Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

A SIMPLE, ALTERNATIVE SYNTHESIS OF L-ERYTHROSE

LÉON M. LERNER
Department of Biochemistry, State University of New York, Downstate Medical Center, Brooklyn, New York 11203 (U.S.A.)

(Received April 16th, 1968; in revised form, June 15th, 1968)

ABSTRACT

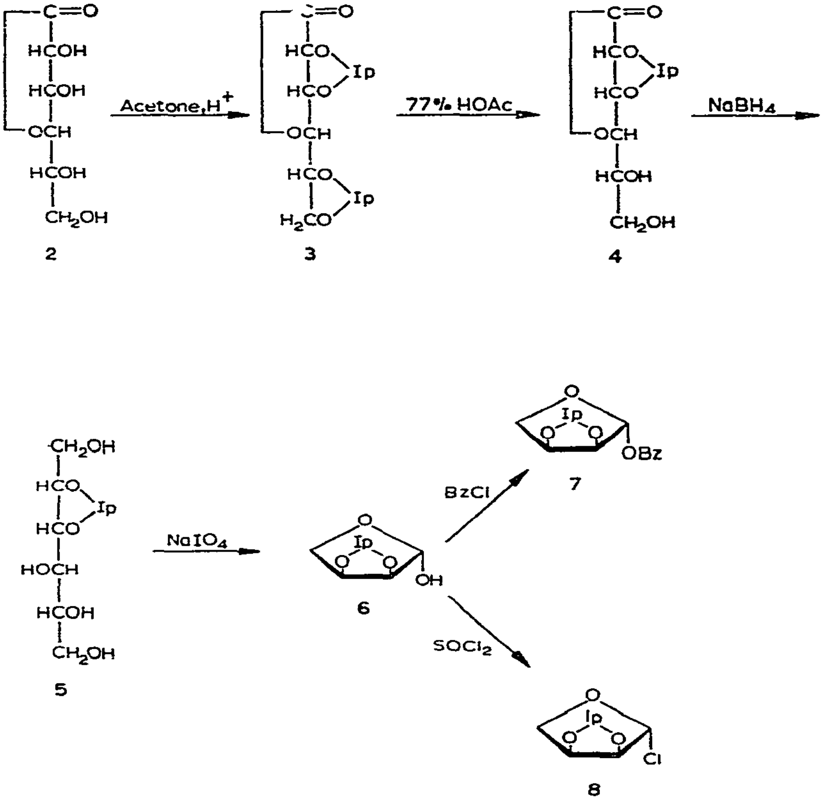
A new route for the synthesis of 2,3-*O*-isopropylidene-L-erythrofuranose and L-erythrose starting from D-gulono-1,4-lactone is presented. The intermediate, 2,3-*O*-isopropylidene-D-gulono-1,4-lactone, was prepared in a very high yield from 2,3:5,6-di-*O*-isopropylidene-D-gulono-1,4-lactone. In addition, 1-*O*-benzoyl-2,3-*O*-isopropylidene-β-L-erythrofuranose and 2,3-*O*-isopropylidene-β-L-erythrofuranosyl chloride were prepared.

INTRODUCTION

The need for a fairly large quantity of 2,3-*O*-isopropylidene-L-erythrofuranose (**6**) has arisen recently in this laboratory. The usual preparation[1] of **6** starts from 6-deoxy-2,3-*O*-isopropylidene-L-mannofuranose (**1**) and proceeds by reduction of C-1 to the alditol followed by oxidative cleavage of C-5 and C-6. This preparation suffers from several disadvantages, the main one being the synthesis of the starting substance **1** which is difficult to obtain in a pure, crystalline form. When 6-deoxy-L-mannose was treated in this laboratory with acetone in the presence of sulfuric acid, a syrupy mixture was obtained which contained at least five components. The best preparation of **1** appeared to start from 6-deoxy-L-mannose which is treated with acetone in the presence of cupric sulfate and a small amount of sulfuric acid[2]. However, purification of **1** by vacuum distillation caused extensive degradation and, in the author's hands, success was best achieved by trituration of the syrup with water followed by decantation of the aqueous solution from the insoluble substance. The aqueous solution could then be utilized directly for the preparation of **6**. The present report describes an excellent new route to **6** starting from commercially available D-gulono-1,4-lactone (**2**).

RESULTS

2,3:5,6-Di-*O*-isopropylidene-D-gulono-1,4-lactone (**3**) was prepared in excellent yield by treatment of **2** with acetone and sulfuric acid[3,4]. The important intermediate, 2,3-*O*-isopropylidene-D-gulono-1,4-lactone (**4**), was synthesized by Hulyalkar and Jones[3] in a satisfactory yield by treatment of **3** with warm 83% acetic acid. Application to **3** of the procedure of Gramera *et al.*[5] gave **4** in very high yield. After reduction of **4** with sodium borohydride, 4,5-*O*-isopropylidene-L-glucitol (**5**) was not isolated, but instead directly treated with sodium periodate. The resulting product **6** was identified by comparison of its properties with those of an original sample[6,7]. Free L-erythrose can be easily obtained by hydrolysis[1]. In addition, 1-*O*-benzoyl-2,3-*O*-isopropylidene-β-L-erythrofuranose (**7**) and 2,3-*O*-isopropylidene-β-L-erythrofuranosyl chloride (**8**), which was desired for nucleoside synthesis, were prepared.



The anomeric configuration of the L-erythrose derivatives was established by investigation of the n.m.r. spectra of **6** and **8**. The spectrum of **6** was identical to that published by Perlin[7]. The benzoate **7** was also assigned the β configuration, since no inversion of the anomeric carbon was expected during benzoylation; the high positive specific rotation (+104°) supports this assignment. Compound **8** had a very high positive rotation ($[\alpha]_D$ +168°), and the n.m.r. spectrum showed a sharp singlet for H-1 at τ 3.87, which was farther downfield than that found in **6** due to the electronegative effect of the chlorine atom. This sharp signal for H-1 of **8** was similar to that found previously for H-1 of 2,3:5,6-di-*O*-isopropylidene-D-gulofuranosyl[4] and -D-mannofuranosyl[8] chloride, both of which give sharp singlets near τ 3.90. Compound **8** was, therefore, given a β assignment.

EXPERIMENTAL

Elementary analyses were performed by the Spang Microanalytical Laboratory, Ann Arbor, Michigan. Melting points were determined on a Kofler hot-stage and correspond to corrected values. T.l.c. was performed on silica gel G plates of 0.25-mm thickness, prepared with Desaga equipment. Spots were located with a chromic acid spray followed by careful application of heat from a hot plate. Optical rotations were determined on a Rudolph Model 200 spectropolarimeter, and infrared spectra were recorded on a Perkin–Elmer Model 21 spectrophotometer by preparation of films of the compounds on sodium chloride plates. N.m.r. spectra were determined in $CDCl_3$ by Dr. Harry Agahigian of the Baron Consulting Co., Orange, Connecticut. Evaporations were performed *in vacuo* at a bath temperature of 40–45°.

2,3-O-Isopropylidene-D-gulono-1,4-lactone (**4**). — 2,3:5,6-Di-*O*-isopropylidene-D-gulono-1,4-lactone[4] (**3**, 14 g) was dissolved in a mixture of acetic acid (224 ml) and water (67.2 ml). This mixture was poured into a crystallizing dish and was left to evaporate for 3–4 days. The residual solid (11.6 g) was suspended in a large volume of ethyl acetate and heated under reflux. A small amount of undissolved D-gulono-1,4-lactone was filtered off; m.p. 180–182°, $[\alpha]_D^{24}$ $-56.8°$ (*c* 4.03, water); lit.[9] m.p. 182–185°, $[\alpha]_D^{20}$ $-57.1°$ (*c* 4.0, water).

