\alpha]_D^{20}$ −18 6° (*c* 0 46, pyridine); ν_{max}^{KBr} 1590 (C=N), 1670 (CONH), and 3450 cm^{-1} (OH), λ_{max}^{EtOH} 204, 240, and 320 nm (log ε 4 24, 4 10, and 4 45); λ_{min}^{EtOH} 221 and 262 nm (log ε 3 0 and 3 0), it gave a characteristic, brown color with ferric chloride solution and consumed one mole of periodic acid per mole.

Anal Calc for $C_{19}H_{20}N_4O_5$ C, 59 4, H, 5 2, N, 14 6 Found C, 59 9, H, 5 0 N, 14 2

*1-p-Anisidino-1-deoxy-*D*-fructose oxime* — A solution of 1-*p*-anisidino-D-fructose (1 g), hydroxylamine hydrochloride (0 3 g), and sodium acetate (0 6 g) in 2M acetic acid (1 ml) and ethanol (10 ml) was heated for 10 min and cooled 1-*p*-Anisidino-1-deoxy-D-fructose oxime, which separated, was filtered off after 24 h, and crystallized from ethanol in plates, yield 0 6 g, m p 154–156° (dec.), $[\alpha]_D^{20}$ −56° (*c* 0 67, ethanol); ν_{max}^{KBr} 1030, 1245 (OMe), 1620 (C=N), 2160 (NH), and 3450 cm^{-1} (OH); λ_{max}^{EtOH} 208, 241, and 306 nm (log ε 4 12, 4 14, and 3 50), λ_{min}^{EtOH} 219 and 257 nm (log ε 3 79 and 3 34)

Anal. Calc. for $C_{13}H_{20}N_2O_6$ C, 52.0; H, 6 7, N, 9.3 Found C, 51.9; H, 6 8; N, 9.2.

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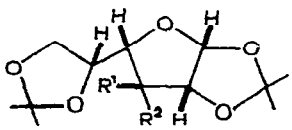
Carbohyd. Res, 8 (1968) 243–248

PRELIMINARY COMMUNICATION

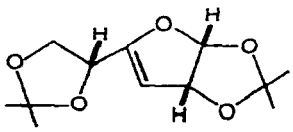
3-Deoxy-3-fluoro-D-galactose

In extending studies of fluorinated carbohydrates[1] as potential antitumour agents and enzyme inhibitors, we now report on the synthesis of 3-deoxy-3-fluoro-D-galactose

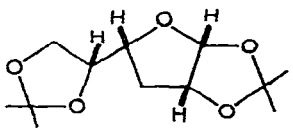
Treatment of 1,2 5,6-di-*O*-isopropylidene-3-*O*-toluene-*p*-sulphonyl-α-D-gulofuranose[2] (1) with tetrabutylammonium fluoride in methyl cyanide[1] for 48 h at 60° gave a mixture of 3-deoxy-3-fluoro-1,2 5,6-di-*O*-isopropylidene-α-D-galactofuranose (2) and 3-deoxy-1,2·5,6-di-*O*-isopropylidene-α-D-*erythro*-hex-3-enofuranose[3] (3) in the ratio *ca.* 1 3 1 The chromatographic mobilities of compounds 2 and 3 were too similar to permit convenient, large-scale separation. However, after hydrogenation of the mixture at room temperature over palladised charcoal, the fluoro derivative 3 {m p 48–49°, $[\alpha]_D$ -30° (*c* 1 0, chloroform) (Found C, 55 4 H, 7 35, F, 7 3 $C_{12}H_{19}FO_5$ calc C, 55 0, H, 7 3, F, 7 3%)} and 3-deoxy-1,2 5,6-di-*O*-isopropylidene-D-*xylo*-hexofuranose[3] (4) were readily separable by elution from Kieselgel (Merck, 7734) with benzene–ether mixtures



1 R^1=H, R^2=OTs
2 R^1=F, R^2=H

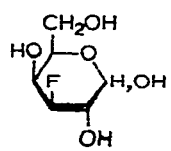


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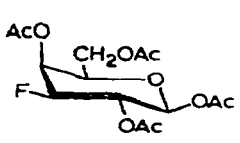


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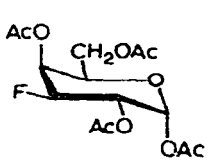
Treatment of the diacetal 2 with an aqueous ethanolic suspension of Amberlite IR-120(H^+) resin gave 3-deoxy-3-fluoro-D-galactose {5, m p 114–116°, $[\alpha]_D$ + 76° (equil , *c* 0 9, water) (Found C, 39 5, H, 5 9, F, 10 9 $C_6H_{11}FO_5$ calc C, 39 6, H, 6 05, F 10 5%)} which, with boiling acetic anhydride and sodium acetate, gave the β-tetra-acetate 6 {m p 126–127°, $[\alpha]_D$ + 35° (*c* 0 8, chloroform) (Found C, 48 15, H, 5 25, F, 5 4 $C_{14}H_{19}FO_9$ calc C, 48 0; H, 5 4, F, 5 4%)} Isomerisation of the latter compound with zinc chloride and acetic anhydride[4] gave the α-tetra-acetate 7 {m p 97–99°, $[\alpha]_D$ + 126° (*c* 1 1, chloroform) (Found C, 48 3, H, 5 3%)}



5



6



7

The *galacto* configuration for compound 2, expected on mechanistic grounds[5] and by analogy[1], was established on the basis of n m r data obtained for solutions of derivatives in deuteriochloroform with a Varian HA-100 spectrometer operating in the

"frequency-sweep" mode for ^{1}H resonances and (at 94 MHz) in the "locked, field-sweep" mode for ^{19}F resonances. The relevant data are given below, a full analysis will be published elsewhere. 1,2,4,6-Tetra-*O*-acetyl-3-deoxy-3-fluoro β-D-galactopyranose (**6**) $J_{1,2}$ 8.1, $J_{2,3}$ 9.5, $J_{3,4}$ 3.8, $J_{4,5}$ ~ 0.5, $J_{F,2}$ 11.5, $J_{F,3}$ 47.4, $J_{F,4}$ ~ 5.6 Hz. α-Tetra-acetate **7** $J_{1,2}$ 3.6, $J_{2,3}$ 10.0, $J_{3,4}$ 3.6, $J_{4,5}$ ~ 0.5, $J_{F,1}$ ~ 4.8, $J_{F,2}$ 11.4, $J_{F,3}$ 47.5, and $J_{F,4}$ ~ 6.4 Hz. The values of $J_{F,2}$ and $J_{F,4}$ which are characteristic[6] for an *eq* fluorine atom coupled to vicinal *ax* and *eq* protons, respectively, confirm the *galacto* configuration of compounds **6** and **7** and, hence, of the diacetal **2**.

Similar steric situations exist in the reactions of the sulphonate **1** and 1,2:5,6-di-*O*-isopropylidene-3-*O*-toluene-*p*-sulphonyl-α-D-allofuranose[1] with tetrabutylammonium fluoride. The latter compound undergoes[1] an S_N2 type displacement with inversion of configuration to give 3-deoxy-3-fluoro-1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose, and it is noteworthy that, although it reacts less readily, apparently no unsaturated product is formed.

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4 M. L. Wolfrom and A. Thompson, *Methods Carbohyd. Chem.*, 2 (1963) 213.
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Book review

Dérivés hydraziniques des glucides (Hydrazine Derivatives of Sugars); by LASZLO MESTER, Institut de Chimie des Substances Naturelles au C N R.S , Gif-sur-Yvette Part 9 of a collection on the Chemistry of Natural Substances, edited by E LEDERER, University of Paris Hermann, Paris, 1967, 179 pp , 20 Tables, 17 5 × 24 cm, paperback Frs 54.00

This excellent monograph covers, in five chapters, a topic that has, in recent years, attracted the attention of many carbohydrate chemists For a better understanding of the reactions of hydrazines with sugars, the author starts his book with a chapter (17 pages) on general aspects of the reactions of amines with sugars, as initiated by nucleophilic attack of a nitrogen atom on the reducing group The mode of preparation, structure, and reactions of glycosylamines, together with their transformations through the Amadori rearrangement, are discussed in the light of recent postulations Chapter II (18 pages) deals with hydrazones of the sugars Their preparation, mechanism of formation, and reactions (especially with periodic acid) are described, and uses of these hydrazones are enumerated All along, the existence of cyclic forms of hydrazones and of their reaction products are discussed in a clear manner Chapter III (26 pages) deals with the formazan derivatives of saccharides. and contains much of the author's own research work He deals first with the preparation and structure of the formazan derivatives of aldoses, and follows with the formazan derivatives of periodate-oxidized mono-, di-, and polysaccharides Next there is a long chapter (64 pages) on the phenylosazones of saccharides It begins with the mechanism of formation of osazones, a subject still open to controversy because of the existence of different, competing processes Transhydrazonation, a process that has complicated these interpretations, is then discussed, and is followed by a study in greater detail of the different osazone derivatives and transformation products, including the esters, ethers, anhydro derivatives, formazans, and triazoles The structures of osazones and of some of their derivatives are then discussed in the light of their physical properties It is shown how the use of ultraviolet, infrared, and n.m r. spectroscopy, and X-ray crystallography have contributed to the elucidation of their structures and to a better understanding of the type of chelation existing in these compounds A description of the degradation of sugars by phenylhydrazine then follows in a short chapter (8 pages) which covers the interesting work of Barry on the degradation of polysaccharides, the work of the author on the periodate-oxidized dehydro-osazones, and the degradation of sulfonylated and phosphorylated derivatives of sugars

The melting points and optical rotations of sugar osazones, formazans, tetrazoles, and phenylosotriazoles, together with similar data for the phenylhydrazides of aldonic acids, are given in 7 Tables The work includes a bibliography of 511 references

covering the period from Emil Fischer's discovery of phenylhydrazine until 1965, and this list is followed by an Author Index and a Subject Index

The book is written in a clear and simple manner by an authority in the field, and will be extremely valuable to carbohydrate chemists and research students interested in nitrogen-containing saccharide derivatives The paper and printing are good, and the book contains remarkably few misprints The reader should however, be cautioned that, for the purpose of simplification, the author sometimes uses common names, such as D-glucosazone, that are not inagreement with the Rules of Carbohydrate Nomenclature

H EL KHADEM (*Alexandria*)

Carbohyd Res, 8 (1968) 251–252

Carbohydrate Research
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2-DEOXY SUGARS

PART XIII. 1,3-DIDEOXY-D-*erythro*-HEXULOSE*

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(Received May 13th, 1968)

ABSTRACT

The known 3,4,5-tri-*O*-benzoyl-2-deoxy-D-*erythro*-pentose (prepared in three steps from 2-deoxy-D-*erythro*-pentose) was oxidized to 3,4,5-tri-*O*-benzoyl-2-deoxy-D-*erythro*-pentonic acid, which was converted into the corresponding acid chloride. Treatment of the acid chloride with ethereal diazomethane afforded the diazomethyl ketone, which underwent reduction to give crystalline 4,5,6-tri-*O*-benzoyl-1,3-dideoxy-D-*erythro*-hexulose, obtained alternatively, in 46% yield, by the action of diazomethane on 3,4,5-tri-*O*-benzoyl-2-deoxy-D-*erythro*-pentose. Mercaptalation of the benzoylated 1,3-dideoxy-D-*erythro*-hexulose with methanethiol yielded syrupy 4,5,6-tri-*O*-benzoyl-1,3-dideoxy-D-*erythro*-hexulose dimethyl dithioacetal which, on debenzoylation, gave 1,3-dideoxy-D-*erythro*-hexulose dimethyl dithioacetal (likewise a syrup), which was converted into crystalline 6-*O*-adamantoyl-1,3-dideoxy-D-*erythro*-hexulose dimethyl dithioacetal. Debenzoylation of 4,5,6-tri-*O*-benzoyl-1,3-dideoxy-D-*erythro*-hexulose afforded 1,3-dideoxy-D-*erythro*-hexulose as an impure syrup.

INTRODUCTION

C'-Methyl branched-chain nucleosides are those in which a ring proton of the sugar residue has been replaced by a methyl group (the smallest possible alkyl substituent), which may serve as a metabolic impediment. For example, both 2'-methyladenosine[1] and 3'-methyladenosine[1] inhibit the growth of KB cells in culture and the incorporation of hypoxanthine-8-^{14}C into ribonucleic acid by Ehrlich ascites cells, and we have considered that the preparation of nucleosides in which the anomeric proton has been replaced by a methyl group might yield compounds having unexpected

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**NASA Fellow, 1964–1967. This work is the subject of a Ph. D. Thesis to be submitted by Barbara F. West to the School of Graduate Studies, Georgetown University. Present address: Division of Analytical Chemistry, National Bureau of Standards, Washington, D. C. 20234, U. S. A.
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biological properties. Because direct replacement of the anomeric proton of a sugar (to include its glycosyl derivatives) is not possible, we sought to prepare a 1-deoxy-2-ketose, which would be capable of forming glycosyl derivatives having a methyl group attached to the anomeric center. This paper reports the synthesis of the heretofore unknown 1,3-dideoxy-D-*erythro*-hexulose and a crystalline derivative; nucleosides prepared from the new sugar could be considered as being those that contain a 2-deoxy-D-*erythro*-pentofuranose (2-deoxy-D-ribofuranose) residue in which the anomeric proton has been replaced by a methyl group.

DISCUSSION AND RESULTS

Wolfrom *et al.* have shown[2] that the diazomethane synthesis for lengthening the carbon chain of sugars may be employed in the preparation of 1-deoxy-2-ketoses; this suggested a convenient means for the synthesis of 1,3-dideoxy-D-*erythro*-hexulose (**9**). Our synthesis required, as the starting compound, 2-deoxy-D-*erythro*-pentose (**1**), and appears to be the first application of this particular variation of the diazomethane synthesis to 2-deoxy sugars, in which the product would be a 1,3-dideoxy-2-ketose.

2-Deoxy-D-*erythro*-pentose (**1**) was converted, in three steps, into the known 3,4,5-tri-*O*-benzoyl-2-deoxy-D-*erythro*-pentose (**2**) by a procedure described[3] by Zinner *et al.* Normally, the bromine-water oxidation of an aldose is performed with the unsubstituted sugar or its open-chain, acetylated derivative, either of which dissolves in the aqueous medium. The tribenzoate (**2**) is, however, insoluble in water, and an attempt was made to oxidize it in methanolic solution containing suspended calcium carbonate. A crystalline product was obtained which was not, however, the desired aldonic acid (**3**), but its methyl ester (**3a**), instead. A second attempt was made, in which the bromine was slowly added to a solution of **2** in methanol containing 10% of water (with suspended calcium carbonate); this also gave the methyl ester **3a**. To the best of our knowledge, the oxidation of an aldose, in one operation, to an aldonic ester has not been previously reported. When 10:1 water–tetrahydrofuran was substituted for methanol, the benzoylated aldose (**2**) was converted smoothly, and in high yield, into 3,4,5-tri-*O*-benzoyl-2-deoxy-D-*erythro*-pentonic acid (**3**).

On refluxing the acid (**3**) with pure thionyl chloride, the corresponding acid chloride (**4**) was obtained as crystalline material. Reaction of **4** with ethereal diazomethane yielded the diazomethyl ketone (**5**), likewise crystalline, which underwent reduction with hydriodic acid to give, in high yield, 4,5,6-tri-*O*-benzoyl-1,3-dideoxy-D-*erythro*-hexulose (**6**) as a crystalline product.

A more direct route to compound **6** was investigated that involved treatment of the benzoylated aldose (**2**) with diazomethane. Although complicated by the formation of epoxides and homologous ketones, simple, aliphatic aldehydes yield methyl ketones when treated with diazomethane, and Wolfrom *et al.*[2] were successful in converting either D- or L-arabinose tetraacetate, by this method, into 1-deoxy-D- or L-fructose tetraacetate, respectively, in 62% yield. Treatment of **2** with an excess

of ethereal diazomethane, followed by evaporation of the solvent, gave a crystalline product that was identified as the desired benzoylated hexulose (**6**). Contrary to reports of other reactions with diazomethane, a vigorous evolution of nitrogen was not observed, and the best yields (*ca.* 50%) were obtained when freshly prepared diazomethane was used and when the reaction proceeded for no less than one, nor more than two, hours. The crystalline residue, obtained after evaporation of the ethereal diazomethane, had to be scrupulously free of residual diazomethane; otherwise, the colorless product became yellow after being kept overnight. Also, it was necessary to bring the product (**6**) to a high degree of purity without delay, because contaminating side-products of undetermined composition appear to catalyze the decomposition of the product. In several instances, the crude product (**6**) was recrystallized from 95% ethanol, and, from the mother liquors, there could be obtained no additional crystalline material, but only dark-yellow syrups that showed a powerful vesicant action.

A 100-MHz n.m.r. spectrum (see Fig. 1) of **6** in chloroform-*d*, with tetramethylsilane as the internal standard, showed a three-proton singlet for the C-1 methyl protons at τ 7.82; a two-proton doublet ($J_{3,4}$ 6 Hz) at τ 6.97 for the C-3 methylene protons; an asymmetric, two-proton, eight-line pattern centered at τ 5.31

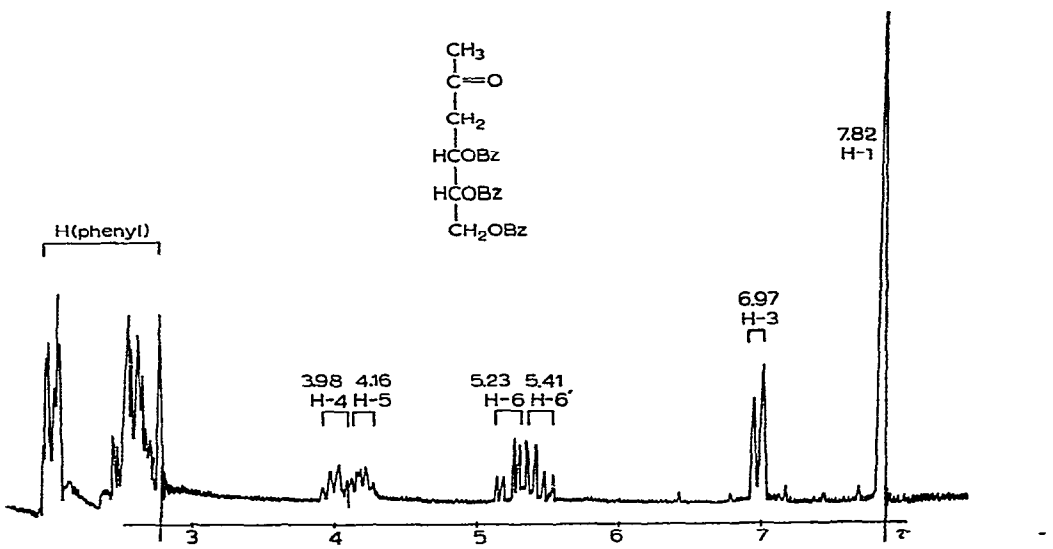


Fig. 1. 100-MHz n.m.r. spectrum of 4,5,6-tri-*O*-benzoyl-1,3-dideoxy-D-*erythro*-hexulose (**6**).

for the two non-equivalent, C-6 methylene protons coupling with the C-5 methine proton ($J_{5,6'}$ 3.4 Hz, $J_{5,6}$ 6.3 Hz); and two overlapping, one-proton multiplets centered at τ 4.16 and τ 3.98 that were assigned to the C-5 and C-4 methine protons, respectively. The two complex multiplets at τ 2.66 and τ 2.10 represented the 15 phenyl protons. That these assignments were correct was proved by decoupling experiments: irradiation at τ 3.98 caused the C-3 methylene doublet to collapse to a singlet at

τ 6.97, whereas irradiation of the proton having the higher-field multiplet at τ 4.16 changed the C-6 methylene peaks from an eight-line pattern to an AB quartet. An AB analysis of this multiplet gave τ (H-6) 5.41 (A) and τ (H-6') 5.23 (B), ($|J_{6,6'}|$ 12.3 Hz). Furthermore, irradiation of the C-3 methylene protons resulted in the collapse of the C-4 methine multiplet to an asymmetric doublet at τ 3.98 ($J_{4,5}$ 6 Hz).

Debenzoylation of **6** with methanolic sodium methoxide gave 1,3-dideoxy-D-*erythro*-hexulose (**9**) as an impure syrup that resisted all efforts to secure it in crystalline form. It gave a positive iodoform test, and its infrared absorption spectrum was concordant with the structure assigned to **9**. Reaction of 4,5,6-tri-*O*-benzoyl-1,3-dideoxy-D-*erythro*-hexulose (**6**) with methanethiol[4] resulted in a syrup that gave analytical results in excellent agreement with those calculated for 4,5,6-tri-*O*-benzoyl-1,3-dideoxy-D-*erythro*-hexulose dimethyl dithioacetal (**7**). Debenzoylation of **7** was effected with methanolic barium methoxide as described by Isbell[5] and modified by Levene and Tipson[6]; this resulted in 1,3-dideoxy-D-*erythro*-hexulose dimethyl dithioacetal (**8**), which could not be obtained in crystalline form. The infrared absorption spectrum of **8** had a striking similarity to that of the known 2-deoxy-D-*erythro*-pentose dimethyl dithioacetal[3].

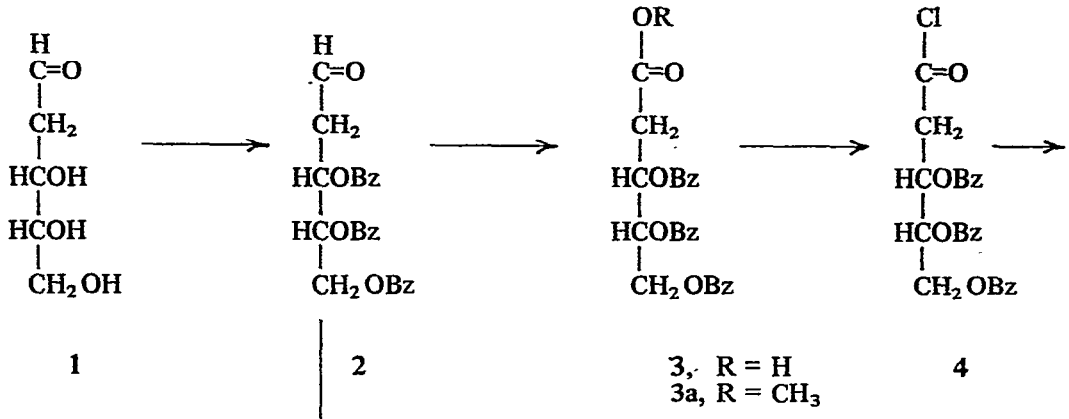
Efforts were made to protect O-6 of the mercaptalated dideoxyketose (**8**) preferentially; this would not only ensure the formation of a furanoid ring on demercaptalation, but could also provide for a crystalline derivative of the ketose (**9**). One approach involved a low-temperature *p*-nitrobenzoylation, successfully applied to the preparation of 2-deoxy-5-*O*-*p*-nitrobenzoyl-D-*erythro*-pentose diisobutyl dithioacetal;[7] however, when we treated **8** in a similar manner, a complex mixture [including unreacted starting-material (**8**)] was obtained, as disclosed by thin-layer chromatography. Similar results were obtained when attempts were made to secure a 6-*p*-toluenesulfonate of **8**, and tritylation gave only a syrupy product.

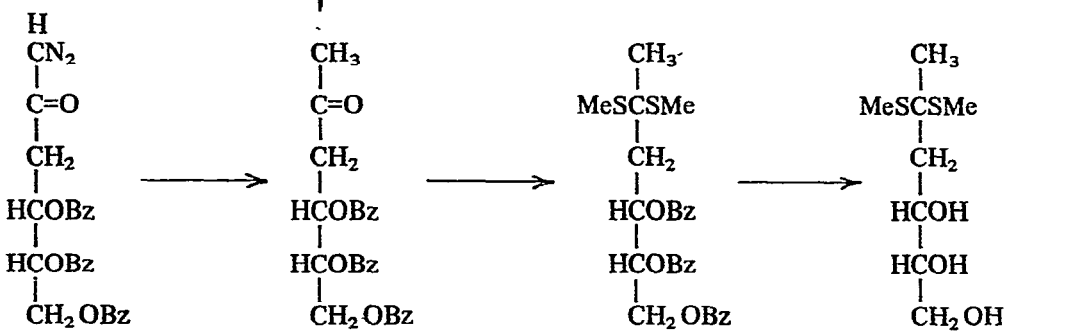
As an alternative, we turned our attention to an adamantoyl derivative. The 1-adamantoyl group has recently been employed for the selective protection of primary hydroxyl groups of nucleosides[8], and, because of its bulk, the group is probably restricted by steric factors to attacking the usually more accessible, primary hydroxyl groups. An adamantoyl ester may be hydrolyzed with 0.25M base to regenerate the free primary hydroxyl group, thus serving as a base-sensitive counterpart to the acid-labile trityl group in syntheses involving carbohydrates and nucleosides. Accordingly, the mercaptalated ketose (**8**) was treated in pyridine at 0° with 1-adamantoyl chloride*, giving 6-*O*-(1-adamantoyl)-1,3-dideoxy-D-*erythro*-hexulose dimethyl dithioacetal (**10**) as a crystalline product.

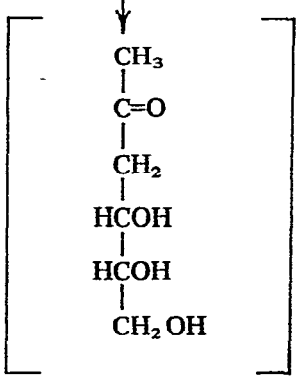
EXPERIMENTAL

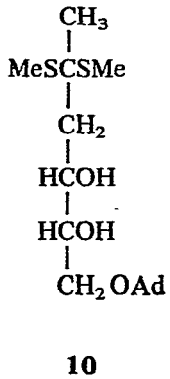
Melting points were determined with a Kofler hot-stage. Thin-layer chromatographic plates were prepared with Kiesel-Gel DF-5 (ultraviolet indicating), and

*The authors thank Dr. J. A. Montgomery, Southern Research Institute, Birmingham, Alabama, for suggesting this reagent.









Bz = PhC(=O); Ad = 1-adamantoyl

detection of all derivatives was made with alkaline potassium permanganate spray reagent. To distinguish between the ketoses and their mercaptalated derivatives, (2,4-dinitrophenyl)hydrazine spray reagent was used.

Optical rotations were determined with a Rudolph Model 80 polarimeter, infrared spectra were recorded with a Perkin–Elmer Model 137B i.r. spectrophotometer, and n.m.r. spectra were recorded with Varian Models T-60 and HA-100 n.m.r. spectrometers, with tetramethylsilane (τ 10.00) as the internal standard.

3,4,5-Tri-O-benzoyl-2-deoxy-D-erythro-pentonic acid (**3**). — A solution of 1.0 g (2.24 mmoles) of[3] **2** in 10 ml of tetrahydrofuran was added, with magnetic stirring, to a solution of 5 g of sodium hydrogen carbonate and 1.0 ml of bromine in 100 ml of water and 10 ml of tetrahydrofuran. The solution was stirred overnight at room temperature, and then adjusted with 2M hydrochloric acid to pH 2.5 (pH test-paper). The white solid was filtered off, and recrystallized from 95% ethanol to yield 579 mg (56%) of **3** as fine, colorless needles, m.p. 145–155°. The mother liquors were concentrated, to yield an additional 391 mg of product, bringing the total yield to 94%. Recrystallization from 95% ethanol raised the melting point of the product to 152.5–155°, $[\alpha]_D^{24}$ +3.30° (*c* 1.0, dichloromethane); $\nu_{max}^{CHCl_3}$ 1725 (carboxylic acid carbonyl) and 1735 cm^{-1} (benzoic ester carbonyl).

Anal. Calc. for $C_{26}H_{22}O_8$: C, 67.53; H, 4.76. Found: C, 67.95; H, 5.16.

Methyl 3,4,5-tri-O-benzoyl-2-deoxy-D-erythro-pentonate (**3a**). — To 10 ml of methanol containing 200 μl of bromine and 2.83 g of calcium carbonate was added 250 mg (460 μmoles) of **2**. The suspension was stirred magnetically for 20 h at room temperature, and the white solid was filtered off (from filtrate *A*) and then suspended in 5 ml of water. To this suspension was added, dropwise, 2M hydrochloric acid to decompose the excess of calcium carbonate, and the residual white solid (insoluble in the acidified solution) was extracted into dichloromethane. The extract was evaporated under diminished pressure to a white solid that was recrystallized from ethanol–water to yield a colorless, crystalline product (**3a**), m.p. 110–113°. The methanol solution (*A*) was concentrated to yield additional product which, when recrystallized, gave a total yield of 130 mg (50%). Recrystallization raised the melting point to 112–113.5°, $[\alpha]_D^{20}$ +0.49° (*c* 1.07, chloroform); $\nu_{max}^{CHCl_3}$ 1725 (aldonic ester carbonyl) and 1735 cm^{-1} (benzoic ester carbonyl); 60-MHz n.m.r.: singlet at τ 6.37 (methoxyl protons).

Anal. Calc. for $C_{27}H_{24}O_8$: C, 68.07; H, 5.04. Found: C, 67.99; H, 4.86.

3,4,5-Tri-O-benzoyl-2-deoxy-D-erythro-pentonyl chloride (**4**). — A solution of 1.50 g (3.42 mmoles) of **3** in 15 ml of *pure* thionyl chloride[9] was refluxed overnight, and concentrated to approximately half its volume under diminished pressure, and 5 ml of dry benzene was added. The solution was reconcentrated under diminished pressure, and this process was repeated three times. The resulting solid residue was dissolved in a large volume of warm cyclohexane, and the solution was concentrated to approximately half its volume under diminished pressure and kept overnight at room temperature. The product (**4**) crystallized in fine, colorless needles, weighing 1.152 g (74%) and having m.p. 118–121° and $[\alpha]_D^{24}$ +17.0° (*c* 0.83, dichloromethane).

Anal. Calc. for $C_{26}H_{21}ClO_7$: C, 65.00; H, 4.38; Cl, 7.29. Found: C, 65.18; H, 4.61; Cl, 6.87.

*4,5,6-Tri-O-benzoyl-1,3-dideoxy-1-diazo-*D-erythro-*hexulose* (**5**). — A solution of 810 mg (1.69 mmoles) of **4** in 75 ml of dry ether was slowly added to 50 ml of a magnetically stirred, freshly prepared solution of diazomethane in ether (containing approximately 30 mg of diazomethane per ml). The product began crystallizing within 5 min, and the suspension was stirred for 1 h at 0° and then refrigerated overnight. The product was filtered off, and dissolved in dry acetone; some acetone-insoluble, flocculent material was removed by filtration, and 2 ml of dry ether was added to the clear filtrate, followed by 2 ml of petroleum ether (b.p. 30–60°). The solution was refrigerated overnight, giving 565 mg of fine, pale-yellow granules. Concentration of the mother liquor, followed by the addition of a little ether, yielded an additional 170 mg, bringing the total yield of product (**5**) to 735 mg (95%), m.p. 137.5–144° (with darkening and decomposition), $[\alpha]_D^{24}$ −28.4° (*c* 1.0, dichloromethane).

*4,5,6-Tri-O-benzoyl-1,3-dideoxy-*D-erythro-*hexulose* (**6**). — (*a*) *From the diazomethyl ketone* (**5**). To a solution of 735 mg (1.51 mmoles) of **5** in 10 ml of chloroform was added 5 ml of 47% hydriodic acid[10], and the resulting solution was vigorously shaken for 5 min. It was washed successively with 3 ml of water, 3 ml of saturated, aqueous sodium thiosulfate (freshly prepared), and three 3-ml portions of water. The chloroform solution was dried with sodium sulfate, the suspension was filtered, and the filtrate was evaporated under diminished pressure to a yellow syrup that crystallized on trituration with ether. The crystals were dissolved in 95% ethanol, and the suspension was filtered through a bed of Celite to remove colloidal sulfur. The filtrate, on being cooled, yielded 581 mg (84%) of **6** as fine, colorless needles, m.p. 119.5–123°, $[\alpha]_D^{24}$ +21.8° (*c* 1.0, dichloromethane); $\nu_{max}^{CHCl_3}$ 1725 (C-2 carbonyl) and 1735 cm^{-1} (benzoic ester carbonyl); n.m.r. data: see discussion in text.

Anal. Calc. for $C_{27}H_{24}O_7$: C, 70.43; H, 5.24. Found: C, 70.53; H, 5.53.

(*b*) *From 3,4,5-tri-O-benzoyl-2-deoxy-*D-erythro-*pentose* (**2**). — To a solution of 92 mg (205 μmoles) of **2** in 2 ml of dry benzene, stirred magnetically (ice-bath), was added 5 ml of a solution of diazomethane in ether (containing approximately 30 mg of diazomethane per ml). The solution was kept for 2 h at 0°, and then evaporated under diminished pressure to a syrup, which was re-evaporated several times with dry ether to remove excess of diazomethane. The crude product, a white solid, was recrystallized from ether–pentane, to yield **6** as fine, colorless needles; yield 43 mg (46%), m.p. 119–123°, $[\alpha]_D^{24}$ +21.8° (*c* 1.0, dichloromethane).

*4,5,6-Tri-O-benzoyl-1,3-dideoxy-*D-erythro-*hexulose dimethyl dithioacetal* (**7**). — To 969 mg (2.11 mmoles) of **6** (cooled in an ice-salt bath to −18°) was added 40 ml of methanethiol, precooled to −18°. Hydrogen chloride was bubbled through the solution for 1 h, and the solution, saturated with hydrogen chloride, was kept for 2 h at −18°. The solution was allowed to warm slowly to room temperature to permit volatilization of the methanethiol, and the residue, a clear syrup, was repeatedly co-evaporated with dry ether to remove traces of methanethiol. The yield of syrup was 1.14 g (100%), $[\alpha]_D^{24}$ +17.5° (*c* 0.96, dichloromethane); C-2 carbonyl absorption

absent from i.r. spectrum; 100-MHz n.m.r.: singlet at τ 8.44 (C-1 methyl protons), singlets at τ 8.99 and τ 7.97 (3 protons each from C-2 methylthio groups), and a two-proton multiplet at τ 7.60 (C-3 methylene protons); R_F 0.55 [upper layer of 10:6:5:3 (v/v) ethyl acetate–methanol–water–heptane].

Anal. Calc. for $C_{29}H_{30}O_6S_2$: C, 64.68; H, 5.58; S, 11.90. Found: C, 64.64; H, 5.82; S, 12.65.

1,3-Dideoxy-D-erythro-*hexulose dimethyl dithioacetal* (**8**). — To a solution of 1.97 g (3.66 mmoles) of **7** in 50 ml of dry methanol, stirred in an ice-bath, was added 0.7 ml of 0.25M barium methoxide in methanol (immediately after the addition of barium methoxide, the odor of methyl benzoate was noted). The solution was kept for 20 min at 0° and for 24 h at room temperature. An excess of solid carbon dioxide was added to decompose the barium methoxide, and the mixture was briefly warmed. On cooling the mixture, barium carbonate was precipitated from the solution; the suspension was filtered, and the clear filtrate was evaporated under diminished pressure to a syrup. This was dissolved in absolute ethanol, and the solution was evaporated under diminished pressure to remove methyl benzoate and traces of water, giving the product (**8**) as a syrup; $[\alpha]_D^{24}$ −37.8° (*c* 7.4, chloroform); R_F 0.088 [10:1 (v/v) chloroform–methanol].

1,3-Dideoxy-D-erythro-*hexulose* (**9**). — To 176 mg (38 μmoles) of **6** suspended in 50 ml of dry methanol was added, with magnetic stirring, 10.2 mg of freshly cut sodium. Within 10 min, the suspended solid had dissolved, and the solution was kept for 20 h at room temperature. T.l.c. [10:1 (v/v) chloroform–methanol] indicated a major component, and a minor component of higher R_F value. The methanol solution was concentrated under diminished pressure to approximately one-third its volume, 1 g of mixed-bed ion-exchange resin [Fisher Rexyn-300 (H^+, OH^-)] was added to the solution, and the suspension was stirred for 0.5 h. The resin was filtered off, and the filtrate was evaporated under diminished pressure to a syrup. The syrup was dissolved in water, and the solution was washed once with ether, and evaporated to a clear syrup that weighed 35 mg. T.l.c. of the impure **9** disclosed a major component having R_F 0.805 [upper layer of 10:6:5:3 (v/v) ethyl acetate–methanol–water–2,2,4-trimethylpentane]; $\nu_{max}^{CHCl_3}$ 1725 cm^{-1} (C-2 carbonyl).

6-O-(*1-Adamantoyl*)-*1,3-dideoxy*-D-erythro-*hexulose dimethyl dithioacetal* (**10**). — To a solution of 256 mg (800 μmoles) of **8** in 5 ml of dry benzene (ice-bath), was added slowly, with magnetic stirring, a solution of 226 mg (1.14 mmoles) of 1-adamantoyl chloride in 2 ml of pyridine. The solution was kept for 4 days at 0°, the pyridinium chloride was filtered off, and the filtrate was washed with 5 ml of dilute, aqueous sodium hydrogen carbonate. To the mixture was added 5 ml of ethyl acetate, the mixture was shaken, and the aqueous phase was separated. The ethyl acetate solution was washed with 5 ml of water, dried with sodium sulfate, and evaporated to a syrup which began crystallizing after standing for one week at room temperature. The product (**10**) was recrystallized from 2:1 (v/v) ether–hexane to yield 26 mg (8.4%), m.p. 89–92°, $[\alpha]_D^{24}$ −26.5° (*c* 0.804, chloroform); $\nu_{max}^{CHCl_3}$ 1725 cm^{-1} (adamantoyl carbonyl); 100-MHz n.m.r.: singlet at τ 8.84 (C-1 methyl protons), a two-proton

multiplet at τ 7.99 (C-3 methylene protons), singlets at τ 7.98 and τ 7.92 (C-2 methylthio protons), and multiplets at τ 8.29 and τ 8.09 (adamantoyl protons); R_F 0.64 [10:1 (v/v) chloroform–methanol].

Anal. Calc. for $C_{19}H_{33}O_4S_2$: C, 58.61; H, 8.48; S, 16.45. Found: C, 58.61; H, 8.35; S, 16.0.

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THE *O*-ACETYL GROUPS OF THE SPECIFIC SUBSTANCE FROM *Pneumococcus* TYPE 34 (U.S. TYPE 41)†

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ABSTRACT

Partial, acid hydrolysis of the specific substance, S.34, of *Pneumococcus* Type 34 (U.S. Type 41) yields 2-*O*-α-D-glucopyranosyl-D-galactose (**2**). When deacetylated S.34 is degraded by periodate oxidation followed by reduction with borohydride, partial hydrolysis with acid yields 2-*O*-α-D-glucopyranosyl-L-arabinose (**4**); a similar degradation of S.34 itself yields a mixture of disaccharides **2** and **4**. Taken with earlier work, these observations show that 6(or 5)-*O*-acetyl-2-*O*-α-D-glucopyranosyl-β-D-galactofuranose occurs as part of the repeating unit of S.34. Deacetylation of S.34 by ammonia treatment causes a partial loss of immunological activity.

INTRODUCTION

Earlier work from this laboratory[1,2] has shown that the type-specific substance from Type 34 *Pneumococcus* (S.34) consists of a pentasaccharide repeating unit (**1**) linked by phosphodiester bridges. In addition, an *O*-acetyl group is present on about half of the units, in such a position that periodate oxidation of a grouping $HOCH_2CH(OH)$- is prevented[2]. This paper, and the related one by Roy and Glaudemans[3], describe further work on the location of the *O*-acetyl group and the effect of deacetylation on the immunological activity of S.34.

EXPERIMENTAL

Materials. — The capsular substance from *Pneumococcus* Type 34 was supplied by Dr. Rachel Brown[4]. It was the same preparation (No. 1) that we have used previously[1,2]. The preparation contained *ca.* 20% of C-substance[1,2] and, for most of the experiments, was used without further purification.

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†Because of an editorial oversight, the publication of this paper, which should have appeared alongside the related paper by Roy and Glaudemans [*Carbohyd. Res.*, 8 (1968) 214], has been delayed.

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The S.34 rabbit antiserum was kindly given to us by Dr. Erna Lund, Staten Seruminstitut, Copenhagen. It was produced in 1941 and had a capsular titre of 256.

Methods. — Paper chromatography was carried out with Whatman No. 1 or No. 4 paper and butyl alcohol–pyridine–water[5] (6:4:3, by vol.). The glycosides were detected by the periodate–Schiff reagents[6,7].

Immunological investigation. — Preparations were examined by the Ouchterlony double-diffusion technique[8]. The gel was made up from 2% Ionagar (Oxoid) in 0.05M glycine buffer (pH 7.4) containing 0.9% of sodium chloride. In the centre well was S.34 antiserum (10 μl) and, in the outer wells, 6 μl of the samples to be tested.

In one experiment, the unpurified[1–3] S.34 (1 mg/ml) was compared with a sample (1 mg/ml) purified by ion-exchange chromatography[2]. The samples were indistinguishable.

In a second experiment, the unpurified S.34 (2 mg) was dissolved in water (1 ml), conc. aqueous ammonia (sp. gr. 0.88, 1 ml) was added, and the solution was kept for 24 h at 37°. Ammonia was removed by aeration, the remaining solution was freeze-dried, and the residue was dissolved in water (1 ml). The solution was compared immunologically with untreated S.34 (1 mg/ml). Whereas authentic S.34 gave a sharp line of precipitation, the sample which had been treated with ammonia gave a weak, diffuse band, which crossed that from S.34.

Partial, acid hydrolysis of S.34. — S.34 (6 mg) was hydrolysed with 0.1N hydrochloric acid for 1 h at 100°. The hydrolysate was evaporated to dryness *in vacuo*, and the residue was examined by paper chromatography. The only product giving a yellow colour with periodate and Schiff's reagent[7] was the compound previously identified[1] as 2-*O*-α-D-glucopyranosyl-D-galactose.

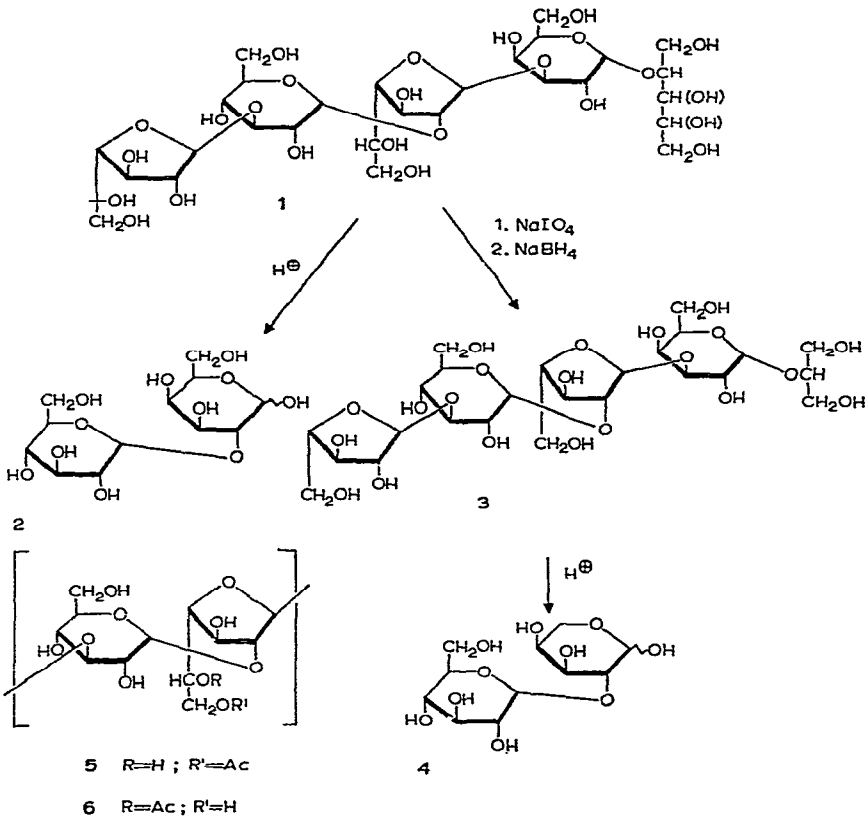
Smith degradation of O-*deacylated S.34.* — A sample of S.34 (6 mg), which had been treated with ammonia as described above, was oxidised with 0.05M aqueous sodium metaperiodate (1 ml) at 20° in the dark for 24 h. Sodium borohydride (10 mg) was added, the solution was kept for 16 h at 20°, and a few drops of acetic acid were added to decompose the excess of borohydride. The solution was passed through a column of Dowex-50 (H^+) resin (3 ml) and evaporated to dryness *in vacuo*, and boric acid was removed from the residue by several evaporations with methanol. The product was hydrolysed with 0.1N hydrochloric acid for 1 h at 100°, the solution was evaporated to dryness *in vacuo*, and the residue was examined by paper chromatography. The only product giving a yellow colour with periodate and Schiff's reagent was chromatographically indistinguishable from 2-*O*-α-D-glucopyranosyl-L-arabinose[1].

Smith degradation of S.34. — When the above degradation sequence was carried out on a sample of S.34 (6 mg), approximately equal quantities of the glucosyl-galactose and glucosylarabinose were detected.

RESULTS AND DISCUSSION

It was shown previously[1] that partial, acid hydrolysis of the pentasaccharide **1** resulted in the preferential cleavage of the glycosidic linkages of the galactofuranose

residues *A* and *B* to yield galactose, 2-*O*-α-D-glucopyranosyl-D-galactose (2) and 2-*O*-α-D-galactopyranosylribitol. The disaccharide **2** could be recognised readily on paper chromatograms by its colour reactions[1,7]. When the pentasaccharide **1** was oxidised briefly with periodate and the resulting polyaldehyde reduced with borohydride, partial hydrolysis[9] of the resulting polyalcohol **3** with acid gave, as one of the products, 2-*O*-α-D-glucopyranosyl-L-arabinose (4), with none of the disaccharide **2** as a contaminant[1].



When S.34 was hydrolysed with 0.1N hydrochloric acid for 1 h, disaccharide **2** was a major product. A sample of S.34, which had been deacetylated with ammonia[2], was oxidised briefly with periodate and then reduced with borohydride, and the product was subjected to partial hydrolysis with acid; the glucosylarabinose **4** was observed on paper chromatograms in place of disaccharide **2**.

When the same degradation was carried out on a sample of S.34 which had not been treated with ammonia, approximately equal quantities of the disaccharides **2** and **4** were produced. In the structure of S.34, it is necessary to account for 0.44 mol. proportions of *O*-acetyl group for each repeating unit[2]. Our present result can be interpreted in terms of partial structures **5** or **6**, in which this part of the repeating

unit carries an *O*-acetyl group on the C-6 or C-5 hydroxyl group in approximately half of the units.

S.34, which had been treated with ammonia, showed a much weaker reaction, of altered specificity, towards S.34 antiserum, when examined by the gel-diffusion technique[8]. Apart from loss of *O*-acetyl groups, no further degradation of S.34 by ammonia would be expected. None is apparent from the analytical figures[2], and Roy and Glaudemans[3] have further shown that when the ammonia-treated product is dialysed only the non-dialysable fraction shows immunological interaction with Type 34 antiserum. It appears, therefore, that an *O*-acetyl group located on the galactofuranose unit, as in structure **5** or **6**, is intimately concerned with the immunological specificity of S.34. The related paper by Roy and Glaudemans[3] provides strong confirmatory evidence, and identifies the acyl group as acetyl and places it on the C-6 hydroxyl group of a galactofuranose residue. We conclude that partial structure **5** represents the position of the *O*-acetyl group in S.34.

O-Acetyl groups are present in a number of other type-specific substances from Pneumococci, *e.g.*, S.1 (Ref. 10), S.11A (Ref. 11), and S.18 (Ref. 12), and, in these three cases, are concerned in the immunological specificity. The fact that, in our preparation of S.34, there is less than 1 mol. proportion of *O*-acetyl group for each repeating unit may be due to loss of ester groups during growth or isolation[13], in spite of the mild conditions used[4,14].

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REACTION OF STARCH WITH CARBOHYDRATE *trans*-CARBONATES*

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ABSTRACT

Mono- and poly-saccharides possessing certain substituted, vicinal, *trans*-hydroxyl groups are readily substituted onto starch. Such substitution occurs when a *trans*-fused, cyclic carbonate derivative of the saccharide is added to starch in the presence of a basic catalyst. Excellent yields of products result on addition of methyl 4,6-*O*-benzylidene-α-D-glucopyranoside 2,3-carbonate (or 2,3-thionocarbonate), and methyl 2,6-di-*O*-(methylsulfonyl)-α-D-gl· opyranoside 3,4-carbonate to starch in the presence of triethylamine. Dextri. onate of degree of substitution (DS) 0.40 and dextran carbonate of DS 0.31 re. with starch under various conditions, in the presence of different catalysts, to give corresponding polysaccharide copolymers.

INTRODUCTION

Sensitivity to alkali and relative stability to acid have made the carbonate group a useful protecting agent in many organic syntheses. In the preparation of selectively substituted carbohydrates, the carbonate group can frequently be used to protect certain hydroxyl groups while a reaction is conducted at other hydroxyl groups in the molecule. Except for subsequent removal of the protecting group by hydrolysis, few examples of further transformations of the carbonate group in carbohydrates have been reported.

Recently, we prepared a cyclic carbonate from vicinal *trans*-hydroxyl groups on pyranoid rings both by oxidation of the analogous thionocarbonate[1] and by treatment of the *trans*-diol with ethyl chloroformate and triethylamine[2]. By the latter method, methyl 4,6-*O*-benzylidene-α-D-glucopyranoside 2,3-carbonate (**1**) was prepared in 90% yield from the corresponding 2,3-diol. This novel *trans*-carbonate group readily underwent opening of the carbonate ring on treatment of **1** with a simple alcohol, amine, or thiol[3]. For example, treatment of **1** with methanol and

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triethylamine at 25° gave an immediate and quantitative conversion into methyl 4,6-*O*-benzylidene-2(and 3)-*O*-(methoxycarbonyl)-α-D-glucopyranosides.

Further consideration of the strained, *trans*-fused, carbonate group suggested that conditions might be found which would permit reaction of this group with hydroxyl groups in more complex molecules, such as starch. Such reaction might provide a facile route for the preparation of polysaccharides having pendant sugar residues. Variation in structure of the carbohydrate moiety that contains the *trans*-fused carbonate grouping would permit formation of various starch derivatives having a broad spectrum of properties. The present work was undertaken to determine whether polysaccharides would undergo reaction with carbohydrate *trans*-carbonates.

RESULTS AND DISCUSSION

Studies were made initially with starch and the 2,3-carbonate **1** in a homogeneous system. Starch and **1** (0.5 mole per mole of D-glucopyranosyl residue of starch) were dissolved in methyl sulfoxide, and triethylamine was added. The mixture was stirred in a closed vessel, and portions were removed periodically for analysis by i.r. spectroscopy. Initially, carbonyl absorption was observed only in the region near 1825 cm^{-1} assigned[1] to the cyclic carbonate group of **1** (Fig. 1A). As the reaction

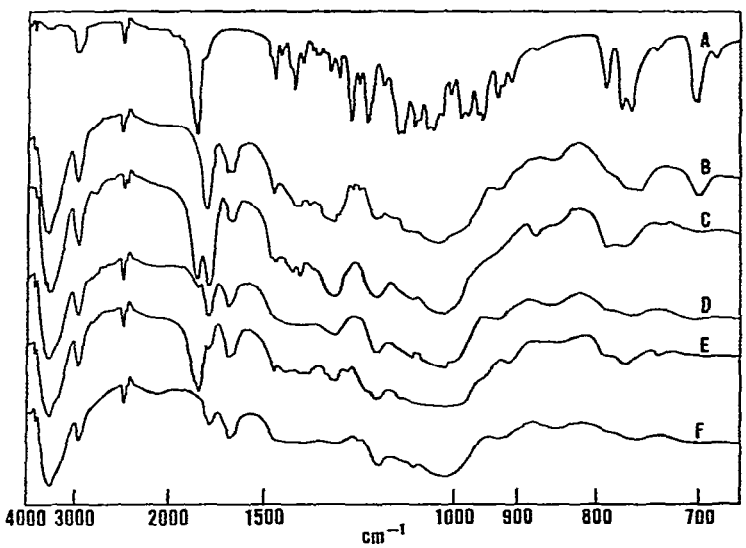
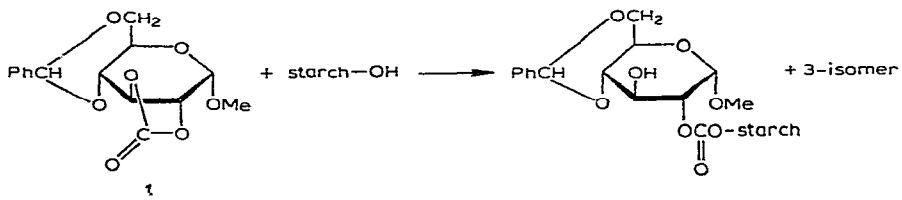


Fig. 1. Infrared spectra in KBr: (A) methyl 4,6-*O*-benzylidene-α-D-glucopyranoside 2,3-carbonate (**1**); (B) reaction product of starch with **1**; (C) dextrin carbonate (**4**); (D) reaction product of starch with **4**; (E) dextran carbonate (**5**); (F) reaction product of starch with **5**.

progressed, absorption appeared near 1750 cm^{-1} (acyclic carbonate), while absorption near 1825 cm^{-1} decreased in intensity. After 16 h, there was carbonyl absorption at 1750 cm^{-1} only. Methyl sulfoxide and amine were removed under diminished pressure, and the residue was extracted with acetone. The i.r. spectrum of the residue

showed carbonyl absorption at 1750 cm^{-1} and phenyl absorption at 700 cm^{-1} (Fig. 1B). Based on the weight of the residue and on the content of carbonate group, about 80% of the carbonate **1** had reacted with starch. Some hydrolysis of **1** to methyl



1

4,6-*O*-benzylidene-α-D-glucopyranoside also occurred during the reaction, as was revealed by t.l.c. of the acetone extract.

When 3 moles of carbonate **1** per mole of D-glucopyranosyl residue of starch was used in the reaction, much unreacted carbonate **1** remained after 16 h. Increase of the reaction time beyond 16 h caused little additional change in the ratio of carbonyl absorptions at 1825 and 1750 cm^{-1}. The starch product isolated from this reaction had a DS for carbonate of 0.74. Since a higher DS was not obtained, it appeared that reaction might have occurred selectively at the less sterically hindered, primary hydroxyl groups in starch, and that little or no reaction had taken place at the hydroxyl groups on C-2 and C-3. However, this possibility was rejected when it was found that 6-*O*-tritylstarch reacts readily with **1** to give a product having a DS as high as 0.9. No further attempts were made to determine the points of attachment between starch and the monosaccharide.

No reaction occurred between starch and **1** in methyl sulfoxide when triethylamine was replaced by pyridine; but, surprisingly, the extent of reaction was substantial when the amine was replaced by aqueous potassium hydroxide. Solutions of starch and **1** (0.8 mole of **1** per mole of D-glucopyranosyl residue of starch) in methyl sulfoxide were treated with various proportions of potassium hydroxide (see Table II, in Experimental Section). The DS of the starch product increased from 0.17 to 0.31 as the amount of alkali added was decreased from 1 mmole to 50 μmole. Further decreases in the amount of alkali to 12 μmole lowered the DS of the product to 0.03.

Although the extent of reaction between starch and **1** was considerable in the methyl sulfoxide system, the reaction was found to be even more efficient when conducted in water. An aqueous suspension of gelatinized starch containing triethylamine was treated with an acetone solution of **1** (0.2 mole per mole of D-glucopyranosyl residues). After vigorous mixing for 10 min, acetone was added, and the precipitate was collected. Both the yield and the carbonate content of the precipitate showed that all of the carbonate **1** added had reacted with the starch. The proportion of triethylamine added is critical. For example, a quantitative yield was obtained when 20 ml of a 2% suspension of starch contained 40 μl of triethylamine. When as much as 1 ml of amine was used in a similar reaction, about 60% of **1** reacted to give the

starch product, but 40% of the **1** was hydrolyzed to the diol. A small amount of amine ($<10\ \mu l$) resulted in low yields of product even after 1 h, and most of the **1** added was recovered unchanged.

Replacement of triethylamine with sodium carbonate in the aqueous system afforded good yields of starch product, whereas replacement with sodium hydrogen carbonate or pyridine gave none.

Methyl 4,6-*O*-benzylidene-α-D-glucopyranoside 2,3-thionocarbonate (**2**) and methyl 2,6-di-*O*-(methylsulfonyl)-α-D-glucopyranoside 3,4-carbonate (**3**) also reacted with starch in methyl sulfoxide to form their respective starch products in 70–80% yields. Whereas the thionocarbonate (**2**) reacted at a rate similar to that of the 2,3-carbonate **1**, reaction of the 3,4-carbonate **3** was more rapid. After the solution of starch and **3** in methyl sulfoxide had been stirred for 2 h, only acyclic carbonyl absorption at 1750 cm^{-1} was detected. If the reaction between starch and **2** or **3** was conducted under aqueous conditions, the yields of product were low.

The methods employed for recovery of the products formed on treatment of starch with **1**, **2**, and **3** also recover unreacted starch. For the purpose of the present study, the primary concern was the extent to which the monosaccharide carbonates would react with starch, and no attempts were made to determine the proportion of unreacted starch that might have been present in the materials isolated.

Reaction between starch and *trans*-carbonates is not restricted to monosaccharide carbonates, but also proceeds well with polysaccharide carbonates. The *trans*-carbonate group was introduced into a starch dextrin of molecular weight about 5,000 and into a dextran of molecular weight 15,000–20,000. Formation of polymeric carbonates was accomplished by treating solutions of the polysaccharides in methyl sulfoxide with triethylamine and ethyl chloroformate. The products were isolated after a reaction time of a few min. The dextrin carbonate (**4**) had absorptions at 1825 and 1750 cm^{-1} (Fig. 1C) that indicated that cyclic and acyclic carbonate groups were both present. The ratio of these two peaks showed a slight preponderance of the acyclic form. The DS of total carbonate was near 0.4.

The dextran carbonate (**5**) had carbonyl absorption mostly for cyclic carbonate groups (Fig. 1E); the DS of the product was 0.31. The difference in the ratio of cyclic to acyclic carbonate groups in **4** *versus* **5** might be explained on the basis of the difference in structure of the two polymers. Dextran is an α-D-(1→6)-linked polymer of D-glucopyranosyl residues and has only vicinal hydroxyl groups available for reaction, whereas dextrin is a D-glucopyranosyl polymer consisting mostly of α-D-(1→4)-linked residues, and it has an isolated hydroxyl group on C-6, in addition to vicinal diol groups available for reaction.

Both polysaccharide carbonates **4** and **5** gave clear solutions in water. Since these polymers contained many cyclic carbonate groups, it was expected that, on reaction with starch, an insoluble, cross-linked product would result. Indeed, a gelatinous precipitate was formed rapidly when a dispersion of starch was treated with an aqueous solution of **4** or **5**, followed by addition of a small proportion of triethylamine. The precipitates were collected, and the content of the carbonate group

was determined. The i.r. spectrum of each showed carbonyl absorption for acyclic carbonate groups only (see Figs. 1D and F).

Table I shows the yields and degrees of substitution of the precipitates when aqueous suspensions of gelatinized starch were treated with various proportions

TABLE I

YIELDS AND DS[a] OF PRODUCT OBTAINED ON REACTION OF STARCH[b] WITH DEXTRAN CARBONATE (5)

Centrifuge tube	*Weight of 5 added, mg*	*Weight of product, mg*	*DS[a] of product*
A	400	450	0.10
B	200	385	0.10
C	100	330	0.08
D	200[c]	0	0

[a]Degree of substitution. [b]Gelatinized-starch suspensions (2%, 20 ml). [c]Triethylamine was not added. [d]DS determined from the content of carbonate.

of 5. The DS of the carbonate group indicates that, in each experiment, the product consists of about 2 parts of starch and 1 part of dextran. Based on this indication, the proportion of starch precipitated decreased from about 75% when equal amounts of starch and 5 were mixed to about 55% when 1 part of 5 was added to 4 parts of starch. In tube D, triethylamine was omitted, and no precipitate formed.

When an aqueous solution of 5 was treated with triethylamine, the acyclic carbonate was formed readily, but did not precipitate from solution. Also, no precipitate formed when this solution of dextran acyclic carbonate was added to a starch suspension. These observations support the supposition that interpolymer crosslinks are formed during treatment of starch suspensions with 5 in the presence of triethylamine. It is conceivable that the precipitates formed might entrap unreacted polysaccharide, but repeated extraction with water failed to remove significant proportions of material.

It is not known how many of the acyclic carbonate groups are in the form of crosslinks between starch and dextran. Probably, most of the acyclic carbonates are formed exclusively within the dextran molecule, and relatively few are crosslinks between the two polysaccharides. This probability would explain why some of the starch was not precipitated, even though the aqueous suspension remaining after removal of the precipitate showed strong carbonyl absorption for the acyclic carbonate groups.

EXPERIMENTAL

I.r. spectra were recorded with a Perkin–Elmer* Model 137 spectrophotometer. Commercial corn-starch was used. The dextrin and dextran were kindly furnished by

*The mention of firm names or trade products does not imply that they are endorsed or recommended by the Department of Agriculture over other firms or similar products not mentioned.

Dr. Allene R. Jeanes of this Laboratory. Dextrin having an average degree of polymerization of 30 was obtained from an acid hydrolyzate of waxy maize. The dextran was an industrial grade (molecular weight 15,000–20,000) from Pharmachem Corp., Bethlehem, Pa. For t.l.c., Silica Gel G was the adsorbent, ethyl acetate was the solvent, and 18:1 (v/v) methanol–sulfuric acid was the spray reagent.

Reactions of starch in methyl sulfoxide with **1**. — *A*. To a solution of starch (250 mg, 1.54 mmoles) in methyl sulfoxide (5 ml), **1** (238 mg, 770 μmoles) and triethylamine (1.5 ml) were added. After the mixture had been stirred overnight, the solvent and amine were evaporated off under diminished pressure at 60°. The product was washed successively with ether and acetone, and dried to constant weight; yield, 438 mg; λ_{max}^{KBr} 1750 cm^{-1} [acyclic O(C=O)O, see Fig. 1B]. Digestion of the product (100 mg) with 20 mM barium hydroxide (5 ml) for 1 h on a steam bath followed by titration of excess base with 10 mM sulfuric acid revealed a DS of 0.38 (a second determination gave 0.39).

T.l.c. of the acetone washings showed a component having an R_F value identical with that of methyl 4,6-*O*-benzylidene-α-D-glucopyranoside.

B. To a solution of starch (125 mg, 770 μmoles) in methyl sulfoxide (12 ml) were added **1** (710 mg, 2.31 mmoles) and triethylamine (2 ml). The mixture was stirred overnight, and the product was isolated as in part *A*; yield, 340 mg; λ_{max}^{KBr} 1750 cm^{-1} [O(C = O)O]; DS 0.74.

C. Solutions of starch (250 mg) and **1** (250 mg) in methyl sulfoxide (5 ml) were treated with different concentrations of aqueous potassium hydroxide (0.5 ml). The alkali was added dropwise during 5 min, after which time the mixture was kept for an additional 5 min. Several volumes of 18:1 (v/v) acetone–acetic acid were added, and the resulting precipitate was washed successively with ethanol and ether, and dried. For each concentration, the yield of product ranged from 320 to 340 mg. The DS of the products was determined for each concentration of alkali added (see Table II).

TABLE II

EFFECT OF CONCENTRATION OF POTASSIUM HYDROXIDE ON THE DS OF THE STARCH PRODUCT

KOH added (μmoles)	1000	500	250	50	25	12
DS of product	0.17	0.23	0.24	0.31	0.18	0.03

When the experiment with 50 μmoles of potassium hydroxide was repeated, but with 25 ml of methyl sulfoxide, the DS of the product was 0.22.

A DS of 0.37 was realized when 50 μmoles of potassium hydroxide was used with 5 ml of methyl sulfoxide and the reaction mixture was kept for 5 min at 50 or 68°.

Reaction of gelatinized starch with **1**. — Starch (10 g, 61.7 mmoles) was slurried in water (500 ml), and the suspension was heated to 95° with stirring. After being kept for 1 h at this temperature, the dispersion was cooled, and water lost during

heating was replaced. To 20 ml of this dispersion (containing 400 mg of starch) in a microblender was added 1 ml of triethylamine solution (0.1 ml of triethylamine in 5 ml of water). The mixture was agitated while a solution of **1** (152 mg) in acetone (3 ml) was slowly added. After the mixture had been blended for 5 min, amine solution (1 ml) was added. Blending was continued for 5 min, and the mixture was neutralized to the phenolphthalein end-point with 5M acetic acid. After acetone (300 ml) had been added, the precipitate was collected, washed successively with acetone and ether, and dried at 80° to constant weight; yield 550 mg (*ca.* 100%); λ_{max}^{KBr} 1750 cm^{-1} [O(C=O)O]. Digestion of the product (100 mg) with barium hydroxide as described previously revealed a DS of 0.20.

When the reaction between starch and **1** was repeated, but with less amine (1 ml of 0.2% triethylamine in water), the yield was lower, and the product had only slight spectral absorption at 1750 cm^{-1}. Unreacted **1** (about 75% of the amount added) was recovered from the acetone washings. The use of a longer reaction time (1 h) failed to increase the yield of the starch product.

When the reaction was conducted with more triethylamine (for example, 1 ml), about 60% of **1** reacted with the starch, and 40% was hydrolyzed to methyl 4,6-*O*-benzylidene-α-D-glucopyranoside. The latter compound was recovered from the acetone washings, and identified by t.l.c. and i.r. spectrum.

Treatment of the starch suspension (18 ml) with potassium hydroxide (100 mM, 1 ml) and **1** (400 mg in 1 ml of acetone) for 5 min gave a product having a DS of 0.15.

Replacement of triethylamine by aqueous sodium carbonate gave good yields of starch product, whereas, with sodium hydrogen carbonate or pyridine, the precipitated starch showed no spectral absorption for a carbonyl group.

Reaction of starch with **2**. — The reaction of starch (250 mg, 1.54 mmoles) with **2** (250 mg, 770 μmoles) in methyl sulfoxide (5 ml) was conducted in a manner similar to that used for **1** (part *A*). The yield of starch product was 440 mg, which indicated that about 76% of the **2** had reacted with the starch. The i.r. spectrum (KBr) of the product showed absorption at 1280 cm^{-1} [O(C=S)O]. The product contained 3.92% of sulfur, corresponding to a DS of 0.33.

Reaction of starch with **3**. — A solution of starch (250 mg, 1.54 mmoles) in methyl sulfoxide (5 ml) was treated with **3** (290 mg, 770 μmoles) and triethylamine (1.5 ml). After the mixture had been stirred for 2.5 h, no cyclic carbonate absorption (1825 cm^{-1}) for **3** was apparent in the i.r. spectrum of the reaction mixture. The starch product was isolated as described for **1** part, *A*; yield 450 mg; λ_{max}^{KBr} 1750 cm^{-1} [O(C=O)O]. The dried product contained 8.05% of sulfur, corresponding to a DS of 0.39.

When an aqueous dispersion of starch was treated with **3** in a manner similar to that used for **1**, yields of the starch product were very low.

Reaction of 6-O-tritylstarch with **1**. — A solution of 6-*O*-tritylstarch (5%, 5 ml) and **1** (250 mg) in methyl sulfoxide was triturated in a mortar with potassium hydroxide solution (0.5 ml, 100 mM) added dropwise during 5 min. After being kept for an additional 5 min at 25°, the product was precipitated with about 200 ml of water,

centrifuged, and successively washed thoroughly with acetone, ether, alcohol, and ether, to yield 210 mg of starch derivative having a DS of 0.36. A DS of 0.9 was obtained when the experiment was repeated with a higher concentration of alkali (500 mM) and a reaction temperature of 55°.

Preparation of dextrin carbonate (**4**), *and reaction with starch.* — Dextrin carbonate (**4**) was prepared as follows: Dextrin (5 g) was dissolved in methyl sulfoxide (50 ml), and triethylamine (25 ml), and *p*-dioxane (15 ml) were added portionwise. The mixture was cooled to 5°, and ethyl chloroformate (10 ml) was added dropwise, with stirring, during 20 min. The dextrin product was precipitated with ether, and washed several times with ether and then with three 50 to 100-ml portions of 90% ethanol, with subsequent washing with absolute ethanol and ether. The yield for several preparations of **4** lay between 3.5 and 4.0 g; it had a DS of 0.35–0.40 (cyclic plus acyclic carbonate); λ_{max}^{KBr} 1825 cm^{-1} and 1750 cm^{-1} in the ratio of 2:3 (see Fig. 1C).

Dextrin carbonate (**4**, 250 mg) in water (1 ml) was added to a gelatinized starch suspension (2%, 10 ml) together with one drop of triethylamine. After the mixture had been stirred for 2–3 min at 25°, it was centrifuged, and the precipitate was washed with three 25-ml portions of water, twice with alcohol, and then with ether; yield, 185 mg; DS 0.12; λ_{max}^{KBr} 1750 cm^{-1} (carbonyl) (see Fig. 1D). In some preparations, compound **4** was obtained as a horny powder that would not dissolve completely in water; therefore, it might be advantageous to treat the starch directly after washing **4** with 90% ethanol.

Preparation of dextran carbonate (**5**), *and reaction with starch.* — Dextran carbonate (**5**) was prepared as follows. To a solution of dextran (2 g) in methyl sulfoxide (20 ml) were added *p*-dioxane (3 ml) and triethylamine (10 ml). The mixture was cooled in an ice–water bath and stirred while ethyl chloroformate (4 ml) was added dropwise. After 2 min, sufficient hydrochloric acid (5M) was added to bring the pH to 6, and then the mixture was added to 400 ml of aqueous ethanol (90%) in a blender. The resultant precipitate was successively washed with 90% ethanol, absolute ethanol, and ether, and dried; yield, 1.9 g. The i.r. spectrum (see Fig. 1E) of the product (**5**) showed strong absorption for the cyclic carbonate group (near 1825 cm^{-1}). Digestion of a sample of **5** with barium hydroxide gave a product having a DS of 0.31.

When the reaction was repeated, but with more ethyl chloroformate, the DS of cyclic carbonate was higher. However, these products were not water-soluble, whereas **5** dissolved readily in water.

Portions (20 ml) of a 2% suspension of gelatinized starch were placed in each of four 40-ml centrifuge tubes. To tubes A, B, and C was added water (3 ml) which contained **5** (400 mg, 200 mg, and 100 mg, respectively). The contents of the tubes were stirred, and 0.1 ml of triethylamine was added. Tube D contained water (3 ml) and **5** (200 mg), but no triethylamine. All of the tubes were immediately centrifuged for about 1 min, and the gelatinous precipitates that formed in tubes A, B, and C were collected. No precipitate had formed in D. Each precipitate was successively washed with water, ethanol, and ether, and dried to constant weight. The amounts

of product formed and the DS of carbonate, as determined by digestion with barium hydroxide, are shown in Table I.

In a separate, control experiment, an aqueous solution of **5** was treated with triethylamine. No precipitate was formed, even though the solution was kept for several h. Several volumes of acetone were added, and the precipitate that resulted exhibited carbonyl absorption at 1750 cm^{-1} only.

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PROBLEMS OF LACTONISATION IN THE ANALYSIS OF URONIC ACIDS

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ABSTRACT

Uronic acids are shown to be subject to irreproducible lactonisation under conditions used in hydrolysis of polysaccharides. Methods have been devised for hydrolysis of such lactones and for their quantitative separation from reducing sugars without alkaline degradation or rearrangement.

INTRODUCTION

Uronic acid constituents of animal and plant polysaccharides are normally identified by acid hydrolysis and subsequent paper chromatography[1], ionophoresis[2], or chromatography on ion-exchange resins[3]. In all such cases, it is highly desirable to separate uronic acids from neutral and amino sugars which are also commonly present in the hydrolysate. The hydrolytic procedures, however, cause extensive lactonisation of uronic acids, and the lactones, behaving as neutral substances during ion-exchange separations, are frequently obtained in the neutral sugar fraction with resultant confusion of chromatographic results. This situation is not readily avoided by hydrolysis of the lactones with alkali, since both the neutral sugars and the lactones are very readily degraded and rearranged by mild alkalies[4a,4b,5].

For the quantitative determination of uronic acids in polysaccharide hydrolysates, recent g.l.c. methods[6] show considerable promise in comparison with previous methods. The hexuronic acids are reduced to the corresponding aldonic acid with sodium borohydride, and the trimethylsilyl ethers of the aldono-1,4-lactones are subjected to g.l.c. Such a quantitative procedure, however, would be invalidated by incomplete prior separation of lactonised uronic acids from neutral sugars.

This paper reports the development of procedures for the quantitative hydrolysis and recovery of uronic acid lactones under conditions which minimise alkaline degradation. The procedures will be applied to polysaccharide hydrolysates in our subsequent work.

EXPERIMENTAL

Reagents and samples. — Ion-exchange resins, Amberlite IR-120(H^+), IRA-400(Ac^-), and IR-45(OH^-) (Rohm and Haas), were analytical grade and were

prepared as recommended[7]. Acetic acid and acetic anhydride were distilled prior to use. Anhydrous pyridine was prepared by standing reagent-grade pyridine over potassium hydroxide pellets for 12 h at room temperature and then distilling from phosphoric oxide. Uronic acids and D-glucurono-6,3-lactone were commercial preparations (B. D. H. Biochemicals) used without further purification. Solvents for paper chromatography on Whatman No. 1 paper were ethyl acetate–pyridine–water (8:2:1) (Solvent *A*), and ethyl acetate–acetic acid–formic acid–water (18:3:1:4) (Solvent *B*). Distilled and subsequently deionised water was used throughout.

Acid-induced lactonisation of D-*glucuronic and* D-*galacturonic acids.* — Hexuronic acid (*ca.* 30 mg) was dissolved in 72% sulphuric acid[8] (0.1 ml) and heated for 1 h at 30 ±0.5°. This will be referred to as the "primary treatment". Dilution with 2.8 ml of water was followed by "secondary treatment" in a sealed tube immersed in a boiling-water bath for 3 h.

Ion-exchange separation of lactone and free acid on Amberlite IRA-400(Ac⁻) resin. — The above solutions were passed through columns of 0.9-cm diameter (prepared with 8 ml of resin) at *ca.* 1 ml/min, and elution of neutral components was completed by washing with 4–5 bed-volumes of water. Control experiments demonstrated that these conditions resulted in complete sorption of uronic acid. Uronic acids were recovered quantitatively by eluting the column with either 6 bed-volumes of N sulphuric acid or 16 bed-volumes of 10% acetic acid (v/v).

The phenol–sulphuric acid procedure[9] was used for colorimetric measurement both of lactone and free acid in aliquot portions of the respective column effluents. Total combined recovery of acid and lactone was 95–96%. The results are shown in Table I.

TABLE I

EXTENT OF LACTONISATION FOLLOWING TREATMENTS WITH SULPHURIC ACID

Sample	*Primary treatment*	*Secondary treatment*
D-Glucuronic acid	89.2%	64.2%
D-Galacturonic acid	38.7%	51.7%

When the neutral effluent was examined after the ion-exchange separation of the components of the secondary treatment, it was found to contain only the corresponding hexuronic acid lactone. In each case, the lactone was identified as the corresponding acid by co-chromatography on paper with authentic samples using the solvent *B* for 60–70 h.

Lactonisation of D-*glucuronic acid in aqueous solution.* — From an 0.25% solution of D-glucuronic acid maintained at 25°, aliquot portions (4 ml) were analysed at different time intervals as follows. One sample was passed through a 2-ml column of Amberlite IRA-400(Ac⁻), and the effluent was analysed colorimetrically as D-glucuronolactone. The results are shown in Table II. A second sample, taken at the same

time, was evaporated (Buchi Rotavapor) at 40°, and the resultant syrup was kept at room temperature for different time intervals before dissolution in water and subsequent ion-exchange treatment similar to the above. The results are shown in Table III. During the period when the syrup was kept at room temperature, partial crystallisation of the lactone occurred.

TABLE II

LACTONISATION OF D-GLUCURONIC ACID AT 25° IN AQUEOUS SOLUTION

Time (h)	0.0	1.0	2.5	4.0	8.2	19.3	34.9
% Lactone in neutral effluent	0.4	0.0	0.4	0.6	0.7	0.75	1.4

TABLE III

LACTONISATION OF D-GLUCURONIC ACID AS A SYRUP

Time (h)	9.8	21.3	25	26.5	28	29
% Lactone	3.3	15.9	14.9	22.2	20.3	19.5

Hydrolysis of D-*glucuronolactone.* — (*a*) *With barium carbonate.* Samples (10 ml) of a solution containing 1.51 g of D-glucuronolactone/100 ml were pipetted into flasks containing barium carbonate (1 g) at 25°. The flasks were shaken at 25 ±1° for different time intervals before filtration into tared vessels, and the aliquot portion of the original solution recovered as filtrate was determined by weighing. Passage through successive, combined columns of Amberlite resins IR-120(H^+) and IRA-400(Ac^-) (3 ml of each), separated by a plug of cotton wool, yielded D-glucuronolactone in the neutral effluent. This was measured as previously described. The results are shown in Fig. 1.

An attempt to accelerate the hydrolysis by refluxing the solution in the presence of barium carbonate (Analar reagent) was abandoned when a strong yellow colour developed in the solution.

(*b*) *Hydrolysis using the weak-base anion-exchange resin Amberlite IR-45*(OH^-). A solution of D-glucuronolactone (100 mg) in water (100 ml) was gently stirred with 2 ml of resin at 25°. Samples (1 ml) were taken periodically and analysed colorimetrically as D-glucuronolactone. The results are shown in Fig. 1.

(*c*) *Use of excess ammonium hydroxide.* The addition of an excess of 0.19N ammonium hydroxide (50 ml) to D-glucuronolactone (701.5 mg) in 50 ml of water at 25° gave an almost immediate yellow colouration and a final pH value between 10.0 and 10.5. Analysis by the ion-exchange procedure indicated that 90% of the glucuronic acid was retained on the anion-exchange resin. Paper chromatography of the free acid fraction recovered from the column revealed only glucuronic acid. Examination

of the neutral effluent by paper chromatography, however, revealed a component which migrated at the same rate as galactose in Solvent *B* but was somewhat slower in Solvent *A*. It had R_G 0.75 in the latter solvent (*cf.* 0.82 for galactose). This product was not investigated further.

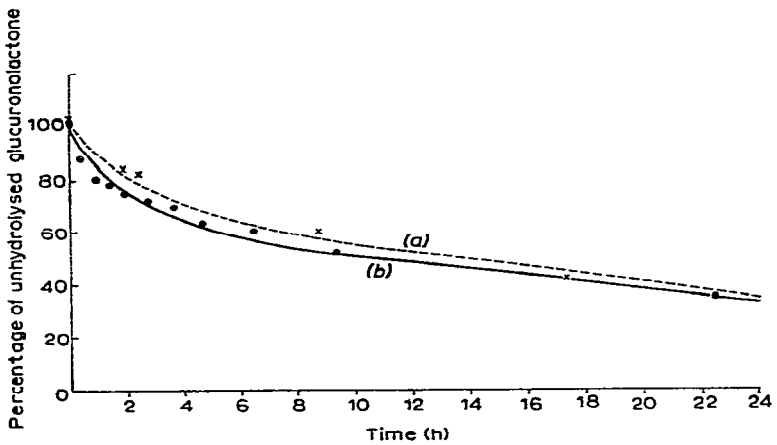


Fig. 1. Hydrolysis of D-glucuronolactone at 25°: (*a*) – – – with barium carbonate; (*b*) ——— with Amberlite IR-45.

(*d*) *Titration with alkali.* D-Glucuronolactone (1 mequiv.) was dissolved in N sulphuric acid (*i.e.*, a solution comparable to that obtained by hydrolysis of a polysaccharide) and titrated with 0.1N potassium hydroxide solution. The change in pH was observed with a pH meter. Addition of the alkali was regulated so that the pH remained between 7.5 and 9.0. This procedure was slow, but, at the final pH of 7.8, good stoichiometry was observed. This was further confirmed by passing an aliquot portion successively through Amberlite IR-120(H^+) and IRA-400(Ac^-). Elution of D-glucuronic acid from the anion-exchange resin with sulphuric acid, and subsequent colorimetry, demonstrated quantitative conversion of lactone into acid.

Gas–liquid chromatography. — A 0.3M solution (8 ml) of sodium borohydride was added to D-glucuronolactone (139.0 mg), and the solution was kept at room temperature overnight. Glacial acetic acid was added to give a pH of 3.5–4.0, and the solution was passed through a column of Amberlite resin IR-120(H^+) (6 ml). After concentration to dryness at diminished pressure, boric acid was removed by repeated distillation of dry methanol (5 × 20 ml). The sample was then vacuum dried for two h over phosphoric oxide at 40°. A mixture of acetic anhydride and pyridine (2 ml, 1:1 v/v) was added, together with arabinitol penta-acetate (50 mg) as internal standard. Immersion of the stoppered vessel in a boiling-water bath for a few minutes ensured rapid dissolution, and the reaction mixture was injected into the gas chromatograph after a further hour at room temperature.

An Aerograph Autoprep Model 705 gas chromatograph was modified for analysis by closing the collector exit with a silicone-rubber septum and so ensuring

that all effluent passed through the detector. Nitrogen carrier-gas flow was 45 ml/min and hydrogen, 24 ml/min. Operating temperatures were column 184°, injector 260°, and detector 240°. The column was coiled glass (5′ × 0.25″ i.d.) containing 3% ECNSS-M phase[10] on Gas Chrom Q (100–120 mesh) (supplied precoated by Applied Sciences Laboratories). Conversion of glucuronolactone to glucitol was 76.4% of theory after application of calibration constants determined with pure glucitol, A typical chromatogram is shown in Fig. 2.

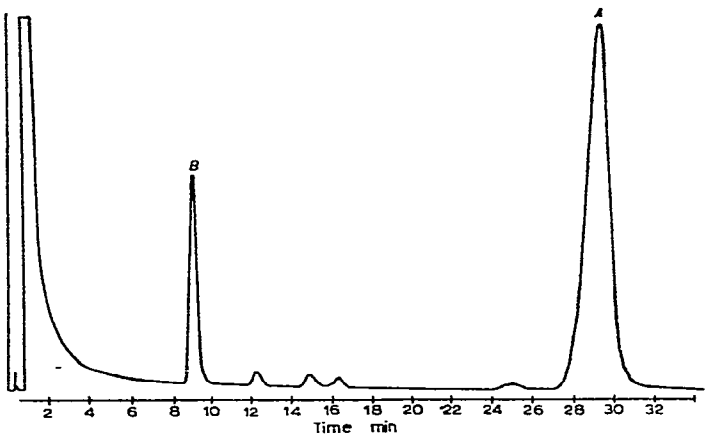


Fig. 2. G.l.c. of glucitol hexa-acetate (*A*) produced by direct reduction of D-glucuronolactone with sodium borohydride. Arabinitol penta-acetate (*B*) was added as an internal standard.

RESULTS AND DISCUSSION

As a model treatment to represent typical polysaccharide hydrolysis conditions, we have chosen a first treatment at 30° with 72% sulphuric acid and a second treatment at 100° with *ca.* N sulphuric acid[11]. Table I shows the extent of lactonisation of galacturonic and glucuronic acids under such conditions. Apparently, galacturonic acid is partly lactonised during the primary treatment and further lactonised during the secondary treatment, whereas glucuronic acid is very extensively lactonised during the first treatment, and the lactone is partly hydrolysed during the second treatment so that the ultimate extent of lactonisation is similar to that of galacturonic acid. Evidently, in both cases, at least half of the uronic acid originating from a polysaccharide hydrolysis would be obtained in the lactone form.

Further experiments were carried out to determine extents of lactonisation under conditions other than the simulated hydrolysis conditions. Table II shows that glucuronic acid lactonises extremely slowly in water at 25°, but Table III shows that a dry sample of the acid in syrupy form is appreciably lactonised on standing overnight at room temperature. The extent of such lactonisation is rather variable, and, since the process is accompanied by crystallisation of the lactone, it may be speculated that the rate of lactonisation is influenced by random nucleation of the syrup.

It is concluded that the products of acid hydrolysis of a polysaccharide contain-

ing uronic acids will be extensively lactonised, and that the extent of the lactonisation will be influenced by the detailed experimental procedure and will probably be irreproducible. For quantitative work of this type, we have emphasised the necessity of separating completely the uronic acids from the reducing sugars. The only feasible small-scale separation process is illustrated above with a strong-base ion-exchange resin in the acetate form. A comparable resin in the free-base form will produce artefacts from reducing sugars[4a,12], whereas a weak-base resin will significantly sorb the reducing sugars[13]. Yet the acetate-form resin will not sorb the uronic acid lactones. The lactones must therefore be converted into the free acids or salts under conditions which preclude alkaline degradation of the reducing sugars or the uronic acid lactones.

Several methods of hydrolysis were attempted with glucuronolactone, which is known to be particularly sensitive to alkali[5] and to produce coloured products very rapidly under such conditions. Hydrolysis of the lactone with water at 25° was extremely slow in the presence of barium carbonate or a weak-base ion-exchange resin (Fig. 1). These methods are obviously not feasible for an analytical process. Boiling an aqueous solution of glucuronolactone in the presence of barium carbonate resulted in rapid formation of colour, as did treatment with 0.1N ammonium hydroxide at 25°. These treatments were therefore assumed to cause excessive alkaline degradation and were abandoned.

However, titration with potassium hydroxide solution (rate of addition controlled to maintain pH 7.5–9.0) resulted in quantitative conversion into the potassium salt with no colour formation. The resultant solution from such a procedure applied to a polysaccharide hydrolysate would be in a suitable form for further analysis as uronic acid and neutral sugar fractions, and the titration method is suitable for automation. This procedure will therefore be adopted in our future work with polysaccharides.

Previously, an accelerated hydrolysis of glucuronolactone in potassium phosphate buffer at pH 7.2 has been reported[14]. The presence of such a buffer, however, would considerably complicate subsequent ion-exchange separations.