The filtrate was concentrated and **4** crystallized as large, long needles, 9.8 g (89% yield), m.p. 142–143.5°, $[\alpha]_D^{24}$ $-74.5°$ (*c* 2.75, acetone); lit.[3] m.p. 142°, $[\alpha]_D$ $-76.5°$. T.l.c. in ethyl acetate showed that the product was homogeneous, R_F 0.17. D-Gulono-1,4-lactone barely migrated in this solvent, whereas **3** had R_F 0.56.

2,3-O-Isopropylidene-β-L-erythrofuranose (**6**). — Compound **4** (9.8 g) was dissolved in water (400 ml), and the solution was added dropwise to a mixture of sodium borohydride (4 g) in water (200 ml), chilled with an ice-bath. After addition of the entire solution, the flask was kept for 4 h at room temperature, and the pH was adjusted to 6.0–6.2 with acetic acid. The flask was cooled again in an ice-bath and sodium periodate (10.8 g) was added in small portions over a 15-min period. The solution was kept in the dark for 3 h at room temperature and concentrated to a volume of 75–100 ml after addition of nonyl alcohol (5–10 drops). The white precipitate was filtered off and washed with ethyl acetate (150 ml). The aqueous layer of the filtrate was extracted twice with 100-ml portions of ethyl acetate. The ethyl acetate portions were combined, washed twice with 50-ml portions of water, and dried ($MgSO_4$). The solution was evaporated to an oil (3.63 g), which was distilled to give 2.44 g of a clear, colorless oil (**6**), b.p. 67–74° (0.45 mm Hg), $[\alpha]_D^{23}$ $+83.2°$ (*c* 4.36, ethyl acetate). A sample of **6** prepared from 6-deoxy-2,3-*O*-isopropylidene-L-mannofuranose, had b.p. 62–67° (0.40 mm Hg) and $[\alpha]_D^{22}$ $+82.5°$ (*c* 4.46, ethyl acetate). Lit.[6] b.p. 50–70° (bath temperature) at 2 mm Hg, $[\alpha]_D$ $+72°$ (*c* 2.4, methanol). Both samples migrated as identical, homogeneous spots on thin-layer plates in 1% methanol–chloroform, R_F 0.14. The i.r. spectra were identical: ν_{max}^{film} 3400 (anomeric OH), 1375 (*gem*-dimethyl), 1160, 1100, 1068, 1045 cm^{-1} (C-O, C-O-C, dioxolane ring).

1-O-Benzoyl-2,3-O-isopropylidene-β-L-erythrofuranose (**7**). — To an ice-cold solution of **6** (517 mg) in dry pyridine (20 ml) was added benzoyl chloride (1.14 ml). The solution was kept for 45 min at 0°, and then for 17 h at room temperature. The reaction mixture was poured as a thin stream into a stirred mixture of ice and saturated sodium bicarbonate solution. This mixture was stirred for 4 h, and the white crystals were filtered off and washed with water (640 mg), m.p. 108–112°. The product was recrystallized from methanol–water to give feathery, white plates (540 mg), m.p. 108–108.5°, $[\alpha]_D^{23}$ +104° (c 4.01, chloroform); homogeneous on t.l.c. in 1% methanol–chloroform, R_F 0.75; ν_{max}^{film} 1730 (benzoate carbonyl), 1385 (doublet, *gem*-dimethyl), 1205, 1175, 1160 (C-O, C-O-C, dioxolane ring), 712 cm^{-1} (monosubstituted phenyl).

Anal. Calc. for $C_{14}H_{16}O_5$: C, 63.63; H, 6.10. Found: C, 63.70; H, 6.04.

2,3-O-Isopropylidene-β-L-erythrofuranosyl chloride (**8**). — Following Freudenberg *et al.*[10], compound **6** (2 g) was dissolved in dry chloroform (12 ml) and dry pyridine (5 ml). The mixture was cooled in an ice-bath, thionyl chloride (2.1 ml) was added, and the reaction mixture was kept for 5 h at 0°. The mixture was poured on ice, and the chloroform layer was separated. The aqueous layer was extracted twice with chloroform, and the chloroform extracts were combined and washed three times with ice-cold M sodium hydroxide, twice with ice-cold water, and dried ($MgSO_4$). Evaporation of the chloroform resulted in an orange, semisolid residue which sublimed slowly under these conditions. The product (1.01 g) was obtained by sublimation at 0.6 mm Hg 40–60°, as irregular plates which turned into long, rectangular plates just before the m.p. was reached; m.p. 59–60°, $[\alpha]_D^{26}$ +168° (c 2.0, chloroform); ν_{max}^{film} 1380 (*gem*-dimethyl), 1230, 1208, 1160 (C-O, C-O-C, dioxolane ring), 705 cm^{-1} (C-Cl); n.m.r. data: (τ) 8.67, 8.53 (singlets, $-CMe_2$), 5.83 ($-CH_2$), 5.08 (H-2, H-3), 3.87 (sharp singlet, H-1). Compound **8** gave an instantaneous alcoholic silver nitrate test. It is quite stable and can be stored in a vial at room environment for several weeks without noticeable deterioration. The analytical sample was prepared by resublimation.

Anal. Calc. for $C_7H_{11}ClO_3$: C, 47.07; H, 6.21; Cl, 19.85. Found: C, 47.17; H, 6.28; Cl, 19.76.

ACKNOWLEDGMENT

This work was supported by Research Grant No. T-442 from the American Cancer Society.

REFERENCES

1 A. S. Perlin, *Methods Carbohyd. Chem.*, 1 (1962) 67.
2 B. R. Baker, *Methods Carbohyd. Chem.*, 2 (1963) 441.
3 R. K. Hulyalkar and J. K. N. Jones, *Can. J. Chem.*, 41 (1963) 1898.
4 L. M. Lerner, B. D. Kohn, and P. Kohn, *J. Org. Chem.*, 33 (1968) 1780.
5 R. E. Gramera, A. Park, and R. L. Whistler, *J. Org. Chem.*, 28 (1963) 3230.
6 J. N. Baxter and A. S. Perlin, *Can. J. Chem.*, 38 (1960) 2217.
7 A. S. Perlin, *Can. J. Chem.*, 42 (1964) 1365.
8 J. B. Lee and T. J. Nolan, *Tetrahedron*, 23 (1967) 2789.
9 J. U. Nef, *Ann.*, 403 (1914) 269.
10 K. Freudenberg, A. Wolf, E. Knopf, and S. H. Zaheer, *Ber.*, 61 (1928) 1743.

Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

REAKTIONEN DER DI-*O*-BENZYLIDEN-PENTONSÄURE-METHYLESTER

H. ZINNER, H. VOIGT UND J. VOIGT
Institut für Organische Chemie der Universität Rostock (D.D.R.)

(Eingegangen den 13. April, 1968)

ABSTRACT

Treatment of the methyl esters of di-*O*-benzylidenepentonic acids with methanolic hydrazine affords the corresponding hydrazides. With ammonia, the amides are obtained, and these, on reduction with lithium aluminium hydride and subsequent acetylation, yield 1-acetamido-di-*O*-benzylidene-1-deoxypentitols. Dehydration of the amides with benzenesulphonyl chloride in pyridine yields the di-*O*-benzylidenepentononitriles. With Grignard reagents, the esters afford 1,1-dialkyl- and 1,1-diaryl-di-*O*-benzylidenepentitols.

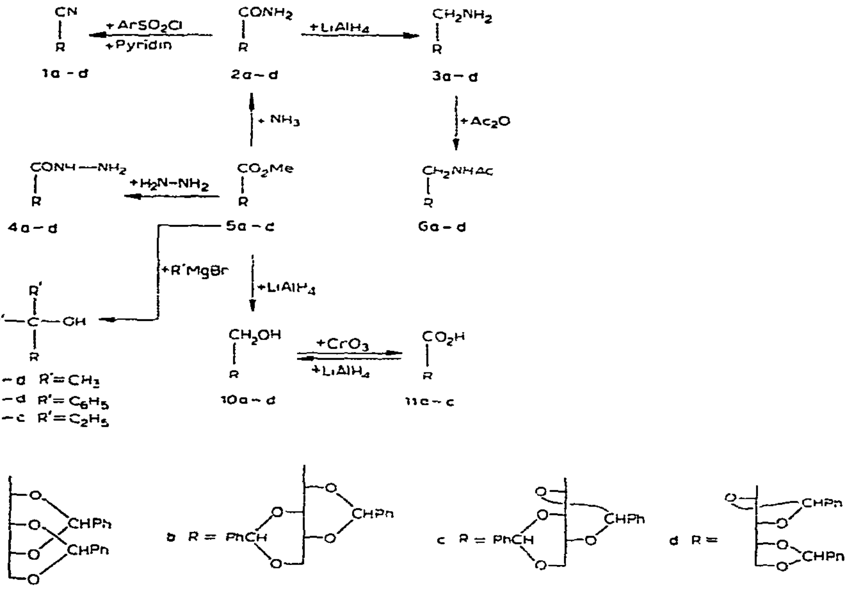
ZUSAMMENFASSUNG

Aus Di-*O*-benzyliden-pentonsäure-methylestern werden durch Kochen mit Hydrazinhydrat in Methanol Di-*O*-benzyliden-pentonsäure-hydrazide dargestellt. Mit Ammoniak erhält man Di-*O*-benzyliden-pentonsäure-amide, die sich durch Reduktion mit Lithiumaluminiumhydrid und anschließendes Acetylieren in 1-Acetamido-di-*O*-benzyliden-1-deoxy-pentite überführen lassen. Aus den Amiden entstehen durch Abspalten von Wasser mit Benzolsulfonsäurechlorid–Pyridin Di-*O*-benzyliden-pentonsäure-nitrile. Die Ester bilden mit Grignard-Verbindungen 1,1-Dialkyl- bzw. 1,1-Diaryl-di-*O*-benzyliden-pentite.

DISKUSSION

Die kürzlich beschriebenen[1] Di-*O*-benzyliden-pentonsäure-methylester (**5a–d**) sind ein geeignetes Ausgangsmaterial für Synthesen anderer Pentonsäure-Derivate. Da die Benzyliden-Gruppen gegen Basen beständig sind und nur von Säuren hydrolytisch abgespalten werden, kann man mit den Estern **5** die üblichen Ester-Reaktionen durchführen, soweit kein saures Medium erforderlich ist.

Beim Kochen der Di-*O*-benzyliden-pentonsäure-methylester mit 85-proz. Hydrazinhydrat in Methanol entstehen die Di-*O*-benzyliden-pentonsäure-hydrazide (**4a–d**), die man nach mehrmaligem Umkristallisieren mit Ausbeuten von 65–79% rein gewinnt. Die Hydrazide der D-Ribonsäure (**4a**), D-Xylonsäure (**4b**) und der D-Lyxonsäure (**4c**) ändern beim Erhitzen auf dem Mikroheiztisch nach Boëtius etwa 15° unter dem Schmelzpunkt ihre Kristallform und schmelzen dann unter Zersetzung. Das Di-*O*-benzyliden-D-arabinonsäure-hydrazid (**4d**) zeigt dieses Verhalten nicht.



Die Umsetzung der Di-*O*-benzyliden-pentonsäure-methylester mit Ammoniak in Methanol führt zu den Di-*O*-benzyliden-pentonsäure-amiden (**2a–d**), die gut kristallisieren und mit hoher Ausbeute gewonnen werden. Sie lassen sich in Dioxan–Äther mit Lithiumaluminiumhydrid zu den 1-Amino-di-*O*-benzyliden-1-deoxy-pentiten (**3a–d**) reduzieren. Diese kristallisieren nur schlecht und zersetzen sich beim Umkristallisieren teilweise. Es gelang uns nicht, die Verbindungen analysenrein darzustellen. Daher acetylierten wir die rohen 1-Amino-1-deoxy-Verbindungen **3** mit Acetanhydrid in Pyridin zu den 1-Acetamido-di-*O*-benzyliden-1-deoxy-pentiten (**6a–d**). Diese sind beständig und lassen sich durch Umkristallisieren gut reinigen.

Aus den Di-*O*-benzyliden-pentonsäure-amiden entstehen durch Abspalten von Wasser die Di-*O*-benzyliden-pentonsäure-nitrile (**1a-d**). Als wasserentziehendes Mittel sind hier Diphosphorpentoxyd, Phosphorpentachlorid und Phosphoroxychlorid ungeeignet, weil durch diese sauren Verbindungen die Benzyliden-Gruppen abgespalten werden. Mit gutem Erfolg benutzten wir Benzolsulfonsäurechlorid in Pyridin als wasserentziehendes Mittel. Die damit gewonnenen Di-*O*-benzyliden-pentonsäurenitrile kristallisieren gut, man erhält sie je nach Vertreter mit Ausbeuten von 64–80%.

Die Di-*O*-benzyliden-pentonsäure-methylester sind auch ein geeignetes Ausgangsmaterial für Synthesen mit Grignard-Verbindungen. Es ist hier die Möglichkeit gegeben, in die Reihe der 1,1-Dialkyl- und 1,1-Diaryl-di-*O*-benzyliden-pentite zu gelangen. Wir setzten die Methylester mit Methyl-, Phenyl- und Äthyl-magnesiumhalogenid in Tetrahydrofuran um und erhielten 1,1-Dimethyl- (**7a–d**), 1,1-Diphenyl- (**8a–d**) bzw. 1,1-Diäthyl-di-*O*-benzyliden-pentite (**9a–c**). Der 1,1-Diäthyl-2,3:4,5-di-*O*-benzyliden-D-arabinit kristallisierte nicht, wir konnten die Verbindung nicht rein isolieren.

Wenn man die Di-*O*-benzyliden-pentonsäure-methylester mit Lithiumaluminiumhydrid in Dioxan behandelt, erhält man die Di-*O*-benzyliden-pentite (**10a–d**), die zum Teil schon früher aus den Di-*O*-benzyliden-*aldehydo*-pentosen durch Reduktion mit komplexen Metallhydriden dargestellt wurden[1–3]. Beim Umkristallisieren des 2,3:4,5-Di-*O*-benzyliden-D-arabinits (**10d**) aus Äthanol–Wasser erhielten wir die Verbindung als Monohydrat.

Die Di-*O*-benzyliden-pentite können auch aus den Di-*O*-benzyliden-pentonsäuren (**11a–d**) durch Reduktion mit Lithiumaluminiumhydrid in Tetrahydrofuran erhalten werden. Die Reduktion der Säuren **11** verläuft aber langsamer als die der Methylester **5**, die Ausbeuten an Di-*O*-benzyliden-pentiten liegen erheblich tiefer.

Wie kürzlich gezeigt wurde[1], lassen sich Di-*O*-benzyliden-*aldehydo*-pentosen mit Chromtrioxyd in Dimethylformamid sehr gut zu Di-*O*-benzyliden-pentonsäuren oxydieren. Dieses Oxydationsmittel ist auch zur Oxydation von freien prim. Hydroxyl-Gruppen geeignet. Wir oxydierten die Di-*O*-benzyliden-pentite (**10a–c**) zu den bekannten Di-*O*-benzyliden-pentonsäuren (**11a–c**). Die Ausbeuten liegen hier etwa gleich wie bei der analogen Oxydation der Di-*O*-benzyliden-*aldehydo*-pentosen[1].