In order to illustrate the confusion which can arise in g.l.c. analysis of a polysaccharide hydrolysate containing both uronic acid lactones and neutral sugars, glucuronolactone was reduced, acetylated, and chromatographed by conventional methods[10]. The results indicated a 76% conversion into glucitol hexa-acetate. Such a product would of course be included to an unpredictable extent in the same product obtained from any glucose present among the neutral sugars.

Since the extent of lactonisation of a uronic acid such as glucuronic acid is probably affected by both crystallisation of the lactone and time of standing of the acid–lactone mixture, it becomes necessary to reconvert all of the mixture into the salt form if sodium borohydride reduction to the aldonic acid is contemplated. The resistance of D-glucuronolactone to hydrolysis is seen in its reduction to D-glucitol in very high yield in the presence of this reagent.

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MASS SPECTROMETRY OF URONIC ACID DERIVATIVES

PART III*. THE FRAGMENTATION OF METHYL ESTER METHYL GLYCOSIDES OF METHYLATED URONIC AND ALDOBIOURONIC ACIDS

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ABSTRACT

The mass spectra of methyl ester methyl glycosides of methylated uronic acids, methyl ester methyl glycosides of methylated aldobiouronic acids of different types, and of methyl ester methyl glycosides of acetylated aldobiouronic acids having 4-*O*-methyl-D-glucuronic acid as the acidic component were obtained. Fragmentation patterns of these compounds, characteristic features of their mass spectra, and their importance for analytical applications are discussed.

INTRODUCTION

Methylation analysis is a conventional method for the structural analysis of polysaccharides and other natural products that contain saccharide units. Recent, substantial improvements involve refinement of the methylation procedures[1,2] and new methods for separation of the products of hydrolysis[3-6]. One of the most important contributions to this development is the identification of monomer units by using mass spectrometry. Heyns[7], and Chizhov and Kochetkov, by a detailed study of the fragmentation of the fully methylated derivatives of pentoses[8,9], hexoses[8,10,11], and methylpentoses[8,12], using specifically labelled compounds containing trideuteromethyl groups in various positions, have elucidated the fragmentation patterns of permethylated monosaccharides and established the structures of ions corresponding to the most important peaks in their mass spectra. Thus, it is now possible to use mass spectrometry for the identification of partially methylated monosaccharides and to establish the significant structural features, *viz.*, ring size, chain length, and molecular weight.

The fragmentation studies of methyl glycosides of methylated, neutral disaccharides possessing different linkages between the monomeric units[13] indicate the possibility of unambiguously determining the type of linkage.

The mass-spectrometric behaviour of permethylated acidic components of polysaccharides, methyl esters of permethylated uronic acids, and methyl esters of permethylated aldobiouronic acids has not been described. In this paper, we report on the fragmentation patterns of these and related types of compound.

*Parts I and II: *Collection Czech. Chem. Commun.*, 33 (1968) in press.

EXPERIMENTAL

The following compounds were studied: methyl (methyl 2,3,4-tri-*O*-methyl-D-galactopyranosid)uronate (**1**), methyl (methyl 2,3,4-tri-*O*-methyl-D-glucopyranosid)uronate (**2**), methyl 3,4-di-*O*-methyl-2-*O*-(methyl 2,3,4-tri-*O*-methyl-α-D-glucopyranosyluronate)-α-D-xylopyranoside (**4**), methyl 3,4,6-tri-*O*-methyl-2-*O*-(methyl 2,3,4-tri-*O*-methyl-β-D-glucopyranosyluronate)-β-D-mannopyranoside (**3**), methyl 2,3,6-tri-*O*-methyl-4-*O*-(methyl 2,3,4-tri-*O*-methyl-α-D-galactopyranosyluronate)-α-D-galactopyranoside (**5**), methyl 2,3,4-tri-*O*-methyl-6-*O*-(methyl 2,3,4-tri-*O*-methyl-β-D-glucopyranosyluronate)-β-D-galactopyranoside (**6**), and methyl 3,4-di-*O*-acetyl-2-*O*-(methyl 2,3-di-*O*-acetyl-4-*O*-methyl-α-D-glucopyranosyluronate)-α-D-xylopyranoside (**7**).

Compounds **1–7** were prepared[14–17] by methylation and acetylation of uronic and aldobiouronic acids isolated from the partial hydrolysates of polysaccharides from plant gums and hemicelluloses.

The mass spectra were obtained with a MCH 1306 mass spectrometer (U.S.S.R.), modified for direct introduction of the sample, at an ionising potential of 70 eV. The temperature in the inlet system depended on the volatility of the sample and was in the range 20–70°, the temperature in the ionising chamber being 130–160°. The intensities of the peaks in the spectra (Tables I and II) are expressed in relation to the base peak, which was assigned an arbitrary value of 100%.

TABLE I

MASS SPECTRA OF METHYL ESTER METHYL GLYCOSIDES OF METHYLATED URONIC ACIDS

m/e	*Relative intensity*, %		*Symbol*	m/e	*Relative intensity*, %		*Symbol*
	1	**2**			**1**	**2**	
233	0.08	0.2	A_1	99	1.8	—	
205	0.8	1.2	E_1	89	7.5	7.0	
201	0.04	3.6	A_2	88	100	69.2	H_1
176	3.8	0.5	B_1	87	6.1	13.8	
173	3.0	1.9	$E_2 + C_2$	85	8.2	12.8	K_2
169	0.05	0.05	A_3	83	4.0	—	
163	0.2	16.1	D_1	75	49.4	49.2	J_1
161	0.5	0.4		74	8.1	12.6	
145	3.0	2.1		73	26.1	21.8	H_2
141	1.2	2.6	C_3	71	10.2	11.1	
135	2.1	—		61	9.8	2.2	
131	2.2	3.1	B_2	60	7.1	—	
127	0.5	0.5		59	27.2	11.2	
117	0.8	1.5		58	9.0	3.8	
115	0.9	2.5		57	26.8	11.7	
113	1.2	0.8		56	12.1	2.0	
111	2.0	3.1	E_3	55	18.2	8.4	
102	3.5	5.8		47	8.0	3.2	
101	65.9	100	F_1	45	42.5	37.2	

TABLE II

MASS SPECTRA OF METHYL ESTER METHYL GLYCOSIDES OF METHYLATED ALDOBIOURONIC ACIDS

m/e	*Relative intensity, %*				*Symbol*
	3	4	5	6	
405	0.5	0.4	—	—	baA_2
394	0.9	0.4	1.0	—	baB_1
367	—	—	—	4.8	baD_1
361	—	0.4	—	—	baH_2
319	3.4	3.7	1.8	—	baF_1
287	1.0	1.4	—	—	
279	7.8	—	6.3	3.1	abJ_1
235	—	10.2	—	—	abJ_1
233	2.7	5.3	10.0	2.0	aA_1
219	1.5	—	0.7	10.0	bA_1
201	58.0	46.0	7.5	15.0	aA_2
187	7.0	—	2.4	1.9	bA_2
175	—	10.0	—	—	bA_1
173	5.9	4.7	6.1	2.5	aC_2
169	5.3	6.0	2.2	2.1	aA_3
161	—	—	2.9	0.2	
159	5.1	2.3	6.5	3.2	bC_2*
157	3.8	5.3	2.7	3.1	
145	3.9	2.4	4.4	7.4	
143	—	6.9	—	—	bA_3
141	12.0	13.0	7.8	5.4	aC_3
131	—	4.2	7.6	4.7	
129	4.3	5.6	8.1	5.0	
127	4.1	—	6.5	5.8	bC_3
117	4.6	4.2	3.4	2.9	
115	2.3	9.1	4.1	4.2	bC_2**
113	2.5	3.5	4.1	2.3	
111	7.0	8.4	7.6	3.7	
102	6.0	3.2	5.3	5.8	bK_1*
101	100.0	100.0	78.0	100.0	F_1, G_1
89	8.3	9.8	14.0	9.8	
88	50.0	51.0	100.0	96.0	H_1
85	14.0	22.0	19.0	8.0	aK_2
83	—	4.9	—	—	bC_3
75	41.0	37.0	54.0	69.0	bJ_1
74	8.2	6.1	—	—	
73	12.0	14.0	14.0	11.0	H_2
71	19.0	16.0	20.0	16.0	
59	10.0	14.0	18.0	6.4	
58	—	12.0	2.1	3.2	bK_1**
57	6.6	7.7	20.4	3.2	
55	—	9.1	19.3	2.7	
45	45.0	49.0	70.0	26.0	

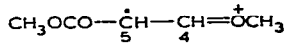
*The symbol refers only to the ions from compounds 3, 5, and 6 having hexose as neutral component.
**The symbol refers only to the ions from compound 4 having pentose as neutral component.

To denote the fragment ions, the symbols *A–K*, employed by Kochetkov[18], were used. In the case of aldobiouronic acids, the structure of which can be expressed

by the general formula **8**, in addition to the *A–K* symbolism, lower-case letters are used. The first lower-case letter characterises the part of the molecule from which the



8



9

fragment originated, and the second designates the unchanged part of the molecule, *e.g.*, baF_1 denotes an ion that has arisen from cleavage of ring *b* following the pathway *F*, and is substituted by ring *a*.

RESULTS AND DISCUSSION

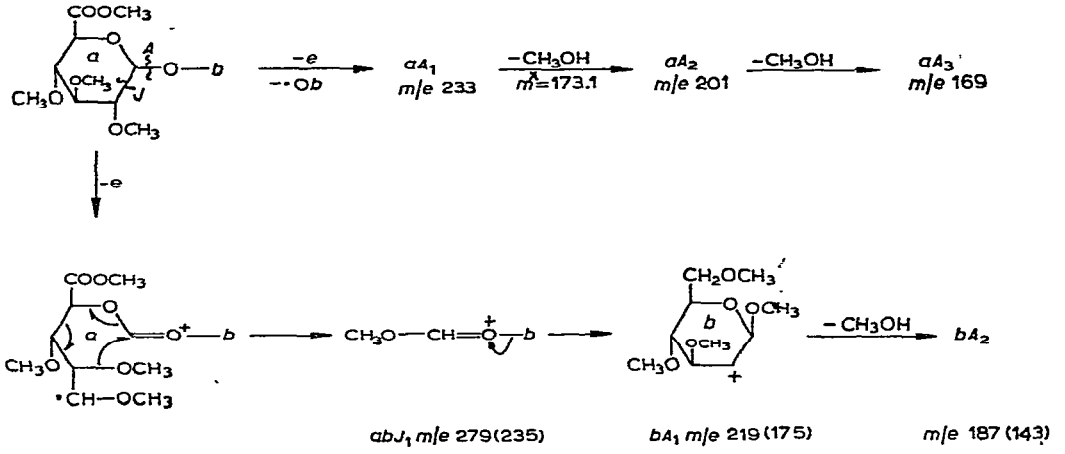
The fragmentation of methyl ester methyl glycosides of the methylated uronic acids. — The mass spectra of compounds **1** and **2** (Table I) contain peaks for all of the ions arising by fragmentations analogous to those of permethylated hexopyranosides[14], the absence of the ions K_1 containing C-5 and C-4 (with the appropriate substituents) being the single exception. In the case of compounds **1** and **2**, they would have structure **9** and *m/e* 116. Their low stability and disintegration to the ions in the lower mass range of the spectrum is presumably due to the presence of the electron-accepting methoxycarbonyl group. The most intense peaks are afforded by the ions F_1 (*m/e* 101), H_1 (88), J_1 (75), and $CH_2{=}\overset{+}{O}CH_3$ (45). Peaks containing C-6, when compared with those of permethylated hexopyranosides, are shifted by 14 mass units (difference between $COOCH_3$ and CH_2-OCH_3 groups) to the upper mass range. The relevant species are A_1 (*m/e* 233), A_2 (201), A_3 (169), C_2 (173), C_3 (141), and D_1 (163).

Considerable differences could be observed among the relative intensities of peaks corresponding to the ions A_2, B_1, D_1, H_1, and F_1 in the spectra of compounds **1** and **2**, even if the spectra were run under identical conditions. The differences are probably due to the configuration of substituents at C-4 and allow compounds **1** and **2** to be distinguished.

The fragmentation of methyl ester methyl glycosides of the methylated aldobiouronic acids. — The mass spectra of compounds **3–6** (Table II) do not contain peaks for the molecular ion. The fragmentation proceeds independently on each unit of the dimer and will be discussed from the point of view of the fragmentation series[18].

Ions of the *A* series are formed by cleavage of the glycosidic bond between the acid and the neutral moiety to give rise to ions aA_1 at *m/e* 233, which, after elimination of methanol, afford ions aA_2 and aA_3 at *m/e* 201 and 169. The abundance ratio aA_2:aA_1 differs considerably in the spectrum of compound **5** (containing D-galacturonic acid) when compared with the spectra of compounds **3**, **4**, and **6** (containing D-glucuronic acid). Regardless of the configuration at C-1 in the uronic acid part of the compounds, the abundance ratio aA_2:aA_1 is < 1 when the acid part is galacturonic acid and $\gg 1$ when glucuronic acid is involved.

Ions bA_1 are produced from abJ_1 ions[13], and, after splitting off of methanol, give rise to ions bA_2. The *m/e* values of ions abJ_1, bA_1, and bA_2 depend on the number of carbon atoms in the chain of the neutral component and can be used for its determination. Their values in the case of a neutral pentose component are given in parenthesis (Scheme 1).

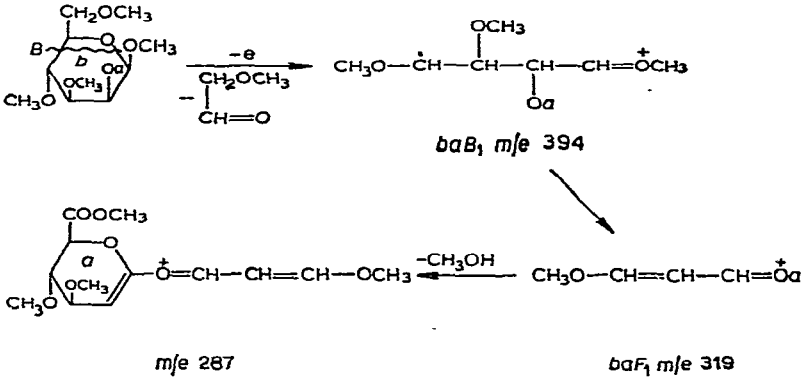


SCHEME 1

Values of *m/e* for the A_1 ions of the *a* and *b* parts of the molecule can be used for an indirect determination of the molecular weight of methyl ester methyl glycosides of methylated aldobiouronic acids, following the equation; $M = aA_1 + bA_1 + 16$.

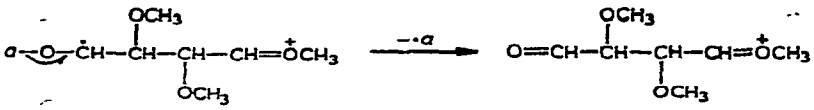
In the spectra of compounds **3** and **4**, a less pronounced peak for baA_2 occurs at *m/e* 405 (361), formed after elimination of methanol from the species baA_1.

Ions of the *B* series (baB_1) are formed only from aldobiouronic acids having (1→2)- and (1→4)-linkages (**3**–**5**). Destruction of baB_1 ions at *m/e* 394 affords the ions baF_1 at *m/e* 319, which, presumably after elimination of methanol, give rise to ions at *m/e* 287 (Scheme 2).



SCHEME 2

The spectrum of compound **5**, which contains a (1→4) glycosidic linkage contains ions at *m/e* 161. These ions are produced by the splitting off of ring *a* as a radical from the ions baB_1, and cannot be formed with dimers containing a (1→2)-linkage (Scheme 3).

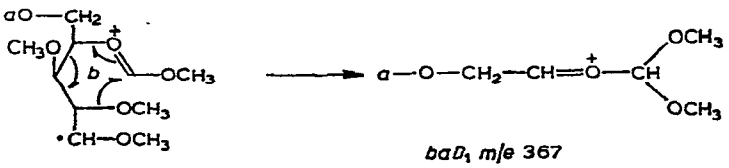


baB_1 *m/e* 394 *m/e* 161

SCHEME 3

Series *C* is represented by the ions C_2 and C_3, namely ions aC_2 at *m/e* 173, aC_3 at *m/e* 141, bC_2 at *m/e* 159 (115), and bC_3 at *m/e* 127 (83), originating from fission of both rings.

Series *D* is characteristic for compound **6**, which contains a (1→6)-linkage, and is represented by ions baD_1 at *m/e* 367 (Scheme 4).



baD_1 *m/e* 367

SCHEME 4

Peaks belonging to the *E* series have not been found in the mass spectra of the compounds examined.

Series *F*, *G*, and *H* afford the most abundant peaks at *m/e* 101 and 88. The abundance ratio of the peaks *m/e* 101:88 in the spectra of compounds **3**, **4**, and **6** (containing glucuronic acid) is > 1, regardless of the configuration at C-1 of the uronic acid, whereas, in the spectrum of compound **5** (containing galacturonic acid), the ratio is < 1. This phenomenon, combined with the characteristic abundance ratio of the peaks aA_2:aA_1, can be used for the differentiation of these acids as components of aldobiouronic acids.

Series *J* is represented by ions abJ_1 at *m/e* 279 (235), and ions bJ_1 at *m/e* 75.

Series *K* contains the peaks bK_1 at *m/e* 102 (58), bK_2 at *m/e* 71, and aK_2 at *m/e* 85.

The fragmentation of methyl ester methyl glycosides of the acetylated aldobiouronic acids containing 4-O-*methyl*-D-*glucuronic acid.* — 4-*O*-Methyl-D-glucuronic acid occurs frequently as a moiety of aldobiouronic acids. In order to differentiate this acid from D-glucuronic acid, we investigated the fragmentation patterns of methyl 3,4-di-*O*-acetyl-2-*O*-(methyl 2,3-di-*O*-acetyl-4-*O*-methyl-α-D-glucopyranosyl-uronate)-α-D-xylopyranoside (**7**, Fig. 1).

Disproportionation of the molecular ion proceeds *via* several pathways. The loss of the $CH_3OCH{=}O$ group from the glycosidic moiety of ring *b* gives rise to

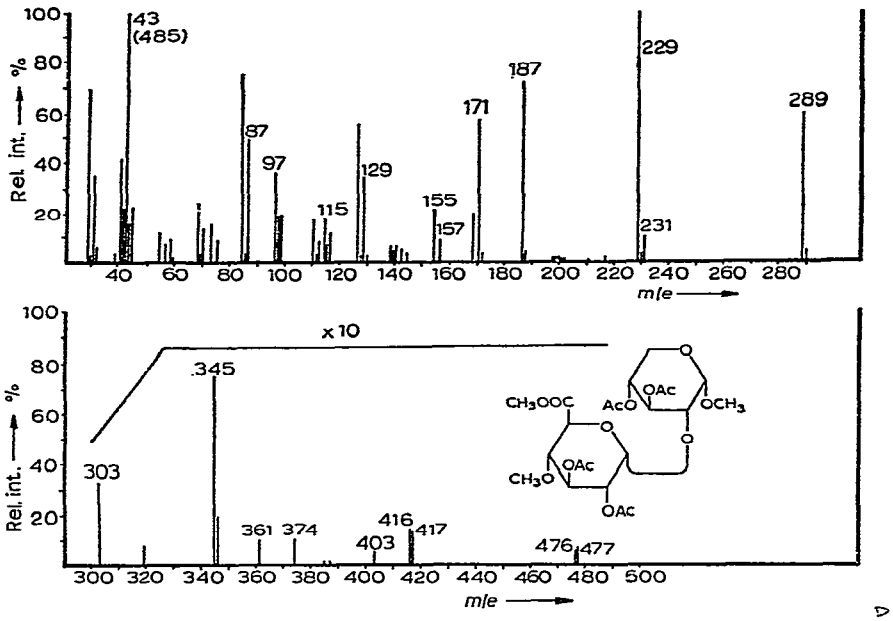
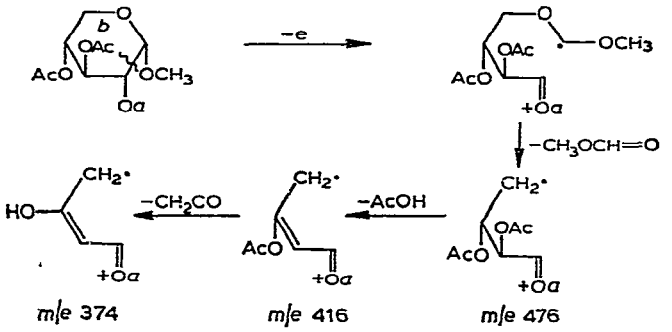


Fig. 1. Mass spectrum of methyl 3,4-di-*O*-acetyl-2-*O*-(methyl 2,3-di-*O*-acetyl-4-*O*-methyl-α-D-glucopyranosyluronate)-α-D-xylopyranoside (7).

the species $(M-60)^+$ at *m/e* 476, which is subsequently degraded by elimination of CH_3COOH and $CH_2=C=O$ (Scheme 5).

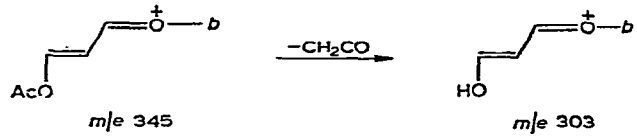


SCHEME 5

The peak (M−59) at *m/e* 477 presumably represents the ions abE_1 generated after the loss of methoxycarbonyl group from ring *a*. These ions, after elimination of CH_3COOH, afford the ions abE_2 at *m/e* 417. The fragments at *m/e* 403 and 361 are attributed to the ions baF_1, typical for the aldobiouronic acids containing (1→2)- and (1→4)-linkages.

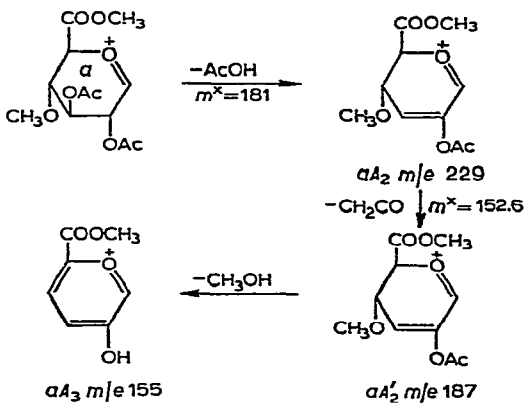
The additional species of the *F* series arise from ring *a*, and their number and values are given by combination of the substituents OAc, OCH_3, and OH (*m/e* 157, 129, 115, and 87).

For the ions represented by the peaks at *m/e* 345 and 303, the following structures could be proposed:



m/e 345 *m/e* 303

Fission of the glycosidic bonds generates the ions aA_1, and the subsequent reactions could be explained as in Scheme 6.



SCHEME 6

The different fragmentation pattern of the *A* series, when compared with the fragmentation of acetylated monosaccharides[19], having an OCH_3 group at C-2 or C-3, enables assignment of the position of the methoxyl group and consequently the identification of 4-*O*-methylglucuronic acid as a component of aldobiouronic acids.

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MASS SPECTROMETRY OF URONIC ACID DERIVATIVES

PART IV. THE FRAGMENTATION OF METHYL ESTER METHYL GLYCOSIDES OF METHYLATED ALDOTRIOURONIC ACIDS

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ABSTRACT

The fragmentation pattern of methyl *O*-(methyl 2,3,4-tri-*O*-methyl-β-D-glucopyranosyluronate)-(1 → 6)-*O*-(2,3,4-tri-*O*-methyl-β-D-galactopyranosyi)-(1 → 6)-2,3,4-tri-*O*-methyl-β-D-galactopyranoside and its application in the structural study of acidic trisaccharides are discussed.

INTRODUCTION

The recent study of the mass spectra of methyl ester methyl glycosides of methylated uronic acids[1] showed that their fragmentation followed the same pattern as that of methylated methyl hexopyranosides. Due to the differences in the relative intensities of some peaks, it is possible to distinguish between glucuronic and galacturonic acids.

The fragmentation pattern of methyl ester methyl glycosides of aldobiouronic acids possessing different types of linkage[1] permitted the molecular weights to be calculated indirectly, the length of the carbon chain of the neutral moiety to be determined, glucuronic or galacturonic acid to be identified as the acidic components, and the type of linkage between the neutral and acidic parts of the molecule to be established.

We now report on the first study of the mass-spectrometric behaviour of a methyl derivative of an oligosaccharide higher than a disaccharide. The compound studied was methyl *O*-(methyl 2,3,4-tri-*O*-methyl-β-D-glucopyranosyluronate)-(1 → 6)-*O*-(2,3,4-tri-*O*-methyl-β-D-galactopyranosyl)-(1 → 6)-2,3,4-tri-*O*-methyl-β-D-galactopyranoside (**1**), in order to demonstrate the usefulness of the application of mass spectrometry for structural studies of trisaccharides.

EXPERIMENTAL

The compound studied was obtained by methylation of *O*-(β-D-glucopyranosyluronic acid)-(1→6)-*O*-(β-D-galactopyranosyl)-(1→6)-β-D-galactose resulting from partial hydrolysis of the polysaccharides of the gum of the peach tree *Prunus persica* (L.) Batsch. The isolation, methylation procedures, and identification of the aldotriouronic acid have been reported[2].

The mass spectrum was measured at a potential of 70 eV by using a Hitachi RMU-6D mass spectrometer fitted with a direct inlet system at a temperature of 160°.

Symbols *A–K* were used throughout this work to designate the various series of ions. The symbols were further refined with lower-case letters *a*, *b*, and *c*, depicting the single rings in the trimer, as shown in Fig. 2. The first lower-case letter preceding the ion symbol designates that part of the molecule from which the ion originated. The second and third lower-case letters designate the uncleaved part of the molecule substituting the fragment, *e.g.*, $cabD_1$ stands for the ion arising by a fission of ring *c* following the pathway *D*, and being substituted by the rings *a* and *b*.

RESULTS AND DISCUSSION

The mass spectrum of compound **1** (Fig. 1) did not contain a peak for the molecular ion. Disintegration of the molecular ions proceeded in all three rings. The proposed fragmentation scheme (Fig. 2), which contains the tentative structures of all of the ions of the spectrum, was corroborated by application of the rules of fragmentation shown to be valid for methyl glycosides of the methylated mono- and disaccharides, and methyl ester methyl glycosides of the methylated uronic and aldobiouronic acids[1].

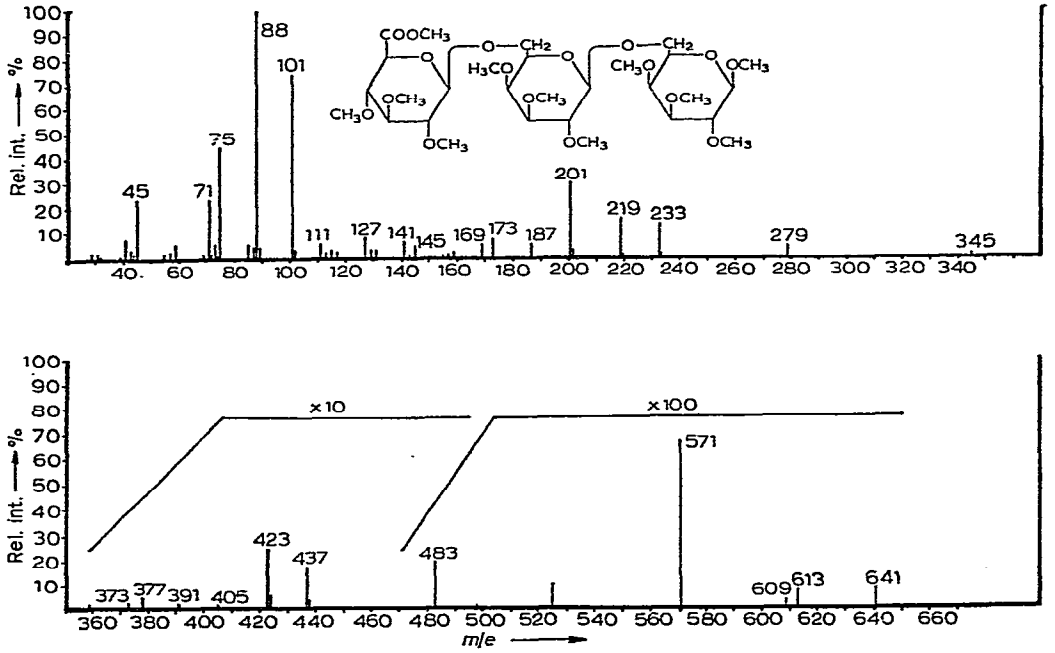


Fig. 1. Mass spectrum of trisaccharide **1**.

The peaks of the ions discussed further afforded important information on the structural features of the aldotriouronic acid.

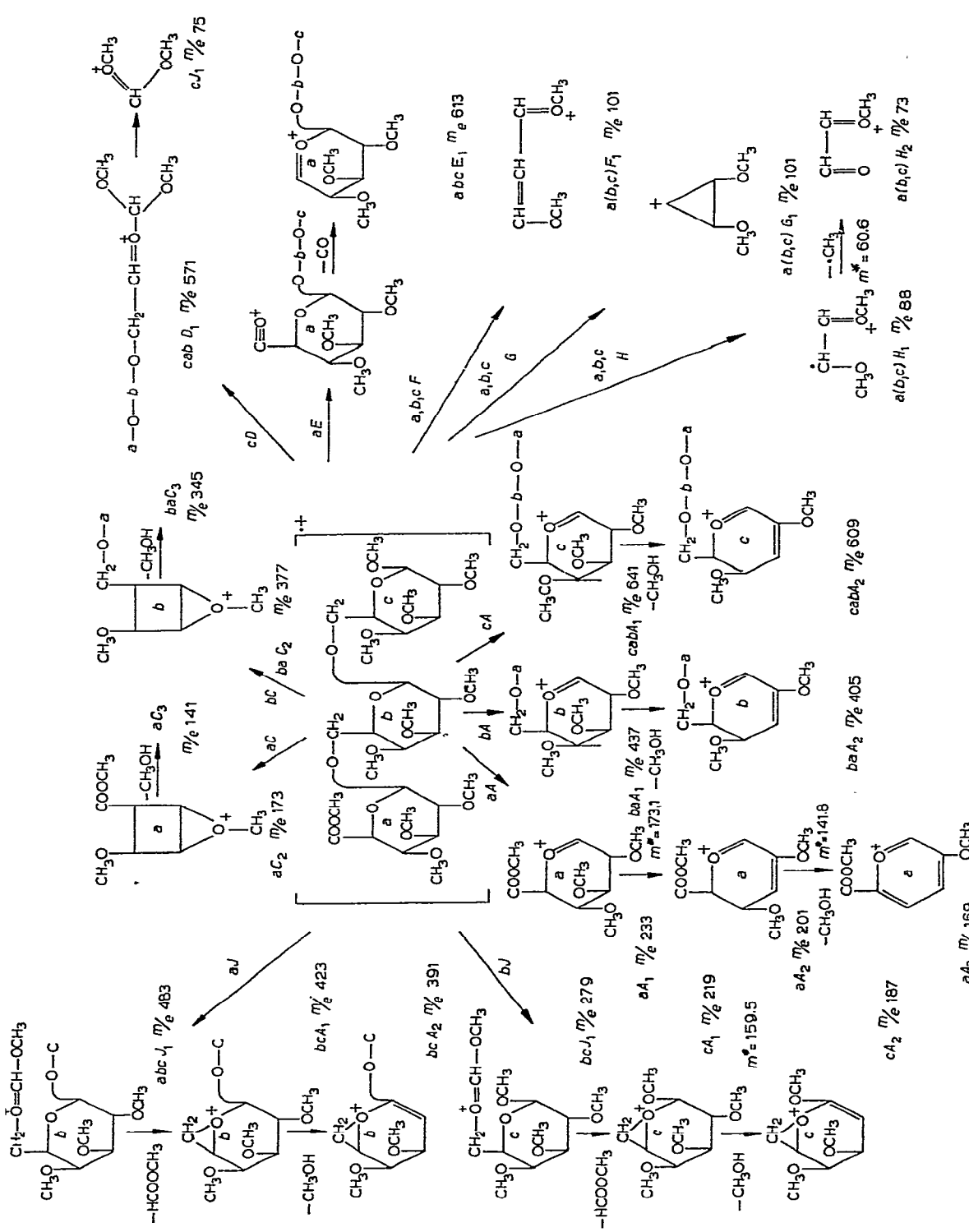


Fig. 2. Fragmentation patterns of trisaccharide 1.

The presence of the species $cabA_1$ enabled the molecular weight to be determined. As the abundance of the peak was negligible, we suggest an indirect way to calculate the molecular weight from *m/e* values of the fragments A_1:

$$M = aA_1 + bcA_1 + 16 \text{ or } M = cA_1 + baA_1 + 16,$$

where the symbols of the ions stand for their *m/e* values.

From the molecular weight as well as from *m/e* values of the ions $cabA_1$, $abcE_1$, $abcJ_1$, and $cabD_1$, the number of hexose and pentose units in the trimer could be determined.

The number of carbon atoms forming ring *c* of a trimer determines the *m/e* values of the species cA_1 and bcJ_1, whereas the presence of an acidic group in ring *a* was substantiated by the *m/e* value of the species aA_1. Following the equation:

$$baA_1 - aA_1 - 16 = b, \text{ or } bcA_1 - cA_1 - 16 = b,$$

it became possible to determine the mass of the ring *b* and consequently the number of its carbon atoms.

These data could also be used to establish the sequence of monomer units in the trimer.

The presence of the fragments $cabD_1$ was found to be characteristic of the (1→6)-linkage between the neutral parts *b* and *c* of the trimer. On the other hand, the (1→6)-linkage between the acidic part *a* and the neutral part *b* could not be unequivocally established on the basis of the mass spectrum.

The ratio of the relative abundance of the ions aA_2:aA_1 allowed differentiation between glucuronic and galacturonic acids as the components of the trimer, in the same way as described with methyl ester methyl glycosides of the methylated aldobiouronic acids[1].

ACKNOWLEDGMENT

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STUDIES OF CARBOHYDRATE DERIVATIVES BY NUCLEAR MAGNETIC DOUBLE-RESONANCE

PART II*. A DETERMINATION OF THE SIGNS OF PROTON–PROTON COUPLING-CONSTANTS OF SATURATED AND UNSATURATED CARBOHYDRATES**

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ABSTRACT

A brief introduction is given to the utilization of both the "selective irradiation" and the "spin-tickling" methods for determining the relative signs of proton–proton coupling-constants of carbohydrate derivatives. For most of the saturated carbohydrates studied, the vicinal ^1H–^1H couplings have been found to be *positive*, and the *geminal* couplings *negative* in sign. For unsaturated derivatives, both the *vicinal* and the *geminal* couplings are positive. The signs of the couplings of an episulfide derivative are the same as those of the saturated carbohydrates, whereas the signs for the epoxide resemble those of unsaturated carbohydrates. These findings are in accord with previous experimental and theoretical results. It has been demonstrated that the signs of long-range (^4J) couplings of saturated derivatives exhibit a regular stereospecificity, those between two equatorially oriented protons ($^4J_{e,e}$) being positive and those between an axial and an equatorial proton ($^4J_{e,a}$) being negative in sign. These findings are contrasted with the previous theoretical predictions, and their potential as an adjunct to stereochemical studies is discussed. For unsaturated carbohydrate derivatives **1**, **6**, and **7**, the long-range couplings are all negative in sign.

INTRODUCTION

Nuclear magnetic resonance (n.m.r.) spectroscopy is at present the most powerful spectroscopic method available for elucidating the structures and conformations of monosaccharide derivatives[3,4]. The most useful adjunct to the basic n.m.r. method is undoubtedly the technique of nuclear magnetic double-resonance (n.m.d.r.). Thus far, applications of n.m.d.r. have been confined to the confirmation of spectral assignments, as we have indicated previously[1]. It is the purpose of this and subsequent papers in this series to describe other applications likely to be of interest

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to carbohydrate chemists. In the present paper, attention will be directed towards the use of the n.m.d.r. technique for determining the relative signs of ^1H–^1H coupling constants in a variety of carbohydrate derivatives.

The early papers by Maher and Evans[5] and Freeman and Whiffen[6] stimulated considerable interest in the problem of determining the relative signs of coupling constants by the n.m.d.r. technique, and, during this initial period, the signs of a large variety of coupling constants, particularly ^1H–^1H couplings, were determined. However, the majority of these studies took little or no cognizance of the relative stereochemistry of the nuclei involved; this particular facet of the problem has been generally overlooked, despite the fact that several important theories of ^1H–^1H coupling constants predict[7–9] changes of sign for some particular stereo-relationships. As the carbohydrate field includes a wide variety of compounds having protons held in many different relative orientations, it seemed appropriate to study the signs of the coupling constants of a selection of carbohydrate derivatives. Besides offering some check on the accuracy of certain of the theories currently available for predicting the absolute magnitudes of coupling constants, these determinations of sign should be of interest to those attempting computer analyses of complex proton magnetic resonance (p.m.r.) spectra of carbohydrate derivatives. Should any of the couplings evidence a stereospecific dependence of the sign, this feature would offer a further approach to the determination of conformations by the p.m.r. method. As will be seen, the long-range couplings of saturated carbohydrate derivatives do indeed exhibit such a sign dependence.

Before discussing our results, it is relevant to indicate briefly what is implied by a "positive" or a "negative" coupling-constant. The simplest model for rationalizing the observation that spin-coupling constants can differ in sign is based on the McConnell extension[10] of the Dirac–Van Vleck model[11]. This approach is based on two quite reasonable sets of assumptions; first, that the spin of a bonding electron will tend to be opposite to that of its associated nucleus and that any two electrons in a bonding orbital will have paired spins (the Pauli principle); and second, that, in saturated systems, ^1H–^1H spin couplings, are transferred predominantly *via* the *s*-shell electrons of the nuclei involved (the so-called Fermi contact interaction). The model then states that any spin–spin coupling between two nuclei having *antiparallel* spins will be defined as *positive*, and that if the nuclear spins are parallel, the coupling constant will be negative. On this basis, it is seen how it is possible for the couplings between the protons of an sp^3-hybridized system to alternate in sign depending upon the number of bonds separating the protons. If ⇑ and ⇓ indicate the two possible senses of a nuclear spin and ↓ and ↑ indicate the senses of an electron spin, the most stable arrangement of nuclear and electron spins for the directly bonded ^{13}C–^1H system is shown in (*A*); it is seen that the two nuclear spins are oriented antiparallel

$$^{13}C \Uparrow \downarrow \quad \uparrow \Downarrow {}^1H \text{ (positive)} \ldots \qquad (A)$$

and thus, by definition[12,13], $J(^{13}C$–$^1H)$ is taken to be positive. Extension of this symbolism to *geminal* and *vicinal* ^1H–^1H couplings, as shown in (*B*) and (*C*), res-

pectively, leads to the expectation that *geminal* couplings should be *negative* in

$$^{1}H \downarrow \uparrow {}^{12}C \uparrow \downarrow {}^{1}H \text{ (negative)} \ldots \quad (B)$$

$$^{1}H \downarrow \uparrow {}^{12}C \uparrow \downarrow {}^{12}C \downarrow \uparrow {}^{1}H \text{ (positive)} \ldots \quad (C)$$

sign, whereas *vicinal* couplings should be *positive*. A further extension to include "long-range" couplings across four bonds leads to the prediction that such couplings should be *negative* in sign. As will be seen later, this alternating vector model successfully predicts the relative signs of *geminal* and *vicinal* couplings for the saturated systems encountered in carbohydrate derivatives, but is unsuccessful for the long-range couplings. It is also not suitable for those couplings transmitted *via* π-electrons (*vide infra*). In spite of these limitations, together with some more fundamental shortcomings, this model provides a conceptually simple rationale for the observation that coupling constants can vary in sign.

EXPERIMENTAL

All p.m.r. spectra were measured with a Varian HA-100 spectrometer operating in the "frequency-sweep" mode, with tetramethylsilane as the internal reference and an "autoshim" unit for "y-gradient-drift" control. The double- or triple-resonance experiments were performed with a Hewlett Packard 204 B audio-oscillator and a modified[1] Hewlett Packard 200 AB audio-oscillator. Spectra were measured by use of whichever sweep-width was required to ensure adequate resolution of the multiplets under investigation, generally the 250-, 100-, or 50-Hz sweep-widths, and with appropriate sweep-rates (0.5 Hz/sec or less). Extensive use was made of the "difference 1" and "difference 2" calibration-modes of the instrument to ensure that the decoupling frequency had been correctly positioned. In all cases, the radiofrequency attenuator was 20 db down from 0.5 watt, and the observing field (H_1) and receiver band-width controls were adjusted for the optimal ratio of signal to noise.

The derivatives investigated in this paper were either prepared by standard procedures in the literature or by methods to be published elsewhere[14].

5,6-Dideoxy-1,2-*O*-isopropylidene-α-D-*xylo*-hex-5-enofuranose (**1**), the corresponding acetate (**6**) as a syrup[15], and 5,6-anhydro-1,2-*O*-isopropylidene-α-D-glucofuranose (**3**) were gifts from Professor A. Rosenthal and Mr. G. Kan of this Department. Compound **1** had m.p. 56–58° (lit.[16] m.p. 61–65°) and **3** had m.p. 130–132° (lit.[17] m.p. 133.5°).

1,6-Anhydro-β-D-idopyranose triacetate (**2**) and the corresponding D-*manno* isomer (**4**) were gifts from Dr. N. K. Richtmyer. A detailed n.m.r. study of these compounds will be reported elsewhere[18].

5,6-Dideoxy-5,6-epithio-1,2-*O*-isopropylidene-β-L-idofuranose (**5**) was prepared by Mrs. Liane Evelyn (née Kristensen), following the method of Hall, Hough, and Pritchard[16].

D-Glucal triacetate (**7**) was a commercial sample from the Aldrich Chemical Co., Milwaukee, Wisconsin, which was purified by vacuum distillation and subsequently recrystallized from aqueous ethanol, m.p. 54–56°, (lit.[19] m.p. 55°; 54–55°).

Methyl tetra-*O*-acetyl-*β*-D-glucopyranoside (**8**) was prepared by acetylating a commercial sample of methyl *β*-D-glucopyranoside in the usual way; it had m.p. 106–107° (lit.[20] m.p. 104°).

β-D-Ribopyranose tetraacetate (**9**) had m.p. 112°, lit.[21] m.p. 110°.

Tetra-*O*-acetyl-2-deoxy-*β*-D-*arabino*-hexopyranose (**10**) had m.p. 93° (lit.[22] m.p. 92.2–93.2°). The synthesis of compounds **11** to **16** is to be described elsewhere[14].

β-D-Xylopyranose tetraacetate (**17**) had m.p. 127° (lit.[23] m.p. 126°).

Methyl *β*-D-glucopyranuronate tetraacetate was kindly provided by Dr. G. G. S. Dutton, Dr. P. Reid, and Mr. K. Gibney, of this Department.

RESULTS AND DISCUSSION

All of the determination of sign by n.m.d.r. utilized either the "selective irradiation"[24,25] or the "spin tickling"[25,26] method. A typical application of each

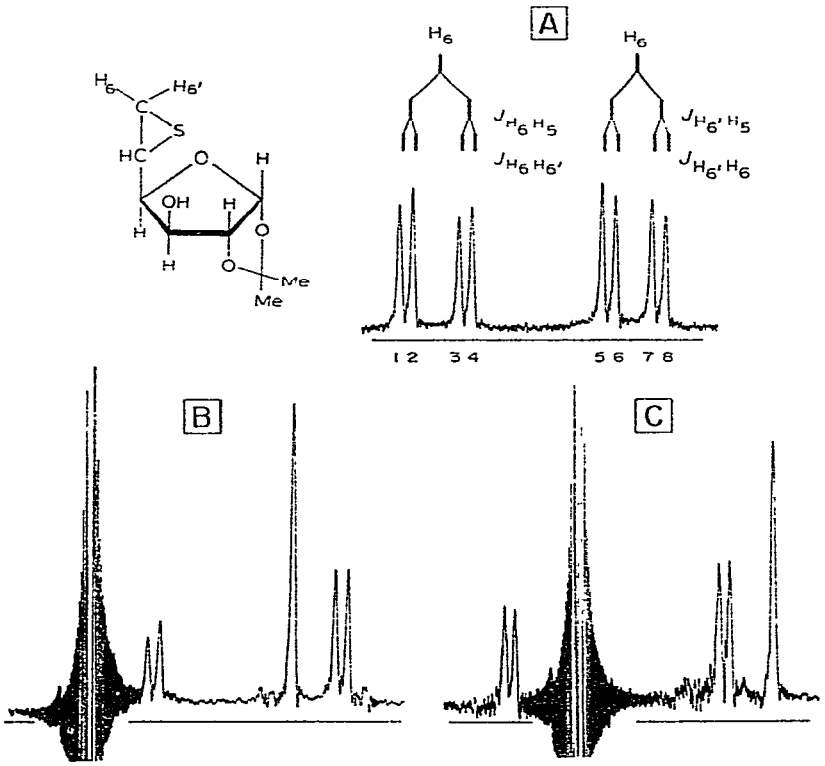


Fig. 1. (A) 100-MHz spectrum of the H-6 and H-6′ resonances of 5,6-dideoxy-5,6-epithio-1,2-*O*-isopropylidene-*β*-L-idofuranose (**5**) in chloroform-*d* solution. (B) Selective irradiation of transitions 1,2. (C) Selective irradiation of transitions 3,4. The "zero-beat" pattern in (B) and (C) indicates the precise point of irradiation.

of these approaches is outlined below. Both approaches are only applicable to systems having at least three mutually coupled spins, such as an AMX or an ABX system. In effect, the spin states of one nuclear species are used to predict the course of a double-resonance experiment involving the other two nuclei.

The first approach, "selective irradiation", involves the simultaneous irradiation of the transitions associated with a particular spectral splitting, and results in the collapse of associated transitions in another resonance, thus simplifying the spectrum. This method will be discussed with reference to the determination of the relative signs of $J_{5,6}$ and $J_{5,6'}$ of 5,6-dideoxy-5,6-epithio-1,2-*O*-isopropylidene-β-L-ido-furanose (**5**). The complete spectrum of this compound has been published[27], and Fig. 1A shows only the C-6 methylene proton resonances. The equal spacings 1,2, 3,4, 5,6, and 7,8 correspond to the characteristically small, *geminal* coupling-constant of epithio derivatives, and thus the splittings 1,3 and 2,4 correspond to $J_{5,6}$, and the splittings 5,7 and 6,8 correspond to $J_{5,6'}$.

If the usual convention is followed, and the two possible spin-states of each proton are represented as "α" and "β", the spin-state assignment for the transitions of the H-6 and H-6′ resonances can be made; this is shown in Table I. *This Table has been drawn up on the assumption that each of the couplings $J_{5,6}$, $J_{5,6'}$, and $J_{6,6'}$ have the same relative sign.* It effectively labels each of the transitions 1 to 8 in terms of the spin-states of its neighboring protons.

TABLE I

SPIN-STATE ASSIGNMENT[a] FOR THE H-6 AND H-6′ RESONANCES OF 5,6-DIDEOXY-5,6-EPITHIO-1,2-*O*-ISOPROPYLIDENE-β-L-IDOFURANOSE (**5**)

Spin states of neighboring protons	*H-6*		*H-6′*	
	Transition number			
	1 2	*3 4*	*5 6*	*7 8*
H-5	$\alpha\ \alpha$	$\beta\ \beta$	$\alpha\ \alpha$	$\beta\ \beta$
H-6			$\alpha\ \beta$	$\alpha\ \beta$
H-6′	$\alpha\ \beta$	$\alpha\ \beta$		

[a]Based on the assumption that of the couplings $J_{5,6}$, $J_{5,6'}$, and $J_{6,6'}$ have the same relative signs.

Consider now the experiment represented in Fig. 1B, in which transitions 1 and 2 are selectively irradiated with a radiofrequency field ($\mathbf{H}_2$) slightly greater in magnitude than $J_{6,6'}$, that is $\simeq 2$ Hz*. According to Table I, these two transitions

*Radiofrequency field-strengths are given in frequency units as $\gamma\mathbf{H}_2/2\pi$; for brevity, we have used the symbol $\mathbf{H}_2$. The strength of the decoupling field ($\mathbf{H}_2$) is readily determined by the method of Bloch and Siegert[28]. Consider a double-resonance experiment in which any resonance, A, is irradiated, while some other resonance, X, is observed; with $\mathbf{H}_2 = 0$, let the separation between A and X be $\Delta\nu_0$ Hz. When $\mathbf{H}_2$ is turned on, the X resonance will be displaced away from the point of irradiation, and the new frequency-separation between A and X will be $\Delta\nu_D$ Hz. Both $\Delta\nu_0$ and $\Delta\nu_D$ can be measured directly from the "normal" and "decoupled" spectra, respectively, and the strength of the decoupling field (in Hz) is given by $(\Delta\nu_D^2 - \Delta\nu_0^2)^{1/2}$ (see also, ref. 29).

correspond to H-5 in the "α" spin-state, and therefore the two transitions of the H-6′ resonance (which also correspond to H-5 = "α"), that is, transitions 5,6, should collapse. It can be clearly seen in Fig. 1B that this is so, and thus the relative signs of $J_{5,6}$ and $J_{5,6'}$ are the same, as had originally been assumed in Table I. Had the two *vicinal* couplings differed in sign, the above experiment would have resulted in the collapse of the H-6′ transitions corresponding the H-5 "β", namely, transitions 7,8. Fig. 1C shows the confirmatory experiment in which irradiation of transitions 3,4 causes a selective collapse of transitions 7,8, each of which corresponds to H-5 = "β".

This experimental method for the determination of sign was the first to be developed, and had several experimental limitations; for example, in the above experiment, it was only possible to determine the relative signs of two of the three coupling-constants possible. Fortunately, this limitation does not apply to the so-called "spin-perturbation" or "spin-tickling" procedure. The basic approach for this alternative method also involves the use of one set of nuclear-spin states as the reference for a double-resonance experiment. However, for spin-tickling experiments, a comparatively weak radiofrequency field ($\mathbf{H}_2 \ll J$, $\mathbf{H}_2 \simeq$ width of the observed line) is applied to a *single transition*. This perturbs the two energy-levels associated with that particular transition; this, in turn, results in the "doubleting" of any other transitions in the spectrum that have an energy level in common with either of the two energy-levels perturbed*. The determination of the relative signs of $J_{4,5e}$, $J_{4,5a}$, and $J_{5e,5a}$ of 1,2,3,4-tetra-*O*-acetyl-β-D-xylopyranose (**17**) provides a clear example of this approach.

The spectrum of this tetraacetate (**17**) is shown in Fig. 2A, and the corresponding spin-state assignment for the H-4, H-5*e*, and H-5*a* resonances is shown in Table II.

TABLE II

SPIN-STATE ASSIGNMENT[a] FOR THE SPECTRUM OF 1,2,3,4-TETRA-*O*-ACETYL β-D-XYLOPYRANOSE (**17**)

Spin states of neighboring protons	*H-4*								*H-5*e				*H-5*a			
	Transition number															
	9	*10*	*11*	*13*	*12*	*14*	*15*	*16*	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*
H-3	α	α	β	α	β	α	β	β								
H-4									α	β	α	β	α	β	α	β
H-5*e*	α	β	α	α	β	β	α	β					α	α	β	β
H-5*a*	α	α	α	β	α	β	β	β	α	α	β	β				

[a]Based on the assumption that $J_{4,5e}$, $J_{4,5a}$, and $J_{5e,5a}$ all have the same relative sign.

In order to simplify the region at *ca.* τ 5, a strong decoupling field was first applied to the H-1 resonance; this caused the H-2 quartet to collapse to a doublet, thus

*The reader is referred to ref. 26 for a discussion of this point. Briefly, the decoupling field $\mathbf{H}_2$ makes possible those transitions corresponding to $\Delta F_z = 0$ that are normally forbidden. Thus, transitions corresponding to both $\Delta F_z = \pm 1$ and 0 are observed.

allowing the H-4 transitions to be seen more clearly. Following this, a rather weak decoupling field ($\mathbf{H}_2 \simeq 0.6$ Hz) was applied to the highest field transition of the H-5*a* resonance (see Fig. 2B); this caused just two of the H-5*e* transitions (2,4 in Table II) to be split into doublets, while the other two transitions (1,3) remained unperturbed. A subsequent experiment (see Fig. 2C) demonstrated that irradiation of the lowest field transition of H-5*a* resulted in the selective perturbation of transitions 1 and 3.

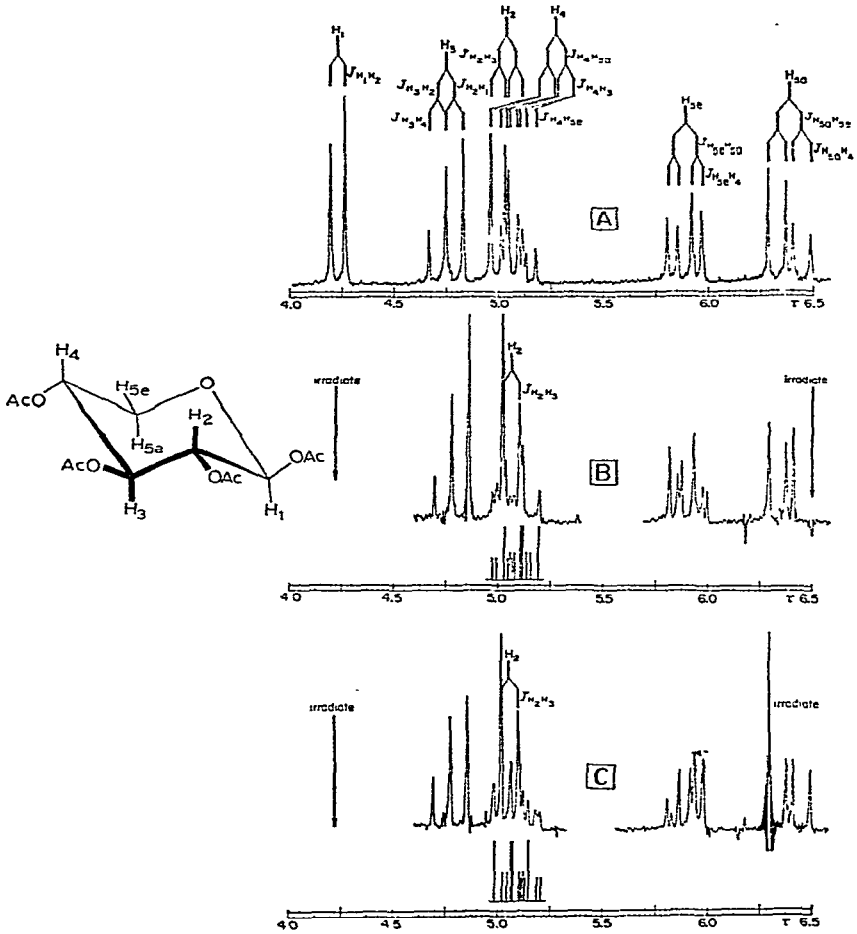


Fig. 2. (A) Partial 100-MHz spectrum of 1,2,3,4-tetra-*O*-acetyl-β-D-xylopyranose (**17**) in acetone-d_6 solution; (B) and (C), frequency-sweep, triple-resonance experiments in which H-1 is irradiated with a strong field ($\mathbf{H}_2 = 18$ Hz) while a single transition of the H-5*a* resonance is irradiated with a weak field ($\mathbf{H}_2 \simeq 0.6$ Hz).

The rationale of these observations is as follows. Let us assume that the relative signs of $J_{4,5e}$ and $J_{4,5a}$ are identical, and consider the case in which the highest field H-5*a* transition is irradiated. This transition (8) corresponds to H-4 = "β", and thus only those H-5*e* transitions which also have H-4 = "β" should be perturbed,

that is, transitions 2 and 4. As the experiment (see Fig. 2B) follows just this sequence, it follows that $J_{4,5e}$ and $J_{4,5a}$ do in fact have the same relative signs. Again, this result was quite simply confirmed by a further experiment in which an H-4 = "α" line was irradiated (see Fig. 2C). A further relevant observation for these two experiments concerns the relative resolution of the two doublets arising from the perturbed transitions. It may be seen quite clearly that, in each case, one of the doublets is more completely resolved than the other: in Fig. 2B, the doublet arising from the original transition 4 is resolved to the base-line, whereas the other doublet-arising from transition 2, is only partially resolved. This observation can also be rationalized[26]: the doublet will be sharply resolved whenever the "non-connective" spin-states of the two transitions involved in the experiment are the same (both "α", or both "β"), and will be less sharply resolved when the "non-connective" spin-states are different. This can be seen in the spin-state diagram for the experiment in which transition 8 is irradiated; this transition has the "connective" spin-state H-4 = "β" and, as already seen, this means that transitions 2 and 4 are the ones that will be perturbed, as they also have H-4 = "β". Now, transition 8 corresponds to H-5*e* = "β", and transition 4 corresponds to H-5*a* = "β"; thus, these two "non-connective" spin-states are the same, namely, both are "β"; and, therefore, transition 4 should become split into a sharply resolved doublet. The "non-connective" spin-states for transitions 8 and 2 are, however, different, and accordingly, transition 2 is perturbed to a poorly resolved doublet.

Because, in both of the spin-tickling experiments depicted in Fig. 2, only a single transition was irradiated, sign information can also be extracted from the perturbations caused in the H-4 resonance, as well as from the H-5*e* resonance. In this particular case, these perturbations are, however, less obvious, because the H-4 resonance is complicated by coupling with H-3, as well as with H-5*e* and H-5*a*; furthermore, some of the H-4 transitions are obscured by the H-2 resonance, even when H-1 is strongly irradiated. Nevertheless, the perturbations produced by irradiating single transitions in H-5*a* (which are depicted schematically below the spectra in Fig. 2), are closely related to the spin-state assignments in Table II, and show that the relative signs of $J_{5e,5a}$ and $J_{5e,4}$ are *opposite*. This result is most clearly visible if attention is directed towards the outermost transitions of the H-4 resonance, namely transitions 9 and 16. Irradiation of line 8 (an H-5*e* = "β" transition) perturbs line 9 (which is an H-5*e* = "α" transition), and this gives the sign relationship mentioned above. Thus, for this β-D-xylose derivative (17), the *relative* signs of the coupling constants are found to be $\pm J_{4,5e}$, $\pm J_{4,5a}$, and $\mp J_{5e,5a}$.

The choice as to which of the above methods should be used for a particular determination of sign is governed by the relative magnitudes of the various couplings under consideration. If a pair of transitions are clearly separated from the remainder of the multiplet, as well as from all other related transitions, and the coupling to be irradiated is fairly small (less than 5 Hz), the selective-irradiation technique is *suitable*. However, each decoupling experiment can lead to the determination of only *two* relative signs, and, therefore, we prefer the spin-tickling method, which can generally

lead to all three signs, even though the end result is a spectrum that is more complicated than the original.

Any experimental determination of sign by n.m.r.d. yields only the *relative* signs of the couplings under consideration. These may, however, be placed on an *absolute* basis as follows. Early experiments showed[13,30] that *vicinal* ^1H–^1H couplings have the same *relative* signs as ^{13}C–^1H couplings. If the assumption is made that the ^{13}C–^1H coupling is *absolutely positive*, it follows[12,13] that *vicinal* ^1H–^1H couplings are also *absolutely positive*. Confirmation of this assignment was claimed by Buckingham and McLauchlan[31] on the basis of an n.m.r. study of *p*-nitrotoluene in the presence of a powerful electrostatic field; the *vicinal* proton coupling was found to be *absolutely positive*. Although the conclusions of this particular experiment have been questioned[32], a recent study of cyclopropane and cyclobutane in nematic solutions has independently shown[33] *vicinal* couplings to be *absolutely positive*. In the present study, our assignments have been based on the assumption that *all vicinal proton–proton couplings are absolutely positive*. The results that we have obtained are summarized in Table III.

*The signs of vicinal and geminal couplings.** — The early theoretical studies[7,35] of vicinal and geminal couplings predicted that both sets of couplings should have the same sign. Subsequent determinations of sign established[13,30] that *vicinal* couplings are *absolutely* positive, whereas *geminal* couplings can have either sign[36], depending on the precise hybridization of the particular carbon atom; they are usually *negative* for sp^3-hybridized systems.

The results obtained for the derivatives **8** and **10** are in accord with the above expectation. In contrast to this finding, derivatives **1** and **6** have both *vicinal* and *geminal* couplings *positive*. An interesting variation in the signs of geminal couplings follows from a comparison of the couplings of the epoxide **3** and episulfide **5**. It is evident that, whereas the sign of the episulfide (**5**) coupling is typical of an sp^3-hybridized system, that of the epoxide (**3**) coupling is typical of an sp^2-hybridized system. Previous comparative studies of epi derivatives have revealed[40] this inversion of sign and, on this basis, it seems highly probable that the *geminal* coupling of the 5,6-epimine derivative related to **3** and **5** should be positive in sign.

Although this inversion of sign should not invalidate the stereospecific dependences outlined by Buss *et al.*[41] for the *vicinal* couplings of epi derivatives, these dependences should at present be treated with some caution.**

*The signs of long-range coupling constants.**** — According to the McConnell extension of the Dirac–Van Vleck vector model, all long-range couplings across four bonds in saturated systems (^4J) should be negative in sign. A more recent theoretical study by Barfield[9] has indicated that, *if the sole mechanism for long-range coupling is* via *the bonding electrons* (the" indirect" mechanism), $^4J_{e,e}$ *and* $^4J_{a,e}$

*For a recent review of the theory of nuclear spin–spin coupling-constants, see ref. 34.
**We are currently re-investigating this problem in collaboration with Mr. A. Tracey.
***For a preliminary discussion of these couplings, see ref. 42.

TABLE III

THE SIGNS OF PROTON–PROTON SPIN-COUPLING CONSTANTS OF CARBOHYDRATE DERIVATIVES[a]

Compound	*Signs and magnitudes of relevant coupling constants (Hz)*
3-*O*-Acetyl-5,6-dideoxy-1,2-*O*-isopropylidene-α-D-*xylo*-hex-5-enofuranose (1)[b]	$J_{5,6}$ +10.0; $J_{5,6'}$ +17.4; $J_{6,6'}$ +2.5
1,6-Anhydro-β-D-idopyranose triacetate (2)[c]	$J_{4,5}$ +4.3; $J_{4,6}$ +1.1; $J_{5,6}$ +4.3
5,6-Anhydro-1,2-*O*-isopropylidene-α-D-glucofuranose (3)[b,d]	$J_{5,6}$ +4.2; $J_{5,6'}$ +2.8; $J_{6,6'}$ +4.85; $J_{4,6'}$ −0.3
1,6-Anhydro-β-D-mannopyranose triacetate (4)[b]	$J_{1,3}$ +1.6; $J_{3,5}$ +1.5; $J_{1,2}$ +2.0; $J_{2,3}$ +5.5; $J_{3,4}$ +1.6; $J_{4,5}$ +1.6
5,6-Dideoxy-5,6-epithio-1,2-*O*-isopropylidene-β-L-idofuranose (5)[b,e]	$J_{5,6}$ +6.55; $J_{5,6'}$ +5.3; $J_{6,6'}$ −1.45
5,6-Dideoxy-1,2-*O*-isopropylidene-α-D-*xylo*-hex-5-enofuranose (6)[b,f]	$J_{4,5}$ +4.8; $J_{5,6}$ +10.0; $J_{5,6'}$ +17.3; $J_{6,6'}$ +2.5; $J_{4,6}$ −1.6; $J_{4,6'}$ −1.4
D-Glucal triacetate (7)[g]	$J_{1,2}$ +6.4; $J_{2,3}$ +3.2; $J_{1,3}$ −1.3
Methyl tetra-*O*-acetyl-β-D-glucopyranoside (8)[g]	$J_{5,6}$ +4.85; $J_{5,6'}$ +2.4; $J_{6,6'}$ −12.3
β-D-Ribopyranose tetraacetate (9)[g]	$J_{3,4}$ +3.2; $J_{2,3}$ +3.2; $J_{2,4}$ +0.8
Tetra-*O*-acetyl-2-deoxy-β-D-*arabino*-hexopyranose (10)[g]	$J_{1,2e}$ +2.4; $J_{1,2a}$ +9.8; $J_{2,e2a}$ −12.4; $J_{2e,3}$ +5.0; $J_{2a,3}$ +11.0
3,4,6-Tri-*O*-acetyl-1-*O*-benzoyl-2-chloro-2-deoxy-α-D-glucopyranose (11)[b]	$J_{1,3}$ −0.4; $J_{1,5}$ |0.6|; $J_{1,2}$ +3.6; $J_{2,3}$ +10.7
3,4,6-Tri-*O*-acetyl-1-*O*-benzoyl-2-deoxy-2-iodo-α-D-mannopyranose (12)[g]	$J_{1,3}$ −0.65; $J_{1,5}$ |0.65|; $J_{1,2}$ +1.6; $J_{2,3}$ +4.3
3,4,6-Tri-*O*-acetyl-2-bromo-2-deoxy-α-D-glucopyranosyl fluoride (14)[b]	$J_{1,3}$ −0.4; $J_{1,2}$ +2.5; $J_{2,3}$ +10.7
3,4,6-Tri-*O*-acetyl-2-bromo-2-deoxy-α-D-mannopyranosyl fluoride (13)[b]	$J_{2,4}$ −0.3; $J_{2,3}$ +3.95; $J_{3,4}$ +9.50
3,4,6-Tri-*O*-acetyl-2-deoxy-2-iodo-α-D-glucopyranosyl fluoride (15)[b]	$J_{1,3}$ −0.2; $J_{1,5}$ |0.2|; $J_{1,2}$ +2.4; $J_{2,3}$ +11.1
3,4,6-Tri-*O*-acetyl-2-deoxy-2-iodo-α-D-mannopyranosyl fluoride (16)[g]	$J_{1,3}$ −0.5; $J_{2,4}$ −0.45; $J_{1,5}$ |0.2|; $J_{1,2}$ +1.5; $J_{2,3}$ +4.3; $J_{3,4}$ +9.5
β-D-Xylopyranose tetraacetate (17)[g]	$J_{4,5e}$ +4.9; $J_{4,5a}$ +8.5; $J_{5e,5a}$ −11.8

[a] All relative signs in this Table were determined by the spin–tickling method. [b] In chloroform-*d* solution; [c] In benzene-d_6 solution; [d] The spectrum of this compound was measured in both chloroform and acetone solution. The magnitudes of the couplings were solvent-dependent. [e] Selective irradiation was also used for determining the relative signs of $J_{5,6}$ and $J_{5,6'}$. [f] The value of $J_{6,6'}$ is an average value. [g] In acetone-d_6 solution.

couplings should both be positive in sign, whereas $^4J_{a,a}$ *couplings should be negative in sign.* The respective magnitudes of these three couplings were predicted to be +1.16, +0.65, and −0.2 Hz. The suggestion has also been made[43,44] that $^4J_{e,e}$ couplings are, in fact, transmitted through space (the "direct" mechanism) as a result of overlap of the "tail" lobes of the two C–H bonds. Although these conflicting views have been on record for some time, a definitive study of this point has been delayed by the difficulty of finding derivatives that would give spectra showing the appropriate couplings in a form suitable for determinations of relative sign. Fortunately, pyranoid carbohydrate derivatives are suitable for this purpose, and a study

of derivatives **2**, **4**, **9**, and **11–16** has, with no exceptions, shown that $^4J_{e,e}$ couplings are *positive* (+0.8 to +1.6 Hz), and $^4J_{e,a}$ couplings are *negative* in sign (−0.2 to −0.7 Hz). Thus, the sign and magnitude of the $^4J_{e,e}$ couplings are in extremely close agreement with Barfield's calculations, and this result implies that such couplings occur *via* the "indirect" instead of the "direct" mechanism, which had previously, and erroneously, been assumed[43]. Although the magnitudes of the $^4J_{e,a}$ couplings of pyranoid sugar derivatives are in accord with the predicted value, their signs are opposite: we do not feel competent to comment on this discrepancy*. This inversion of sign should provide an extremely valuable extension of the selection of stereospecific dependences that are available for configurational and conformational assignments. In particular, it should find some use in studies of derivatives that show ^4J couplings whose magnitudes lie between those normally found for $^4J_{e,e}$ and $^4J_{e,a}$ couplings. Since these studies were completed, Pachler and Underwood have reported[45] that the ^4J couplings of eight pterocarpan derivatives exhibit an inversion of sign similar to that observed by us for pyranoid derivatives. It is noteworthy that the magnitudes of the long-range couplings observed in the present study are considerably smaller than those found for strained-ring systems[46]; we are currently investigating this point. It is also of some interest that we find little evidence for $^4J_{a,a}$ coupling. The sole *possible* example we have found is an apparent coupling of +0.75 Hz between the H-2 and H-4 resonances of methyl β-D-glucopyranuronate tetraacetate. Although this spectrum was clearly beyond the first-order limits of Musher and Corey[47], the fact that the coupling changed from +0.75 Hz in chloroform to +0.60 Hz in acetone solution suggests that it would be unwise to base any discussion on this one example.

Long-range couplings occur so widely[48] in furanoid derivatives that it is seldom possible to resolve and observe them directly. In a detailed n.m.d.r. study of 3-*O*-acetyl-1,2-*O*-isopropylidene-α-D-xylofuranurononitrile, Pachler and Wessels[49] showed that the signs of all the ^4J couplings were *negative*.

The long-range couplings of the unsaturated derivatives **1**,**6**, and **7** were all found to be negative in sign. It is interesting that the epoxide **3** exhibits a long-range coupling of magnitude similar to that of that shown by the 5,6-ene (**6**), whereas no such couplings are observed for the episulfide **5**. This result supports the similarity previously observed between the couplings of **3** and **6**. The mechanism of proton–proton coupling in unsaturated systems**, has been discussed previously[8,9].

Although this study represents the first extensive investigation of the signs of carbohydrate coupling-constants, there have been previous (isolated) studies[50–52]. In particular, Coxon, Jennings, and McLauchlan have recently determined[53] the signs of couplings of 3,4-dichloro-2,3,4-trideoxy-D-*glycero*-pent-2-enopyranosyl derivatives. Current studies in this laboratory involve the use of carbohydrates substi-

*Professor M. Barfield has informed us that he is currently re-investigating the theoretical analysis of long-range couplings.

**See ref. 44 for an excellent review.

tuted by such other "magnetic" nuclei as ^{19}F (see ref. 54) and ^{31}P (see ref. 55) as model systems for a general investigation of the signs and stereospecific dependences of a variety of coupling constants.

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SYNTHESIS OF GLUCOSYLGLYCEROLS AND DIGLUCOSYLGLYCEROLS, AND THEIR IDENTIFICATION IN SMALL AMOUNTS

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ABSTRACT

The four *O*-β-D-glucopyranosyl(1→*x*)-*O*-β-D-glucopyranosyl(1→1)-D-glycerols (*x* = 2, 3, 4, or 6) have been synthesized and shown to be distinguishable by paper and gas–liquid chromatographic techniques. Synthesis of the four *O*-D-glucopyranosyl-(1→1)-glycerols and *O*-α-kojibiosyl-(1→1)-L-glycerol showed that anomers, but not all diastereomers, are distinguishable by these methods. The application of these results to general problems in structural carbohydrate chemistry is outlined, and the procedure has been used in the identification of small amounts of glycosides derived from bacterial glycolipids.

INTRODUCTION

Glycosylglycerols and diglycosylglycerols have been isolated as deacylation products of glycolipids from Gram-positive bacteria[1] and plants[2], and as degradation products of various carbohydrates[3–5] and teichoic acids[6]. For three of the glycolipids, confirmation of structures derived from degradative studies has been provided by synthesis[7–9]. However, as the amount of glycoside isolated from bacteria[10] is usually small, a microscale method of identification, based on a comparison with synthetic compounds, seemed desirable.

DISCUSSION

Gas–liquid chromatography of glycosyl- and diglycosyl-glycerols (obtained from bacterial glycolipids[10]), as their trimethylsilyl ethers[11] on SE52 at 185° and 255°, respectively, indicated that this method might be useful for the identification of related glycosides, especially when taken in conjunction with paper chromatography. The procedure has been examined for a particularly accessible series, the *O*-β-D-glucopyranosyl-*O*-β-D-glucopyranosyl-(1→1)-D-glycerols, and for diastereomeric glucosyl- and diglucosyl-glycerols. The results (Table I) indicated that the four positional isomers of diglucosylglycerol are distinguishable by g.l.c. and paper chromatography, and provide proof that the glycoside obtained by deacylation of the glycolipids of *Bacillus subtilis*, *Staphylococcus aureus* H, *Staph. aureus* B4, *Staph. saprophyticus* I2,

Staph. lactis (*albus*) N.C.T.C. 7944, and *Staph. lactis* I3 is, in all cases, *O*-β-gentiobiosyl-(1→1)-glycerol (**1**). The glycoside from *Lactobacillus casei* A.T.C.C. 7469 is identical to that[7] from *Pneumococcus* 1-192R, A.T.C.C. 12213, and was shown

TABLE I

PAPER AND GAS–LIQUID CHROMATOGRAPHIC PROPERTIES OF SYNTHETIC AND NATURAL DIGLYCOSYLGLYCEROLS

Glycoside	R_{Glc}[a]	T[b]
From *Pneumococcus glycolipid*[26] (**2**)	0.43	1.00
From *Lactobacillus casei* glycolipid[10]	0.43	1.01
Synthetic Gal-Glc-Gly[7] (**2**)	0.43	1.00
O-β-Sophorosyl-(1→1)-D-glycerol (**10**)	0.65	0.93
O-α-Sophorosyl-(1→1)-D-glycerol (**9**)	0.57	1.06
O-Laminaribiosyl-(1→1)-D-glycerol (**8**)	0.79	0.91
O-β-Cellobiosyl-(1→1)-D-glycerol (**6**)	0.43	1.10, 0.87[c]
O-β-Gentiobiosyl-(1→1)-D-glycerol (**7**)	0.49	1.33
From *B. subtilis* glycolipid[10]	0.49	1.33
From *Staph. aureus* H glycolipid[10]	0.49	1.33
From *Staph. aureus* B4 glycolipid	0.49	1.33
From *Staph. saprophyticus* I2 glycolipid[10]	0.49	1.33
From *Staph. lactis* (albus) glycolipid[10]	0.49	1.33
From *Staph. lactis* I3 glycolipid[8]	0.49	1.33
From *Micrococcus lysodeikticus* glycolipid[10]	0.51	0.76
From *Strep. faecalis* glycolipid[10]	0.53	1.06
O-α-Kojibiosyl-(1→1)-L-glycerol (**4**)	0.52	1.07

[a]Paper-chromatographic mobility relative to that of glucose (Ref. 24). [b]Elution time of *O*-trimethylsilyl derivative relative to that of glycoside **2**, 13.25 min. [c]Impurity, presumably of α-cellobioside, present as 4% of preparation.

previously[7] by synthesis to be *O*-α-D-galactopyranosyl-(1→2)-*O*-α-D-glucopyranosyl-(1→1)-D-glycerol (**2**). The chemical degradation of the glycoside from *L. casei* confirms this structure[12]. The glycoside from the glycolipid of *Streptococcus faecalis* 9, characterized[13] for a different strain as *O*-α-D-glucopyranosyl-(1→2)-*O*-α-D-glucopyranosyl-(1→1)-glycerol (**3**) (presumably the D-glycerol form, by biosynthetic reasoning), chromatographed identically with the synthetic diastereomer of L-glycerol (**4**), suspected as a degradation product of a novel teichoic acid under investigation in this laboratory[14]. Thus, it appears that the method will not distinguish diastereomeric glycerol glycosides. The same conclusion was reached with the four synthetic *O*-D-glucopyranosyl-(1→1)-glycerols (Table II).

Isomers of *O*-β-D-glucopyranosyl-*O*-β-D-glucopyranosyl-(1→1)-D-glycerol were synthesized by condensation of the appropriate hepta-*O*-acetyldisaccharide bromides and methylenebis-2-*O*-(3-*O*-benzoyl-D-glycerol)[9] (**5**) in methylene chloride in the presence of mercuric oxide and mercuric bromide[15]. Hepta-*O*-acetyl-α-sophorosyl bromide gave a high proportion of the α-sophoroside, in accordance with the previous conclusion that the reason for the usual production of *trans*-glycosides in the Koenigs–Knorr synthesis is the participation of a neighbouring acyl group on C-2 of the

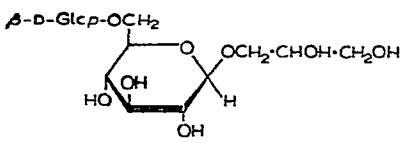
acylglycosyl halide[7,16–18]. It is interesting that the β-sophoroside trimethylsilyl ether emerged from the g.l.c. column before the α-sophoroside derivative, and that the β-sophoroside had the lower melting point and the faster mobility on paper chromatography. Construction of the molecular models indicated that the β-glycoside is the more hindered of the two anomers.

TABLE II

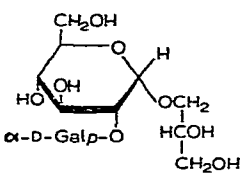
PAPER AND GAS–LIQUID CHROMATOGRAPHIC PROPERTIES OF SYNTHETIC AND NATURAL GLYCOSYLGLYCEROLS

Glycoside	R_{Glc}[a]	T[b]
O-α-Glucosyl-(1→1)-D-glycerol	1.00	1.00
O-β-Glucosyl-(1→1)-D-glycerol	1.02	1.36
O-α-Glucosyl-(1→1)-L-glycerol	1.00	1.00
O-β-Glucosyl-(1→1)-L-glycerol	1.00	1.36
O-α-Galactosyl-(1→1)-D-glycerol	0.86	0.97
O-β-Galactosyl-(1→1)-D-glycerol	0.85	1.08
From *Pneumococcus* minor glycolipid[26]	1.00	1.00
From *Staph. aureus* B4 minor glycolipid	1.01	1.36
From partial acidic hydrolysis of 8	—	1.00, 1.36

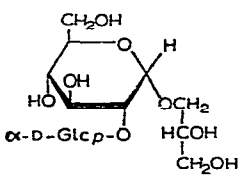
[a]Paper-chromatographic mobility relative to that of glucose (Ref. 24). [b]Elution time of *O*-trimethylsilyl derivative relative to that of *O*-α-D-glucopyranosyl-(1→1)-D-glycerol, 17.05 min.



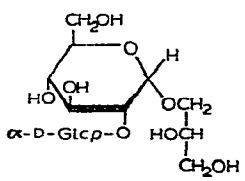
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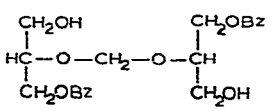
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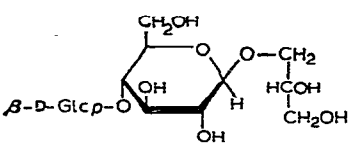
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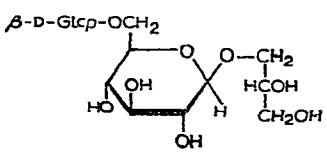
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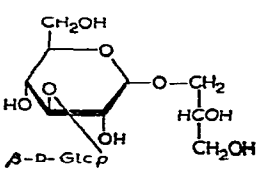
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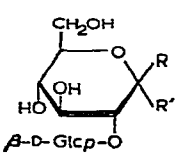
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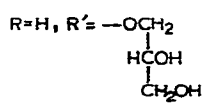
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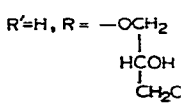


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The β-cellobioside **6** and β-gentiobioside **7** were easily prepared in a pure condition[8]. On the other hand, the laminaribioside **8** was obtained as a mixture of the α- and β-anomers, and these were not resolved by g.l.c. or paper chromatography. This may be due to the steric effect between the bifunctional aglycon **5** and the hepta-*O*-acetyl-α-laminaribiosyl bromide in which the tetra-*O*-acetyl-D-glucopyranosyl substituent is still near the C-1 reaction centre. Support for the view that an aglycon can be partly responsible for influencing the anomeric configuration in the synthesis of a glycoside comes from a study of the reaction between compound **5** and tetra-*O*-acetyl-α-D-glucopyranosyl bromide, when 29% of β-D-glucoside and 6% of α-D-glucoside were obtained. Wickberg's synthesis[9] of the corresponding anomeric galactosides was repeated, and his similar observation was confirmed. By contrast, condensation of tetra-*O*-acetyl-α-D-glucopyranosyl bromide with 2,3-di-*O*-benzyl-L-glycerol yielded only a trace of the α-D-glucoside, a result consistent with Wickberg's findings[9] in the synthesis of the corresponding anomeric L-glycerol galactosides. We synthesized *O*-α-D-glucopyranosyl-(1→1)-L-glycerol by condensation of 2,3-di-*O*-benzyl-L-glycerol with 1 molar proportion of 3,4,6-tri-*O*-acetyl-β-D-glucopyranosyl chloride[19]. Subsequent addition of a second molar proportion of the chloride to the reaction mixture yielded *O*-α-D-glucopyranosyl-(1→2)-*O*-α-D-glucopyranosyl-(1→1)-L-glycerol (**4**). Sequential addition of hexose residues in this way has found use recently in the synthesis of substituted cerebrosides[20,21], but this is the first report of the synthesis of *cis*-glycosidic linkages by this procedure.

The g.l.c. and paper-chromatographic procedure described here has been used successfully on small amounts (less than 2 mg) of a number of glycosides of glycerol obtained from earlier work[10] on the structure of bacterial glycolipids. When appropriate samples of synthetic glycosides are available, it could be used for the identification of glycosylglycerols obtained from teichoic acids and from the degradation of other carbohydrates, especially in the application of the Smith degradation[23] to polysaccharides. The method would also be valuable in the identification of glycosylglycerols obtained by partial hydrolysis of diglycosylglycerols with acid and in the determination of purity of anomeric glycerol glycosides. Its sensitivity is shown in an examination of the glycoside preparation from the total glycolipid of *Staph. aureus* B4; g.l.c. of the trimethylsilyl ethers revealed the presence of *O*-β-D-glucopyranosyl-(1→1)-glycerol and *O*-β-D-gentiobiosyl-(1→1)-glycerol (**1**) in the molar ratio 1:100.

O-β-D-Glucopyranosyl-(1→1)-D-glycerol, the L-glycerol diastereomer, and *O*-β-gentiobiosyl-D-glycerol (**7**) were examined by Prof. E. F. Gale as possible[22] bacterial 'amino acid incorporation factors'. The first glycoside was highly active, whereas the other two were totally inactive.

EXPERIMENTAL

General Methods. — Gas–liquid chromatography of glycosylglycerols as their trimethylsilyl ethers, prepared by addition of Pierce 'TRI-SIL' reagent (1 ml/1 mg

of glycoside), was carried out with a Pye Series 104 Chromatograph on a column (150 cm) of 3% SE-52 on Celite (80–100 mesh) with N_2 as carrier[11] at a pressure of 1.06 Kg/cm^2 and a temperature of 185°. Diglycosylglycerols were examined at 255°. Mixtures arising from partial hydrolysis of diglycosylglycerols with acid were examined by a programme using an initial period of 30 min at 185°, followed by maximal rate of column heating to 255° and a final period of 30 min.

Paper chromatography of glycosides was performed on Whatman No. 1 paper in the solvent system[24] butyl alcohol–pyridine–water (6:4:3 by vol.). Compounds were detected by the periodate–Schiff reagents for α-glycols[25] and the modified[26] silver nitrate reagent for sugars and polyols[27].

Thin-layer chromatography of glycoside acetates was performed on Silica gel G (Merck) with the solvent system benzene–ethanol (19:1). Compounds were detected by spraying the plates with 4% sulphuric acid followed by heating in an oven for 20 min at 150°.

*Methylenebis-2-*O*-(3-*O*-benzoyl-*D*-glycerol)* (**5**). — This compound, prepared by the literature method[7], had m.p. 107°, $[\alpha]_D$ −61 ±1° (*c* 0.8, chloroform).

*2,3-Di-*O*-benzyl-*L*-glycerol.* — This compound, prepared as required by acid hydrolysis[9] of its 1-trityl ether, had m.p. 85°, $[\alpha]_D$ −9° (*c* 0.8, chloroform).

*3,4,6-Tri-*O*-acetyl-2-*O*-(2,3,4,6-tetra-*O*-acetyl-β-*D*-glucopyranosyl)-α-*D*-glucopyranosyl bromide.* — Methyl 4,6-*O*-benzylidene-2-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-α-D-glucopyranoside[28] (1.43 g) was dissolved in acetic anhydride (4 ml), a mixture of acetic anhydride and sulphuric acid (14:1 by vol., 4 ml) was added, and the acetolysis product was recovered[29]. The syrup was dissolved in 47% hydrogen bromide in acetic acid (10 ml) and kept for 3 h at room temperature. The mixture was poured into ice–water (20 ml), and the product was extracted with chloroform (3 × 20 ml). The combined extracts were washed with ice-cold, saturated, aqueous sodium hydrogen carbonate (3 × 20 ml), dried (Na_2SO_4), and evaporated to dryness. The residue was recrystallized twice from ethyl acetate to give the bromide (1.13 g, 69%), m.p. 192°, $[\alpha]_D$ +94 ±2° (*c* 0.5, chloroform).

*2,4,6-Tri-*O*-acetyl-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-*D*-glucopyranosyl)-α-*D*-glucopyranosyl bromide.* — Laminaribiose was synthesized[30], and the resulting reaction mixture was chromatographed on a column (35 × 6 cm) of charcoal–Celite (1:1, w/w)[31]. D-Glucose was removed by elution with water (10 l), and then portions (3 l) of increasing concentrations of ethanol in water (respectively 2, 4, 6, 8, and 10%, v/v) were applied to the column. Laminaribiose (1.5 g), containing a trace of D-glucose, was obtained from the 8% ethanol fraction. The material could not be crystallized, presumably due to the D-glucose impurity, and it was rechromatographed. The pure disaccharide was converted, in the usual manner, into the hepta-*O*-acetyl bromide *via* its crystalline octa-acetate. It had m.p. 175°, $[\alpha]_D$ +80 ±2° (*c* 0.8, chloroform).

*2,3,6-Tri-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-*D*-glucopyranosyl)-α-*D*-glucopyranosyl bromide.* — This was prepared by bromination of cellobiose octa-acetate. It had m.p. 184°, $[\alpha]_D$ +93 ±2° (*c* 1.0, chloroform).