EXPERIMENTELLER TEIL

Die Schmelzpunkte wurden auf dem Mikroheiztisch nach Boëtius bestimmt.

Darstellung der Di-O-benzyliden-pentonsäure-hydrazide (**4**). — Man kocht 0.89 g (0.0025 Mol) eines Di-*O*-benzyliden-pentonsäure-methylesters[1] mit 10 ml 85-proz. Hydrazinhydrat in 25 ml Methanol 3 Stdn. unter Rückfluß, läßt dann 16 Stdn. bei 0° stehen, filtriert das Rohprodukt ab und kristallisiert um. Die Eigenschaften und Analysen der einzelnen Vertreter sind in der Tabelle zusammengestellt.

Darstellung der Di-O-benzyliden-pentonsäure-amide (**2**). — Man löst 0.89 g (0.0025 Mol) eines Di-*O*-benzyliden-pentonsäure-methylesters[1] in 150 ml Methanol, kühlt auf 0° ab, leitet 15 Min. Ammoniak ein (etwa 4 Blasen pro Sek.), läßt unter Verschluß 12 Stdn. bei 20° stehen, destilliert das Lösungsmittel ab und kristallisiert den Rückstand unter Verwendung von Aktivkohle um. Die Eigenschaften und Analysen der einzelnen Vertreter zeigt die Tabelle.

Darstellung der 1-Acetamido-di-O-benzyliden-1-deoxy-pentite (**6**). — Ein Di-*O*-benzyliden-pentonsäure-amid (0.85 g, 0.0025 Mol) wird in 120 ml Dioxan–Äther (1:1) mit 0.3 g $LiAlH_4$ 7 Stdn. unter Rückfluß gerührt. Dann kühlt man auf 0° ab, fügt tropfenweise 5.0 ml Wasser hinzu, filtriert die anorganischen Bestandteile ab, wäscht dreimal mit je 40 ml heißem Chloroform, dampft die vereinigten Filtrate ein und trocknet die zurückbleibenden, rohen 1-Amino-di-*O*-benzyliden-1-deoxy-pentite (**3a–d**) im Vakuum. Dann löst man das Rohprodukt **3** (0.57–0.64 g) in 20 ml Pyridin, gibt 3.0 ml Acetanhydrid hinzu, läßt 16 Stdn. bei 20° stehen, gießt in 200 ml

TABELLE I

EIGENSCHAFTEN UND ANALYSEN DER DI-*O*-BENZYLIDEN-PENTONSÄURE-HYDRAZIDE (4), DI-*O*-BENZYLIDEN-PENTONSÄURE-AMIDE (2), 1-ACETAMIDO-DI-*O*-BENZYLIDEN-1-DEOXY-PENTITE (6), DI-*O*-BENZYLIDEN-PENTONSÄURE-NITRILE (1), DER 1,1-DIMETHYL- (7), 1,1-DIPHENYL- (8) UND 1,1-DIÄTHYL-DI-*O*-BENZYLIDEN-PENTITE (9) UND DER DI-*O*-BENZYLIDEN-PENTITE (10)

Verbindung	*Ausbeute* (%)	*Umkristall. aus*	*Schmp.* (°)	$[\alpha]^{20}$ (°)	*Formel*	*Analyse*	*C*	*H*	*N*
2,4:3,5-Di-*O*-benzyliden-D-ribonsäure-hydrazid (4a)	73	Äthanol	254–257	−45.6 *c* 0.66, DMF	$C_{19}H_{20}N_2O_5$	Ber. Gef.	64.03 64.26	5.66 5.92	7.86 7.69
2,4:3,5-Di-*O*-benzyliden-D-xylonsäure-hydrazid (4b)	79	Äthanol	237–238	±0 *c* 1.32, DMF	$C_{19}H_{20}N_2O_5$	Ber. Gef.	64.03 64.30	5.66 5.88	7.86 8.09
2,4:3,5-Di-*O*-benzyliden-D-lyxonsäure-hydrazid (4c)	65	Äthanol	229–232	±0 *c* 1.47, DMF	$C_{19}H_{20}N_2O_5$	Ber. Gef.	64.03 63.82	5.66 5.83	7.86 7.77
2,3:4,5-Di-*O*-benzyliden-D-arabinonsäure-hydrazid (4d)	67	Äthanol	144.5–147.5	−39.8 *c* 2.40, DMF	$C_{19}H_{20}N_2O_5$	Ber. Gef.	64.03 64.00	5.66 5.94	7.86 8.16
2,4:3,5-Di-*O*-benzyliden-D-ribonsäure-amid (2a)	92	Aceton–Wasser	239–241	−45.5 *c* 1.34, DMF	$C_{19}H_{19}NO_5$	Ber. Gef.	66.84 66.74	5.61 5.46	4.10 4.19
2,4:3,5-Di-*O*-benzyliden-D-xylonsäure-amid (2b)	92	Aceton–Wasser	290.5–291.5	−7.4 *c* 1.21, DMF	$C_{19}H_{19}NO_5$	Ber. Gef.	66.84 66.96	5.61 5.91	4.10 4.26
2,4:3,5-Di-*O*-benzyliden-D-lyxonsäure-amid (2c)	91	Aceton–Wasser	286–288	−41.4 *c* 1.91, DMF	$C_{19}H_{19}NO_5$	Ber. Gef.	66.84 66.65	5.61 5.85	4.10 4.30
2,3:4,5-Di-*O*-benzyliden-D-arabinonsäure-amid (2d)	79	Äthanol–Wasser	133–135	−54.3 *c* 2.25, DMF	$C_{19}H_{19}NO_5$	Ber. Gef.	66.84 66.62	5.61 5.32	4.10 4.19
1-Acetamido-2,4:3,5-di-*O*-benzyliden-1-deoxy-D-ribit (6a)	63	Aceton–Wasser	234–236	−57.2 *c* 0.80, DMF	$C_{21}H_{23}NO_5$	Ber. Gef.	68.27 68.37	6.28 6.27	3.80 4.07
1-Acetamido-2,4:3,5-di-*O*-benzyliden-1-deoxy-D-xylit (6b)	62	Aceton–Petroläther	250.5–252.5	−38.6 *c* 1.34, DMF	$C_{21}H_{23}NO_5$	Ber. Gef.	68.27 68.31	6.28 6.27	3.80 4.02
1-Acetamido-2,4:3,5-di-*O*-benzyliden-1-deoxy-D-lyxit (6c)	58	Aceton–Wasser	284–285.5	*a*	$C_{21}H_{23}NO_5$	Ber. Gef.	68.27 68.48	6.28 6.50	3.80 4.06
1-Acetamido-2,3:4,5-di-*O*-benzyliden-1-deoxy-D-arabinit (6d)	61	Äthanol	156	+1.0 *c* 2.40, DMF	$C_{21}H_{23}NO_5$	Ber. Gef.	68.27 68.19	6.28 6.57	3.80 3.93
2,4:3,5-Di-*O*-benzyliden-D-ribonsäure-nitril (1a)	78	Aceton–Wasser	184.5–186.5	−39.9 *c* 2.09, DMF	$C_{19}H_{17}NO_4$	Ber. Gef.	70.56 70.86	5.30 5.35	4.33 4.60
2,4:3,5-Di-*O*-benzyliden-D-xylonsäure-nitril (1b)	80	Aceton–Wasser	210.5–213	+1.0 *c* 2.94, DMF	$C_{19}H_{17}NO_4$	Ber. Gef.	70.56 70.53	5.30 5.37	4.33 4.53
2,4:3,5-Di-*O*-benzyliden-D-lyxonsäure-nitril (1c)	69	Aceton–Wasser	205–206	−1.5 *c* 2.07, DMF	$C_{19}H_{17}NO_4$	Ber. Gef.	70.56 70.32	5.30 5.57	4.33 4.56

2,3:4,5-Di-*O*-benzyliden-D-arabinonsäure-nitril (**1d**)	64	Äthanol	97.5	−25.4 *c* 2.74, DMF	$C_{19}H_{17}NO_4$	Ber. Gef.	70.56 70.35	5.30 5.19	4.33 4.59
2,4:3,5-Di-*O*-benzyliden-1,1-dimethyl-D-ribit (**7a**)	64	Aceton–Wasser	132–136	−39.3 *c* 3.44, Chlf.	$C_{21}H_{24}O_5$	Ber. Gef.	70.77 70.62	6.79 6.85	
2,4:3,5-Di-*O*-benzyliden-1,1-dimethyl-D-xylit (**7b**)	85	Chloroform–Petroläther	170–173	−32.7 *c* 1.66, Chlf.	$C_{21}H_{24}O_5$	Ber. Gef.	70.77 70.76	6.79 6.78	
2,4:3,5-Di-*O*-benzyliden-1,1-dimethyl-D-lyxit (**7c**)	42	Aceton–Wasser	125–128	+10.8 *c* 1.67, Chlf.	$C_{21}H_{24}O_5$	Ber. Gef.	70.77 70.69	6.79 6.54	
2,3:4,5-Di-*O*-benzyliden-1,1-dimethyl-D-arabinit (**7d**)	70	Aceton–Benzin	69.5–74	+15.9 *c* 2.41, Chlf.	$C_{21}H_{24}O_5$	Ber. Gef.	70.77 70.98	6.79 6.73	
2,4:3,5-Di-*O*-benzyliden-1,1-diphenyl-D-ribit (**8a**)	78	Aceton–Benzin	186–190	+0.0 *c* 1.74, Chlf.	$C_{31}H_{28}O_5$	Ber. Gef.	77.46 77.36	5.87 5.60	
2,4:3,5-Di-*O*-benzyliden-1,1-diphenyl-D-xylit (**8b**)	48	Aceton–Wasser	183	+98.8 *c* 1.37, Chlf.	$C_{31}H_{28}O_5$	Ber. Gef.	77.46 77.50	5.87 5.98	
2,4:3,5-Di-*O*-benzyliden-1,1-diphenyl-D-lyxit (**8c**)	39	Aceton–Wasser	229–231	+26.2 *c* 0.70, Chlf.	$C_{31}H_{28}O_5$	Ber. Gef.	77.46 77.33	5.87 6.09	
2,3:4,5-Di-*O*-benzyliden-1,1-diphenyl-D-arabinit (**8d**)	92	Aceton–Wasser	162–164	+1.7 *c* 2.59, Chlf.	$C_{31}H_{28}O_5$	Ber. Gef.	77.46 77.37	5.87 6.13	
1,1-Diäthyl-2,4:3,5-di-*O*-benzyliden-D-ribit (**9a**)	89	Benzin	106–109.5	−45.9 *c* 1.44, Chlf.	$C_{23}H_{28}O_5$	Ber. Gel.	71.84 71.77	7.34 7.34	
1,1Diäthyl-2,4:3,5-di-*O*-benzyliden-D-xylit (**9b**)	47	Aceton–Wasser	170–173.5	−25.1 *c* 1.42, Chlf.	$C_{23}H_{28}O_5$	Ber. Gef.	71.84 71.98	7.34 7.45	
1,1-Diäthyl-2,4:3,5-di-*O*-benzyliden-D-lyxit (**9c**)	41	Aceton–Wasser	107–110	+25.9 *c* 1.69, Chlf.	$C_{23}H_{28}O_5$	Ber. Gef.	71.84 71.80	7.34 7.20	
2,4:3,5-Di-*O*-benzyliden-D-ribit (**10a**)	nach (*a*) 84 nach (*b*) 49	Aceton–Wasser	174.5–176[b]	−32.4 *c* 1.67, Chlf.[b]	Lit.[2]				
2,4:3,5-Di-*O*-benzyliden-D-xylit (**10b**)	nach (*a*) 81 nach (*b*) 55	Aceton–Wasser	187–188[c]	−36.9 *c* 1.46, Pyridin[c]	Lit.[3]				
2,4:3,5-Di-*O*-benzyliden-D-lyxit (**10c**) (=1,3:2,4-Di-*O*-benzyliden-D-arbinit)	nach (*a*) 76 nach (*b*) 52	Aceton–Wasser	175–176	−34.3 *c* 2.76, Chlf.	$C_{19}H_{20}O_5$	Ber. Gef.	69.49 69.61	6.14 6.40	
2,3:4,5-Di-*O*-benzyliden-D-arabinit-hydrat (**10d** · H_2O)	nach (*a*) 72	Äthanol–Wasser	81–85	+2.5 *c* 2.60, Pyridin	$C_{19}H_{22}O_6$	Ber. Gef.	65.87 66.05	6.40 6.27	

---

[a] Wegen zu geringer Löslichkeit der Verbindung in allen üblichen Lösungsmitteln konnte die spezif. Drehung nicht exakt bestimmt werden. [b] Lit.[2]: Schmp. 176–177°, $[\alpha]_D^{27}$ −35.7° (*c* 4, Chloroform). [c] Lit.[3]: Schmp. 187–188°, $[\alpha]_D^{20}$ −55.7° (*c* 0.26, Pyridin); die hier angegebene spezif. Drehung von −55.7° beruht auf einem Druckfehler, der richtige Wert beträgt −35.7°.

Wasser, filtriert den Niederschlag ab, wäscht mit Wasser und kristallisiert um. Die einzelnen Vertreter zeigt die Tabelle.

Darstellung der Di-O-benzyliden-pentonsäure-nitrile (**1**). — Man löst 0.85 g (0.0025 Mol) eines Di-*O*-benzyliden-pentonsäure-amids in 40 ml Pyridin, läßt bei 0° unter Umschütteln 2.5 ml Benzolsulfonsäurechlorid zutropfen, läßt 72 Stdn. bei +5° und 2 Stdn. bei 20° stehen und gibt dann unter Rühren soviel einer gesättigten Natriumhydrogencarbonat-Lösung hinzu, bis keine Kohlendioxyd-Entwicklung mehr stattfindet. Dann gießt man in 100 ml Eiswasser, filtriert das Rohprodukt ab, wäscht mehrfach mit Wasser nach, trocknet über Schwefelsäure, bis das Rohprodukt nicht mehr nach Pyridin riecht, und kristallisiert unter Verwendung von Aktivkohle um. Die einzelnen Vertreter sind in der Tabelle aufgeführt.

Darstellung der 1,1-*Dimethyl-* (**7**), 1,1-*Diphenyl-* (**8**) *und der* 1,1-*Diäthyl-di-O-benzyliden-pentite* (**9**). — Man bereitet aus 0.01 Mol Methyljodid (1.42 g), Brombenzol (1.57 g) oder Äthyljodid (1.56 g) und 0.24 g (0.01 Mol) Magnesium in 20 ml Äther eine Grignard-Lösung, fügt eine Lösung von 0.89 g (0.0025 Mol) eines Di-*O*-benzyliden-pentonsäure-methylesters[1] in 50 ml Tetrahydrofuran hinzu, erwärmt 4 Stdn. bei 50°, hydrolysiert anschließend mit einem Gemisch von 40 ml Wasser und 5 ml Essigsäure, extrahiert dreimal mit je 50 ml Chloroform, wäscht die vereinigten Extrakte mit Wasser, trocknet über Natriumsulfat, dampft die Lösung ein und kristallisiert den Rückstand um. Die Tabelle zeigt einzelne Vertreter.