3,4,6-Tri-O-acetyl-β-D-glucopyranosyl chloride. — This was prepared[19] from the 2-trichloroacetate[32]. It had m.p. 134°, $[\alpha]_D$ +41 ±1° (*c* 0.4, chloroform).

O-β-D-Glucopyranosyl-O-β-D-glucopyranosyl-(1→1)-D-glycerols. — These were prepared by a general procedure. A mixture of compound **5** (500 mg), mercuric oxide (400 mg), mercuric bromide (40 mg), and Drierite (1.5 g) was stirred in dry methylene chloride (5 ml) for 2 h. A solution of the appropriate acetylated disaccharide bromide (1.85 g) and iodine (300 mg) in dry methylene chloride (7 ml) was added during 5 min, and the mixture was stirred for 72 h at room temperature. The glycosides were recovered[9] and separated[33] on a column (30 × 2.5 cm) of Dowex-1 (OH^- form) resin.

The sophorosyl bromide yielded *O-β-D*-glucopyranosyl-(1→2)-*O-α-D*-glucopyranosyl-(1→1)-D-glycerol (**9**) (160 mg, 16%), which, after two recrystallizations from moist ethanol, had m.p. 190°, $[\alpha]_D$ +66.5 ±1.0° (*c* 0.1, water) [*Anal.* Calc. for $C_{15}H_{28}O_{13}$: C, 43.25; H, 6.8. Found: C, 43.25; H, 6.85]; and *O-β-D*-glucopyranosyl-(1→2)-*O-β-D*-glucopyranosyl-(1→1)-D-glycerol (**10**) (283 mg, 27.5%), which, after two recrystallisations from moist ethanol, had m.p. 186°, $[\alpha]_D$ −20.0 ±1.0° (*c* 0.1, water) [*Anal.* Calc. for $C_{15}H_{28}O_{13}$: C, 43.25; H, 6.8. Found: C, 43.3; H, 6.9].

"The laminaribiosyl bromide (1.59 g) yielded 45 mg (4.8%) of gum **8**, $[\alpha]_D$ +10.0 ±0.2° (*c* 1.5, water)." The nona-acetate was also non-crystalline and, when examined by t.l.c., gave two compounds, R_F 0.22 and 0.235. A degradation described later shows that the preparation is a mixture of the two anomeric laminaribiosides.

The cellobiosyl bromide (1.93 g) gave a mixture of non-crystalline cellobiosides (139 mg, 12%), $[\alpha]_D$ −8.7° (*c* 1.2, water), estimated by g.l.c. of their trimethylsilyl ethers to be present in the ratio $\beta:\alpha$ = 93:7. The isomers were not separable by chromatography on a column of Dowex-1 (OH^- form) resin, but the preparation ran on a paper chromatogram as an intense spot having a faint tail. The mixture afforded nona-*O*-acetyl-*O-β-D*-glucopyranosyl-(1→4)-*O-β-D*-glucopyranosyl-(1→1)-D-glycerol which, after four recrystallizations from ethanol, had m.p. 148°, $[\alpha]_D$ −5.8 ±1.0° (*c* 0.5, chloroform).

Anal. Calc. for $C_{33}H_{46}O_{22}$: C, 49.9; H, 5.7. Found: C, 49.9; H, 5.8.

Saponification of the acetate and g.l.c. investigation of the liberated glycoside as its trimethylsilyl ether showed the preparation to contain 4% of the α-anomer as impurity (Table I).

The preparation of nona-*O*-acetyl-*O-β-D*-glucopyranosyl-(1→6)-*O-β-D*-glucopyranosyl-(1→1)-D-glycerol has already been published[8].

The anomeric O-D-*galactopyranosyl-(1→1)-D-glycerols.* — These were prepared as described in the literature[9] and separated by chromatography on a column of Dowex-1 (OH^- form) resin. The α-anomer had m.p. 152°, $[\alpha]_D$ +158 ±3° (*c* 0.3, water); the β-anomer had m.p. 138–40°, $[\alpha]_D$ 0 ±1° (*c* 1.0, water).

The anomeric O-D-*glucopyranosyl-(1→1)-D-glycerols.* — These were prepared from tetra-*O*-acetyl-α-D-glucopyranosyl bromide (2.25 g) as described above for the corresponding galactosides.

The α-anomer (82 mg, 6%) had $[\alpha]_D$ +93 ±2° (*c* 0.2, water) and was character-

ized as hexa-*O*-acetyl-*O*-α-D-glucopyranosyl-(1→1)-D-glycerol, crystallized twice from aqueous ethanol, m.p. 90°, $[\alpha]_D$ +87 ±2° (*c* 0.9, methanol).

Anal. Calc. for $C_{21}H_{30}O_{14}$: C, 49.9; H, 5.9. Found: C, 49.95; H, 5.7.

The β-anomer (397 mg, 29%) had $[\alpha]_D$ −32 ±1° (*c* 0.5, water) and was characterized as hexa-*O*-acetyl-*O*-β-D-glucopyranosyl-(1→1)-D-glycerol; crystallized twice from ethanol, m.p. 144°, $[\alpha]_D$ −4.4 ±1.0° (*c* 0.4, chloroform).

Anal. Calc. for $C_{21}H_{30}O_{14}$: C, 49.9; H, 5.9. Found: C, 49.8; H, 5.8.

O-β-D-*Glucopyranosyl-(1→1)-*L*-glycerol.* — This was prepared from tetra-*O*-acetyl-α-D-glucopyranosyl bromide (4.5 g) and 2,3-di-*O*-benzyl-L-glycerol (2.5 g) as described[9] for the corresponding galactoside. Chromatography of the crude glycoside mixture on a column (30 × 2.5 cm) of Dowex-1 (OH^- form) resin gave 12.4 mg of impure α-D-glucoside and 1.19 g (51%) of pure β-D-glucoside, $[\alpha]_D$ −24.5 ±0.5° (*c* 0.4, water), which was characterized as hexa-*O*-acetyl-*O*-β-D-glucopyranosyl-(1→1)-L-glycerol, crystallized twice from ethanol, m.p. 107°, $[\alpha]_D$ −54.6 ±1.0° (*c* 0.5, chloroform).

Anal. Calc. for $C_{21}H_{30}O_{14}$: C, 49.9; H, 5.9. Found: C, 49.9; H, 6.1.

O-α-D-*Glucopyranosyl-(1→1)-*L*-glycerol.* — A solution of 2,3-di-*O*-benzyl-L-glycerol (0.5 g) in dry ether (20 ml) was stirred in the dark for 2 h with silver carbonate (1.5 g), silver perchlorate (60 mg), and Drierite (2 g). A solution of the 3,4,6-tri-*O*-acetyl-β-D-glucosyl chloride (0.62 g, 1 mol. prop.) in ether (10 ml) was added in one portion, and the mixture was stirred for 4 h at room temperature. The reaction mixture was filtered through a pad of Celite, and the filtrate was evaporated to a syrup. This was redissolved in methanol (30 ml) and hydrogenated in the presence of 10% palladium-on-charcoal (0.5 g) until reaction was complete. After filtration, a little sodium methoxide in methanol was added to the filtrate, and the mixture was kept overnight. Water (60 ml) was added, and the solution was passed through a short column (10 ml) of Dowex-50 (H^+ form) resin, and the eluate was evaporated. Chromatography of the crude glycoside mixture on a column (20 × 3 cm) of Dowex-1 (OH^- form) resin yielded 141 mg (30%) of pure α-anomer, $[\alpha]_D$ +142 ±3° (*c* 0.3, water); and 64 mg (14%) of pure β-anomer. The α-anomer was characterized as hexa-*O*-acetyl-*O*-α-D-glucopyranosyl-(1→1)-L-glycerol; crystallised twice from aqueous ethanol, m.p. 56°, $[\alpha]_D$ +89 ±2° (*c* 1.5, chloroform).

Anal. Calc. for $C_{21}H_{30}O_{14}$: C, 49.9; H, 5.9. Found: C, 49.9; H, 6.2.

O-α-D-*Glucopyranosyl-(1→2)-*O*-α-D-glucopyranosyl-(1→1)-*L*-glycerol* (**4**). — The first step of the preparation described above was repeated on a scale four times greater. After the addition of a molar proportion of the chloride, the reaction mixture was stirred in the dark for 24 h, and then a further amount of silver carbonate (6.0 g) was added, followed by a solution of the chloride (2.48 g, 1 mol. prop.) in dry ether (40 ml). The reaction mixture was stirred for a further 72 h, and the glycosides were recovered as described above.

Examination of the glycoside mixture by paper chromatography revealed the presence of glycerol and glucosylglycerol in small proportions, and larger proportions of two diglucosylglycerols. The crude mixture (2.5 g) was chromatographed on

a column (30 × 3 cm) of Dowex-1 (OH^- form) resin with automatic collection of fractions (20 ml each). Fractions 21–25 yielded 619 mg of material having a large, positive optical rotation which, when examined by paper chromatography, was seen to consist of a mixture of glucosyl- and diglucosyl-glycerol (R_{Glc} 0.52). Examination of a portion (1 mg) of the mixture, after trimethylsilylation, by g.l.c. showed that the glucosylglycerol was entirely β-, whereas the diglucosylglycerol consisted of one major and two minor components.

The mixture was dissolved in water (500 ml), digested with β-D-glucosidase (0.1 g, Koch–Light) for two weeks at 37°, and then passed through a short column (10 ml) of Dowex-1 (OH^- form) resin. The recovered mixture was rechromatographed as described before on Dowex-1 (OH^- form) resin, and fractions 21–25 were again combined and evaporated to yield 503 mg (17%) of material, $[\alpha]_D$ +87 ±2° (*c* 1.1, water). Examination of this material by g.l.c. showed the presence of two diglucosylglycerols in the ratio 15:85. Attempts to purify the major component by crystallization of the glycoside or its acetate were unsuccessful. Degradation, described below, yielded α- and β-D-glucosylglycerols in the ratio, α:β = 77.23.

Degradation of diglycosylglycerols to glycosylglycerols as a test of their purity. — A sample of the appropriate diglycosylglycerol (5 mg) was heated in 0.1N HCl for 40 min at 100° and evaporated *in vacuo* over potassium hydroxide pellets[34]. Trimethylsilylating reagent (1 ml) was added, and portions (2 μl) of the reaction mixture were examined in the g.l.c. programme. The ratio of α-D-glucosylglycerol to β-D-glucosylglycerol was obtained directly from the peaks on the chart. All compounds described as pure in this section showed only one peak in this procedure. The laminaribiosylglycerol **8** gave the ratio α:β-D-glucosylglycerol as 42:58, the 'α-gentiobiosylglycerol' reported earlier[8] gave the ratio α:β-D-glucosylglycerol as 29:71, and the kojibiosylglycerol **4** gave the ratio α:β-D-glucosyiglycerol as 77:23. Samples of pure glycosylglycerols were not anomerized by the partial hydrolysis conditions.

Examination of the lipid from Staph. aureus *B4.* — A sample of lipid was provided by Prof. E. F. Gale. It was dissolved in chloroform–methanol (4 ml, 1:1 v/v), and 0.1N sodium methoxide in methanol (1 ml) was added[35]. After 10 min, water (5 ml) was added, and the mixture was passed through a column (4 ml) of Dowex-50 (H^+ form) resin and then through a column (4 ml) of Dowex-1 (OH^- form) resin. The separated aqueous layer was evaporated to yield 35 mg of a gum. Examination of the material by paper chromatography showed a single compound (R_{Glc} 0.49) which co-chromatographed with the diglucosylglycerol **7**. Examination of a portion (1 mg) of the material by the programmed g.l.c. method showed the presence of *O*-β-D-glucosylglycerol and the *O*-β-gentiobiosylglycerol **1** in the molar ratio 1:100.

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THE EFFECT OF pH ON THE INTERACTION OF SERUM ALBUMIN WITH HYALURONIC ACID

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ABSTRACT

The formation of soluble complexes of hyaluronic acid and bovine serum albumin under conditions of Tiselius electrophoresis was studied in four different buffer solutions at three different hydrogen-ion concentrations. The appearance of a third boundary (Pi complex) having a mobility intermediate between that of albumin and hyaluronic acid was observed in barbital (Veronal) buffer at pH 8.6, borate buffer at pH 8.5, and phosphate buffer at pH 7.4. Pi-complex formation was not observed in acetate buffer, either at pH 7.1 or 5.3. The mobilities of the boundaries observed when each component was analyzed separately, compared with those observed in patterns obtained on solutions that contained both components, suggested formation of a complex in acetate buffer also. The data suggest that complex-formation which occurs at high pH and which results in the appearance of a third electrophoretic boundary may represent a type of binding different from that which occurs at lower pH; the latter is probably due to electrostatic binding.

INTRODUCTION

In previous studies, hyaluronic acid and bovine serum albumin were shown to form a soluble complex demonstrable under conditions of Tiselius electrophoresis, where the complex appears as a boundary having a mobility intermediate between that of the two components. This boundary was termed the Pi complex[1-3].

In early studies, complete resolution of the Pi complex from the albumin boundary was not achieved. In later studies, the use of a longer electrophoretic cell gave essentially complete resolution of these two boundaries[4]. In the ascending limb of the electrophoresis cell, the Pi boundary appeared in about 180 min and remained well defined for periods of up to 395 min, after which it migrated beyond the limits of the optical system of the instrument. The persistence and definition of the boundary suggest that it represents a relatively stable complex and that it is not a reaction boundary or the result of frictional interaction, as proposed by Davies, Nichol, and Ogston[5].

Our previous studies were made in Veronal buffer of ionic strength 0.1 (0.1 μ) at pH 8.6. Attempts to demonstrate the Pi complex in phosphate buffer were not

successful; this observation suggested that the Veronal buffer might be responsible for the interaction of albumin with hyaluronic acid. The present studies were undertaken for the purpose of determining the conditions for formation of the Pi complex relative to pH, ionic strength, and composition of the buffer.

MATERIALS

Hyaluronic acid was prepared from bovine synovial fluid collected from the astrogalotibial joint by a modification of the electrodialysis method of Roseman and Watson [6]. The hyaluronic acid was stored at $-10°$ in the form of a gel. Prior to use, a weighed sample was diluted with the appropriate buffer, and stirred overnight at 2–4°. A small proportion of Celite was added, and the solution was stirred for approximately one h. The solution was centrifuged at $43000 \times g$ for 30 min, and the supernatant fluid was decanted. The product contained less than 4% of protein. Its intrinsic viscosity in 0.1 μ Veronal buffer, determined at 30° in a Cannon–Manning semi-micro-capillary viscometer, was 55 dl/g. Crystalline, bovine-plasma albumin was purchased from Armour Laboratories. Buffer solutions were prepared from commercial, reagent-grade chemicals without further purification.

METHODS

The hyaluronic acid solution to which bovine-plasma albumin had been added was dialyzed for 18–48 h at 0° against the appropriate buffer solution. The concentration of each of the buffer solutions used for dialysis was adjusted to give a specific resistance of approximately 342 ohms (the same as the resistance of 0.1 μ Veronal buffer, pH 8.6, which contained 2.797 g of 5,5-diethylbarbituric acid and 20.6 g of the monosodium salt of 5,5-diethylbarbituric acid). In every case, the electrical conductivity after dialysis was the same as that of the buffer against which the solution had been dialyzed. The concentrations of the buffers were 110mM for Veronal, 330mM for borate, 80mM for phosphate, 80mM for acetate (pH 7.1), and 100mM for acetate (pH 5.3). Because dialysis against phosphate buffer for 48 h resulted in depolymerization of the hyaluronic acid (as judged from its viscosity), the solution used in one experiment was prepared by adding, to the aqueous solution of hyaluronic acid and albumin, sufficient phosphate buffer to produce a phosphate concentration of 80mM (specific resistance, 342 ohms). Electrophoresis was conducted at 0° immediately after admixture.

The electrical resistance was measured with a specially designed, conductivity cell having a capacity of 2 ml. The conductivity bridge was a Leeds–Northrop portable model. A Perkin–Elmer Model 38, Tiselius-type of electrophoresis apparatus was used with a standard, 6-ml cell. The mobilities of the electrophoretic boundaries, calculated from micrometer measurements, were determined with a Nikon shadowgraph.

An aliquot of the solution for electrophoretic study was analyzed for protein

by the Lowery phenol method[7]. Hyaluronic acid was determined by analysis for uronic acid by the carbazole method, with anhydrous D-glucuronic acid as the standard[8].

RESULTS

Boundaries. — In Veronal buffer at pH 8.6, bovine-plasma albumin migrated as a single boundary that had a mobility* of 6.6 in both the ascending and descending electrophoretic patterns, as shown in Fig. 1-A. Hyaluronic acid likewise gave a

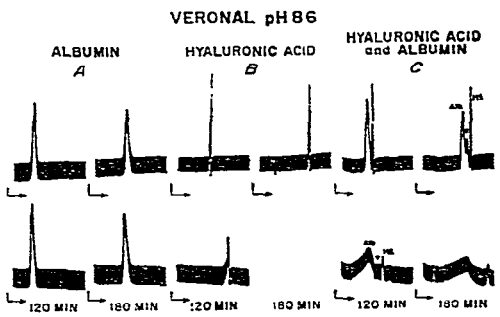


Fig. 1. Electrophoretic patterns of hyaluronic acid and bovine plasma albumin in 110mM Veronal buffer, pH 8.6. R = 345 ohms. Top patterns are from the ascending limb. Bottom patterns are from the descending limb. A. 420 mg of bovine plasma albumin per 100 ml. B. 106 mg of hyaluronic acid per 100 ml. C. 107 mg of hyaluronic acid + 358 mg of bovine plasma albumin per 100 ml.

single boundary, having mobilities of 9.6 and 14.0 in the ascending and descending patterns, respectively, as shown in Fig. 1-B. The boundary for hyaluronic acid could not be observed in the descending limb at 180 min, because it had migrated beyond the limits of the optical system of the instrument.

Typical results that were obtained when solutions containing both hyaluronic acid and bovine-plasma albumin were analyzed electrophoretically are shown in Fig. 1-C. After 120 min, two boundaries were observed in the ascending limb. The faster-migrating boundary had a mobility of 9.3, and the slower, a mobility of 8.2. After the elapse of 180 min, the leading boundary remained sharp and had a mobility of 9.1, but the slower-moving boundary was partially resolved into two components having mobilities of 8.6 and 7.9, which correspond to the Pi complex and to albumin, respectively. The limited length of the electrophoretic cell precluded a more complete resolution of the boundaries.

Although the boundaries in the descending limb were not so sharp as those in the ascending limb, they were well defined at 120 min, as shown in Fig. 1-C. There were three major boundaries having mobilities of 11.8, 10.5, and 8.4, These mobilities are somewhat greater than the comparable boundaries in the ascending limb, but they apparently represent hyaluronic acid, Pi complex, and albumin, respectively.

*All mobilities are expressed as $\times 10^{-5}$ cm^2V^{-1} sec^{-1}.

The mobility of hyaluronic acid in the descending limb, in either the absence or presence of albumin, has repeatedly been observed to be greater than in the ascending limb. A fourth boundary of small magnitude, having a mobility slightly greater than that of hyaluronic acid is also sometimes seen.

The results of a similar experiment, conducted in borate buffer at pH 8.6, are shown in Fig. 2. Analysis of solutions that contained either hyaluronic acid or albumin yielded only a single boundary. Solutions that contained both hyaluronic

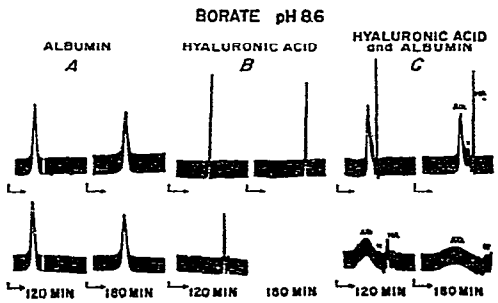


Fig. 2. Electrophoretic patterns of hyaluronic acid and bovine plasma albumin in 330mM borate buffer, pH 8.6. R = 348 ohms. Top patterns are from the ascending limb. Bottom patterns are from the descending limb. A. 400 mg of bovine plasma albumin per 100 ml. B. 90 mg of hyaluronic acid per 100 ml. C. 87 mg of hyaluronic acid + 389 mg bovine plasma albumin per 100 ml.

acid and bovine-serum albumin contained three electrophoretically demonstrable peaks similar to those observed with Veronal buffer. The Pi complex was apparent in both the ascending and the descending patterns. The mobilities of the boundaries are listed in Table I. The boundary in the descending pattern that has a mobility greater than that of hyaluronic acid appears to arise from a contaminant in the preparation of hyaluronic acid.

TABLE I

MOBILITIES OF HYALURONIC ACID, Pi COMPLEX, AND ALBUMIN BOUNDARIES IN TISELIUS ELECTROPHORESIS[a]

Composition of solution	*Boundary measured*	*Veronal, pH 8.6*		*Borate, pH 8.5*		*Phosphate, pH 7.4*		*Acetate, pH 7.1*		*Acetate, pH 5.3*	
		A	*D*	*A*	*D*	*A*	*D*	*A*	*D*	*A*	*D*
Hyaluronic acid	hyaluronic acid	9.4	14.0	9.5	13.4	12.0	16.6	10.1	15.8	9.6	15.0
Albumin	albumin	6.6	6.6	6.3	6.2	5.7	5.3	4.5	4.5	2.2	22
Hyaluronic acid plus albumin	hyaluronic acid	9.2	11.9	9.6	12.5	11.9	15.3	10.3	15.4	9.6	
	Pi	8.6	10.5	8.7	11.1	8.2	14.9				13.6
	albumin	8.0	8.5	7.5	6.9	8.1	4.8	7.1	3.0	4.6	1.7

[a]A denotes ascending limb; D denotes descending limb.

When a solution containing hyaluronic acid and albumin was dialyzed against phosphate buffer (pH 7.4) for 48 h, the hyaluronic acid underwent extensive depolymerization, as judged from determinations of viscosity. In the electrophoretic patterns obtained from these solutions, the Pi complex is indicated in the ascending limb by asymmetry of the albumin boundary, as shown in Fig. 3. In the descending pattern, a well-defined boundary characteristic of the Pi complex was seen at 120 min. At

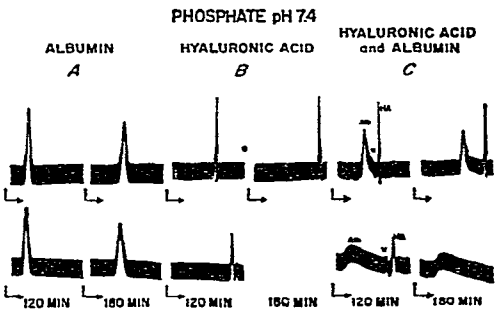


Fig. 3. Electrophoretic patterns of hyaluronic acid and bovine plasma albumin in 80mM phosphate buffer, pH 7.4. R = 346 ohms. Top patterns are from the ascending limb. Bottom patterns are from the descending limb. A. 392 mg of bovine plasma albumin per 100 ml. B. 96 mg of hyaluronic acid per 100 ml. C. 102 mg of hyaluronic acid + 334 mg bovine plasma albumin per 100 ml.

180 min, the Pi boundary had apparently been destroyed by diffusion, and the hyaluronic acid boundary had migrated beyond the field of visual observation. The electrophoretic patterns shown in Fig. 4 were obtained for a solution of hyaluronic acid and albumin to which phosphate buffer had been added directly, without

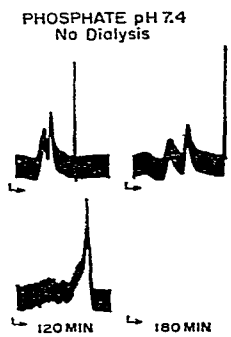


Fig. 4. Electrophoretic pattern of 100 mg of hyaluronic acid and 357 mg of bovine plasma albumin per 100 ml in 80mM phosphate buffer, pH 7.4. R = 346 ohms. Top patterns are from the ascending limb. Bottom patterns are from the descending limb.

dialysis. There was no evidence for depolymerization of hyaluronic acid in this solution. A sharp, well-defined boundary corresponding to the Pi complex was observed in the ascending pattern. Although the hyaluronic acid boundary in the descending pattern observed at 120 min was very asymmetric, suggesting the presence

of a diffuse boundary adjacent to it, no clearly defined Pi complex could be identified. By 180 min, the boundary had migrated out of the field of observation.

In acetate buffer, discrete boundaries representative of the Pi complex were not observed. However, at pH 7.1, the albumin boundaries were asymmetric in both the ascending and descending patterns, as shown in Fig. 5, suggesting the presence of

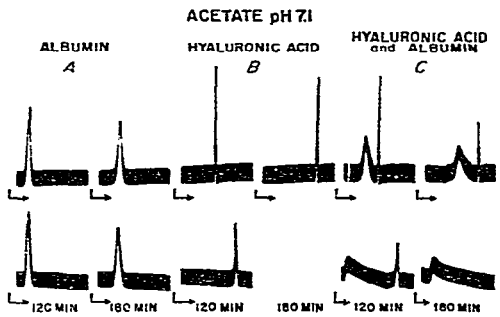


Fig. 5. Electrophoretic pattern of hyaluronic acid and bovine plasma albumin in 80mM acetate buffer, pH 7.1. R = 340 ohms. Top patterns are from the ascending limb. Bottom patterns are from the descending limb. A. 355 mg of bovine plasma albumin per 100 ml. B. 114 mg of hyaluronic acid per 100 ml. C. 128 mg of hyaluronic acid + 375 mg bovine plasma albumin per 100 ml.

an unresolved boundary. At pH 5.3, free hyaluronic acid in a solution to which no albumin had been added gave, in the descending pattern, a sharp peak which was similar to that shown in Fig. 1 for hyaluronic acid in Veronal buffer; it had a mobility of 15.0. In the presence of albumin, however, a boundary representative of hyaluronic acid was not observed, but a broad peak having a mobility of 13.6 appeared, as shown in Fig. 6-C.

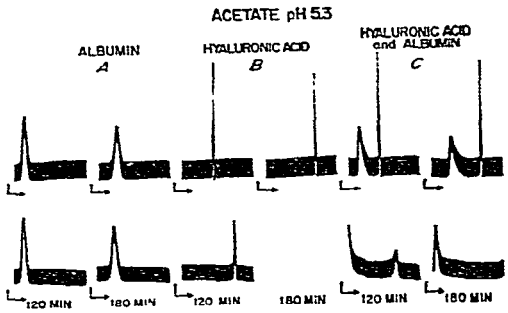


Fig. 6. Electrophoretic patterns of hyaluronic acid and bovine plasma albumin in 100mM acetate buffer, pH 5.3. R = 338 ohms. Top patterns are from the ascending limb. Bottom patterns are from the descending limb. A. 360 mg of bovine plasma albumin per 100 ml. B. 99 mg of hyaluronic acid per 100 ml. C. 95 mg of hyaluronic acid + 340 mg bovine plasma albumin per 100 ml.

Mobilities. — The electrophoretic mobilities of the boundaries observed are listed in Table I. Either in the absence of added serum albumin or in its presence, the mobility of the hyaluronic acid boundary was greater in the descending than in the

ascending pattern. This difference was observed in all of the buffers and at all pH values used in these studies. In the descending patterns, the mobility of the hyaluronic acid boundary was always decreased by the presence of albumin.

In the absence of hyaluronic acid, the mobility of the albumin boundary was essentially the same in the ascending as in the descending patterns, and decreased as a function of the pH of the buffer. In the presence of hyaluronic acid, the mobility of the albumin boundary in the ascending patterns was increased. In the descending patterns, the mobility of albumin was also increased by the presence of hyaluronic acid at high pH, but was decreased at lower pH.

Concentration dependence. — As already noted, the mobility of hyaluronic acid was consistently observed to be higher in the descending than in the ascending limb of the electrophoresis cell. This observation suggested that the electrophoretic mobility of hyaluronic acid might be dependent upon concentration.

Solutions containing hyaluronic acid in concentrations ranging from 0.3 to 1.4 g/l were prepared in 0.1 μ Veronal buffer (pH 8.6). The mobility of hyaluronic acid was determined in each solution after electrophoresis had proceeded for 120 min.

The results obtained in four experiments on three different preparations of hyaluronic acid are shown in Fig. 7. In experiments A, B, and C, the mobility of

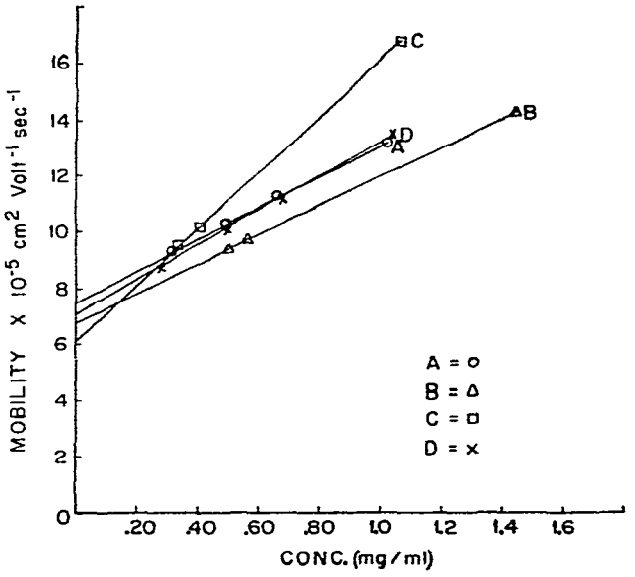


Fig. 7. Mobility of hyaluronic acid in the descending limb of Tiselius electrophoresis as a function of concentration. Intrinsic viscosity (dl/g) of A = 58, B = 57, C = 65, D = 57.

hyaluronic acid in the most concentrated solution was determined first. After removal from the electrophoretic cell, the solution was diluted with Veronal buffer, mixed thoroughly, and returned to the electrophoretic cell, and the mobility was redetermined. This procedure was repeated sequentially, to obtain each of the successively more-dilute solutions. Experiment D was performed on the same hyaluronic acid preparation

as for experiment B, but all dilutions were prepared simultaneously from a stock preparation of hyaluronic acid, prior to determination of the electrophoretic mobility. The data from experiments B and D were comparable, indicating that the lower mobilities observed in succeeding analyses on sequentially diluted solutions were not due to changes that might have occurred during the previous electrophoretic analysis. The hyaluronic acid used in experiments B and D had an intrinsic viscosity of 57 dl/g. The results are comparable with those of experiment A in which hyaluronic acid having an intrinsic viscosity of 58.5 was used. The dependence on concentration of the hyaluronic acid preparation used in experiment C, which had an intrinsic viscosity of 64.5, was much greater than that observed for the other two preparations. The mobility of hyaluronic acid in the ascending limb was not influenced by concentration.

DISCUSSION

The results of these studies indicate that formation of a Pi complex is not limited to solutions that contain Veronal buffer at pH 8.6. Similar electrophoretic boundaries were also observed in borate buffer at essentially the same pH and, under certain conditions, in phosphate buffer at pH 7.4. Dialysis of solutions of hyaluronic acid and albumin against phosphate buffer (pH 7.4) resulted in a marked decrease in the viscosity of the solution, and subsequent failure to observe a Pi-complex boundary in the electrophoretic patterns. Gramling *et al.*[4] were unable to demonstrate formation of a Pi complex with hyaluronic acid of low, mean molecular weight. When the conductivity of the solution was adjusted by the direct addition of phosphate (instead of by dialysis), and the electrophoresis was performed before extensive depolymerization had occurred, Pi-complex formation was demonstrated. Formation of a Pi complex in phosphate buffer has been observed by Pigman *et al.*[1]; they minimized depolymerization of the polysaccharide in phosphate buffer by including 10% of ethanol in the solution for analysis. In acetate buffer, formation of a Pi complex was not observed at either pH 5.3 or 7.1. Failure to observe formation of a Pi complex in these buffers could not be attributed to depolymerization of the hyaluronic acid during dialysis. As Pi-complex formation was observed at pH 7.1 in phosphate buffer, but not in acetate buffer at the same pH, it is suggested that the acetate ion may interfere specifically with the reaction.

Davies *et al.*[5] applied the mathematical treatment derived by Gilbert and Jenkins[9] to the data on Pi-complex formation reported by Gramling *et al.*[4], and concluded that the Pi complex is the result of frictional effects and that it does not represent a chemical complex. In view of the dependence of electrophoretic mobility on the concentration of hyaluronic acid reported in the present studies, the application of this mathematical treatment to these data may not be justified.

In a recent report, Bettelheim, Laurent, and Pertoft[10] demonstrated the complexing of albumin with chondroitin sulfate and D-galacturonan, and attempted to equate their results with those obtained by Pigman *et al.*[1] for the binding of albumin with hyaluronic acid. With chondroitin sulfate in 50mM and 150mM acetate buffer and with

D-galacturonan in 150mM acetate buffer solution, no evidence of Pi-complex formation was seen. They observed only two boundaries in solutions containing albumin and the polysaccharide; these were apparently similar to the patterns obtained in the present study with solutions that contained hyaluronic acid and albumin in acetate buffer. The criteria of Bettelheim *et al.* for binding of albumin to the polysaccharide were: (*1*) difference in size of the peak representing the polysaccharide in the ascending pattern and of albumin in the descending pattern in the presence and absence of the second constituent; and (*2*) an increase in the mobility of the albumin boundary in the ascending patterns because of the presence of polysaccharide in the solution. Evidence was presented for the appearance of a third boundary, only in the ascending pattern obtained with D-galacturonan and albumin in 50mM acetate buffer between pH 5.5 and 7.5. Because of the dependence of complex-formation on pH and ionic strength of the buffer, these investigators concluded that the complexes are the result of electrostatic forces. Evidence for complex-formation above pH 7.0 was not obtained. In addition to the appearance of the Pi boundary, evidence for the binding of albumin to hyaluronic acid was also provided in the present studies, on the basis of the above criteria even at pH 8.6 at ionic strength 0.1.

Bettelheim *et al.*[10] concluded from their observations that the interaction between serum albumin and hyaluronic acid observed by Pigman and associates at pH 8.6 and ionic strength 0.1 was not electrostatic, and was probably the result of frictional effects. Some of the results of the present study are compared with those of Bettelheim *et al.*[10] in Table II. There is sufficient disparity between the observations

TABLE II

COMPARISON OF THE ELECTROPHORETIC BEHAVIOR OF CHONDROITIN SULFATE AND D-GALACTURONAN WITH HYALURONIC ACID

Parameter measured	*Chondroitin sulfate or D-galacturonan*[13]	*Hyaluronic acid*
Change in mobility of polysaccharide with pH (ascending)	no change	no change
Effect of albumin on mobility of polysaccharide (ascending)	no change	no change
Effect of albumin on mobility of polysaccharide (descending)	no change	lower in presence of albumin
Mobility of polysaccharide in ascending relative to descending pattern	higher in ascending pattern	higher in descending pattern; concentration dependent
Effect of polysaccharide on mobility of albumin (ascending)	higher in presence of polysaccharide	higher in presence of hyaluronic acid
Effect of polysaccharide on mobility of albumin (descending)	no effect except at low pH where mobility was increased	increased at high pH; decreased at low pH

to suggest that the electrophoretic behavior of D-galacturonan and chondroitin sulfate do not reflect the electrophoretic properties of hyaluronic acid. Of particular interest is the higher electrophoretic mobility of hyaluronic acid in the descending limb as compared with that in the ascending limb, both in the presence and absence of albumin, and the dependence of the mobility of hyaluronic acid in the descending limb on the concentration. The latter observation appears to preclude the application of any currently available mathematical treatment to the interpretation of the data[9]. The increased mobility of albumin in the ascending patterns due to the presence of hyaluronic acid in the solution is consistent with the observations of Bettelheim and associates[10] for chondroitin sulfate and D-galacturonan. In the descending patterns, however, the presence of hyaluronic acid results in an increase in the mobility of albumin at high pH and a decrease at low pH. Were these changes in mobility the result of complex-formation involving electrostatic forces, the opposite effect would be expected. Bettelheim *et al.*[10] observed no effect of chondroitin sulfate or D-galacturonan on the mobility of albumin in the descending patterns, except at low pH, where they noted an increase in the mobility of albumin due to the presence of the polysaccharide.

The results of the present studies suggest that several mechanisms may be involved in the formation of albumin–hyaluronate complexes. The first of these appears to involve electrostatic interaction, and it predominates at low pH. This type of complex does not result in the formation of a third boundary in electrophoresis, and it can be detected only by measuring the relative areas of the boundaries, or on the basis of changes in mobility. The second type of complex appears to preponderate at higher pH, and it results in the formation of a third boundary in electrophoresis. Because of its low rate of diffusion and its persistence under the conditions of electrophoresis for periods in excess of 6 h, and for other reasons discussed previously[3], it is probably the result of chemical bonds stronger than those attributable to electrostatic forces, and is probably not the result of frictional effects as suggested by Davies *et al.*[5]. Bettelheim[11] suggested that Pi-complex formation might be due to hydrophobic interaction between the protein moiety of our preparations of hyaluronic acid and serum albumin. He further postulated that hydrophobic interaction might occur between the protein moieties of the hyaluronic acid chains; this type of interaction would decrease the molecular surface in the region where relatively little formal charge exists, giving rise, therefore, to an increase in the number of charges per unit of surface area. A dependence of mobility on concentration of hyaluronic acid similar to that observed in the present study could be explained on the basis of such an interaction.

The physiological significance of complexes of hyaluronic acid with serum albumin is uncertain. The failure of Bettelheim *et al.*[10] to observe interaction at neutral pH and at high ionic concentrations suggests that these complexes do not occur under physiological conditions. Barnhart *et al.*[12], however, observed, in synovial fluid from patients with rheumatoid arthritis, microscopic strands of fibrous material that combined with fluorescent antibody to albumin; they concluded that these

strands of material most probably represent a complex composed of a mucopolysaccharide and albumin, similar to the complexes described earlier by Pigman *et al.*[2].

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METHYLENE-INSERTION REACTIONS WITH UNSATURATED SUGARS. SYNTHESIS AND REACTIVITY OF 4-*C*-CYCLOPROPYL-D-*xylo*-TETROFURANOSE*†

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ABSTRACT

Insertion of methylene into the double bond of 5,6-dideoxy-1,2-*O*-isopropylidene-α-D-*xylo*-hex-5-enofuranose (**1**) by use of the Simmons–Smith reaction gives 4-*C*-cyclopropyl-1,2-*O*-isopropylidene-α-D-*xylo*-tetrofuranose (**2**), further characterized as the 3-acetate (**5**) and 3-(trimethylsilyl) ether (**6**). The cyclopropyl group in **2** is resistant to hydrogenolysis over palladium or platinum, but is cleaved exclusively between the methylene groups by hydrogenolysis over Raney nickel, to give the isopropyl derivative **7**. The cyclopropyl derivative **2** is stable to base and to sodium azide in *N*,*N*-dimethylformamide. Cleavage of the acetal group from **2** by mild acid gives 4-*C*-cyclopropyl-α,β-D-*xylo*-tetrofuranose (**3**); considerably more severe conditions are required for acid-catalyzed scission of the cyclopropyl ring. The structure of **3** and its derived alditol (**4**) were verified by periodate oxidation, and by acetylation of **3** to give crystalline 1,2,3-tri-*O*-acetyl-4-*C*-cyclopropyl-α-D-*xylo*-tetrofuranose (**9**) and its syrupy β-D anomer (**10**). The conformations of the products were studied by n.m.r. spectroscopy.

INTRODUCTION

A program in this laboratory[1–4] is concerned with synthesis of unsaturated sugars, with their reactions with electron-deficient species, including cations, free radicals, carbenes, sulfenes, and nitrenes, and with reactions of the resultant species. Earlier reports have described the synthesis of unsaturated sugars from *cis*- and *trans*-diol precursors[2,3], and the reaction of unsaturated sugars with acid has been studied in detail[3,4]. Photochemical procedures for synthesis have been introduced into the carbohydrate field in a route from an unsaturated sugar to a 5-deoxy-6-thiohexose system[2,5], and for transformations of thio sugars[6] and related derivatives[7]

*Part VIII in the series Synthesis and Reactions of Unsaturated Sugars. For Part VII, see ref. 1. Preliminary report: Ester L. Albano, D. Horton, and C. G. Tindall, Jr., *Abstracts Papers Amer. Chem. Soc. Meeting*, 153 (1967) C13.

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into deoxy sugars. The present report describes the generation of a cyclopropyl group in the carbon-atom chain of a carbohydrate by a carbenoid type of reaction with an unsaturated sugar. The stability of the cyclopropyl residue, under reaction conditions commonly used in synthetic interconversions of sugars, has been assessed.

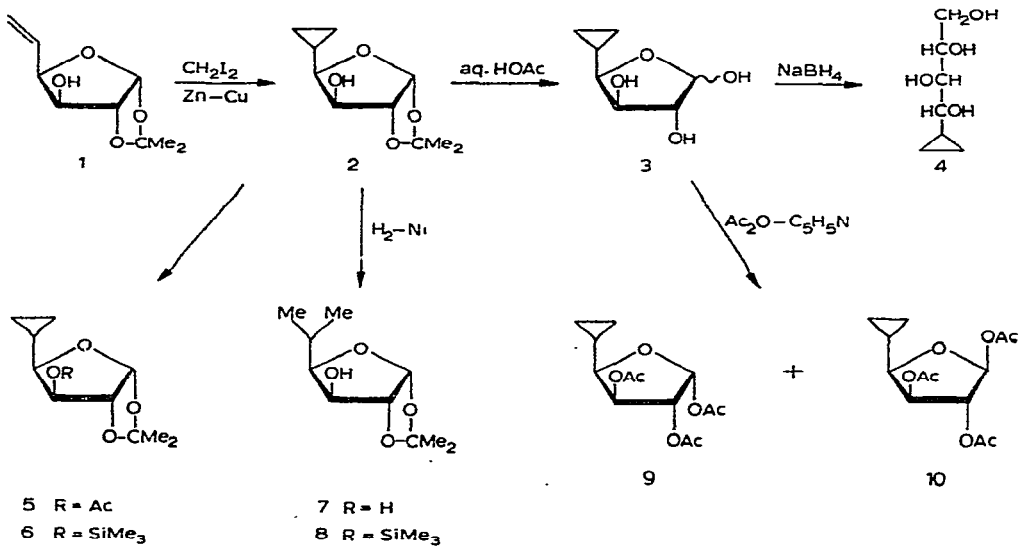
RESULTS AND DISCUSSION

A method was sought for introducing a cyclopropyl group as part of the carbon-atom chain of a sugar derivative, under mild conditions and without the necessity of protecting all of the hydroxyl groups in the sugar derivative. The insertion of a carbenoid species into the carbon–carbon double bond of an unsaturated sugar derivative provides the simplest route for this purpose. The direct insertion into a double bond of singlet methylene, as obtained by photolysis of diazomethane[8], is not a promising route because of competing insertion reactions[9] into carbon–hydrogen, carbon–oxygen, and oxygen–hydrogen bonds. Insertion of dihalocarbene[10] followed by reductive removal[11] of the halogen atoms is a practical route to cyclopropane derivatives of sugars[12], but the initial sugar derivative must withstand the strong base used to generate the reagent, and also the conditions for reductive cleavage of the halogen atoms. The use of a halomethylzinc halide[13,14] for insertion of a methylene group into a double bond offered promise, because the reaction is known[15] to be favored and directed by the presence of hydroxyl or ether groups in the unsaturated substrate, and because the reagent, prepared *in situ* from dihalomethane and zinc–copper couple by the method of Simmons and Smith[14], reacts with the alkene under mild conditions that do not require the use of strong base or acid. Various methods have been described[16,17] for preparing the zinc–copper couple; in the present work, the method of LeGoff[17] led to the highest yields of product.

Treatment of 5,6-dideoxy-1,2-*O*-isopropylidene-α-D-*xylo*-hex-5-enofuranose[2] (**1**) in ether with a 2–3 molar excess of diiodomethane in the presence of an excess of zinc–copper couple[17] gave the crystalline cyclopropyl derivative **2** in 60–70% yield after recrystallization. The n.m.r. spectrum of **2** (see Fig. 1 and Tables I and II) shows the presence of a monosubstituted cyclopropyl group and an exchangeable proton (3-OH), and the signals of the four methine protons are completely first-order. Acetylation of **2** with acetic anhydride–pyridine gave the crystalline 3-acetate (**5**), whose n.m.r. spectrum (see Tables I and II) resembled that of **2**, except for the absence of the 3-OH signal, the presence of an acetoxy resonance, and the downfield shift (~1.1 p.p.m.) of the H-3 signal. The i.r. spectra of **2** and **3** both showed absorption characteristic[18] of cyclopropyl C–H stretching (3.23 and 3.30 μm, respectively, with no absorption at ~6.0 μm), together with the usual absorptions for other functional groups (see Experimental section). Deacetylation of **5** by catalytic transesterification with methanolic sodium methoxide regenerated the starting alcohol **2**.

Reductive cleavage of the cyclopropane ring in **2** was effected with hydrogen at 2000 lb. in^{-2} over Raney nickel at 140°, to give 71% of a crystalline product formulated as 4-*C*-isopropyl-1,2-*O*-isopropylidene-α-D-*xylo*-tetrofuranose (**7**). The

n.m.r. spectrum of 7 (Tables I and II) showed no signals above τ 9.15 (proving that the cyclopropyl group was absent), and two 3-proton doublets, at τ 8.91 and 9.04, were present. The latter signals can clearly be assigned to the methyl protons of an isopropyl group, indicating that reductive scission had taken place between the methylene groups. The data rule out a possible alternative structure having an *n*-propyl group, which would have resulted had cleavage occurred between C-5 and one of the methylene groups.



The course of the reduction of **2** to **7** was monitored conveniently by g.l.c. of the respective 3-(trimethylsilyl) ethers **6** and **8**. Under the conditions used, the retention time of **8** was approximately half that of **6**. At the point of complete reduction of **2**, the trimethylsilylated product contained ~95% of product having the retention time of **8** and only a trace of a second component.

Treatment of the cyclopropane derivative **2** with 60% aqueous acetic acid for 3 h at 90° cleaved the *O*-isopropylidene group to give 4-*C*-cyclopropyl-D-*xylo*-tetrofuranose (**3**), which crystallized in essentially quantitative yield. No mutarotation was observed for this product in water, and the n.m.r. spectrum in deuterium oxide (see Tables I and II), in addition to confirming the gross structure of **3**, indicated that an equilibrium mixture containing approximately equal proportions of the α-D and β-D furanose anomers was present immediately after dissolution. On oxidation with an excess of periodate, compound **3** rapidly consumed 3 moles of oxidant per mole with the production of 3 moles of formic acid per mole.

Reduction of the cyclopropyl aldose **3** with aqueous sodium borohydride gave crystalline 4-*C*-cyclopropyl-D-*xylo*-tetritol (**4**) in almost quantitative yield. Periodate oxidation of **4** led to the very rapid uptake of 3 moles of oxidant per mole and the formation of 2 moles of acid per mole.

TABLE I

CHEMICAL-SHIFT DATA FOR CYCLOPROPYL AND RELATED DERIVATIVES

Compound	*Chemical shifts*[a] (τ)								
	H-1	*H-2*	*H-3*	*H-4*	*H-5*	*H-6*	*H-6'*	*CMe_2*	*Others*
2	4.10 (d)	5.48 (d)	5.93 (d)	6.58 (q)	8.99 (m)	−9.16–9.83 (m)−		8.55 (s), 8.70 (s)	7.70 (s, OH)[b]
5[c]	4.13 (d)	5.46 (d)	4.80 (d)	6.45 (q)	8.99 (m)	−9.24–9.78 (m)−		8.51 (s), 8.68 (s)	7.88 (s, Ac)
7[c]	4.12 (d)	5.52 (d)	5.92 (d)	6.34 (q)	8.07 (m)	8.91 (d)	9.04 (d)	8.50 (s), 8.68 (s)	~8.0 (s, OH)[b]
3α[d,e]	4.60 (d)	−5.76−6.03 (m)−		~6.60 (q)	9.04 (m)	−9.28–9.90 (m)−			
3β[e,f]	4.90 (d)	−5.76−6.03 (m)−		~6.60 (q)	9.04 (m)	−9.28–9.90 (m)−			
9	3.57 (d)	4.64 (q)	4.52 (t)	6.30 (q)	9.02 (m)	−9.28–9.84 (m)−			7.85 (s) 7.90 (s), 7.92 (s) (Ac)
10[c]	3.90 (s)	4.72 (s)	4.65 (m)	6.25 (m)	9.02 (m)	−9.28-9.84 (m)−			~7.90 (m) (Ac)

[a]At 100 MHz in chloroform-*d*, unless noted otherwise. Peak multiplicity given in parentheses: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet. Ring-proton assignments were verified by spin decoupling. [b]Disappears on deuteration. [c]At 60 MHz. [d]α-D Anomer of 3. [e]In deuterium oxide. [f]β-D Anomer of 3.

TABLE II

FIRST-ORDER COUPLING-CONSTANTS FOR CYCLOPROPYL AND RELATED DERIVATIVES

Compound	*First-order couplings*[a] (*Hz*)					*Compound*	*First-order coupling*[a] (*Hz*)				
	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6}$		$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6}$
2	3.7	~0	2.5	8.5		**3β**[d,e]	1.6		4.0	9.0	
5[b]	3.5	~0	3.0	7.0		**9**	4.5	6.0	6.0	8.8	
7[b]	4.0	~0	2.2	11.8	6.0	**10**	~0	~0	~4	~8	
3α[c,d]	3.6		4.0	9.0							

[a]Measured at 100 MHz in chloroform-*d*, unless noted otherwise. [b]At 60 MHz. [c]α-D Anomer of 3. [d]In deuterium oxide. [e]β-D Anomer of 3.

Acetylation of the cyclopropyl aldose **3**, either with acetic anhydride–pyridine or acetic anhydride–zinc chloride, gave a mixture of 1,2,3-tri-*O*-acetyl-4-*C*-cyclopropyl-α- and β-D-*xylo*-tetrofuranose (**9** and **10**) in approximately equal proportions.

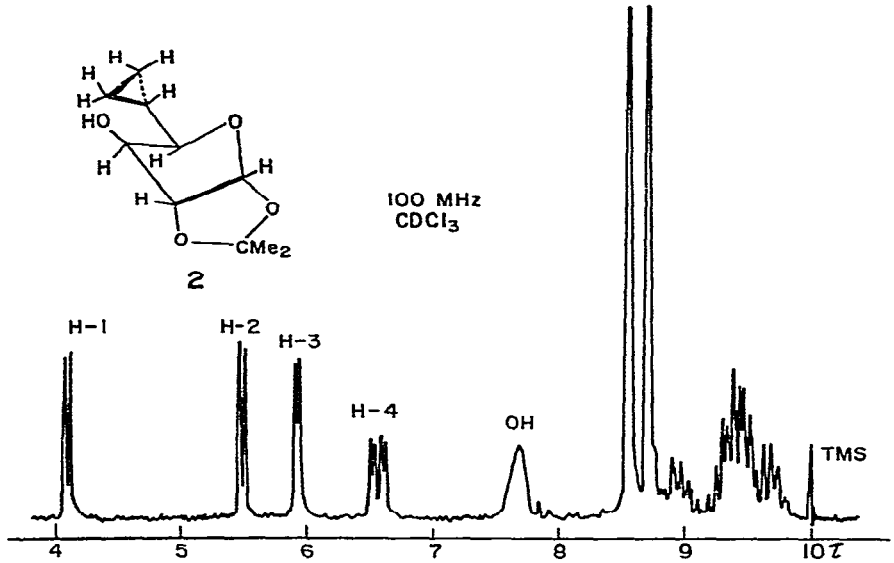


Fig. 1. The n.m.r. spectrum of 4-*C*-cyclopropyl-1,2-*O*-isopropylidene-α-D-*xylo*-tetrofuranose (**2**) at 100 MHz in chloroform-*d*.

Crystallization from ether–petroleum ether gave the pure α-D anomer **9**. The n.m.r. spectrum of **9** (see Fig. 2 and Tables I and II) is in complete agreement with the structure assigned. The mother liquors from the crystallization of **9** contained the β-D anomer (**10**), contaminated with a little of **9**, and the n.m.r. data for **10** (see Tables I and II) were determined from the spectrum of this mixture by subtraction of the signals for **9**.

The spin-coupling between H-4 and H-5 in **2**, **3**, **5**, **9**, and **10** is, in each case, large (7–9 Hz), indicating that the favored C-4–C-5 rotamer is that having H-4 and H-5 antiparallel; this is the rotamer in which the steric interactions between the cyclopropyl group and the furanose ring-system are at a minimum. The $J_{4,5}$ value for the isopropyl derivative **7** is also large (11.8 Hz), indicating that the rotamer having H-4 and H-5 antiparallel is strongly favored, and that the two methyl groups are magnetically nonequivalent[19,20].

The n.m.r. spectra of the cyclopropyl derivative **2**, its 3-acetate **5**, and the isopropyl derivative **7** are all very similar in the region for the furanoid-ring protons. The three compounds all contain the 4-*C*-substituted 1,2-*O*-isopropylidene-α-D-*xylo*-tetrofuranose ring-system and exhibit almost identical chemical shifts for H-1 and H-2 (τ 4.1 and 5.5, respectively). The $J_{1,2}$, $J_{2,3}$, and $J_{3,4}$ couplings (3.5–4.0, ~0.0, and 2.2–3.0 Hz, respectively) closely resemble those[2,21] of a series of derivatives of 1,2-*O*-isopropylidene-α-D-glucofuranose, for which a T_2^3 (twist) conformation,

having C-2 below and C-3 above the plane of C-1, C-4, and O-4, has been proposed[21]. In such a conformation, viewed along the C-2–C-1 bond, the H-2 atom is projected within the angle of H-1 and O-1.

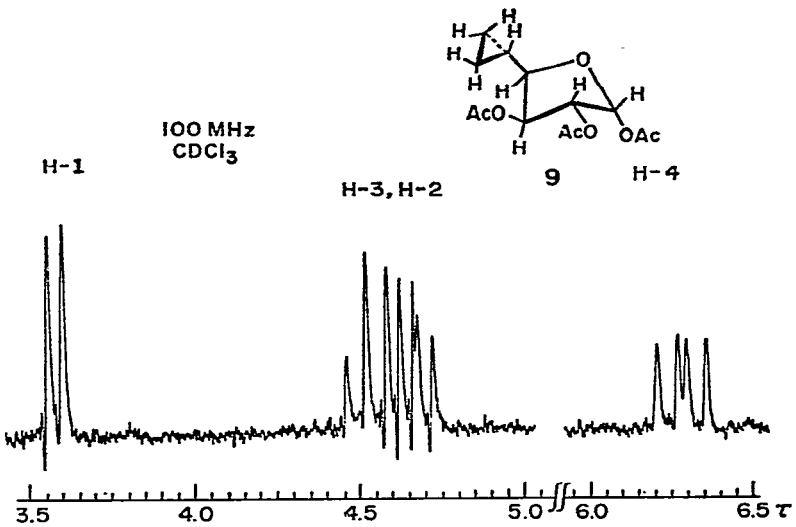


Fig. 2. The low-field portion of the n.m.r. spectrum of 1,2,3-tri-*O*-acetyl-4-*C*-cyclopropyl-α-D-*xylo*-tetrofuranose (**9**) at 100 MHz in chloroform-*d*.

A similar conformation is suggested for the β-D triacetate (**10**) from the fact that the $J_{1,2}$ and $J_{2,3}$ couplings are both approximately zero; the H-1–H-2 and H-2–H-3 dihedral angles are approximately right angles for the β-D configuration in a T_2^3 conformation. Such a conformation would allow the 1-acetoxy group to adopt a quasi-axial orientation, for maximum relief from polar destabilization (anomeric effect)[22].

A marked contrast is observed in the α-D triacetate (**9**), where $J_{1,2}$ is 4.5 Hz, and $J_{2,3}$ and $J_{3,4}$ are both large (6.0 Hz). In this example, a T_2^3 conformation would lead to an unfavorable polar situation, because the C-1 acetoxy group would be equatorially attached. The coupling data can best be accommodated by a conformation[23] which, viewed along the C-2–C-1 bond, would have H-2 projected within the angle of H-1 and O-4. This arrangement would place the 1-acetoxy group in a quasi-axial orientation and the ring in the V_1 or T_3^2 conformation (either an envelope conformer having C-1 below the plane of C-2, C-3, C-4, and O-4, or a twist form having C-2 above and C-3 below the plane of C-1, C-4, and O-4).

It is noteworthy that the β-D anomer of the cyclopropyl aldose (**3**) exhibits a non-zero, $J_{1,2}$ coupling (1.6 Hz).

The n.m.r. spectrum of the cyclopropyl alditol (**4**) in deuterium oxide (see Experimental section) showed a quartet for H-4 at higher field than the H-1, H-2, and H-3 signals, and spacings of 9.1 and 6.0 Hz could be assigned to $J_{4,5}$ and $J_{3,4}$,

respectively. The observed $J_{4,5}$ value accords with the antiparallel disposition anticipated for H-4 and H-5 in the favored rotamer state. The $J_{3,4}$ value observed indicates that H-3 and H-4 are not exclusively *gauche*, as would be the case were the chain of carbon atoms in a planar, zig-zag arrangement, and shows that there is a substantial contribution from that rotamer having H-3 and H-4 antiparallel. This behavior is in accord with observations from this laboratory[24], which show that the planar, zig-zag arrangement of an alditol chain is not favored if it brings a pair of hydroxyl groups on β-carbon atoms (2-OH and 4-OH in **4**) into a parallel arrangement.

In order to determine the stability of the cyclopropyl group to reactions commonly used in synthetic work in the carbohydrate field, the cyclopropyl derivative **2** and the free sugar **3** were subjected to the conditions of various common reactions. The cyclopropyl acetal **2** was found to be completely inert to methanolic sodium methoxide or aqueous sodium hydroxide for 3 days at reflux, and it was also unaffected by treatment with sodium azide in boiling acetonitrile or *N*,*N*-dimethylformamide for 3 days. Compound **2** was also inert to hydrogenolysis with hydrogen at 50 lb.in^{-2} over platinum or palladium at room temperature. Treatment of the cyclopropyl triol **3** with 0.5M aqueous sulfuric acid at 100° led to negligible decomposition during 2 h, but gradual decomposition of **3** took place at longer times of reaction.

The results show that the cyclopropyl group in these carbohydrate derivatives is unaffected under conditions of acylation catalyzed by bases or Lewis acids, deacylation by the Zemplén procedure, treatment with strong aqueous or alcoholic base, treatment with powerful nucleophiles, conditions for acid hydrolysis of acetals or glycosides, hydrogenolysis of benzyl ethers, benzylidene acetals, and *N*-benzyloxycarbonyl groups, and borohydride reduction of aldoses to alditols.

EXPERIMENTAL

General methods. — Solutions were evaporated below 50° under diminished pressure. Melting points were determined with a Thomas–Hoover "Unimelt" apparatus (Arthur H. Thomas Co., Philadelphia, Pennsylvania) and are uncorrected. Specific rotations were determined 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 or 100 MHz with Varian A-60 or HA-100 n.m.r. spectrometers. Chemical shifts are given on the τ-scale, with tetramethylsilane (τ = 10.00) or sodium 4,4-dimethyl-4-silapentane-1-sulfonate (τ = 10.00) as internal standards for chloroform-*d* and deuterium oxide solutions, respectively. Signal assignments were confirmed by spin-decoupling. 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. Microanalyses were made by W. N. Rond. T.l.c. of substituted sugars was performed with Silica Gel G (E. Merck, Darmstadt, Germany), activated at

110°, as the adsorbent. The developing solvent was 3:1 (v/v) chloroform–ether, and indication was effected with sulfuric acid. Chromatography of free sugars was performed on Whatman No. 1 paper, or by t.l.c. on microcrystalline cellulose[25] (Avicel, technical grade, American Viscose Division of Food Machinery Corp., Marcus Hook, Pennsylvania), with 4:1:1 (v/v) butyl alcohol–ethanol–water as the developer, and alkaline silver nitrate as the indicator. G.l.c. was conducted with a Wilkens "Autoprep" Model 705 gas chromatograph (Varian Aerograph, Walnut Creek, California), equipped with a flame-ionization detector and a stainless-steel column (10 ft × 0.25 in.) packed with 5% STAP on Chromosorb W-AW DMSC (Steroid Analysis Phase on acid-washed, dichlorodimethylsilane-treated, diatomaceous earth; Varian Aerograph, Walnut Creek, California). Nitrogen was used, at a flow rate of 80 ml/min, as the carrier gas.

*4-C-Cyclopropyl-1,2-O-isopropylidene-α-*D-xylo-*tetrofuranose* (**2**). — Granular zinc–copper couple (3.5 g, 54 mmoles), freshly prepared by the method of LeGoff[17], was covered with 25 ml of anhydrous ether in a 125-ml flask fitted with an addition funnel and a reflux condenser. Diiodomethane (3.8 g, 14 mmoles) was placed in the funnel, and a few drops were introduced into the flask to initiate reaction with the metal. A solution of 5,6-dideoxy-1,2-*O*-isopropylidene-α-D-*xylo*-hex-5-enofuranose[2] (**1**, 1.0 g, 5.4 mmoles) in anhydrous ether was placed in the funnel, and mixed with the remainder of the diiodomethane. The resulting solution was introduced into the flask during 20 min, and the mixture was then heated for 5 h at reflux. T.l.c. at 30-min intervals showed complete disappearance of starting material (R_F 0.33) after 4.5 to 5 h of refluxing, and the appearance of a major product (R_F 0.36) and a very minor side-product (R_F 0.82). The mixture was cooled, treated with 50 ml of saturated aqueous ammonium chloride, and extracted with two 50-ml portions of ether. The combined extracts were successively washed with saturated aqueous potassium carbonate (50 ml) and saturated aqueous sodium chloride (50 ml), dried (magnesium sulfate), and evaporated to give a crystalline solid. Recrystallization of the solid from ether (10 ml) and petroleum ether (b.p. 60–110°, 5 ml) gave **2**; yield (in two crops) 0.62 g (61%), m.p. 145–146°. Sublimation at 50°/50 mtorr gave **2** having m.p. 147–148°, $[\alpha]_D^{20}$ −26 ±1° (*c* 4, chloroform); R_F 0.36 (t.l.c.); λ_{max}^{KBr} 2.92 (OH), 3.23, 3.32 (cyclopropyl C–H), 7.19, and 7.25 μm (CMe_2); n.m.r. data in chloroform-*d* at 100 MHz* (see Fig. 1 and Tables I and II); X-ray powder diffraction data: 9.45 vs (1), 5.19 vs (2,2), 5.00 vs (2,2), 4.82 vw, 4.54 s (3), 4.35 s, 4.06 m, 3.52 m, 3.22 s, 3.11 vw, and 2.91 vw.

Anal. Calc. for: $C_{10}H_{16}O_4$: C, 59.98; H, 8.05. Found: C, 60.29; H, 7.92.

*3-O-Acetyl-4-C-cyclopropyl-1,2-O-isopropylidene-α-*D-xylo-*tetrofuranose* (**5**). — A solution of **2** (0.87 g) in anhydrous pyridine (10 ml) was treated with acetic anhydride (3 ml), and the mixture was kept for 12 h at 25°. Chloroform (25 ml) was added, and the solution was washed with two 25-ml portions of water and evaporated to a syrup.

*The authors thank Professor L. D. Hall, University of British Columbia, Vancouver 8, B. C., Canada, for measurement of this spectrum.

Toluene (5 × 25 ml) and then carbon tetrachloride (3 × 25 ml) were evaporated from the syrup. The resulting yellow syrup, which crystallized spontaneously, was distilled at 130° (bath)/25 mtorr to give the acetate **5** as a crystalline solid, yield 1.049 g (98%), m.p. 45–47°. Recrystallization from ether–petroleum ether (b.p. 60–110°) gave pure **5**, m.p. 49–50°, b.p. 85°/25 mtorr, $[\alpha]_D^{20}$ −21 ±1° (*c* 4, chloroform); λ_{max}^{KBr} 3.30, 3.32 (cyclopropyl CH), 5.72 (OAc), and 7.30 μm (CMe_2); n.m.r. data in chloroform-*d*, see Tables I and II; X-ray powder diffraction data: 9.04 m (2), 6.10 w, 5.33 vw, 5.00 s (1), 4.70 vw, 4.53 vw, 4.18 w, 3.76 w, and 3.62 m.

Anal. Calc. for $C_{12}H_{18}O_5$: C, 59.48; H, 7.48. Found: C, 59.15; H, 7.45.

Deacetylation of **5** *under the Zemplén conditions.* — A solution of **5** (300 mg) in anhydrous methanol (10 ml) was treated with 1 ml of 0.5M methanolic sodium methoxide for 45 min at room temperature. The solution was decationized with an excess of Amberlite IR-120 (H^+) ion-exchange resin, and evaporated to give the crystalline alcohol **2**; yield 220 mg (89%), identical with an authentic sample by mixed m.p., and i.r. and n.m.r. spectra.

4-C-Cyclopropyl-1,2-O-isopropylidene-3-O-(trimethylsilyl)-α-D-xylo-tetrofuranose (**6**). — The cyclopropyl derivative **2** (5 mg) was dissolved in 100 μl of Tri-Sil*, and the solution was kept for 5 min at 25°. G.l.c. of the resulting solution (3 μl) at 150° revealed the presence of **6** as the sole component, retention time 9.8 min (except for peaks resulting from the reagent).

4-C-Isopropyl-1,2-O-isopropylidene-α-D-xylo-tetrofuranose (**7**). — A solution of the cyclopropyl derivative **2** (250 mg) in anhydrous methanol (30 ml) was shaken with 0.2 g of Raney nickel catalyst (W-4 grade, prepared by the method of Pavlic and Adkins[26]) under hydrogen at 2000 lb.in^{-2} for 48 h at 140°. The catalyst was filtered off, and the solution was evaporated to give crude **7** as a white solid; yield 195 mg. Recrystallization from ether–petroleum ether (b.p. 60–110°) gave pure **7** as prisms; yield 180 mg (71%), m.p. 112–114°, $[\alpha]_D^{20}$ −16.5 ±2° (*c* 1.4, chloroform); λ_{max}^{KBr} 2.96 (OH), 7.20, and 7.28 (CMe_2); for n.m.r. data, see Tables I and II; X-ray powder diffraction data: 9.58 vs (1), 5.28 vs (2), 5.03 s (3), 4.76 w, 4.58 m, 4.39 w, 4.17 vw, 4.00 w, 3.52 w, 3.33 w, 3.19 vw, 2.75 w, 2.52 w, and 2.38 w.

Anal. Calc. for $C_{10}H_{18}O_4$: C, 59.38; H, 8.97. Found: C, 59.72; H, 8.99.

Trimethylsilylation of the product, by the method used for **6**, gave 4-*C*-isopropyl-1,2-*O*-isopropylidene-3-*O*-(trimethylsilyl)-α-D-*xylo*-tetrofuranose (**8**) which, on g.l.c. under the conditions used for **6**, had a retention time of 4.8 min. Trimethylsilylation of crude **7**, followed by g.l.c., indicated that **8** was the major component (>95%); very minor proportions (2–3%) of components having retention times of 9 and 6 min, respectively, were present.

4-C-Cyclopropyl-α,β-D-xylo-tetrofuranose (**3**). — A solution of the cyclopropyl derivative **2** (1.02 g) in 60% aqueous acetic acid (20 ml) was heated for 3 h at 90°. The solution was evaporated, and five 15-ml portions of toluene and three 15-ml

*Pre-mixed hexamethyldisilazane, chlorotrimethylsilane, and pyridine; Pierce Chemical Company, Rockford, Illinois.

portions of carbon tetrachloride were successively added to and evaporated from the residue. The resultant solid was recrystallized from acetone–toluene to give **3**; yield 0.80 g (98%), m.p. 109–110°, $[\alpha]_D^{20}$ +16 ±2° (*c* 1.2, water; no mutarotation observed between 3 min and 48 h); λ_{max}^{KBr} 3.05 μm (OH); for n.m.r. data, see Tables I and II; X-ray powder diffraction data: 15.05 m, 7.80 m, 5.17 vw, 4.88 s (2), 4.40 vs (1), 4.21 vw, 3.73 s, 3.06 w, 2.70 vw, and 2.60 w.

Anal. Calc. for $C_7H_{12}O_4$: C, 52.49; H, 7.55. Found: C, 52.28; H, 7.41.

Integration of the n.m.r. spectrum in the region of the H-1 signals indicated that the product contained 57% of the α-D anomer and 43% of the β-D anomer.

A 2mM aqueous solution of **3** containing a tenfold excess of sodium metaperiodate was kept in the dark at room temperature, and the consumption of oxidant was determined by the Müller–Friedberger procedure[27] on aliquots taken at various time-intervals. The results (time, moles of oxidant consumed per mole of **3**) were: 15 min, 2.6; 1 h, 2.7; 2 h, 2.8; 12 h, 2.9; 24 h, 2.9. Titration after 24 h of an aliquot with mM sodium hydroxide to an end-point with Methyl Red indicated the production of 2.85 moles of formic acid per mole of **3**.

*4-C-Cyclopropyl-*D-xylo-*tetritol* (**4**). — A solution containing **3** (2.5 g, 16 mmoles) and sodium borohydride (1.5 g, 40 mmoles) in water (100 ml) was kept for 5 h at room temperature. Acetic acid was added to bring the pH to 5, and the solution was decationized with Amberlite IR-120 (H^+) resin. The solution was evaporated, and methanol (3 × 50 ml) and toluene (3 × 50 ml) were successively added to and evaporated from the residue. The crystalline, crude product, $R_{Compound\ 3}$ 0.9, was homogeneous by t.l.c. on microcrystalline cellulose[25]. Recrystallization from methanol–ether gave pure **4**; yield 2.1 g (81%), m.p. 84.5–85.5°, $[\alpha]_D^{20}$ +13.9 ±2° (*c* 2, water); λ_{max}^{KBr} 3.0 μm (OH); n.m.r. data (100 MHz, deuterium oxide): τ 5.94–6.17 and 6.27–6.48 (multiplets, 4 protons, H-1,2,3), τ 7.12 (1-proton quartet, $J_{3,4}$ 6.0 Hz, $J_{4,5}$ 9.1 Hz), τ 8.86–9.23 (1-proton multiplet, H-5), τ 9.31–9.80 (4-proton multiplet, H-6,6'); X-ray powder diffraction data: 11.81 vs (1), 8.91 m, 6.63 m, 5.74 w, 5.45 w, 4.76 vs (2), 4.45 vs (3), 4.12 m, 3.85 s, 3.74 s, 3.54 s, 3.38 w, 3.18 vw, 3.08 m, 2.93 s, 2.72 s, and 2.54 s.

Anal. Calc. for $C_7H_{14}O_4$: C, 51.83; H, 8.70. Found: C, 51.51; H, 8.82.

Periodate oxidation of **4**, under the conditions used with **3**, led to the uptake of 3.01 moles of oxidant per mole of **4** after 5 min, unchanged after 24 h; and 2.01 moles of formic acid per mole of **4** were produced after 24 h.

*1,2,3-Tri-*O*-acetyl-4-*C*-cyclopropyl-α and β-*D*-xylo-tetrofuranose* (**9** and **10**). — A solution of the triol **3** (122 mg) in dry pyridine (10 ml) and acetic anhydride (2 ml) was kept for 12 h at 25°, and then poured into ice–water. The mixture was stirred for 1 h, and extracted with chloroform (100 ml), and the dried (magnesium sulfate) extract was evaporated to a syrup from which 25 ml of toluene was evaporated to remove pyridine. The n.m.r. spectrum of the syrup indicated that it contained the anomers **9** and **10** in the ratio of approximately 1:1. Distillation of the syrup at 150° (bath)/25 mtorr gave pure **9**+**10**; yield 205 mg (92.5%). A solution of the anomeric mixture of **9** and **10** in ether–petroleum ether (b.p. 60–110°) crystallized slowly to

give prisms of the α-D anomer **9**, yield 95 mg (43.4%), m.p. 94–95°, $[\alpha]_D^{20}$ +150 ±2° (*c* 4.0, chloroform); R_F 0.95; λ_{max}^{KBr} 3.23, 3.30 (cyclopropyl C-H), 3.34, 3.42 (C-H), and 5.73 μm (OAc); for n.m.r. data, see Fig. 2 and Tables I and II; X-ray powder diffraction data: 9.06 s, 7.19 s, 5.02 vs (1), 4.62 w, 4.34 m, 4.01 vw, 3.81 s (2), 3.58 s, 3.34 s, 3.19 vw, 3.08 vw, and 3.01 s.

Anal. Calc. for $C_{13}H_{17}O_7$: C, 54.54; H, 6.34. Found: C, 54.51; H, 6.23.

Evaporation of the mother liquor gave a syrup, the n.m.r. spectrum of which (see Tables I and II) indicated that it was the β-D anomer (**10**), contaminated by a small proportion of the α-D anomer (**9**). T.l.c. with a variety of solvents gave no separation of the anomers. The β-D anomer **10** was not obtained crystalline.

Acetylation of **3** (2.0 g) with acetic anhydride (50 ml) and freshly fused zinc chloride (0.22 g) for 2 h at 0° followed by 2 h at 25°, and 0.5 h at 50°, with conventional processing of the mixture, gave 3.46 g (95%) of a mixture of **9** and **10**, whose i.r. spectrum was identical with that of the syrupy product after acetylation of **3** in pyridine. The n.m.r. spectrum of the product indicated that 48% of **9** and 52% of **10** were present.

Treatment of 4-C-cyclopropyl-1,2-O-isopropylidene-α-D-xylo-tetrofuranose (**2**) *with strong base. — A. With methanolic sodium methoxide.* A solution of **2** (60 mg) in M methanolic sodium methoxide was boiled for 3 days under reflux. No evidence for decomposition of **2** during this period was observed by t.l.c., and crystalline **2** (56 mg), identical by mixed m.p. and i.r. spectrum with authentic **2**, was isolated at the end of the experiment.

B. With aqueous sodium hydroxide. The preceding experiment was repeated, with 56 mg of **2**, but with the use of M aqueous sodium hydroxide for 3 days at reflux. No evidence for decomposition of **2** was indicated by t.l.c., and 50 mg of crystalline **2** was recovered.

Treatment of **2** *with sodium azide. — A. In acetonitrile.* A solution of **2** (50 mg, 250 μmoles) in acetonitrile (25 ml) containing sodium azide (0.3 g, 4.6 mmoles) and ammonium chloride (0.1 g, 1.9 mmoles) was boiled for 48 h under reflux. The solution was concentrated almost to dryness, water (25 ml) was added, and the solution was extracted with two 50-ml portions of ether. The extracts were combined and evaporated to give **2** (50 mg), homogeneous by t.l.c., and identical with the starting material by mixed m.p. and i.r. spectrum.

B. In N,N-*dimethylformamide.* — Repetition of the foregoing experiment, but with *N,N*-dimethylformamide as the solvent gave no evidence (t.l.c.) for decomposition of **2**.

Treatment of **2** *with hydrogen and palladium or platinum.* — A sample of **2** (50 mg) in anhydrous methanol (5 ml) containing 0.1 g of 5% palladium-on-carbon was stirred for 3 days under hydrogen at 1 atm pressure. T.l.c. revealed no change in the starting compound, which was recovered quantitatively at the end of the experiment. Similar results were obtained when the experiment was repeated, but with the pressure of hydrogen raised to 3 atm, and also when platinum (generated from platinum oxide) was used instead of palladium.

Treatment of 4-C-cyclopropyl-α,β-D-xylo-tetrofuranose (**3**) *with 0.5M sulfuric*

acid. — A solution of 3 (11 mg) in 0.5M sulfuric acid (5 ml) was heated at 100°, and samples were withdrawn at various time-intervals, rendered neutral (barium carbonate), and examined by paper chromatography. For times up to 2 h, only unchanged starting material (R_G 6.6) could be detected; but, after longer times of reaction, three additional components, R_G 4.5, 7.9, and 0.25 (major) gradually appeared. After 24 h, all of the starting material (3) had disappeared, and a black precipitate was observed in the reaction flask.

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Notes

Substituted cerebrosides
Part V. Synthesis of a dihydroceramide trihexoside*

The isolation of several ceramide trihexosides from natural sources has been described[1]. They include a digalactosylglucosylceramide isolated from normal human kidney[2] and an apparently similar compound which accumulates in that organ in Fabry's disease[3]. We have recently described the synthesis of a number of ceramide–(and dihydroceramide)–dihexosides[4—8], and we now wish to report the first synthesis of a dihydroceramide–trihexoside. This compound may serve as a model intermediate in studies of the synthesis of oligosaccharide–lipids with structures similar to that of the gangliosides.

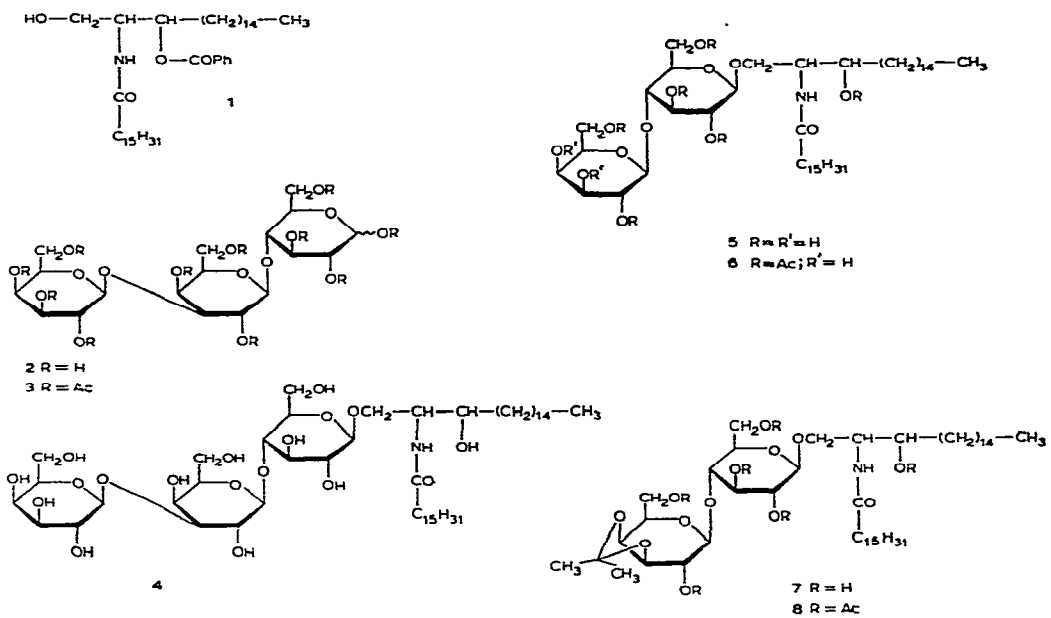
Two approaches were investigated for the preparation of the trihexoside. One approach was the attachment of a monosaccharide unit to a suitably protected β-lactosyl dihydroceramide, whereas the other was the direct condensation of a glycosyl bromide of a trisaccharide with 3-*O*-benzoyl-*N*-hexadecanoyl-DL-dihydrosphingosine[9] [*N*-(DL-*erythro*-3-benzoyloxyoctadecan-2-yl)hexadecanamide] (**1**).

Acetylation of the trisaccharide *O*-(β-D-galactopyranosyl)-(1→3)-*O*-(β-D-galactopyranosyl)-(1→4)-D-glucose[10] (**2**) afforded *O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→3)-*O*-(2,4,6-tri-*O*-acetyl-β-D-galactopyranosyl)-(1→4)-1,2,3,6-tetra-*O*-acetyl-D-glucopyranose (**3**). This crystalline undecaacetate was converted into the syrupy decaacetylglycosyl bromide, which was condensed with **1** in the usual manner[4—9]. After catalytic deacetylation, the trihexoside 1-*O*-[(*O*-β-D-galactopyranosyl)-(1→3)-*O*-(β-D-galactopyranosyl)-(1→4)-(β-D-glucopyranosyl)]-*N*-hexadecanoyl-DL-dihydrosphingosine—*N*-{DL-*erythro*-1-[*O*-(β-D-galactopyranosyl)-(1→3)-*O*-(β-D-galactopyranosyl)-(1→4)-(β-D-glucopyranosyl)]oxyoctadecan-2-yl}hexadecanamide (**4**)–was isolated in low yield.

We have recently described the conversion of a β-lactoside into a trisaccharide in which the 3′-*O*-position of the lactose residue had been glycosylated[10]. The method used involved the stepwise acetonation of benzyl β-lactoside to give the 3′,4′-acetal, acetylation of the remaining free hydroxyl groups, mild acid hydrolysis of the acetal, and glycosylation of the product under such conditions that the relatively more reactive 3′-hydroxyl group was attacked preferentially to the alternative, unprotected, 4′-group. In this series of conversions, the structures of all intermediates were proved unequivocally. Application of analogous reactions to lactosyldihydroceramide **5** as star-

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ting-material gave 1-*O*-[*O*-(2,6-di-*O*-acetyl-β-D-galactopyranosyl)-(1→4)-(2,3,6-tri-*O*-acetyl-β-D-glucopyranosyl)]-*N*-hexadecanoyl-DL-dihydrosphingosine — *N*-{DL-*erythro*-3-acetoxy-1-[*O*-(2,6-di-*O*-acetyl-β-D-galactopyranosyl)-(1→4)-*O*-(2,3,6-tri-*O*-acetyl-β-D-glucopyranosyl)]octadecan-2-ylhexadecanamide (**6**)}—in good yield. Treatment of **6** with 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide in slight excess, followed by catalytic deacetylation, afforded the trihexoside **4** in an overall yield of 20% from **6**. This trihexoside was identical with the one described in the preceding paragraph, on the basic of m.p., optical rotation, i.r. spectrum, and t.l.c.



EXPERIMENTAL

Melting points were determined on substances between glass slides with a Fisher–Johns apparatus and were corrected. Rotations were determined in semimicrotubes, using a Perkin–Elmer 141 polarimeter. N.m.r. spectra were recorded with a Varian A-60 n.m.r. spectrometer using tetramethylsilane as internal standard and chloroform-*d* as the solvent. "Silica gel" refers to silica gel Davison, grade 950, 60–200 mesh, used without pretreatment; the flowing method was used, and elution was stepwise, in order of increasing polarity of the solvents. The proportion of weight of substance added to the column to weight of adsorbent was 1 to 50–100. The fractions eluted were 2 ml/g of adsorbent. "Silicic acid" refers to Bio-Rad silicic acid, used after a preliminary heating for 60 min at 120°, and the chromatographic columns were prepared using chloroform–methanol (19:1). Thin-layer chromatograms were performed on Kieselgel G (E. Merck, Darmstadt).

O-(2,3,4,6-Tetra-O-acetyl-β-D-galactopyranosyl)-(1→3)-O-(2,4,6-tri-O-acetyl-β-D-galactopyranosyl)-(1→4)-1,2,3,6-tetra-O-acetyl-D-glucopyranose (**3**). — A solution of compound **2** (1.1 g) in pyridine (20 ml) and acetic anhydride (20 ml) was kept for 1 h at 100°. The solution was concentrated *in vacuo* to a syrup which crystallized from aqueous ethanol in prisms; m.p. 108–110°, $[\alpha]_D^{25}$ +17.2° (*c* 0.61, chloroform); homogeneous on t.l.c. in 4:1 benzene–methanol, R_F 0.1.

Anal. Calc. for $C_{40}H_{54}O_{27}$: C, 49.69; H, 5.63. Found: C, 49.33; H, 5.56.

N-{DL-erythro-1-[(O-β-D-Galactopyranosyl)-(1→3)-O-(β-D-galactopyranosyl)-(1→4)-(β-D-glucopyranosyl)]oxyoctadecan-2-yl}hexadecanamide (**4**). — A solution of compound **3** (300 mg) in chloroform (20 ml) containing hydrogen bromide in glacial acetic acid (4 ml, 50%) was kept for 2.5 h at 0–5°. The chloroform solution was washed several times with a cold aqueous solution of sodium bicarbonate (5%), dried with calcium chloride, and concentrated *in vacuo* to a syrup. This syrup was added to a stirred mixture at 70° of *N*-(DL-*erythro*-3-benzoyloxyoctadecan-2-yl)hexadecanamide (0.13 g, 0.2 mmole) and mercuric cyanide (0.05 g, 0.2 mmole) in nitromethane (20 ml) and benzene (10 ml), which had been previously dried as described in earlier publications[4–9], and the reaction mixture was maintained overnight at 70°. The cooled mixture was diluted with benzene (50 ml), and processed in the usual manner[4–9]. Catalytic deacylation with sodium methoxide in anhydrous methanol, followed by the addition of a few drops of cold water, led to the precipitation of an amorphous solid which was dissolved in 19:1 chloroform–methanol and chromatographed on silicic acid. After elution of unreacted dihydroceramide with 19:1 chloroform–methanol, 9:1 chloroform–methanol eluted fractions that crystallized from aqueous acetone, m.p. 257–258°; homogeneous on t.l.c. in 80:20:3 chloroform–methanol–water (R_5 0.5); $[\alpha]_D^{20}$ +5.7° (*c* 0.77, pyridine).

Anal. Calc. for $C_{52}H_{99}NO_{18}.H_2O$: C, 59.80; H, 9.75. Found: C, 60.02; H, 9.95.

N-{DL-erythro-1-O-[(3,4-O-Isopropylidene-β-D-galactopyranosyl)-(1→4)-(β-D-glucopyranosyl)]oxyoctadecan-2-yl}hexadecanamide (**7**). — A stirred suspension of compound **5** (500 mg, previously dried overnight at 100° in high vacuum over phosphorus pentaoxide) and anhydrous *p*-toluenesulfonic acid (50 mg) in dry acetone (200 ml) was maintained for 5 h at b.p. under reflux with careful exclusion of moisture. The mixture was cooled to room temperature, and unreacted **5** (170 mg) separated by filtration. The clear filtrate was neutralized with anhydrous sodium carbonate, and insoluble material removed by filtration. Evaporation of the solvent *in vacuo* gave a solid residue which was crystallized from acetone–methanol, yield 300 mg (56%), m.p. indeterminate, $[\alpha]_D^{20}$ +14.0° (*c* 0.62, pyridine). The product was homogeneous on t.l.c. in 3:1 benzene–methanol, R_5 1.2.