Darstellung der Di-O-benzyliden-pentite (**10**). — (*a*) Man rührt eine Lösung von 1.78 g (0.005 Mol) eines Di-*O*-benzyliden-pentonsäure-methylesters[1] in 120 ml Dioxan–Äther (1:1) mit 0.5 g $LiAlH_4$ 6 Stdn. unter Rückfluß, kühlt auf 0° ab, fügt 5.0 ml Wasser hinzu, neutralisiert mit 3N HCl, saugt die anorganischen Bestandteile ab, wäscht den Filterrückstand dreimal mit je 60 ml warmem Aceton, dampft die vereinigten Filtrate ein und kristallisiert den Rückstand um.

(*b*) Eine Di-*O*-benzyliden-pentonsäure (1.71 g, 0.005 Mol) wird in 70 ml Tetrahydrofuran mit 0.7 g $LiAlH_4$ unter Rühren 6 Stdn. bei 50° reduziert. Dann arbeitet man auf, wie unter (*a*) angegeben. Die einzelnen Vertreter sind in der Tabelle aufgeführt.

Oxydation der Di-O-benzyliden-pentite zu Di-O-benzyliden-pentonsäuren (**11**). — Man gibt 0.33 g (0.001 Mol) eines Di-*O*-benzyliden-pentits in eine Lösung von 0.40 g Chromtrioxyd und 0.2 ml konz. Schwefelsäure in 15 ml Dimethylformamid, läßt 30 Stdn. bei 20° stehen und arbeitet auf, wie früher für die Oxydation der Di-*O*-benzyliden-*aldehydo*-pentosen zu **11** beschrieben[1].

LITERATUR

1 H. Zinner, H. Voigt und J. Voigt, *Carbohyd. Res.*, 7 (1968) 38.
2 D. J. J. Potgieter und D. L. MacDonald, *J. Org. Chem.*, 26 (1961) 3934.
3 H. Zinner, K.-H. Rohde und A. Mattheus, *Ann.*, 677 (1964) 160.

Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

STUDIES OF CARBOHYDRATE DERIVATIVES BY NUCLEAR MAGNETIC DOUBLE-RESONANCE

PART III*. THE SIGNS OF VICINAL AND GEMINAL, FLUORINE–PROTON COUPLING-CONSTANTS**

L. D. HALL AND J. F. MANVILLE†
Department of Chemistry, The University of British Columbia, Vancouver 8, British Columbia (Canada)

(Received August 7th, 1967; in revised form, July 8th, 1968)

ABSTRACT

The "selective-irradiation" and "spin-tickling", nuclear magnetic double-resonance (n.m.d.r.) techniques have been used for determining the *relative* signs of the *vicinal* and *geminal* ^{19}F–^{1}H couplings of a series of glycopyranosyl fluorides. In all cases, these two couplings have the same *relative* signs. Heteronuclear decoupling experiments on 3,4,6-tri-*O*-acetyl-2-bromo-2-deoxy-α-D-mannopyranosyl fluoride (**2**) show that, for this derivative, the *vicinal* coupling between H-1 and H-2 and the *geminal* coupling between H-1 and F have the same relative sign. On this basis, an *absolute* positive sign has been assigned to both the *vicinal* and the *geminal* ^{19}F–^{1}H couplings; this result is in accord with data for other derivatives.

INTRODUCTION

In the previous paper in this series[1], we used the nuclear magnetic double-resonance (n.m.d.r.) technique to determine the relative signs of the ^{1}H–^{1}H coupling constants of a series of carbohydrate derivatives. We now describe the extension of that study to the determination of the relative signs of ^{19}F–^{1}H coupling constants. This information was of interest to us because recent results from this [3–5] and other laboratories [6–8] have indicated that *vicinal* ^{19}F–^{1}H and ^{1}H–^{1}H couplings exhibit a fundamentally similar angular dependence. As will be seen later, the *vicinal* ^{19}F–^{1}H and ^{1}H–^{1}H couplings that we have examined are all *absolutely positive* in sign, whereas *geminal* ^{19}F–^{1}H couplings are *positive* and *geminal* ^{1}H–^{1}H couplings are *negative.*

EXPERIMENTAL

All homonuclear n.m.d.r. experiments were performed as described previously[1]. For the heteronuclear decoupling experiment, a Varian Dual-Purpose spectro-

---

*For Part II, see ref. 1.
**Presented, in part, at the 152nd National Meeting of the American Chemical Society, New York, New York, September 11–16, 1966. For a preliminary communication, see ref. 2.
†Recipient of a National Research Council of Canada Studentship, 1966–67.

meter having a V-4311 radiofrequency unit tuned to 60 MHz was used, together with a Varian V-3521A Integrator/Decoupler for base-line stabilization. The ^{19}F decoupling frequency was obtained from a Nuclear Magnetic Resonance Specialities* SD-60 Decoupler by use of a basic, crystal oscillator (25.547 MHz), in conjunction with a variable-frequency oscillator (V.F.O.) of *ca.* 5.350 MHz. The overall output-frequency of this unit was monitored indirectly by beating the output of the V.F.O. against a standard, 5.365-MHz crystal oscillator, available as the N.M.R.S. Electronic Counter Adaptor (EC-60), and by counting the resultant, audiofrequency, "beat" signal with a Hewlett–Packard 3734A frequency-counter. In normal operation, the output frequency of the V.F.O., and hence that of the whole unit, is somewhat unstable. Professor F.A.L. Anet (University of California, Los Angeles) kindly gave us details of a modification whereby the V.F.O. may be effectively stabilized, and we used this modification for the ^{1}H-(^{19}F) experiments here described.

At the start of this study, we had no facility for measuring the "absolute" output frequency of our decoupler, and we obtained an "absolute" calibration indirectly, as follows. As part of another study, we had shown[3] that the ^{19}F chemical shift [which has subsequently been remeasured in chloroform with use of trichlorofluoromethane (Freon 11) as the internal standard] of tetra-*O*-acetyl-*β*-D-glucopyranosyl fluoride (6) is +137.8 p.p.m., and we have now determined that the chemical shift of 3,4,6-tri-*O*-acetyl-2-bromo-2-deoxy-*α*-D-mannopyranosyl fluoride (2), also in chloroform with Freon 11 as the internal standard, is +123.2 p.p.m. The 60-MHz p.m.r. spectrum of each of these derivatives was monitored in turn while the ^{19}F resonance at *ca.* 56.4 MHz was simultaneously irradiated. The V.F.O. was tuned until the H-1 resonance of each derivative was completely decoupled from the fluorine resonance. The nominal, audio-beat frequencies were respectively 20.584 and 19.931 kHz. Thus, *high* values of the audio-beat frequency correspond to irradiation of the *high*-field regions of the fluorine magnetic resonance spectrum. This "indirect" calibration was subsequently confirmed both by direct measurement of the radiofrequency output of the SD-60 decoupler, by use of a Hewlett–Packard 524B radiofrequency counter fitted with a 10–100-MHz counter Adaptor (No. 525A), and by inspection of the resulting ^{1}H spectra (*vide infra*).

The 60-MHz, field-swept, homonuclear decoupling of derivative 2 utilized the Varian Dual-purpose spectrometer with the Varian V-3521A Integrator/Decoupler, as instructed by Johnson[9].

Synthesis of the derivatives used in this study has been published elsewhere, or will be the subject of future communications: the structures are not the subject of any controversy.

RESULTS AND DISCUSSION

As, in the previous paper[1] of this series, we gave a detailed discussion of the "selective-irradiation"[10,11] and the "spin-tickling"[11,12] methods for determining the

---

*1410 Greensburg Road, New Kensington, Pa., U.S.A.

relative signs of coupling constants, it is sufficient to make only brief mention here of the general approach. In contrast to the determination of ^1H–^1H signs, where we found "spin-tickling" to be the preferred method, selective irradiation is often suitable for the determination of the ^{19}F–^1H signs. Because the large (*ca.* 50 Hz) *geminal* ^{19}F–^1H coupling effectively separates the fluorine spin-state "α" from the fluorine spin-state "β" transitions of the proton resonance, one set can readily be irradiated without perturbing the other. This conclusion is illustrated in the following discussion.

The spectra given in Fig. 1 show typical examples of sign determination by the frequency-sweep, selective-irradiation technique. Table I shows the corresponding spin-state assignments for the H-1 and H-2 resonances of the compounds involved. Fig. 1A shows the partial p.m.r. spectrum of 2,3-di-*O*-acetyl-4,6-*O*-benzylidene-α-D-glucopyranosyl fluoride (**3**, R′ = H, R″ = F), and Fig. 1B shows the effect of irradiating the $^{19}F = \alpha$ transitions (5,6) of the H-1 resonance. It is clearly evident that this treatment results in the collapse of transitions 9,10 and 11,12, which also

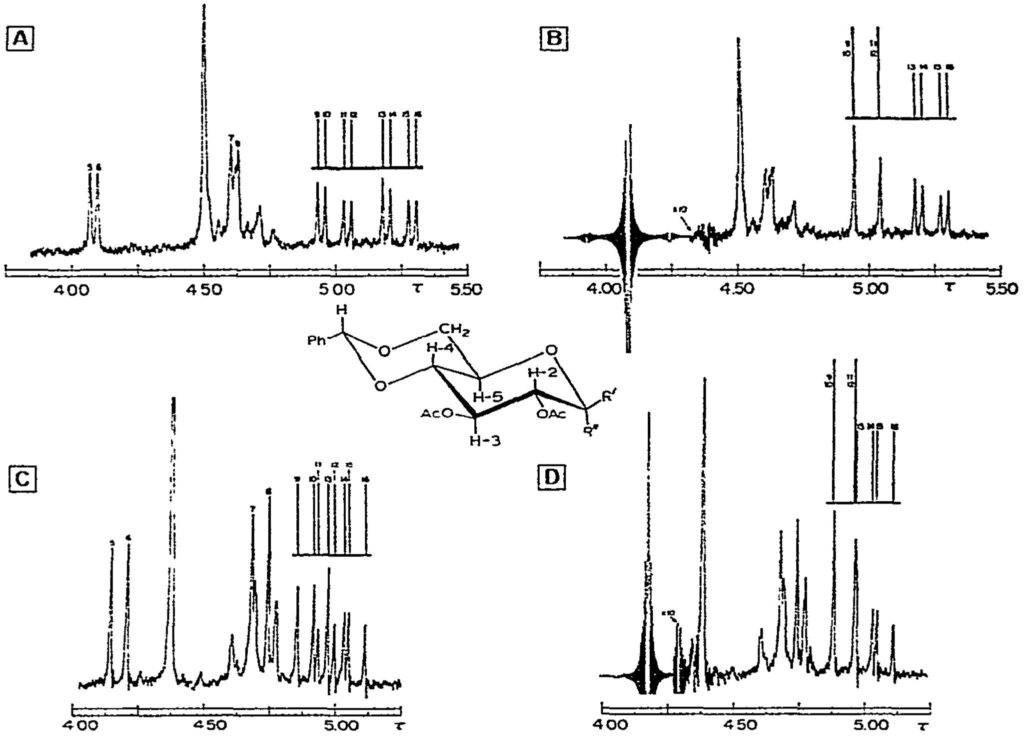


Fig. 1. Partial, 100-MHz p.m.r. spectra of 2,3-di-*O*-acetyl-4,6-*O*-benzylidene-α-D-glucopyranosyl fluoride (**3**: R′ = H, R″ = F) in acetone-d_6 solution; (A) is the "normal" spectrum, and (B), with irradiation of transitions 5,6. 2,3-Di-*O*-acetyl-4,6-*O*-benzylidene-β-D-glucopyranosyl fluoride (**4**: R′ = F, R″ = H) in acetone-d_6 solution; (C) is the "normal" spectrum, and (D), with irradiation of transitions 5,6.

TABLE I

SPIN-STATE ASSIGNMENTS[a] FOR THE ^{19}F, H-1, AND H-2 RESONANCES OF (A) 2,3-DI-*O*-ACETYL-4,6-*O*-BENZYLIDENE-α-D-GLUCOPYRANOSYL FLUORIDE (3), AND (B) 2,3-DI-*O*-ACETYL-4,6-*O*-BENZYLILYDENE-β-D-GLUCOPYRANOSYL FLUORIDE (4)

Spin states of neighboring nuclei	*^{19}F*				*H-1*				*H-2*							
	Transition number															
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*
Set A																
^{19}F					α	α	β	β	α	α	α	α	β	β	β	β
H-1	α	α	β	β					α	β	α	β	α	β	α	β
H-2	α	β	α	β	α	β	α	β								
H-3									α	α	β	β	α	α	β	β
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*13*	*12*	*14*	*15*	*16*
Set B																
^{19}F					α	α	β	β	α	α	α	β	α	β	β	β
H-1	α	α	β	β					α	β	α	α	β	β	α	β
H-2	α	β	α	β	α	β	α	β								
H-3									α	α	β	α	β	α	β	β

[a] All signs are assumed to be positive.

have ^{19}F = α spin-states. Hence, it follows that the *relative* signs of $J_{F,1}$ and $J_{F,2}$ are the same. Figs. 1C and 1D show, respectively, the "normal" and "irradiated" spectra of 2,3-di-*O*-acetyl-4,6-*O*-benzylidene-β-D-glucopyranosyl fluoride (4, R′ = F,

TABLE II

THE SIGNS OF THE ^{19}F–^{1}H COUPLING CONSTANTS OF A SERIES OF GLYCOPYRANOSYL FLUORIDES

Fluoride	*Sign and magnitude of relevant coupling constants (Hz)*
3,4,6-Tri-*O*-acetyl-2-bromo-2-deoxy-β-D-glucopyranosyl (1)[a]	$J_{1,F}+50.3$; $J_{2,F}+10.0$
3,4,6-Tri-*O*-acetyl-2-bromo-2-deoxy-α-D-mannopyranosyl (2)[a]	$J_{1,F}+50.2$; $J_{2,F}+2.85$; $J_{2,F}+1.50$
2,3-Di-*O*-acetyl-4,6-*O*-benzylidene-α-D-glucopyranosyl (3)[b]	$J_{1,F}+53.0$; $J_{2,F}+24.6$
2,3-Di-*O*-acetyl-4,6-*O*-benzylidene-β-D-glucopyranosyl (4)[b]	$J_{1,F}+53.4$; $J_{2,F}+11.6$
Tetra-*O*-acetyl-α-D-glucopyranosyl (5)[a]	$J_{1,F}+52.8$; $J_{2,F}+23.8$
Tetra-*O*-acetyl-β-D-glucopyranosyl (6)[b]	$J_{1,F}+52.5$; $J_{2,F}+12.0$
Tri-*O*-acetyl-β-D-arabinopyranosyl (7)[b]	$J_{1,F}+54.0$; $J_{2,F}+23.9$
Tri-*O*-acetyl-2-deoxy-α-D-*arabino*-hexopyranosyl (8)[a]	$J_{1,F}+51.4$; $J_{2e,F}+5.0$; $J_{2a,F}+33.0$; $J_{1,2e}+1.6$; $J_{1,2a}+2.85$; $J_{2e,2a}-13.9$; $J_{2e,3}+5.2$; $J_{2a,3}+10.8$
Tri-*O*-acetyl-β-D-ribopyranosyl (9)[b]	$J_{1,F}+49.5$; $J_{2,F}+7.1$
Tri-*O*-acetyl-α-D-xylopyranosyl (10)[b]	$J_{1,F}+53.8$; $J_{2,F}+23.7$

[a] Measured in chloroform solution. [b] Measured in acetone solution.