Anal. Calc. for $C_{49}H_{93}NO_{13}$: C, 65.2; H, 10.3. Found: C, 65.0; H, 10.4.

N-{DL-erythro-3-Acetoxy-1-[O-(2,6-di-O-acetyl-3,4-O-isopropylidene-β-D-galactopyranosyl)-(1→4)-O-(2,3,6-tri-O-acetyl-β-D-glucopyranosyl)]oxyoctadecan-2-yl}hexadecanamide (**8**). — A solution of compound **7** (200 mg) in pyridine (10 ml) and acetic anhydride (15 ml) was kept overnight at room temperature. The pyridine and acetic

anhydride were removed by evaporation *in vacuo* followed by co-evaporation with toluene. The residual syrup crystallized from cold methanol; yield 230 mg (83%;) m.p. 70–71°; n.m.r.: 7.9 τ (15H, 5-OAc groups), τ 8.6 (6H, -C(CH_3)$_2$); $[\alpha]_D^{20}$ +1.67° (*c* 0.54, chloroform).

Anal. Calc. for $C_{61}H_{105}NO_{19}$: C, 63.4; H, 9.9. Found: C, 63.0; H, 9.6.

N-{DL-erythro-*3-Acetoxy-1*-[O-(*2,6-di*-O-*acetyl*-β-D-*galactopyranosyl*)-(*1*→*4*)-O-(*2,3,6-tri*-O-*acetyl*-β-D-*glucopyranosyl*)]*oxyoctadecan-2-yl*}*hexadecanamide* (**6**). — The isopropylidene group of compound 7 was hydrolyzed by treatment with 80% aqueous acetic acid (200 ml/g of 7) for 4 h at 100°. The acetic acid was removed by several codistillations with water, and the residual water removed by coevaporation with absolute alcohol. The residual solid was crystallized from anhydrous methanol, m.p. 95–97°, t.l.c. in 9:1 benzene–methanol, R_7 0.2. In a typical experiment, 95 mg of 7 afforded 79 mg (87%) of **6**.

Anal. Calc. for $C_{58}H_{101}NO_{19}$: C, 62.4; H, 9.1. Found: C, 62.2; H, 9.1.

N-{DL-erythro-*1*-[O-(β-D-*Galactopyranosyl*)-(*1*→*3*)-O-(β-D-*galactopyranosyl*)-(*1*→*4*)-(β-D-*glucopyranosyl*)]*oxyoctadecan-2-yl*}*hexadecanamide* (**4**). — A solution of compound **6** (578 mg) in nitromethane–benzene (2:1) was stirred in the usual manner with mercuric cyanide and 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide. After catalytic deacetylation and processing, including chromatography on silicic acid, the product crystallized from aqueous acetone; yield 108 mg (20%); m.p. 257–258°; $[\alpha]_D^{20}$ +5.7 (*c* 1, pyridine); i.r. spectrum identical with that of **4**. The product was indistinguishable from **4** described in the preceding paragraphs on t.l.c. in 80:20:3 chloroform–methanol–water.

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Enzymic preparation of L-fructose*

L-Fructose, a sugar which has not yet been detected in Nature, was first synthesized by Fischer[1], who treated α-acrose with phenylhydrazine. The resulting DL-glucose phenylosazone was isolated, and hydrolyzed to the glycosulose (osone), which was then reduced to DL-fructose. The D-fructose was then fermented with yeast, to yield a solution of L-fructose which was identified by formation from it of L-*arabino*-hexose phenylosazone, but it was not, itself, isolated in crystalline form.

L-Fructose was later synthesized by Wolfrom and Thompson[2] by the following sequence of reactions: L-arabinonic acid tetraacetate→L-arabinonoyl chloride tetraacetate→1-deoxy-1-diazo-*keto*-L-fructose tetraacetate→*keto*-L-fructose pentaacetate →L-fructose. The L-fructose was crystallized, and identified by elemental analysis, polarimetry, and derivatives. To date, this procedure is the only satisfactory method available for the synthesis of L-fructose.

The present report describes an alternative and more simplified procedure for the preparation of L-fructose. It involves the isomerization of L-mannose to L-fructose catalyzed by an enzyme present in cell-free extracts of *Aerobacter aerogenes* grown on L-rhamnose. L-Fructose has been isolated in crystalline form, and identified by paper chromatography, polarimetry, and derivatives.

The enzymic method for the preparation of L-fructose described here is considerably shorter than the chemical method previously described[2]. It involves only the isomerization of L-mannose, a compound which can be readily prepared or purchased; the enzyme does not need to be purified, but can be used directly as it occurs in cell-free extracts. Unreacted L-mannose may be recovered from the equilibrium mixture by regeneration from its phenylhydrazone. The yield (28–32%) of L-fructose is equal to or better than that obtained with the chemical method starting from L-arabinonic acid tetraacetate[2].

Although the isomerization of L-mannose to L-fructose represents a new enzymic reaction, the isolation of L-fructose is an adaptation of a standard procedure. Mannose (D or L) phenylhydrazone is well known for its low solubility in water; thus, it has been used to separate mannose quantitatively from other hexoses. Sowden and Fischer[3] succeeded in separating L-mannose from L-glucose through their corresponding phenylhydrazones, and we have succeeded in separating L-mannose from L-fructose by the same means. Two precautions may be specifically noted. First, a high excess of phenylhydrazine should be avoided, because L-fructose and L-fructose phenylhydrazone readily react with the excess reagent to produce insoluble L-*arabino*-hexulose phenylosazone[4], thus decreasing the yield of L-fructose. Second, mixed-bed resins possessing strong anionic groups should not be used for deionizing solutions of fructose because they cause partial isomerization to glucose.

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Since the extent of isomerization is based solely on establishing equilibrium, the amount of L-fructose that can be obtained is limited only by the amount of L-mannose available; the time required to reach equilibrium is inversely proportional to the proportion of enzyme. Thus, larger quantities of L-fructose could be prepared, simply by increasing the initial amounts of the reaction components.

EXPERIMENTAL

Materials. — L-Mannose was prepared by the method of Sowden and Fischer[3] and possessed the following properties: m.p. 129–135°, $[\alpha]_{578}^{24}$ −14.8° (*c* 5, water). (It is commercially available from Calbiochem, Los Angeles, California.) L-Fructose for use as seed crystals was a generous gift from Professor M. L. Wolfrom, The Ohio State University. Duolite resins C-25 (Na^+) and A-6 (Cl^-) were purchased from the Diamond Alkali Company, Redwood City, California; they were converted into the H^+ and OH^- forms, respectively, and, prior to preparation of the column, were mixed in the ratio of cation:anion of 3:5 (wet weight).

Analytical. — L-Fructose was determined by the Roe method[5]. Polarimetric measurements were made with a Zeiss photoelectric polarimeter (courtesy of Dr. J. C. Speck, Jr.). Protein was measured[6] at 210 nm. L-*arabino*-Hexulose phenylosazone was prepared by the procedure of Garard and Sherman[7], and *arabino*-hexulose phenylosotriazole by the procedure of Hann and Hudson[8]. Chromatography was performed on Whatman No. 1 paper with (1) water-saturated phenol, and (2) 15:5:2 (v/v) butanone–acetic acid–water; the sugars on the chromatograms were located with a bath of silver nitrate[9], or sprays of orcinol[10] and *N*,*N*-dimethyl-*p*-phenylenediamine monohydrochloride[11].

Preparation of cell-free extracts. — *A. aerogenes* PRL-R3 was grown aerobically at 30° in 500 ml of a medium consisting of 0.71% of Na_2HPO_4, 0.15% of KH_2PO_4, 0.3% of $(NH_4)_2SO_4$, 0.01% of $MgSO_4$, 0.0005% of $FeSO_4 \cdot 7H_2O$, and 0.4% of L-rhamnose (autoclaved separately). The cells were harvested by centrifugation after growing for 10–12 h in a Fernbach flask on a rotary shaker. They were washed with cold water, recentrifuged, and suspended in 15 ml of 20 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride buffer (pH 7.6). They were disrupted by sonication for 4 min in a Raytheon 10-kHz sonic oscillator equipped with an ice-water cooling-jacket. The cellular debris was removed by centrifugation at 31,000 *g* for 10 min. The supernatant solution was the cell-free extract.

Enzymic preparation of L-*fructose.* — The reaction mixture (75 ml) consisted of the following: 28 mmoles of L-mannose, 5 mmoles of cobalt chloride, 12 mmoles of 2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride buffer (pH 7.6), and cell-free extract (300–350 mg of protein); it was incubated for 2–3 h at 30°, or until equilibrium was established. At equilibrium, the ratio of L-mannose:L-fructose was 19:31. The reaction mixture was then heated in a boiling-water bath for several minutes, and the denatured protein was removed by centrifugation at 31,000 *g* for 15 min.

L-Mannose was separated from L-fructose by adding a solution containing 3.1 g of phenylhydrazine in 7.0 ml of glacial acetic acid, and keeping the resulting mixture overnight at 4°. The L-mannose phenylhydrazone which crystallized was removed by suction filtration. The filtrate was concentrated under vacuum to about 50–60 ml, and stored an additional 2–3 h at 4°. The precipitate that formed was removed by suction filtration.

Free L-fructose was regenerated from the dissolved phenylhydrazone by refluxing the filtrate for 2–3 h with 13 ml of ethanol, 8 ml of benzaldehyde, and 0.8 g of benzoic acid. After the suspension had been cooled to room temperature, the liquid phase was decanted from the insoluble 2-benzyl-2-phenylhydrazone, washed with three 100-ml portions of chloroform, and decolorized with Darco G-60 carbon. This solution (about 60 ml) was deionized by passage through a column (2.7 × 30 cm) of mixed-bed resins consisting of Duolite C-25 (H^+) and A-6 (OH^-). About 500 ml of effluent was collected, which provided a 96% recovery of L-fructose. The neutral effluent was evaporated under diminished pressure to a syrup, which was dissolved in warm absolute alcohol; the solution was nucleated with crystals of L-fructose, and kept for 24 h at room temperature. The fine, needle-like crystals of L-fructose were collected by gravity filtration, and the mother liquor was reprocessed to give additional product. The overall yield of L-fructose was 1.4–1.6 g (28–32%), m.p. 93–95°, $[\alpha]_{578}^{24}$ +91.0° (*c* 2.5, water)[12]. Heating the crystals for 2 h in an Abderhalden vacuum dryer at 83° or 110° resulted in a weight loss of no more than 1.5%; thus, the crystals were considered to be essentially anhydrous.

When chromatographed on paper, with two different solvent-systems, L-fructose migrated with the authentic D-enantiomorph, and was free from detectable L-mannose. L-Fructose was converted into L-*arabino*-hexulose phenylosazone, m.p. 205–208°, a value in good agreement with that recorded by Garard and Sherman[7]. The osazone was subsequently converted into L-arabino-hexulose phenylosotriazole, m.p. 193–197°, $[\alpha]_{578}^{24}$ +80.0° (*c* 4, pyridine); these values are in good agreement with those given by Hann and Hudson[8].

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Recovery of useful carbohydrates in nucleoside syntheses: 2,5-di-*O*-benzoyl-3-deoxy-β-D-*erythro*-pentofuranose*

The mercury derivative of 2-acetamido-6-chloropurine[1] was first employed in nucleoside synthesis for the preparation of the anomers of 2′-deoxy-6-thioguanosine and related compounds. The method has been extended[2] to the preparation of 3′-deoxy-6-thioguanosine, L-thioguanosine, and some related nucleosides. The proposed[2] generality of this synthetic route and the interesting biological properties attributed to various nucleosides derived from thioguanine[3], which necessitates their preparation in amounts sufficient for chemotherapeutic evaluation, prompt us to record our further observations on this synthetic method.

In previous work[4] on the synthesis of moderately large quantities of the anomers of 2′-deoxy-6-thioguanosine[1], we have examined the coupling mixture after removal of the nucleosides and the unreacted purine starting-materials, and have found the protected, (1→1)-linked, disaccharide of 2-deoxy-D-*erythro*-pentofuranose to be a major artifact in this reaction. This compound has proved valuable from a practical standpoint in that it is readily reconvertible into a glycosyl halide that could be re-used in the nucleoside synthesis, thus decreasing the cost and effort necessary to prepare quantities of these compounds sufficient for biological evaluation. The formation of the disaccharide was attributed to the presence, or generation, of water during the nucleoside synthesis or to the formation of a labile purine–mercury–sugar complex, which was decomposed during the isolation and purification process. The exact nature of this mechanism has remained in doubt largely because of the obscure nature of the mercury complex of 2-acetamido-6-chloropurine[1], wherein the elements Hg and O_2 are added to give a complex of unknown structure. In the preparation of 2-acetamido-6-chloro-9-(2,5-di-*O*-benzoyl-3-deoxy-β-D-*erythro*-pentofuranosyl)-9*H*-purine[2] by the coupling of the mercury complex of 2-acetamido-6-chloropurine and 2,5-di-*O*-benzoyl-3-deoxy-β-D-*erythro*-pentofuranosyl chloride[2], it was found[2] that the addition of molecular sieves** is useful. It has further been observed that the mode of addition of the molecular sieves is important. The overall yield in the coupling is materially increased when the azeotropically dried mercury

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**Linde, Type 4A, 1/16″ pellets.

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**Linde, Type 4A, 1/16″ pellets.

complex is first treated with a portion of the sieves in refluxing xylene, and the glycosyl halide and the remainder of the sieves are then added. The residues from the reaction mixture, after removal of the coupling product and the unreacted purine starting-material, yielded a syrup that showed a prominent hydroxyl band in its i.r. spectrum, in contrast to the 2-deoxy-D-*erythro*-pentofuranosyl derivatives, which show no such absorption. Chromatography of the crude syrup on neutral alumina yielded a main, crystalline component whose microanalysis and molecular weight were in agreement for that of a di-*O*-benzoyl-deoxy-pentose. The n.m.r. spectrum indicated that the material was mainly the β-D anomer (singlet for H-1′) contaminated with a trace of what was presumed to be the α-D anomer (doublet for H-1′). Recrystallization from methanol–water afforded the pure β-D anomer in approximately 10% yield from the crude syrup. For recycling, it was not necessary to go through the wasteful process of isolation; instead, the crude syrup was treated with acetyl chloride–hydrogen chloride as in the preparation of the glycosyl chloride[2] from methyl 2,5-di-*O*-benzoyl-3-deoxy-β-D-*erythro*-pentofuranose[5]. The crude, syrupy chloride thus obtained was coupled with an equivalent of the 2-acetamido-6-chloropurine–mercury complex to give an additional 21% of 2-acetamido-6-chloro-9-(2,5-di-*O*-benzoyl-3-deoxy β-D-*erythro*-pentofuranosyl)-9*H*-purine for an overall yield of 52%, as compared with a literature[2] yield of 24% *with* isolation of the intermediate chloride. The experience in these two nucleoside series would indicate that the neutral portion of the nucleoside coupling-mixture may constitute a rich source of re-usable sugar derivatives. Rather low yields (20–50%) are commonly realized in coupling reactions, and it would appear that the bulk of the material is usually lost or discarded in processing the reaction mixture. Despite the supposedly sensitive nature often attributed to the glycosyl halides, it would appear that, actually, they are rather stable under the reaction conditions, and often undergo but little decomposition during the nucleoside synthesis. It may, therefore, prove useful to examine routinely nucleoside coupling-mixtures with a view toward recovery of re-usable carbohydrate intermediates.

EXPERIMENTAL

*2-Acetamido-6-chloro-9-(2,5-di-*O*-benzoyl-3-deoxy-β-*D*-*erythro*-pentofuranosyl)-9H-purine*[2]. — The mercury complex of 2-acetamido-6-chloropurine[1] (235 mmoles) on Celite was suspended in dry xylene (6 liters), and a portion of the solvent (~ 800 ml) was distilled off, so as to dry the mixture. Molecular sieves (Linde, Type 4A, 1/16″ pellets, 80 g) were added, and the mixture was stirred for 3–5 min under reflux. The crude glycosyl chloride[2], prepared from methyl 2,5-di-*O*-benzoyl-3-deoxy-β-D-*erythro*-pentofuranose[5] (85.6 g, 235 mmoles), and a second portion of molecular sieves (80 g) were then added. The mixture was stirred for 6 h under reflux, and then filtered hot, and the filtrate was diluted with petroleum ether (b.p. 30–60°, 12 liters). The filter cake was washed with chloroform, and the washings (*A*) were preserved. 2-Acetamido-6-chloropurine could be recovered from the cake as previously described[4]. The material precipitated by petroleum ether was recovered by filtration, and dissolved in the chloroform washings (*A*). The solution of the crude,

protected nucleoside was extracted twice with 30% aqueous potassium iodide (to free it from mercury), and the organic layer was washed with water and dried (magnesium sulfate). The filtrate was evaporated to dryness *in vacuo*, and the residue was triturated with a small volume of benzene to yield crystals (38.7 g, 31%); m.p. 195–198°, $[\alpha]_D^{24.5}$ +25.5° (*c* 0.5, chloroform) {lit.[2] m.p. 197.5–203.5°, $[\alpha]_D^{23}$ +24° (*c* 0.5, chloroform)}. The u.v. spectral data were in good accord with the literature[2] values.

Recovery of 2,5-di-O-benzoyl-3-deoxy-β-D-erythro-pentofuranose. — The combined, petroleum ether filtrates from the coupling of 176 g of crude glycosyl chloride were evaporated *in vacuo* to a syrup which was dissolved in chloroform, and freed of mercury by extraction with 30% aqueous potassium iodide. The organic layer was washed with water, dried (magnesium sulfate), and evaporated to dryness *in vacuo* to a heavy syrup (127.5 g). A portion of this syrup (10 g) was dissolved in the minimal volume of benzene, and the solution was applied to a column of Woelm neutral alumina (activity I, 300 g). Elution with benzene and ethyl acetate–benzene yielded syrups until 1:1 benzene–ethyl acetate was reached, whereupon material began to be eluted that crystallized. Continued elution, first with 1:10 chloroform–ethyl acetate, and then with chloroform, yielded a total of 3.8 g of crude, crystalline solid. Recrystallization from methanol–water (carbon) gave colorless crystals; m.p. 89–91°. The elementary analysis, molecular-weight determination, and n.m.r. spectrum indicated that this was essentially pure 3,5-di-*O*-benzoyl-3-deoxy-β-D-*erythro*-pentofuranose containing a trace of what was presumed to be the α-D anomer, as evidenced by the n.m.r. spectrum (doublet, δ 5.87, $J_{1',2'}$ 3.4 Hz). The material was recrystallized once more, yielding the pure β-D anomer (1.0 g); m.p. 89–91°, $[\alpha]_D^{22.5}$ +23.8° (*c* 1.0, chloroform); n.m.r. data (chloroform-*d*, tetramethylsilane as internal standard, Varian A-60A spectrometer): δ 8.1, 7.5 (10-proton multiplet, aromatic), δ 5.58 (1-proton singlet, H-1), δ 5.45, 5.35 (1-proton pair of doublets, H-2, *J* 2.5 Hz), δ 4.5 (3-proton multiplet, H-4, H-5), δ 4.1 (one-proton, broad singlet, exchanges, OH), δ 2.3 (2-proton multiplet, H-3). The compound moved as a single spot on t.l.c. (silica gel–chloroform).

Anal. Calc. for $C_{19}H_{18}O_6$: C, 66.66; H, 5.30; O, 28.04. Found: C, 66.64; H, 5.39; O, 27.86.

A portion of the syrup recovered from the petroleum ether solution (177 g) was dissolved in acetic acid (400 ml) and treated by the literature[2] procedure for preparation of the glycosyl chloride. The crude chloride was coupled with 2-acetamido-6-chloropurine–mercury complex (125.7 g, 285 mmoles), and processed as already described, to give the protected nucleoside (32.6 g, 21.5%), m.p. 197.5–200°, having spectral and chromatographic behavior identical with that of the sample already prepared.

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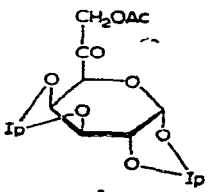
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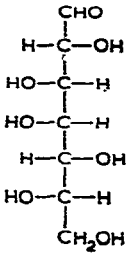
Carbohyd. Res., 8 (1968) 347–350

Un nouvel heptose, le L-*glycéro*-D-*galacto*-heptose

Nous avons décrit récemment[1] la synthèse commode d'un heptulose protégé, le 7-*O*-acétyl-1,2:3,4-di-*O*-isopropylidène-α-D-*galacto*-heptos-6-ulose (**1**) par allongement à l'extrémité non réductrice du D-galactose. Nous avons obtenu, avec un rendement final de 43%, une huile recristallisable dans l'éther isopropylique par réduction d'une solution éthérée de **1** par $LiAlH_4$. Ce traitement doit transformer la partie acétate α-cétonique de la molécule en α-diol. Les cristaux obtenus paraissent homogènes, mais n'ont pas pu être obtenus avec la composition attendue. Après une hydrolyse acide, nous n'avons pu isoler qu'un seul produit cristallisé, dont toutes les propriétés examinées sont celles de l'heptose énantiomorphe **2** du D-*glycéro*-L-*galacto*-heptose déjà connu[2] (Tableau I). Le rendement est de 24% à partir de l'heptulose protégé **1**. Par réduction de l'heptose avec $NaBH_4$, on retrouve le L-*glycéro*-D-*galacto*-heptitol, polyol déjà préparé par une voie différente[3] (Tableau II).



1



2

Le L-*glycéro*-D-*galacto*-heptose cristallise facilement à partir du méthanol, mais nous n'avons pas de preuves sur la structure cyclique de ces cristaux. Il est raisonnable d'admettre que c'est un dérivé sur C-6 du D-galactopyranose *C 1*, la chaîne $CHOH\text{-}CH_2OH$ adoptant la position équatoriale. La mutarotation monotone décroissante suggère une anomérisation α→β. Ceci pourrait être confirmé par

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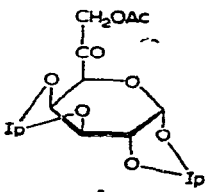
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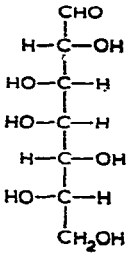
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l'examen du spectre de n.m.r. immédiatement après dissolution. En fait, pour pouvoir observer la région des protons anomères, dans l'oxyde de deutérium, il est nécessaire d'éliminer au maximum DHO, par des traitements (évaporations répétées) qui donnent finalement une solution en équilibre. Nous pouvons interpréter le spectre de la façon maintenant classique : un singulet élargi, δ 5.33 (H-1 anomère équatorial dans la forme α, faiblement couplé avec H-2 axial); un doublet δ 4.55 et 4.68, $J_{7,5}$ (H-1 anomère axial dans la forme β, fortement couplé avec H-2 axial). L'intensité totale de ces deux signaux correspond à un proton. On sait aussi que l'anomérisation $\alpha \rightarrow \beta$ se traduit par un déplacement vers les champs forts. Le reste du spectre est un massif, de δ 3.51 à 4.10, correspondant aux autres protons non échangeables, d'intensité totale égale à sept.

Le rendement global de notre préparation du L-*glycéro*-D-*galacto*-heptose est de 7% à partir du D-galactose. L'énantiomorphe a été préparé[2] avec 19% de rendement à partir du D-gulose, qui, toutefois, n'est pas directement accessible.

PARTIE EXPÉRIMENTALE

Méthodes générales. — On a fait les chromatographies sur couche mince (c.c.m.) sur plaque de gel de silice fluorescent, les chromatographies gaz–liquide (c.g.l.) sur une colonne chromosorb W, 5% SE 30 de 150 cm (Aérograph Co.), à 187°, avec l'azote sous 690 mm comme gaz porteur. On a déterminé les points de fusion avec le microscope Reichert. Les spectres de n.m.r. sont mesurés à 60 NHz et rapportés en p.p.m. (δ) à partir du tétraméthylsilane.

TABLEAU I

COMPARAISON DES PROPRIÉTÉS DES L-*glycéro*-D-*galacto*-, D-*glycéro*-L-*galacto*- ET D-*glycéro*-D-*galacto*-HEPTOSE

	L-glycéro-D-galacto-*Heptose*	D-glycéro-L-galacto-*Heptose*[a]	D-glycéro-D-galacto-*Heptose*[b]
Sucre libre			
p.f.	178–182°	185°	145°
[α]	+106 (7 min.)→ + 66° (équilibre)[c]	−120.5°→ − 65.1°[d]	
R_G	0.73		0.78
Héxacétate			
p.f.	115–122°	117–118°; 150–155°	106–107°

[a]D'après les réf. 2 et 4. [b]D'après les réf. 5, 6 et 7. [c]λ 578 nm; t, 20°. [d]λ 589 nm, t 20°; R_G migration relative au D-glucose dans alcool butylique–pyridine–eau (6:4:3).

Réduction par $LiAlH_4$ du 7-O-acétyl-1,2:3,4-di-O-isopropylidène-α-D-galacto-heptos-6-ulose (1). — On ajoute en 30 min le cétoacétate **1** (1.27 g; 3.8 mmoles) dans l'éther (150 ml) à une suspension de $LiAlH_4$ (16 mmoles) dans l'éther (10 ml). On

TABLEAU II

DÉRIVÉS DU L-*glycéro*-D-*galacto*- ET DU D-*glycéro*-D-*galacto*-HEPTITOL

	L-glycéro-D-galacto-*Heptitol*		D-glycéro-D-galacto-*Heptitol*[b]
	Valeurs trouvées	*Valeurs publiées*[a]	
Polyol libre			
p.f.	141–142°	141–142°	187–188°
$[\alpha]^c$	+20°	+26°	
R_F[d]	0.37		0.32
R_{Gal}	0.90		0.84
Heptaacétate			
p.f.	101–102°		119–121°
R_F[e]	0.68		0.73

[a]D'après la réf. 3. [b]D'après les réf. 8 et 9 en partie; [c][α] pouvoir rotatoire spécifique (*c* 0.4 dans une solution aqueuse à 5% de $(NH_4)_2MoO_4$, à 20°, λ 578 nm). [d]Sur papier, dans le phénol saturé d'eau; R_{Gal} : migration relative au galactose, mêmes conditions. [e]Couche mince de silice, chloroforme–acétate d'éthyle (1:3).

fait bouillir à reflux 25 min avec agitation. Après refroidissement, on détruit avec précaution l'excès d'hydrure par la quantité calculée d'eau. On filtre, évapore à sec le filtrat et sèche le résidu sur P_2O_5. L'huile résiduelle (1.04 g; 94%) cristallise par trituration avec de l'éther isopropylique. On recristallise deux fois et l'on obtient des cristaux, p.f. 98–101, 5° (43%) qui paraissent homogènes en c.c.m., soit avec le système chloroforme–acétate d'éthyle (1:1), R_F 0.29, soit avec le système alcool butylique–pyridine–eau (3:1:1), R_F 0.65. Nous considérons que le composé obtenu est principalement le diol, 1,2:3,4-di-*O*-isopropylidène-L-*glycéro*-α-D-*galacto*-heptose.

L-glycéro-D-galacto-*Heptose* (**2**). — On fait bouillir à reflux 30 min une solution de l'heptose protégé précédent (121 mg) dans l'acide acétique à 30% (10 ml). On évapore à sec et l'on entraîne les dernières traces d'acide par additions et évaporations successives d'eau. On sèche le résidu sur l'hydroxyde de potassium et l'on recristallise dans le méthanol (53 mg, 56%), p.f. 178–182°; $[\alpha]^{20}_{578}$ +106° (7 min)→ +66° (équilibre, *c* 0.25, eau); chromatographie sur papier (éluant : alcool butylique–pyridine–eau, 6:4:3); révélateur $AgNO_3$–NaOH) : R_G 0.73; c.g.l. après silylation par l'hexaméthyldisilazane et triméthylchlorosilane dans la pyridine[10] : trois pics, 9 min 12 sec (très prépondérant), 12 min 20 sec, 14 min 45 sec.

Anal. Calc. pour $C_7H_{14}O_7$: C, 40.00; H, 6.71. Trouvé : C, 40.22; H, 6.88.

L-glycéro-D-galacto-*Heptose hexaacétate*. — On dissout l'heptose ci-dessus (86 mg) dans la pyridine (0.8 ml) et l'anhydride acétique (1 ml). On agite jusqu'à dissolution (24 h). On verse sur la glace, extrait au benzène, sèche l'extrait et reprend à l'alcool chaud. Les cristaux blancs obtenus au refroidissement, p.f. 115–122°, sont apparemment un mélange d'α et de β acétate, car ils donnent deux taches en c.c.m., R_F 0.84 (prépondérante) et R_F 0.59 (éluant : chloroforme–méthanol, 9:1; révélation par $AgNO_3$ 0.7% dans l'acétone, puis soude alcoolique 0.5M).

Anal. Calc. pour $C_{19}H_{26}O_{13}$: C, 49.35; H, 5.67. Trouvé : C, 49.23; H, 5.88.

L-glycéro-D-galacto-*Heptitol.* — On ajoute $NaBH_4$ (50 mg) à une solution de l'heptose (50 mg) dans l'eau (8 ml). Après 48 h, on fait passer à travers une colonne de Dowex-50 (H^+, g), évapore à sec et élimine l'acide borique par additions de méthanol et évaporations. On recueille 52 mg de cristaux blancs, recristallisables dans l'alcool, p.f. 141–2°, $[\alpha]_{578}^{20}$ +20° [*c* 0.40, solution aqueuse à 5% de $(NH_4)_2\,MoO_4$]. La chromatographie sur papier a été faite avec le phénol saturé d'eau comme éluant. On sèche la feuille 3 jours à l'air, puis 3 h à 100°, et l'on révèle avec le réactif permanganate–periodate. Les taches apparaissent après 24 h : R_F 0.37; R_{Gal} 0.90. Pour un échantillon de perséitol (D-*glycéro*-D-*galacto*-heptitol) examiné simultanément, les valeurs sont R_F 0.32; R_{Gal} 0.84. Le L-*glycéro*-D-*galacto*-heptitol n'est totalement silylé qu'après 2 h de réaction et donne alors un seul pic en c.g.l., 21 min 35 sec.

L-glycéro-D-galacto-*Heptitol heptaacétate.* — Ce composé a été obtenu par agitation pendant 2 jours à température ambiante, dans la pyridine et l'anhydride acétique, suivie du traitement habituel et de recristallisation dans le méthanol, p.f. 101–102°; c.c.m. : éluant chloroforme–acétate d'éthyle (3:1); révélation par traitements successifs par la soude alcoolique 0.5M et le réactif permanganate–periodate, R_F 0.68. On observe simultanément pour l'heptaacétate de perséitol, R_F 0.73.

REMERCIEMENT

Les auteurs remercient le Professeur J. E. Courtois pour le don d'un échantillon de perséitol.

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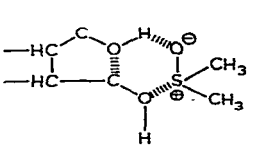
The preparation of 1,6-anhydro-β-D-glucopyranose (levoglucosan) by using methyl sulfoxide

INTRODUCTION

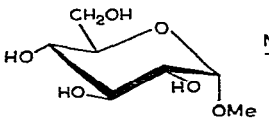
A few years ago, an attempt was made in the laboratory of the late Dr. Fred Smith to carry out the codextrinization of amylopectin with D-xylose in methyl sulfoxide (Me_2SO). Acid hydrolysis of the product and subsequent paper chromatography of the hydrolyzate revealed the presence of a component having a mobility far greater than that of any of the expected products. At the same time, there was a diminution in the number of hydroxyl groups in the product.

At approximately the same time, reports[1,2] appeared of the formation of cyclic ethers by heating certain diols in methyl sulfoxide under comparable conditions, leading us to speculate as to whether there was a direct relationship between these phenomena and whether useful products, including modified polysaccharides, could be obtained in this way. Earlier observations[3] that benzylic and tertiary alcohols are dehydrated to mixtures of olefins, and that 2,3-diphenyl-2,3-butanediol undergoes a pinacol rearrangement, were suggestive of a carbonium-ion process—an unattractive mechanism for our purposes. This view was re-inforced further, when a possible thermal-elimination mechanism was shown to be invalid[3].

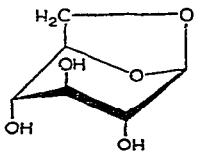
It appeared that other factors might be operative, however, since unexpectedly high yields of tetrahydrofuran derivatives were obtained from primary, secondary, and tertiary 1,4-diols. These results prompted Gillis and Beck[2] to postulate a cyclic transition-state (**1**) to rationalize this selectivity. The fact that the yields of cyclic



1



2



3

ethers[1] decreased as the number of carbon atoms separating the hydroxyl groups was increased from two to four is in agreement with this proposal. A less favorable, cyclic transition-state would be anticipated with this increase in the size of the quasi-ring form. The ensuing observation that the dehydration of 5-butyl-5-nonanol is unaffected by aniline or sodium octoxide led Traynelis and co-workers[1] to suggest that methyl sulfoxide facilitates the ionization of alcohols; this view of the role of methyl sulfoxide in dehydration appeared compatible with Gillis and Beck's postulate[2], and encouraged continuance of our investigation.

DISCUSSION

Methyl α-D-glucopyranoside (2) was selected for study, as it was considered to be a model for polysaccharides and it lacks vicinal, *cis*-hydroxyl groups. The decomposition[4] of methyl sulfoxide has been shown to be accelerated by the presence of certain 1,2- and 1,3-diols, which are converted into dioxolane and *m*-dioxane derivatives, respectively.

The color changes that ensued during the heating of the glycoside 2 in methyl sulfoxide at 160°, an arbitrarily chosen temperature, were typical of carbohydrate pyrolysis. For this reason, the identification of 1,6-anhydro-β-D-glucopyranose (levoglucosan, 3) as a product of this reaction, by paper chromatography and isolatively by cellulose-column chromatography, was not unexpected. Levoglucosan has frequently been reported[5–8] to be among the products resulting from the pyrolysis of D-glucose and related di- and poly-saccharides. Temperatures considerably higher than that utilized here are commonly used, and diminished pressures are often employed. Yields[9] as high as 62% have been reported, although yields below 40% are more common. The methods and conditions that have been used in many pyrolytic studies are not readily amenable to scaling-up for commercial purposes.

Rate data and information on pH changes were obtained for the conversion of 2 in Me_2SO. Samples were removed periodically, and the components were converted into their per(trimethylsilyl) ethers; these were separated and assayed by g.l.c. with D-mannitol as an internal standard. The 50% yield of "anhydro" sugars, 85% as 3, suggests that the reaction may be a useful route to 3, as especially it involves a homogeneous reaction-system, which lends itself to considerable variation in reaction conditions and additives.

Fig. 1 is a first-order plot of the disappearance of 2. The initial deviation from these kinetics probably resulted from the fact that the acidity had not reached a level

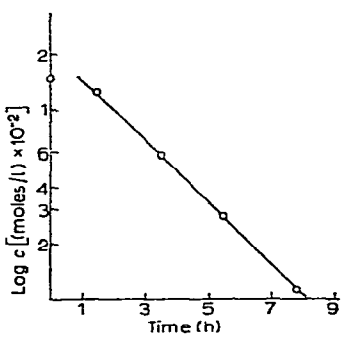


Fig. 1. A first-order plot of disappearance of methyl α-D-glucopyranoside, log *c* *vs.* *t*.

necessary to catalyze the reaction. The concentration of 3 increased (see Table I) during the course of the reaction, while the content of total carbohydrate decreased, as measured by unreacted 2 and the anhydro sugars produced. The remainder had

formed dark, resinous materials and, probably, volatile, non-carbohydrate products. No oligosaccharides were detected by g.l.c. or by paper chromatography.

TABLE I

THE CONVERSION OF METHYL α-D-GLUCOPYRANOSIDE (2) INTO ANHYDRO SUGARS

	Reaction time (h)					
	0	0.5[a]	1.5	3.5	5.5	7.75
	Components (mmoles/liter)					
2	143		120	56	27	12
Anhydro sugars	0		5	38	60	71
Total carbohydrate	143		125	94	87	83
pH	76.5[b]	7.33	4.01	3.58	3.36	3.20	3.31

[a]Time required to reach 160° from room temperature. [b]pH without glycoside.

A gas chromatogram of the reaction mixture at the completion of the reaction is shown in Fig. 2. Component *B* was identified as 1,6-anhydro-β-D-glucofuranose, a product that accompanies **3** during pyrolysis of D-glucans[7,10]. The furanose deriva-

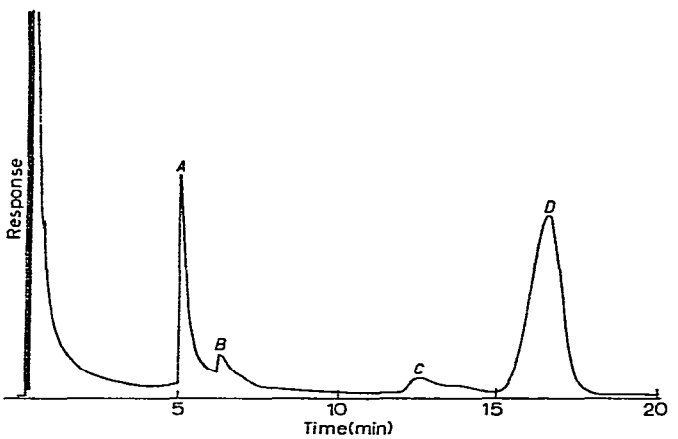


Fig. 2. Gas chromatogram of reaction mixture as per(trimethylsilyl) ethers. *A*, 1,6-anhydro-β-D-glucopyranose (**3**); *B*, 1,6-anhydro-β-D-glucofuranose; *C*, methyl α-D-glucopyranoside (**2**); *D*, internal standard (D-mannitol).

tive was isolated from the reaction mixture by degrading the other components by periodate oxidation and converting the resultant aldehydes into acids by oxidation with bromine. The acids were removed by an anion-exchange resin, and 1,6-anhydro-β-D-glucofuranose was isolated, and identified as its triacetate. After a reaction time of 30 min, a compound having a retention time identical with that of the anhydrofuranose derivative was the only product, other than **2**, detectable by g.l.c. Curiously, the concentration of the anhydrofuranose fluctuated very little during the remainder of the reaction.

The shoulder on the peak (*c*) for methyl α-D-glucopyranoside (Fig. 2) did not appear until elapse of 5 h of reaction, and it is designated tentatively as the β-D-glycoside derivative. Per(trimethylsilyl)ated methyl β-D-glucopyranoside has the same retention time as this component, alone or in admixture with the α-D anomer on this column.

It is apparent from the decrease in pH as the reaction progresses that an acid-catalyzed process is very probably responsible for the course of the reaction. The presence of calcium carbonate as an acid scavenger prohibited the reaction. The insolubility of this salt in methyl sulfoxide eliminated the possibility that inhibition was caused by complexing of the calcium ion with the carbohydrate. Although the pyrolysis of cellulose is known to produce acids[11,12], neither heating in the dry state nor in 2-butoxyethanol solution for 4 h at 160° brought about any change in the glycoside **2**. Heating of **2** in bis(2-methoxyethyl) ether containing 0.015% of anhydrous *p*-toluenesulfonic acid (initial pH 3.70) did result in formation of **3**.

Although the heating of methyl sulfoxide alone failed to produce any acidic materials, repetition of the preparation of 4-(hydroxymethyl)-1,3-dioxolane[4] also resulted in a reaction mixture having a decreased pH (7.09→3.38). The acids were removed by an anion-exchange resin and, after exhaustive washing of the resin, they were recovered as their ammonium salts. The i.r. spectrum of the viscous syrup showed bands typical[13] of carbonyl (1710 cm^{-1}), carboxylate anion (1575 cm^{-1}, 1400 cm^{-1}), and sulfoxide (1050 cm^{-1}). The acids were recovered by treatment with cation-exchange resin, and they had a spectrum lacking the carboxylate anion bands, but showing bands at 1730 and 1720 cm^{-1}, and two peaks in the sulfoxide region (1065 cm^{-1} and 1045 cm^{-1}). Sulfur was detected qualitatively[14].

Cyclic transition-states need not be involved to explain the product[1]. An acid-catalyzed process would result in cleavage of the aglycon, with the carbonium ion produced at C-1 being stabilized by the polar methyl sulfoxide. There is sufficient thermal energy to produce the inversion of the conformer, to permit attack of the 6-hydroxyl group on C-1 and formation of the anhydro ring.

Both D-glucose and starch yield **3** under comparable conditions.

EXPERIMENTAL

General. — Methyl α-D-glucopyranoside (**2**) was dried *in vacuo* over phosphorus pentaoxide. Methyl sulfoxide was dried over calcium hydride and distilled under diminished pressure. All reactions were effected in an atmosphere of dry nitrogen. Paper chromatography was performed on Whatman No. 1 paper with 6:4:3 (v/v) butanol–pyridine–water as solvent, and ammoniacal silver nitrate[15] for detection. The spots were intensified by treating the chromatograms with Kodak F-24 fixing solution[16] 20 min after dipping in the Tollens reagent. The gas chromatograph was an F & M Model 810 equipped with a flame-ionization detector (Hewlett–Packard).

Treatment of methyl α-D-glucopyranoside (**2**) *with methyl sulfoxide.* — A solution of **2** (5 g) in methyl sulfoxide (100 ml) was stirred and heated for 2.5 h at

160°. Paper chromatography of an aliquot (2 μl) of the reaction mixture revealed the presence of unreacted **2** and a component having a mobility identical with that of an authentic sample[17] of levoglucosan (**3**). The reaction mixture was evaporated *in vacuo* to a dark-brown syrup (5.92 g).

The components of a portion (1.235 g) of the syrup were separated by cellulose-column chromatography. The solvent was removed from that fraction corresponding to **3**, whereupon the residue crystallized. Recrystallization from methanol gave **3**, m.p. 182–183°, mixed m.p. with an authentic sample 184–185°, $[\alpha]_D^{24}$ −65° (*c* 1.1, water); [lit.[5] m.p. 178°, $[\alpha]_D$ −66.5° (water)].

Acetylation of **3** with a mixture of acetic anhydride and sodium acetate[18] gave 2,3,4-tri-*O*-acetyl-1,6-anhydro-β-D-glucopyranose, m.p. 112–114°, mixed m.p. 112–113°.

Analysis of the progress of the reaction with time. — The reaction was repeated exactly as before, but on 3 times the scale. Aliquots (12 ml) were removed at time zero, 0.5 h (the temperature had reached 160°), 1.5, 3.5, 5.5, and 7.75 h, and were chilled immediately in an ice bath. A portion (10 ml) of each aliquot was diluted with an equal volume of distilled water, and the pH was determined after the temperature had been readjusted to 25°.

A portion (300 μl) of each sample was added to a weighed amount of D-mannitol, and the weight of the reaction mixture was determined. Dry pyridine, hexamethyldisilazane, and trifluoroacetic acid were added to convert the components into their per(trimethylsilyl) ethers, and the mixture was shaken for 15 min before injection onto the column. A stainless-steel column (6 ft × 3/16 in O.D., wall thickness 0.028 in) was coated with hexamethyldisilazane, and packed with 3% SE-52 on Gas Chrom Z, 80–100 mesh (Applied Science Labs., State College, Pa.). Helium was used as the carrier gas at a flow rate of 90 ml/min. The areas under the peaks were determined by planimetry.

Identification of 1,6-anhydro-β-D-glucofuranose. — The reaction mixture was evaporated *in vacuo* at 60° (bath temperature), and the residue was acetylated with a mixture of acetic anhydride and pyridine for 4 h at 60–70°. The mixture was poured into ice–water, and the product was extracted into chloroform. The dried chloroform solution was evaporated to yield a syrup that partially crystallized. The entire residue was subjected to short-path, vacuum distillation. The material distilling at 165–172° (bath temperature)/0.13 torr was collected, and crystalline 2,3,4-tri-*O*-acetyl-1,6-anhydro-β-D-glucopyranose was isolated from the distillate.

The part of the distillate that did not crystallize was deacetylated with a catalytic amount of sodium methoxide. A qualitative gas-chromatogram of the deacetylated, trimethylsilylated material established that three components (*A*, *B*, and *C*) were present. This mixture was oxidized with periodic acid as described by Dimler *et al.*[10]; however, the resulting "dialdehydes" were not treated with phenylhydrazine, but were oxidized with bromine to the corresponding carboxylic acids. The pH of the oxidation solution was maintained between 5.5 and 7 by the intermittent addition of 100 mM sodium hydroxide. The solution was deionized by passage through columns

of Amberlite IR-120 cation-exchange resin and Duolite A-4 anion-exchange resin. The eluate was evaporated *in vacuo* to a syrup that, by g.l.c., gave a single peak having a retention time identical with that of component *B*, alone or in admixture with **3**. The syrup did not crystallize, and it was therefore acetylated with acetic anhydride and pyridine; the product, devoid of hydroxyl bands in its i.r. spectrum, crystallized after being nucleated with an authentic sample of 2,3,5-tri-*O*-acetyl-1,6-anhydro-β-D-glucofuranose (kindly supplied by Dr. R. J. Dimler, Northern Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture). The crystals were triturated with ice-cold 1:1 (v/v) methanol–water and then had m.p. 81–83°, $[\alpha]_D^{23}$ −16.9° (*c* 2.0, chloroform) [lit.[10] m.p. 82.5–83.5, $[\alpha]_D^{25}$ −15.3° (*c* 2, chloroform)].

Screening reactions. — *A.* All reactions were conducted with the glycoside **2** (3 g) in a solvent (100 ml) for 4 h at 160°. An aliquot (2 μl), equivalent to 60 μg of **2**, was chromatographed on paper. It was possible to detect less than 1 μg of **3**. The solvents used were: methyl sulfoxide containing (*a*) anhydrous calcium carbonate (10 g); (*b*) 2-butoxyethanol; (*c*) *p*-toluenesulfonic acid monohydrate (15 mg) that had been dissolved in dry xylene (40 ml), the solvent (35 ml) distilled to remove the water azeotropically, and the solution concentrated *in vacuo* and then diluted with bis(2-methoxyethyl) ether.

B. Methyl α-D-glucopyranoside (2.7 mg) was placed on a Fisher–Johns melting-point apparatus and heated for 4 h at 160°. The material was dissolved in water (0.2 ml), and an aliquot (5 μl) was chromatographed.

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Carbohyd. Res., 8 (1968) 354–360

Sulphated polysaccharides of the Grateloupiaceae family Part II[1]. Isolation of 4-*O*-methyl-L-galactose, 6-*O*-methyl-D-galactose, and two disaccharides from hydrolysates of aeodan

Previous work on aeodan[1], a sulphated polysaccharide from *Aeodes orbitosa*, revealed the presence of D-galactose (58%), 2-*O*-methyl-D-galactose (10%), xylose (trace), glycerol (trace), and sulphate (27.5%) in the polymer. The polysaccharide contains only (1→3) and (1→4) links, with most of the (1→4)-linked galactose residues being free of sulphate. The major sulphated units are (1→3)-linked galactose 2-sulphate and probably (1→3)-linked galactose 2,6-disulphate. In addition, the presence of some sulphate-free (1→3)-linked units was established.

We now report the isolation of small proportions of two additional monomethylgalactoses and two galactosyl-galactoses from hydrolysates of aeodan. Elution of a hydrolysate of aeodan from a cellulose column afforded, in addition to D-galactose and 2-*O*-methyl-D-galactose, a mixture which was shown by paper chromatography to consist of 4-*O*-methylgalactose (minor sugar), xylose (minor sugar), 2-*O*-methylgalactose (major sugar), and 6-*O*-methylgalactose. A portion of the syrup was separated by paper chromatography, and the 4-*O*-methyl- and 6-*O*-methyl-galactoses were isolated. The 4-methyl ether, $[\alpha]_D^{17}$ −84°, had m.p. 203–206°, alone and in admixture with authentic 4-*O*-methyl-L-galactose[2]. This is the second, reported instance of the isolation of 4-*O*-methyl-L-galactose from a natural source. Araki *et al.*[3] isolated a small proportion of this sugar from the hydrolysate of the agar of *Gelidium amansii*. The 6-*O*-methylgalactose, $[\alpha]_D^{17}$ +76°, had m.p. 119–120° alone and in admixture with authentic 6-*O*-methyl-D-galactose. The presence of 6-*O*-methyl-D-galactose in the hydrolysate of desulphated, periodate-oxidised aeodan suggests that this sugar is (1→3)-linked in the polymer. It is interesting to note that Araki *et al.*[4] isolated small proportions of 4-*O*-methyl-D-galactose and 2-*O*-methyl-L-galactose and its 3,6-anhydride from the sulphated polysaccharide of *Grateloupia elliptica* (Grateloupiaceae).

Further evidence for the presence of (1→3)- and (1→4)-linked galactose in aeodan was obtained by the isolation of two disaccharides (*A* and *B*) from a partial, acid hydrolysate of the polysaccharide. The crystalline disaccharide *A*, $[\alpha]_D^{17}$ +73.2°, m.p. 205–208°, gave only galactose on hydrolysis. It was methylated and methanolysed, and the resulting mixture was separated by g.l.c. Peaks corresponding to methyl 2,3,4,6-tetra-*O*-methylgalactoside and methyl 2,3,6-tri-*O*-methylgalactoside were observed. These results indicate that this disaccharide is 4-*O*-β-D-galactopyranosyl-D-galactose. The amorphous disaccharide *B*, which also gave only galactose on hydrolysis, was methylated and hydrolysed, and the products were separated by paper chromatography. 2,3,4,6-Tetra-*O*-methyl-D-galactose ($[\alpha]_D^{17}$ +90.5°; "anilide", m.p. 188–190°) and 2,4,6-tri-*O*-methyl-D-galactose ($[\alpha]_D^{17}$ +92.5°; "anilide", m.p. 170–171°) were isolated. These results indicate that this disaccharide is 3-*O*-D-galactopyranosyl-

D-galactose. The configuration of the linkage in this disaccharide is uncertain, as the specific rotation (+60°) was measured on a cloudy solution, and all attempts to produce an optically clear solution failed.

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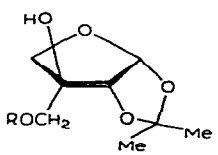
Preliminary Communications

Methyl 2,3-*O*-isopropylidene-4-thio-β-D-apio-D-furanoside; a branched-chain 4-thioaldofuranose*

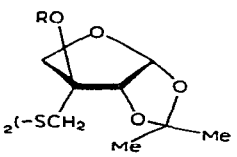
There has been considerable interest in the synthesis of sugars in which the oxygen atom of the hemiacetal ring is replaced by another atom, particularly nitrogen or sulfur[1-5] Several such derivatives have been incorporated into nucleosides [6,7]. Recent work has involved the synthesis of nucleosides of branched-chain sugars [8-10]

We have been concerned with the synthesis of nucleosides that incorporate analogs of the branched-chain sugar apiose in which the ring-oxygen atom is replaced by sulfur or nitrogen. This Communication describes some novel reactions involved in the synthesis of the title compound, the first example of a branched-chain 4-thioaldofuranose.

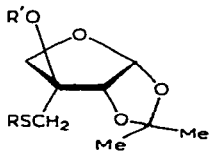
Treatment of 1,2-*O*-isopropylidene-α-D-apio-L-furanose[11] (**1**) with *p*-toluene sulfonyl chloride in pyridine yielded the 5-*p*-toluenesulfonate† **2**, m.p. 137–138°; $[\alpha]_D^{29}$ + 47° (*c* 1.0, chloroform)[12]



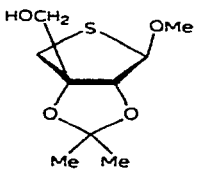
1 R = H
2 R = Ts



3 R = Ac
6 R = H



4 R = R' = Ac
5 R = H, R' = Ac
7 R = Ac, R' = H
8 R = R' = H



9

Treatment of **2** with 2 molar proportions of potassium thiolacetate in *N,N*-dimethylformamide at 100°, or in refluxing acetone, gave a mixture of three components: bis(3-*O*-acetyl-5-deoxy-1,2-*O*-isopropylidene-α-D-apio-L-furanose-5-yl) 5,5'-disulfide (**3**), m.p. 151–152°, $[\alpha]_D^{25}$ + 170° (*c* 0.78, chloroform), ν_{max}^{KBr} 1735 cm^{-1} (OAc); 3-*O*-acetyl-5-*S*-acetyl-1,2-*O*-isopropylidene-5-thio-α-D-apio-L-furanose (**4**) as an analytically pure syrup, $[\alpha]_D^{25}$ + 97° (*c* 0.76, chloroform), $\nu_{max}^{CHCl_3}$ 1735 (OAc) and 1680 cm^{-1} (SAc); and 3-*O*-acetyl-1,2-*O*-isopropylidene-5-thio-α-D-apio-L-furanose (**5**), characterized by oxidation with iodine to disulfide **3**. Compound **3** showed absorption at λ_{max} 250 nm, characteristic of disulfides, and gave no color reaction with alkaline sodium nitroprusside [13,14] until it had been reduced with aqueous potassium cyanide[15], or lithium aluminum hydride in ether[16]. Thiol **5** gave an immediate purple color in the cold with sodium nitroprusside.

*Paper IV of a series of publications from this laboratory concerning the chemistry of apiose.
† Satisfactory chemical analyses were obtained for all crystalline compounds reported. Melting points are uncorrected.

Similar treatment of 2 with KSAc in refluxing ethanol resulted in an almost quantitative yield of bis(5-deoxy-1,2-*O*-isopropylidene-α-D-apio-L-furanose-5-yl) 5,5'-disulfide (**6**),m.p. 128–129°, $[\alpha]_D^{26}$ + 182°(*c* 0.77, ethanol). This compound had λ_{max} 250 nm, and only gave a positive reaction with sodium nitroprusside after reduction with aqueous KCN or $LiAlH_4$ in ether.

The use of $HCONMe_2$ or acetone as the reaction solvent results in S→O acetyl migration in the 5-*S*-acetyl-1,2-*O*-isopropylidene-5-thio-α-D-apio-L-furanose (**7**) initially formed, and the resulting thiol (**5**) can form the disulfide 3 by oxidation, or the fully acetylated derivative 4 by *S*-acetylation. Indeed, treatment of 5 with excess KSAc in $HCONMe_2$ gave 4 as the major product.

When ethanol is used as the solvent, the acetylthio derivative initially formed loses its acetyl group to the solvent, and the resulting thiol is oxidized to disulfide 6 under the reaction conditions used.

Reduction of disulfide 6 with $LiAlH_4$ in ether afforded thiol 8, a small proportion of which reverted to the corresponding disulfide during isolation. It is noteworthy that thiol 8 is much more susceptible to atmospheric oxidation than is the 3-acetate (**5**), which may to some extent be stabilized by hydrogen bonding between the thiol group and the carbonyl group of the adjacent *O*-acetyl group.

Treatment of thiol 8 with 2% methanolic HCl at ~ 25° afforded a stable, crystalline compound, m.p. 52–53°, $[\alpha]_D^{26}$ - 264° (*c* 0.8, ethanol). The compound showed no thiol activity with sodium nitroprusside either before or after treatment with aqueous KCN and was shown, by analytical and n.m.r. spectral data, to be methyl 2,3-*O*-isopropylidene-4-thio-β-D-apio-D-furanoside (**9**); n.m.r. data (60 MHz, chloroform-*d*; internal standard, Me_4Si): τ 5.09 (1-proton doublet, $J_{1,2}$ ~ 0.7 Hz, H-1), τ 5.47 (1-proton doublet, H-2), τ 6.26 (2-proton singlet, methylene protons of CH_2OH), τ 6.67 (3-proton singlet, OMe), τ 7.06 (center of a 2-proton quartet, $J_{4,4'}$ 12.5 Hz, H-4, 4'), τ 7.79 (1-proton singlet, OH), τ 8.45, 8.59 (6 protons, CMe_2). After addition of trichloroacetyl isocyanate (rapid, exothermic reaction), the 1-proton singlet at τ 7.79 disappeared, and a 1-proton singlet at τ 1.52 (NH) was observed. The methylene protons of the CH_2O group now appeared as an AB quartet centered at τ 5.51 (*J* 11.5 Hz).

The formation of the title compound (**9**) from thiol 8, which involves a migration of the acetal group from the 1,2- to the 2,3-oxygen atoms of the sugar, is in the process of further study. Treatment of 1,2-*O*-isopropylidene-α-D-apio-L-furanose (**1**) with methanol and Dowex-50W (H^+) ion-exchange resin also resulted in migration of the acetal group and concomitant change of configuration at C-3, and afforded methyl 2,3-*O*-isopropylidene-α-D-apio-D-furanoside in 65% yield[17].

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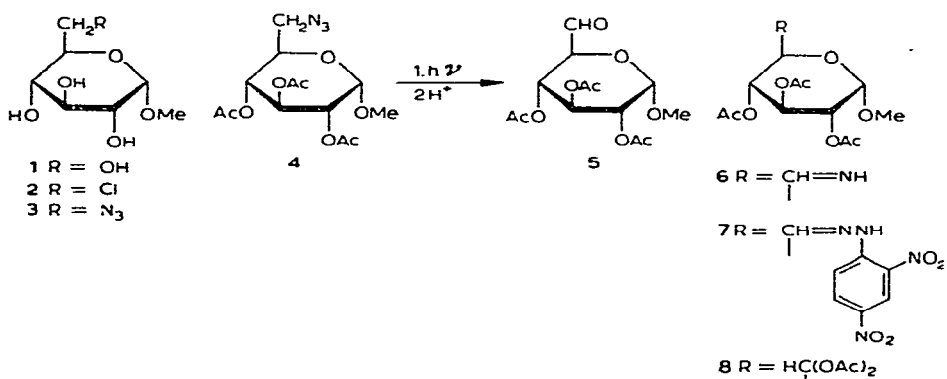
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Generation of an aldehyde group in a protected sugar by photolysis of a primary azide

Effective routes to protected *ω-aldehydo*-dialdose derivatives[1,2] are required in this laboratory for use in chain-extension reactions employing Grignard reagents[1,3]. Photochemical procedures have been found useful for effecting a number of preparative transformations in the carbohydrate field[4,5], and the fact that simple, primary, aliphatic azides have been observed[6] to give imines upon irradiation suggested that photolysis of azido sugars might provide a useful route to *aldehydo* and *keto* sugars. This Communication demonstrates that a 6-azido-6-deoxyhexose derivative, easily prepared from the parent hexose, can readily be converted into the corresponding 6-aldehyde by photolysis and mild hydrolysis.

Methyl α-D-glucopyranoside (**1**, 50 g) was treated with methanesulfonyl chloride in *N,N*-dimethylformamide, and the product was saponified to the 6-chloro-6-deoxy analog **2** according to the procedure of Evans, Long, and Parrish[7]. Compound **2** was heated for 3 h at 150° with an excess of sodium azide in *N,N*-dimethylformamide to give the 6-azido-6-deoxy derivative **3**, which was acetylated to afford the known[8] methyl 2,3,4-tri-*O*-acetyl-6-azido-6-deoxy-α-D-glucopyranoside (**4**), yield 46.4 g, m.p. 101–102°. Irradiation of **4** (6.8 g) in cyclohexane under nitrogen, with unfiltered light from a mercury arc[4] for 6 h at room temperature led to decomposition of all of the starting material (as shown by t.l.c.) and formation of an amorphous precipitate (4.8 g), presumably the imine **6**. A solution of the latter (120 mg) in methanol (10 ml) was mixed with M hydrochloric acid (1.5 ml), (2,4-dinitrophenyl)hydrazine (73 mg) was added, and the mixture was stirred for 18 h at room temperature. The resultant, partially deacetylated product was acetylated (acetic anhydride-sodium acetate) to give methyl 2,3,4-tri-*O*-acetyl-6-*aldehydo*-α-D-*gluco*-hexodialdo-1,5-pyranoside 6-[(2,4-dinitrophenyl)hydrazone] (**7**), yield 120 mg (50%, based on the azide **4**), m.p. 167–168°, n.m.r. data (chloroform-*d*): τ 2.50 (1-proton doublet, $J_{5,6}$ 6 Hz, H-6), τ 5.48 (1-proton quartet, $J_{4,5}$ 10 Hz, H-5), τ 7.89, 7.95, and 8.00 (acetyls).



The amorphous photolysis product was treated for 15 min at room temperature with 500 mM hydrochloric acid, and the solution was rendered neutral with sodium hydrogen carbonate and evaporated. Acetylation of the resultant syrup with acetic anhydride and pyridine gave, after purification by chromatography on silica gel with 3:1 dichloromethane-ether as eluant, the chromatographically homogeneous and analytically pure peracetylated aldehydrol 8, together with an unidentified, crystalline side-product, m.p. 178–182°. The 100-MHz n.m.r. spectrum of 8 in chloroform-*d* showed acetate resonances at τ 7.90 (2), 7.94, 7.96, and 8.00, the H-6 signal as a doublet at τ 3.13 ($J_{5,6}$ 2 Hz), and the H-5 signal as a quartet at τ 5.91 ($J_{4,5}$ 10 Hz).

The results show that photolysis of the azide 4, followed by mild hydrolysis, gives the aldehyde 5, which has been characterized by means of the (2,4-dinitrophenyl)-hydrazone 7 and the acetylated aldehydrol 8.

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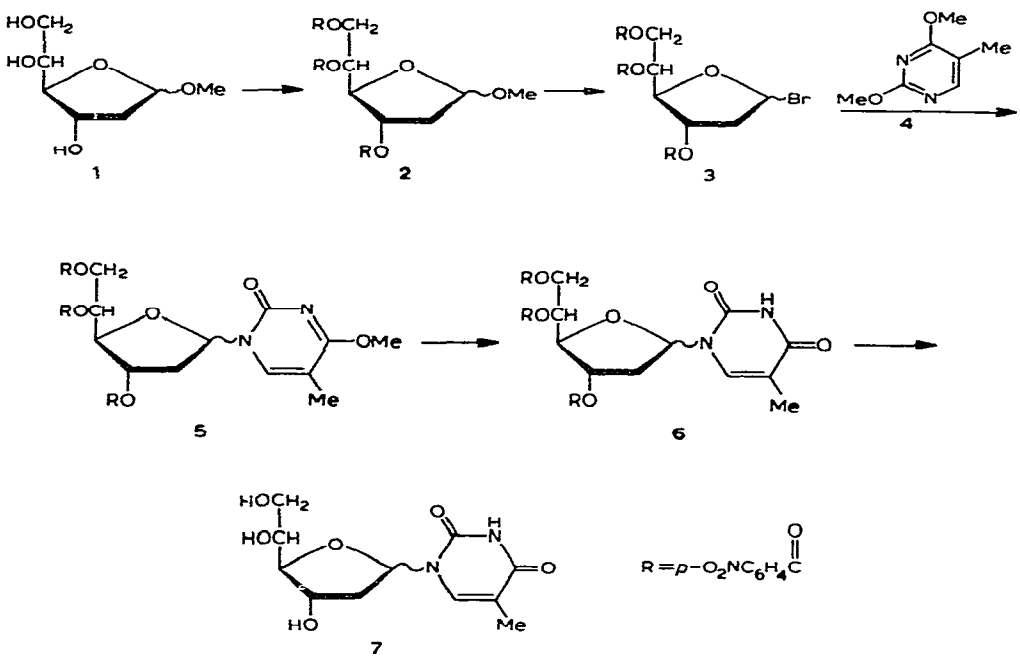
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2-Deoxy sugars
Part XIV. 1-(2-Deoxy-D-*ribo*-hexofuranosyl)thymine

We report the preparation of a crystalline *O*-acylglycofuranosyl halide (3) of the non-naturally occurring 2-deoxy-D-*ribo*-hexose[1], and the utility of 3 in the synthesis of a pyrimidine nucleoside containing 2-deoxy-D-*ribo*-hexofuranose as the carbohydrate residue.

Treatment of methyl 2-deoxy-D-*ribo*-hexofuranoside[2] (1) with *p*-nitrobenzoyl chloride resulted in the *p*-nitrobenzoylated furanoside (2) as a crystalline mixture of the anomers, m.p. 136–150°, $[\alpha]_D^{25}$ +128° (*c* 0.456, acetone), and reaction of 2 with an excess of hydrogen bromide in dichloromethane afforded 2-deoxy-3,5,6-tri-*O*-*p*-nitrobenzoyl-D-*ribo*-hexosyl bromide (3), m.p. 93–95° (dec.), $[\alpha]_D^{25}$ +120° (*c* 0.489, dichloromethane). Fusion of 3 with 2,4-dimethoxy-5-methylpyrimidine[3] (4) according to the procedure of Hilbert and Johnson[4] gave low yields (15–30%) of the protected nucleoside (5), m.p. 211–215°, $[\alpha]_D^{25}$ 0° (*c* 0.344, dichloromethane); therefore, an alternative procedure, described by Aaronoff, Babson, and Zambito[5], was employed, in which 3 and an excess of 4 were dissolved in dichloromethane, and the solvent was evaporated to give a syrupy residue that was heated *in vacuo* for 2 h at 50°, giving 5 in 60% yield. Compound 5 underwent demethylation in methanolic hydrogen chloride to yield 1-(2-deoxy-3,5,6-tri-*O*-*p*-nitrobenzoyl-D-*ribo*-hexosyl)thymine (6), m.p. 133–137°, $[\alpha]_D^{25}$ +32.8° (*c* 0.458, dichloromethane), and methoxide-catalyzed deacylation of 6 yielded 1-(2-deoxy-D-*ribo*-hexofuranosyl)thymine (7), m.p. 162–168°, $[\alpha]_D^{25}$ +9.5° (*c* 0.14, methanol).



The n.m.r. spectrum (60 MHz) of the new nucleoside (7) shows, for the anomeric protons, a septuplet centered at τ 3.78, suggestive of an anomeric mixture in which substantial proportions of both anomers are present. The β-D anomer of 7 may be considered as being 5′-*C*-(hydroxymethyl)thymidine.

Satisfactory elemental analyses have been obtained for all of the compounds reported herein.

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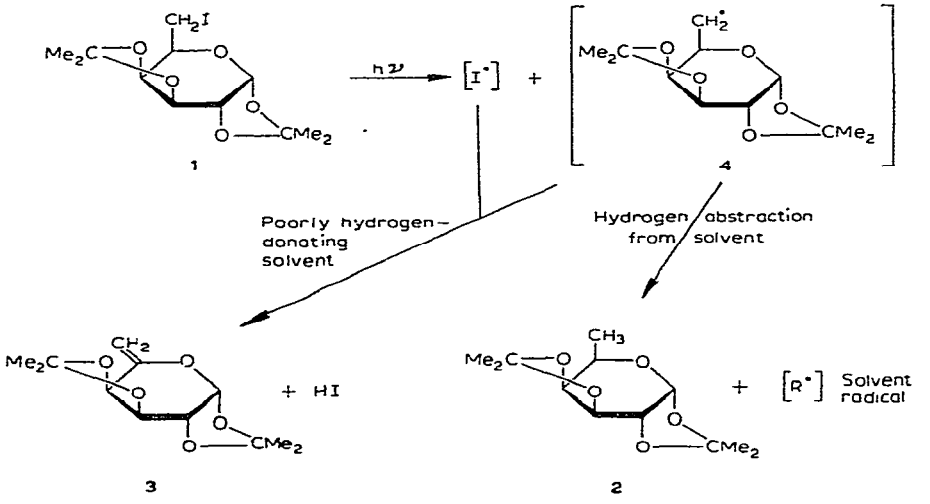
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Photochemical reactions of carbohydrates. I. The irradiation of 6-deoxy-6-iodo-1,2:3,4-di-*O*-isopropylidene-α-D-galactopyranose

Practical photochemical reactions of carbohydrates in the laboratory have been described only recently[1]. We show herein that irradiation of 6-deoxy-6-iodo-1,2:3,4-di-*O*-isopropylidene-α-D-galactopyranose (**1**) gives the corresponding 6-deoxy analog **2** in high yield, thus providing an alternative to the conventional reductive procedure[2] for this conversion.

Irradiation of **1** (1 mmole) in 40mM methanolic NaOH at 25.0° under nitrogen with a 100-watt, Hanovia SOL 608A-36 mercury arc causes complete decomposition of **1** in 2.0 h. No reaction occurs in the dark. Chromatography of the product on Florisil gave 83% of 6-deoxy-1,2:3,4-di-*O*-isopropylidene-α-D-galactopyranose[3] (**2**); b.p. 59–61°/0.2 torr, $[\alpha]_D^{20}$ -62° (a supercooled melt), n_D^{20} 1.45053, identical with authentic[4] **2** by microanalysis, t.l.c., and i.r. spectrum. Hydrolysis of **2** gave 6-deoxy-D-galactose as elongated plates, m.p. 139–142°, $[\alpha]_D^{20}$ +76° (*c* 1, water), identical with an authentic specimen. A second, unidentified photoproduct was isolated in 11% yield.



Photolysis of **1** in a 40mM solution of NaOH in 2-methyl-2-propanol (a poorly hydrogen-donating solvent) gave, after chromatography, compound **2** (32%) and a second product (in 36% yield) found to be 6-deoxy-1,2:3,4-di-*O*-isopropylidene-L-*arabino*-hex-5-enopyranose[5] (**3**), identical with authentic **3** by mixed m.p. and i.r. spectrum.

A mechanism suggested for the observed conversions of **1** in methanol and 2-methyl-2-propanol involves homolysis of a carbon-iodine bond (a well known photochemical process[6]) as the first step. In the second step, the radical species **4** abstracts a hydrogen atom from the effective hydrogen donor, methanol[7], to produce **2** and a hydroxymethyl

radical. In 2-methyl-2-propanol, where abstraction of hydrogen is more difficult[7-9], hydrogen transfer from 4 to an iodine atom, to produce 3, is able to compete effectively with the first route. This reaction scheme is tentative, and a possible contribution to product formation from chain reactions initiated by iodine or hydroxymethyl radicals exists. Further studies to explore the various mechanistic possibilities are in progress.

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Linkage analysis of heparin and related mucopolysaccharides by use of *N*-(2,4-dinitrophenyl) derivatives

"Linkage analysis", the elucidation of the nature of the intersugar linkages in a polysaccharide, is frequently achieved by graded depolymerisation of the macromolecule and the isolation and determination of the structures of oligosaccharide fragments. However, linkage analysis of mucopolysaccharides is made difficult by the resistance towards acid hydrolysis of glycuronidic linkages, which are present initially, and also, in the case of substances like heparin that contain a labile sulphoamino group, by acid-resistant 2-amino-2-deoxyglycosidic bonds formed during the early stages of hydrolysis. Thus, gross chemical modification of the polysaccharide may be necessary before suitable oligosaccharides can be obtained[1,3].

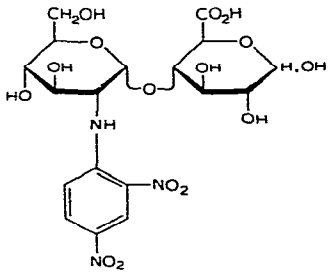
We wish to report a method of linkage analysis based upon the formation of *N*-(2,4-dinitrophenyl) (DNP) derivatives of de-*N*-acylated mucopolysaccharides. Studies on simple glycosides and disaccharides containing DNP-2-amino-2-deoxy-D-glucose residues[4] have shown that the DNP group not only facilitates the smooth and complete hydrolysis of an adjacent 2-amino-2-deoxyglycosidic linkage but is itself completely resistant to cleavage by acids. Earlier work[5,6] has demonstrated that DNP derivatives of amino sugars (which are coloured yellow with λ_{max} 358 nm) readily lend themselves to separation, analysis, and synthetic manipulations, and are usually crystalline. Thus, by appropriate variation in the order of (*a*) hydrolysis and (*b*) *N*-(2,4-dinitrophenylation) of the *N*-de-acylated polysaccharide or modified (*e.g.*, de-*O*-sulphated, carboxyl-reduced) polysaccharide, it is to be expected that a comprehensive range of oligosaccharides will be formed.

The recent publication[7] on the enzymic degradation of heparin prompts us to report on our work on the chemical fragmentation of heparin by this dinitrophenylation method.

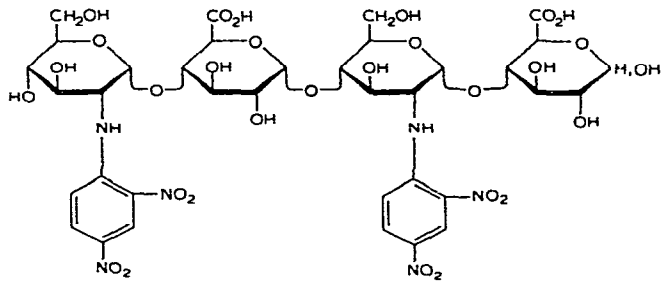
Two different, commercial specimens of heparin were used in our studies and gave similar results: they were purified initially by the method of Wolfrom *et al.*[8]. The heparin was de-*N*-sulphated with acid, under conditions designed to minimise concurrent de-*O*-sulphation, and the product, on treatment with 1-fluoro-2,4-dinitrobenzene, was converted into the DNP derivative[9]. The DNP-polysaccharide was depolymerised by treatment with N sulphuric acid for 90 min at 100°, and the products were separated by successive column analyses on Sephadex G-25 and G-10. The main coloured products consisted of DNP-2-amino-2-deoxy-D-glucose (1% of the total weight of products), a crystalline disaccharide (8%), and a crystalline mono-*O*-sulphated tetrasaccharide (26%), together with a mixture of higher oligosaccharides (46%) (which have not yet been resolved or investigated further) and an unidentified, non-reducing product of low molecular weight (1%).

Analyses, determination of the reducing power, and electrophoretic, chromatographic, optical rotational, periodate-oxidation, spectrophotometric (u.v. and i.r.), and hydrolysis studies on the disaccharide, and also on its fully *O*-methylated derivative, have indicated that it has the probable structure **1**. Similar studies have been made on the tetrasaccharide which is tentatively represented as a mono-*O*-sulpho derivative of structure **2**.

Methylation and hydrolysis of tetrasaccharide 2 gave 3,6-di-*O*- and 3,4,6-tri-*O*-methyl derivatives of 2-deoxy-2-(2,4-dinitrophenylamino)-D-glucose, the nature of which was verified by comparison with authetic specimens[9]. Although from this data, it is clear that the sulphate group is located on a uronic acid residue, its precise position has yet to be determined.



1



2

An interesting feature of the above results is that the oligosaccharides 1 and 2 had D-glucuronic acid residues at the reducing end, which, in view of our preliminary studies[4], was unexpected. Work in progress on modified heparins should clarify these results. Also, in order that the DNP method may be applied to *N*-acetylated mucopolysaccharides, investigations on improved chemical and possible enzymic de-*N*-acetylation of chondroitin sulphates and similar polysaccharides are being made.

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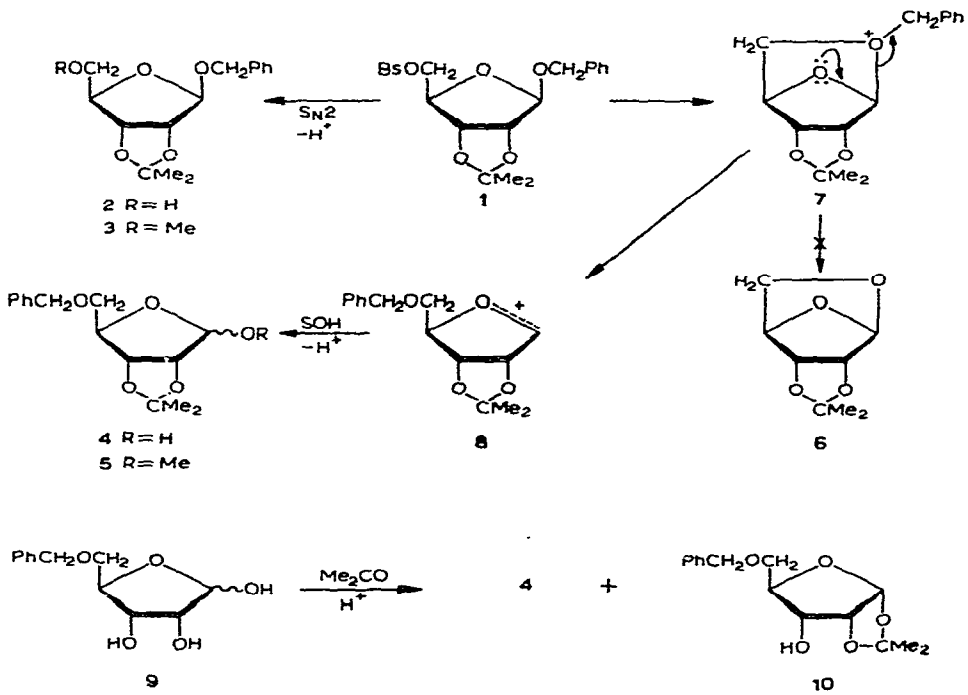
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The solvolysis of benzyl 5-*O*-*p*-bromobenzenesulphonyl-2,3-*O*-isopropylidene-β-D-ribofuranoside: a benzyloxy group migration

In previous cases[1, 3] of participation by neighbouring benzyloxy groups in solvolytic displacements of carbohydrate sulphonates, preferential cleavage of the $\dot{O}$-benzyl bond in the cyclic oxonium ion intermediates has afforded anhydro sugars as products. On solvolysis of benzyl 5-*O*-*p*-bromobenzenesulphonyl-2,3-*O*-isopropylidene-β-D-ribofuranoside (**1**) {m.p. 76–77°, $[\alpha]_D$ -45° (*c* 2, chloroform)} in boiling 50% aqueous methanol–sodium acetate, a benzyloxy group migration occurs.



The solvolysis of sulphonate **1** was essentially complete after 116 h to give a complex mixture of products which was shown (g.l.c.) not to contain any 1,5-anhydro-2,3-*O*-isopropylidene-β-D-ribofuranose[4] (**6**). Chromatography on silica gel (*n*-hexane–ethyl acetate, 3:1) separated the mixture into two inhomogeneous fractions. Fractional crystallisation and further chromatography of the second fraction afforded benzyl 2,3-*O*-isopropylidene-β-D-ribofuranoside[5] (**2**, 15%) and another crystalline compound (24%) {b.p. 110–115°(bath)/0.01 mm, m.p. 54–55° (from ether–*n*-hexane), $[\alpha]_D$ *ca.* 0° (*c* 0.6, chloroform)} that was shown by elemental analysis to be isomeric with compound **2**. Hydrogenolysis of this compound over palladised charcoal gave 2,3-*O*-isopropylidene-D-ribofuranose[6a], and acid hydro-

lysis afforded a reducing sugar that could not be distinguished on paper chromatograms (R_{Ribose} 1.82, butyl alcohol–ethanol–water, 2:1:1) from the product, presumed to be 5-*O*-benzyl-D-ribose (**9**) {$[\alpha]_D$ + 39°(*c* 1.1, chloroform)}, resulting from similar hydrolysis of benzyl 5-*O*-benzyl-2,3-*O*-isopropylidene-β-D-ribofuranoside {b.p. 156–162° (bath)/ 0.01 mm, $[\alpha]_D$ -75° (*c* 1.1, chloroform)}. On this evidence, and from the appearance of H-1 as a singlet ($J_{1,2}$ <0.5 Hz) at τ 4.70 in its n.m.r. spectrum ($CDCl_3$ containing a few drops of D_2O), the structure 5-*O*-benzyl-2,3-*O*-isopropylidene-β-D-ribofuranose (**4**, β-anomer) can be assigned to this compound.

This structure was also established by synthesis. Acetonation of 5-*O*-benzyl-D-ribose (**9**), in the presence of phosphoric acid, gave compound **4**, m.p. and mixed m.p. 54–55°, together with a small proportion of 5-*O*-benzyl-1,2-*O*-isopropylidene-α-D-ribofuranose (**10**), m.p. 80–81°, $[\alpha]_D$ + 42° (*c* 0.3, chloroform). The structure of acetal **10** was proved by its conversion into 1,2-*O*-isopropylidene-3,5-di-*O*-toluene-*p*-sulphonyl-α-D-ribofuranose*, m.p. 123–124° (lit.[6b], m.p. 122–123°), following hydrogenolysis and sulphonylation.

Distillation of the first fraction recovered from the original separation removed a number of minor components, but the distillate, b.p. 185–190° (bath)/ 0.6 mm, was shown by n.m.r. spectroscopy ($CDCl_3$) to be essentially a mixture of benzyl 2,3-*O*-isopropylidene-5-*O*-methyl-β-D-ribofuranoside[5] (**3**) and methyl 5-*O*-benzyl-2,3-*O*-isopropylidene-β-D-ribofuranoside[8] (**5**, β-anomer), although the presence of a small proportion of the α-anomer of **5** could not be discounted. Catalytic hydrogenolysis of the mixture, followed by acidic hydrolysis, afforded two reducing sugars that were indistinguishable on paper chromatograms from 5-*O*-methyl-D-ribose and D-ribose, respectively. The mixture of ribosides (18%) could not be resolved by either g.l.c. or t.l.c.

Whereas compounds **2** and **3** are probably formed by direct solvolytic displacement of the sulphonic ester group (*cf.* references 1–3), the rearranged products **4** and **5** presumably arise by way of an oxonium ion intermediate **7**, formed as a consequence of benzyloxy group participation. Opening of the ion **7** at C-1 can be ascribed to the mesomeric effect of the ring-oxygen atom and is analogous to that observed with participating methoxy[9] and methylthio[10] groups which form part of an acetal system. The resultant carboxonium ion **8** could then react with solvent to give a mixture of compounds **4** and **5**.

New compounds had satisfactory elemental analyses and spectroscopic data.

ACKNOWLEDGMENTS

We thank Professor M. Stacey, C.B.E., F.R.S., for his interest, Dr. N. A. Hughes for a sample of 1,5-anhydro-2,3-*O*-isopropylidene-β-D-ribofuranose, and the University of Birmingham for financial assistance (to O.A.C.).

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*Mills[7] has pointed out that this compound was mistakenly formulated[6b] as a 2,3-*O*-isopropylidene-1,5-di-*O*-toluene-*p*-sulphonyl-D-ribofuranose.

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Book review

Advances in Carbohydrate Chemistry, Volume 21: edited by M. L. WOLFROM and R. S. TIPSON. Academic Press Inc., New York, 1966, xi + 572 pp., 16 × 23.5 cm, cloth. $19.50.

The invaluable *Advances in Carbohydrate Chemistry* series celebrates its "coming of age" in this issue with a biographical memoir of Emil Fischer by one of his most notable students, Karl Freudenberg. This article presents the modern reader with a rare personal glimpse of the great master and his monumental contributions to carbohydrate chemistry. The clear exposition, in familiar terminology, of Fischer's classic work will undoubtedly be appreciated by many readers for whom the older nomenclature and style in Fischer's original papers imposes a real barrier to comprehension.

N. K. Kochetkov and O. S. Chizov provide the first major review of the applications of mass spectrometry to carbohydrate chemistry, in a survey that covers the literature through early 1966. Basic principles of the technique, its scope and limitations, and applications to acyclic, monocyclic, and polycyclic derivatives of monosaccharides, are discussed.

The extensive but scattered literature on the glycofuranosides is brought together for the first time in *Advances* in a useful chapter by J. W. Green. The subject of the deoxy sugars is brought up to date by S. Hanessian in a review of the natural occurrence, synthesis, and chemical and physical properties of the various mono-, di-, and tri-deoxy sugars; major emphasis is on more-recent developments, and the chapter includes tabulated mass-spectrometric and n.m.r. data.

The structures of the teichoic acids, complex polymers occurring in association with the cell wall of Gram-positive bacteria and containing glycerol phosphate or ribitol phosphate, D-alanine, and a sugar, have been unfolded over the last ten years, largely through the work of Baddiley and his colleagues. In this volume, the chemistry of these polymers forms the subject of an excellent review by A. R. Archibald and J. Baddiley.

In a chapter on synthetic cardenolides (cardiac glycosides), W. W. Zorbach and K. V. Bhat discuss general methods for coupling aldoses and 2-deoxyaldoses to steroidal aglycons, and tabulate cardenolides that have been synthesized; the contribution of the carbohydrate component to the physiological activity of these glycosides is also described. The chemical synthesis of polysaccharides by condensation and addition polymerization, and characterization of the resultant polymers, is the subject of a chapter by I. J. Goldstein and T. L. Hullar.

The two remaining chapters each focus material from widely divergent sources on a specific topic in carbohydrate chemistry. J. A. Rendleman, Jr., in a chapter on

the complexes formed between carbohydrates and alkali metals or alkaline earth metals, brings together data that will be of especial interest to the chemist concerned with problems of large-scale separation of carbohydrates, and the biologist or biochemist interested in membrane phenomena. A chapter by H. W. Hilton includes contributions from plant physiology, agricultural chemistry, botany, and agronomy, in assessing the effect of plant-growth substances on carbohydrate systems. The discussion is concerned with the effects of various growth promoters and herbicides on the biosynthesis of starch, soluble carbohydrates, and cell-wall polysaccharides.

The book is well indexed. The Subject Index, prepared by L. T. Capell, is a model of what a good index should be, and it adds greatly to the usefulness of the volume.

The *Advances in Carbohydrate Chemistry* series has, over the years, been characterized by a standard of editorial excellence that is extremely rare in any multi-authored work. The present volume is no exception, and any errors of editing or typography are quite insignificant.

The field of carbohydrate chemistry is indeed fortunate in having such a fine series of authoritative reviews as are presented in the *Advances*. This volume, together with its predecessors, should be in every chemical library; and it is an indispensable item in the personal library of anyone working in the carbohydrate field.

The Ohio State University D. HORTON

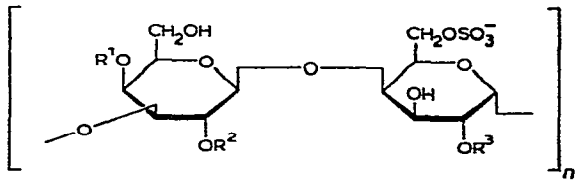
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Corrigenda

Carbohydrate Research, 7 (1968).

Page 35 line 4 up for 1720 read 720.

The formula on p. 470 should be as follows:



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REACTIONS, CONFORMATIONS, AND ACIDITY OF BIS(ETHYLSULPHONYL)-β-D-RIBOPYRANOSYLMETHANE AND RELATED DERIVATIVES

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ABSTRACT

Bis(ethylsulphonyl)-β-D-ribopyranosylmethane (9) has been prepared by oxidation of the diethyl dithioacetals of D-allose and D-altrose. The conformations of compound 9 and of its triacetate (11) have been studied by periodate oxidation, p.m.r. spectroscopy, and deuterium exchange. In connection with studies of their nucleophilic substitution reactions, pK_a values of several cyclic carbohydrate disulphones have been measured and compared in ammonium hydroxide solution. These reactions have been investigated by paper chromatography, polarimetry, and p.m.r. spectroscopy, and the dependence of their rates on inductive effects is noted. The periodate-oxidation reactions of various carbohydrate dithioacetals and disulphones have been analyzed. The periodate uptake was found to be more characteristic of the ring structure of the disulphone when the oxidation was conducted at 3°.

INTRODUCTION

Oxidations of aldohexose and aldoheptose diethyl dithioacetals with peroxyacids or hydrogen peroxide have frequently given major yields of bis(ethylsulphonyl)-glycopyranosylmethane derivatives formed by cyclization of a 1-alkene intermediate[1–6]. These cyclic disulphones are useful in the conversion of aldoses into their next lower homologues[1,2,6,7], and also in studies of mechanism[8–10] and conformation[1,3,4,6,9]. The scope and limitations of the aldose-degradation method have not been thoroughly investigated. Since the only cyclic disulphones of simple aldohexoses that have thus far been prepared are those derived from the diethyl dithioacetals of D-glucose (or D-mannose) and D-galactose[1,2], it was appropriate to investigate the structures of the oxidation products of D-allose and D-altrose diethyl dithioacetals. However, reactions of the 3-acetamido-3-deoxy analogues of these latter dithioacetals with peroxypropionic acid have been studied previously[4,5]. This paper describes the reactions, properties, and synthesis of a number of cyclic disulphones from the point

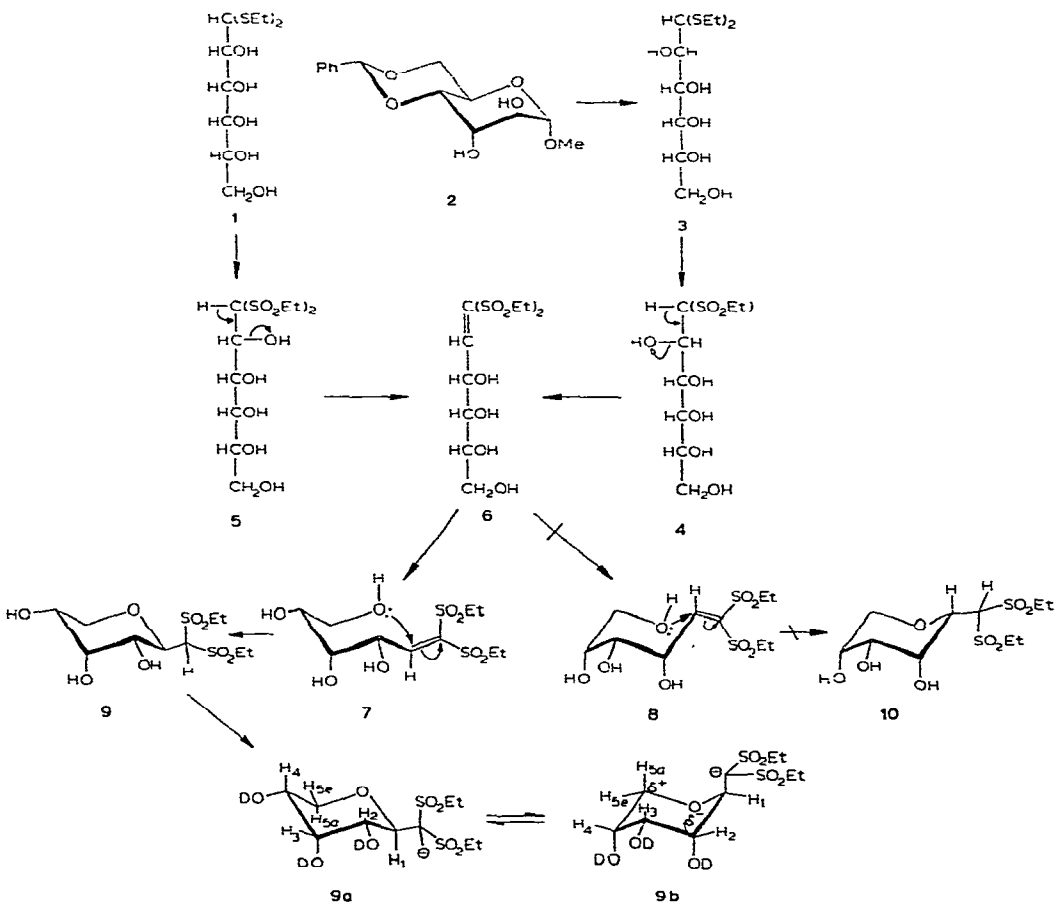
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of view of their nucleophilic substitution, the application of periodate-oxidation techniques to these and related compounds, the determination of conformation by p.m.r. spectroscopy, and characterization of the acidic properties[9,10] of the disulphones by measurements of pK_a.

DISCUSSION

D-Allose diethyl dithioacetal (**1**) was prepared by treatment of the free sugar with ethanethiol and hydrochloric acid, followed by chromatography of the resulting mixture of products. Direct treatment of methyl 4,6-*O*-benzylidene-α-D-altropyranoside (**2**) with ethanethiol and hydrochloric acid afforded a convenient synthesis of D-altrose diethyl dithioacetal (**3**). A similar preparation from methyl α-D-altropyranoside has been described previously[11].

Oxidation of compounds **1** and **3** with cold, methanolic, aqueous peroxypropionic acid yielded, in each case, the same crystalline disulphone, which must therefore be either the 1-alkene **6**, formed by elimination of a molecule of water from



1,1-bis(ethylsulphonyl)-D-*altro*-2,3,4,5,6-hexanepentol (**4**), or its D-*allo* epimer (**5**), or a cyclic disulphone (for example, **9** or **10**) resulting from cyclization of alkene **6**. As the oxidation product gave a triacetate on acetylation, but displayed no vinylic proton resonance in its n.m.r. spectrum (D_2O), the unsaturated structure **6** could be discounted. In view of previous work[1-6], the bis(ethylsulphonyl)-D-ribopyranosylmethanes **9** and **10** were considered to be the most plausible structures. The cyclic disulphones that have been formed in similar reactions are those anomers that would be predicted by traditional conformational analysis to be thermodynamically the more stable, that is, in chair conformations having an equatorial bis(ethylsulphonyl)methyl group, and the minimum number of axial interactions[1,3-6]. It has usually been tacitly assumed that, either the anomeric pyranosyl structures (*e.g.*, **9** and **10**) are in equilibrium, with the 1-alkene (*e.g.*, **6**) as an intermediate, or that cyclization of the 1-alkene is directed toward the more stable conformation because the geometry of the transition state (**7** or **8**) resembles that of the final product. When the oxidation product of compounds **1** and **3** was treated with sodium metaperiodate, either in unbuffered solution at 3°, or under buffered conditions[12] (at pH 7), two molar proportions of periodate were rapidly consumed, with the liberation of one molar proportion of formic acid (determined under unbuffered conditions). The resulting product then suffered slow over-oxidation. No discontinuity was observed after uptake of one molar proportion of periodate. These data show, firstly, that the disulphone is cyclic, but does not have a furanosyl structure; and secondly, that its pyranosyl ring cannot exist to any great extent in the *1C*(D) conformation (*e.g.*, **10**) which has the C-2, C-3, and C-4 hydroxyl groups in *ax*, *eq*, and *ax* orientations, respectively. Under the neutral conditions mentioned, Barker and Shaw[12] found that the formation of a comparatively stable terdentate complex of one molecule of periodate with one molecule of a *cis,cis*-1,2,3-triol grouping on a six-membered ring was diagnostic for an *ax-eq-ax* arrangement of the respective hydroxyl groups. Thus, 1,6-anhydro-β-D-allopyranose, which probably does not assume any other conformation, showed a discontinuous uptake of periodate at one mole per mole[12]. The disulphone was, therefore, oxidized in the alternative chair conformation **9**, in which the specific stereochemical requirements for terdentate complex formation are absent, namely, bis(ethylsulphonyl)-β-D-ribopyranosylmethane. Although a *cis,cis*-1,2,3-triol may adopt an unfavorable conformation in order to afford a terdentate complex with periodate, this evidently did not occur for **9**, probably because of the marked tendency of the bis(ethylsulphonyl)methyl group to assume the equatorial orientation.

The assignment of the β-D anomeric configuration **9** to the disulphone is supported by its molecular rotation (−4,850°), because application of the Isorotation Rule (B = −260°; obtained from the molecular rotations of methyl α- and β-D-ribopyranosides[13]) gave a negative value for A (−4,590°). The p.m.r. spectrum (D_2O, 60 MHz) of **9** was not informative in this regard.

The foregoing assignments of configuration and conformation to the disulphone **9** are entirely supported by the p.m.r. spectrum (see Fig. 1*a*) of its triacetate measured at 60 MHz. Acetyl methyl signals at τ 7.82, 7.98, and 7.99 suggested the

presence of one axial and two equatorial acetoxy substituents, respectively[14–16], and the spectral assignments shown in Fig. 1*a*. yielded first-order coupling-constants $J_{1,\beta}$ 1.4, $J_{1,2}$ 10.8, $J_{2,3}$ 2.3, $J_{3,4}$ 2.7, $J_{4,5e}$ 5.8, $J_{4,5a}$ 10.8, and $J_{5e,5a}$ 11.4 Hz. These values compare favourably with those reported previously[17] for acetamido-(2,3,4-tri-*O*-acetyl-β-D-ribopyranosyl)methane. The large values of $J_{1,2}$ and $J_{4,5a}$ show that both H-1 and H-2, and H-4 and H-5a, exist predominantly in a diaxial relationship[14]. Since a triacetate of the α-D disulphone **10** would be expected to show a small value of $J_{1,2}$ in either of its chair conformations, this result demonstrates that the bis-(ethylsulphonyl)methyl derivative has the β-D anomeric configuration, and that, in chloroform-*d*, it exists mainly in the C_1^4 conformation **11**. The small value of $J_{1,\beta}$ indicates that the favored rotamer about the C-1–C-β bond is one or both of those in which H-1 and H-β are in *gauche* orientation[6], as depicted in structure **11***.

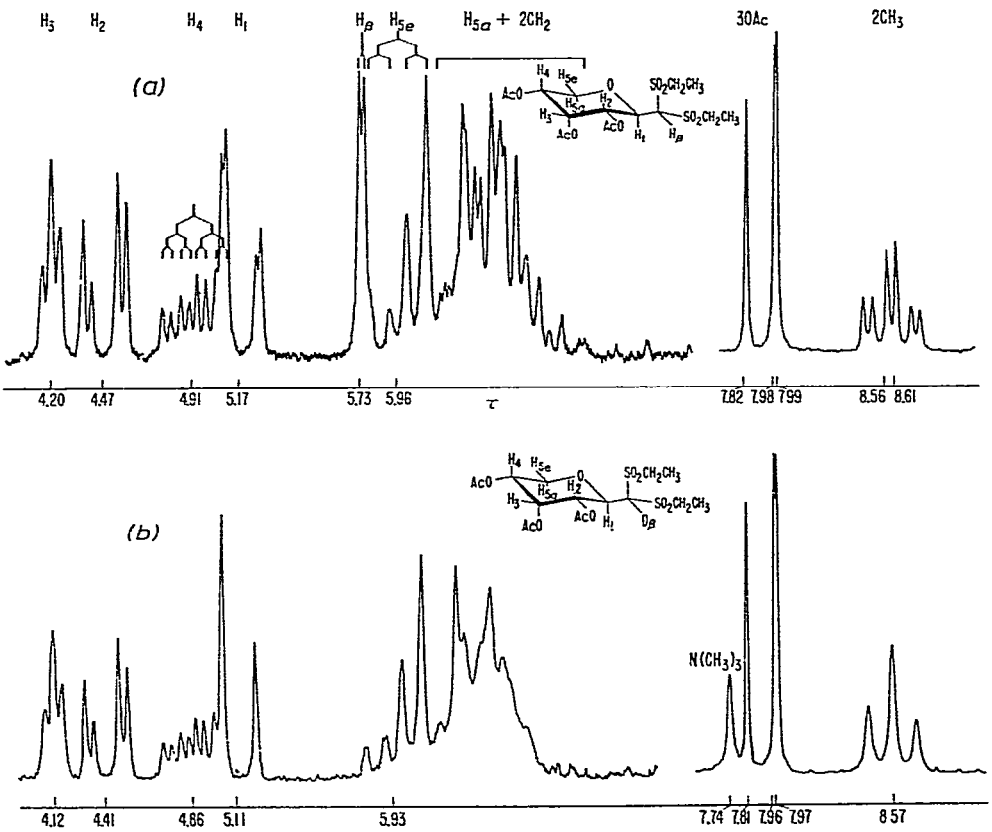
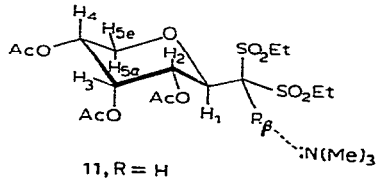


Fig. 1. P.m.r. spectra in chloroform-*d* at 60 MHz, with the methyl resonances at diminished amplitude: (*a*) bis(ethylsulphonyl)-(2,3,4-tri-*O*-acetyl-β-D-ribopyranosyl)methane (**11**), (*b*) bis(ethylsulphonyl)-(2,3,4-tri-*O*-acetyl-β-D-ribopyranosyl)methane-*d* (**12**) plus one third of a molecular equivalent of trimethylamine.

*The methane carbon atom and its hydrogen substituent are labelled as either C-α and H-α, or C-β and H-β, respectively, according to whether the glycosyl group to which this carbon atom is attached has the α- or the β-D anomeric configuration[10].

Recently, it has been observed that the favored chair conformation of many saturated[17,18], and some unsaturated[19], pentopyranosyl derivatives having an electron-rich atom bonded to C-1 often contains this atom in axial orientation, irrespective of the steric arrangement of the remaining substituents on the ring. These results have been attributed to the dominance of the anomeric effect of the 1-substituent[17–19].



11, R = H
12, R = D

In simple 2-halotetrahydropyran derivatives, the anomeric effect of the halogen substituent amounts to 2.7–3.2 kcal/mole, or more[20]. The observation that bis-(ethylsulphonyl)-(2,3,4-tri-*O*-acetyl-β-D-ribopyranosyl)methane adopts mainly the C_1^4 conformation **11** lends further support to the view[17] that the conformation assumed by ribopyranosyl derivatives is frequently determined by the presence, or absence, of an anomeric effect. Although it has previously[17] been pointed out that the electron-deficient α-carbon atom in the $-CH(SO_2Et)_2$ substituent should show a small, reverse, anomeric effect[21], the separation of the dipolar tendency from the steric tendency to assume the equatorial orientation is difficult as we lack a suitable *A*-value for this substituent.

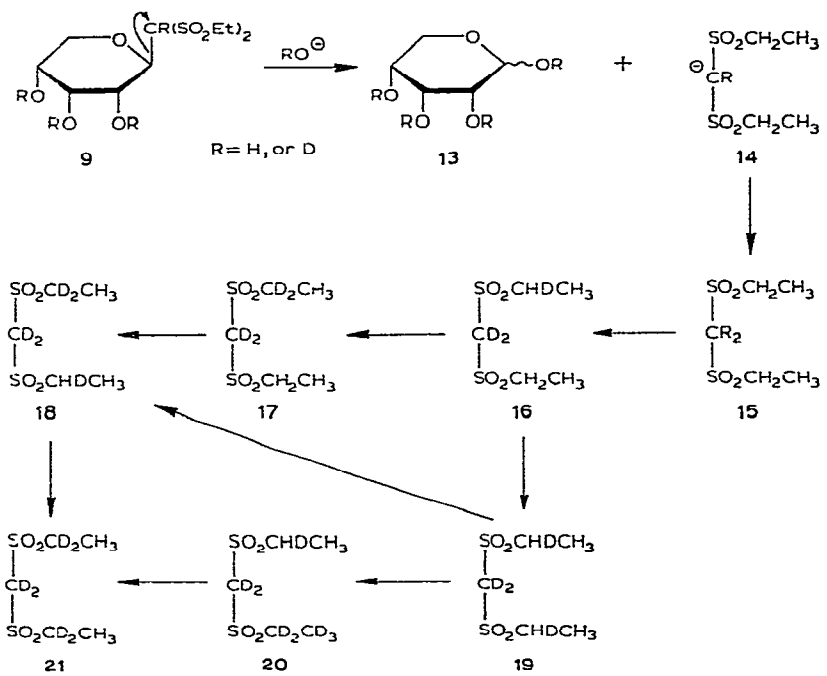
In order to confirm the assignments of the H-1 and H-β multiplets in the spectrum (see Fig. 1*a*) of the triacetate **11**, the deuterium exchange of its weakly acidic H-β atom was investigated. Exchange did not occur readily under uncatalysed conditions, although, with very vigorous mixing of the chloroform-*d* and deuterium oxide layers, H-α in the α-D-*lyxo* analogue[1] of **11** underwent exchange slowly (half-time, 1 h). In the presence of a small proportion of trimethylamine, exchange of H-β in compound **11** was rapid, as indicated by the disappearance of its doublet at τ 5.73, and the simplification of the H-1 signal to a doublet that no longer contained the small $J_{1,\beta}$ coupling (see Fig. 1*b*). When isolation of the *C*-β-deuterated disulphone **12** was attempted, its deuterium was lost at a surprisingly high rate, owing, perhaps, to retention of some trimethylamine (by salt formation with the disulphone). Whilst the spectrum (Fig. 1*a*) of **11** shows the methyl protons of the ethyl groups as non-equivalent triplets, in the spectrum (see Fig. 1*b*) of **12**, measured in chloroform-*d* with one third of a molecular equivalent of trimethylamine present, these protons are equivalent. That this difference is not due to a deuterium isotope-effect on the chemical shift of the ethyl groups was demonstrated by the fact that a sample of **12**, prepared by equilibration of a chloroform-*d* solution of **11** with aqueous sodium deuteroxide solution, gave a spectrum in which the methyl protons were still non-equivalent. This result was confirmed by the observation that treatment of the chloroform-*d* solution of **11** with trimethylamine, alone or in water, also caused the non-equivalence of the methyl groups to disappear, and, of course, produced chemical exchange of

H-β. It seems possible, therefore, that the chemical-shift effect in chloroform-*d* solution is due to some specific association between the trimethylamine nitrogen atom and H-β or D-β in **11** and **12**, respectively.

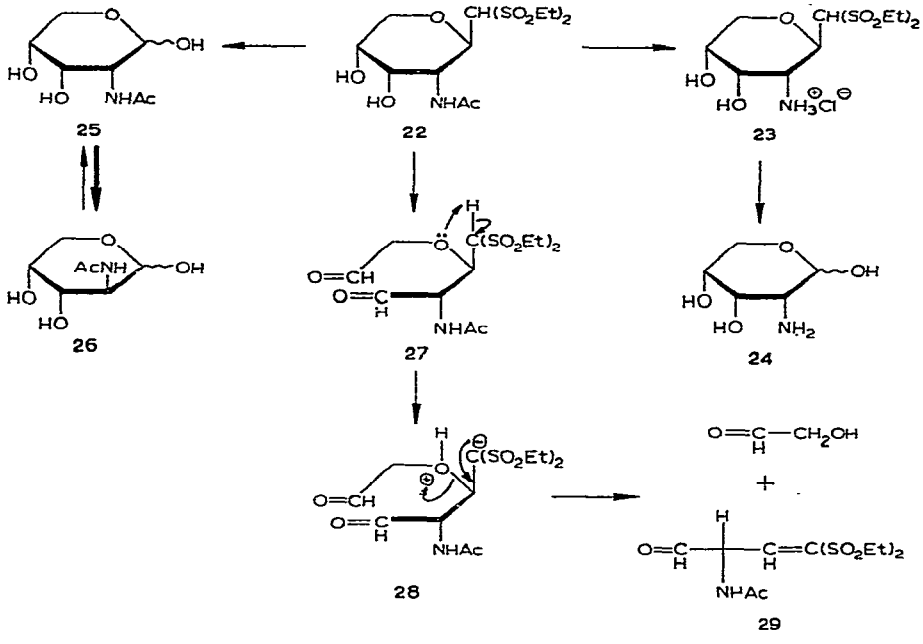
Previous investigations[8-10] have indicated that the base-catalysed cleavage of bis(ethylsulphonyl)glycopyranosylmethane derivatives to bis(ethylsulphonyl)-methane and the next lower aldose probably proceeds by nucleophilic substitution of the bis(ethylsulphonyl)methyl group by hydroxide ion, *via* an intermediate oxonium ion.

In the present work, the reaction of sulphone **9** with ammonium hydroxide was investigated by polarimetry, paper chromatography, and p.m.r. spectroscopy. In 0.73M ammonium hydroxide at 25°, cleavage of **9** to D-ribose (**13**) and bis(ethylsulphonyl)methane (**15**) was practically complete in seven days. A plot of $\log_{10}(\alpha_\infty - \alpha)$ against time yielded a straight line, and a pseudo-unimolecular rate-constant[8-10], $k_{25} = 6.1 \times 10^{-6}$ sec^{-1}. In ammonium hydroxide-d_5 at pD *ca.* 12 and 42°, cleavage of **9** was much more rapid. The complex series of changes observed in the p.m.r. spectrum of the solution is best interpreted in terms of the reaction sequence involving, initially, rapid deuterium exchange of H-β in **9**, followed by comparatively fast cleavage to **13** and bis(ethylsulphonyl)methane-d_2 (**15**, R = D). There is then a slower, but progressive, deuterium exchange of all of the methylene protons of the ethyl groups, finally giving bis(ethylsulphonyl)methane-d_6 (**21**). The latter material was characterized by means of its melting point, and i.r. and p.m.r. spectra.

As part of a comparative study, the base-catalysed cleavage reactions of the 2-amino-2-deoxy and 2-acetamido-2-deoxy analogues of sulphone **9** were investigated.



Polarimetric data from the reaction of (2-amino-2-deoxy-β-D-ribopyranosyl)bis-(ethylsulphonyl)methane hydrochloride (**23**) with 0.73M ammonium hydroxide again gave a straight-line plot of $\log_{10}(\alpha_\infty - \alpha)$ against time, and a pseudo-unimolecular rate-constant, $k_{25} = 5.3 \times 10^{-6}$ sec^{-1}, that was very similar to that of **9**. After eight days, formation of the carbohydrate product of the reaction was essentially complete; this compound was identified by paper chromatography as a 2-amino-2-deoxyribose (**24**). The kinetic data for compounds **9** and **23** are consistent with the oxonium-ion mechanism mentioned above. Their rate constants are of the same order as those measured previously for bis(ethylsulphonyl)-α-D-lyxopyranosylmethane under similar conditions[8].



The reaction of the 2-acetamido analogue **22** with 0.73M ammonium hydroxide was slower and more complex, and it was not possible to measure a simple rate-constant. Paper chromatography revealed that the initial product of cleavage of **22** was a 2-acetamido-2-deoxyribose (**25**), but that, under the alkaline conditions of the reaction, this was epimerized to a 2-acetamido-2-deoxyarabinose (**26**). The ready equilibration in dilute alkaline solution of these[22] and other[23–25] epimeric pairs of 2-acetamido-2-deoxy sugars has been ascribed to the inductive effect of the acetamido substituent[22]. It is obvious, therefore, that, if the method involves the formation of a cyclic disulphone and its subsequent alkaline degradation, the disulphone method for shortening the carbon chain of aldoses is unsuitable for the preparation of a pure sample of an aldose that contains a strongly electrophilic substituent at C-2. In all cases, the cyclic disulphones have been found to react with base more slowly than their acyclic, saturated analogues[6,7]. The reaction of **22** was particularly slow, in that

it had proceeded for 48 h before **25** was detectable, and required 15 days to approach completion. It has been noted[8–10] that these reactions are sensitive to small electronic effects, and it is reasonable to presume that the inductive effect of the acetamido substituent in **22**, being greater than that of the amino group in the free base derived from **23**, or the 2-hydroxyl group in **9**, tends to prevent recession of the electrons in the C-1–C-β bond of **22**, and hence inhibits formation of the oxonium ion and degradation of the disulphone. Although neighboring-group participation may increase[26] the unimolecular reactivity in this situation, the lessened reactivity of **22** suggests that anchimerically assisted displacement of the bis(ethylsulphonyl)methyl group by the oxygen atom of the acetamido group is not a major reaction-pathway, possibly because such a process would require these groups to become *trans*-diaxial in a conformation that is destabilized by the strong tendency of the bis(ethylsulphonyl)-methyl group to remain equatorial.

The pK_a values of five bis(ethylsulphonyl)glycopyranosylmethane derivatives were obtained by potentiometric titration, and are compared in Table I with literature values for two related sulphones. Activity corrections were applied by using the Debye–Hückel theory[27a]. The titration curves for all of the cyclic carbohydrate disulphones listed in Table I displayed a recognizable inflexion at the neutralization point, whereas the curve of bis(ethylsulphonyl)methane did not. No pK_a value for the latter compound was available, but that of its methyl homologue has been reported[28] to be 14. The carbohydrate disulphones showed pK_a values in the range of 9.4 to 10.1, and the development of appreciable acidity is due to the electron-attracting, glycopyranosyl substituent. The main influence must be the inductive effect of the ring oxygen-atom, as this electronegative atom is only three bonds removed from the ionizing hydrogen atom. It is apparent from the values in Table I that the inductive effect of the substituent at C-2 also influences the pK_a of the disulphone, although to only a minor extent. Thus, the least acidic compounds of those examined were the β-D-*ribo* disulphone **9** and its α-D-*lyxo* stereoisomer, both of which have a hydroxyl group at C-2. With a pK_a of 9.6, bis(ethylsulphonyl)-(2-*O*-methyl-α-D-arabinopyranosyl)methane is more acidic, and the value may be contrasted with those of methoxyacetic acid[29] (pK_a 3.5), and glycolic acid[29,30] (pK_a 3.8). Presumably, hydrogen

TABLE I

pK_a VALUES OF SUBSTITUTED BIS(ALKYLSULPHONYL)METHANE DERIVATIVES

Compound	pK_a
Bis(methylsulphonyl)methane[28]	14
Bis(ethylsulphonyl)-α-D-lyxopyranosylmethane[1]	10.1
Bis(ethylsulphonyl)-β-D-ribopyranosylmethane (**9**)	9.9
Bis(ethylsulphonyl)-(2-*O*-methyl-α-D-arabinopyranosyl)methane	9.6
(2-Amino-2-deoxy-β-D-ribopyranosyl)bis(ethylsulphonyl)methane hydrochloride (**23**)	9.5 5.9
(2-Acetamido-2-deoxy-β-D-ribopyranosyl)bis(ethylsulphonyl)methane (**22**)[4,5]	9.4
Tris(ethylsulphonyl)methane[31]	< 1

bonding of the hydroxyl group increases the anionoid character of the oxygen atom, and hence diminishes its effective electronegativity. The 2-acetamido-2-deoxy disulphone **22** (pK_a 9.4) is appreciably more acidic than the 2-hydroxy compound **9** (pK_a 9.9), and this substantiates the foregoing conclusions concerning the influence of the inductive effect of the acetamido substituent on the rate of nucleophilic substitution of **22**. Because the 2-amino-2-deoxy analogue of **9** was titrated as its hydrochloride salt **23**, two pK_a values were obtained. The value (9.5) due to ionization of H-β is, unexpectedly, somewhat lower than that of **9**, but it may not be fully independent of the ionization of the ammonium group[27b].

It may be noted that the carbohydrate disulphones are less acidic than tris(ethylsulphonyl)methane[31]. Many of the reactions of cyclic carbohydrate disulphones with dilute ammonium hydroxide have been conducted in solutions having a pH that is 0.5–2.1 units greater than the pK_a value of the disulphone. Under these conditions, the disulphone will be ionized to the extent[27c] of 76–99%, and the optical rotation measured will be mainly that of the anion of the disulphone. For example, in ammonium hydroxide-d_5 solution at pD 12, *ca.* 99% of **9** would be present as the anion (*e.g.*, **9a** or **9b**). Whilst the equatorial tendency of the un-ionized bis(ethylsulphonyl)methyl substituent may include a small, reverse, anomeric effect, the anionic bis(ethylsulphonyl)methyl group would, conversely, be expected to show a *normal* anomeric effect. Depending on its magnitude, this effect could well counteract the steric tendency of the group, and cause the ionized molecules to exist to some degree in that chair conformation in which the bis(ethylsulphonyl)methyl group anion is axially attached. Some evidence for such an effect was seen in the p.m.r. spectrum of the solution of **9** in ammonium hydroxide-d_5, which showed, as its signal at lowest field, a sharp doublet (J 7.4 Hz) at τ 5.17 that disappeared as the disulphone was degraded. If this doublet is assigned to H-1 in, for the most part, the anion of **9**, the fact that its spacing is appreciably less than the value $J_{1,2}$ 10.8 Hz in **11** may indicate that a proportion of the anion adopts a conformation in which H-1 and H-2 are in *gauche* orientation, for example, diequatorial in the chair conformation **9b** in which the bis(ethylsulphonyl)methyl anion is axial. It has been proposed[9] that the anions probably do not play any part in the mechanism of nucleophilic degradation of the disulphones.

As an aid to structural characterization, periodate-oxidation reactions of the disulphones **9**, **22**, and **23** were studied, together with the reactions of some of the hexose diethyl dithioacetals from which they had been prepared. Many sulphur derivatives of carbohydrates are oxidized by periodate in a non-Malapradian manner[32,33]. The consumption of 9.6 moles of periodate per mole, and the liberation of 4.7 moles of titratable acid by **3** during its oxidation with periodate for 57 h suggests that each carbon–carbon bond of the hexose chain had been cleaved, together with extensive oxidation of the ethylthio groups to sulphur acids[32]. The diethyl dithioacetals of 3-acetamido-3-deoxy-D-altrose and 3-acetamido-3-deoxy-D-allose each rapidly consumed 4 moles of periodate per mole, with the liberation of 1 mole of formic acid, followed by slow over-oxidation. Three moles of periodate per mole

are undoubtedly consumed in the cleavage of the C-1–C-2, C-4–C-5, and C-5–C-6 bonds, with the production of one mole each of formic acid, formaldehyde, and 2-acetamidomalonaldehyde. The latter compound is a common intermediate in the periodate oxidation of acetamido-deoxy sugars[34], and hydroxylation of its active hydrogen atom at C-2, combined with some oxidation of the sulphur atoms, probably accounts for the consumption of the fourth mole of periodate in these reactions[35,36]. The rate of the hydroxylation process was assessed by oxidation of the D-ribose derivative **25**, which quickly consumed 4 moles of periodate per mole, with the liberation of 2.2 moles of formic acid. Since even the *aldehydo* form of **25** requires only 2 moles of periodate per mole to afford 2-acetamidomalonaldehyde, the hydroxylation of the latter compound must occur extremely rapidly and be facilitated by the electrophilic acetamido group[34,37]. Further cleavage of the carbon chain may then take place.

Previous periodate-oxidation reactions of bis(ethylsulphonyl)glycopyranosylmethanes at room temperature have shown marked overoxidation, due to disproportionation of the dialdehyde products[1]. By performing these reactions at 3°, the over-oxidation has been lessened considerably, giving results more characteristic of pyranosyl structures. Under these conditions, bis(ethylsulphonyl)-α-D-lyxopyranosylmethane, **9**, and **23** reacted rapidly with only 2 moles of periodate per mole, with the simultaneous liberation of 1 mole of acid. Over-oxidation then proceeded slowly, until, after two days, 3.1–3.5 moles of periodate had been consumed. However, compound **22** underwent a marked and rapid over-oxidation[4], even at 3°, which must be attributed to the influence of its acetamido group. That this overoxidation does not proceed *via* hydroxylation of the acidic H-β (as for activated methylene groups[35,36]) was suggested by the observation that (2-acetamido-3,4-di-*O*-acetyl-2-deoxy-β-D-ribopyranosyl)bis(ethylsulphonyl)methane[4,5] does not react with periodate during 24 h. The H-β atom of the dialdehyde **27** from **22** would be expected[9] to migrate to the adjacent, ethereal oxygen atom, with facilitation by the electron-attracting acetamido substituent, thus accelerating the rate of disproportionation of the zwitterion **28** to glycolaldehyde and the unsaturated disulphone **29**, and their subsequent oxidation by periodate.

EXPERIMENTAL

General. — Solutions were concentrated under diminished pressure. Melting points were determined on either a Kofler hot stage, or in a Thomas–Hoover apparatus. Polarimetry was conducted at 24 ±1°, unless indicated otherwise. Decolorizations were effected in alcoholic solution, with charcoal that had been washed with water and alcohol. Paper chromatography was performed by the descending method on Whatman No. 1 paper with the solvent mixtures: (*i*) 10:3:3 (v/v) butyl alcohol–pyridine–water; (*ii*) 9:2:2 (v/v) ethyl acetate–acetic acid–water; and (*iii*) 40:11:19 (v/v) butyl alcohol–ethanol–water. Compounds were detected with the spray reagents: (*a*) 1% (w/v) ninhydrin in butyl alcohol (for amino derivatives); (*b*) 0.02M sodium

metaperiodate solution, followed after 5 min by 4% (w/v) ammoniacal silver nitrate (polyhydric alcohols)[8]; (*c*) 4% (w/v) ammoniacal silver nitrate alone (reducing sugars and dithioacetals); and (*d*) *p*-anisidine hydrochloride in butyl alcohol–ethanol–water (reducing sugars). Migration rates are given relative to those of rhamnose (R_{Rha}), or of the solvent front (R_F). T.l.c. was conducted on glass plates coated with Silica Gel G (Merck), and the developed layers were sprayed, and heated with either 5% or 50% H_2SO_4 as necessary. I.r. spectra were recorded (Nujol mulls) on either a Unicam S.P. 100, a Perkin–Elmer 137, or a Beckman IR-5A spectrometer, unless stated otherwise. Selected absorption maxima are given in cm^{-1}, and are described as strong (s), or medium–weak (m-w) intensity, with probable assignments. P.m.r. spectra were recorded on a Varian A-56/60 spectrometer at 60 MHz, with either tetramethylsilane (TMS, τ 10.00) as an internal reference, or, for deuterium oxide solutions, a capillary of 1:1 (v/v) TMS–carbon tetrachloride (τ' 10.00) as an external reference. Correct sweep-calibration was confirmed by the observation of a resonance at 436 Hz from TMS when a 2% (v/v) solution of benzene in carbon tetrachloride was inserted into the probe[38]. Multiplet splittings were measured at a sweep width of 250 Hz.

Potentiometric titrations. — A solution of each disulphone derivative (145–285 mg; dried *in vacuo* over sodium hydroxide to remove any traces of volatile acids) in water (10 ml) was titrated potentiometrically with 0.1M sodium hydroxide. An E.I.L. direct-reading pH-meter, standardized against buffer at pH 9, was used. Bisection of the linear portion of the inflexion on the plot of pH *versus* volume of base added gave the neutralization point, from which neutralization equivalents were calculated. The pK_a values of the disulphones were determined from the half-neutralization point on the titration curve by means of the equation[27d]

$$pK_a^T = \mathrm{pH} + \log \frac{[\mathrm{HA}]}{[\mathrm{A}^-]} \pm \frac{0.505\, I^{\frac{1}{2}}}{1+1.6\, I^{\frac{1}{2}}},$$

where I is the ionic strength at half-neutralization of either the bis(ethylsulphonyl)-pentopyranosylmethyl anion (positive activity term), or the pentopyranosylammonium cation **23** (negative activity term).

D-*Allose diethyl dithioacetal* (**1**). — A mixture of D-allose (0.96 g), ethanethiol (3 ml), and conc. hydrochloric acid (2 ml) was shaken vigorously for 15 min at room temperature; it was then cooled in ice–water, and a few chips of ice were added. The mixture was diluted with methanol, neutralised (basic lead carbonate), and filtered through Filter Cel. Concentration of the pale-yellow filtrate gave a syrup which was dried by addition and evaporation of ethanol. Crystals, m.p. > 320°, were removed from an ethanolic suspension of the product, which was then freed of the remaining lead by saturation of its aqueous solution with hydrogen sulphide.

Filtration and concentration of the filtrate yielded a semi-solid mass (1.43 g) which, by paper chromatography in solvent (*iii*) and by t.l.c. in 5:1 (v/v) ethyl acetate–ethanol, consisted mainly of a fast-moving major component and a slower-moving minor component. As the product could not be recrystallized, it was dissolved in ethanol (20 ml) and chromatographed on silica gel (150 g; Davison, grade 950,

60–200 mesh) suspended in petroleum (b.p. 60–80°). Elution with ethyl acetate, followed by evaporation of the eluate, then yielded chromatographically homogeneous **1** as colourless sheaves of needles (0.412 g), m.p. 94–95°, $[\alpha]_D^{26}$ −27.4° (*c* 1.84, methanol).

Anal. Calc. for $C_{10}H_{22}O_5S_2$: C, 41.93; H, 7.74; S, 22.38. Found: C, 42.11; H, 7.64; S, 22.24.

D-*Altrose diethyl dithioacetal* (**3**). — Methyl 4,6-*O*-benzylidene-α-D-altropyranoside[39] (**2**) (3 g) was shaken with ethanethiol (8 ml) and conc. hydrochloric acid (8 ml) for 7.5 h at room temperature. The mixture was then diluted with methanol (80 ml), and neutralized with lead carbonate, and the insoluble residue was filtered off and washed with hot methanol (150 ml). The filtrate and washings were combined, and evaporated to an emulsion that crystallized from methanol–ether; needles of **3** (2.23 g; 73%), m.p. 96–98°, raised to 98–100° on recrystallization from the same solvent pair; $[\alpha]_D$ +11.7° (*c* 2.42, water), R_F 0.81 [solvent (*i*), sprays (*b*) and (*c*) (white-centred spot)]; lit.[11], m.p. 103–106°, $[\alpha]_D$ +12.3°.

Bis(ethylsulphonyl)-α-D-ribopyranosylmethane (**9**). — (*a*) *From* D-*altrose diethyl dithioacetal* (**3**). A solution of **3** (1.11 g) in 1:1 (v/v) *p*-dioxane–water was cooled to 0°, an excess of aqueous peroxypropionic acid was carefully added, and the mixture was kept for 80 min at room temperature. The solution was then evaporated to a clear syrup, from which traces of peroxypropionic acid were removed by repeated dissolution in methanol and re-evaporation. The syrup (1.37 g) crystallized after several days, affording square prisms (0.95 g, 74%) of **9**, m.p. 151–158°. Recrystallized twice from methanol–ether, it had m.p. 155–158°, $[\alpha]_D^{28}$ −14.6° (*c* 0.86, methanol), R_{Rha} 1.44 [solvent (*ii*), spray (*a*)], R_F 0.73 [solvent (*i*)].

Anal. Calc. for $C_{10}H_{20}O_8S_2$: C, 36.13; H, 6.07; S, 19.29; mol. wt., 332. Found: C, 36.40; H, 6.00; S, 18.30; neutralisation equivalent, 321.

I.r. data: ν_{max} 3413s and 3300s (OH), 1314s and 1117s ($-SO_2-$). P.m.r. data in deuterium oxide; triplets ($J_{CH_3-CH_2}$ ~7 Hz) at τ' 8.40 and 8.42 (nonequivalent CH_3 groups). A series of complex multiplets in the τ' 5.2–6.5 region indicated the presence of the remaining protons.

Oxidation of **3** in methanol for 4 h at −10° also yielded **9**.

(*b*) *From* D-*allose diethyl dithioacetal* (**1**). Ice-cold peroxypropionic acid (8 ml) was added dropwise, with shaking, to a cooled solution of the dithioacetal **1** (0.412 g) in methanol (15 ml). The solution was kept for 2.25 h at room temperature, and evaporated to a colorless solid which was freed of peroxyacid by several distillations with methanol, followed by drying at 30° over sodium hydroxide pellets; yield 0.5 g, m.p. 154–160°. Paper chromatography of the product in solvent (*iii*) (spray *b*) indicated that it contained a ribose as a minor impurity, and, as this was not removed by two recrystallizations from ethanol, a solution of the product in water (35 ml) was extracted continuously with chloroform for 25 h. The extraction was monitored by paper chromatography. Evaporation of the extract gave a syrup which crystallized from ethanol–ether; square prisms of chromatographically homogeneous **9**, 0.15 g, m.p. 148–157°; on recrystallization from ethanol–ether, m.p. 155–156°, $[\alpha]_D^2$ −15.8°

(*c* 0.86, methanol). It had undepressed mixture m.p., and i.r. spectrum and R_F value identical with those of the disulphone prepared from **3**.

*Bis(ethylsulphonyl)-(2,3,4-tri-*O*-acetyl-β-*D*-ribopyranosyl)methane* (**11**). — The disulphone **9** (0.20 g) was heated with a mixture of acetic anhydride (5 ml) and conc. sulphuric acid (2 drops) for 0.5 h at 100°. The resulting, pale-brown solution was cooled, and poured into ice–solid sodium hydrogen carbonate, and the suspension was extracted with chloroform (2 × 50 ml); the extracts were combined, washed with water, dried ($MgSO_4$), decolorized, and evaporated to dryness, yielding a clear syrup (0.30 g). T.l.c. (wet ether) indicated the presence of a fast-moving impurity, and the p.m.r. spectrum in chloroform-*d* showed some small, spurious peaks in the acetyl methyl region.

A solution of the syrup in dry ether (10 ml) was chromatographed on silica gel (20 g; Mallinckrodt, 100 mesh) with ether as the eluant. Evaporation of the second fraction yielded a homogeneous syrup (0.257 g) which crystallized when its solution in chloroform was allowed to evaporate slowly at room temperature. Crystallization from ethanol gave the triacetate **11** as colorless, rectangular prisms (0.167 g), m.p. 140–141°, $[\alpha]_D^{29}$ +1.5° (*c* 2.61, chloroform).

Anal. Calc. for $C_{16}H_{26}O_{11}S_2$: C, 41.91; H, 5.72; S, 13.98. Found: C, 41.70; H, 5.75; S, 13.85.

I.r. data: ν_{max} 1757s and 1736s (ester C=O). P.m.r. data in chloroform-*d*: τ 4.20 (triplet, $J_{2,3} \approx J_{3,4} \approx 2.7$ Hz, H-3); 4.47 (quartet, $J_{2,3}$ 2.3, $J_{1,2}$ 10.8 Hz, H-2); 4.91 (octet not completely resolved, $J_{3,4}$ 2.7, $J_{4,5e}$ 5.8, $J_{4,5a}$ 10.8 Hz, H-4); 5.17 (quartet, $J_{1,\beta}$ 1.4, $J_{1,2}$ 10.8 Hz, H-1); 5.73 (doublet, $J_{1,\beta}$ 1.4 Hz, H-β); 5.96 (quartet, $J_{4,5e}$ 6.0, $J_{5e,5a}$ 11.4 Hz, H-5*e*); 6.2–6.8 (complex multiplets, H-5*a* and two ethyl CH_2); 7.82 (singlet, AcO-3, axial); 7.98 and 7.99 (singlets, AcO-2 and AcO-4, equatorial); 8.56 and 8.61 (triplets, $J_{CH_3-CH_2}$ 7.5 Hz, two ethyl CH_3).

*Deuterium exchange of the acidic hydrogen atom in bis(ethylsulphonyl)-(2,3,4-tri-*O*-acetyl-β-*D*-ribopyranosyl)methane* (**11**). — (*a*) *With trimethylamine as catalyst.* A solution of **11** (0.109 g) in chloroform-*d* (0.45 ml) was treated with deuterium oxide (0.3 ml), followed by 25% aqueous trimethylamine (0.03 ml). The mixture was shaken for 2–3 min, and then spun in the n.m.r. spectrometer to assist the separation of the layers. The aqueous phase was removed, and the organic phase was shaken with deuterium oxide (1 ml). Examination of the p.m.r. spectrum of the chloroform-*d* layer then disclosed that the H-β doublet of **11** had disappeared, and that its H-1 quartet had collapsed to a sharp doublet ($J_{1,2}$ 10.5 Hz) at τ 5.11. In addition, the methyl protons of the two ethyl groups were no longer non-equivalent, since they appeared as a single, 6-proton triplet at τ 8.57. The spectrum integral of the chloroform-*d* layer indicated that the molar ratio of disulphone to trimethylamine in this layer was 7:2. Attempted separation of the *C*-β-deuterated disulphone **12** from the solvents led only to material that had lost almost all of the deuterium, as indicated by the reappearance of the H-β doublet and the H-1 quartet in the p.m.r. spectrum. In the absence of trimethylamine, the methyl groups in the ethylsulphonyl moieties were once again non-equivalent.

Repetition of the experiment, with trimethylamine–water instead of deuterium oxide, gave a chloroform-*d* solution whose p.m.r. spectrum displayed a sharp doublet ($J_{1,2} \sim 10.2$ Hz) at τ 5.17 (H-1, decoupled by chemical exchange of H-β), and a broad singlet at 5.42 (H-β) which, while the sample was kept in the n.m.r. spectrometer for 2 h, slowly moved downfield until it lay inside the H-1 doublet. In the presence of trimethylamine and water, or alternatively, a six-fold molar excess of anhydrous trimethylamine, the methyl protons of the ethyl groups were equivalent.

(*b*) *With sodium deuteroxide as catalyst.* To a solution of **11** (0.08 g) in chloroform-*d* was added deuterium oxide (1 ml), followed by a solution (0.06 ml) of sodium hydroxide (0.158 g) in deuterium oxide (1 ml). The mixture was shaken for 3 min, the layers were allowed to separate, and a p.m.r. spectrum was recorded for the chloroform-*d* layer. The H-β signal was found to have disappeared, and the H-1 quartet had collapsed to a sharp doublet ($J_{1,2} \sim 10.2$ Hz), but the signals for the methyl groups remained as non-equivalent triplets at τ 8.55 and 8.60. Because appreciable increase of the spectrum amplitude-control was necessary, it was apparent that some of the **11** had dissolved in, or reacted with, the sodium deuteroxide solution.

When a solution of **11** in chloroform-*d* was kept in contact with deuterium oxide for 28 h with occasional shaking, no change was observed in its p.m.r. spectrum.

In a comparative experiment, a chloroform-*d* solution of the related bis(ethylsulphonyl)-(2,3,4-tri-*O*-acetyl-α-D-lyxopyranosyl)methane[1] was subjected to vigorous, continuous, mechanical shaking with deuterium oxide. After 1 h, the H-α doublet at τ 5.83 had diminished to half its original intensity, and, after 24 h, had almost disappeared, with concomitant collapse of the H-1 quartet at τ 5.28 to a sharp doublet ($J_{1,2}$ 10.4 Hz).

*Reactions of bis(ethylsulphonyl)-β-*D*-ribopyranosylmethane* (**9**) *with ammonium hydroxide.* — (*a*) *Analyzed by polarimetry and paper chromatography.* Polarimetry of a solution of the disulphone **9** (0.262 g) in 0.73M ammonium hydroxide (10.0 ml) gave the following data (length of tube, 1 dm):

Time (h)	0.08	3.5	6.25	25	28	31.5	50	56	74	82	104	126	168	192
α_D (degrees)	−0.60	−0.57	−0.55	−0.45	−0.43	−0.41	−0.34	−0.33	−0.31	−0.30	−0.26	−0.24	−0.22	−0.22

Paper chromatography after 6 h revealed that **9** (R_F 0.76) was undergoing degradation to a ribose **13** [R_F 0.36 in solvent (*ii*) with sprays (*b*) and (*d*) (red spot)]. The density of the latter spot increased rapidly, and cleavage of **9** was essentially complete in 7 days. The solution then showed $[\alpha]_D$ −18.5° (constant; calc. as pentose), in good agreement with the value of −19.0° obtained after treatment of authentic D-ribose (0.163 g) with 0.73M ammonium hydroxide (10 ml) for 7 days.

During the reaction of **9** with this hydroxide, bis(ethylsulphonyl)methane (**15**, R=H) crystallized out; after recrystallization from methanol, it had m.p. and mixed m.p. 103°.

(*b*) *Analysed by p.m.r. spectroscopy.* The disulphone **9** (0.091 g) was thrice evaporated to dryness with portions (2–3 ml) of deuterium oxide. The exchanged material was then dissolved in dilute ammonium hydroxide-d_5 (0.4 ml) (prepared by

boiling a mixture of aluminium nitride and deuterium oxide and then passing the effluent vapors into deuterium oxide until, according to narrow-range test paper, there sulting solution had pD *ca.* 12–13). The solution of **9** was kept in the probe of the n.m.r. spectrometer at 42°, and p.m.r. spectra were recorded periodically. Initially (10 min), the spectrum of the solution displayed mainly the signals of the anion of **9**; a pair of overlapping triplets ($J_{CH_3-CH_2}$ 7.4 Hz) at τ_s' 8.55 and 8.56 (non-equivalent methyl groups), two overlapping quartets ($J_{CH_3-CH_2}$ 7.4 Hz) at τ' 6.55 and 6.65 (non-equivalent ethyl group methylene protons), a complex series of multiplets between τ_s' 5.3 and 6.25, and a doublet ($J_{1,2}$ 7.4 Hz) at τ' 5.17 (H-1). As the reaction proceeded, the intensities of the triplets decreased, and the triplets were replaced by a single triplet ($J_{CH_3-CH_2}$ 7.4 Hz) at τ' 8.43 (CH_3-CH_2 in **15**). Concurrently, the quartets were gradually replaced by three lines that were part of a single quartet ($J_{CH_3-CH_2}$ and J_{CH_3-CHD} 7.4 Hz) at τ' 6.37 (CH_3-CH_2 or CH_3-CHD in **16–20**), and the intensity of the doublet decreased. The centre lines of the quartet then slowly developed some small, extra splittings. The appearance of a small peak at τ' 8.59 suggested that the methylene protons of the ethylsulphonyl groups in **9** had undergone deuterium exchange to a very minor extent, before **9** was degraded. After 43 min, a doublet (J_{CH_3-CHD} 7.4 Hz) appeared at τ' 8.42 (CH_3-CHD in **16**, and **18–20**). The intensities of the triplet at τ' 8.43 and the quartet at τ' 6.37 increased until they reached a maximum at 1.25 h, and then declined, so that, after 6 h, they were negligible. Spectrum integrals indicated that the intensity lost from the latter quartet reappeared in the HOD resonance at τ' 5.9. Maximum intensity of the doublet at τ' 8.42 was reached after 2.7 h, and it then declined slowly until, after 11 h, it had been completely replaced by a broad singlet at τ' 8.45 (CH_3-CD_2 in **17**, **18**, **20**, and **21**). After 5 h, a quartet ($J_{1,2}$ 8.7, and $J_{2,3}$ 2.8 Hz) was visible at τ' 6.54 (H-2 in β-D-ribopyranose[40]). The spectrum was unchanged after 25 h, and, at 50 h, the sample tube was removed from the probe, whereupon the compound crystallized spontaneously. The contents of the tube (pD *ca.* 11.5–12) were washed out with deuterium oxide (3 × 1 ml), and chloroform (15 ml). The layers were then shaken together, the aqueous layer was re-extracted with chloroform (5 ml), and the extracts were combined and evaporated to dryness to give a solid (0.052 g), m.p. 102°. Recrystallization from boiling deuterium oxide (1.5 ml) gave clusters of colourless plates (0.029 g) of bis(ethylsulphonyl)methane-d_6 (**21**), m.p. 102°. I.r. data in chloroform: ν_{max} 2250 (m–w) and 2170 (m–w) (C-D stretch). P.m.r. data in chloroform-*d**: intense, broad singlet at 8.55 (CH_3-CD_2), weak quartet (*J* 7.5 Hz) at τ 6.57 (residual CH_3-CH_2 and CH_3-CHD).

(2-Amino-2-deoxy-β-D-ribopyranosyl)bis(ethylsulphonyl)methane hydrochloride (**23**). — (2-Acetamido-2-deoxy-β-D-ribopyranosyl)bis(ethylsulphonyl)methane[4,5] (**22**) (0.61 g) was heated with 4M hydrochloric acid (20 ml) for 2 h at 100°. Evaporation of the solution gave a semi-crystalline, brown mass which, when decolorized, and

*A saturated solution of **15** (R = H) in chloroform-*d* showed a triplet (*J* 7.5 Hz) at τ 8.55 (CH_3-CH_2), a quartet (*J* 7.5 Hz) at τ 6.56 (CH_3-CH_2), and a singlet at τ 5.52 [$CH_2(SO_2)_2$][41]. In deuterium oxide solution, the chemical shifts of these multiplets were τ' 8.40, 6.32, and 5.24, respectively.

recrystallized from methanol–ether, yielded the hydrochloride **23** as cubes (0.43 g, 72%), m.p. 175–185° (with sintering and decomposition from 160°), $[\alpha]_D$ −9° (*c* 1.04, water), R_F 0.71 [solvent (*i*)], 0.70 [solvent (*ii*), sprays (*a*) (pink spot) and (*b*)].

Anal. Calc. for $C_{10}H_{22}ClNO_7S_2$: C, 32.64; H, 6.03; Cl, 9.64; N, 3.81; S, 17.43; mol. wt., 368. Found: C, 32.30; H, 6.00; Cl, 10.10; N, 3.80; S, 18.00; neutralisation equivalent, 368, 184.

Treatment of (2-amino-2-deoxy-β-D-ribopyranosyl)bis(ethylsulphonyl)methane hydrochloride (**23**) *with ammonium hydroxide.* — Initially, the solution of **23** (0.105 g) in 0.73M ammonium hydroxide (10.0 ml) had pH 10.4. Polarimetry (1-dm tube) gave

Time (h)	0.08	5	23	29	52	72	81	96	124	168	182	206
α_D (degrees)	−0.21	−0.20	−0.14	−0.13	−0.07	−0.04	−0.03	−0.02	−0.01	0.00	0.01	0.01

After 29 h, paper chromatography of the reaction mixture in solvent (*ii*) revealed that, in addition to the amino-disulphone (R_{Rha} 1.44), an amino sugar was present. This product [R_{Rha} 0.62, sprays (*a*) (brown spot), (*b*), and (*d*) (orange–yellow spot)] was identified as a 2-amino-2-deoxyribose (**24**) by chromatographic comparison with an authentic sample prepared by acid hydrolysis of 2-acetamido-2-deoxy-D-ribose[4,5] (**25**). Cleavage of the disulphone was essentially complete after 8 days.

Treatment of (2-acetamido-2-deoxy-β-D-ribopyranosyl)bis(ethylsulphonyl)methane (**22**) *with ammonium hydroxide.* — The disulphone[4,5] (**22**) (0.179 g) was dissolved in 0.73M ammonium hydroxide (10.0 ml). The optical rotation of the solution (1-dm tube) changed as follows:

Time (h)	0.25	13.5	21.0	49	117	153	169	190	214	289	312	382
α_D (degrees)	−1.090	−1.070	−1.055	−0.987	−0.810	−0.750	−0.688	−0.675	−0.610	−0.530	−0.495	−0.450

Pentose derivatives could *not* be detected by paper chromatography when the reaction had proceeded for 21 h; however, after 48 h, a 2-acetamido-2-deoxyribose[4,5] (**25**) [R_{Rha} 1.0, solvent (*i*), spray (*b*)] was just detectable. After 117 h, in addition to **22** (R_{Rha} 1.7) and **25**, a further reducing sugar, a 2-acetamido-2-deoxyarabinose[22] (**26**) [R_{Rha} 0.81, spray (*b*)], was detected, together with an unknown, non-reducing compound [R_{Rha} 0.48, spray (*b*)]. After 12 days, these four spots were of approximately equal intensity. Cleavage of **22** was essentially complete after 15 days, at which time a preponderance of **26** was present. An experiment in which a greater concentration of **22** (0.256 g in 10 ml of 0.73M ammonium hydroxide) was used gave similar results. After 33 h, this solution had pH 10.45.

Periodate oxidations. — The carbohydrate derivatives (15–35 mg) were oxidized in unbuffered 0.015M sodium metaperiodate (100 ml), unless stated otherwise. The consumption of periodate was determined by the neutral thiosulphate method[42], and the liberated acid by addition of ethylene glycol (2 ml) to an aliquot (5 or 10 ml) of the solution, followed by titration after 10 min with 0.01M sodium hydroxide, with 1% Methyl Red (screened with Methylene Blue) as the indicator. The formaldehyde released was determined by the chromotropic acid method[43].

At room temperature. —

D-*Altrose diethyl dithioacetal* (3)

Time (h)	*0.25*	*1*	*3*	*23.5*	*57*
Periodate consumed (moles/mole)	5.78	6.43	6.95	8.89	9.64
Acid liberated (moles/mole)	2.31	2.76	3.34	4.11	4.70

3-Acetamido-3-deoxy-D-*altrose diethyl dithioacetal*[4]

Time (h)	*0.25*	*0.75*	*1.5*	*3*	*8.25*	*21*	*53*	*117*	*243*
Periodate consumed (moles/mole)	3.05	3.36	3.43	3.62	4.09	4.30	4.62	5.01	5.76
Acid liberated (moles/mole)	0.41	0.75	1.02	1.06	1.06	1.13	1.23	1.40	1.40
Formaldehyde (moles/mole)	0.97	1.00	1.11	—	—	—	—	—	—

3-Acetamido-3-deoxy-D-*allose diethyl dithioacetal*[5]

Time (h)	*0.25*	*0.75*	*1.51*	*3.25*	*5.00*	*29*	*50*	*105*
Periodate consumed (moles/mole)	3.01	3.25	3.58	3.74	4.01	4.47	4.55	5.17
Acid liberated (moles/mole)	0.67	0.85	0.90	0.93	1.03	1.08	1.18	1.41

2-Acetamido-2-deoxy-D-*ribose*[4,5] (22)

Time (h)	*0.25*	*0.75*	*2.50*	*7.00*	*27.00*	*55*	*104*
Periodate consumed (moles/mole)	1.95	2.25	3.04	3.98	4.30	4.40	4.42
Acid liberated (moles/mole)	0.36	0.66	1.39	2.24	2.60	2.60	2.60

At 3 ±2°. —

Bis(ethylsulphonyl)-α-D-*lyxopyranosylmethane*[1]

Time (h)	*0.25*	*1.50*	*4.00*	*9.00*	*24.00*	*49.00*
Periodate consumed (moles/mole)	1.43	1.62	1.84	2.11	2.70	3.27
Acid liberated (moles/mole)	0.32	0.46	0.74	1.18	2.04	2.90

Bis(ethylsulphonyl)-β-D-*ribopyranosylmethane* (9)

Time (h)	*0.25*	*0.75*	*1.50*	*4.50*	*11.00*	*25.50*	*49.50*
Periodate consumed (moles/mole)	1.71	1.98	2.25	2.36	2.69	3.17	3.50
Acid liberated (moles/mole)	0.59	0.91	1.00	1.30	1.78	2.51	3.22

(2-Amino-2-deoxy-β-D-*ribopyranosyl)bis(ethylsulphonyl)methane hydrochloride* (23)

Time (h)	*0.25*	*0.75*	*2.00*	*5.00*	*10*	*24*	*48*
Periodate consumed (moles/mole)	1.81	1.90	1.96	2.27	2.34	2.63	3.08
Acid liberated (moles/mole)	1.26	1.43	1.51	1.65	1.79	2.04	2.24

(2-Acetamido-2-deoxy-β-D-ribopyranosyl)bis(ethylsulphonyl)methane[4,5] **(22)**

Time (h)	*0.25*	*0.75*	*1.50*	*3.25*	*6.50*	*11*	*25*	*49*
Periodate consumed (moles/mole)[a]	0.64	0.96	1.32	1.54	2.00	2.16	2.96	3.51
Acid liberated (moles/mole)	0.12	0.12	0.14	0.35	0.51	0.79	1.19	1.61

At room temperature in phosphate buffer at pH 6.9. — Bis(ethylsulphonyl-β-D-ribopyranosylmethane **(9)**

Time (h)	*0.025*	*0.06*	*0.10*	*0.17*	*0.33*	*0.67*	*1.33*	*2.33*	*4.00*
Periodate consumed (moles/mole)[b]	0.98	1.46	1.63	1.91	2.09	2.28	2.52	2.82	3.07

[a]Initial periodate concentration: 0.009M. [b]Periodate determined by arsenite method[44].

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NUCLEOPHILIC REPLACEMENT REACTIONS OF SULPHONATES

PART IV[1]. TRANSFORMATION OF 2-AMINO-2-DEOXY-D-GLUCOSE INTO DERIVATIVES OF 2-AMINO-2-DEOXY-D-GALACTOSE AND 2-AMINO-2,4,6-TRIDEOXY-D-*xylo*-HEXOSE

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ABSTRACT

Methyl 2-acetamido-3-*O*-acetyl-2-deoxy-4,6-di-*O*-methanesulphonyl-α-D-glucopyranoside (**1**) undergoes displacement of both sulphonate groups with sodium benzoate in *N*,*N*-dimethylformamide to give, after saponification, methyl 2-acetamido-2-deoxy-α-D-galactopyranoside (**5**), thereby providing a convenient synthesis of this galactosamine derivative The 6-methanesulphonate group of compound **1** is very easily replaced by a chlorine atom when treated with pyridinium chloride in pyridine, so that the 6-chloro derivative (**2**) is encountered as a by-product in the preparation of compound **1**. The disulphonate also undergoes displacement with the thiocyanate anion to give the corresponding 4,6-dithiocyanato-galactopyranoside **6**, together with the disulphide **7** derived from the dithiocyanate by hydrolysis followed by oxidative ring-closure. The desulphurisation of either of these derivatives gives, after hydrolysis, 2-amino-2,4,6-trideoxy-D-*xylo*-hexose hydrochloride (**11**).

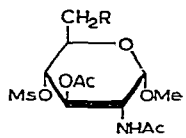
INTRODUCTION

4-*O*-Sulphonyl derivatives of α-D-glucopyranosides and α-D-galactopyranosides undergo S_N2 displacement reactions in *N*,*N*-dimethylformamide with such nucleophilic anions as benzoate, azide, thiocyanate, etc., to give, with inversion of configuration at C-4, the corresponding 4-substituted α-D-galactopyranosides and α-D-glucopyranosides, respectively[2,3]. The use of benzoate provides a convenient method of inverting the configuration of the 4-hydroxyl group, and the use of thiocyanate can be used as a route to the synthesis of 4-thio derivatives, and, by desulphurisation, 4-deoxy derivatives. Since 2-amino-2-deoxy-D-galactopyranose (galactosamine), a constituent of many biological products[4], is difficultly accessible from natural sources, a convenient method of synthesis involving inversion of configuration at C-4 of the readily available 2-amino-2-deoxy-D-glucose was considered feasible by methods analogous to those described previously[2,3].

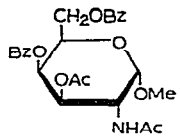
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RESULTS AND DISCUSSION

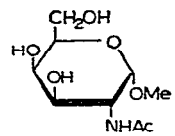
Methyl 2-acetamido-3-*O*-acetyl-2-deoxy-4,6-di-*O*-methanesulphonyl-α-D-glucopyranoside (**1**) was required as starting material for this synthesis. This compound was prepared (60%) from methyl 2-acetamido-3-*O*-acetyl-4,6-*O*-benzylidene-2-deoxy-α-D-glucopyranoside[5] by sequential hydrolysis with mild acid, to remove the benzylidene substituent, and methanesulphonylation. It was essential that the final stage be carried out at low temperature, because, at room temperature, a substantial proportion of a side product was formed. This by-product was isolated by chromatography; the n.m.r. spectrum showed that it was a monomethanesulphonate, and elemental analysis revealed it to be a monochloro derivative to which the 6-chloro structure **2** was assigned. Chlorination has been observed on several occasions during sulphonylation reactions of carbohydrates[6], generally at the primary position, although it is normally observed only when the reaction is carried out at elevated temperatures or for a prolonged time. The same chloro derivative was also obtained by treatment of the dimethanesulphonate **1** with pyridine hydrochloride in hot pyridine. The first-order coupling constants derived from the n.m.r. spectrum of **2** (Table I) were in complete accord with the *gluco* but not the *galacto* configuration, hence showing that it was the 6-chloro derivative and not the 4-chloro compound.



1 R = OMs
2 R = Cl
3 R = OBz



4



5

Treatment of the disulphonate **1** with sodium benzoate in *N*,*N*-dimethylformamide afforded a complex mixture, from which a 41% yield of the known methyl 2-acetamido-2-deoxy-α-D-galactopyranoside (**5**) could be isolated after saponification, thereby providing a convenient synthesis of this galactosamine derivative. The complexity of the original reaction mixture was probably a result of the presence of partially de-esterified products. The two major components of the reaction mixture were isolated by chromatography; one was the expected 4,6-dibenzoate **4** (31% yield), and the other was a related monobenzoate (4%). Their relationship was established by de-esterification to the same compound, namely, methyl 2-acetamido-2-deoxy-α-D-galactopyranoside (**5**). The presence of the monobenzoate was of some interest because it could have arisen by neighbouring-group participation by either the 3-acetoxy or the 6-benzoyloxy group in the displacement of the 4-sulphonate group in the initially formed methyl 2-acetamido-3-*O*-acetyl-6-*O*-benzoyl-2-deoxy-4-*O*-methanesulphonyl-α-D-glucopyranoside (**3**), followed by solvolysis of the intermediary acyloxonium ion. However, we have no evidence as to whether the monobenzoate arises in this way or whether it is formed by partial de-esterification of the 4,6-dibenzoate **4** during the course of the reaction. The same mixture of dibenzoate **4** and monobenzoate was

obtained when the 6-chloro-4-sulphonate **2** was treated similarly with sodium benzoate in *N,N*-dimethylformamide. Two related syntheses of galactosamine derivatives have been described previously, by using 4,6-disulphonates of benzyl glycosides of 2-amino-2-deoxy-D-glucose[7,8], but the sulphonate groups were replaced by acetolysis with potassium acetate in mixtures of acetic acid and acetic anhydride.

TABLE I

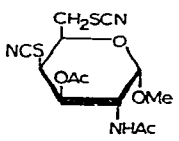
CHEMICAL SHIFTS (τ-VALUES) IN CHLOROFORM-*d* AND FIRST-ORDER COUPLING CONSTANTS

Compound	**2**	**4**	**6**	**7**	**9**[a]
H-1	5.28d[b]	5.13d	5.19d	—	5.15d
H-2	—	5.31q	—	—	—
H-3	4.73q	4.78q	4.69q	4.46q	4.71s
H-4	5.32t	4.27d	5.82q	6.00q	7.97 8.52
H-5	—	—	—	—	—
H-6	—	—	~6.7	~6.62	8.90d
OMe	6.57s	6.59s	6.57s	6.58s	6.8s
OAc	7.93s	8.13s	7.86s	7.95s	7.93s
NAc	8.07s	8.18s	8.06s	8.06s	8.03s
OMs	6.96s	—	—	—	—
$J_{1,2}$	3.5	3.0	3.5	—	3.5
$J_{2,3}$	11.0	11.0	10.5	10.0	11.0
$J_{3,4}$	9.0	3.0	3.5	5.0	3,4*a*, 11.0 3,4*e*, 5.0
$J_{4,5}$	9.0	<1	2.0	2.5	—
$J_{5,6}$	—	—	—	—	6.0

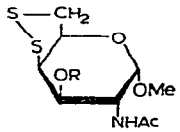
[a]In pyridine-d_5.
[b]d = doublet, s = singlet, q = quartet, t = triplet.

The dimethanesulphonate **1** also underwent replacement with the thiocyanate anion in dry *N,N*-dimethylformamide to give, *inter alia*, two products which could be readily separated by fractional crystallisation. The former was the expected galacto-pyranoside 4,6-dithiocyanate **6** (13%), and the latter was the corresponding disulphide (4%) formed by hydrolysis of compound **6** followed by oxidation. This result is analogous to those obtained with methyl 2,3-di-*O*-acetyl-4,6-di-*O*-methanesulphonyl-α-D-glucopyranoside[3] from which both the dithiocyanate and the disulphide were isolated; the respective yields depended upon the amount of water present in the reaction mixture, since water favours hydrolysis of the dithiocyanate and hence formation of the disulphide. In agreement with this, when the reaction was carried out in the presence of 5% of water, only the disulphide **7** (18%) could be isolated. The structures of the two products **6** and **7** were assigned from their n.m.r. spectra. For the dithiocyanate **6**, the resonance due to H-3 was seen to low field (τ 4.82) of all other ring protons, because of the deshielding by the acetoxy group. It occurred as a quartet with splittings of 10.5 and 3.5 Hz which indicated an *eq*, *ax*, *ax*, arrangement of H-4, H-3, and H-2, respectively, in agreement with the *galacto* configuration.

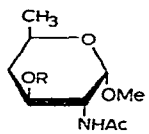
The signal for H-1 occurred as a doublet at τ 5.31 (J 3.5 Hz) and that for H-4 as a quartet at τ 5.92 (J 3.5 and 2.0 Hz). The small value of $J_{4,5}$ is a characteristic feature of galactopyranosides[9]. In the spectrum of the disulphide **7**, the resonance due to H-3 was again clearly discernible as a quartet at τ 4.39, with coupling constants of 10.0 and 5.0 Hz, and H-4 was observed at τ 6.10 as a quartet with splittings of 5.0 and 2.5 Hz. Treatment of either the dithiocyanate **6** or the 3-*O*-acetyl-disulphide **7** with sodium ethoxide afforded the de-acetylated disulphide **8**.



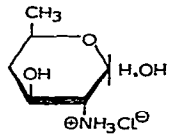
6



7 R = Ac
8 R = H



9 R=Ac
10 R=H



11

Desulphurisation of either the dithiocyanate **6** or the 3-*O*-acetyl-disulphide **7** with Raney nickel gave methyl 2-acetamido-3-*O*-acetyl-2,4,6-trideoxy-α-D-*xylo*-hexopyranoside (**9**), the structure of which was confirmed by its n.m.r. spectrum. The signal for H-3 was observed at lowest field (τ 4.71) as a well-defined sextet with $J_{2,3}$ 11.0, $J_{3,4e}$ 5.0, and $J_{3,4a}$ 11.0 Hz, and H-1 as a doublet at τ 5.15 with $J_{1,2}$ 3.5 Hz. The protons at C-4 produced a complex multiplet at about τ 8.5, and the assignment of other ring protons was not possible with any certainty. Deacetylation of the product afforded methyl 2-acetamido-2,4,6-trideoxy-α-D-*xylo*-hexopyranoside (**10**) which, upon acid hydrolysis, gave the crystalline 2-amino-2,4,6-trideoxy-D-*xylo*-hexose (4,6-dideoxy-glucosamine) hydrochloride (**11**). Although a number of aminotrideoxy-hexoses have been isolated from natural sources[10], some of which have been synthesised[11], this represents the first preparation of such a compound having the basic group at C-2.

EXPERIMENTAL

For general notes on experimental procedures, see Ref. 2.

Methyl 2-acetamido-3-O-acetyl-2-deoxy-4,6-di-O-methanesulphonyl-α-D-gluco-pyranoside (**1**). — A mixture of methyl 2-acetamido-3-*O*-acetyl-4,6-*O*-benzylidene-2-deoxy-α-D-glucopyranoside[5] (48.5 g), 50% acetic acid (500 ml), and acetone (150 ml) was heated for 25 min at 90° (reaction complete by t.l.c.) and then concentrated to a syrup which was extracted with light petroleum (to remove benzaldehyde) and then dried thoroughly *in vacuo*. A solution of the syrup in pyridine (600 ml) was cooled in an ice-bath and treated with methanesulphonyl chloride (31 ml; 3 mol. proportions) in three portions (temperature $\neq 0°$). After being kept for three days at 0°, the reaction mixture was decomposed by the addition of water and then concentrated to give a black syrup. A solution of this product in chloroform (600 ml) was washed successively with N hydrochloric acid, aqueous sodium hydrogen carbonate, and water. The organic

layer was then decolorised with charcoal, dried, and evaporated. Recrystallisation of the residue from ethanol–light petroleum gave disulphonate **1** as colourless crystals (32 g; 60%), m.p. 151–152°, $[\alpha]_D$ +70° (*c* 0.92) (Found: C, 36.2; H, 5.25; N, 3.15; S, 14.8. $C_{13}H_{23}NO_{11}S_2$ calc.: C, 36.05; H, 5.3; N, 3.25; S, 14.8%).

In an early preparation, carried out at room temperature with a large excess of the acid chloride, the yield of the diester **1** was only 22%, and t.l.c. showed a major impurity to be present. After isolation by chloroform extraction, the mixture was fractionated on a column of silica gel with chloroform–acetone (8:1). The impurity (12% yield) was identified as the 6-chloro derivative **2**, identical with the product obtained below.

*Methyl 2-acetamido-3-O-acetyl-6-chloro-2,6-dideoxy-4-O-methanesulphonyl-α-*D*-glucopyranoside* (**2**). — A solution of disulphonate **1** (5 g) in pyridine (240 ml) was heated for 48 h at 70° with pyridine hydrochloride (10 g), whereupon t.l.c. showed the reaction to be complete. The mixture was diluted with water, and the product was isolated by chloroform extraction in the usual way to give 2.63 g (58%) of compound **2** (from ethanol–light petroleum), m.p. 165–167°, $[\alpha]_D$ +80° (*c* 1.24) (Found: C, 38.6; H, 5.45; N, 3.75; S, 8.65; Cl, 9.5. $C_{12}H_{20}ClNO_8S$ calc.: C, 38.6; H, 5.35; N, 3.75; S, 8.6; Cl, 9.5%).

Treatment of disulphonate **1** *with sodium benzoate in* N,N*-dimethylformamide.* — The dimethanesulphonate **1** (3.2 g) and sodium benzoate (3 g) were heated in *N,N*-dimethylformamide (40 ml) for 24 h at 130°. The resulting mixture was then diluted with chloroform (200 ml), filtered, and concentrated. A solution of the black, syrupy residue in chloroform was washed successively with N sulphuric acid, aqueous sodium hydrogen carbonate, and water, and then decolorised with charcoal and dried (Na_2SO_4). Evaporation gave a syrup (2.8 g) which was shown by t.l.c. to be a complex mixture. The product was subjected to chromatography on silica gel with chloroform–acetone (8:1) as eluant. The first component eluted was crystallised from ethanol–light petroleum to give 0.88 g of the 4,6-dibenzoate **4**, m.p. 191–193°, $[\alpha]_D$ +100° (*c* 1.1) (Found: C, 61.6; H, 5.6; N, 2.9. $C_{25}H_{27}NO_9$ calc.: C, 61.9; H, 5.55; N, 2.85%).

The major, slower-moving component crystallised from ethanol–light petroleum to give 97 mg (3.5%) of a monobenzoate, m.p. 212–214°, $[\alpha]_D$ +86° (*c* 0.61) (Found: C, 56.65; H, 5.95; N, 3.7. $C_{18}H_{23}NO_8$ calc.: C, 56.7; H, 6.05; N, 3.65%).

A comparable result was obtained when the 6-chloro derivative **2** was treated in the same way.

*Methyl 2-acetamido-2-deoxy-α-*D*-galactopyranoside* (**5**). — (*a*) The 4,6-dimethanesulphonate **1** (1 g) and sodium benzoate (0.9 g) were heated for 18 h at 130° in *N,N*-dimethylformamide (25 ml) and then under reflux for a further hour. The black reaction mixture was cooled, diluted with chloroform (50 ml), filtered, and concentrated. A solution of the resulting black syrup in chloroform was decolorised with charcoal and dried (Na_2SO_4). After filtration, concentration gave a syrup which was dissolved in ethanol (25 ml) and treated with M ethanolic sodium ethoxide (1 ml). After 4 h, water was added, and the solution was deionised with Amberlite IR-120(H^+) resin and then extracted with ether to remove benzoic acid. Evaporation of the aqueous

layer gave a solid, recrystallisation of which from ethanol–light petroleum gave 36 mg (41%) of the galactopyranoside **5**, m.p. 218–221°, $[\alpha]_D$ +168° (*c* 1.03); lit.[12], m.p. 217–218°, $[\alpha]_D$ +170°.

(*b*) Methyl 2-acetamido-3-*O*-acetyl-4,6-di-*O*-benzoyl-2-deoxy-α-D-galactopyranoside (**4**) (0.72 g) was dissolved in ethanol (20 ml), and M ethanolic sodium ethoxide solution was added. After being kept overnight, the reaction mixture was processed as above to give the same product (**5**) (80% in two crops), m.p. 219–220°.

(*c*) The monobenzoate (0.1 g) obtained in the above replacement reaction was dissolved in ethanol (10 ml) and saponified as above to give compound **5** (47 mg; 80%), m.p. 217–220°, identical with the two previous samples.

Treatment of disulphonate **1** *with potassium thiocyanate in* N,N-*dimethylformamide.* — (*a*) A mixture of 5.65 g of disulphonate **1** and 11.3 g of potassium thiocyanate was thoroughly dried *in vacuo* and then heated for 24 h at 145° in dry *N*,*N*-dimethylformamide (45 ml), whereupon the reaction was judged to be complete by t.l.c. The mixture was then concentrated to dryness, and the product was isolated in the usual way by chloroform extraction. The resulting solid was recrystallised twice from ethanol–light petroleum to give methyl 2-acetamido-3-*O*-acetyl-2,4,6-trideoxy-4,6-dithiocyanato-α-D-galactopyranoside (**6**) as long, colourless needles (0.61 g; 13%), m.p. 161–163°, $[\alpha]_D$ +80° (*c* 0.75) (Found: C, 43.4; H, 4.8; N, 11.85; S, 17.7. $C_{13}H_{17}N_3O_5S_2$ calc.: C, 43.45; H, 4.7; N, 11.7; S, 17.8%).

Concentration of the mother liquors from the first recrystallisation gave a syrup which crystallised from ethanol to give methyl 2-acetamido-3-*O*-acetyl-2,4,6-trideoxy-4,6-epidithio-α-D-galactopyranoside (**7**) as pale-yellow crystals (4%) identical with the product obtained below.

(*b*) A mixture of 3 g of disulphonate **1** and 6 g of potassium thiocyanate was heated for 22 h at 145° in a mixture of *N*,*N*-dimethylformamide (25 ml) and water (2 ml); after 20 h, more water (2 ml) was added. The product was isolated in the usual way by chloroform extraction. Recrystallisation of the resulting product from ethanol–light petroleum gave the disulphide **7** as pale-yellow crystals (0.36 g; 18%), m.p. 211–212°, $[\alpha]_D$ +129° (*c* 0.82), λ_{max} 222 (ε 419) and 336 nm (ε 85) (Found: C, 42.8; H, 5.25; N, 4.55; S, 20.65. $C_{11}H_{17}NO_5S_2$ calc.: C, 43.0; H, 5.55; N, 4.55; S, 20.85%).

*Methyl 2-acetamido-2-deoxy-4,6-epidithio-α-*D*-galactopyranoside* (**8**). — (*a*) The 3-acetate **7** (0.1 g) was dissolved in ethanol (20 ml) and treated with M ethanolic sodium ethoxide (2 ml). After 3 h at room temperature, when t.l.c. indicated the reaction to be complete, the mixture was diluted with water, de-ionised with Amberlite IR-120(H^+) resin, and then concentrated to a yellow solid. Recrystallisation from ethanol–light petroleum gave 44 mg (60%) of disulphide **8** as yellow needles, m.p. 252–255°, $[\alpha]_D$ +188° (*c* 0.32 in ethanol), λ_{max} 220 (ε 1103) and 336 nm (ε 74) (Found: C, 40.6; H, 5.75; N, 5.5; S, 24.3. $C_9H_{15}NO_4S_2$ calc.: C, 40.75; H, 5.65; N, 5.3; S, 24.15%).

(*b*) The dithiocyanate **6** (0.12 g) was dissolved in 20 ml of M methanolic sodium methoxide and heated for 20 min under reflux. After dilution with water, the solution was de-ionised with Amberlite IR-120(H^+) resin, decolorised with charcoal, and

evaporated to dryness. The resulting yellow solid was recrystallised from ethanol to give the disulphide **8** (38 mg; 42%), identical with the product from (*a*).

*Methyl 2-acetamido-3-*O*-acetyl-2,3,6-trideoxy-α-*D*-xylo-hexopyranoside* (**9**). — (*a*) The 3-*O*-acetyl-disulphide **7** (0.67 g) was dissolved in ethanol (70 ml) and heated under gentle reflux with Raney nickel (*ca.* 10 g) for 3 h. After filtration, the nickel was washed well with ethanol, and the combined filtrates were concentrated to dryness. Recrystallisation of the residue from ethanol–light petroleum gave compound **9** as colourless crystals (0.27 g, 51%), m.p. 171–173°, $[\alpha]_D$ +85° (*c* 0.86) (Found: C, 53.8; H, 7.9; N, 5.5. $C_{11}H_{19}NO_5$ calc.: C, 53.9; H, 7.75; N, 5.70%).

(*b*) Methyl 2-acetamido-3-*O*-acetyl-2,4,6-trideoxy-4,6-dithiocyanato-α-D-galactopyranoside (**6**) (0.52 g) was desulphurised as described in (*a*). The resulting product was, however, found to be a more-complex mixture, and only 59 mg of compound **9** could be obtained.

*Methyl 2-acetamido-2,4,6-trideoxy-α-*D*-xylo-hexopyranoside* (**10**). — The 3-acetate **9** (0.26 g) was dissolved in ethanol (20 ml), and M ethanolic sodium ethoxide (1 ml) was added. After 2 h, a small amount of water was added, and concentration of the solution gave a solid mass, the bulk of which dissolved in boiling ethyl acetate. After removal of the insoluble inorganic material, the ethyl acetate extract was concentrated to a smaller bulk, whereupon compound **10** was obtained as three crops of crystals (0.36 g; 80%), m.p. 168–170° (decomp.), $[\alpha]_D$ +99° (*c* 0.89) (Found: C, 53.2; H, 8.65; N, 6.8. $C_9H_{17}NO_4$ calc.: C, 53.2; H, 8.35; N, 6.9%).

*2-Amino-2,4,6-trideoxy-*D*-xylo-hexopyranose hydrochloride* (**11**). — Compound **10** (0.26 g) was dissolved in 2N hydrochloric acid (5 ml) and heated on a boiling-water bath for 2 h, and then concentrated to a syrup which readily crystallised. The hydrochloride **11** was isolated by trituration with a 1:1 mixture of ethanol–acetone, to give 102 mg of colourless crystals. A further crop (77 mg) was obtained by decolorisation of the mother liquors, followed by concentration and trituration; total yield, 76%. Recrystallisation from methanol–acetone–light petroleum afforded an analytical sample of hydrochloride **11**, m.p. 166–168° (decomp.), $[\alpha]_D$ +98 (2 min)→ +54° (2 h, const.) (*c* 1.54 in water) (Found: C, 39.25; H, 7.85, Cl; 19.45; N, 7.65. $C_6H_{14}ClNO_3$ calc.: C, 39.25; H, 7.65; Cl, 19.35; N, 7.65%).

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METHYL 2,3,6-TRIDEOXY-2,3-EPIMINO-α-D-ALLOPYRANOSIDE

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ABSTRACT

The reduction of methyl 2-benzamido-2-deoxy-3,4,6-tri-*O*-methanesulphonyl-α-D-glucopyranoside with lithium aluminium hydride gave methyl 2,3,6-trideoxy-2,3-epimino-α-D-allopyranoside. The structure of the epimine was proved by an unequivocal synthesis from methyl 2,3-acetylepimino-4,6-*O*-benzylidene-2,3-dideoxy-α-D-allopyranoside by removal of the benzylidene substituent with *N*-bromosuccinimide, followed by reductive dehalogenation of the resulting 6-bromo derivative and the removal of the 4-*O*-benzoyl and *N*-acetyl substituents.

INTRODUCTION

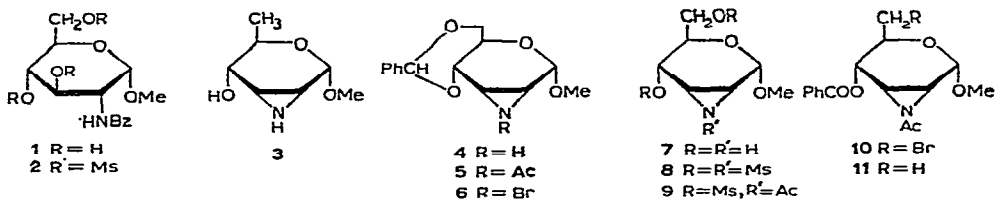
Carbohydrate epimines, unknown before 1960, are potentially useful intermediates for the synthesis of diamino sugars[1], amino-halo sugars[2,3], aminodideoxy sugars[2], unsaturated sugars[4], etc., by way of ring-opening reactions with various nucleophiles. Several methods are available for their preparation[3,5–8], one of which[7] employs the action of lithium aluminium hydride on a compound containing a benzamido substituent *trans* to a vicinal sulphonyloxy group. We[8] have previously described the preparation of methyl 3,4,6-trideoxy-3,4-epimino-α-L-galactopyranoside from methyl 3-benzamido-3,6-dideoxy-2,4-di-*O*-mesyl-α-L-glucopyranoside by this method, and we now wish to report a related synthesis of an isomeric epimine, methyl 2,3,6-trideoxy-2,3-epimino-α-D-allopyranoside.

RESULTS AND DISCUSSION

The action of lithium aluminium hydride on methyl 2-benzamido-2-deoxy-3,4,6-tri-*O*-methanesulphonyl-α-D-glucopyranoside (**2**) gave a product (20% yield) which possessed the properties expected of a 2,3-epimino-allopyranoside (**3**). In order to confirm this structure, an unequivocal synthesis of epimine **3** from the known[3,7]

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methyl 4,6-*O*-benzylidene-2,3-dideoxy-2,3-epimino-α-D-allopyranoside (**4**) was pursued. Initially, it was hoped that methyl 2,3-dideoxy-2,3-epimino-α-D-allopyranoside hydrochloride (**7**·HCl), which is readily prepared[3] from compound **4**, could be converted into the 6-deoxy analogue by standard methods. Thus, methanesulphonylation of the hydrochloride of **7** gave the corresponding tri-*O*-methanesulphonyl derivative **8**, but none of the required 6-deoxy-epimine (**3**) could be detected chromatographically when this compound was treated with lithium aluminium hydride. We have recently found[9] that, whereas the epimine ring is resistant to ring-opening by lithium aluminium hydride, the methanesulphonylepimino ring is readily opened. In an alternative approach, the epimine hydrochloride (**7**·HCl) was *N*-acetylated[10], and then methanesulphonylated. Treatment of the resulting disulphonate **9** with lithium aluminium hydride gave mainly methyl 2,3-dideoxy-2,3-epimino-α-D-allopyranoside (**7**) (identified chromatographically), and only a trace of the required 6-deoxy derivative could be detected. Selective replacement of the 6-sulphonyloxy group of compound **9** by thiocyanate, which might provide an alternative route to the 6-deoxy-epimine **3**, was also unsuccessful.



A recent method of removing 4,6-*O*-benzylidene substituents from hexopyranosides involves treatment with *N*-bromosuccinimide to give the corresponding 4-*O*-benzoyl-6-bromo-6-deoxyhexopyranosides and thence[11] the 6-deoxy derivatives. When this reaction was applied to the free epimine **4** with about 1 mole of reagent, the benzylidene ring was not ruptured; instead, *N*-bromination took place to give methyl 4,6-*O*-benzylidene-2,3-bromoepimino-2,3-dideoxy-α-D-allopyranoside (**6**) in 25% yield. This product was more conveniently formed (73% yield) when the epimine **4** was treated with sodium hypobromite. When the reaction was applied to the acetylepimine **5**, however, it followed the normal course to give the required methyl 2,3-acetylepimino-4-*O*-benzoyl-6-bromo-2,3,6-trideoxy-α-D-allopyranoside (**10**) as a syrup. Reductive dehalogenation of the bromo derivative with Raney nickel in ethanol yielded the crystalline 6-deoxy-4-benzoate **11**, which, on treatment with sodium methoxide, gave methyl 2,3,6-trideoxy-2,3-epimino-α-D-allopyranoside (**3**) identical with the product obtained from the 3,4,6-trimethanesulphonate **2**.

The structures of the 6-bromo-4-benzoate **10** and the derived 6-deoxy derivative (**11**) were proved by their 100 MHz n.m.r. spectra. Previous n.m.r. data for epiminopyranosides[8,12] have shown (*i*) that protons situated at the bridgehead positions of the three- and six-membered rings are coupled to adjacent protons on the pyranoside ring only if they are *cis* and (*ii*) that these protons are to high field of most other ring-protons (τ 6.9–7.9). The bromo derivative **10** showed (Fig. 1) a

complex 2-proton multiplet at τ 6.76 associated with the epimine ring. The low-field multiplet at τ *ca.* 5 was assigned to H-1 and H-4, and this was clearly demonstrated by irradiating at the frequency of H-2,3, when the multiplet collapsed to a broad doublet (H-4; J 9.5 Hz) and a broad singlet (H-1). The H-5 resonance was clearly observed at τ 5.9 as a septet, and the C-6 protons as the AB part of an ABX system (Fig. 1). The 6-deoxy-4-benzoate **11** had a similar spectrum, except that the C-6 protons occurred as a doublet at τ 8.78. H-2 and H-3 were observed at τ 6.82, and H-1 and H-4 as overlapping multiplets at τ *ca.* 5.16; H-5 gave an octet at τ 6.0 similar to that observed for H-5 in methyl 3,4,6-trideoxy-3,4-epimino-α-D-galacto-pyranoside[8] and its derivatives. Irradiation at the frequency of H-2,3 collapsed the multiplet at τ 5.16 into a singlet (H-1) and a doublet (H-4; J 9.0 Hz), and irradiation at the frequency of H-6 collapsed the H-5 octet into what was almost a doublet (J 9.0 Hz).

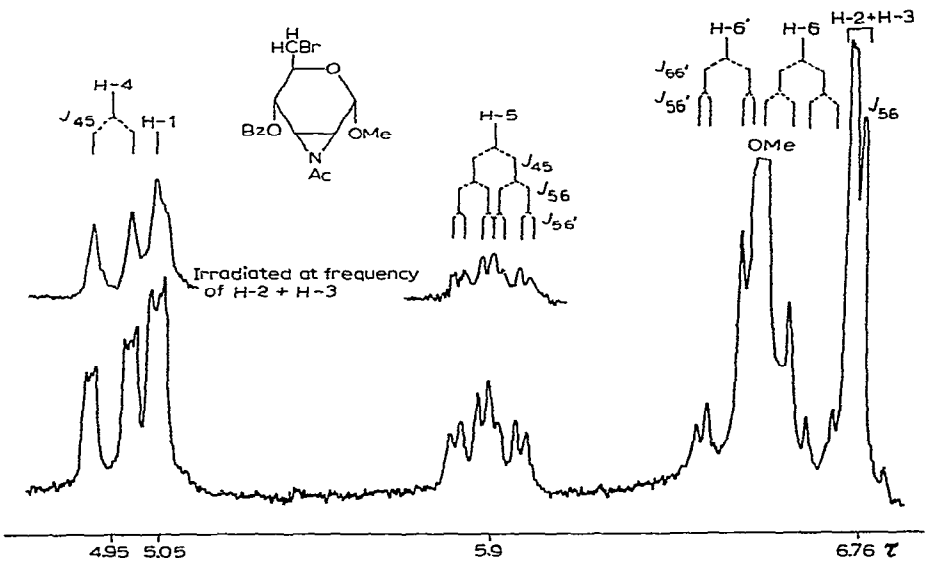


Fig. 1. The 100 MHz n.m.r. spectrum of methyl 2,3-acetylepimino-4-*O*-benzoyl-6-bromo-2,3,6-trideoxy-α-D-allopyranoside (**10**) with the results of decoupling experiments.

EXPERIMENTAL

M.p.s. were measured on a Kofler microstage. Optical rotations were determined at 20° in chloroform on a Perkin–Elmer 141 polarimeter, unless otherwise stated. P.m.r. spectra were measured on a Varian HA-100 spectrometer with tetramethylsilane as internal reference.

Methyl 2-benzamido-2-deoxy-3,4,6-tri-O-methanesulphonyl-α-D-glucopyranoside (**2**). — A solution of 17 g of methyl 2-benzamido-2-deoxy-α-D-glucopyranoside[13] (**1**) in dry pyridine (300 ml) was cooled in an ice bath, and methanesulphonyl chloride (50 g) was added slowly (5 min) with mechanical stirring. The solution was left

for 6 h at room temperature and cooled, and ice (50 g) was then added. After 1 h at room temperature, the mixture was evaporated to a thick slurry which was extracted with chloroform (5 × 100 ml). The chloroform extract was washed with water (100 ml), dried ($CaSO_4$), and evaporated. The residue crystallised from a mixture of methanol (100 ml) and ethanol (30 ml) as two crops. The trisulphonate (17.6 g, 58%) had m.p. 151° (decomp.) and $[\alpha]_D$ +85° (*c* 0.3) (Found: C, 38.4; H, 4.8; N, 2.65; S, 18.1. $C_{17}H_{25}NO_{12}S_3$ calc.: C, 38.4; H, 4.7; N, 2.65; S, 18.1%).

*Methyl 2,3,6-trideoxy-2,3-epimino-α-*D*-allopyranoside* (**3**). — To a solution of 8 g of the trimethanesulphonate **2** in dry tetrahydrofuran (350 ml) was cautiously added lithium aluminium hydride (11.2 g), and the solution was heated under reflux for 3.5 h. The reaction mixture was cooled to 0°, and a saturated solution (100 ml) of Rochelle salt was added slowly. The precipitate was filtered off, and washed with boiling chloroform, and tetrahydrofuran. The combined filtrate and washings were evaporated to a syrup which was distilled at 125°/0.2 mm. The distillate was dried *in vacuo* over phosphorus pentaoxide, and crystallised from ether–light petroleum, to yield the epimine **3** (0.48 g, 20%), m.p. 123–125°, $[\alpha]_D$ +177° (*c* 0.4) (Found: C, 52.4; H, 8.6; N, 8.1. $C_7H_{13}NO_3$ calc.: C, 52.8; H, 8.2; N, 8.8%).

*Methyl 2,3-dideoxy-4,6-di-*O*-methanesulphonyl-2,3-methanesulphonylepimino-α-*D*-allopyranoside* (**8**). — To a solution of 1.1 g of methyl 2,3-dideoxy-2,3-epimino-α-D-allopyranoside hydrochloride[3] (**7**·HCl) in dry pyridine (10 ml), was added methanesulphonyl chloride (2 ml), and the solution was left for 18 h at room temperature. Ice–water was added to precipitate the product which was collected, dried *in vacuo*, and dissolved in hot acetone, and the solution was filtered. Addition of ether, with cooling, caused crystallisation of the trisulphonate **8** (1 g, 50%), m.p. 173–174°, $[\alpha]_D$ +156° (*c* 0.5) (Found: C, 29.5; H, 4.6; N, 3.5. $C_{10}H_{19}NO_{10}S_3$ calc.: C, 29.3; H, 4.65; N, 3.5%). The infrared spectrum showed strong absorption at 1170 cm^{-1} and a broad band at 1310–1360 cm^{-1}, and no band assignable to NH stretching.

*Methyl 2,3-acetylepimino-2,3-dideoxy-4,6-di-*O*-methanesulphonyl-α-*D*-allopyranoside* (**9**). — A solution of methyl 2,3-dideoxy-2,3-epimino-α-D-allopyranoside hydrochloride[3] (1 g) in methanol–acetic anhydride (3:1, 250 ml) was shaken overnight with silver acetate (2 g). The filtered solution was evaporated to a syrup which was co-concentrated twice with toluene and twice with dry ethanol. The resulting, syrupy *N*-acetylepimine, although pure by t.l.c., could not be crystallised. To a solution of this syrup in pyridine (4 ml), was added methanesulphonyl chloride (2 ml) with cooling, and the solution was kept for 24 h at room temperature. To the resulting black solution was added ice–water, and the mixture was extracted with chloroform (3 × 10 ml). The chloroform extract was washed with aqueous sodium carbonate and water, boiled with decolourising charcoal, and evaporated. On cooling, an ethanolic solution of the residue deposited a syrup which crystallised on standing at room temperature to yield the product **9** (0.6 g, 34%), m.p. 102–105°, $[\alpha]_D$ +160° (*c* 2) (Found: C, 35.5; H, 5.0; N, 3.7. $C_{11}H_{19}NO_9S_2$ calc.: C, 35.4; H, 5.1; N, 3.8%). Attempts to recrystallise the compound always produced a syrup which crystallised on standing.

Methyl 4,6-O-benzylidene-2,3-bromoepimino-2,3-dideoxy-α-D-allopyranoside (**6**). — (*a*) To the epimine **4** (25 mg) in carbon tetrachloride (1 ml) was added *N*-bromosuccinimide (18.6 mg, 1.1 mol.) and barium carbonate (30 mg). The suspension was heated under reflux for 1 h, when t.l.c. (chloroform–ether, 1:1 v/v) indicated that reaction was complete, giving one major, fast-moving product. Recrystallisation from ethanol gave a white powder, but caused considerable decomposition to give back starting material, which remained in the mother liquors. Further recrystallisation from ethyl acetate yielded the product (8 mg, 25%), m.p. 174–175°, $[\alpha]_D$ +99° (*c* 0.6) (Found: C, 49.0; H, 4.6; Br, 23.5; N, 4.1. $C_{14}H_{16}BrNO_4$ calc.: C, 49.1; H, 4.7; Br, 23.5; N, 4.1%).

Attempts to repeat this reaction on a larger scale led to mixtures which could not be crystallised.

(*b*) A solution of sodium hypobromite was prepared by the addition of bromine (6.1 g) to a solution of sodium hydroxide (3 g) in water (10 ml). Portions of this solution were added dropwise with shaking to a solution of the epimine **4** (100 mg) in chloroform (0.5 ml) until a distinct yellow colour remained. More chloroform was added to redissolve the precipitated product, and an equal volume of water was added. The organic layer was washed twice with water and evaporated at room temperature to give a white solid, which was triturated with light petroleum and collected (crude yield, 95 mg, 73%). Recrystallisation from ethyl acetate afforded fine needles, m.p. 174–175°, identical (i.r. and mixed m.p.) with the product obtained above.

The compound was unstable in solution, an undried chloroform solution becoming discoloured after about 30 min.

Methyl 2,3-acetylepimino-4-O-benzoyl-6-bromo-2,3,6-trideoxy-α-D-allopyranoside (**10**). — Methyl 2,3-acetylepimino-4,6-*O*-benzylidene-2,3-dideoxy-α-D-allopyranoside (**5**) (2.5 g), *N*-bromosuccinimide (3 g), and barium carbonate (5 g) were suspended in carbon tetrachloride (100 ml), and the mixture was heated under reflux in the presence of broken glass for 15 min. The solution, which contained bromine, was filtered and evaporated to a syrup. T.l.c. indicated that reaction was complete, yielding a faster moving product together with considerable proportions of slow-moving impurities. Separation on a column of silica gel, with chloroform–ether (1:1, v/v) as eluent, yielded the 6-bromo derivative as a syrup which was dried *in vacuo* over silica gel and paraffin wax. Crystallisation from ethanol–light petroleum, with seeding, afforded granular crystals, m.p. 88–89°, $[\alpha]_D$ +144° (*c* 1) (Found: C, 50.2; H, 4.8; Br, 20.7; N, 3.6. $C_{16}H_{18}BrNO_5$ calc.: C, 50.0; H, 4.7; Br, 20.8; N, 3.6%).

The use of equimolar proportions of the acetylepimine and *N*-bromosuccinimide led to incomplete reaction, and, owing to the similarity in mobilities of the product and starting material, chromatographic purification of the product was not practicable in this case.

Methyl 2,3-acetylepimino-4-O-benzoyl-2,3,6-trideoxy-α-D-allopyranoside (**11**). — The 6-bromo-4-benzoate **10** (600 mg) was dissolved in ethanol (10 ml), and silver

carbonate (300 mg) and Raney nickel (2 spatula loads) were added. The mixture was heated under reflux for about 6 h, and then filtered and evaporated to a syrup. The product and starting material could not be distinguished chromatographically. After purification by column chromatography with chloroform–ether (1:1, v/v), the resulting syrup crystallised spontaneously. Recrystallisation from ethanol–light petroleum yielded the product (200 mg, 40%), m.p. 107–108°, $[\alpha]_D$ +182° (*c*, 0.6) (Found: C, 62.6; H, 6.3; N, 4.6. $C_{16}H_{19}NO_5$ calc.: C, 63.0; H, 6.2; N, 4.6%).

*Methyl 2,3,6-trideoxy-2,3-epimino-α-*D*-allopyranoside* (**3**) *from* **11**. — To a solution of compound **11** (84 mg) in methanol (2 ml), was added a solution of sodium (10 mg) in methanol (2 ml), and the mixture was heated under reflux for 5 min, when chromatography (chloroform–methanol, 4:1 v/v) indicated the presence of a product that was co-incident with the product obtained previously by lithium aluminium hydride reduction of compound **2**.

The solution was evaporated to half its original volume, and an equal amount of chloroform was added. The mixture was passed through a short column of silica gel, by using chloroform–methanol (1:1), to remove excess of sodium methoxide. After evaporation of the solvent, the residue was crystallised from ether, yielding a sample (15 mg, 35%) identical (i.r. and mixed m.p.) with that obtained from the reaction of lithium aluminium hydride with the trimethanesulphonate **2**.

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CHEMICAL MODIFICATION OF TREHALOSE

PART I. SELECTIVE SULPHONYLATION OF TREHALOSE, AND DETERMINATION OF THE CONFORMATION OF HEXA-*O*-ACETYL-6,6′-DIDEOXYTREHALOSE

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ABSTRACT

Selective esterification of α,α-trehalose with sulphonyl chlorides has been investigated. Selective dimethanesulphonylation of the disaccharide gave a mixture of the 6-ester and the 6,6′-diester, but in poor yield. Selective toluene-*p*-sulphonylation of trehalose gave the 6,6′-diester, readily isolable in 39% yield as the hexa-acetate, which is a useful starting material for the preparation of 6,6′-disubstituted derivatives of α,α-trehalose. The p.m.r. spectrum of hexa-*O*-acetyl-6,6′-dideoxy-α,α-trehalose, which may be prepared from the ditoluene-*p*-sulphonate, can be interpreted by first-order analysis and confirms that the D-glucopyranose units are physically equivalent and that they adopt the expected C_1^4 conformation.

INTRODUCTION

As a result of its simple, two-fold axis of symmetry, α,α-trehalose (α-D-glucopyranosyl α-D-glucopyranoside) (1) is unique inasmuch as the two D-glucopyranose units should be indistinguishable by chemical and physical means. Therefore, unlike most disaccharides, the reactions of trehalose should be comparatively simple, resembling those of methyl α-D-glucopyranoside. Hence, all the chemical transformations described starting with this glycoside should be applicable to trehalose, and we are embarking upon a programme of research aimed at the chemical modification of this disaccharide along these lines. As a necessary prelude to this study, we have investigated the selective sulphonylation of trehalose, since the resulting sulphonic esters should be useful intermediates in the synthesis of other trehalose analogues.

RESULTS AND DISCUSSION

Helferich and von Stryk[1] have described the selective methanesulphonylation of anhydrous trehalose to give the 6-ester (isolated as the hepta-acetate in 70% yield). However, we have been unable to repeat this reaction because of the insolubility of anhydrous trehalose under the reaction conditions[2]. With the dihydrate, which is

more soluble in pyridine, some selectivity was observed, but the yields were low. Thus, treatment of the dihydrate with 1.5 equivalents of methanesulphonyl chloride at $-10°$ afforded, in addition to much unchanged trehalose, a mixture of two products, from which could be isolated α,α-trehalose 6-methanesulphonate (*ca.* 3%) and α,α-trehalose 6,6′-dimethanesulphonate (**2**) (8%) after chromatography. The latter compound was characterised as the hexa-acetate (**3**) which was identical with the product obtained by methanesulphonylation of 2,3,4,2′,3′,4′-hexa-*O*-acetyl-α,α-trehalose[3] (**4**). Modification of the reaction conditions, such as increasing the amount of acid chloride, did not improve the yields, so it was envisaged that the use of a more bulky sulphonyl chloride might favour greater selectivity. In agreement with this, treatment of the dihydrate with 4.7 equivalents of toluene-*p*-sulphonyl chloride, followed by acetylation, afforded the highly crystalline hexa-*O*-acetyl-6,6′-di-*O*-toluene-*p*-sulphonyl derivative (**6**) which was readily isolated in 39% yield. The disulphonate **6** has been prepared previously[3], in low overall yield, by a tedious route. The selective ditoluene-*p*-sulphonylation of trehalose has been described previously, but the product was not isolated as the hexa-acetate, and only a poor yield was obtained after chromatography[4].

The disulphonate **6** is a useful precursor of 6,6′-substituted derivatives. It underwent replacement with azide in hexamethylphosphoric triamide to give the crystalline 6,6′-diazide (**7**), which was deacetylated to give 6,6′-diazido-α,α-trehalose (**8**). The disulphonate also undergoes replacement with iodide[5] to give the 6,6′-di-iodo derivative (**9**), which can be reductively dehalogenated to the 6,6′-dideoxytrehalose hexa-acetate[5] (**10**).

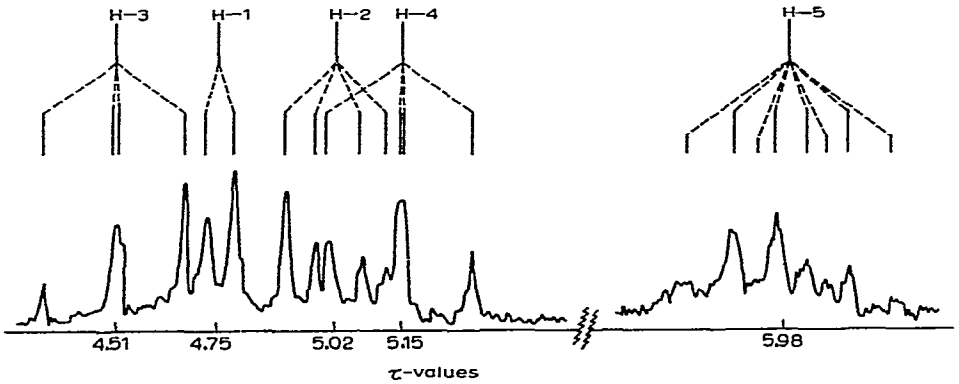
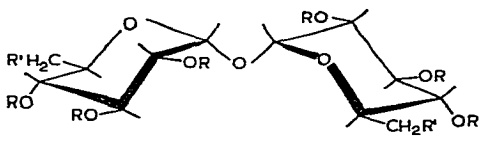


Fig. 1. The low-field portion of the n.m.r. spectrum of 6,6′-dideoxy-α,α-trehalose hexa-acetate (**10**) showing the resonances due to single ring protons.

The n.m.r. spectrum of the dideoxy derivative **10** showed that the two D-gluco-pyranose rings are equivalent and that they adopt the C_1^4 conformation (Fig. 1). The resonance due to the C-5 methyl groups was observed as a doublet at τ 8.85, and the H-5 protons gave rise to an octet at τ 5.98, similar to that given by other 6-deoxy-hexopyranosides[6]. This assignment was confirmed by irradiation of the resonance at τ 8.85, which caused collapse of the H-5,5′ resonance to a doublet. The resonances

due to the other ring protons were overlapped, but could be elucidated with the help of a further decoupling experiment. Irradiation at the frequency of H-5,5′ caused a triplet at τ 5.15 (J 10 Hz) to collapse, showing it to be due to H-4. The lowest-field triplet at τ 4.51 (J 10 and 9.5 Hz) was therefore assigned to H-3,3′, and the H-1,1′ doublet was assigned at τ 4.75 (J 4 Hz). Of the remaining peaks unassigned, only a quartet remained at τ 5.02 which must therefore be due to H-2. The derived first-order coupling constants [$J_{1,2}$ 4.0, $J_{2,3}$ 9.5, $J_{3,4}$ 10.0, $J_{4,5}$ 10.0, and $J_{5,6}$ 6.0 Hz] provide unequivocal support for the anticipated C_1^4 conformation of the two D-gluco-pyranose rings.



1 R=H, R'=OH
2 R=H, R'=OMs
3 R=Ac, R'=OMs
4 R=Ac, R'=OH
5 R=H, R'=OTs
6 R=Ac, R'=OTs
7 R=Ac, R'=N_3
8 R=H, R'=N_3
9 R=Ac, R'=I
10 R=Ac, R'=H

EXPERIMENTAL

Thin-layer chromatography was performed on plates coated with Kieselgel H, and n.m.r. spectra were determined on a Perkin–Elmer R-10 spectrometer operating at 60 MHz. "Analar" pyridine was used throughout.

The 6-methanesulphonate and the 6,6′-dimethanesulphonate (**2**) *of α,α-trehalose.* — A solution of α,α-trehalose dihydrate (20 g) in pyridine (220 ml) was cooled to −10°, treated with cold (−10°) methanesulphonyl chloride (6.0 ml, 1.5 mol. proportions), and stored for 4 days at −10°. T.l.c. showed the presence of much unreacted trehalose, together with two other components. The mixture was then evaporated to dryness to give a syrup which was taken up in a little water and applied to a charcoal–Celite (1:1) column (3 × 54 cm). The column was first eluted with water, and the eluate (25-ml fractions) was monitored by t.l.c. and by optical activity. After the elution of 40 fractions with water, the solvent was changed to 50% methanol. Fractions 14–16 contained trehalose, and fractions 31–68 contained α,α-trehalose 6-methanesulphonate, which was isolated as a syrup (0.59 g, 2.6%), $[\alpha]_D$ +159° (*c* 0.6, water); lit.[1] $[\alpha]_D$ +139° (Found: C, 37.05; H, 6.15; S, 7.15. $C_{13}H_{24}O_{13}S$ calc.: C, 37.15; H, 5.75; S, 7.6%).

Fractions 100–160 contained 6,6′-di-*O*-methanesulphonyl-α,α-trehalose (**2**) which was also obtained as a syrup (2.1 g, 8%), $[\alpha]_D$ +144° (*c* 0.25, water) (Found: C, 33.45; H, 5.45; S, 13.3. $C_{14}H_{26}O_{15}S_2$ calc.: C, 33.75; H, 5.25; S, 12.85%).

The intermediate fractions contained mixtures of both components from which more of the pure components could be obtained by further chromatography.

*Hexa-*O*-acetyl-6,6′-di-*O*-mesyl-α,α-trehalose* (**3**). — (*a*). A solution of the above dimethanesulphonate (**2**) in pyridine was treated with acetic anhydride (6 ml)

and kept overnight at room temperature. It was then poured into ice–water (100 ml), and the resulting precipitate was filtered off, and recrystallised from ethanol to give compound **3** (0.61 g, 69%), m.p. 168–169.5°, $[\alpha]_D$ +143° (*c* 1.6, chloroform) (Found: C, 41.5; H, 5.1; S, 8.5. $C_{26}H_{38}O_{21}S_2$ calc.: C, 41.6; H, 5.1; S, 8.5%).

(*b*) The hexa-*O*-acetyltrehalose[3] (**4**) (2 g) was dissolved in pyridine (12 ml) and treated with mesyl chloride (4 ml) with cooling. The mixture was kept for 70 min at room temperature and then poured into ice–water, and the resulting precipitate was collected and recrystallised from ethanol to give compound **3** (1.69 g, 67%) which was identical with the product obtained in (*a*).

Hexa-O-*acetyl-6,6'-di*-O-*toluene*-p-*sulphonyl-α,α-trehalose* (**6**). — Trehalose dihydrate (30 g) was dissolved in pyridine (400 ml), and toluene-*p*-sulphonyl chloride (70 g) was added portionwise to the solution with vigorous, continuous shaking; the temperature rose to 30–40°. After 35 min, reaction was complete (t.l.c.), and the mixture was treated with acetic anhydride (400 ml) and kept overnight at room temperature. The mixture was poured into ice–water, and the resulting precipitate was collected and recrystallised from methanol to give compound **6** (28 g, 39%), m.p. 140–150°, $[\alpha]_D$ +132° (*c* 0.5, chloroform). A further recrystallisation from methanol gave material having m.p. 170–172°. This product was identical with a sample prepared by the method of Bredereck[3], who reported m.p. 170–172°, $[\alpha]_D$ +133°.

6,6'-Di-O-*toluene*-p-*sulphonyl-α,α-trehalose* (**5**). — A solution of hexa-*O*-acetyl-6,6'-di-*O*-toluene-*p*-sulphonyl-α,α-trehalose (5 g) in acetone (25 ml) was treated with 5 ml. of 0.51M methanolic sodium methoxide and kept for 15 min at room temperature. The pH was adjusted to 7 by shaking with Permutit Biodeminrolite mixed-bed, ion-exchange resin (CO_3^{2-} form), and the solution was evaporated to a hard glass (3.1 g, 87%), which crystallised from hot water containing methanol (*ca.* 20%) to give the disulphonate (**5**) (2.2 g, 62%), m.p. 106–110°, $[\alpha]_D$ +90° (*c* 0.2, 1:9 methanol–chloroform) (Found: C, 47.7; H, 5.65; $C_{26}H_{34}O_{15}S_2$ calc.: C, 48.0; H, 5.3%); lit.[4] $[\alpha]_D$ +110°.

Hexa-O-*acetyl-6,6'-diazido-6,6'-dideoxy-α,α-trehalose* (**7**). — Hexa-O-acetyl-di-*O*-toluene-*p*-sulphonyl-α,α-trehalose (1 g) was dissolved in hexamethylphosphoric triamide (5 ml) and heated with sodium azide for 5 h on a boiling-water bath. The mixture was then diluted with water, and the precipitate was collected and recrystallised from methanol to give the diazide **7** (0.36 g, 51%). Two further recrystallisations from methanol gave pure material, m.p. 114–116°, $[\alpha]_D$ +134° (*c* 0.5, chloroform) (Found: C, 44.7; H, 5.1; N, 13.15. $C_{24}H_{32}N_6O_{15}$ calc.: C, 44.7; H, 5.0; N, 13.0%).

6,6'-Diazido-6,6'-dideoxy-α,α-trehalose (**8**). — The hexa-acetate **7** (3.5 g) was dissolved in methanol (340 ml) and treated with 3.5 ml of 0.57M methanolic sodium methoxide. After 25 min, the pH of the solution was adjusted to 7 with resin as before, and the solution was then concentrated to dryness to give a crystalline mass (1.17 g, 55%). Recrystallisation from methanol gave the diazide, m.p. 209–211°, $[\alpha]_D$ +158° (*c* 0.7, water) (Found: C, 36.65; H, 5.2; N, 21.4. $C_{12}H_{20}N_6O_{19}$ calc.: C, 36.75; H, 5.15; N, 21.45%).

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BIOSYNTHESIS OF STARCH

PART XI*. INHIBITION EFFECTS OF THIOL REAGENTS ON PHOSPHORYLASE ACTION**

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ABSTRACT

The inhibition effects of the reagents *p*-chloromercuribenzoate (*p*CMB), iodoacetamide (IAA), and *N*-ethylmaleimide (NEMI) in the reaction catalysed by phosphorylase were investigated. The dissociation constants of the complexes of the various inhibitors formed with the enzyme were established. The values of K_i were 4.4×10^{-6} (*p*CMB), 1.2×10^{-2} (IAA), and 8.7×10^{-2} (NEMI). It was thus inferred that latent SH groups play an essentially greater role than do free SH groups. Although it is unlikely that they participate in the formation of the enzyme–substrate complex, they presumably stabilise a definite enzyme orientation.

It was found that the reagents tested cause irreversible inhibition, and that the substrates exert a protecting effect.

INTRODUCTION

In our earlier communications, we reported on the synthesis of polysaccharides catalysed by potato phosphorylase and on the reaction kinetics of phosphorolysis[1,2]. It was established, on the basis of reaction kinetics, that, in the active centre of the enzyme, the substrates are bound to different functional groups, and thus they do not inhibit the binding of each other. The temperature dependence of the pK values and other experiments indicate that the binding of substrates appears to be associated with the imidazole and α-amino groups of the active centre[2]. Investigations of reaction kinetics, carried out with various protein inhibitors, played a prominent role in elucidating the mechanism of the enzymic reaction.

Very few data are available concerning the structure of the protein of potato phosphorylase. The enzyme contains[3,4] 2 moles of bound organic phosphate per molecule as pyridoxal 5-phosphate. These results are in accordance with those[5,6] obtained in the investigation of muscle phosphorylase. Decomposition by chymotrypsin afforded[7,8] a different amino acid residue, and the binding site of pyridoxal 5-phosphate is still uncertain, but it is likely to involve a thiol, amino, imidazole, or hydroxyl group[9]. Also, the number of SH groups of the enzyme molecule has been determined

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by various methods. In the dialysed enzyme protein, Lee[10] found 5.7–6.0 and 5.4 moles of SH, respectively, by amperometric titration with 1×10^{-3}M silver nitrate and 1×10^{-3}M mercury chloride, whereas titration with *p*-chloromercuribenzoate (*p*CMB) indicated the presence of 6.8 moles of SH group per molecule of enzyme. These values are, however, lower than those (18.5–18.7 moles of SH) obtained for muscle phosphorylase[5,11].

Vulfson and Skolysheva[12] detected two different types of SH group in muscle phosphorylase *b*. According to their investigations, five SH groups readily reacted with *p*CMB and *N*-ethylmaleimide (NEMI), whereas four other SH groups reacted only slowly with *p*CMB and did not react at all with NEMI. The SH groups of the latter type play an important role in the stabilisation of the enzyme and in the catalytic activity.

The role of the SH groups of phosphorylase is still not clear. In studying the mechanism of enzyme action, it appeared necessary to investigate whether the SH groups actually participate in the binding of substrates or whether they act solely as structure stabilisers. This problem can only be solved with the aid of SH-specific protein inhibitors.

According to the experiments carried out by Lee, a tenfold excess of *p*CMB causes irreversible, full inhibition, in contrast to muscle phosphorylase. 10^{-2}M Iodoacetamide (IAA) does not cause any inhibition, whereas heavy metals and mercury compounds proved to be potent inhibitors[3], although to a slighter extent than in the case of muscle phosphorylase[5,13–15].

However, the above data are not sufficient for the elucidation of the role of the SH groups. Moreover, it is rather difficult to evaluate the experimental values, due to the multiple nature of effects. Inhibition is appreciably affected, for example, by the concentration of substrates, the pH value, the dielectric constant, the salt concentration, the ionic strength, and contaminants.

In the course of the present experiments, the conditions of inhibition and the constants K_i which characterise the inhibition were investigated with *p*CMB, IAA, and NEMI as SH reagents.

MATERIALS AND METHODS

Potato phosphorylase was prepared[16] by chromatography on DEAE-cellulose. The activity of the enzyme was 54 enzyme units per ml and its specific activity was 450 enzyme units per mg of protein.

D-Glucopyranosyl dipotassium phosphate (analytical grade REANAL Finomvegyszergyár, Budapest), contained 73.9% of organic phosphate and 0.2% of inorganic phosphate contaminant. Amylopectin was prepared[17] from potato starch by fractionation with butyl alcohol.

0.1M Citrate buffer: the pH value of the disodium hydrogen citrate solution was adjusted to 6.0 $\pm$0.1 with 0.05N sodium hydroxide.

p-Chloromercuribenzoate (Light and Co. Ltd.), *N*-ethylmaleimide (Fluka A. G.), and iodoacetamide (Fluka A. G.) were analytical grade reagents.

Liberated inorganic phosphate was determined after interruption of the enzymic reactions by heating for 10 min at 100°. The precipitated proteins were removed, and the inorganic phosphate in the clear filtrate was determined by the colorimetric method of Fiske–Subbarow[18], as modified by Butyenko–Kirsch[19]. D-Glucopyranosyl phosphate, formed during phosphorolysis, was determined in a similar manner by a modification[20] of the method of Sugino and Miyoshi[21].

The first-order reaction rate (k) was determined, in the case of synthesis, from the amount of inorganic phosphate liberated, whereas, in the case of phosphorolysis, it was determined from the D-glucopyranosyl phosphate liberated. The correlation of the equilibrium constant with temperature and pH value has been described[20]. The initial reaction rate (v_0) can be calculated from these data.

EXPERIMENTAL AND DISCUSSION

Effect of inhibitor concentration on the reaction rate. — The specific SH inhibitors were chosen in such a way as to enable determination of whether the free and/or the latent SH groups participate in the enzymic reaction. The following inhibitors were employed: *N*-ethylmaleimide (reacts only with the free SH groups), iodoacetamide (reacts mainly with the free, but to a small extent also with the latent, SH groups), and *p*-chloromercuribenzoate (reacts both with the free and with the latent SH groups).

The experiments were carried out at 30° at pH 6.0. The inhibitors, the enzyme, and the substrates were dissolved in a 0.1M citrate buffer. In the synthesis tests, the donor substrate concentration was 10 mmoles of D-glucopyranosyl phosphate per litre of buffer, whereas the acceptor substrate concentration was 0.95 mmole of amylopectin per litre of buffer. The final volume of the reaction mixture was 2.0 ml, the applied enzyme concentration was 17 E units, and the reaction period was 10 min. The relevant data, together with the applied inhibitor concentrations, are given in Table I.

In the decomposition tests, the degree of inhibition was investigated only at one given inhibitor concentration. The experimental conditions (35°, 0.1M citrate buffer, pH 6.0) were the same as those for synthesis. The donor substrate concentration was 50 mmoles of inorganic phosphate per litre of buffer, and the acceptor substrate concentration was 1.18 mmoles of amylopectin per litre of buffer. The final volume of the reaction mixture was 4.0 ml, the applied enzyme concentration was 34 E units, and the reaction period was 5 h. The data are given in Table II.

On employing various inhibitor concentrations in the synthesis tests, the value of K_i (the dissociation constant of the enzyme–inhibitor complex) can be established with the aid of the graphic method[22]. The equation applied in the graphic method can be derived, by a simple rearrangement, from the general formula:

$$v_i = v_m \cdot \frac{[S]}{[S] + K_s\{1 + [I]/K_i\}},$$

where v_i is the rate of the inhibited reaction, v_m the maximal rate of the reaction, $[S]$ the substrate concentration, K_s the dissociation constant of the enzyme–substrate

TABLE I

EFFECT OF CHANGES IN INHIBITOR CONCENTRATION ON THE RATE OF SYNTHESIS AND THE DEGREE OF INHIBITION

Inhibitors	*Inhibitor conc. (mole/litre)*	*Initial rate* $v_0 . 10^{-3}$ *(μmoles/min)*	*Inhibition (%)*
*p*CMB	2.0×10^{-5}	90	52.6
	1.5×10^{-5}	105	44.8
	1.0×10^{-5}	121	36.4
	5.0×10^{-6}	145	23.7
	1.0×10^{-6}	174	8.4
	1.0×10^{-7}	190	0
IAA	2.0×10^{-2}	128	32.7
	1.5×10^{-2}	139	27.1
	1.0×10^{-2}	132	20.0
	5.0×10^{-3}	166	8.8
	1.0×10^{-3}	174	8.4
	1.0×10^{-4}	190	0
NEMI	2.0×10^{-2}	162	14.8
	1.5×10^{-2}	167	12.0
	1.0×10^{-2}	170	10.5
	5.0×10^{-3}	172	9.1
	1.0×10^{-3}	174	8.4
	1.0×10^{-4}	190	0
Without inhibitor	—	190	0

TABLE II

EFFECT OF VARIOUS INHIBITORS ON THE RATE OF PHOSPHOROLYSIS AND THE DEGREE OF INHIBITION

Inhibitors	*Inhibitor conc. (mole/litre)*	*Initial rate* $v_0 . 10^{-3}$ *(μmoles/min)*	*Inhibition (%)*
*p*CMB	10^{-5}	0.86	70.5
NEMI	10^{-1}	2.30	20.6
IAA	10^{-1}	1.67	42.6
Without inhibition	—	2.90	—

complex, $[I]$ the inhibitor concentration, and K_i the dissociation constant of the enzyme–inhibitor complex. The equation can be rearranged as follows:

$$\frac{1}{v_i} = \frac{1}{V_m}\left\{1 + \frac{K_s}{[S]} + \frac{K_s[I]}{V_m[S]K_i}\right\},$$

where, on plotting $1/v_i$ against the inhibitor concentration $[I]$, a straight line is obtained, and the value of K_i (Figs. 1–3) can be determined from the slope. The K_i values established in this way are listed in Table III. The mean K_i values are 4.4×10^{-6} (*p*CMB), 1.20×10^{-2} (IAA), and 8.7×10^{-2} (NEMI).

TABLE III

K_i VALUES DETERMINED WITH VARIOUS INHIBITORS[a]

Inhibitors	K_i	
	With acceptor substrate	*With donor substrate*
*p*CMB	3.5×10^{-6}	4.6×10^{-6}
	4.6×10^{-6}	5.5×10^{-6}
IAA	9.2×10^{-3}	1.3×10^{-2}
	1.16×10^{-2}	1.4×10^{-2}
NEMI	7.5×10^{-2}	8.8×10^{-2}
	8.7×10^{-2}	1.0×10^{-1}

[a]Determined from axis intersection of slope.

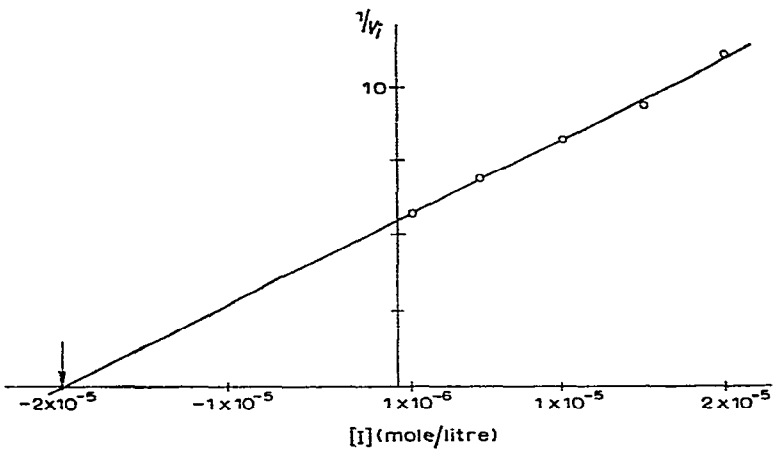


Fig. 1 Values of $1/v_i$ plotted against inhibitor concentration for *p*-chloromercuribenzoate.

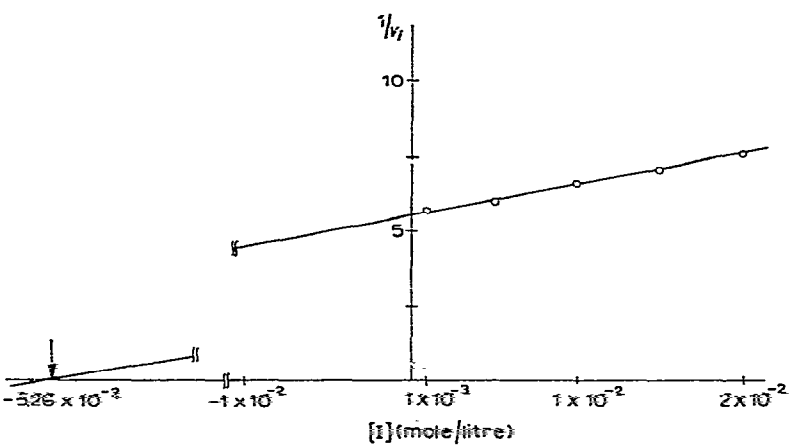


Fig. 2 Values of $1/v_i$ plotted against inhibitor concentration for iodoacetamide.

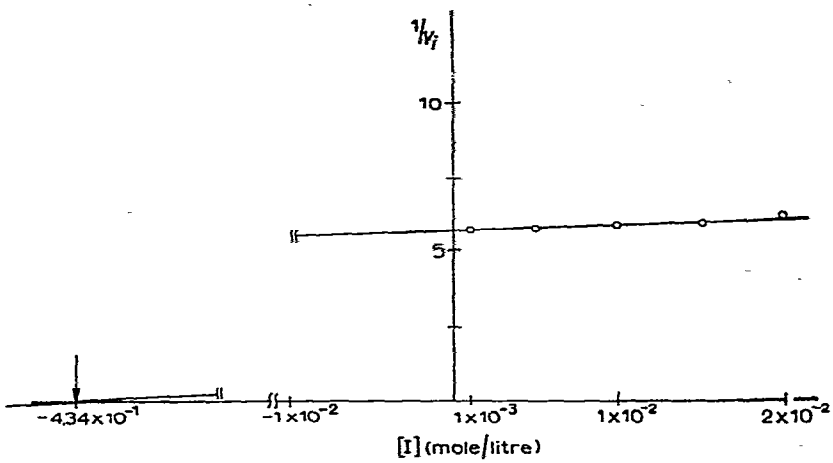


Fig. 3. Values of $1/v_i$ plotted against inhibitor concentration for *N*-ethylmaleimide.

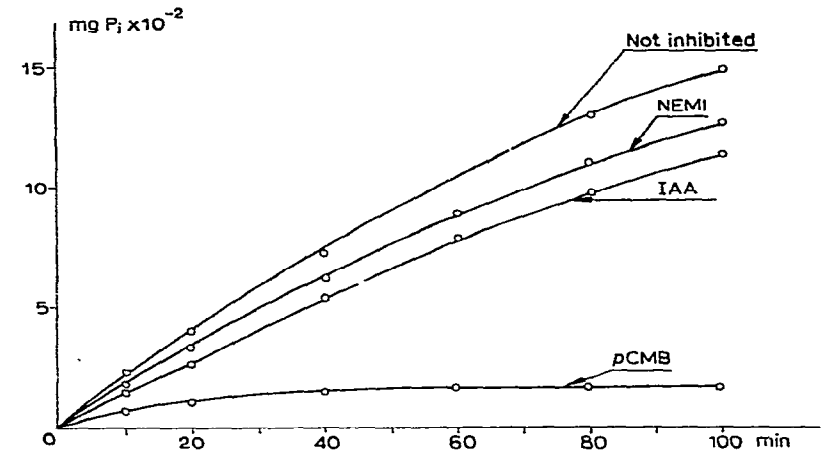


Fig. 4. Inhibition of the reaction catalysed by potato phosphorylase, plotted against time with various SH inhibitors.

It can be seen from the above data that *p*CMB is by far the strongest inhibitor of the enzymic reaction. Thus, a solution having a concentration lower by *ca.* 3 orders of magnitude than those of IAA and NEMI showed the same inhibition. The inhibitor NEMI, although specific in respect to the free SH groups, proved to be the weakest. IAA was also a weak inhibitor, although it reacts to a small extent also with the latent SH group. *p*CMB was found to be the strongest inhibitor, reacting with both types of SH group. It follows from the experimental results that the free SH groups play an essentially smaller role in the enzymic reaction than the latent SH groups. It is unlikely that latent SH groups would play any appreciable role in the development of the enzyme–substrate complex, and it may be presumed that they stabilise the spatial structure of the enzyme, very probably through disulphide bonds. On binding

the SH groups, this spatial structure is decomposed, the specific enzyme conformation is altered, and enzymic catalysis is inhibited.

Changes of the inhibition process. — Since both IAA and NEMI are very weak inhibitors, it appeared desirable to investigate the inhibition process as a function of time, because it is likely that the affinity of the various inhibitors for the SH groups of the enzyme is different, and that the degree of inhibition may be a function of time.

This series of experiments was carried out under the above conditions, with the following inhibitor concentrations: *p*CMB, 5×10^{-4} moles/litre; IAA, 1×10^{-1} mole/litre; and NEMI, 1×10^{-1} mole/litre; 17 E units of enzyme were used, and the end volume of the reaction mixture was 8.0 ml. During the synthesis, samples (1.0 ml) were withdrawn at various intervals, and the amount of inorganic phosphate liberated was determined. The results are shown in Fig. 4, from which it can be seen that the degree of inhibition increases with the progress of reaction, and, again, *p*CMB proved to be a stronger inhibitor than NEMI or IAA.

Investigation of the protecting effect of substrates and of irreversibility. — Owing to the differences in the inhibition effect, and particularly to the facts that the inhibitors IAA and NEMI showed effects weaker than expected, the protecting action of the applied substrates was investigated.

The enzymic reaction was carried out with enzyme samples previously incubated with inhibitors and substrates. The preincubation period was 20 min at 35°. Inhibitor concentrations applied were: *p*CMB, 5×10^{-5} moles/litre; NEMI, 1×10^{-2} mole/litre; and IAA, 1×10^{-2} mole/litre. The concentration of the donor substrate was

TABLE IV

CHANGES IN REACTION RATES AND THE DEGREE OF INHIBITION ON EMPLOYING ENZYME SAMPLES PREINCUBATED UNDER VARIOUS CONDITIONS

Sample	*Inhibitors*	*Initial rate* $v_0 \cdot 10^{-3}$ (*μmoles/min*)	*Inhibition* (%)
Freincubated without inhibition	—	125	0
With inhibitor, without preincubation	*p*CMB	84.7	32.2
	IAA	113.0	9.6
	NEMI	121.0	3.2
Incubated with inhibitor	*p*CMB	44.1	64.7
	IAA	16.1	87.2
	NEMI	97.0	22.4
Preincubated with D-glucopyranosyl phosphate	*p*CMB	101	19.0
	IAA	119.2	9.6
	NEMI	110.5	19.6
Preincubated with amylopectin	*p*CMB	109	12.8
	IAA	119.2	4.8
	NEMI	110.5	11.6

20 mmoles/litre of D-glucopyranosyl phosphate, and the concentration of the acceptor substrate 0.237 mmole/litre of amylopectin. The amount of enzyme employed was 17 E units. The reaction period was 20 min at 35°. On interrupting the enzymic reaction, the liberated inorganic phosphate, the initial reaction rate, and the degree of inhibition were determined (Table IV).

The data in Table IV show that inhibition was weaker in the reactions carried out with enzymes preincubated with substrates. This is presumably due to the protecting effect of substrates.

Irreversibility was proved by the very low values of reaction rates measured in tests carried out with samples preincubated with substrates.

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ZUM MECHANISMUS DER TRANSGLYKOSIDIERUNG VON GLYKOSYLAMINEN UND UNTERSUCHUNGEN ZUR MUTAROTATION

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(Eingegangen den 7. März, 1968)

ABSTRACT

Under different conditions, the rates of transglucosylation of *N*-*p*-tolyl-D-glucopyranosylamine (**1a**) and *N*-*p*-nitrophenyl-β-D-glucopyranosylamine (**1b**) were measured with labelled *p*-toluidine and labelled *p*-nitroaniline, respectively. Some possible mechanisms can be excluded. Depending on the acid concentration, the reaction shows a distinct rate maximum which is different for **1a** and **1b**. The acid catalysis is specific. Under most conditions, the mutarotation is faster. General acid catalysis has little influence. In contrast to the transglucosylation, the mutarotation is catalyzed by bases. The Schiff's bases **12** and **13** exchange the amines much faster than do glucosylamines; the rate for 4,6-*O*-benzylidene-*N*-*p*-tolyl-D-glucosylamine (**11**) is intermediate.

ZUSAMMENFASSUNG

Die Geschwindigkeit der Transglucosidierung von *N*-*p*-Tolyl-D-glucopyranosylamin (**1a**) wurde mit radioaktiv markiertem *p*-Toluidin und die von *N*-*p*-Nitrophenyl-β-D-glucopyranosylamin (**1b**) mit markiertem *p*-Nitranilin unter verschiedenen Bedingungen gemessen. Es werden einige mögliche Mechanismen ausgeschlossen. Die Reaktion ist spezifisch säurekatalysiert und zeigt in Abhängigkeit von der Säurekonzentration ein ausgeprägtes Geschwindigkeits-Maximum, das für **1a** und **1b** verschieden ist. Die ebenfalls unter Transglucosidierungsbedingungen gemessene Mutarotation ist meist wesentlich rascher, die allgemeine Säurekatalyse spielt nur eine geringe Rolle. Sie ist im Gegensatz zur Transglucosidierung auch alkalisch katalysierbar. Die Schiffschen Basen **12** und **13** zeigen einen viel rascheren Aminaustausch als obige Glucosylamine. 4,6-*O*-Benzyliden-*N*-*p*-tolyl-D-glucosylamin (**11**) nimmt eine Mittelstellung ein.

EINLEITUNG

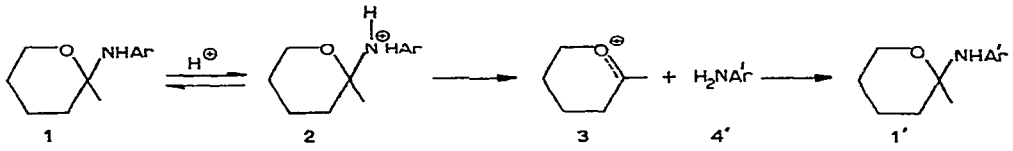
In der Literatur wurde die Übertragung des Glykosidrestes eines Glykosylamins auf ein freies Amin (Gleichung 1) unter präparativen[1–4], mechanistischen[4] und

*Postanschrift : D 8050 Freising-Weihenstephan.

biochemischen[5] Gesichtspunkten beschrieben. Von Bognár und Nánási[4] wurden darüber hinaus die durch Gleichung (*2*) und (*3*) wiedergegebenen Reaktionstypen beschrieben (G = Glykosylrest, H_2NAr = aromatisches Amin). Kinetische Daten

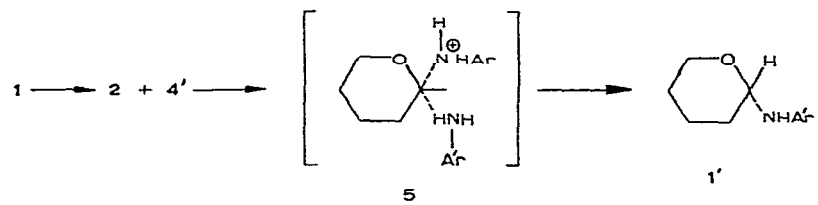
$$\text{G-NHAr} + \text{H}_2\text{NAr}' \rightleftharpoons \text{G-NHAr}' + \text{H}_2\text{NAr} \quad (1)$$
$$\text{G-NHAr} + \text{G}'\text{-OH} \rightleftharpoons \text{G}'\text{-NHAr} + \text{G-OH} \quad (2)$$
$$\text{G-NHAr} + \text{G}'\text{-NHAr}' \rightleftharpoons \text{G}'\text{-NHAr}' + \text{G-NHAr} \quad (3)$$

sind unseres Wissens nicht bekannt und über den Mechanismus wurden bisher nur von Bognár und Nánási[4] Angaben gemacht. Danach soll sich aus dem Glykosylamin **1** ein Glykosylaminkation **2** und daraus ein Carboniumion **3** bilden, das dann mit dem freien Amin reagiert (Mechanismus *A*).



Mechanismus *A*

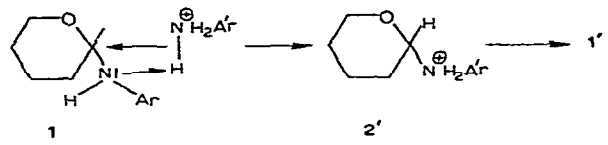
Bei den Reaktionstypen (*2*) und (*3*) sollen protonisierte Spezies über intermediär gebildete mesomere Carboniumionen Aminmoleküle miteinander austauschen[4]. Nach unseren Untersuchungen zur Hydrolyse von Glykosylaminen[6] interessierten wir uns aus mechanistischen und präparativen Gründen für die Transglykosidierung des Typs (*1*) und (*2*). Neben dem Reaktionsablauf nach Bognár und Nánási[4] sind noch folgende Mechanismen denkbar. Das am Stickstoff protonisierte Glykosylamin **2** wird in einer S_N2-Reaktion von freiem Amin mit Übergangszustand **5** verdrängt (Mechanismus *B*).



Mechanismus *B*

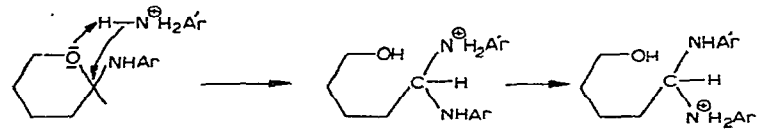
Ein cyclischer Elektronenübergang könnte direkt über **2'** zu **1'** führen. Weiterhin könnte in einer Art S_Ni-Reaktion **1** ein protonisiertes Amin unter Ringöffnung zu einem Bis-*N*-acetal **6** addieren, das sodann nach Umprotonisierung zu **7** unter Amin und Protonabspaltung zu **1'** cyclisiert. (Mechanismus *C*).

In Analogie zur Hydrolyse von Glykosylaminen könnte zunächst der Ringsauerstoff zu **8** protonisiert werden. Anschließende Ringöffnung ergibt die Schiffsche



1 2'

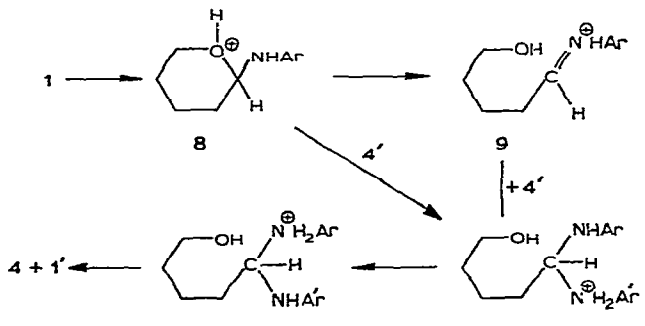
oder



6 7 1'

Mechanismus C

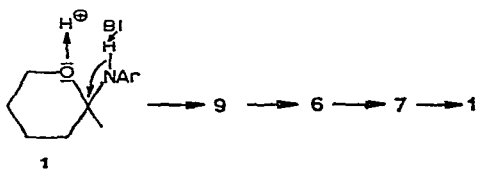
Base **9**, die das freie Amin unter Bildung von **6** addiert, das wie oben angegeben über **7** in **1'** übergeht. Auch ein direkter Übergang **8**→**6** wäre möglich (Mechanismus *D*).



8 9 7 6

Mechanismus D

Schließlich könnte eine Säure-Base-Katalyse eine Rolle spielen. Dabei würde in einem intermolekularen Schritt der Ring unter Bildung einer Schiffschen Base geöffnet, die dann wie unter Mechanismus *D* weiter reagieren kann (Mechanismus *E*).



1

Mechanismus E

Es sollte möglich sein, einige dieser Mechanismen auszuschließen, wenn die Reaktionsgeschwindigkeit (RG) der Transglykosidierung in Abhängigkeit von der Säurekonzentration, bei konstantem pH in Abhängigkeit von der Pufferkonzentration, sowie im Vergleich mit der Mutarotation kinetisch untersucht würde. Weiterhin wäre von Interesse, die Austauschgeschwindigkeit von Schiffschen Basen im Vergleich

zu Glykosylaminen zu kennen. Sollten erstere langsamer reagieren als letztere, könnten die Mechanismen *D* und *E* ausgeschlossen werden.

ZUR METHODIK

Eine relativ einfache Versuchsanordnung ergibt sich durch Austauschreaktionen, bei denen das gebundene und freie Amin chemisch gleich sind, jedoch aufgrund von Isotopenmarkierung unterschieden werden können. Daher wurde *N*-*p*-Tolyl-β-D-glucopyranosylamin (**1a**) mit Tritium-markiertem *p*-Toluidin (**4a**) und *N*-*p*-Nitrophenyl-β-D-glucopyranosylamin (**1b**) mit Tritium-markiertem *p*-Nitranilin (**4b**) umgesetzt. Bei der Untersuchung von **1a** wurde **4a** in Form des Hydrochlorids isoliert und analysiert. Im Falle von **1b** wurde entweder **1b** (Zunahme der Radioaktivität von Null bis zur Gleichverteilung) oder **4b** (Verminderung der ursprünglichen Radioaktivität bis zur Gleichverteilung) wieder isoliert.

Die RG-Konstanten wurden rechnerisch oder graphisch bestimmt. Zur rechnerischen Ermittlung wurde die Gleichung

$$-\ln\left(1-\frac{X_t}{X_\infty}\right) = 2akt$$

verwendet. Dabei bedeuten: X_t = die molare Radioaktivität des Glucosylamins zur Zeit t, falls zur Zeit Null nur das freie Amin markiert war; X_∞ = die molare Radioaktivität des Glucosylamins bzw. Amins nach der Gleichverteilung; a = die Konzentration des Amins bzw. Glucosylamins; und k = RG-Konstante.

Da im vorliegenden Fall stets mit äquimolaren Lösungen gearbeitet wurde, ist $X_\infty = A_0/2$, wenn A_0 die molare Ausgangsaktivität des Amins darstellt. Für den Zusammenhang zwischen RG-Konstante und Halbwertszeit gilt: $t_{0.5} = (\ln 2/2ak)$. Bei den Experimenten, bei denen das eingesetzte markierte Amin nach der Reaktionszeit t isoliert worden war, wurde aus dessen Radioaktivität die molare Radioaktivität des Glucosylamins errechnet. Zur graphischen Ermittlung der Halbwertszeit wurde log(100−% Austausch) gegen die Zeit aufgetragen. Aus den sich ergebenden Geraden (vgl. Abb. 1) wurde die Halbwertszeit bestimmt. Für den prozentualen Austausch gilt für den Fall, daß das freie Amin markiert ist und das Amin isoliert wird: % Austausch $= 100\,(A_0-A_t)/A_\infty$. Dabei bedeuten A_0, A_t und A_∞ die spezifischen Radioaktivitäten des Amins zur Zeit 0, zur Zeit t und nach Gleichverteilung. Falls das freie Amin markiert ist und das Glucosylamin isoliert wird, gilt: % Austausch $= 100\ X_t/(A_0-X_\infty)$. (Bedeutung der Symbole siehe oben.)

Eine besondere Schwierigkeit ergab sich daraus, daß nicht in Wasser oder wasserhaltigen Medien gearbeitet werden konnte, da die Hydrolyse z.T. von vergleichbarer Geschwindigkeit wie die Transglykosidierung verläuft und außerdem die RG in 95proz. und wasserfreiem Äthanol bei gleicher Säurekonzentration stark unterschiedlich ist. Die Alkoholyse von **1a** und **1b** war vernachlässigbar. Wie sich herausstellte, ist die Mutarotation von **1a** nur bei schwach saurem pH und bei einer höheren Konzentration von **1a** und **4a**, wie üblicher Weise verwendet, langsamer als

die Transglucosidierung. Daher wurde, von einer Ausnahme abgesehen, nur die β-Form der D-Glykosylamine eingesetzt. Über Kontrollversuche, Aufarbeitungsbedingungen etc. siehe Beschreibung der Versuche.

ERGEBNISSE

Abhängigkeit der RG von der Konzentration. — In Abb. 1 ist die Abhängigkeit der Austauschgeschwindigkeit von **1b** bei äquimolaren Konzentrationen von **1b** und **4b** dargestellt und in Abb. 2 die Abhängigkeit der Halbwertszeit von der reziproken Konzentration. Es zeigt sich ein linearer Verlauf, d.h. die Reaktion ist erster Ordnung in bezug auf Glucosid und Amin.

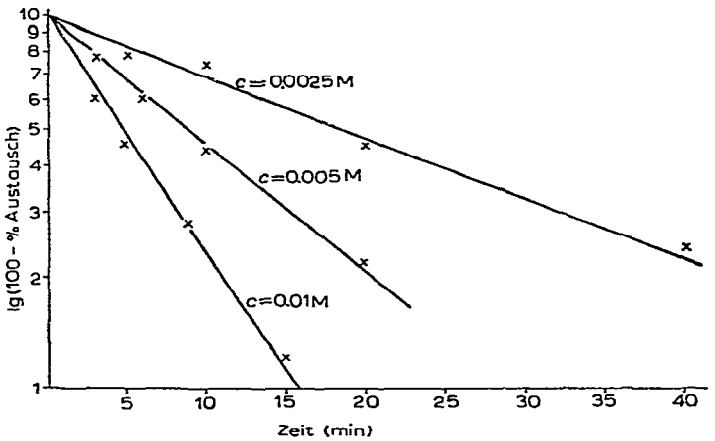


Abb. 1. Austauschgeschwindigkeit von *N-p*-Nitrophenyl-D-glucopyranosylamin mit Nitranilin-*t* bei verschiedenen äquimolaren Konzentrationen der Reaktionspartner in 0.1N äthanolischer Salzsäure bei 0.2°.

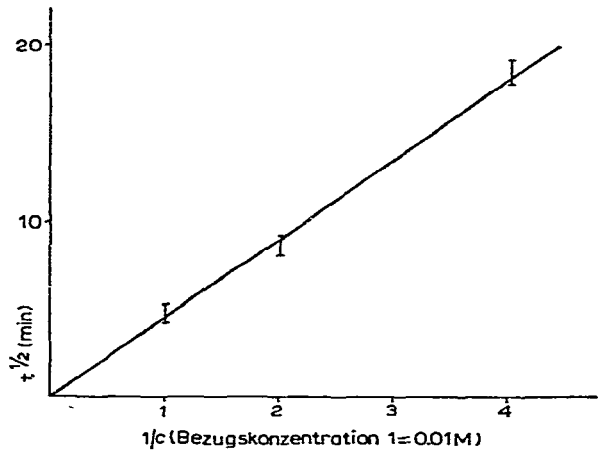


Abb. 2. Abhängigkeit der Halbwertszeit des Austauschs zwischen *N-p*-Nitrophenyl-D-glucopyranosylamin und Nitranilin-*t* von der reziproken Konzentration. Bedingungen wie bei Abb. 1 angegeben.

Abhängigkeit der RG von der Säurekonzentration. — In Abb. 3 ist der Logarithmus der RG-Konstanten der Transglucosidierung (2. Ordnung) von **1a** und **1b** in Abhängigkeit vom negativen Logarithmus der Chlorwasserstoffkonzentration in absolutem Äthanol dargestellt. Für **1a** liegt das RG-Maximum nach dieser Art der Wiedergabe bei *ca.* 0.005N HCl und für **1b** zwischen 0 und 1. Das scharfe Maximum von **1a** kommt daher, daß durch das *p*-Toluidin die freie HCl-Konzentration unter 0.01M wesentlich geringer ist, als angegeben. Berechnet man die Konzentration des freien Chlorwasserstoffs, wie er bei den Verhältnissen in Wasser vorliegen würde und trägt diese Werte auf die Abszisse auf, so ergibt sich die mit ⊗ gekennzeichnete Kurve, d.h. das Maximum liegt bei einer Säurekonzentration von 10^{-4} bis 10^{-5}. In den Tab. I und II sind die Halbwertszeiten der Transglucosidierung unter dem

TABELLE I

ABHÄNGIGKEIT DER RG DER TRANSGLUCOSIDIERUNG VON *N*-*p*-TOLYL-β-D-GLUCOPYRANOSYLAMIN (**1a**) VON VERSCHIEDENEN SÄUREN UND KONZENTRATIONEN IN ABSOLUTEM ÄTHANOL. KONZENTRATION VON **1a** UND **4a** JE 0.01M TEMP. 0.2±0.1°.

Katalysator	*Konzentration* (N)	*Halbwertszeit* (*Min*)
HCl	2	708 ±65
HCl	1	624 ±60
HCl	0.1	504 ±50
HCl	0.01	29.7±3
HCl	0.005	8.5±0.9
HCl	0.004	10.0±1.0
HCl	0.001	43 ±4
HCl	0.0001	720 ±60
Benzoesäure	0.01	228 ±18
m-Chlorbenzoesäure	0.01	100 ±10
m-Nitrobenzoesäure	0.01	38.5±3
p-Nitrobenzoesäure	0.01	38.5±3

TABELLE II

ABHÄNGIGKEIT DER RG DER TRANSGLUCOSIDIERUNG VON *N*-*p*-NITROPHENYL-β-D-GLUCOPYRANOSYLAMIN (**1b**) BEI VERSCHIEDENEN ÄQUIMOLAREN KONZENTRATIONEN VON **1b** UND **4b** UND VERSCHIEDENEN SÄUREN UND KONZENTRATIONEN IN ABSOLUTEM ÄTHANOL. TEMP. 0.2 ±0.1°

Molare Konzentration der Reaktionspartner	*Katalysator Konzentration* (N)	*Halbwertszeit (Min)*
0.005	2 HCl	10.8±1
0.005	1 HCl	9.2±0.9
0.01	0.1 HCl	4.7±0.5
0.005	0.1 HCl	8.3±0.8
0.0025	0.1 HCl	18.5±1.6
0.005	0.01 HCl	47.5±4
0.005	0.001 HCl	960 ±80
0.005	kein	kein Austausch nach 7 Stdn.
0.005	0.1 Essigsäure	kein Austausch nach 5 Stdn.
0.005	1 Essigsäure	kein Austausch nach 50 Stdn.

Einfluß einiger anderer Säuren im Vergleich mit Chlorwasserstoff bei verschiedenen Konzentrationen und bei **1b** auch in Abhängigkeit von der Eigenkonzentration wiedergegeben.

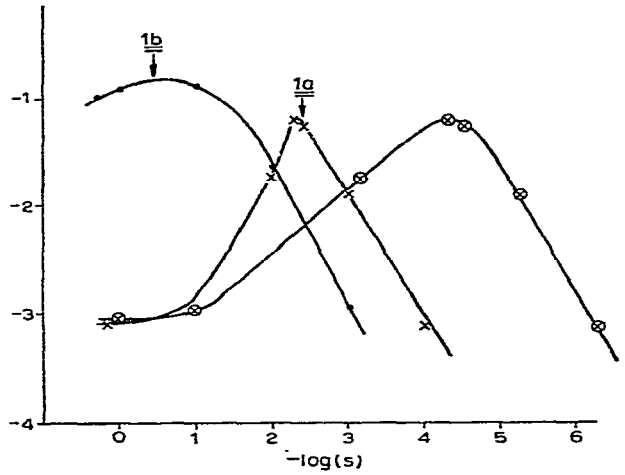


Abb. 3. Logarithmus der RG-Konstanten (2. Ordnung) der Transglucosidierung von **1a** und **1b** in Gegenwart äquimolarer Konzentration von **4a** bzw. **4b** in Abhängigkeit vom negativen Logarithmus der Chlorwasserstoffkonzentration in absolutem Äthanol bei 0.2°. Die mit ⊗ gezeichnete Kurve ergibt sich für **1a** unter Berücksichtigung der freien HCl-Konzentration (vgl. Text).

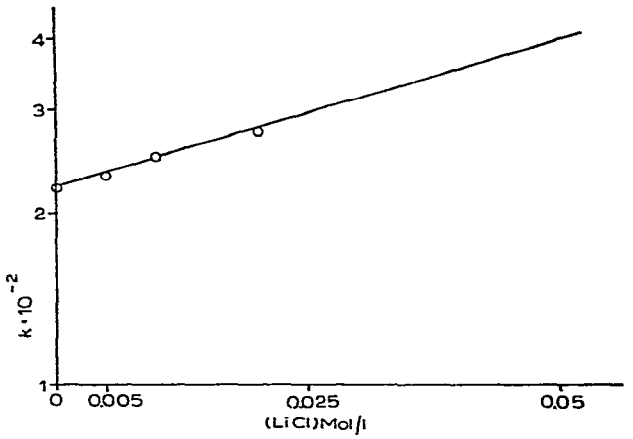


Abb. 4. Abhängigkeit der RG der Transglucosidierung von steigenden Mengen Lithiumchlorid bei konstanter Pufferkonzentration (Essigsäure 0.1M, Natriumacetat 0.05M) in Äthanol. Die Extrapolation bis 0.05M Lithiumchlorid zeigt, welche Wirkung dem Puffer aufgrund seiner Ionenstärke zukommt.

Art der Säurekatalyse und Temperaturabhängigkeit der Transglykosidierung. — Wie Abb. 4 zeigt, ist die Ionenstärke auf die RG von Einfluß. Die Pufferkonzentration konnte aber nur über einen kleinen Bereich variiert werden, da in absolutem

Äthanol nur wenig Lithiumchlorid löslich ist. Tab. III zeigt, daß bei konstanter Ionenstärke die RG bei verschiedenen Pufferkonzentrationen konstant ist. Diese Befunde, zusammen mit der Tatsache, daß der α-Wert der logarithmischen Bronsted-Gleichung sehr nahe 1 ist, zeigt, daß es sich bei der Reaktion um spezifische Säurekatalyse handelt.

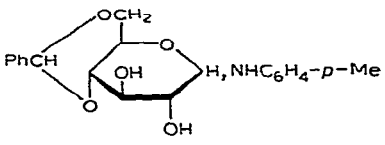
TABELLE III

RG DER TRANSGLUCOSIDIERUNG VON **1a** IN WASSERFREIEM ÄTHANOL IN 0.01M LÖSUNG BEI 20.0° BEI VERSCHIEDENEN PUFFERKONZENTRATIONEN UND KONSTANTER IONENSTÄRKE

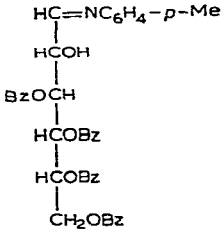
Essigsäure	*Natriumacetat*	*Lithiumchlorid*	*Ionenstärke*	$k.10^2$ *sec*
0.08	0.040	0.010	0.050	1.86
0.06	0.030	0.020	0.050	1.98
0.04	0.020	0.030	0.050	1.86

Die Messungen bei 0; 11.4; 15.0 und 20.0° in einem Puffer Essigsäure 0.1M und Natriumacetat 0.05M ergaben RG-Konstanten von 4.81; 13.4; 18.6 und 22.8×10^{-3} Konz.$^{-1}$sec^{-1}. Daraus berechnet sich eine Aktivierungsenergie von 12.8 $\pm$1.3 kcal/Mol. Die Aktivierungsentropie beträgt $-5.3 \pm\frac{9.7}{4.4}$(cal/Grad.Mol).

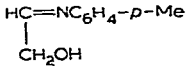
Vergleich der Austauschgeschwindigkeit von Glykosylaminen und entsprechenden Schiffschen Basen. — Die Halbwertszeiten des Austauschs für **1a**, 4,6-*O*-Benzyliden-*N*-*p*-tolyl-D-glucosylamin (**11**), und die Schiffschen Basen **12** und **13** in *p*-Dioxan–



11



12



13

Äthanol (1:1, v/v) sind in Tab. IV wiedergegeben. Es ist anzunehmen, daß der Anteil der Schiffschen Base an **11** bereits wesentlich höher ist als bei **1a**. Entsprechende

TABELLE IV

HALBWERTSZEITEN DES AMINAUSTAUSCHS IN *p*-DIOXAN–ÄTHANOL (1:1, V/V). SALZSÄUREKONZENTRATION 0.1M, KONZENTRATION DER REAKTIONSPARTNER 0.05M. TEMP. 0.2°

Verbindung	*Halbwertszeit (Min)*
N-*p*-Tolyl-β-D-glucopyranosylamin (**1a**)	108
4,6-*O*-Benzyliden-*N*-*p*-tolyl-D-glucosylamin (**11**)	4
Schiffsche Base **12**	~0.5
Schiffsche Base **13**	sehr rasch

Kontrollversuche zeigten, daß **12** und insbesondere **13** bereits unter den alkalischen Aufarbeitungsbedingungen Austausch zeigen.

Zur Gleichgewichtslage bei Transglykosidierungen. — Da bei der Transglykosidierung das glykosidisch gebundene und freie Amin im allgemeinen chemisch verschieden sind, erhebt sich die Frage nach der Gleichgewichtslage. Wir untersuchten daher die Reaktion **1a**+**4b** ⇌ **1b**+**4a** in 0.01M Lösung der Reaktionspartner in 0.1N und 1.0N äthanolischer Salzsäure von beiden Seiten. In 0.01N HCl ergab sich sowohl ausgehend von **1a** wie ausgehend von **1b** eine Gleichgewichtskonstante von 81 und in NHCl eine Konstante von 2,400. Das *N*-Glucosyl-Derivat mit der schwächeren Base ist unter diesen Bedingungen stabiler.

Das Gleichgewicht stellte sich in 0.01N HCl bei 0.2° in 1–2 Stdn. ein und in N HCl in *ca.* 6 Stdn.

Versuche, aus **1a** und *N*-Methyl-*p*-nitranilin durch Transglykosidierung *N*-Methyl-*p*-nitrophenyl-glucopyranosylamin darzustellen, waren unter den im Versuchsteil angegebenen Bedingungen nicht erfolgreich. Die Methode ist offensichtlich nicht universell.

Zur Frage der Transglykosidierung des Typs 2. — Die Transglykosidierung vom Typ 2 mit **1b** (0.01M) und markierter D-Glucose (0.01M) in wasserfreiem Äthanol ergab, daß die Reaktion bei 0° und 0.01N HCl nach 49 Stdn. nicht oder weniger als zu 0.02% abgelaufen war. Wir halten es für zweifelhaft, ob die Reaktion überhaupt abläuft. Unter den von Bognár und Nánási[4] angegebenen Bedingungen (Siedehitze und Verwendung von Glycosylaminen, die rasch Amadori-Umlagerung ergeben, und dann unter Wasserabspaltung weiter reagieren) kann selbst bei ursprünglich wasserfreiem Arbeiten genügend Wasser für eine Hydrolyse entstehen. In diesem Zusammenhang ist es von Interesse, daß sich Glykosylamine von sekundären schwachen Aminen sehr schwer bilden. So ist es uns nach den üblichen Darstellungsmethoden für **1a** oder **1b** und wesentlich drastischeren Bedingungen (vgl. Beschreibung der Versuche) nicht gelungen, die *N*-Glucosyl-Derivate von *N*-Methyl-*p*-nitranilin oder *N*-Methyl-*p*-toluidin darzustellen. Da zur Transglykosidierung nach Typ 2 ebenfalls ein sekundäres Amin von einem freien Zucker angegriffen werden müßte, spricht auch diese Erfahrung mit den *N*-Methylderivaten von *p*-Toluidin und *p*-Nitranilin gegen diese Reaktion.

Bestimmung der Mutarotationsgeschwindigkeit, Art der Säurekatalyse unter Transglucosidierungsbedingungen und Vergleich mit der Geschwindigkeit der Transglucosidierung. — Da Messungen der Mutarotation von **1a** unter den Bedingungen der Transglucosidierung nicht bekannt sind, bestimmten wir die RG unter verschiedenen Bedingungen. Die Ergebnisse sind in Tab. V wiedergegeben.

Auch für die Mutarotation untersuchten wir die Art der Säurekatalyse. Die Ergebnisse mit einer Reihe von Phenol- und Benzoesäurederivaten sind in Tab. VI zusammengestellt. Das Bronsted-Diagramm (Abb. 5), in dem der log der RG gegen die log der Ka-Werte der Säuren aufgetragen ist, ergibt eine Steigung α von 0.98 $\pm$0.02, was zeigt, daß die allgemeine Säurekatalyse nur eine untergeordnete Rolle spielt.

TABELLE V

HALBWERTSZEIT DER MUTAROTATION VON *N*-*p*-TOLYL-D-GLUCOPYRANOSYLAMIN (0.0371M) UNTER VERSCHIEDENEN BEDINGUNGEN (VERSUCHE NR. 1–15 MIT α-FORM, VERSUCHE 16–18 MIT β-FORM

Vers. Nr.	*Lösungsmittel*	*Temp.* (°)	*Katalysator und Bedingungen*	*Halbwertszeit (Min) und Bemerkungen*
1	Methanol	25.0	—	keine Veränderung nach 3 Stdn.
2	Methanol–Wasser (1:1)	25.0	—	99
3	Methanol–Wasser (1:1)	25.0	Wasser wurde von gelöstem CO_2 befreit	160
4	Methanol	25.0	CH_3COOH/CH_3COONa = 1:1, 0.04M	14
5	Methanol–Wasser (1:1)	0.2	CH_3COOH/CH_3COONa = 1:1, 0.04M	~1
6	Methanol	−10.0	HCl 0.005N	<0.2
7	Methanol	−20.0	HCl 0.005N	<0.2
8	Methanol	20.0	HCl 0.0005N	92
9	Methanol	−20.0	HCl 0.5N	sehr schnell
10	Methanol	−20.0	HCl 8N	sehr schnell
11	Methanol	25.0	CH_3COONa 0.08M	1930
12	Methanol	23.5	CH_3COOH/CH_3COONa = 1:1, 0.04M	11.2[a]
13	Methanol	23.5	CH_3COOH/CH_3COONa = 1:1, 0.04M und *p*-Toluidin[b]	10.5[a]
14	Methanol	23.5	CH_3COOH/CH_3COONa 1:1, 0.08M	6.8
15	Methanol	23.5	CH_3COOH/CH_3COONa = 1:1, 0.08M und *p*-Toluidin[b]	6.5
16	Methanol	20.0	NaOH 0.01N	2400
17	Methanol	20.0	NaOH 0.1N	200
18	Methanol	20.0	NaOH 1N	20

[a]Unter diesen Bedingungen ist die Halbwertszeit der Transglucosidierung für die α-Verbindung 5.9 ±0.4 Min. und für die β-Verbindung 13.3 ±1.8 Min.
[b]0.0371M.

TABELLE VI

MUTAROTATION VON *N*-*p*-TOLYL-β-D-GLUCOPYRANOSYLAMIN IN WASSERFREIEM METHANOL BEI 20.0° UNTER DEM EINFLUSS VERSCHIEDENER SÄUREN. KONZENTRATION = 1.00%

Katalysator	*Konzentration* (M)	*Halbwertszeit* (*Min*)
Benzoesäure	5.0×10^{-4}	9.75; 9.90; 9.60
Benzoesäure	5.0×10^{-3}	0.91; 0.92
p-Chlorbenzoesäure	5.0×10^{-4}	3.75; 3.87; 3.81
m-Nitrobenzoesäure	5.0×10^{-4}	1.0; 1.0; 1.0
p-Nitrobenzoesäure	5.0×10^{-4}	0.87; 0.85; 0.86
Phenol	5.0×10^{-2}	1020
m-Nitrophenol	5.0×10^{-2}	44
m-Nitrophenol	5.0×10^{-3}	500
p-Nitrophenol	5.0×10^{-3}	77.2; 68.0
2,4-Dinitrophenol	5.0×10^{-4}	0.325; 0.325

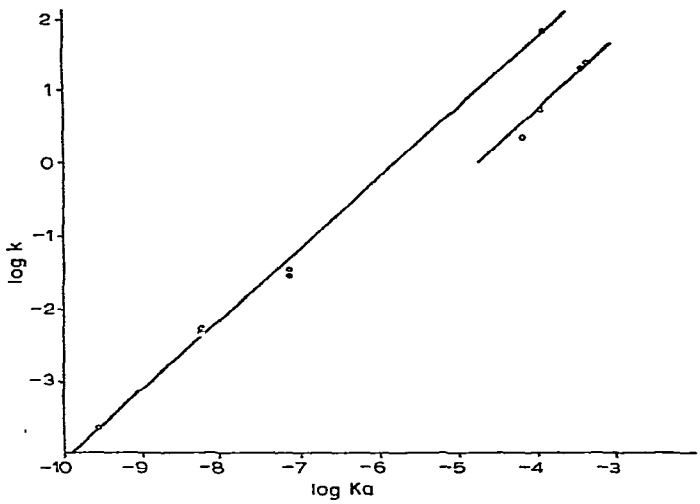


Abb. 5. Abhängigkeit des Logarithmus der RG der Mutarotation von *N*-*p*-Tolyl-β-D-glucopyranosylamin in 1-prozentiger methanolischer Lösung bei 20° von den Logarithmen der Dissoziationskonstanten verschiedener Phenole und Benzoesäuren. Obere Gerade von links unten nach rechts oben: Phenol, *m*-Nitrophenol, *p*-Nitrophenol, 2,4-Dinitrophenol. Untere (kurze) Gerade von links unten nach rechts oben: Benzoesäure, *p*-Chlorbenzoesäure, *m*-Nitrobenzoesäure, *p*-Nitrobenzoesäure.

DISKUSSION

Die von Bognár und Nánási[4] angegebene Reaktionsfolge entspricht einem SN1-Mechanismus. Die Austauschgeschwindigkeit sollte daher nullter Ordnung in bezug auf freies Amin sein. Wie die Abb. 1 und 2 bzw. die Tab. II zeigen, ist die Transglucosidierung erster Ordnung in bezug auf das Glucosylamin und das freie Amin. Unabhängig davon wären bei einem Carboniumion als Zwischenstufe u.a. Solvolyseprodukte zu erwarten, da nicht anzunehmen ist, daß das Carboniumion besonders selektiv ist. Das Äthanol weist eine *ca.* 1700fach höhere Konzentration auf.

Wie wir fanden, tritt bei **1a** unter Bedingungen, bei denen die Transglucosidierung eine Halbwertszeit zwischen 5 und 10 Min. hat, innerhalb von 3 bzw. 4 Stdn. keine meßbare (d.h. <2%) Solvolyse auf. Diese Befunde schließen den Mechanismus *A* aus.

Für eine S_N2-Reaktion (Mechanismus *B*) spricht die Konzentrationsabhängigkeit der Transglucosidierung. Auch die ausgeprägte Abhängigkeit der RG von der Säurekonzentration läßt sich erklären.

Für den S_N2-Mechanismus sollte für die RG gelten:

$$RG = k[GNH_2^+Ar]\,[H_2NAr]$$

Da jedoch die glucosidisch gebundenen Anilinderivate um *ca.* 4 p_K-Einheiten schwächere Basen sind, als die entsprechenden freien Anilinderivate[7], durchläuft die RG in Abhängigkeit von der Säurekonzentration ein Maximum, das bei einem p_H-Wert zwischen dem p_K-Wert des freien Amins und des Glucosylamins liegen sollte. Dies wäre, falls man die für Wasser gültigen p_K-Werte heranzieht, für **1a** bei einer Säurekonzentration von 10^{-3} und für **1b** von 10^{1} zu erwarten. Die beobachteten Werte liegen bei Konzentrationen, die 1–2 Größenordnungen tiefer liegen. Da diese Messungen in Äthanol durchgeführt wurden, wurde eine quantitative Beziehung nicht aufgestellt.

Sollte der S_N2-Mechanismus gültig sein, so sollte die Reaktion mit einer Konfigurationsumkehr an C-1 verbunden sein, d.h. daß die Mutarotation z.B. von **1a** in Gegenwart von freiem *p*-Toluidin erhöht ist. Eine entsprechende Untersuchung ist jedoch nur unter Bedingungen möglich, bei denen die RG der Mutaroration geringer oder vergleichbar mit der der Transglucosidierung ist. Ein Vergleich der Versuche 12 und 13 bzw. 14 und 15 in Tab. V zeigt, daß die RG der Mutatotation von **1a** in der α-Form durch freies Amin nicht signifikant erhöht wird, wie es zu erwarten wäre, wenn die Transglucosidierung, die unter diesen Bedingungen eine Halbwertszeit von 5.9 ±0.4 Min. hat, unter Konfigurationsumkehr erfolgen würde. Man muß aus diesen Versuchen weiter folgern, daß bei der Transglucosidierung von α-**1a** ausschließlich oder stark bevorzugt wieder α-D-Glycosylamin entsteht. Sonst müßte, wegen der unter diesen Verhältnissen rascheren Transglucosidierung, die Mutarotation doch scheinbar rascher gehen. Die Halbwertszeit ist für die β-Form von **1a** unter diesen Bedingungen *ca.* 3mal länger als für die α-Form.

Der von **1** über **2'** zu **1'** führende Weg des Mechanismus *C* ist mit den Ergebnissen der Mutarotation der α-Form von **1a** mit und ohne *p*-Toluidinzusatz gut verständlich. Der Austausch würde unter Konfigurationserhalt erfolgen. Auch die Abhängigkeit der Reaktionsgeschwindigkeit von der Säurekonzentration steht damit in Einklang. Das Maximum der RG sollte bei einer Säurekonzentration liegen, bei der das freie Amin vollkommen protonisiert ist, ohne daß Protonisierung am Glykosylamin erfolgt wäre. Bei einem p_H-Wert, der absolut um eine Einheit kleiner ist als der p_K-Wert, ist das freie Amin fast völlig protonisiert und das Glykosylamin jedoch erst in Spuren[7]. Allerdings wäre für diesen Mechanismus für die stark eingeengte Bewegungsfreiheit zwischen den Reaktionspartnern im Übergangszustand eine höhere negative Aktivierungsentropie als −5 bis −10 (cal/Mol. Grad) zu erwarten.

Der über **6** und **7** verlaufende Weg könnte unter Bedingungen eine Rolle spielen bei denen die Mutarotation rascher verläuft als die Transglykosidierung (vgl. hierzu auch das zum Mechanismus *D* Gesagte).

Mechanismus *E* kann aufgrund der gefundenen spezifischen Säurekatalyse für die Transglucosidierung keine wesentliche Rolle spielen. Es wurde im System Methanol Essigsäure–Natriumacetat keine Abhängigkeit der RG von der Pufferkonzentration gefunden. Für die Mutarotation ist dieser Mechanismus jedoch wahrscheinlich, da die Mutarotation auch alkalisch zu katalysieren ist (Tab. V, Vers. 16–18) und die Halbwertszeit bei Verdoppelung der Pufferkonzentration fast halbiert wird (Tab. V, Vers. 12 und 14).

Recht wahrscheinlich ist Mechanismus *D* für die p_H-Bereiche, in denen die Mutarotation sehr viel rascher erfolgt als die Transglucosidierung. Hier dürften die Übergänge **8**→**9**→**6** oder **8**→**6** stattfinden. Die Schiffsche Base reagiert dann rasch nach einem Additions-Eliminierungsmechanismus. Die stark zunehmende Austauschgeschwindigkeit in der Reihe **1a**, **11**, **12** bzw. **13** zeigt, daß dieser letzte Schritt sehr rasch verläuft.

Die Substanz **11** reagiert deshalb rascher als **1a**, da die ringoffene Form im Gleichgewicht gegenüber der Ringform relativ zu **1a** bevorzugt sein dürfte. Bei **12** und **13** ist nur die Schiffsche Base möglich. Die p_H-Abhängigkeit ist wie folgt zu erklären. Die Mutarotation steigt mit zunehmender Säurekonzentration rasch an (Vers. 1–9, Tab. V) und nimmt auch bei hohen Konzentrationen nicht wieder ab (Vers. 10, Tab. V). D.h., daß der Übergang **8**→**9** mit zunehmender Säurekonzentration rascher wird. In dem Maße, wie das freie Amin protonisiert wird, ist jedoch eine Addition an die Schiffsche Base nicht mehr möglich. Damit nimmt zwar die Mutarotation mit zunehmender Säurekonzentration zu, die RG der Transglucosidierung wird jedoch nach Erreichen eines Maximums wieder geringer.

BESCHREIBUNG DER VERSUCHE

1. *Allgemeines.* — Die Schmelzpunkte sind unkorrigiert.

Zur Radioaktivitätsanalyse wurde *p*-Nitranilin oder sein Glucosid in Schöniger-Kolben[8] verbrannt und die Lösungen in einem Flüssigkeits-Szintillations-Zähler Marke TRI CARB vermessen. Dazu wurden 5 ml der anfallenden 10 ml Absorptionslösung (4proz. Äthanolamin in Methanol) mit 15 ml einer Lösung von 4.0 g "Omnifluor" der Firma New England Nuclear Chicago pro l Toluol vermischt. Das *p*-Toluidin wurde als Hydrochlorid in Mengen von 6–7 mg der obigen Mischung gelöst und gemessen. Häufig wurden Doppelbestimmungen durchgeführt.

Die Mutarotationen wurden mit dem Polarimeter 141 der Firma Perkin–Elmer in thermostatisierten Küvetten vermessen und auf einem Schreiber aufgezeichnet. Bei Drehwerten $<2°$ war die Anzeige besser als $\pm 0.002°$.

2. *Lösungsmittel.* — Käufliches wasserfreies, benzolvergälltes Äthanol wurde über Calciumhydrid unter Rückfluß gekocht und destilliert. Vor- und Nachlauf wurden verworfen. Methanol wurde über eine 1.5-m Kolonne destilliert, über

Magnesiumspänen unter Rückfluß erhitzt und sodann erneut destilliert. Die Reinigung von Dioxan erfolgte nach lit. cit.[9]. Die alkoholischen Salzsäuren wurden unter peinlichem Feuchtigkeitsausschluß durch Einleiten von HCl-Gas aus einer Stahlflasche in die wasserfreien Alkohole dargestellt.

3. *Darstellung der Verbindungen.* — *N-p*-Tolyl-β-D-glucopyranosylamin (**1a**) wurde nach Weygand[10] und die α-Verbindung nach Ellis und Honeyman[11] gewonnen. Ersteres zeigte nach zweimaligem Umkristallisieren aus 96proz. Äthanol einen Schmp. von 133–134°; $[\alpha]_D^{20}$ −107.3° (*c* 1, abs. Methanol). Bei der Darstellung der α-Verbindung wurde auf strikten Feuchtigkeitsausschluß geachtet. Im Gegensatz zu Befunden in lit. cit.[11] gelang es, die Verbindung aus wasserfreiem Äthanol umzukristallisieren. Schmp. 140°, $[\alpha]_D^{20}$ +210° (*c* 1, abs. Methanol).

N-p-Nitrophenyl-β-D-glucopyranosylamin (**1b**) wurde nach Weygand und Mitarbb.[12] gewonnen. Nach Umkristallisieren aus Methanol und Trocknen bei 110° im Vakuum über P_2O_5. Schmp. 185–188°, $[\alpha]_D^{25}$ −164° (*c* 1, abs. Methanol).

p-Nitranilin-*t* und *p*-Toluidin-*t* wurden nach lit. cit.[6] erhalten und mit nichtmarkiertem Material auf eine Radioaktivität von *ca.* 1000–2000 ipm*/mg verdünnt.

4,6-*O*-Benzyliden-*N-p*-tolyl-D-glucopyranosylamin wurde aus 4,6-*O*-Benzyliden-D-glucose, die nach L. Zervas[13] dargestellt worden war, und *p*-Toluidin nach Micheel und Frowein[14] gewonnen.

Die Schiffsche Base **12** wurde aus 3,4,5,6-Tetra-*O*-benzoyl-D-glucose nach Brigl[15] mit *p*-Toluidin nach Micheel und Dijong[16] dargestellt.

Die Schiffsche Base **13** : 600 mg Glykolaldehyd (10 mmole) wurden in 100 ml Äthanol unter Erwärmen gelöst und noch warm mit 1071 mg (10 mmole) *p*-Toluidin versetzt. Nach kurzem Aufkochen kristallisierten nach 24stündigem Stehen im Kühlschrank 1010 mg (67%) aus, Schmp. 144°

$C_9H_{11}NO$ (149.02) Gef.: C, 72.6; H, 7.66; N, 9.42.
Ber.: C, 72.5; H, 7.40; N, 9.40.

4. *Allgemeine Arbeitsmethodik für die Austauschreaktionen.* — Das Glucosylamin wurde gelöst und die Lösung in einem Thermostaten auf Reaktionstemperatur (±0.1°) gebracht. Im gleichen Thermostaten wurde eine dem Glucosylamin entsprechende äquimolare Lösung des Amins im gleichen Lösungsmittel zusammen mit dem Katalysator temperiert. Durch Vereinigen der beiden Lösungen wurde die Reaktion gestartet. Nach passenden Zeiten wurden 50 ml Proben gezogen und unter Kühlung durch Einfließenlassen in wäßrige Natronlauge auf p_H 9–10 gebracht. Bei Untersuchungen von **1a** wurde daraus nach Zugabe von weiteren 50 ml Wasser bei *ca.* 50° Badtemperatur und 20 Torr das wasserdampfflüchtige Toluidin in 15 ml alkoholische 2N Salzsäure überdestilliert. Das Destillat wurde am Rotationsverdampfer zur Trockne gebracht, 20 ml Äthanol zum Rückstand gegeben und erneut abdestilliert. Das hinterbleibende *p*-Toluidin-hydrochlorid wurde über KOH und P_2O_5 getrocknet und sodann analysiert.

Bei Untersuchungen von **1b** wurde nach dem Stoppen der Reaktion die Lösung

*imp = Impulse pro Minute.

am Rotationsverdampfer unter Vakuum fast bis zur Trockene eingeengt. Der Rückstand wurde in 30 ml Wasser aufgenommen, wobei sich das Glucosylamin löste und der größte Teil des *p*-Nitranilins ungelöst blieb. Falls das Amin analysiert werden sollte, wurde dieses abgesaugt und durch Umkristallisation aus Wasser–Methanol gereinigt. Zur Isolierung des Glucosylamins wurde die wäßrige Lösung zweimal mit 20 ml Äther ausgeschüttelt, die wäßrige Phase bis zur Trockene schonend eingeengt, der Rückstand in 10 ml heißen Methanol aufgenommen, filtriert und das umkristallisierte **1b** abgesaugt. Nach Waschen mit Methanol–Äther und Trocknen war es analysenrein.

Kontrollversuche zeigten, daß unter diesen Aufarbeitungsbedingungen sowohl die RG der Transglucosidierung und der Hydrolyse vernachlässigbar sind. Eine Ausnahme machte die Schiffsche Base **13**. Die Tritium-Markierung der Amine war unter diesen Bedingungen völlig stabil.

5. *Beispiel einer Austauschkinetik.* — Ein Reaktionsansatz von 300 ml wasserfreiem Äthanol (HCl = 0.01N) enthielt **1b** und **4b** in einer Konzentration von 0.005M bei +0.2°. Nach 30, 60, 90, 120 und 180 Min. wurden Proben entnommen. Die Radioaktivität des eingesetzten *p*-Nitranilins betrug 134.9 ipm/μMol und das nach den angegebenen Zeiten isolierte **1b** 23.4; 38.7; 51.0; 55.2 und 60.9 ipm/μMol. Dies entspricht einem Austausch von 34.7; 57.4; 75.7; 81.9 und 90.4%. Die graphisch ermittelte Halbwertszeit betrug 47.5 Min.

6. *Versuche zur Ermittlung des Gleichgewichts* **1a**+**4b** $\rightleftharpoons$ **1b**+**4a**. — Konzentration der Reaktionspartner (**1a**+**4b**) 0.01M in 0.1N äthanolischer HCl. Nach 5; 10; 60 und 780 Min. wurden Proben entnommen und das in Freiheit gesetzte **4a** durch Verdünnungsanalyse bestimmt. Nach den angegebenen Zeiten hatten sich 37.5; 62.5; 86.0 und 90.0% **1b** gebildet. Bei dem entsprechenden Gegenversuch (**1b**+**4a**) wurden nach 80; 240 und 1320 Min. Proben genommen. Nach diesen Zeiten hatten sich 6.5; 10.5 und 11.0% **1a** gebildet.

In N äthanolischer HCl wurde entsprechend verfahren.

7. *Bestimmung der Mutarotation.* — Die Lösung des Glucosylamins und die des Katalysators wurden getrennt auf die Reaktionstemperatur gebracht und je 5 ml vereinigt (Zeit = 0). Das Gemisch wurde sodann rasch in die thermostatisierte Küvette gefüllt und vermessen. Der Gleichgewichtszustand wurde für **1a** bei $[\alpha]_D^{20}$ −45.5° gefunden, was mit lit. cit.[11] übereinstimmt. In N NaOH wurde allerdings $[\alpha]_D^{20}$ −51.2° für den Gleichgewichtszustand und −119° fur den Ausgangsdrehwert bestimmt. Die RG-Konstanten wurden aus der Beziehung $\ln(A-A_\infty)/(A_t-A_\infty) = kt$ berechnet.

8. *Versuche zur Darstellung von* N-*Glucosyl-Derivate des* N-*Methyl*-p-*toluidins und* N-*Methyl*-p-*nitranilins.* — Die Methoden nach Weygand[10], Ellis und Honeyman[11] sowie Weygand und Mitarbb.[12] führten auch bei wesentlich längeren Versuchszeiten und erhöhten Temperaturen nicht zum Ziel.

Auch die Umsetzung von Tetra-*O*-acetyl-α-D-glucopyranosylbromid mit **4a** bzw. **4b** in Pyridin oder Nitromethan in Gegenwart von Barium-carbonat führte nicht zum Erfolg.

Die Transglucosidierung von *N*-*p*-Tolyl-β-D-glucopyranosylamin (**1a**) und *N*-Methyl-*p*-nitranilin führte auch nach 12 Tagen in 0.1N äthanolischer Salzsäure bei 0° nicht zum Ziel. Von dem sekundären Amin hatten sich weniger als 4% umgesetzt. Bei Raumtemperatur wurden die Reaktionsansätze schnell stark dunkelbraun.

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STUDIES ON URONIC ACID MATERIALS

PART XXX*. EXAMINATION OF THREE FRACTIONS OBTAINED FROM *Acacia drepanolobium* GUM

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ABSTRACT

Dissolution of *A. drepanolobium* gum Fraction *C* (the water-insoluble gel) has been attempted with eight solvents. Sodium borohydride solution (1%) was found to be the best solvent, giving a 69% yield of Soluble-fraction *C*. Molecular-sieve chromatography with Bio-Gel P300 indicated that Soluble-fraction *C* has a much higher molecular weight than the cold water-soluble gum (Fraction *A*), and this has been confirmed by light-scattering measurements. Smith-degradation and methylation analyses have indicated that Fractions *A*, *B*, and Soluble-fraction *C* are structurally very similar.

INTRODUCTION

In a previous paper[2], Fraction *A* (water soluble), Fraction *B* (soluble in M sodium chloride) and Fraction *C* (the insoluble gel) of *A. drepanolobium* gum were compared analytically. Four aldobiouronic acids were also characterised after hydrolysis of Fraction *A* with N sulphuric acid. In this Paper, methods for dissolving Fraction *C* are explored. After dissolution in 1% sodium borohydride solution, Soluble-fraction *C* is compared with Fractions *A* and *B* in terms of (*a*) Smith degradation and methylation analyses, and (*b*) molecular weight measurements, in order to ascertain whether the insolubility of Fraction *C* arises from structural or from molecular weight differences.

MATERIALS AND METHODS

The gum nodules from *A. drepanolobium* Harms ex Sjöstedt were collected by Mr. W. M. C. Bagshawe, Provincial Forest Officer, at Tabora, Western Province, Tanganyika, in July 1961. Powdered *A. drepanolobium* gum (45 g) was fractionated as described previously[2] to give Fractions *A* (35 g), *B* (3.5 g), and *C* (5.0 g).

The standard analytical methods have been described previously[3]. Paper chromatography was carried out on Whatman No. 1 paper with the following solvent

*For Part XXIX, see Ref. 1.

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*) butyl alcohol–acetic acid–water (4:1:5, upper layer); (*f*) butanone–water–conc. ammonia (200:17:1). R_G values of methylated sugars refer to distances moved relative to 2,3,4,6-tetra-*O*-methyl-D-glucose in solvent (*d*). G.l.c. of mixtures of *O*-methyl sugars was carried out as described previously[4] on columns of (*i*) 15% by weight of poly(butane-1,4-diol succinate) on 60–70 mesh Celite (120 × 0.5 cm) at 175°, and (*ii*) 15% by weight of poly(ethylene glycol adipate) on 60–70 mesh 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. Polysaccharides were methylated successively with methyl sulphate and sodium hydroxide solution, and with methyl iodide and silver oxide. Methanolyses were carried out under reflux for 7 h with 5% methanolic hydrogen chloride.

Light-scattering measurements were made with a SOFICA Model 42000 Photo Gonio Diffusometer, using unpolarised green light (5460 Å). Weight-average molecular weights, $\overline{M}_w$, were calculated as described previously[5,6].

RESULTS

Attempts to dissolve Fraction C. — It has already been shown[2] that Fraction *C* is soluble in N sodium hydroxide to the extent of 1% (w/v). This method of dissolution is not entirely satisfactory, since alkaline degradation of the polysaccharide will probably occur. Different methods of dissolution were therefore attempted. Samples of Fraction *C* (120 mg) were extracted with (*i*) distilled water; (*ii*) 3M sodium chloride; (*iii*) 3M magnesium chloride; (*iv*) 7M urea; (*v*) 2% disodium ethylenediaminetetraacetate solution (w/v); (*vi*) 2% sodium hexametaphosphate solution (w/v); (*vii*) 1% sodium borohydride solution (w/v); (*viii*) 0.1M phosphate buffer, pH 9.0; (*ix*) 3% sodium borate solution (w/v). Each extract was filtered, dialysed against tap water for 48 h, and freeze-dried. Table I shows the percentage of Fraction *C* dissolved by these solvents. All of the freeze-dried products were water-soluble.

TABLE I

SOLUBILITY OF FRACTION *C* IN DIFFERENT SOLVENTS

Solvents	*Percentage of Fraction* C *dissolved*
Distilled water	1
3M Sodium chloride	1.5
3M Magnesium chloride	1.5
7M Urea	2
3% Solution (w/v) of sodium borate	7
0.1M Phosphate buffer, pH 9.0	10.5
2% Solution (w/v) of sodium salt of ethylenediamine tetraacetate	17.5
2% Solution (w/v) of sodium hexametaphosphate	47
1% Solution (w/v) of sodium borohydride	69

The best solvent for Fraction *C* is undoubtedly 1% sodium borohydride solution (w/v). Fraction *C* (3.75 g) was therefore extracted with this solvent (400 ml), filtered, dialysed against tap water for 48 h, electrodialysed, and freeze-dried, to give Soluble-fraction *C* (2.25 g), for which the analytical data are shown in Table II. Hydrolysis (N sulphuric acid for 7 h at 100°) of Soluble-fraction *C*, followed by paper chromatography, indicated the presence of galactose, arabinose, rhamnose, and the same four aldobiouronic acids which were detected[2] in Fractions *A*, *B*, and *C*.

TABLE II

ANALYTICAL DATA FOR SOLUBLE-FRACTION *C*

	Soluble-fraction C
Moisture, %	10.2
Nitrogen, %	0.85
Intrinsic viscosity, cm^3g^{-1}	11.1
$[\alpha]_D$ (*c* 1.0, water)	+79.5°
Equivalent weight	1955
Hence, uronic acid anhydride, %	9.0
Galactose, %	51
Arabinose, %	39
Rhamnose, %	1
Formic acid released on periodate oxidation (moles/g × 10^3)	1.05
Periodate reduced (moles/g × 10^3)	3.95

In order to check whether Fraction *C* had been degraded by treatment with 1% sodium borohydride solution (w/v), Fractions *A*, *B*, and Soluble-fraction *C* were examined by molecular-sieve chromatography with a previously calibrated[3,7] "Bio-Gel P300" column (45 × 4.5 cm). The elution volume of Soluble-fraction *C* was less than that of "Blue Dextran 2000"; Fractions *A* and *B* were eluted at the same elution volume as "Blue Dextran" (Pharmacia, Uppsala, Sweden). This suggested that Soluble-fraction *C* had a higher molecular weight tnan Fractions *A* and *B*. Light-scattering measurements subsequently gave weight-average molecular weights of 9.5×10^5, 10.2×10^5, and 22.0×10^5, for Fractions *A*, *B*, and Soluble-fraction *C*, respectively.

Methylation analysis of Fractions A, B, *and Soluble-fraction* C. — The three fractions (300 mg) were methylated exhaustively. Yields, specific rotations, and methoxyl contents (not raised on further attempted methylation) of the products are shown in Table III. On methanolysis and g.l.c. examination of the mixture of methyl glycosides, the methylated products of each fraction gave identical traces by using columns (*i*) and (*ii*). Results of g.l.c. examination are shown in Table IV; retention times are comparable with those for methyl glycosides from authentic *O*-methyl sugars. A portion of the methanolysate from each methylated product was hydrolysed with N sulphuric acid for 4 h on a boiling-water bath. The cooled solutions were neutralised with barium carbonate, filtered, treated with Amberlite resin IR-

120(H^+), and concentrated. Paper-chromatographic examination of the hydrolysates in solvents (*d*) and (*f*) showed the presence of 2-*O*-methylgalactose and 4-*O*-methylarabinose, in addition to those *O*-methyl sugars already identified by g.l.c. of their methyl glycosides (Table IV).

TABLE III

DATA FOR METHYLATED FRACTIONS *A*, *B*, AND SOLUBLE-FRACTION *C*

	Fraction A	*Fraction* B	*Soluble-fraction* C
Yield[a] (mg)	240	250	200
$[\alpha]_D$ (*c* 1.0, chloroform)	+60°	+61°	+59°
OMe, %	40.8	40.7	41.0

[a]From 300 mg of each fraction.

TABLE IV

EXAMINATION OF METHANOLYSIS AND HYDROLYSIS PRODUCTS FROM METHYLATED FRACTIONS *A* AND *B*, AND METHYLATED SOLUBLE-FRACTION *C*

Relative retention times (T) *of methyl glycosides on columns*[a]: (*i*)	(*ii*)	R_G *in solvent* (d)	O-*Methyl sugars*
0.48	(0.50)		2,3,4-tri-*O*-methyl-L-rhamnose
0.55 ; 0.73	(0.50); 0.64	0.97	2,3,5-tri-*O*-methyl-L-arabinose
(1.05)	(0.82)	0.79	2,3,4-tri-*O*-methyl-L-arabinose
1.80 ; 3.20	1.26 ; (2.20)	0.82	2,5-di-*O*-methyl-L-arabinose
(1.05); (2.36)	(0.82); 1.76	0.82	3,5-di-*O*-methyl-L-arabinose
1.98	1.43	0.56	3,4-di-*O*-methyl-L-arabinose
1.68	1.65	0.88	2,3,4,6-tetra-*O*-methyl-D-galactose
(3.05); (3.89); (4.22)	2.44 ; (3.00); (3.44)	0.73	2,3,6-tri-*O*-methyl-D-galactose
(3.89); (4.22)	(3.00); (3.44)	0.73	2,4,6-tri-*O*-methyl-D-galactose
6.42	5.08	0.73	2,3,4-tri-*O*-methyl-D-galactose
14.6 ; 16.4	9.9 ; 11.4	0.52	2,4-di-*O*-methyl-D-galactose
(2.36); (3.05)	(2.20); 2.75		2,3,4-tri-*O*-methyl-D-glucuronic acid[b]
		0.32	2-*O*-methyl-D-galactose
		0.35	4-*O*-methyl-L-arabinose

[a]Figures in parentheses indicate *T* values of components which are incompletely resolved. [b]As methyl ester methyl glycoside.

Smith degradation[8] *of Fractions*, A, B, *and Soluble-fraction* C. — The purified fractions (1.5 g of polysaccharide) were dissolved in water (37.5 ml), and 0.25M sodium metaperiodate (37.5 ml) was added. The oxidation mixtures were left in darkness for 48 h at room temperature, when the reactions were stopped by addition of ethylene glycol (2 ml). The solutions were dialysed against tap water for 48 h. Sodium borohydride (0.5 g) was added to each solution, and the mixtures were kept for 30 h at room temperature and then dialysed for a further 48 h. The three poly-

alcohols were hydrolysed in N sulphuric acid for 48 h at room temperature, and the hydrolysates were neutralised with barium carbonate, filtered, and deionised with Amberlite resin IR-120 (H^+). The solutions were each dialysed against distilled water (1 litre) for 24 h, and then against tap water for 48 h. The three Smith-degraded polysaccharides were isolated as freeze-dried products, and the dialysates were concentrated to syrups.

Yields and analytical data for the three Smith-degraded polysaccharides are shown in Table V. Hydrolysis of the Smith-degraded polysaccharides (N sulphuric acid for 7 h at 100°), followed by paper chromatography, indicated the presence of only galactose and arabinose. The Smith-degraded polysaccharides were hydrolysed with 0.5N sulphuric acid for 1 h on a boiling-water bath. Paper-chromatographic examination 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*), 0.54 in solvent (*c*)] and 6-*O*-*β*-D-galactopyranosyl-D-galactose [R_{Gal} 0.31 in solvent (*a*), 0.38 in solvent (*c*), major component].

TABLE V

ANALYTICAL DATA FOR THE SMITH-DEGRADED POLYSACCHARIDES

	Fraction A	*Fraction* B	*Soluble-fraction* C
Moisture, %	11.9	10.5	10.8
Nitrogen, %	1.01	0.98	0.32
Yield of polysaccharide (g)	1.11	1.14	1.12
Yield, %	55.5	57	56
Galactose, %	43	44	44
Arabinose, %	57	56	56
$[\alpha]_D$ (*c* 1.0, water)	+45°	+45°	+45°
Formic acid released on periodate oxidation [mole/g] × 10^3	0.94	0.96	0.97
Periodate consumed [mole/g] × 10^3	3.38	3.47	3.45
Ratio of $\frac{\text{periodate consumption}}{\text{formic acid released}}$	3.59	3.62	3.56

Chromatographic examination of the three dialysates in solvents (*a*) and (*c*) showed the presence of glycolaldehyde and glycerol, and traces of ethylene glycol and threitol. The traces of ethylene glycol would be expected from non-reducing, arabinopyranosyl end-groups. The traces of threitol would be expected from D-galactopyranosyl residues having a substituent on C-4, as in the α-D-(1→4)-linked aldobiouronic acids previously detected in the hydrolysates of Fractions *A*, *B*, and *C*.

Methylation analysis of Smith-degraded products of Fractions A, B, *and Soluble-fraction* C. — The three degraded products (200 mg) were methylated exhaustively by using the methods of Haworth and Purdie. Yields, specific rotations, and methoxyl contents (not raised on further attempted methylation) of the products are shown in Table VI. On methanolysis and g.l.c. examination of the mixtures of methyl

glycosides with columns (*i*) and (*ii*), the three methylated products gave identical results. The results of the g.l.c. examination are shown in Table VII. A portion of each methanolysate was hydrolysed (N sulphuric acid for 4 h at 100°). The cooled solutions were neutralised with barium carbonate, filtered, treated with Amberlite resin IR-120(H^+), and concentrated. Paper-chromatographic examination of the hydrolysates in solvents (*d*) and (*f*) showed the presence of 2,6-di- and 2-*O*-methyl-galactose, in addition to those *O*-methyl sugars already identified by g.l.c. of their methyl glycosides (Table VII).

TABLE VI

DATA FOR METHYLATED, SMITH-DEGRADED FRACTIONS *A*, *B*, AND SOLUBLE-FRACTION *C*

	Fraction A	*Fraction* B	*Fraction* C
Yield[a] (mg)	156	150	155
$[\alpha]_D$ (*c* 1.0, chloroform)	+39°	+39°	+38°
OMe, %	40.1	40.0	40.1

[a]From 200 mg of each fraction.

TABLE VII

EXAMINATION OF METHANOLYSIS AND HYDROLYSIS PRODUCTS FROM METHYLATED, SMITH-DEGRADED POLYSACCHARIDES

Relative retention times (T) *of methyl glycosides on columns:* — (*i*)	(*ii*)	R_G *in solvent* (d)	*Hence,* O-*methyl sugars identified*
0.56; 0.74	0.50; 0.63	0.97	2,3,5-tri-*O*-methyl-L-arabinose
1.06; 2.36	0.81; 1.76	0.82	3,5-di-*O*-methyl-L-arabinose
1.80; 3.22	1.26; 2.20	0.82	2,5-di-*O*-methyl-L-arabinose
1.68	1.65	0.88	2,3,4,6-tetra-*O*-methyl-D-galactose
3.90; 4.22	3.00; 3.45	0.73	2,4,6-tri-*O*-methyl-D-galactose
6.45	5.08	0.73	2,3,4-tri-*O*-methyl-D-galactose
14.6; 16.4	9.9; 11.4	0.52	2,4-di-*O*-methyl-D-galactose
		0.55	2,6-di-*O*-methyl-D-galactose
		0.32	2-*O*-methyl-D-galactose
1.53	1.05		unknown sugars

DISCUSSION

A. drepanolobium gum Fraction *C* is sparingly soluble (≯ 1% w/v) in M sodium hydroxide. This method of dissolution is not satisfactory, since degradation may occur under such alkaline conditions. Eight different aqueous solvents for Fraction *C* were therefore investigated (Table I). 3M Sodium chloride, 3M magnesium chloride, and 7M urea were unsuccessful solvents; hydrogen bonding between polysaccharide molecules is therefore unlikely to be the major reason for the insolubility of Fraction *C*. 3% Sodium borate solution and 0.1M phosphate buffer dissolved Fraction *C* to the

extent of 7 and 10.5%, respectively; these solvents have pH 9, and this may account for the slight solubilisation effected. Fraction *C* was dissolved to a greater extent by 2% disodium ethylenediaminetetra-acetate solution (17.5%) and by 2% sodium hexametaphosphate solution (47%). The action of these solvents is probably dependent on the chelation of calcium and magnesium[9,10], and cross-linking by polyvalent metal ions might contribute to the insolubility of Fraction *C* in aqueous solutions. The higher solubility in sodium hexametaphosphate solution is expected, since its optimum conditions[11] are at neutral pH and room temperature (*i.e.*, the conditions of extraction); the optimum conditions for disodium ethylenediaminetetra-acetate solution[9] are at pH 10 and 60–70°.

The best solvent found for Fraction *C* is 1% sodium borohydride solution. This has pH 9, and contains borate ions, but these facts cannot account for the dissolution action of this solvent in view of the results reported above for sodium borate solution and for phosphate buffer. Dilute sodium borohydride solution has also been found[12] to be an effective solvent for water-insoluble gums from the genera *Combretum*, *Lannea*, and *Teclea*.

Fraction *C* was extracted with 1% sodium borohydride solution in a large-scale experiment to yield Soluble-fraction *C* as the freeze-dried, electrodialysed product. The yield of solubilised gum was 69%, based on the estimated dry weight of the insoluble gel. This represents virtually complete recovery of the polysaccharide present; all of the bark and other foreign matter in the gum sample had inevitably become concentrated in the gel (Fraction *C*).

Analysis showed Soluble-fraction *C* to have the same sugar percentages as Fraction *C*; on periodate oxidation, the two fractions released the same amount of formic acid and reduced the same amount of periodate.

Fractions *A*, *B*, and Soluble-fraction *C* were compared by using methylation analysis. The methylated products were obtained in similar yields, and had the same specific rotations. On methanolysis and g.l.c. examination, the three methylated products gave identical results. On Smith degradation, Fractions *A*, *B*, and Soluble-fraction *C* gave similar yields of Smith-degraded products. The analytical data (Table V) for the Smith-degraded polysaccharides are almost identical, and methylation analyses of the three degraded polysaccharides gave identical results. There is therefore a close structural similarity between Fractions *A*, *B*, and Soluble-fraction *C*.

Soluble-fraction *C* has a lower intrinsic viscosity than that obtained[2] in M sodium hydroxide solution for Fraction *C*. Molecular-sieve chromatography on a previously calibrated Bio-Gel P300 column was therefore carried out to determine if the decrease in viscosity was the result of degradation of the polysaccharide during the extraction with sodium borohydride solution. Soluble-fraction *C* was eluted before the elution volume of "blue dextran"; in contrast, Fractions *A* and *B* were eluted at the same elution volume as for "blue dextran". Soluble-fraction *C* could therefore differ from Fractions *A* and *B* in having *either* a much higher molecular weight, *or* a different molecular shape[13]. Differences in shape imply differences in molecular structure, such as the frequency or pattern of branching. Since the Smith degradation

and methylation analyses showed that the three fractions are closely similar structurally, it seems unlikely that the differences between Fractions *A*, *B*, and Soluble-fraction *C* are due to differences in molecular shape. Light-scattering measurements confirmed that Soluble-fraction *C* has a much higher weight-average molecular weight (22.0×10^5) than Fraction *A* (9.5×10^5) and Fraction *B* (10.2×10^5). The precise reasons why sodium borohydride solution dissolves Fraction *C* are not known; the interactions which cause the polysaccharide molecules to aggregate seem to be counteracted irrevocably, since, after freeze-drying, the product (Soluble-fraction *C*) remains water-soluble. Although it appears that gross degradation does not occur during the solubilising process, the possibility of some degradation's occurring cannot be excluded. The true molecular weight of the gel (Fraction *C*) may beconsiderably higher than that indicated by the experiments involving Soluble-fraction *C*.

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STUDIES ON URONIC ACID MATERIALS

PART XXXI*. SOME STRUCTURAL FEATURES OF THE WATER-SOLUBLE FRACTION OF *Acacia drepanolobium* GUM

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ABSTRACT

The *O*-methyl derivative of the water-soluble fraction of the gum from *Acacia drepanolobium* Harms ex Sjöstedt yielded 2,3,5- and 2,3,4-tri-, and 2,5-, 3,5-, and 3,4-di-*O*-methyl-L-arabinose, 2,4-di-*O*-methyl-D-galactose, 2,3,4-tri-*O*-methyl-D-glucuronic acid, and small proportions of 2,3,4-tri-*O*-methyl-L-rhamnose, 4-*O*-methyl-L-arabinose, 2,3,4,6-tetra-, 2,4,6-, 2,3,6-, and 2,3,4-tri-, and 2-*O*-methyl-D-galactose. The water-soluble gum was subjected to five successive Smith-degradations, and the *O*-methyl derivatives of the degraded polysaccharides were analysed, after methanolysis, by gas–liquid partition chromatography. The structural evidence obtained indicates that arabinose side-chains, some of which are considerably longer than in other *Acacia* gums studied to date, are attached to a compact, branched galactan framework.

INTRODUCTION

The gum exudate from *A. drepanolobium* Harms ex Sjöstedt is not completely soluble in cold distilled water[2]. Extraction of the gum with water gives Fraction *A* (*ca.* 80%), and extraction of the residue with M sodium chloride solution gives Fraction *B* (*ca.* 8%), leaving the insoluble gel, Fraction *C* (*ca.* 12%). A comparative examination of these three Fractions has been reported[1,3]; a study of Fraction *A* by partial, acid hydrolysis has indicated a close similarity to *A. arabica* gum[4]. In this Paper, five successive Smith-degradations, and methylation analyses, are utilized to establish the main features of the molecular structure of Fraction *A* of *A. drepanolobium gum.*

RESULTS AND DISCUSSION

The gum exudate from *A. drepanolobium* Harms ex Sjöstedt was collected by Mr. W. M. C. Bagshawe, Provincial Forest Officer, at Tabora, Western Province, Tanganyika, in July 1961. After aqueous extraction and isolation[3], the gum Fraction *A* had the specific rotation and sugar composition shown in Table I. Samples of Fraction *A* were examined by zone electrophoresis on cellulose acetate film, and by

*For Part XXX, see Ref. 1.

ion-exchange chromatography on DEAE-cellulose[5]; no sharp discontinuities in properties were indicated. It is therefore possible that Fraction *A* of *A. drepanolobium* gum exhibits the same kind of heterogeneity as the gum exudates of *A. arabica*[6], *A. laeta*[7], and *A. senegal*[8], comprising a continuous spectrum of related molecular species.

TABLE I

DATA FOR *A. drepanolobium* GUM FRACTION *A* AND SMITH-DEGRADED POLYSACCHARIDES *A–E*

Polysaccharide	$[\alpha]_D$ (*degrees*)	*Constituent sugars* (%)					*Yield* (%)	*Periodate reduced* (*mmoles/g*)	*Formic acid released* (*mmoles/g*)
		Gal	*Ara*	*Rha*	*GlcA*	*4-O-Me GlcA*			
Fraction *A*	+78	38	52	1	7	2	—	3.96	1.05
A	+45	43	57	—	—	—	55.5	3.38	0.94
B	+20	46	54	—	—	—	42	4.18	1.47
C	−2	33	67	—	—	—	26.7	3.78	1.15
D	−21	32	68	—	—	—	20.3	1.87	0.53
E	−36	29	71	—	—	—	29.5	1.05	0.35

An examination of the *O*-methyl derivative of Fraction *A* showed the presence of 2,3,5- and 2,3,4-tri-, and 2,5-, 3,5-, and 3,4-di-*O*-methyl-L-arabinose, 2,4-di-*O*-methyl-D-galactose, 2,3,4-tri-*O*-methyl-D-glucuronic acid, and small proportions of 2,3,4-tri-*O*-methyl-L-rhamnose, 4-*O*-methyl-L-arabinose, and 2,3,4,6-tetra-, 2,4,6-, 2,3,6-, and 2,3,4-tri-, and 2-*O*-methyl-D-galactose (See Table II).

TABLE II

EXAMINATION OF METHANOLYSIS AND HYDROLYSIS PRODUCTS FROM METHYLATED FRACTION *A*

Relative retention times (T) *of methyl glycosides*[a]		R_G *in solvent* (d)	*O-Methyl sugars*
Column (i)	*Column* (ii)		
0.48	(0.50)		2,3,4-Tri-*O*-methyl-L-rhamnose
0.55; 0.73	(0.50); 0.64	0.97	2,3,5-Tri-*O*-methyl-L-arabinose
(1.05)	(0.82)	0.79	2,3,4-Tri-*O*-methyl-L-arabinose
1.80; 3.20	1.26; (2.20)	0.82	2,5-Di-*O*-methyl-L-arabinose
(1.05); (2.3)	(0.82); 1.76	0.82	3,5-Di-*O*-methyl-L-arabinose
1.98	1.43	0.56	3,4-Di-*O*-methyl-L-arabinose
1.68	1.65	0.88	2,3,4,6-Tetra-*O*-methyl-D-galactose
(3.05); (3.89); (4.22)	2.44; (3.00); (3.44)	0.73	2,3,6-Tri-*O*-methyl-D-galactose
(3.89); (4.22)	(3.00); (3.44)	0.73	2,4,6-Tri-*O*-methyl-D-galactose
6.42	5.08	0.73	2,3,4-Tri-*O*-methyl-D-galactose
14.6; 16.4	9.9; 11.4	0.52	2,4-Di-*O*-methyl-D-galactose
(2.36); (3.05)	(2.20); 2.75		2,3,4-Tri-*O*-methyl-D-glucuronic acid[b]
		0.32	2-*O*-Methyl-D-galactose
		0.35	4-*O*-Methyl-L-arabinose

[a]Figures in parenthesis indicate *T* values of components which are not completely resolved. [b]As methyl ester methyl glycoside.

The small proportions of monomethyl-galactose and monomethyl-arabinose are not thought to be structurally significant; the presence of 2-*O*-methyl-D-galactose is ascribed to under-methylation, and Smith-degradation studies suggest that the 4-*O*-methyl-L-arabinose arises similarly. The high proportion of 2,4-di-*O*-methyl-D-galactose shows that most of the D-galactose residues are 3,6-di-*O*-substituted. The small proportions of 2,3,4,6-tetra-, and 2,4,6-, 2,3,6-, and 2,3,4-tri-*O*-methyl-D-galactose show that there are a few terminal, non-reducing D-galactose residues, and some 3-*O*-, 4-*O*-, and 6-*O*-substituted D-galactose units. The identification of 2,3,4-tri-*O*-methyl-D-glucuronic acid indicates that D-glucuronic acid (and a small proportion of its 4-methyl ether) occur as terminal, non-reducing units. The presence of 2,3,4-tri-*O*-methyl-L-rhamnose indicates that most, if not all, of the L-rhamnose (1%) is present as non-reducing end-group, and, by analogy with *A. senegal* gum[9,10], it is possible that these residues are attached to some of the D-glucuronic acid residues.

The presence of 2,3,5- and 2,3,4-tri-*O*-methyl-L-arabinose shows that the arabinose side-chains are terminated by either L-arabinofuranose or L-arabinopyranose residues. The identification of some 2,5-di-*O*-methyl-L-arabinose indicates the presence of some 3-*O*-substituted L-arabinofuranose residues in these arabinose chains. The major di-*O*-methyl derivative of arabinose present was 3,5-di-*O*-methyl-L-arabinose, which has previously been isolated from the methylated gums of *A. pycnantha*[11,12], *A. arabica*[6], *A. karroo*[13], and *A. nubica*[14], and detected in the methanolysates of the methylated gums from *A. mearnsii*, *A. giraffae*, *A. decurrens*, and *A. cyanophylla*[15]. The presence of 3,5-di-*O*-methyl-L-arabinose in methylated Fraction *A* shows that there is a large proportion of 2-*O*-substituted L-arabinofuranose residues in the arabinose chains; the detection of 3,4-di-*O*-methyl-L-arabinose indicates that these chains also contain a small proportion of 2-*O*-substituted L-arabinopyranosyl residues. 3,4-Di-*O*-methyl-L-arabinose has previously been isolated from the methylated gums from *A. arabica*[6] and *A. karroo*[13], and a trace of this *O*-methyl sugar has been detected in the methanolysate of the methylated gum from *A. podalyrifolia*[15]. Methylation evidence for Fraction *A* of *A. drepanolobium* gum and for autohydrolysed[4] Fraction *A* suggests that the arabinose chains are attached to a branched galactan framework at the 3 and 6 positions of certain D-galactose residues.

A sample of Fraction *A* was subjected to five successive Smith-degradations; the percentage yields of the products (polysaccharides *A–E*) are shown in Table I. Although these yields are lower than those obtained from successive Smith-degradations of *A. senegal*[8] and *A. laeta* gums[7], they are higher, particularly for the later degradations, than were obtained from *A. arabica* gum[6]. The relative decrease in molecular weight from Fraction *A* to its first Smith-degraded product, polysaccharide *A*, is similar to that reported for *A. senegal* gum[8]. Molecular-sieve chromatography indicates a drastic decrease in molecular size during the second Smith-degradation, *i.e.*, from polysaccharide *A* (M_n *ca.* 95,500) to polysaccharide *B*(M_n *ca.* 9,200). The third, fourth, and fifth Smith-degradations did not cause such relatively large decreases in M_n, but the yields from these degradations are low. The pattern

of this sequence of Smith-degradations differs from that reported for *A. laeta*[7], *A. senegal*[8], and *A. arabica* gums[6]. Four successive Smith-degradations were required to remove all of the arabinose units from *A. senegal* gum[8], and five degradations were required for *A. laeta* gum[7]. For *A. arabica* gum[6], four successive Smith-degradations decreased the arabinose content, but the fourth Smith-degraded product contained a considerable proportion of arabinose; it was concluded[6] that the arabinose chains in *A. arabica* are at least six units long. A similar conclusion was reached for *A. nubica* gum[14]. In contrast, five successive Smith-degradations of Fraction *A* of *A. drepanolobium* gum led to an increase in the arabinose content, from 52% for the gum Fraction *A* to 71% for the fifth degradation product (polysaccharide *E*).

On methanolysis, the *O*-methyl derivatives of the Smith-degradation polysaccharides *A–E* gave the proportions of the methyl glycosides of the methylated sugars shown in Table III. The absence of arabinopyranose residues in polysaccharide *A* suggests that the 4-*O*-methyl-L-arabinose obtained from methylated Fraction *A* is not structurally significant. 3,5-Di-*O*-methyl-L-arabinose is by far the most abundant *O*-methyl sugar in polysaccharide *E*; this permits a calculation that shows that gum Fraction *A* contains chains of at least eight arabinose residues, with the possibility that much longer arabinose chains may be present.

TABLE III

RELATIVE PROPORTION OF *O*-METHYL SUGARS PRESENT IN METHYLATED POLYSACCHARIDES *A–E*

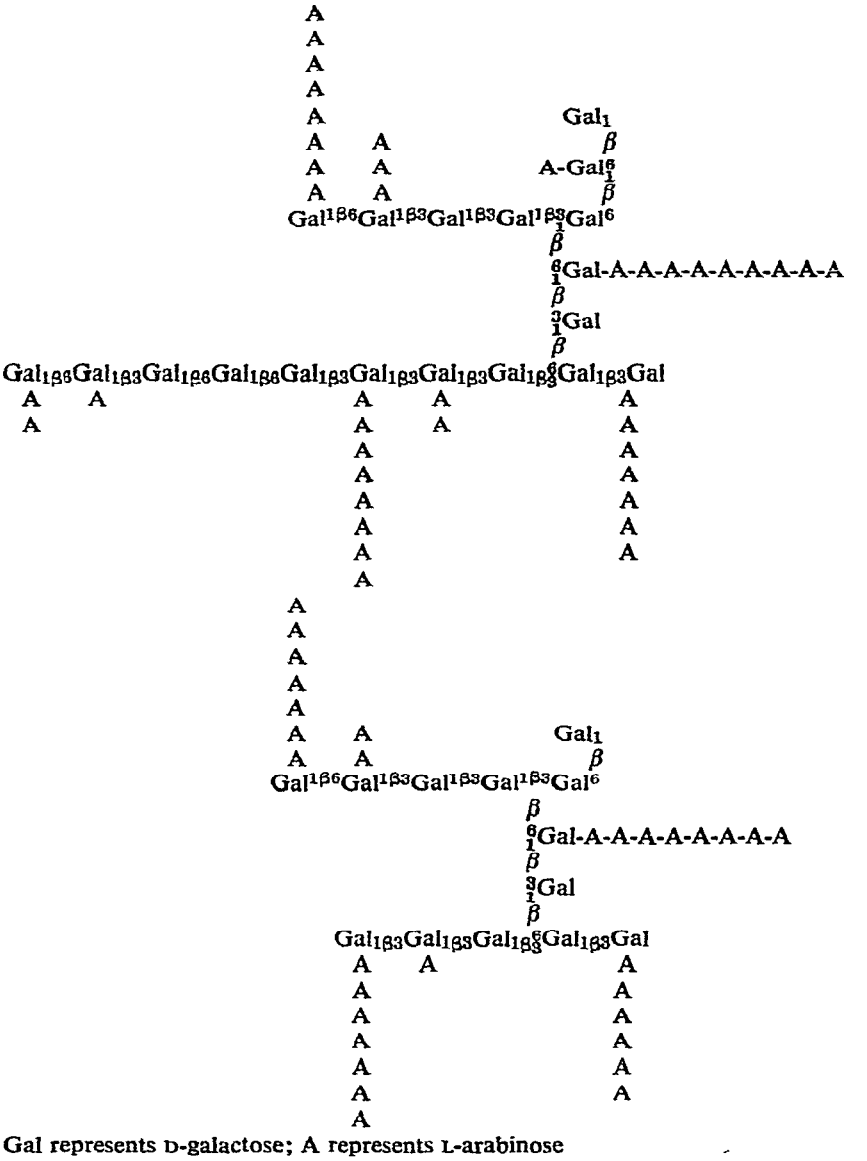
O-Methyl sugar	A	B	C	D	E
2,3,5-Tri-*O*-methyl-L-arabinose	4	2	2	2	4
2,5-Di-*O*-methyl-L-arabinose	4	2	2	1	1
3,5-Di-*O*-methyl-L-arabinose	7	6	8	9	10
2,3,4,6-Tetra-*O*-methyl-D-galactose	trace	trace	1	1	1
2,4,6-Tri-*O*-methyl-D-galactose	3	4	2	2	2
2,3,4-Tri-*O*-methyl-D-galactose	1	2	1	1	1/2
2,4-Di-*O*-methyl-D-galactose	3	3	1	1	1

From the evidence obtained, some of the structural features of Fraction *A* of *A. drepanolobium* gum can be deduced. Smith degradation and methylation analysis of gum Fraction *A* and the autohydrolysed gum[4] indicate that long chains of β-(1→3)-linked D-galactose residues are not such a dominating feature of Fraction *A* of *A. drepanolobium* gum as they are of *A. senegal* gum[8] and *A. laeta* gum[7]. Smith degradation and methylation analysis of the autohydrolysed gum suggest that the branched galactan frameworks of *A. drepanolobium* gum are closely similar to those of *A. arabica* gum, being more compact and highly branched than those of *A. senegal* gum[4]. In these compact frameworks, blocks of periodate-resistant β(1→3)-linked D-galactose residues must be interspersed with blocks of periodate-vulnerable β-(1→6) linked D-galactose residues. The first Smith-degradation of Fraction *A* of *A. drepanolobium* gum appears to proceed similarly to that of *A. senegal* gum[8], although arabinose residues must be removed from the C-3 positions of 6-*O*-substituted D-galactose

residues, since the second degradation causes a large drop in M_n. In the third, fourth, and fifth Smith-degradations, the M_n of the degradation products falls only slightly, although the yields are in the 20–30% range. This implies that, at each of these stages, the majority of the polymer molecules are periodate-vulnerable to such an extent that drastic degradation, resulting in the formation of molecular fragments so small that they can escape during the dialysis process, takes place. In contrast, the minority (*ca.* 30%) of the molecular arrays at each stage are substantially stable, in their inner core, to periodate attack, so that they suffer comparatively little degradation, although a majority of these in turn become periodate-vulnerable at the ensuing stage. This indicates that, although some of the arabinose chains in the whole gum are comparatively long, there are also some short chains of two, three, or four arabinose units.

A scheme such as the following is necessary to explain the unusual increase in the relative arabinose content of the Smith-degraded polysaccharides *B*–*E*. If the first degradation removes single arabinose units and/or arabinose chains attached by 2-*O*-substituted L-arabinopyranose residues to the branched galactan framework, some periodate-vulnerable 6-*O*-substituted D-galactose residues will be formed in the core. The second degradation fragments the molecule drastically; those parts of the galactan framework which resist this periodate oxidation bear a high proportion of the arabinose chains or single arabinose residues. These will be progressively removed from the C-3 positions of 6-*O*-substituted D-galactose units in the subsequent degradations, leaving molecules that will be rapidly fragmented. On successive Smith-degradations, those structures having comparatively long arabinose chains attached to galactan frameworks consisting mainly of β-(1→3)-linked D-galactose residues will tend to survive. Fig. 1 shows one possible molecular array (based on the sugar ratios shown in Table I) that represents generally the broad spectrum of structures possible for the periodate-resistant portions of polysaccharide *D* which, in turn, give the Smith-degraded product, polysaccharide *E*, in small yield. This model illustrates the need to assign some unusually long arabinose chains to Fraction *A* of *A. drepanolobium* gum; if, in Fig. 1, polysaccharide *D* contained eight short chains of arabinose residues, instead of four longer chains averaging at least eight units (some may be considerably longer), the arabinose content of polysaccharide *E* would be less than that of polysaccharide *D*.

The experimental evidence therefore suggests that the typical molecules of Fraction *A* of *A. drepanolobium* gum possess a compact, branched galactan framework, to which are attached non-reducing, end-group residues of (4-*O*-methyl)-D-glucuronic acid and rhamnose. To the galactan framework, which has blocks of β-(1→3)-linked D-galactose residues interspersed with blocks of β-(1→6)-linked D-galactose residues, are attached some single arabinose residues, some short arabinose chains (less than 5 units), and some relatively long arabinose chains (>8 units). From the ratio of 2,5- to 3,5-di-*O*-methyl-L-arabinose obtained from the *O*-methyl derivatives of polysaccharides *A*–*E* (Table III), it seems probable that the short arabinose chains are predominantly 1,3-linked, with the longer chains predominantly 1,2-linked. Linkage analysis[4], methylation analysis, and Smith degradations show that the



Gal represents D-galactose; A represents L-arabinose

Fig. 1. Possible molecular structure of the periodate-resistant portions of polysaccharides *D* (above) and *E*.

structure of Fraction *A* of *A. drepanolobium* gum differs from that of *A. senegal* gum[8], but is similar to that of *A. arabica* gum[6]; the significant molecular difference between Fraction *A* of *A. drepanolobium* gum and *A. arabica* gum involves the arrangement and length of the arabinose chains. The longer arabinose chains in Fraction *A* of *A. drepanolobium* gum may result in its overall, average structure being less compact than that of *A. arabica* gum; this would explain the higher intrinsic viscosity of

Fraction *A* of *A. drepanolobium* gum, and its different behaviour[1,3,6] during molecular-sieve chromatography.

EXPERIMENTAL

The analytical methods have been described elsewhere in more detail[8,16]. Paper chromatography was carried out on Whatman Nos. 1 and 3MM papers with: (*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–acetic acid–formic acid–water (18:8:3:9); (*d*) butyl alcohol–ethanol–water (4:1:5, upper layer); (*e*) butyl alcohol–acetic acid–water (4:1:5, upper layer); (*f*) ethyl acetate–pyridine–water (10:4:3); (*g*) butanone–water–ammonia (sp. gr. 0.880) (200:17:1). R_{Gal} values of sugars and R_G values of *O*-methyl sugars refer to distances moved relative to those of galactose and 2,3,4,6-tetra-*O*-methyl-D-glucose, respectively. G.l.c. (Chromatograph Type S3A, fitted with flame-ionisation detectors; 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 Celite (5 ft × 0.25 in) at 175°; and (*ii*) 15% by weight of poly(ethylene glycol adipate) on Celite (3 ft × 0.25 in) at 260°. Retention times (*T*) are given relative to methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucopyranoside as standard. Polysaccharides were methylated successively with methyl sulphate and sodium hydroxide, and with methyl iodide and silver oxide. Methanolyses were carried out under reflux for 7 h with 5% methanolic hydrogen chloride.

Purification of A. drepanolobium *gum Fraction* A. — The preparation of the electrodialysed, freeze-dried sample of *A. drepanolobium* gum Fraction *A* used in the methylation study has been described previously[3]. A portion of *A. drepanolobium* gum was extracted with cold water, as described previously[3], to yield Fraction *A* (148 g from 180 g). The samples of Fraction *A* had $[\alpha]_D$ +78° (*c* 1.0) (Found: L-arabinose, 52; D-galactose, 38; L-rhamnose, 1; D-glucuronic acid, 7; 4-*O*-methyl-D-glucuronic acid, 2%; all sugar percentages are for the "anhydro forms" and are corrected for moisture and protein contents).

Fraction *A* migrated as a single band in 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[5] on a DEAE-cellulose column (45 × 1.3 cm). Gradient elution with sodium chloride solution (0.0–0.5M) in 0.02M acetate buffer (pH 4.1) yielded a single, slightly asymmetric peak for its elution pattern.

Methylation of A. drepanolobium *gum Fraction* A. — The purified gum (10 g) was methylated by the Haworth and Purdie procedures to give a product (5.9 g), $[\alpha]_D$ +60° (*c* 1.00, chloroform) (Found: OMe, 39.5%, not raised on further attempted methylation). Methanolysis of a portion of this product, followed by g.l.c. examination of the mixture of methyl glycosides, gave the results shown in Table II.

The methylated gum (5 g) was refluxed with 5% methanolic hydrogen chloride (500 ml) for 7 h. The methanolysate was cooled, neutralised with silver carbonate, filtered, and concentrated to a syrup. The methyl glycosides were removed by hydrolysis (N sulphuric acid for 4 h at 100°). The cooled solution was neutralised with barium carbonate, filtered, treated with Amberlite IR-120(H^+) resin, and concentrated to a syrup. This syrup was applied to a column (33 × 3 cm) of Duolite A4 in the formate form. Elution of the column with water (3 l) gave the neutral *O*-methyl sugar fraction; elution with 5% aqueous formic acid (500 ml) gave the acidic *O*-methyl sugar fraction.

The acidic *O*-methyl sugar fraction (165 mg) was refluxed with 5% methanolic hydrogen chloride for 7 h. The derived methyl ester methyl glycosides were reduced by refluxing in dry tetrahydrofuran (40 ml) with lithium aluminium hydride (300 mg) for 2 h. After cooling, the excess hydride was decomposed by addition of ethyl acetate and water. The mixture was evaporated to dryness, extracted exhaustively with chloroform, and concentrated to a syrup. The methyl glycosides were removed by hydrolysis (N sulphuric acid for 4 h at 100°). The cooled solution was neutralised with barium carbonate, filtered, treated with Amberlite IR-120(H^+) resin, and concentrated to a syrup (85 mg). Fractionation of this product on Whatman 3MM papers in solvent (*d*) yielded pure 2,3,4-tri-*O*-methyl-D-glucose (35 mg). The derived "anilide", after recrystallisation from ethyl acetate, had m.p. 146–148°; lit.[18] m.p. 145–150°.

A portion of the neutral *O*-methyl sugar fraction (3.5 g) was chromatographed on a cellulose column (100 × 3.5 cm) with light petroleum (b.p. 100–120°)–butyl alcohol (7:3, saturated with water) as eluant to give six fractions, and with light petroleum (b.p. 100–120°)–butyl alcohol (1:1, saturated with water) as eluant to give a further five fractions.

Fraction **1** (35 mg) from tubes 1–80. Paper chromatography in solvent (*d*) showed the presence of 2,3,4-tri-*O*-methyl-L-rhamnose (R_G 1.03) and 2,3,5-tri-*O*-methyl-L-arabinose (R_G 0.98). Demethylation gave arabinose and rhamnose. A portion (10 mg) was refluxed with 5% methanolic hydrogen chloride for 7 h. G.l.c. examination of the mixture of methyl glycosides indicated the presence of 2,3,4-tri-*O*-methyl-L-rhamnose [(*i*) *T* 0.48; (*ii*) *T* (0.50)] and 2,3,5-tri-*O*-methyl-L-arabinose [(*i*) *T* 0.56, 0.73; (*ii*) *T* (0.50), 0.64].

Fraction **2** (355 mg), from tubes 81–190, had $[\alpha]_D$ −19° (*c* 1.00). It had the same paper-chromatographic mobility [R_G 0.98 in solvent (*d*) and 1.00 in solvent (*g*)] as 2,3,5-tri-*O*-methyl-L-arabinose. It was characterized by conversion into 2,3,5-tri-*O*-methyl-L-arabinonamide which, after recrystallisation from acetone, had m.p. 133–135°; lit.[18] m.p. 134–138°].

Fraction **3** (93 mg), from tubes 191–280, had $[\alpha]_D$ +27° (*c* 0.93). Demethylation gave galactose and arabinose. A portion was refluxed with 5% methanolic hydrogen chloride for 7 h. G.l.c. examination of the mixture of methyl glycosides indicated the presence of 2,3,4,6-tetra-*O*-methyl-D-galactose [(*i*) *T* 1.68; (*ii*) *T* 1.66] and 2,3,5-tri-[(*i*) *T* 0.56, 0.73; (*ii*) *T* 0.50, 0.64], and 2,5- [(*i*) *T* 1.75, 3.29; (*ii*) *T* 1.24, 2.20] and 3,5-di-*O*-methyl-L-arabinose [(*i*) *T* 1.04, 2.40; (*ii*) *T* 0.85, 1.76].

Fraction **4** (770 mg), from tubes 281–420, had $[\alpha]_D$ +26° (*c* 1.00). A portion

(10 mg) was refluxed with 5% methanolic hydrogen chloride for 7 h. G.l.c. examination of the mixture of methyl glycosides indicated that 2,5- [(*i*) *T* 1.76, 3.29; (*ii*) *T* 1.24, 2.20] and 3,5-di-*O*-methyl-L-arabinose [(*i*) *T* 1.04, 2.40; (*ii*) *T* 0.85, 1.76] were the main components of the mixture. A portion (600 mg) was fractionated on Whatman 3MM papers in solvent (*d*) to yield a mixture (390 mg) of 2,5- and 3,5-di-*O*-methyl-L-arabinose. Paper-electrophoretic examination in 0.05M borate buffer confirmed the presence of 2,5- [M_G 0.00, 10 volts/cm, 0.5 mamp/cm] and 3,5-di-*O*-methyl-L-arabinose [M_G 0.68, 10 volts/cm, 0.5 mamp/cm, brown spot (yellow under u.v.) with aniline oxalate[19]]. These components were separated from the syrupy mixture (200 mg) by paper electrophoresis in borate buffer[20] on a preparative scale. Zones were detected by spraying of side strips. Carbohydrate material and borate buffer were eluted from each zone; sodium ions were removed with Amberlite IR-120(H^+) resin, and borate was removed as methyl borate by repeated evaporation with methanol. 2,5-Di-*O*-methyl-L-arabinose (47 mg) was characterized by conversion into 2,5-di-*O*-methyl-L-arabinonamide which, after recrystallisation from ethyl acetate, had m.p. 130–131°; lit.[18] m.p. 122–132°]. 3,5-Di-*O*-methyl-L-arabinose (39 mg) was characterized by conversion into 3,5-di-*O*-methyl-L-arabinonamide which, after recrystallisation from ethyl acetate, had m.p. 139–141°; lit.[18] m.p. 144–145°].

Fraction **5** (181 mg), from tubes 421–650, had $[\alpha]_D$ +100° (*c* 1.81). Demethylation gave arabinose. Paper-chromatographic examination indicated the presence of 2,3,4-tri-*O*-methyl-L-arabinose [R_G 0.78 in solvent (*d*) and 0.54 in solvent (*g*)], in addition to 2,5- and 3,5-di-*O*-methyl-L-arabinose.

Fraction **6** (123 mg), from tubes 651–1200, had $[\alpha]_D$ +98° (*c* 1.23). Demethylation gave galactose and a trace of arabinose. A portion (20 mg) was refluxed with 5% methanolic hydrogen chloride. G.l.c. examination of the mixture of methyl glycosides indicated that 2,4,6- [(*i*) *T* (3.76), (4.20); (*ii*) *T* (3.05), (3.50)], 2,3,6- [(*i*) *T* 3.04, (3.76), (4.20); (*ii*) *T* 2.50, (3.05), (3.50)], and 2,3,4-tri-*O*-methyl-D-galactose [(*i*) *T* 6.45; (*ii*) *T* 5.25] were the main components. The identification of these three trimethylgalactoses was confirmed by paper chromatography involving double development in solvent (*g*).

Fraction **7** (56 mg), from tubes 1201–1520, had $[\alpha]_D$ +97° (*c* 1.12). A portion (6 mg) was refluxed with 5% methanolic hydrogen chloride. G.l.c. examination of the mixture of methyl glycosides showed the presence of 3,4-di-*O*-methyl-L-arabinose [(*i*) *T* 1.90; (*ii*) *T* 1.38]. The remainder of the fraction was chromatographed on Whatman 3MM papers in solvent (*d*) to give a product (10 mg). Demethylation gave arabinose. It had the same paper-chromatographic mobilities [R_G 0.56 in solvent (*d*) and 0.20 in solvent (*g*)] as an authentic specimen of 3,4-di-*O*-methyl-L-arabinose. A portion (4 mg) was converted into the methyl glycosides and methylated with methyl iodide (0.5 ml) and silver oxide (0.5 g) in *N*,*N*-dimethylformamide (0.5 ml). G.l.c. examination of the reaction mixture showed the presence of the methyl glycoside(s) of 2,3,4-tri-*O*-methyl-L-arabinose [(*i*) *T* 1.00; (*ii*) *T* 0.81].

Fraction **8** (170 mg), from tubes 1521–1640, had $[\alpha]_D$ +89° (*c* 0.85). Paper-chromatographic examination in solvent (*d*) showed that 2,4-di-*O*-methyl-D-galactose

was the main component of the mixture. After purification by thick-paper chromatography, it was characterized by conversion into 2,4-di-*O*-methyl-*N*-phenyl-D-galactosylamine which, after recrystallisation from acetone, had m.p. 214–216°; lit.[18] m.p. 214–228°.

Fraction **9** (45 mg), from tubes 1641–1770, had $[\alpha]_D$ +108° (*c* 0.45). Fractionation on Whatman 3MM papers in solvent (*d*) gave a product (15 mg). Demethylation gave arabinose. The *O*-methyl sugar had the same chromatographic mobility [R_G 0.35 in solvent (*d*) and 0.09 in solvent (*g*)] as an authentic specimen of 4-*O*-methyl-L-arabinose. A portion (5 mg) was converted into the methyl glycosides and methylated with methyl iodide (0.5 ml) and silver oxide (0.5 g) in *N*,*N*-dimethylformamide (0.5 ml). G.l.c. examination of the reaction mixture indicated the presence of the methyl glycoside(s) of 2,3,4-tri-*O*-methyl-L-arabinose [(*i*) *T* 1.00; (*ii*) *T* 0.82].

Fraction **10** (46 mg), from tubes 1771–1820, had $[\alpha]_D$ +88° (*c* 0.46). Paper-chromatographic examination in solvents (*d*) and (*g*) indicated that 2-*O*-methyl-D-galactose was the main component of the mixture.

Fraction **11** (14 mg) was obtained from tubes 1821–2000. Paper chromatography in solvents (*a*), (*b*), and (*f*) showed the presence of galactose (major component) and arabinose (minor component).

Preparation, partial hydrolysis with acid, and methylation of polysaccharide A. — The gum (123 g) was dissolved in water (3.1 l), mixed with 0.25M sodium metaperiodate (3.1 l), and left for 48 h. The amount of formic acid released in this time was 1.05 mmoles/g. The corresponding amount of periodate consumed was 3.96 mmoles/g. The reaction was stopped by addition of ethylene glycol (50 ml), and the solution dialysed against running tap-water for 48 h. Sodium borohydride (32 g) was added, and the mixture kept at room temperature for 30 h and then dialysed for a further 48 h. The polyalcohol was hydrolysed in N sulphuric acid for 48 h at room temperature. After dialysis against running tap-water for 48 h, polysaccharide *A* was isolated as the freeze-dried product (68.2 g; yield, 55.5%), $[\alpha]_D$ +45° (*c* 1.63) (Found: galactose, 43; arabinose, 57%). Molecular-sieve chromatography of polysaccharide *A* (20 mg) on a previously calibrated Bio-Gel P300 column[16,21,22] (45×4.8 cm) gave M_n *ca.* 95,500. (See Table I).

Polysaccharide *A* (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, 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 (*f*), minor component] and 6-*O*-β-D-galactopyranosyl-D-galactose [R_{Gal} 0.31 in solvent (*a*) and 0.38 in solvent (*f*), major component], and higher neutral oligosaccharides.

Polysaccharide *A* was methylated (for details, see Table IV), and methanolysis of a sample of the product, followed by g.l.c. examination of the mixture of methyl glycosides, gave the results shown in Table III. Hydrolysis of the methyl glycosides, followed by paper chromatography in solvents (*d*) and (*g*), indicated the presence of

2,6-di- and 2-*O*-methyl-D-galactose, in addition to those *O*-methyl sugars already characterized by g.l.c. of their methyl glycosides.

TABLE IV

METHYLATION OF SMITH-DEGRADED POLYSACCHARIDES

	Polysaccharide				
	A	B	C	D	E
Amount of polysaccharide used (mg)	200	179	136	124	186
Amount of product (mg)	110	85	60	53	65
$[\alpha]_D$ of product (degrees)	+39	+24	+2	−17	−29
OMe of product (%)	40.5	41.2	42.1	41.5	41.0

Preparation, partial hydrolysis with acid, and methylation of polysaccharides B–E. — The following weights of polysaccharide were periodate-oxidised, borohydride-reduced, and hydrolysed, and the corresponding weights of degraded polysaccharides were recovered as for the first Smith-degradation: polysaccharide *A* (67.4 g)→*B* (28.4 g); *B* (27.7 g)→*C* (7.4 g); *C* (6.9 g)→*D* (1.40) g; *D* (1.07 g)→*E* (0.32 g). The amount of periodate consumed and formic acid released on periodate oxidation, and the percentage yields, specific rotations, and constituent sugars of each degraded polysaccharide are given in Table I. Molecular-sieve chromatography of polysaccharides *B*–*E* indicated values for M_n of *ca.* 9,200, 8,000, 7,200, and 6,000, respectively.

Partial hydrolysis of polysaccharides *B*, *C*, and *D* with acid, followed by paper-chromatographic examination of the hydrolysates, indicated the presence of 3-*O*-β-D-galactopyranosyl-D-galactose (minor composent) and 6-*O*-β-D-galactopyranosyl-D-galactose (major component), and higher neutral oligosaccharides. The proportion of the β-(1→3)-linked disaccharide increased steadily from polysaccharide *B* to *D*. Partial hydrolysis of polysaccharide *E* with acid, 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 higher neutral oligosaccharides.

Polysaccharides *B*–*E* were methylated (Table IV). Methanolysis of a sample of the products, followed by g.l.c. examination of the mixtures of methyl glycosides, gave the results shown in Table III.

In the fourth and fifth Smith-degradations, the controlled acid hydrolysates were neutralised with barium carbonate, filtered, treated with Amberlite IR-120(H^+) resin, and dialysed against distilled water (2 l) for 24 h. After further dialysis against running tap-water, the respective degraded products were isolated. The dialysates from the fourth and fifth degradations were deionised and concentrated to syrups (1.75 and 0.35 g, respectively). Paper-chromatographic examination of the syrups indicated the presence of glycerol, glycolaldehyde, arabinose (a trace), and slower, non-reducing glycosides. Hydrolysis of the syrups, followed by paper-chromato-

graphic examination, indicated the presence of glycerol, glycolaldehyde, arabinose, and galactose (a trace).

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STUDIES ON URONIC ACID MATERIALS

PART XXXII*. SOME STRUCTURAL FEATURES OF THE GUM EXUDATE FROM *Acacia seyal* DEL.

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ABSTRACT

The gum polysaccharide exuded by *Acacia seyal* trees contains D-galactose (38%), L-arabinose (45%), L-rhamnose (4%), D-glucuronic acid (7%), and 4-*O*-methyl-D-glucuronic acid (6%). Linkage analysis yields 3-*O*-β-L-arabinopyranosyl-L-arabinose, 3-*O*-β-L-arabinofuranosyl-L-arabinose, 3-*O*-β-D-galactopyranosyl-D-galactose, and 6-*O*-β-D-galactopyranosyl-D-galactose. The *O*-methyl derivatives of *A. seyal* gum and degraded gum *A* (obtained by partial hydrolysis with 0.01N acid) were analysed, after methanolysis, by gas–liquid partition chromatography. Degraded gum *A* was subjected to a Smith degradation, and *A. seyal* gum was subjected to four successive Smith-degradations; the degradation products were examined by methylation and linkage analysis. Degraded gum *A* was also subjected to a controlled Smith-degradation to establish the nature of the reducing end-group. The structural evidence obtained indicates a strong similarity between *A. seyal* gum and *A. arabica* gum[2].

INTRODUCTION

It has already been shown[3] that *A. seyal* gum is not unusual in having a positive specific rotation and a low content of L-rhamnose that does not bear a unit molar correspondence to the uronic acid content. D-Glucuronic acid and its 4-methyl ether have been isolated from the gum and characterized[4]; acid hydrolysis, followed by preparative paper-chromatography, led to the isolation and characterization[4] of four aldobiouronic acids, *viz.*, 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose, 6-*O*-(4-*O*-methyl-β-D-glucopyranosyluronic acid)-D-galactose, 4-*O*-(α-D-glucopyranosyluronic acid)-D-galactose, and 4-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-D-galactose. An internodule analytical study of *A. seyal* gum has shown[5] that D-galactose, L-arabinose, and L-rhamnose are constituent sugars of the gum; for different nodules, the proportions of these sugars were found[5] to vary between 34–42%, 42–47%, and 1–8%, respectively. The results of a detailed structural study of *A. seyal* gum are reported in this Paper.

RESULTS AND DISCUSSION

A single nodule of gum from *A. seyal* Del. was used; it was collected, by the Gum Research Officer to The Republic of Sudan, from a botanically authenticated

*For Part XXXI, see Ref. 1.

tree at Umm Ruaba Forest Reserve, Eastern Kordofan, on March 9th, 1960. After purification, the gum polysaccharide had the specific rotation and sugar composition given in Table I. The gum was examined by zone electrophoresis, and by ion-exchange chromatography on DEAE-cellulose[6]. Since no sharp discontinuities in the properties of the molecular species were indicated by these experiments, it seems probable that *A. seyal* gum exhibits the same kind of heterogeneity that has been suggested for the gums of *A. senegal*[7,8], *A. arabica*[2], *A. laeta*[9], and *A. drepanolobium*[1,10]. If this is the case, *A. seyal* gum may be considered to contain a continuous spectrum of related molecular species.

TABLE I

DATA FOR *A. seyal* GUM AND SMITH-DEGRADED POLYSACCHARIDES α-δ

Polysaccharide	$[\alpha]_D$ (*degrees*)	*Constituent sugars* (%)					*Recovery* (%)	*Periodate* (*mmoles/g*) *reduced*	*Formic acid* (*mmoles/g*) *released*
		Gal	*Ara*	*Rha*	*GlcA*	*4-O-Me-GlcA*			
A. seyal gum	+59	38	45	4	7	6	—	3.89	0.76
Polysaccharide α	0	67	33	—	—	—	51	3.89	1.00
Polysaccharide β	−15	89	11	—	—	—	29	5.58	1.99
Polysaccharide γ	−24	95	5	—	—	—	12.5	4.89	1.56
Polysaccharide δ	−25	100	—	—	—	—	7	—	—

Mild, acid hydrolysis of the gum (0.01N sulphuric acid for 100 h at 100°) released all of the rhamnose, most of the arabinose, and some galactose. Degraded gum *A*, isolated after dialysis, contained D-galactose (82%), L-arabinose (3%), and uronic acid (15%); molecular-sieve chromatography gave an estimated value of 4,900 for its number-average molecular weight ($\overline{M}_n$). Light-scattering measurements gave a value of *ca.* 850,000 for the weight-average molecular weight ($\overline{M}_w$) of *A. seyal* gum. The extensive degradation on mild, acid hydrolysis is therefore much greater than can result from the simple removal of arabinose and rhamnose from the periphery of the molecule, nor can it have been caused by the presence of internal, acid-labile arabinofuranose residues, since acid hydrolysis of borohydride-reduced, degraded gum *A* yielded galactitol but not arabinitol. There is no evidence for any galactofuranose residues in the polysaccharide, although traces of di- and tri-*O*-methyl-D-galactofuranoses would be difficult to detect in the mixtures of methyl glycosides examined by g.l.c. Bouveng and Lindberg[11–15] have observed that certain galactopyranosidic bonds in highly branched arabinogalactans are unusually sensitive to mild, acid hydrolysis. It therefore seems probable that certain galactopyranosidic bonds in *A. seyal* gum are unusually sensitive to mild, acid hydrolysis, as has already been reported for *A. arabica*[2], *A. senegal*[7,8], *A. laeta*[9], and *A. drepanolobium*[1,10] gums.

An examination of methylated, degraded gum *A* showed the presence of 2,3,4,6-tetra- (++), 2,4,6- (++), 2,3,6- (+), 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 (see Table II). This shows that **1** and **2** are structural fragments of degraded gum *A*. Methylation analysis has shown the presence of traces of terminal, non-reducing L-arabinofuranose

L-Ara*f*-(1→ L-Ara*p*-(1→

1 **2**

residues in the autohydrolysed gum from *A. senegal*[7,8], and terminal, non-reducing L-arabinopyranose residues in the degraded gum *A* from *A. arabica*[2]. Traces of terminal, non-reducing L-arabinofuranose and L-arabinopyranose residues have been detected in the degraded gum *A* of *A. laeta*[9] and *A. drepanolobium* gum[10] Fraction *A*. Lithium aluminium hydride reduction of methylated, degraded gum *A*, followed by a Kuhn methylation[16], yielded 2,3,4,6-tetra-*O*-methyl-D-glucose after methanolysis (see Table III). This confirms that D-glucuronic acid and its 4-methyl ether are non-reducing end-groups in degraded gum *A*. After partial methanolysis of methylated, degraded gum *A* and hydrolysis of the methyl glycosides, the methylated aldobiouronic acids were separated from the neutral *O*-methyl sugars by ion-exchange chromatography. Methanolysis of the methylated aldobiouronic acids, followed by g.l.c. 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 **3**–**6** are structural fragments of degraded gum *A*. Similar experiments on the methylated, degraded gum *A* from *A. arabica*[2] and *A. drepanolobium*[10] Fraction *A* have shown that these gums contain fragments **3**, **4**, and **6**, and **3**–**6**, respectively.

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*, and partial hydrolysis with acid 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- (+),

TABLE II

EXAMINATION OF METHANOLYSIS AND HYDROLYSIS PRODUCTS FROM METHYLATED, DEGRADED GUM *A*

Relative retention times (T) *of methyl glycosides*[a]		R_G *in solvent* (d)	*Approximate relative molar proportions*	O-*Methyl sugar*
Column (i)	*Column* (ii)			
0.56; 0.73	0.50; 0.63	0.98	Trace	2,3,5-Tri-*O*-methyl-L-arabinose
1.02	0.82	0.79	Trace	2,3,4-Tri-*O*-methyl-L-arabinose
1.69	1.66	0.90	+ +	2,3,4,6-Tetra-*O*-methyl-D-galactose
(3.02); (3.80); (4.29)	2.50; (3.05); (3.51)	0.72	+	2,3,6-Tri-*O*-methyl-D-galactose
(3.80); (4.29)	(3.05); (3.51)	0.72	+ +	2,4,6-Tri-*O*-methyl-D-galactose
6.40	5.29	0.72	+ + + +	2,3,4-Tri-*O*-methyl-D-galactose
14.4; 16.3	9.9, 11.3	0.53	+ + +	2,4-Di-*O*-methyl-D-galactose
2.40; (3.02)	2.21; 2.72		+ +	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 parenthesis indicate *T* values of components which are not completely resolved. [b]As methyl ester methyl glycoside.

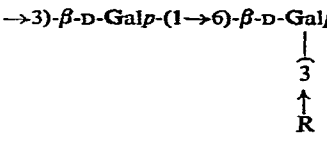
TABLE III

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

Relative retention times (T) *of methyl glycosides*[a]		Rg *in solvent* (d)	*Approximate relative molar proportions*	O-*Methyl sugar*
Column (i)	*Column* (ii)			
0.56; 0.73	0.50; 0.64	0.98	Trace	2,3,5-Tri-*O*-methyl-L-arabinose
(1.01)	0.82	0.79	Trace	2,3,4-Tri-*O*-methyl-L-arabinose
(1.01); 0.40	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
(3.02); (3.79); (4.29)	2.49; (3.05); (3.51)	0.72	+	2,3,6-Tri-*O*-methyl-D-galactose
(3.79); (4.29)	(3.05); (3.52)	0.72	++	2,4,6-Tri-*O*-methyl-D-galactose
6.40	5.29	0.72	+++	2,3,4-Tri-*O*-methyl-D-galactose
14.4; 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]Figures in parenthesis indicate *T* values of components which are not completely resolved.

2,6- (+/2) and 2,4-di- (+), and 2-*O*-methyl-D-galactose (+/2), and a trace of 2,3,4-tri-*O*-methyl-D-glucuronic acid (see Table IV). The presence of a trace of glucuronic acid in degraded gum *B* could arise from incomplete oxidation of degraded gum *A*, and the 2,6-di- and 2-*O*-methyl-D-galactose probably result from undermethylation. The proportion of the *O*-methyl-D-galactoses obtained from methylated, degraded gum *B* indicates that degraded gum *B* is a branched 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 indicates that **7** is a possible structural



7

where R = L-Araf-(1→, L-Arap-(1→, D-Galp-(1→, →4)-D-Galp-(1→, or →6)-D-Galp-(1→

unit of degraded gum *A*. A similar structural unit occurs in the degraded gum *A* of *A. arabica*[2] and *A. drepanolobium*[10] Fraction *A*.

A controlled Smith-degradation[7] of degraded gum *A* gave degraded gum *C*. Periodate oxidation of degraded gum *A* did not yield formaldehyde, and the reducing galactose residue is therefore substituted at C-6. Degraded gum *C* contains arabinitol; at least some of the reducing end-group galactose residues of degraded gum *A* are therefore[17] substituted at C-3.

Mild, acid hydrolysis of degraded gum *A* gave 3-*O*-β-D-galactopyranosyl-D-galactose and 6-*O*-β-D-galactopyranosyl-D-galactose. Two disaccharides obtained from the diffusate of the mild, acid hydrolysis of *A. seyal* gum were characterized as 3-*O*-β-L-arabinofuranosyl-L-arabinose and 3-*O*-β-L-arabinopyranosyl-L-arabinose; since these disaccharides are released under very mild acidic conditions, the reducing arabinose residues were probably present initially in the furanose form. Thus **8**, **9**, **10**, and **11** are structural fragments of *A. seyal* gum.

-β-D-Galp-(1→6)-D-Galp-(1→ **8**

-β-D-Galp-(1→3)-D-Galp-(1→ **9**

-β-L-Araf-(1→3)-L-Araf-(1→ **10**

-β-L-Arap-(1→3)-L-Araf-(1→ **11**

An examination of the *O*-methyl derivative of *A. seyal* gum indicated the presence of 2,3,4-tri-*O*-methyl-L-rhamnose (+/2), 2,3,5- (++) and 2,3,4-tri- (+) and 2,5- (+++++), 3,5- (++), and 3,4-di-*O*-methyl-L-arabinose (+), 2,4-di-*O*-methyl-D-galactose (++++++), 2,3,4-tri-*O*-methyl-D-glucuronic acid (+++), and small proportions of 4-*O*-methyl-L-arabinose, 2,3,4,6-tetra-, 2,3,6-, 2,4,6-, and 2,3,4-tri-, and 2-*O*-methyl-D-galactose, and 2,3-di-*O*-methyl-D-glucuronic acid (see Table V). The presence of 2-*O*-methyl-D-galactose is ascribed to under-methyl-

TABLE IV

EXAMINATION OF METHANOLYSIS AND HYDROLYSIS PRODUCTS FROM METHYLATED, DEGRADED GUM *B*

Relative retention times (T) *of methyl glycosides*		R$_G$ *in solvent* (d)	*Approximate relative molar proportions*	O-*Methyl sugar identified*
Column (i)	*Column* (ii)			
1.68	1.66	0.90	+ +	2,3,4,6-Tetra-*O*-methyl-D-galactose
3.75; 4.25	3.00; 3.44	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
2.57; 3.00	2.21; 2.74		Trace	2,3,4-Tri-*O*-methyl-D-glucuronic acid[a]
		0.59	+/2	2,6-Di-*O*-methyl-D-galactose
		0.33	+/2	2-*O*-Methyl-D-galactose

[a]As methyl ester methyl glycoside.

ation, and subsequent Smith-degradations of the gum suggested that the 4-*O*-methyl-L-arabinose probably arises similarly. The high proportion of 2,4-di-*O*-methyl-D-galactose indicates that most of the D-galactose residues in *A. seyal* gum are 3,6-di-*O*-substituted, although the identification of trace proportions of 2,3,4,6-tetra-, 2,3,6-, 2,4,6-, and 2,3,4-tri-*O*-methyl-D-galactose indicates that there are a few, terminal, non-reducing D-galactose residues, and some 4-*O*, 3-*O*, and 6-*O* substituted D-galactose residues, respectively. The identification of 2,3,4-tri-*O*-methyl-D-glucuronic acid and 2,3,4-tri-*O*-methyl-L-rhamnose suggests that D-glucuronic acid, 4-*O*-methyl-D-glucuronic acid, and L-rhamnose occur as terminal, non-reducing units in the gum, and the 2,3-di-*O*-methyl-D-glucuronic acid suggests that the terminal, non-reducing L-rhamnose is attached to D-glucuronic acid residues[18,19]. The presence of 2,3,5- and 2,3,4-tri-*O*-methyl-L-arabinose indicates that the arabinose chains are terminated either by L-arabinofuranose (**1**) or by L-arabinopyranose residues (**2**). In contrast to *A. arabica* gum[2] and *A. drepanolobium* gum[1,20] Fraction *A*, 2,5-di-*O*-methyl-L-arabinose is the major di-*O*-methyl-L-arabinose obtained from methylated *A. seyal* gum, with a much smaller proportion of the 3,5 isomer. This shows that many L-arabinofuranose residues are 3-*O*-substituted (**12**) in the arabinose chains, which also contain a few 2-*O*-substituted-L-arabinofuranose residues (**13**). The identification of 3,4-di-*O*-methyl-L-arabinose indicates that there is a small proportion of 2-*O*-substituted-L-arabinopyranose residues (**14**) in the arabinose chains, and the

→3)-L-Ara*f*-(1→	→2)-L-Ara*f*-(1→	→2)-L-Ara*p*-(1→
12	**13**	**14**

significance of this will be mentioned later. Methylation analysis of *A. seyal* gum and its degraded gum *A* suggests that the arabinose chains are attached to the branched galactan framework at either the 3 or 6 positions of certain D-galactose residues.

A. seyal gum was submitted to four successive Smith-degradations. Although the yields of the Smith-degraded products are low compared with those obtained during successive Smith-degradations of *A. senegal*[8], *A. laeta*[9], and *A. drepanolobium*[1] gums, they are similar to those obtained from *A. arabica* gum[2]. Molecular-sieve chromatography indicates that there is pronounced fragmentation of the *A. seyal* gum macromolecule during Smith degradations; the cleavage of D-galactose residues is particularly drastic during the second and subsequent Smith-degradations. The virtually complete fragmentation of the macromolecule during successive Smith-degradations shows that long chains of periodate-resistant β-(1→3)-linked D-galactose residues are not such a dominant structural feature of *A. seyal* gum as they are of *A. senegal*[8] and *A. laeta*[9] gums. *A. seyal* gum contains blocks of periodate-resistant β-(1→3)-linked D-galactose residues interspersed with blocks of periodate-vulnerable β-(1→6)-linked D-galactose residues in a highly branched galactan framework, fragmentation of which occurs as the arabinose chains are progressively removed from the 3 positions of 6-*O*-substituted D-galactose units.

Arabinose residues were present in the product obtained from *A. arabica* gum[2] after four successive Smith-degradations, and in that from *A. drepanolobium* gum[1]

TABLE V

EXAMINATION OF METHANOLYSIS AND HYDROLYSIS PRODUCTS FROM METHYLATED *A. seyal* GUM

Relative retention times (T) *of methyl glycosides*[a]		R_G *in solvent* (d)	*O-Methyl sugar identified*
Column (i)	*Column* (ii)		
0.48	(0.50)	1.01	2,3,4-Tri-*O*-methyl-L-rhamnose
0.53; 0.73	(0.50); 0.64	0.98	2,3,5-Tri-*O*-methyl-L-arabinose
(1.03)	(0.82)	0.79	2,3,4-Tri-*O*-methyl-L-arabinose
1.81; 3.20	1.25; 2.21	0.82	2,5-Di-*O*-methyl-L-arabinose
(1.03); (2.36)	(0.82); 1.75	0.82	3,5-Di-*O*-methyl-L-arabinose
1.99	1.44	0.56	3,4-Di-*O*-methyl-L-arabinose
1.68	1.66	0.88	2,3,4,6-Tetra-*O*-methyl-D-galactose
(3.01); (3.89); (4.22)	2.45; (3.00); (3.45)	0.72	2,3,6-Tri-*O*-methyl-D-galactose
(3.89); (4.22)	(3.00); (3.45)	0.72	2,4,6-Tri-*O*-methyl-D-galactose
6.40	5.20	0.72	2,3,4-Tri-*O*-methyl-D-galactose
14.6; 16.4	9.9; 11.3	0.52	2,4-Di-*O*-methyl-D-galactose
(2.36); (3.01)	(2.21); 2.75	—	2,3,4-Tri-*O*-methyl-D-glucuronic acid[b]
		0.32	2-*O*-Methyl-D-galactose
		0.36	4-*O*-Methyl-L-arabinose
7.8; 9.3	6.2; 7.1	—	2,3-Di-*O*-methyl-D-glucuronic acid[b]

[a]Figures in parenthesis indicate *T* values of components which are not completely resolved. [b]As methyl ester methyl glycoside.

after five degradations. In contrast, four successive Smith-degradations removed all of the arabinose residues from *A. seyal* gum; it has already been pointed out that there are, however, some 2-*O*-substituted-L-arabinopyranose residues in the arabinose chains, some of which could therefore be longer than four units; for example, the model shown in Fig. 1 shows that periodate oxidation of the arabinose chain depicted, having a minimum length of five units, would yield the permissible arabinose chains of up to three units in polysaccharide α.

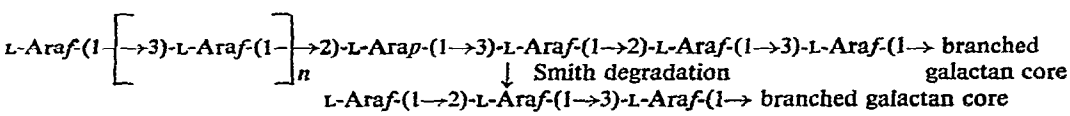


Fig. 1.

As for *A. drepanolobium* gum[1] Fraction *A*, the specific rotation of the Smith-degraded products from *A. seyal* gum decreases to a negative value after four successive degradations; during successive Smith-degradations on *A. arabica* gum[2], the specific rotation decreases, but remains positive. In contrast, *A. senegal*[8] and *A. laeta*[9] gums are levorotatory, whereas their Smith-degradation products are dextrorotatory. It is now clear that the structure of the branched galactan core may vary markedly from one gum species to another within the *Acacia* genus.

On methanolysis, the *O*-methyl derivatives of the Smith-degraded polysaccharides α, β, and γ gave the results shown in Table VI. 2,3,5-Tri-*O*-methyl-L-arabinose

was the only *O*-methyl-L-arabinose to be detected in the methanolysate of methylated polysaccharide γ. The absence of L-arabinopyranose residues in polysaccharide α suggests that the 4-*O*-methyl-L-arabinose identified in methylated *A. seyal* gum is not structurally significant.

TABLE VI

RELATIVE PROPORTIONS OF *O*-METHYL SUGARS PRESENT IN METHYLATED POLYSACCHARIDES α–δ

O-Methyl sugar	*Methylated polysaccharides*			
	α	β	γ	δ
2,3,5-Tri-*O*-methyl-L-arabinose	2	2	trace	—
2,5-Di-*O*-methyl-L-arabinose	2	1/2	—	—
3,5-Di-*O*-methyl-L-arabinose	1/2	trace	—	—
2,3,4,6-Tetra-*O*-methyl-D-galactose	trace	1	1	2
2,4,6-Tri-*O*-methyl-D-galactose	6	6	4	4
2,3,4-Tri-*O*-methyl-D-galactose	2	3	2	2
2,4-Di-*O*-methyl-D-galactose	3	2	1	1

The structural evidence therefore indicates that *A. seyal* gum is very similar to *A. drepanolobium*[1] and *A. arabica*[2] gums, having galactan frameworks that are more highly branched than in *A. senegal*[8] and *A. laeta*[9] gums. To these frameworks are attached uronic acid residues and arabinose chains which, on average, are shorter than those in *A. arabica* gum[2] and considerably shorter than those in *A. drepanolobium* gum[1] Fraction *A*. Molecular-sieve chromatograhpy and viscometry further indicate that molecules of *A. seyal* gum are similar to those of *A. arabica* gum[2] in having a more compact structure than *A. senegal*[8] and *A. laeta*[9] gum molecules.

EXPERIMENTAL

The analytical methods have been described elsewhere in more detail[7]. Paper chromatography was carried out on Whatman Nos. 1 and 3MM papers with (*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*) butyl alcohol–acetic acid–water (4:1:5, upper layer); (*f*) butanone–water–ammonia (sp. gr. 0.880) (200:17:1); (*g*) butanone–acetic acid–water (9:1:1, saturated with boric acid). R_{Gal} values of sugars and R_G values of *O*-methyl sugars refer to distances moved relative to those of galactose and 2,3,4,6-tetra-*O*-methyl-D-glucose, respectively (see Table V). G.l.c. (Chromatograph Type S3A, fitted with flame-ionisation detectors; 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 butane-1,4-diol succinate polyester on 60–70 mesh Celite (5 ft × 0.25 in) at 175°, and (*ii*) 15% by weight of ethylene glycol adipate polyester on 60–70 mesh Celite (3 ft × 0.25 in) at 160°. Retention

times (T) are given relative to methyl 2,3,4,6-tetra-*O*-methyl-D-glucopyranoside as standard. The retention times of the methylated sugars are given in Tables II–V. 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 by the method of Perila and Bishop[22].

Purification of A. seyal *gum.* — The gum (91 g) was dissolved in water (2 l), filtered, and dialysed against running tap-water. The polysaccharide was isolated as the freeze-dried product (87.5 g), $[\alpha]_D$ +59° (*c* 0.50) (Found: moisture, 8.9; N, 0.17; protein, 1.1; OMe, 0.70; D-galactose, 38; L-arabinose, 45; L-rhamnose, 4; D-glucuronic acid, 7; 4-*O*-methyl-D-glucuronic acid, 6%; limiting-viscosity number, 9.9; 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 cellulose acetate film in both 0.1M ammonium carbonate buffer (pH 8.9) and 0.1M acetate buffer (pH 4.7). The gum was chromatographed on a DEAE-cellulose column[6] (45 × 1.3 cm). Gradient elution with sodium chloride solution (0.0M→0.5M) in 0.02M acetate buffer (pH 4.1) gave a single, slightly asymmetric peak.

Preparation of degraded gum A. — The gum (20 g) was hydrolysed with 0.01N sulphuric acid (800 ml) for 100 h on a boiling-water bath. The cooled solution was neutralised with barium carbonate, filtered, treated with Amberlite IR-120(H^+) resin, and dialysed against distilled water (3 l). Dialysis was completed against running tap-water. Degraded gum *A* was isolated as the freeze-dried product (4.4 g; yield, 22%); $[\alpha]_D$ +21° (*c* 0.87) (Found: uronic acid, 15; D-galactose, 82; L-arabinose, 3%). Hydrolysis of degraded gum *A* (20 mg) gave galactose, arabinose, and the four aldobiouronic acids which have been characterized from the whole gum[4] [*viz.*, 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose, 6-*O*-(4-*O*-methyl-β-D-glucopyranosyluronic acid)-D-galactose, 4-*O*-(α-D-glucopyranosyluronic acid)-D-galactose, and 4-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-D-galactose]. Molecular-sieve chromatography with a previously calibrated "Bio-Gel P300" column[23–25] (45 × 5.0 cm) gave an estimated $\bar{M}_n$ for degraded gum *A* of 4,900.

The diffusate was concentrated to a syrup (11.6 g). Chromatographic examination of the syrup showed the presence of galactose, arabinose, rhamnose, and two neutral disaccharides having the mobilities of 3-*O*-β-L-arabinofuranosyl-L-arabinose and 3-*O*-β-L-arabinopyranosyl-L-arabinose.

The first disaccharide (35 mg) was isolated by fractionation of a portion of the diffusate on Whatman 3MM papers in solvent (*f*). This had $[\alpha]_D$ +90° (*c* 0.70) and yielded arabinose on acid hydrolysis. It had the same paper-chromatographic mobility [R_{Gal} 1.25 in solvent (*b*) and 1.44 in solvent (*f*)] as an authentic specimen of 3-*O*-β-L-arabinofuranosyl-L-arabinose. A portion (15 mg) was methylated. G.l.c. examination of the methanolysis products revealed peaks having retention times corresponding to the methyl glycosides of 2,3,5-tri-, and 2,5- and 2,4-di-*O*-methyl-L-arabinose [(*i*) T 2.07, 2.18; (*ii*) T 1.46, 1.56]. 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 0.65).

The second disaccharide (63 mg) was isolated by fractionation of a portion of the diffusate on Whatman 3MM papers in solvent (*b*). This had $[\alpha]_D$ +193° (*c* 0.63) and yielded arabinose on acid hydrolysis. It had the same paper-chromatographic mobility [R_{Gal} 0.77 in solvent (*a*) and 0.71 in 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 peaks having retention times corresponding to the methyl glycosides of 2,3,4-tri-, and 2,5- and 2,4-di-*O*-methyl-L-arabinose [(*i*) *T* 2.08, 2.19; (*ii*) *T* 1.47, 1.56]. 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 0.65). The phenylosazone of the disaccharide was prepared and, after recrystallisation from water, had m.p. 232–234° (decomp.); lit.[26] m.p. 233–235°.

Partial, acid hydrolysis of degraded gum A. — Degraded gum *A* (1.9 g) was hydrolysed with 0.5N sulphuric acid (500 ml) on a boiling-water bath for 1 h. The cooled solution was neutralised with barium carbonate, filtered, treated with Amberlite IR-120(H^+) resin, and concentrated to a syrup (1.8 g). The syrup was chromatographed on Whatman 3MM papers in solvent (*a*) to give two pure disaccharides.

Fraction 1 (30 mg) had $[\alpha]_D$ +62° (*c* 0.60) and yielded galactose on acid hydrolysis. It had the same paper-chromatographic mobility [R_{Gal} 0.47 in solvent (*a*) and 0.53 in solvent (*c*)] as an authentic specimen of 3-*O*-β-D-galactopyranosyl-D-galactose. A portion (10 mg) was methylated. G.l.c. examination of the methanolysis products revealed peaks having 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* 3.98; (*ii*) *T* 3.25]. Hydrolysis of the methyl glycosides, followed by paper chromatography 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 0.88). The disaccharide was crystallised from aqueous acetone to give needles of 3-*O*-β-D-galactopyranosyl-D-galactose monohydrate, which had m.p. and mixed m.p. 157–159°; lit.[26] m.p. 159–160°.

Fraction 2 (44 mg) had $[\alpha]_D$ +31° (*c* 0.44) and yielded galactose on acid hydrolysis. It had the same paper-chromatographic mobility [R_{Gal} 0.30 in solvent (*a*) and 0.38 in solvent (*c*)] as an authentic specimen of 6-*O*-β-D-galactopyranosyl-D-galactose. A portion (15 mg) was methylated. G.l.c. examination of the methanolysis products revealed peaks having retention times corresponding to the methyl glycosides of 2,3,4,6-tetra-, and 2,3,5- [(*i*) *T* 4.05, 5.40; (*ii*) *T* 3.30, 4.38] 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 0.86) and 2,3,4-tri-*O*-methyl-D-galactose.

Borohydride reduction of degraded gum A. — Degraded gum *A* (50 mg) was dissolved in water (10 l), and sodium borohydride (100 mg) was added. After 24 h at room temperature, more sodium borohydride (100 mg) was added. After the solution had been stirred for 6 h, it was dialysed against running tap-water for 2 days

and freeze-dried to yield the product (45 mg). A portion of the product (20 mg) was hydrolysed (N sulphuric acid for 7 h at 100°) to yield galactose, arabinose, and the same four aldobiouronic acids detected in the hydrolysate of degraded gum *A*. In addition, paper chromatography in solvent (*g*) indicated the presence of galactitol. No arabinitol was detected. Degraded gum *A* gave no formaldehyde on periodate oxidation. Some formaldehyde was released on periodate oxidation of reduced, degraded gum *A*.

Methylation of degraded gum A. — Degraded gum *A* (400 mg) was methylated successively with methyl sulphate and sodium hydroxide, and with methyl iodide and silver oxide, to give a product (240 mg), $[\alpha]_D$ −14° (*c* 0.83, chloroform) (Found: OMe, 41.1%, not raised on further attempted methylation). 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 II. Examination of the hydrolysate of the methyl glycosides, 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 characterized by g.l.c. of their methyl glycosides.

Reduction of methylated, degraded gum A. — Lithium aluminium hydride (50 mg) was added to methylated, degraded gum *A* (50 mg) dissolved in tetrahydrofuran (10 ml); the mixture was refluxed for 3 h and cooled. The excess hydride was then destroyed by addition of ethyl acetate and water, and the mixture was evaporated to dryness and extracted with chloroform. The 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 (35 mg), $[\alpha]_D$ −13° (*c* 0.70, chloroform). Methanolysis of the product, followed by g.l.c. examination of the mixture of methyl glycosides, gave the results 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 identified by g.l.c. of their methyl glycosides.

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 with silver carbonate, filtered, and evaporated to a syrup, which was hydrolysed with 0.5N sulphuric acid (10 ml) for 5 h on a boiling-water bath. The cooled solution was neutralised with barium carbonate, filtered, treated with Amberlite IR-120(H^+) resin, and adsorbed on to a column (33 × 3 cm) of Duolite A-4 in the formate form. After elution of the neutral *O*-methyl sugars with water (3 l), the acidic *O*-methyl sugars were eluted with 5% aqueous formic acid (500 ml). Water and formic acid were removed, and paper chromatography of the resulting syrup in solvent (*d*) indicated that it was free of neutral *O*-methyl sugars. The syrup was refluxed with 5% methanolic hydrogen chloride for 7 h. G.l.c. examination of the products revealed peaks having retention times corresponding to the methyl glycosides of 2,3,4-tri-*O*-methyl-D-glucuronic acid, and 2,3,6- and 2,3,4-tri-, and 2,6- and 2,4-di-*O*-methyl-D-galactose. Hydrolysis of the methyl glycosides, followed by paper chromatography in solvents (*d*), (*e*), and (*f*), confirmed these results.

Smith degradation[17] *of degraded gum* A. — Degraded gum *A* (1.0 g) was dissolved in water (25 ml), and 0.5M sodium metaperiodate (25 ml) was added. After 96 h, 7.1 mmoles of periodate was reduced and 3.2 mmoles of formic acid was released. After 96 h, the reaction was stopped by addition of ethylene glycol (2 ml), and the solution was dialysed against running tap-water for 48 h. Sodium borohydride (0.5 g) was added, and the mixture kept for 30 h at room temperature. After dialysis for a further 48 h, the polyalcohol was hydrolysed in N sulphuric acid for 48 h at room temperature. The solution was neutralised with barium carbonate, filtered, treated with Amberlite IR-120(H^+) resin, and dialysed against distilled water (1 l) for 24 h. After further dialysis against running tap-water for 48 h, degraded gum *B* (50 mg; yield, 5%), $[\alpha]_D$ +7.5° (*c* 0.50), was isolated as the freeze-dried product.

The syrup obtained from the dialysate (400 mg) was shown by paper chromatography in solvents (*a*), (*b*), and (*c*) to contain glycerol and glycolaldehyde as main components, together with small proportions of slower-moving, non-reducing glycosides. Hydrolysis of a portion of the dialysate (N sulphuric acid for 7 h at 100°), followed by paper chromatography in solvents (*a*) and (*b*), showed a trace of galactose to be present.

Mild, acid hydrolysis (0.5N sulphuric acid for 1 h at 100°) of degraded gum *B* (10 mg), followed by paper chromatography in 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} 0.31). 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. 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 identified by g.l.c. of their methyl glycosides.

Controlled Smith-degradation[27] *of degraded gum* A. — Degraded gum *A* (1.0 g) was treated in the same way as in the normal Smith-degradation, except that the periodate-oxidation stage was carried out at 2°. Degraded gum *C* was isolated as the freeze-dried product (60 mg; yield, 6%). Hydrolysis of a portion (20 mg) of degraded gum *C*, followed by paper chromatography in solvent (*g*), indicated the presence of galactose and arabinitol, but no galactitol.

Methylation of A. seyal *gum.* — The purified gum (300 mg) was methylated by the Haworth and Purdie procedures to give a product (255 mg), $[\alpha]_D$ +17° (*c* 1.00, chloroform) (Found: OMe, 41.6%, not raised on further attempted methylation). Methanolysis of a portion, followed by g.l.c. examination of the mixture of methyl glycosides, gave the results shown in Table V. The same *O*-methyl sugars were detected as were detected and characterized from methylated *A. drepanolobium* gum[1] Fraction *A*. However, the major di-*O*-methyl-L-arabinose was 2,5-di-*O*-methyl-L-arabinose. Only a small porportion of 3,4-di-*O*-methyl-L-arabinose was detected. Hydrolysis of the methyl glycosides, followed by paper chromatography in solvents (*d*), (*e*), and

(*f*), indicated the presence of 4-*O*-methyl-L-arabinose and 2-*O*-methyl-D-galactose and confirmed the presence of 2,3,4-tri-*O*-methyl-L-arabinose.

Preparation, partial hydrolysis with acid, and methylation of polysaccharide α.— The *A. seyal* gum (54 g) was dissolved in water (1.5 l), mixed with 0.25M sodium metaperiodate (1.5 l) and left for 72 h. The amount of formic acid released in this time was 0.76 mmoles/g. The corresponding amount of periodate reduced was 3.89 mmoles/g. The reaction was stopped by addition of ethylene glycol (20 ml), and the solution was dialysed against running tap-water for 48 h. Sodium borohydride (15 g) was added, and the mixture was 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. After dialysis against running tap-water for 48 h, polysaccharide α was isolated as the freeze-dried product (27.5 g; yield, 51%), $[\alpha]_D$ 0° (*c* 0.50) (Found: galactose, 67; arabinose, 33%).

Polysaccharide α (20 mg) was hydrolysed with 0.5N sulphuric acid (10 ml) for 1 h on a boiling water-bath. Paper chromatography 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} 0.48 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], and higher neutral oligosaccharides.

Polysaccharide α (315 mg) was methylated with methyl sulphate and sodium hydroxide, and with methyl iodide and silver oxide, to give a product (274 mg), $[\alpha]_D$ −31.7° (*c* 0.89, chloroform) (Found: OMe, 41.1%, not raised on further attempted methylation) (See Table VII). 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 VI. 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 characterized by g.l.c. of their methyl glycosides.

TABLE VII

METHYLATION DATA FOR SMITH-DEGRADED POLYSACCHARIDES

Polysaccharide	α	β	γ	δ
Amount of polysaccharide used (mg)	315	232	85	30
Amount of product (mg)	274	190	21	7
$[\alpha]_D$ of product (degrees)	−31.7	−38.6	−39.5	—
OMe of product (%)	41.1	40.8	41.4	—

Preparation, partial hydrolysis with acid, and methylation of polysaccharides α, β, and γ. — The following weights of polysaccharide were periodate-oxidised, borohydride-reduced and hydrolysed, and the corresponding degraded polysaccharides recovered as for the first Smith-degradation. Polysaccharide α (26.6 g) gave polysaccharide β (7.7 g), and β (7.1 g) gave polysaccharide γ (0.93 g). Smith degradation

of polysaccharide γ (0.70 g) gave polysaccharide δ (50 mg). The amount of periodate reduced and formic acid released on periodate oxidation, and the percentage yields, specific rotations, and constituent sugars of each degraded polysaccharide are given in Table I.

Partial, acid hydrolysis of polysaccharide β (20 mg), polysaccharide γ (5 mg), and polysaccharide δ (5 mg), followed by paper-chromatographic examination of the hydrolysates, indicated the presence of 6-*O*-β-D-galactopyranosyl-D-galactose (major component) and 3-*O*-β-D-galactopyranosyl-D-galactose (minor component), and higher neutral oligosaccharides.

Polysaccharides β, γ, and δ were methylated (Table VII). Methanolysis of a sample of each *O*-methyl derivative, followed by g.l.c. examination of the mixtures of methyl glycosides, gave the results shown in Table VI.

Molecular-sieve chromatography of A. seyal *gum and the Smith-degraded products.* — On molecular-sieve chromatography, with a previously calibrated Bio-Gel P300 column (45 × 5.0 cm), both *A. seyal* and polysaccharide α were eluted before the elution volume of "Blue Dextran 2000". This indicates that *A. seyal* gum and polysaccharide α have very high $\overline{M}_n$ values (perhaps as high as 500,000). Light-scattering measurements indicated that *A. seyal* had $\overline{M}_w$ *ca.* 850,000. Molecular-sieve chromatography indicated that polysaccharides β and γ had $\overline{M}_n$ values of *ca.* 8,000 and less than 3,000, respectively.

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PREFERENTIAL TOLUENE-*p*-SULPHONYLATION OF METHYL 6-*O*-TRITYL-α-D-MANNOFURANOSIDE; SYNTHESIS OF 3,5-DI-*O*-METHYL-D-MANNOSE*

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ABSTRACT

Toluene-*p*-sulphonylation of methyl 6-*O*-trityl-α-D-mannofuranoside produced, preferentially, the 2-*O*-toluene-*p*-sulphonyl derivative. Successive methylation, desulphonylation, detritylation, and hydrolysis of this compound yielded 3,5-di-*O*-methyl-D-mannose. A proof of structure for this methylated sugar is presented.

INTRODUCTION

Of the methyl ethers of D-mannofuranose, only the 2,3,5-tri- and 2,3,5,6-tetra-*O*-methyl derivatives are known. In 1963, Urbas *et al.*[1] reported the isolation and characterization of 3,5-di-*O*-methyl-D-mannose from a methylated polysaccharide of birch sap. Based on this identification, the same sugar was detected[2] in the cleavage products of the methylated carbohydrate portion of a glycopeptide from royal jelly. Since then, there have been reports of the isolation[3–5] of this sugar from a number of methylated polysaccharides, and it became important to confirm its identity by synthesis. We now report on a synthesis that shows that the sugar isolated from all of the above sources could not have been 3,5-di-*O*-methyl-D-mannose.

RESULTS AND DISCUSSION

The route followed was similar to that reported for the synthesis of 2,5- and 3,5-di-*O*-methyl-D-galactose[6]. Unlike methyl 6-*O*-trityl-β-D-galactofuranoside, methyl 6-*O*-trityl-α-D-mannofuranoside, when toluene-*p*-sulphonylated, yielded the 2-*O*-tosyl** compound with only minute proportions of a second mono- and a di-*O*-tosyl derivative, and these were removed by chromatography on a column of silica gel. Methyl 2-*O*-tosyl-6-*O*-trityl-α-D-mannofuranoside (overall yield, 84%) was successively methylated, detosylated, detritylated, and hydrolyzed to yield a syrupy dimethyl ether, which, on demethylation, gave mannose. The paper-chromatographic and -electrophoretic behavior, colour reaction with aniline hydrogen phthalate, and

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

specific optical rotation of the ether *disagreed* with those reported for 3,5-di-*O*-methyl-D-mannose[1].

Confirmation that the syrupy product from the above synthesis was 3,5-di-*O*-methyl-D-mannose was obtained as follows. (*i*) Oxidation by bromine water yielded a crystalline *γ*-lactone [ν_{max} for C=O at[7] 1780 cm^{-1}]. (*ii*) Periodate oxidation of the free sugar and its methyl glycoside was followed by paper chromatography and paper electrophoresis. The former compound after 6 and 24 h showed the presence of a product having R_F and M_G values and colour reaction with aniline hydrogen phthalate identical with those of 2,4-di-*O*-methyl-L-arabinose; demethylation produced arabinose. The product was isolated by chromatography on silica gel and was identified by conversion into 2,4-di-*O*-methyl-*N*-phenyl-D-arabinosylamine. The glycoside was resistant to periodate. (*iii*) The di-*O*-methylhexitol (derived from the sugar by reduction with borohydride) consumed one mole of periodate per mole after oxidation for 5 h, and this figure was unchanged after 25 h.

The above data, coupled with the large M_G value and the isolation of an osazone without loss of a methyl group from the parent sugar, establish the parent sugar as 3,5-di-*O*-methyl-D-mannose. The crystalline phenylosazone prepared from our synthetic sugar had m.p. and specific rotation in fair agreement with those reported for 3,5-di-*O*-methyl-D-glucose phenylosazone[8] [3,5-di-*O*-methyl-D-*arabino*-hexosulose bis(phenylhydrazone)].

Thus, it is clear that the 3,5-di-*O*-methyl-D-mannose that we have synthesized is not identical with the compound previously assigned[1] this structure.

EXPERIMENTAL

The general experimental methods have been reported previously[6].

Methyl 6-O-trityl-α-D-mannofuranoside. — Methyl α-D-mannofuranoside[9] (20.0 g, m.p. 118–120°, $[\alpha]_D^{27}$ +106.4°) was dissolved in dry pyridine (200 ml), and chlorotriphenylmethane (29.0 g) was added. After 48 h at room temperature, the solution was poured into ice–water, and the product was isolated as a solid (43 g). The trityl derivative (30.0 g) was fractionated on a column of silica gel to remove small amounts of triphenylmethanol, by eluting first with acetone–chloroform (1:15, 10 litres) to remove the impurity, followed by acetone–chloroform (3:7) to remove the compound as a homogeneous (t.l.c.) solid (26.0 g, 83%), $[\alpha]_D^{27}$ +50.6° (*c* 5.14, chloroform).

Anal. Calc. for $C_{26}H_{28}O_6$: C, 70.57; H, 6.80. Found: C, 70.47; H, 6.73.

Methyl 2-O-tosyl-6-O-trityl-α-D-mannofuranoside. — Methyl 6-*O*-trityl-α-D-mannofuranoside (6.0 g) in dry pyridine (100 ml) was treated dropwise with a solution of tosyl chloride (2.90 g) in dry pyridine (25 ml) at −15° during 26 h. After a further period of 8 h at −15°, the solution was poured into a mixture of ice and water (2 litres). The syrupy product was extracted with chloroform (4 × 250 ml), and the combined extracts were washed successively with water, 10% cupric sulphate solution, and water, and finally dried over sodium sulphate. Removal of chloroform gave a

syrup (7.5 g) that showed, in addition to starting material (R_F 0.20), only one major product (R_F 0.68; t.l.c. in chloroform–acetone, 1:5). The material (7.5 g) was fractionated on silica gel with the same solvent mixture, giving the starting material (1.9 g) and the major fraction (4.6 g, 56%). Small amounts (0.185 and 0.180 g) of a mono- and di-*O*-tosyl compound [R_F values (t.l.c.) of 0.52 and 0.89, respectively] were also recovered.

Methyl 2-*O*-tosyl-6-*O*-trityl-α-D-mannofuranoside had $[\alpha]_D^{27}$ +43° (*c* 5.0, chloroform).

Anal. Calc. for $C_{33}H_{34}O_8S$: C, 67.11; H, 5.80; S, 5.42. Found: C, 67.30; H, 5.95; S, 5.30.

*Methyl 3,5-di-*O*-methyl-2-*O*-tosyl-6-*O*-trityl-α-D-mannofuranoside.* — Methyl 2-*O*-tosyl-6-*O*-trityl-α-D-mannofuranoside (4.4 g) was dissolved in methyl iodide (75 ml), silver oxide (15.0 g) was added, and the reaction mixture was stirred and refluxed for 24 h. Filtration and evaporation gave a product which, after six such further methylations, gave a solid (4.4 g, 95%); $[\alpha]_D^{28}$ +26° (*c* 5.0, chloroform), which was homogeneous (t.l.c.) and had R_F 0.63 (acetone–chloroform, 1:15).

Anal. Calc. for $C_{35}H_{38}O_8S$: C, 67.94; H, 6.19; S, 5.18; OCH_3, 15.05. Found: C, 67.60; H, 6.27; S, 5.06, OCH_3, 14.66.

*Methyl 3,5-di-*O*-methyl-6-*O*-trityl-α-D-mannofuranoside.* — Solutions of methyl 3,5-di-*O*-methyl-2-*O*-tosyl-6-*O*-trityl-α-D-mannofuranoside (1.5 g) in benzene (20 ml) and sodium methoxide [from 2.0 g of sodium in dry methanol (40 ml)] were mixed and refluxed[10] on a sand bath for 7 h. The mixture was cooled to room temperature, water (20 ml) was added, organic solvents were distilled off, and the suspension was extracted with chloroform (4 × 20 ml). The dried (Na_2SO_4) chloroform extract was concentrated to a syrup (1.09 g, 96%), R_F 0.42 (t.l.c.; acetone–chloroform, 1:1), $[\alpha]_D^{29}$ +23.2° (*c* 4.30, chloroform).

Anal. Calc. for $C_{28}H_{36}O_6$: C, 72.39; H, 6.94; OCH_3, 20.04. Found: C, 72.27; H, 6.70; OCH_3, 19.95.

*Methyl 3,5-di-*O*-methyl-α-D-mannofuranoside.* — Methyl 3,5-di-*O*-methyl-6-*O*-trityl-α-D-mannofuranoside (1.0 g) was stirred magnetically, at room temperature, with acetic acid–water (17:13; 30 ml). After 6 h, the precipitate was removed by filtration, the solution was evaporated, and water was distilled from the residue several times (keeping the temperature below 30°). The residue was dissolved in chloroform–acetone (1:1) and was fractionated on silica gel by elution at first with acetone–chloroform (1:15) to remove traces of triphenylmethanol, and then with acetone–chloroform (1:1) to remove the desired compound. The syrupy product (0.40 g, 83%) showed a single component having R_F 0.44 (t.l.c.; chloroform–acetone, 1:1) and had $[\alpha]_D^{26}$ +92.3° (*c* 3.87, water). Paper chromatography of the glycoside showed a single component (R_F 0.62, solvent *A*), giving a blue colour with spray reagent *B*. Found: OCH_3, 41.35. Di-*O*-methylhexoside calc.: 41.9.

*3,5-Di-*O*-methyl-D-mannose.* — Methyl 3,5-di-*O*-methyl-α-D-mannofuranoside (0.32 g) was heated with 0.5N sulphuric acid (4 ml) for 6 h at 100°. The hydrolysate was neutralized with barium carbonate, filtered, and concentrated to a syrup (0.30 g).

The product contained a small amount of a faster moving component besides the main product (t.l.c.). The impurity was removed by chromatography on silica gel with chloroform–acetone (1:1). Elution with acetone–methanol (1:1) gave the product (0.265 g, 95 %), which on paper chromatography and electrophoresis showed a single component having R_F 0.24 and R_G 0.32 (solvent *A*) and a brown colour reaction with spray *A*. The syrupy sugar showed M_G 0.63, and on demethylation produced mannose. The corresponding data, obtained from the same chromatogram and electrophoretogram, for the compound described by Urbas *et al.*[1] were R_F 0.22; R_G 0.28, M_G streak, and a reddish-brown colour with spray reagent *A*. Urbas *et al.*[1] recorded an M_G value (borate) of 0.56, which was not reproduced, and a reddish-brown colour with the *p*-anisidine spray.

The syrupy product showed $[\alpha]_D^{29}$ +4.4° (*c* 5.0, water); Urbas *et al.*[1] reported $[\alpha]_D^{20}$ +14.5° (*c* 1.5, water). Found: OCH_3, 29.78. Di-*O*-methylhexose calc.: 29.8.

*3,5-Di-*O*-methyl-*D*-mannono-1,4-lactone.* — A mixture of 3,5-di-*O*-methyl-D-mannose (0.090 g), water (2 ml), barium carbonate (0.150 g), and bromine (20 drops) was kept in the dark for 70 h. Bromine was removed by aeration, the solution was acidified, and water was removed by aeration. The residue was extracted with chloroform–acetone (1:1), and the soluble portion was fractionated on a small column of silica gel by using the same solvent mixture. The resulting syrup (0.060 g) afforded the lactone, b.p. 165–175° (bath)/0.02 mm. A portion (0.03 g) crystallized from ethanol in the cold to give the title compound, m.p. 163–164°, ν_{max}^{Nujol} 1780 cm^{-1}, $[\alpha]_D^{26}$ +35.2 (initial)→ +32.8° (4 days) (*c* 0.411, water).

Anal. Calc. for $C_6H_{14}O_6$: C, 46.60; H, 6.84. Found: C, 46.94; H, 6.92.

*3,5-Di-*O*-methyl-*D*-mannonamide.* — 3,5-Di-*O*-methyl-D-mannono-1,4-lactone (0.03 g) was dissolved in methanol saturated with ammonia, and the solution was kept for 48 h at 0°. Evaporation of the solvent, with recrystallization of the residue from ethanol–ether, gave the amide, m.p. 161–162°, $[\alpha]_D^{26}$ −2.8° (*c* 0.44, water).

Anal. Calc. for $C_8H_{17}NO_6$: C, 43.04; H, 7.68; N, 6.28. Found: C, 42.60; H, 7.30; N, 6.10.

"*3,5-Di-*O*-methyl-*D*-mannose phenylosazone*". — A mixture of 3,5-di-*O*-methyl-D-mannose (0.05 g), 20% acetic acid (2 ml), sodium bisulphite (0.05 g), and freshly distilled phenylhydrazine (0.5 ml) was heated for 3 h at 100°. The yellow precipitate, obtained on cooling, was washed with 10% aqueous acetic acid and water. Crystallization from aqueous ethanol gave the phenylosazone, m.p. 66–69° with softening at 65°, $[\alpha]_D^{26}$ −105° (*c* 0.40, ethanol); lit.[8], for "3,5-di-*O*-methyl-D-glucose phenylosazone", m.p. 64–65°, $[\alpha]_D^{25}$ −83° (*c* 1.5, ethanol).

*Periodate oxidation of 3,5-di-*O*-methyl-*D*-mannose and 3,5-di-*O*-methyl-*D*-mannitol.* — A 1% solution of 3,5-di-*O*-methyl-D-mannose was mixed with an equal volume of 0.1M sodium metaperiodate solution. Paper chromatography and electrophoresis of this solution at 6 and 24 h revealed the presence of 2,4-di-*O*-methyl-arabinose.

3,5-Di-*O*-methyl-D-mannose (0.055 g) in water (2 ml) was mixed with a solution of sodium borohydride[11] (0.10 g in 1 ml of water), and the solution was left overnight.

Excess of borohydride was decomposed with glacial acetic acid, and the solution was deionized with Rexyn 101 (H^+) and Rexyn 203 (OH^-). The syrupy hexitol (0.050 g) was oxidized in aqueous solution (50 ml) containing 0.15M sodium metaperiodate (35 ml); a blank was run concurrently. The consumption of periodate, determined by the arsenite method[12], was 1.05 moles per mole of hexitol after 5 h. A second determination, after 25 h, gave the same figure.

2,4-Di-O-methyl-N-phenyl-D-arabinosylamine. — The solution (48 ml) remaining from periodate oxidation was concentrated, and deionized with Rexyn exchange resins. The solution was evaporated to dryness, and the residue was extracted with acetone–chloroform (1:1). The soluble portion was chromatographed on silica gel by using the same solvent mixture. On paper chromatography and electrophoresis, the resulting syrup (0.025 g) showed the presence of a pentose having R_F 0.24 (solvent *A*; reddish colour reaction with aniline hydrogen phthalate) and M_G 0.00. These values agreed with those for an authentic sample of 2,4-di-*O*-methyl-L-arabinose, placed on the same chromatogram and electrophoretogram. A portion (0.002 g) of the product, on demethylation[13], gave arabinose. Another portion (0.020 g) in ethanol (1 ml) was heated under reflux with freshly distilled aniline (0.01 g) for 4 h. The ether soluble portion, on being seeded with 2,4-di-*O*-methyl-*N*-phenyl-L-arabinosylamine and cooled, gave crystals of the D-enantiomorph, m.p. 126°; lit.[14,15], for 2,4-di-*O*-methyl-*N*-phenyl-L-arabinosylamine, m.p. 126° and 145°. The infrared spectrum of this aniline derivative and that of authentic 2,4-di-*O*-methyl-*N*-phenyl-L-arabinosylamine were superimposable.

ACKNOWLEDGMENTS

The authors are indebted to Dr. G. A. Adams for a sample of their "3,5-di-*O*-methyl-D-mannose". Analyses were performed by Mr. George Morris, Analytical Chemistry Research Service, Canada Department of Agriculture, and Mr. A. E. Castagne, Division of Biosciences, National Research Council.

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METHODS FOR THE MICROSCALE IDENTIFICATION OF SOME ACIDIC MUCOPOLYSACCHARIDES

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ABSTRACT

Methods for the chemical identification of hyaluronic acid, chondroitin 4-sulphate, chondroitin 6-sulphate, and dermatan sulphate, on a 150-μg scale, have been elucidated. Hyaluronic acid has been degraded with hyaluronidase, the products being further degraded with β-D-glucuronoside glucuronhydrolase and 2-acetamido-2-deoxy-β-D-glucoside acetamidodeoxyglucohydrolase to a disaccharide. An enzyme preparation containing chondroitinase and sulphatases from *Proteus vulgaris* has been applied to chondroitin 4-sulphate, chondroitin 6-sulphate, and dermatan sulphate, to give a sulphate-free, unsaturated disaccharide which gave a characteristic periodate-oxidation product, formylpyruvic acid. The position of linkage in the disaccharides has been established by specific, photometric methods.

INTRODUCTION

In cases of limited supplies of acidic mucopolysaccharides, *e.g.*, from human sources, many workers have relied principally upon quantitative comparison with fully characterised polysaccharides of non-human, mammalian origin as a means of identification. Large samples are required for rigorous identification by classical methods, and such methods have the added disadvantage that they are not quantitative, since conversion into the derivatives is not complete. The results obtained, therefore, do not necessarily account for the whole of the structure under investigation.

In order to overcome these difficulties and to avoid the use of destructive methods, it is necessary to choose a sequential scheme of characterisation and identification in which the products obtained at each stage can be used in the next. Such a sequence provides the maximal amount of information from a given quantity of sample. To this end, fully characterised enzymes can be used, non-destructively, to depolymerise a polysaccharide to a form more convenient for analysis. Gel filtration and ion-exchange chromatography can then be used to fractionate the products, and micro-scale colorimetric assays can be used to monitor the sequences.

We now report on the use of such methods for the micro-scale identification of hyaluronic acid, chondroitin 4- and 6-sulphates, and dermatan sulphate.

METHODS AND RESULTS

2-Amino-2-deoxyhexose identification. — Solutions of hyaluronic acid (Fluka Inc.) and chondroitin 4-sulphate were hydrolysed in 3.9N hydrochloric acid for a series of times, and the 2-amino-2-deoxyhexoses liberated were extracted by the method of Boas[1], on a modified scale. Application of the Elson–Morgan[2,3] spectrophotometric assay for 2-amino-2-deoxyhexoses, on a modified scale, showed that maximal release occurred after 9 h, with less than 4% destruction.

The 2-amino-2-deoxyhexoses were identified by Gardell's method[4]. A column of AG-50Wx8 resin (200–400 mesh; H^+ form; bed volume, 21 ml) was equilibrated at 18° with 0.3N HCl at a flow rate of 0.10 ml/min. The column was then calibrated under the same conditions by separate elution of 2-amino-2-deoxy-D-glucose and 2-amino-2-deoxy-D-galactose. Aliquots of the polysaccharide hydrolysates were fractionated on the calibrated column, the constituent 2-amino-2-deoxyhexoses of hyaluronic acid and chondroitin 4-sulphate being identified as 2-amino-2-deoxy-D-glucose and 2-amino-2-deoxy-D-galactose, respectively. These assignments were confirmed by ninhydrin oxidation[5] of the bulked 2-amino-2-deoxyhexose peak and thin-layer cellulose chromatography of the products, with standards of D-ribose, L-arabinose, D-lyxose, and D-xylose, in butanone–acetic acid–saturated, aqueous boric acid (9:1:1 by volume).

Hyaluronidase hydrolysis. — Hyaluronic acid and chondroitin 4-sulphate were separately dissolved in sterile 0.005M phosphate–citrate buffer (pH 5.0). Solutions of testicular hyaluronidase (Sigma Chemical Co.) in 0.1N sodium chloride were sterilized by using a Millipore filter and were added to the polysaccharide solutions which were then incubated under aseptic conditions for 48 h at 37°. When the Morgan–Elson[6,7] assay for reducing 2-acetamido-2-deoxyhexoses was applied to aliquots of the solutions, the response was positive in the case of hyaluronic acid and negative in the case of chondroitin 4-sulphate.

A column of Biogel P-30 (bed volume, 56 ml) was equilibrated at 4° with the 0.01M phosphate–0.2M sodium chloride buffer (pH 7.3) at a flow rate of 0.33 ml/min. The column was calibrated under identical conditions by the separate elution of testicular hyaluronidase, hyaluronic acid, and hyaluronic acid hexasaccharide (Fig. 1); the recovery from the column was 100% in each case. Aliquots of the enzyme hydrolysates (containing 5–15 μg of uronic acid) were fractionated on the column under conditions identical to those used for calibration. Fractions corresponding to the elution positions of void volume, polymer, and oligomer were collected, and concentrated to render their uronic acid contents detectable by the carbazole[8] assay used on a modified scale. In both cases, only the fractions corresponding to oligomer gave a positive result.

Hydrolyses with 2-acetamido-2-deoxy-β-D-glucoside acetamidodeoxyglucohydrolase and β-D-glucuronoside glucuronhydrolase. — 2-Acetamido-2-deoxy-β-D-glucoside acetamidodeoxyglucohydrolase was extracted from ram testes. The tissues were homogenised in dilute acetic acid and centrifuged to remove debris. These and

subsequent operations were carried out at 4°. The supernatant (Fisons Pharmaceuticals Ltd.) was clarified by high-speed centrifugation, and fractions were precipitated by the addition of ammonium sulphate. The fraction precipitating between 30 and 40% saturation was checked for enzyme activity by incubation at pH 4.5 for 1 h with a solution of *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside. After the resulting solutions had been made alkaline, determination of absorbance at 420 nm demonstrated that *p*-nitrophenol had been liberated by the enzyme preparation alone.

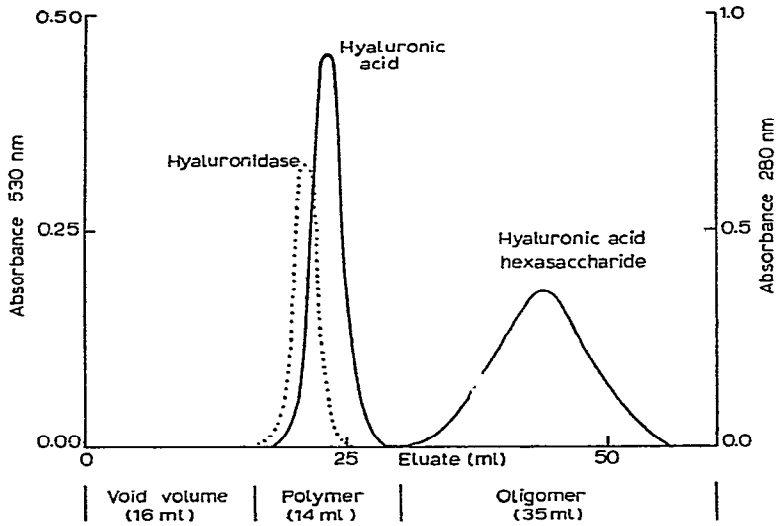


Fig. 1. Calibration of the BioGel P-30 column. (———), carbazole assay; (.....), u.v. assay.

The solution of hyaluronidase-hydrolysed hyaluronic acid was treated with solutions of β-D-glucuronoside glucuronhydrolase and 2-acetamido-2-deoxy-β-D-glucoside acetamidodeoxyglucohydrolase in 0.005M phosphate–citrate buffer (pH 4.0). The resulting solutions, which had a pH of 4.5, were incubated for 24 h at 37°.

A micro column of AG-1x8 resin (200–400 mesh; acetate form; bed volume, 5.5 ml) was calibrated for the elution position of D-glucuronic acid, 2-acetamido-2-deoxy-D-glucose, and the di-, tetra-, and hexa-saccharides obtained from a large-scale digestion of hyaluronic acid with testicular hyaluronidase. For each sample, the column was eluted at 18° with an approximately linear gradient of 0.00–0.30M ammonium acetate at a flow rate of 0.56 ml/min. The fractions were assayed by the carbazole and Morgan–Elson assays, as appropriate (Fig. 2). An aliquot of the doubly digested hyaluronic acid solution (containing 40 μg of carbohydrate) was fractionated under identical conditions, and the fractions were assayed as before (Fig. 3).

The peak responding positively to both assays contained D-glucuronic acid and 2-acetamido-2-deoxy-D-glucose in a unit molar ratio. The fractions containing this peak were combined, and the ammonium acetate was removed by repeated evaporation *in vacuo* at 80° with excess of water. Aliquots of the solution were

hydrolysed with N sulphuric acid for 0–3 h at 100° and assayed by the Boas[1] version of the Elson–Morgan assay, on a modified scale. The absorption maximum (λ_{max})

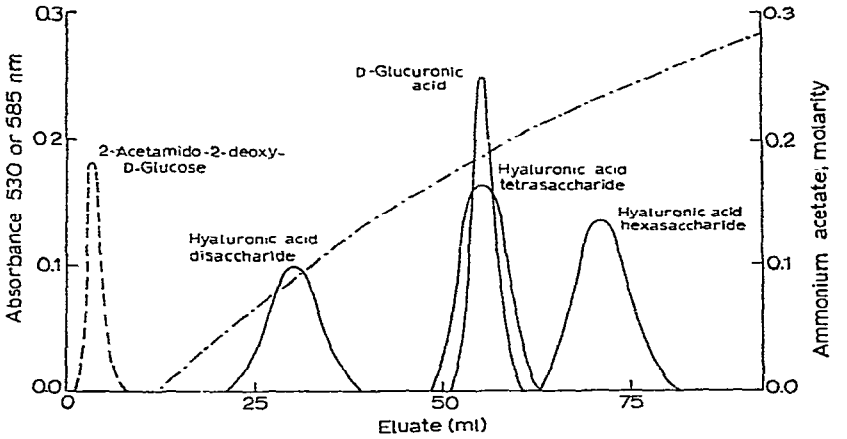


Fig. 2. Calibration of the AG-1x8 column. (– – – –), Morgan–Elson assay; (———), carbazole assay; (–·–·–·–), gradient.

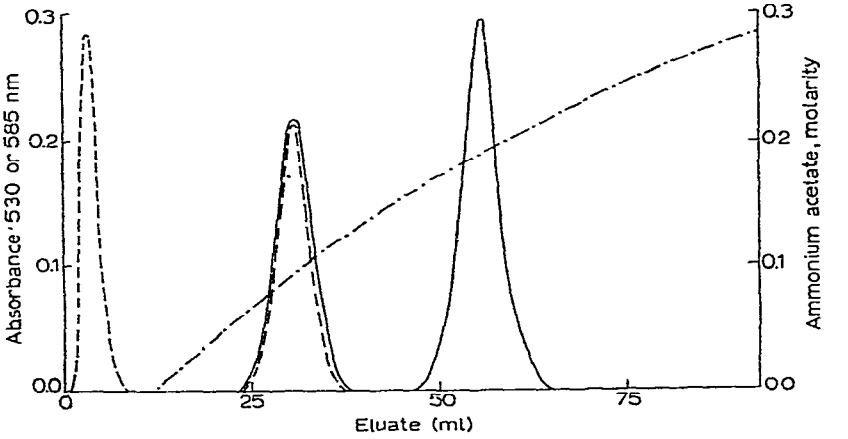


Fig. 3. Fractionation on AG-1x8 of hyaluronic acid after hydrolysis with three enzymes.

of each assay sample and the absorbance at 528 nm were determined (Fig. 4). A sample of 2-amino-2-deoxy-D-glucose subjected to the same hydrolysis conditions was stable and gave an absorption maximum at 540 nm.

Chondroitinase and sulphatase hydrolysis. — *Proteus vulgaris* was grown in pure culture at 37° on a vigorously aerated medium (5 l) consisting of peptone (1.0% w/v), yeast (0.5% w/v), and sodium chloride (0.1% w/v). When the stationary phase was reached, the cells were harvested by centrifugation. The packed cells were resuspended in 0.01M Tris buffer (pH 7.2) and were then shaken at 4° with glass beads in a Mickel disintegrator until microscopic examination showed that a majority of the cells had been broken. After centrifugation, nucleic acids were precipitated

at 4° by the addition of streptomycin sulphate to a final concentration of 1% w/v. After 30 min, the precipitate was centrifuged off, and the clear supernatant was dialysed at 4° against four changes of 0.01M Tris buffer (pH 7.2). Incubation of aliquots of each precipitate and the final dialysed supernatant with a solution of chondroitin 4-sulphate in 0.5M Tris buffer (pH 8.0) for 1 h at 37°, with subsequent analysis for the release of 4-deoxy-L-*threo*-hex-4-enosyluronic acid (by application of the Warren[9] assay), demonstrated that the final, dialysed supernatant contained 97% of the total chondroitinase activity.

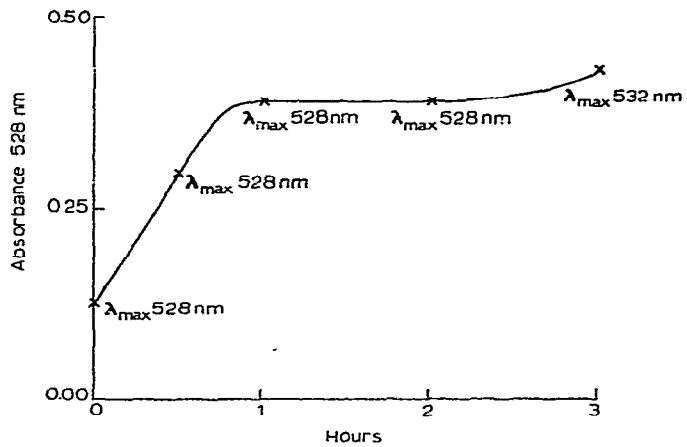


Fig. 4. Elson–Morgan (Boas) behaviour of *N*-acetylhyalobiouronic acid during acid hydrolysis.

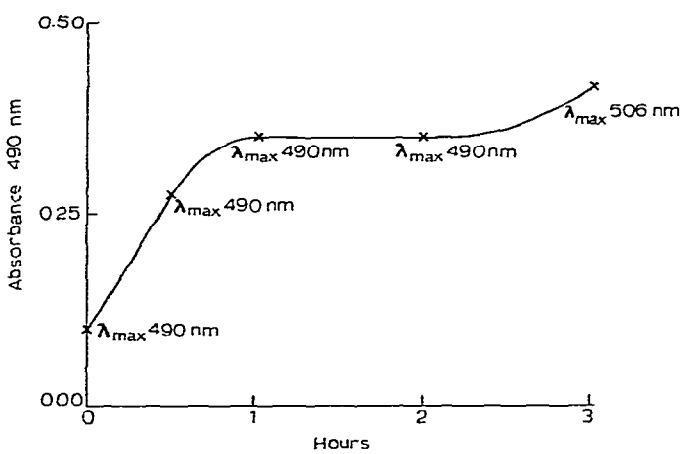


Fig. 5. Elson–Morgan (Boas) behaviour of *N*-acetylchondrosine during acid hydrolysis.

The enzyme preparation was dialysed at 4° against five changes of 0.005M phosphate buffer (pH 8.1) and purified by fractionation on a column of DEAE-cellulose (Cl^- form; bed volume, 110 ml). The column was eluted at the same temperature with successive volumes of 0.005M phosphate buffer (pH 8.1), 0.005M phosphate

buffer (pH 7.6), 0.005M phosphate–0.1M sodium chloride buffer (pH 7.0), and 0.1M phosphate–0.5M sodium chloride buffer (pH 6.0) (500 ml each) at a flow rate of 30 ml/h. The effluent was checked for chondroitinase activity by the procedure just described, and the positive fractions were combined and freeze-dried.

Chondroitin 4-sulphate was dissolved in 0.025M Tris buffer (pH 8.0), a quantity of the freeze-dried powder was added, and the solution was incubated for 48 h at 37°. Fractionation of an aliquot on the calibrated BioGel P-30 column, with assay of the combined fractions in the manner previously described, demonstrated that the polymer had been completely degraded to oligomers. Assay of an aliquot by the Warren method showed that formylpyruvic acid was formed on periodate oxidation. Fractionation of a further aliquot of the digest on the AG-1x8 resin column gave one carbazole-positive peak. The fractions containing this peak were combined, and the ammonium acetate was removed as before. Application of a flame-photometric assay[10] to an aliquot, after hydrolysis in 0.5N HCl for 2 h at 100°, showed that the carbohydrate contained no sulphate. A further aliquot was hydrolysed with N sulphuric acid for 0–3 h at 100° and assayed by the Boas technique, as previously described (Fig. 5).

DISCUSSION

Analyses of acidic polysaccharides for uronic acid, 2-amino-2-deoxyhexose, and sulphate, and identification of the 2-amino-2-deoxyhexose are easily accomplished on a few micrograms of material. The results provide an initial, broad classification, since the repeating unit for hyaluronic acid is (1→4)-*O*-β-D-glucopyranosyluronic acid-(1→3)-2-acetamido-2-deoxy-β-D-glucopyranose, and for chondroitin 4-(and 6-) sulphate is (1→4)-*O*-β-D-glucopyranosyluronic acid-(1→3)-2-acetamido-2-deoxy-4-(6-)*O*-sulpho-β-D-galactopyranose. Dermatan sulphate is the L-idopyranosyluronic acid isomer of chondroitin 4-sulphate.

Since testicular hyaluronidase is specific for a β-D-glucopyranosyluronic acid-(1→3)-2-acetamido-2-deoxy-β-D-hexopyranose linkage[11], hyaluronic acid and chondroitin 4- and 6-sulphates are susceptible, whereas dermatan sulphate is resistant to hydrolysis. The Morgan–Elson reaction can be used to demonstrate this susceptibility only in certain cases, since substitution of the reducing 2-acetamido-2-deoxyhexose at position 4 prevents chromophore production, and hence this assay will not differentiate between chondroitin 4- and dermatan sulphates. Fractionation on the basis of molecular size was therefore chosen to demonstrate enzymic hydrolysis. After incubation with the enzyme, use of a BioGel P-30 column demonstrated that the whole of the polysaccharide structures had been hydrolysed to oligomers, and pre-calibration of the column enabled the fractionation to be carried out on a sample of 10 μg.

In the case of hyaluronic acid, further incubation with 2-acetamido-2-deoxy-β-D-glucoside acetamidodeoxyglucohydrolase and β-D-glucuronoside glucuronhydrolase effected hydrolysis of the oligomers. Ion-exchange fractionation of the products revealed three peaks, respectively containing 2-acetamido-2-deoxy-D-glucose,

D-glucuronic acid, and both saccharides. The last peak was eluted in the same position as 2-acetamido-2-deoxyhyalobiouronic acid, and the presence of a disaccharide was revealed by the fact that it contained D-glucuronic acid and 2-acetamido-2-deoxy-D-glucose in unit molar ratio. Once again, the whole of the structure was accounted for, the two enzymes demonstrating the β-D-configuration of the two linkages.

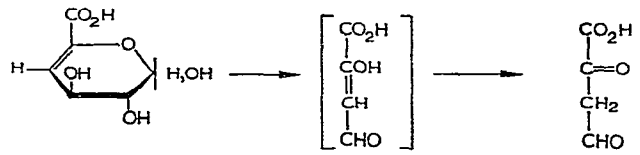
In spite of the fact that 2-acetamido-2-deoxyhyalobiouronic acid contains a glucuronosidic linkage, it is not hydrolysed by β-D-glucuronoside glucuronhydrolase[12]. Hence, another agent was required for demonstrating the structure of the enzyme-resistant disaccharide. This was afforded by the Elson–Morgan reaction, since, in the Boas modification, substitution at position 3 of 2-amino-2-deoxyhexoses results in a shift of λ_{max} from the normal value of 540 nm. However, since the reaction does not apply to 2-acetamido-2-deoxyhexoses, it was necessary to remove the acetyl group without cleavage of the glycosidic bond. It was shown that the *N*-deacetylated disaccharide produced a chromophore having an absorption maximum at 528 nm, and hence that the 2-amino-2-deoxyhexose was substituted at position 3. Since the disaccharide initially gave a low colour yield, the results also demonstrated that it was *N*-acetylated, and that *N*-deacetylation was complete after one hour; during the next hour, no further liberation of 2-amino-2-deoxyhexose was detected. The initial colour yield by the unhydrolysed disaccharide was due to partial *N*-deacetylation during the removal of ammonium acetate at high temperature. Further hydrolysis resulted in an increase in colour yield, but with a chromophore absorption maximum approaching that of 2-amino-2-deoxy-D-glucose, presumably due to partial hydrolysis of the glycosidic bond.

Thus, the method for the identification of hyaluronic acid on a micro scale involved the following stages (the minimal amount of polysaccharide required for each stage being included in parenthesis): 1, demonstration of the molar ratios of 2-amino-2-deoxyhexose, uronic acid, and sulphate to be 1:1:0 (50 μg total); 2, demonstration of the constituent 2-amino-2-deoxyhexose to be 2-amino-2-deoxyglucose (50 μg); 3, demonstration of total degradation by testicular hyaluronidase, indicating the presence of D-glucuronic acid and a β-(1→4) 2-acetamido-2-deoxy-D-hexose to D-glucuronic acid linkage (10 μg); 4, demonstration of the presence of D-glucuronic acid, 2-acetamido-2-deoxy-D-glucose, and the β-D-configuration of the linkages (40 μg); 5, demonstration of a (1→3) D-glucuronic acid to 2-amino-2-deoxy-D-glucose linkage in the enzyme-resistant disaccharide (included in 4). Hence, the method for identification of the chemical structure can be carried out on a sample of 150 μg of the polysaccharide.

Although the nature of the chondroitin sulphates and dermatan sulphate is similar to that of hyaluronic acid, application of a similar scheme was not possible due to their contents of sulphate and 2-amino-2-deoxy-D-galactose. It has been demonstrated that *Proteus vulgaris* contains a chondroitinase and a sulphatase[13,14], and that incubation of chondroitin and dermatan sulphates with *Pr. vulgaris* extracts yields a series of unsaturated oligosaccharides and their sulphuric esters[15]. Exhaustive hydrolysis of chondroitin 4-sulphate yielded two disaccharides[16], subsequently

identified as 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enosyluronic acid)-D-galactose and 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enosyluronic acid)-4-*O*-sulpho-D-galactose[15]. Dermatan sulphate gave rise to the same two disaccharides, whereas chondroitin 6-sulphate afforded the corresponding 6-*O*-sulpho isomer of the sulphated disaccharide as well as the non-sulphated disaccharide. The nature of the 2-acetamido-2-deoxyhexose to uronic acid linkage is disclosed by the specificity of the chondroitinase. It has been demonstrated that the sulphatases of *Pr. vulgaris* are specific for the 4- and 6-*O*-sulpho isomers of the disaccharide[17].

In the present study, it was necessary to devise a method for the easy recognition of chondroitinase which gives rise to a 4-deoxy-L-*threo*-hex-4-enosyluronic acid at the terminal, non-reducing end of the oligosaccharides. Structures (*e.g.*, **1**) which give rise to formylpyruvic acid (**2**) as a periodate-oxidation product can be recognised by the Warren spectrophotometric reaction which involves periodate oxidation, acid hydrolysis, and condensation of the products with 2-thiobarbituric acid. Formylpyruvic acid alone gives a chromophore absorption maximum of 549 nm, and the



1 2

adopted chondroitinase test was based on this sensitive reaction. *Pr. vulgaris* was then grown on a scale that permitted isolation of the enzyme which was then purified by DEAE-cellulose chromatography. It was subsequently demonstrated that the fractions containing the chondroitinase activity also contained the sulphatases.

Chondroitin 4-sulphate was hydrolysed with the chondroitinase–sulphatase preparation, and application of an aliquot to the calibrated BioGel P-30 column revealed that hydrolysis to material of low molecular weight was complete. Assay of a second aliquot by the Warren technique demonstrated the release of 4-deoxy-L-*threo*-hex-4-enosyluronic acid, whilst ion-exchange fractionation of a third aliquot revealed that the carbohydrate was homogeneous. Application of the flame-photometric technique indicated that this carbohydrate contained no sulphate, it being concluded that the enzyme extract contained the chondroitinase and the sulphatases. As in the case of the hyaluronic acid disaccharide, the nature of the glycosidic linkage of the enzyme-resistant disaccharide was determined by the Elson–Morgan assay. Removal of the *N*-acetyl group was complete after one hour, and, as with hyalobiouronic acid, glycosidic cleavage occurred on prolonged hydrolysis. The absorption maximum of the substituted 2-amino-2-deoxyhexose was at 490 nm, indicating a (1→3)-glucuronic acid to 2-amino-2-deoxyhexose linkage in the polysaccharide.

Thus, the method for the identification of the chondroitin and dermatan sulphates on a micro scale involved the following stages: 1, demonstration of the molar ratios of the 2-amino-2-deoxyhexose, uronic acid, and sulphate contents to

be 1:1:1 (50 μg total); 2, demonstration of the constituent 2-amino-2-deoxyhexose to be 2-amino-2-deoxygalactose (50 μg total); 3, demonstration of either total degradation by testicular hyaluronidase, indicating the presence of D-glucuronic acid and a β-(1→4) 2-acetamido-2-deoxy-D-hexose to D-glucuronic acid linkage, or complete resistance to the enzyme, suggesting the presence of L-iduronic acid; and demonstration that the 2-acetamido-2-deoxyhexose was substituted at C-4 in the case of chondroitin 4-sulphate and not at C-2 or -4 in the case of chondroitin 6-sulphate (10 μg); 4, demonstration of a β-(1→4) 2-acetamido-2-deoxy-D-hexose to uronic acid linkage by total chondroitinase hydrolysis, and confirmation of D-glucuronic or L-iduronic acid (40 μg); 5, demonstration of a (1→3) uronic acid to 2-amino-2-deoxyhexose linkage in the enzyme-resistant disaccharide, and thereby that the sulphate was situated at C-4 or -6 of the 2-amino-2-deoxyhexose and that this amino group was *N*-acetylated. Hence, with the results from 3, assignment of the sulphate group to C-4 or -6 (included in 4).

The method for identification of the chemical structure can thus be carried out on 150-μg samples of the polysaccharides. Work involving an application of these methods to the acidic mucopolysaccharides of human skin will be published elsewhere.

ACKNOWLEDGMENTS

The authors thank Professor M. Stacey, C.B.E., F.R.S., for his interest in this work, and Dr. J. A. Cifonelli for provision of chondroitin 4-sulphate. The work was supported by a Medical Research Council research scholarship to J.F.K.

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PREPARATION AND PROPERTIES OF α-AMYLASE CHEMICALLY COUPLED TO MICROCRYSTALLINE CELLULOSE

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ABSTRACT

Methods are reported for the preparation of water-insoluble derivatives of α-amylase, in which the enzyme is covalently linked to microcrystalline cellulose. Coupling was effected *via* diazotised 3-(*p*-aminophenoxy)-2-hydroxypropyl ethers and 2-hydroxy-3-(*p*-isothiocyanatophenoxy)propyl ethers of cellulose having different degrees of substitution; low substitution results in higher retention of α-amylase activity. The water-insoluble enzyme shows greater heat stability than the native α-amylase, 20% of the original activity remaining after 7 days at 45°.

INTRODUCTION

Water-insoluble derivatives of α-amylase (diastase) have been prepared by chemical coupling of the enzyme to (*a*) nitrated copolymers of methacrylic acid, methacrylic acid *m*-fluoroanilide, and divinylbenzene[1,2], and (*b*) nitrated copolymers of methacrylic acid, 4- or 3-fluorostyrene, and divinylbenzene[3]. The enzyme activity of the bound protein in these preparations did not exceed 3% of that of the free enzyme in aqueous solution. Also, the stability of these preparations was reported to be of only the same order as aqueous solutions of α-amylase stored under similar conditions.

It is well known that when an enzyme is attached to an insoluble support the novel microenvironment of the enzyme markedly affects its stability. Hydrophilic features of the carrier tend to enhance the stability of the attached enzyme, whereas hydrophobic features have the opposite effect. Polysaccharide carriers such as fibrous cellulose[4,5] and cross-linked dextran[6] have been shown to be particularly effective in conferring stability to the attached enzyme. The aims of the present studies, therefore, were to investigate the activity and stability of α-amylase chemically coupled to a polysaccharide support. The choice of microcrystalline cellulose was made in view of its fine, particulate nature and consequent high surface-to-volume ratio as compared to fibrous cellulose and cross-linked dextran.

EXPERIMENTAL

Microcrystalline cellulose (Sigmacel type 38) and crystalline α-amylase (*B. subtilis*) were purchased from the Sigma Chemical Company, London, England.

1,2-Epoxy-3-(p-nitrophenoxy)propane (glycidyl p-nitrophenyl ether). — This compound was prepared by a modification of the method of Marle[7]. *p*-Nitrophenol (69.5 g, 0.5 mole) was suspended in a solution of sodium hydroxide (25 g, 0.6 mole) in water (400 ml), and the mixture was heated to 70° to effect dissolution. When the solution was cooled slowly to room temperature with vigorous stirring, finely divided sodium *p*-nitrophenoxide separated. Epichlorohydrin (46 g, 39 ml, 0.5 mole) was added, and the solution was gently stirred for 6 days at room temperature, after which the solid was collected and washed on a filter with distilled water until free of bright-yellow sodium *p*-nitrophenoxide. The damp, off-white solid was dissolved directly in ether (500 ml), and the water layer which separated was discarded. The ether layer was dried with magnesium sulphate and concentrated, whereupon a very pale-yellow solid crystallised. Recrystallisation from light petroleum (b.p. 40–60°) gave the pure ether (25.0 g, 25.5%), m.p. 67° (lit.[7] 67°).

Preparation of 2-hydroxy-3-(p-nitrophenoxy)propyl ethers and 3-(p-aminophenoxy)-2-hydroxypropyl ether hydrochlorides of cellulose. — This method is an adaption of that applied by Axen and Porath[6] to cross-linked dextran.

Samples of microcrystalline cellulose (10 g) were placed in four 50-ml, stoppered, round-bottomed flasks which were subsequently evacuated, left for 1 h, and then filled with nitrogen. After each flask had been warmed to 50°, pre-warmed aliquots (20 ml) of a 10% solution of glycidyl *p*-nitrophenyl ether were added, followed by aliquots (10 ml) of 10% aqueous sodium hydroxide. The contents of the flasks were well mixed, and the stoppered flasks were kept at 50°. All operations were performed under nitrogen. After various times (12, 24, 48, and 96 h), the reaction mixtures were transferred to a mortar and ground lightly with 2N acetic acid before being suspended and stirred for 0.5 h with the same solvent (0.5 l). The 2-hydroxy-3-(*p*-nitrophenoxy)-propyl ethers of cellulose were then collected, washed and ground lightly with acetone, and then stirred with acetone (0.5 l) for 0.5 h. After three washings with distilled water (1 litre) and one further washing with acetone (0.5 l), the pale-yellow ethers were collected and dried.

Reduction of these cellulose ethers was effected by suspending samples (2 g) in a solution of titanous chloride (5%) in hydrochloric acid (6N, 200 ml) for 5 min at 100°. The 3-(*p*-aminophenoxy)-2-hydroxypropyl ether hydrochlorides were collected on a filter and washed with 2N hydrochloric acid until free of titanous chloride. After being ground lightly in a mortar with distilled water, the samples were washed three times with distilled water (0.5 l) and finally with acetone (500 ml), before being collected and dried. The degree of etherification was determined by titration of the hydrochlorides with aqueous sodium hydroxide (Table I).

Preparation of water-insoluble α-amylase derivatives. — *(a) By coupling α-amylase with diazotised 3-(p-aminophenoxy)-2-hydroxypropyl ethers of cellulose.* Samples

(100 mg) of the four cellulose derivatives prepared above were treated, in stoppered test-tubes, with N hydrochloric acid (5 ml), cooled (ice-bath), and magnetically stirred for 15 min. Aliquots (4 ml) of ice-cold sodium nitrite solution (2%) were added. After 15 min, the tubes were centrifuged, the supernatants discarded, aliquots (15 ml) of ice-cold phosphate buffer (0.075M, pH 7.6–7.7) added, and the mixtures magnetically stirred for 15 min. The washing cycle was repeated three times. After the final washings had been decanted, aliquots (1 ml) of α-amylase solution (0.5%) in phosphate buffer (0.075M, pH 7.6–7.7) were added, the mixtures were stirred magnetically for 18 h at 0–5°, and aliquots (5 ml) of an ice-cold solution of 2-naphthol (0.01%) in saturated, aqueous sodium acetate were added. After a further 15 min, the water-insoluble α-amylase derivatives were subjected to five cycles of washing with phosphate buffer (0.02M, pH 6.9, 15 ml) and 0.5M sodium chloride in the same buffer (15 ml). The α-amylase derivatives were finally washed twice with phosphate buffer.

TABLE I

DEGREE OF SUBSTITUTION OF 3-(*p*-AMINOPHENOXY)-2-HYDROXYPROPYL ETHERS OF CELLULOSE

Reaction time of glycidyl *p*-nitrophenyl ether with cellulose (h)	12	24	48	96
μequiv. of 3-(*p*-aminophenoxy)-2-hydroxypropyl ether per g of cellulose	13.0	20.7	31.3	56.2

(*b*) *By coupling at pH 6.3–6.4.* This coupling was performed as described above except that phosphate buffer (0.02M, pH 6.3–6.4) was used in the preliminary washing of the diazotised cellulose derivatives and in the subsequent coupling with α-amylase.

(*c*) *By coupling α-amylase with 3-(p-aminophenoxy)-2-hydroxypropyl ethers of cellulose activated with thiophosgene.* Samples (100 mg) of the four cellulose ether hydrochlorides were magnetically stirred into a slurry with phosphate buffer (3.5M, pH 6.8, 0.5 ml). Aliquots (0.2 ml) of a thiophosgene solution (10%) in carbon tetrachloride were added, stirring was continued for 20 min, and further aliquots (0.2 ml) of thiophosgene solution were then added. After a further 20 min, the 2-hydroxy-3-(*p*-isothiocyanatophenoxy)propyl ethers were washed once with acetone (15 ml), twice with 0.5M sodium hydrogen carbonate (15 ml), and twice with borate buffer (0.05M, pH 8.5, 15 ml). After decantation of the final washings, aliquots (1 ml) of an α-amylase solution (0.5%) in borate buffer (0.05M, pH 8.5) were added, and the mixtures were stirred magnetically for 18 h at 0–5°. The α-amylase derivatives were finally subjected to the washing procedure outlined for the α-amylase derivatives prepared by diazo-coupling.

The effectiveness of the washing procedure was tested on a sample (100 mg) of 3-(*p*-aminophenoxy-2-hydroxypropyl ether hydrochloride of cellulose (56.2 μequiv. of ether/g) which had been washed with phosphate buffer (0.075M, pH 7.0). After centrifugation, the supernatant was discarded, and the cellulose derivative was stirred

for 18 h at 0° with an aliquot (1.0 ml) of α-amylase solution (0.5%) in phosphate buffer (0.075M, pH 7.0).

Determination of bound α-amylase. — The water-insoluble α-amylase derivatives were subjected to hydrolysis with 6N hydrochloric acid for 18 h at 110°, after which the hydrolysates were neutralised and subjected to the ninhydrin assay[8]. The original protein concentration in the hydrolysates was determined by means of a standard graph relating absorbance (570 nm) to protein concentration prior to hydrolysis. The standard graph was obtained by assaying various known amounts of a hydrolysate of free α-amylase.

Determination of α-amylase activity. — α-Amylase activity was determined by measuring the initial rate of hydrolysis at 20° of a solution of soluble starch (1%) in phosphate buffer (0.02M, pH 6.9). All determinations were made with magnetic stirring, the rate of which was kept constant for all determinations. The initial rate of hydrolysis was determined by following the rate of formation of reducing sugar by assaying aliquots of the digests with dinitrosalicylate reagent[9] against a zero-time blank. A minor modification was made in that the procedure of colorimetric determination of the brown reduction product with a green filter was superseded by measurement of the absorbance change (520 nm) with a Unicam SP-500 spectrophotometer. Thus, reducing sugar was determined by reference to a standard graph relating absorbance change (520 nm) to reducing equivalents of maltose.

Stability of water-insoluble α-amylase to heat denaturation. — A sample of water-insoluble α-amylase (preparation 2, 20 mg) was suspended in phosphate buffer (0.02M, pH 6.9, 2 ml) and incubated at 45°. Aliquots (0.2 ml) were withdrawn after 0.3 h, 1, 2, 4, and 7 days and pipetted directly into a magnetically stirred starch solution (10 ml, 1%) in phosphate buffer (0.02M, pH 6.9) at 45°. The activity of the water-insoluble α-amylase sample was then determined by periodic sampling, and assay of the digests with dinitrosalicylate reagent as described previously. Hence, the percentage of the original activity remaining in the water-insoluble α-amylase preparation was determined.

A control incubation was performed in which the water-insoluble α-amylase was replaced by a solution of an equivalent amount of free α-amylase in phosphate buffer (0.02M, pH 6.9, 2 ml). The results are shown in Fig. 1.

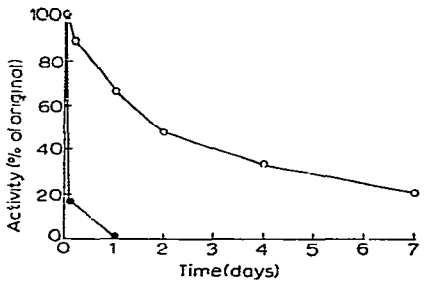


Fig. 1. Loss in activity of α-amylase coupled to microcrystalline cellulose, on incubation at 45°. —O—, α-Amylase coupled to microcrystalline cellulose; —●—, α-amylase control.

Stability of water-insoluble α-amylase to lyophilisation and acetone drying. — An aliquot (0.5 ml) of a suspension of water-insoluble α-amylase (10 mg/ml, preparation 10) in phosphate buffer (0.02M, pH 6.9) was centrifuged, and the supernatant discarded. The remaining water-insoluble derivative was then lyophilised.

A second aliquot (0.5 ml) was stirred magnetically and gradually diluted by addition of acetone (15 ml). The suspension was then centrifuged, the supernatant discarded, and the water-insoluble derivative allowed to dry in air. After storage of both samples overnight at 4°, the suspensions were reconstituted by addition of phosphate buffer (0.02M, pH 6.9). The α-amylase activity in both reconstituted suspensions, together with that of the original suspension of preparation 10, was determined against 1% starch solution at 20° as previously. The activity retained after lyophilisation was 21%, and that retained after acetone drying was 5%.

Deterioration of water-insoluble α-amylase on storage in aqueous suspension. — Suspensions (10 mg/ml) of water-insoluble α-amylase preparations Nos. 1, 2, 3, and 4 in phosphate buffer (0.02M, pH 6.9) were stored for 4 months at 0–5°. The activity of the preparations was then determined in the usual manner (Table II).

TABLE II

RETENTION OF ACTIVITY ON STORAGE OF WATER-INSOLUBLE α-AMYLASE DERIVATIVES

Preparation No.	1	2	3	4
Percentage of original activity remaining after 4 months	66	62	73	71

DISCUSSION

Attachment of an enzyme to a polymeric carrier frequently produces serious steric problems in reactions involving large substrates. Thus, even though the majority of enzymically active sites may be undamaged by the coupling procedure, the catalytic activity retained towards large substrates may be very small. Such steric problems must be in part responsible for the low activities reported previously for the water-insoluble α-amylase preparations.

In the water-insoluble enzymes reported to date, the polymeric carrier has always contained a large proportion of the functional site active in protein binding. For example, Bar-Eli and Katchalski[10] coupled trypsin to a diazotised copolymer of *p*-aminophenyl-DL-alanine and L-leucine (1:2), obtaining water-insoluble derivatives containing up to 29% of enzyme. Such high concentrations of enzyme must serve to increase steric effects, since the enzyme molecules will be tightly packed together on the carrier surface. Also, the increased probability that any given enzyme molecule will be bound by several covalent linkages will mean that the enzyme is more inflexibly bound to the carrier surface, which again must increase steric problems. Similarly, there is an increased risk of chemical coupling destroying the active site of the enzyme.

In order to test whether restriction of the amount of bound enzyme does in fact

improve the catalytic activity retained, a series of 3-(*p*-aminophenoxy)-2-hydroxypropyl ether hydrochlorides of cellulose was prepared, in which the degree of etherification varied as shown in Table I. The primary aromatic amino group was chosen as the functional site potentially active in protein binding, since this group may be conveniently activated either by diazotisation[4,10] or by conversion into the isothiocyanate[6]. It is probable that, at pH >7, diazo-coupling takes place predominantly with the tyrosine residues in the protein, whereas, at pH ⩽7, coupling with histidine and tryptophan residues may become more important, and coupling will be relatively slower. 2-Hydroxy-3-(*p*-isothiocyanatophenoxy)propyl ethers of cellulose may be presumed to couple at the side-chain amino group of lysine[6] and other free amino functions. It was considered important that more than one mode of coupling should be used in the preparation of the water-insoluble derivatives, since a given mode of coupling may be particularly likely to destroy the active site of the enzyme or effect coupling at a prominent amino-acid residue, coincidentally causing the bound enzyme to be orientated on the insoluble matrix in a way that hinders the active site.

Accordingly, α-amylase derivatives were prepared in which the various cellulose ethers had been activated by diazotisation. One series of derivatives was prepared in which the coupling pH was 7.6–7.7 and a second series in which the coupling pH was 6.3–6.4. Further derivatives were prepared in which the cellulose ethers were activated with thiophosgene and coupled with α-amylase at pH 8.5. The results shown in Table III demonstrate that the percentage activity retained by α-amylase on coupling

TABLE III

PROTEIN COUPLED[a] AND ENZYME ACTIVITY IN THE WATER-INSOLUBLE α-AMYLASE PREPARATIONS

Functional group active in protein binding	*Active functional group (μequiv.)/g of cellulose*	*Bound protein (mg)/100 mg of derivative*	*Enzyme units[b]/mg of bound protein*	*Activity retained by enzyme after coupling (%)*	*Preparation No.*
Diazotised 3-(*p*-aminophenoxy)-2-hydroxypropyl, pH 7.6–7.7	13.0	1.04	5.40	6.0	1
	20.7	1.09	4.08	5.3	2
	31.3	1.46	3.37	3.8	3
	56.2	1.66	3.33	3.7	4
Diazotised 3-(*p*-aminophenoxy)-2-hydroxypropyl, pH 6.3–6.4	13.0	0.79	4.57	5.11	5
	20.7	0.83	3.15	3.52	6
	31.3	1.47	1.59	1.78	7
	56.2	1.50	1.45	1.62	8
2-Hydroxy-3-(*p*-isothiocyanatophenoxy)propyl, pH 8.5	13.0	1.36	4.74	5.29	9
	20.7	1.42	3.66	4.09	10
	31.3	1.49	2.24	2.51	11
	56.2	1.50	2.11	2.35	12

[a]In the control experiment to test the efficiency of the washing procedure in removing physically attached protein, the 3-(*p*-aminophenoxy)-2-hydroxypropyl ether of cellulose (56.2 μequiv. of ether linkage/g) had a residual protein content of 0.05 mg/100 mg of carrier, and residual α-amylase activity of 0.10 enzyme units/100 mg of carrier. [b]One α-amylase unit is that which liberates reducing sugar equivalent to 1 mg of maltose in 3 min at 20°.

to the least substituted carriers was approximately double that of the enzyme bound to the most highly substituted carrier, whatever the mode of coupling. It should be noted that the packing of the enzyme molecules on the carrier surface is not just a function of the quantity of bound enzyme but may be due in part to the decreased hydrophilic character of the more substituted derivatives, which in turn causes the cellulose particles to decrease in size and consequently in surface area.

Although the retention of activity on lyophilisation and acetone-drying of water-insoluble α-amylase was disappointing, the activity retained on storage in buffered suspension at 0–5° was a considerable improvement on that of the α-amylase derivatives (based on polymethacrylic acid) described by Manecke[1-3]. Correspondingly, water insoluble α-amylase showed greatly improved stability over unbound α-amylase when subjected to heat denaturation at 45°. Thus, more than a ten-fold increase in the enzyme half-life was observed.

Such increases in stability are of obvious importance with solid-phase enzyme preparations where high stability is necessary to take full advantage of the ease of recovery and consequent re-usability of the preparations. Also, increased stability is important to compensate for activity losses in coupling.

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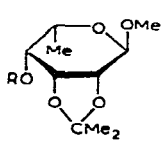
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PRELIMINARY COMMUNICATION

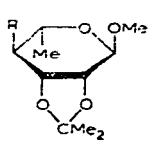
A synthesis of derivatives of L-perosamine (4-amino-4,6-dideoxy-L-mannose)

Perosamine, an amino sugar component of the heptaenic antibiotic perimycin[1] (*syn.*, fungimycin), has been tentatively assigned the structure 4-amino-4,6-dideoxy-D-mannose on the basis of chemical evidence[2]. This assignment of structure was corroborated in a recent communication[3] in which the synthesis of a number of D-perosamine derivatives was reported. We have adopted a different approach to the synthesis of certain L-perosamine derivatives in an attempt to confirm the structure and stereochemistry of natural perosamine.

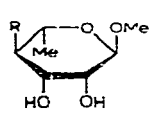
In the absence of steric hindrance[4] caused by a *trans*-axial substituent at C-2, it was anticipated that methyl 6-deoxy-2,3-*O*-isopropylidene-4-*O*-methanesulphonyl-α-L-talopyranoside (2) would undergo direct, bimolecular displacement of the sulphonyloxy group. Methanesulphonate 2 {m.p. 116–117°, $[\alpha]_D$ –19° (*c* 0.6, chloroform)} was prepared by reduction of methyl 6-deoxy-2,3-*O*-isopropylidene-α-L-*lyxo*-hexopyranosid-4-ulose[5,6] with sodium borohydride, and sulphonylation of the resulting alcohol 1, $[\alpha]_D$ –49° (*c* 1.3, methanol). The marked stereoselectivity of the reduction was demonstrated by g.l.c. and by the preponderance of methyl 2,3,4-tri-*O*-acetyl-6-deoxy-α-L-talopyranoside[5] {m.p. 90–91°, $[\alpha]_D$ –77° (*c* 0.6, methanol)} following partial, acid hydrolysis of alcohol 1 and acetylation of the resulting triol; only trace amounts of methyl 2,3,4-tri-*O*-acetyl-6-deoxy-α-L-mannopyranoside were detected by t.l.c.



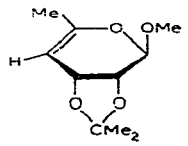
1 R = H
2 R = Ms



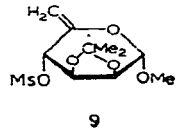
3 R = N_3
4 R = NHAc



5 R = N_3
6 R = NH_2
7 R = NHAc



8



9

On treatment with sodium azide in boiling *N,N*-dimethylformamide for 3 h, methanesulphonate 2 afforded two products, in the ratio of 1:3, which were separated by chromatography on silica gel and identified as methyl 4-azido-4,6-dideoxy-2,3-*O*-isopropylidene-α-L-mannopyranoside (3) {b.p. 66–68°/0.1 mm, $[\alpha]_D$ –28° (*c* 1.8, methanol)} and methyl 4,6-dideoxy-2,3-*O*-isopropylidene-β-D-*erythro*-hexopyran-4-enoside (8) {b.p. 46–48°/0.1 mm, $[\alpha]_D$ –195 ±1° (*c* 1, chloroform)}, respectively. Removal of the acetal group from azide 3 with hot 30% acetic acid gave methyl 4-azido-4,6-dideoxy-α-L-mannopyranoside (5) {m.p. 80–81°, $[\alpha]_D$ –128° (*c* 1, methanol)}, whose structure is readily correlated with that of the D-enantiomer {m.p. 81.5–82.5°, $[\alpha]_D$ +127° (*c* 1, methanol)} obtained by treatment of methyl 3,4-anhydro-6-deoxy-α-D-talopyranoside or its 2-benzoate with lithium azide and ammonium chloride[3]. Catalytic hydrogenation of azide 5 afforded methyl 4-amino-4,6-dideoxy-α-L-mannopyranoside (6) {m.p. 151–152°, $[\alpha]_D$ –82° (*c* 1, methanol)}. Methyl perosaminide[2], prepared by methanolysis of perimycin, had m.p. 150–151°, $[\alpha]_D$ +80° (*c* 1, methanol),

and a synthetic material[3] had m.p. 152–153°, $[\alpha]_D$ +82.5° (*c* 1, methanol). Catalytic hydrogenation of azide 3 gave, after *N*-acetylation, methyl 4-acetamido-4,6-dideoxy-2,3-*O*-isopropylidene-α-L-mannopyranoside (4) {m.p. 82–84°, $[\alpha]_D$ −23° (*c* 1.8, methanol)}, which was converted into methyl 4-acetamido-4,6-dideoxy-α-L-mannopyranoside (7) {m.p. 184–186°, $[\alpha]_D$ +20° (*c* 1, methanol)} by treatment with hot 35% acetic acid. Natural *N*-acetylperosaminide has[3] m.p. 184–185.5°, and a synthetic compound prepared by Stevens *et al.*[3] had m.p. 184–186°

The molecular formula of the major product obtained by treatment of compound 2 with azide ion was given as $C_{10}H_{16}O_4$ by mass spectrometry, and the structure 8 was clearly indicated by infrared and n.m.r. spectroscopy. Alkene 8 is presumably formed by a base-catalysed elimination reaction. Its enantiomer, obtained by an S_N2' displacement with lithium aluminium hydride on the methanesulphonate 9, had[7] b.p. 93–96°/13 mm, $[\alpha]_D$ +193° (*c* 1, chloroform)[7]; the i.r. spectrum was identical with that of 8.

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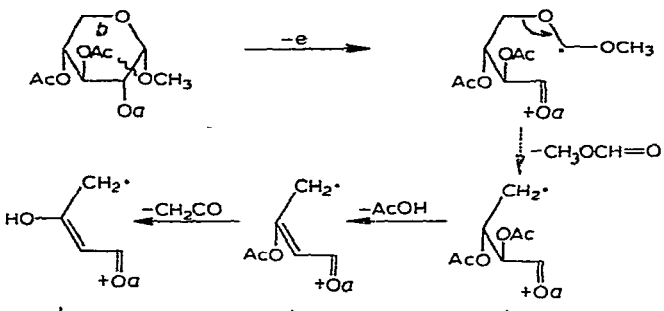
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CORRIGENDUM

Carbohydrate Research, 8 (1968)

Scheme 5 on p. 288 should be as follows:



m/e 374 *m/e* 416 *m/e* 476

SCHEME 5