R″ = H). Again, the *relative* signs of $J_{F,1}$ and $J_{F,2}$ are the same, as irradiation of the low-field transitions (5,6) of the H-1 resonance effects decoupling of the low-field transitions (9,10 and 11,12) of the H-2 resonance.

All of the homonuclear-decoupling experiments performed on derivatives **1** to **10**, summarized in Table II, indicated that the *relative* signs of $J_{F,1}$ and $J_{F,2}$ are the same, and we were interested in placing these observations on an *absolute* basis. Because it was already known[13—16] that *vicinal* ^1H–^1H couplings are *absolutely positive* in sign, this objective could be achieved by determining the *relative* signs of $J_{F,1}$ and $J_{1,2}$. This would involve selective irradiation of part of the ^{19}F spectrum while the H-2 resonance was being observed; 3,4,6-tri-*O*-acetyl-2-bromo-2-deoxy-α-D-mannopyranosyl fluoride (**2**) had spectral properties at 60 MHz that were suited to such an experiment (see Fig. 2A).

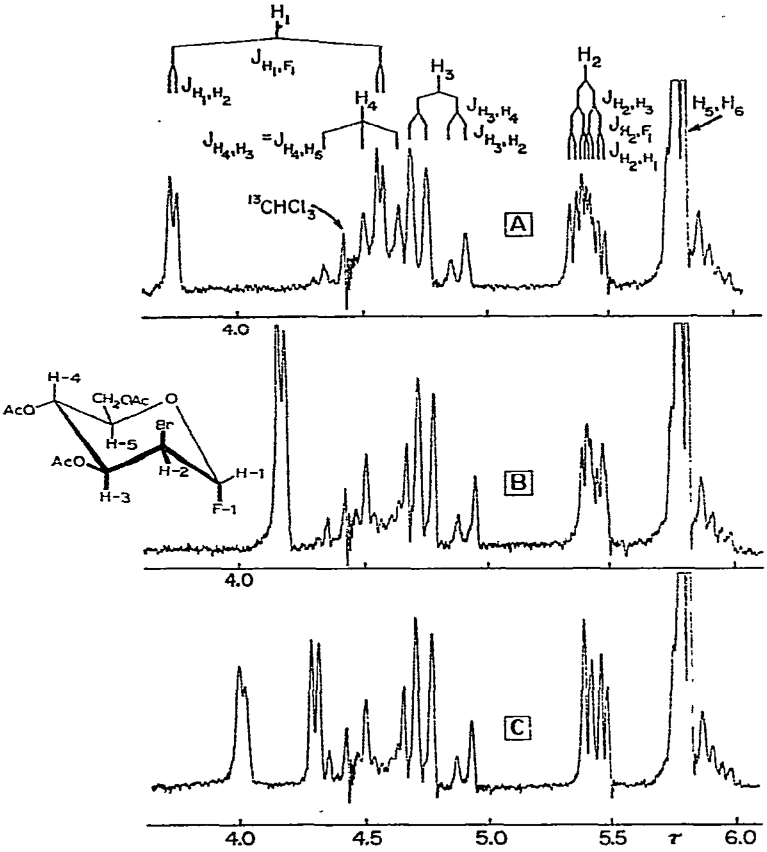


Fig. 2. Partial, 60-MHz p.m.r. spectrum of 3,4,6-tri-*O*-acetyl-2-bromo-2-deoxy-α-D-mannopyranosyl fluoride (**2**) in chloroform solution; (A) normal spectrum; (B) and (C), with irradiation at the fluorine frequency to effect optimal decoupling of the H-1 and H-2 resonances, respectively.

Initially, the ^{19}F resonance frequency of **2** was located by sweeping the ^{19}F decoupler output until complete decoupling of the ^1H spectrum was observed. It may be seen from Figs. 2B and 2C that the power output of our decoupler was not sufficiently great to decouple simultaneously the H-1 and H-2 resonances from the fluorine spectrum*. Thus, in Fig. 2C, where the ^{19}F frequency had been chosen for optimal decoupling of the H-2 resonance, the H-1 resonance is only partially decoupled, with each of the two "parts" of the resonance displaced towards the completely decoupled position, which is shown in Fig. 2B.

Having in this way located the approximate frequency of the ^{19}F resonance, two selective-irradiation experiments were performed; the results are shown in Fig. 3. The ^{19}F frequency was so selected (see Experimental Section) that transitions 3,4

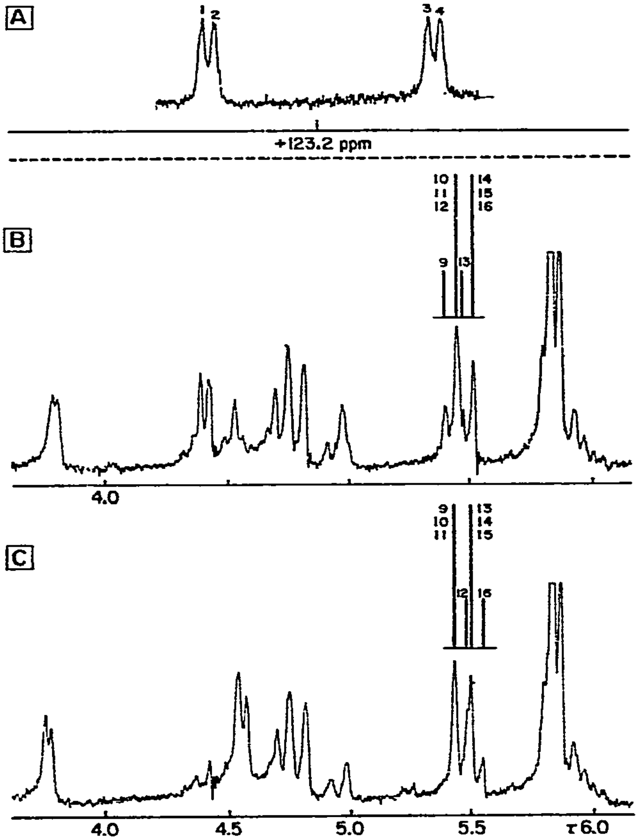


Fig. 3. (A) ^{19}F Resonance of 3,4,6-tri-*O*-acetyl-2-bromo-2-deoxy-α-D-mannopyranosyl fluoride (**2**) in chloroform solution; (B) ^{1}H spectrum of **2**, showing effect of irradiating ^{19}F transitions 3,4; (C) ^{1}H spectrum showing effect of irradiating ^{19}F transitions 1,2.

---

*It should be remembered that these experiments were performed under *field-sweep* conditions, so that the ^{19}F decoupling frequency was swept while the ^{1}H spectrum was scanned. To effect decoupling of both H-1 and H-2 for the same setting of the SD-60 decoupler would require a power output at least as great as the sum of the bandwidth of the ^{19}F resonance (53 Hz), the chemical-shift separation between H-1 and H-2 (76 Hz), and the bandwidth of the H-2 resonance, that is, a total of 138 Hz.

could be irradiated (see Fig. 3B), and the power ouptut ($\mathbf{H}_2$) was so selected that ($\mathbf{H}_2$) > ($J_{F,2}$) + $\sum(J_{F,2} + J_{1,2} + J_{2,3})$, that is ($\mathbf{H}_2$) > 12 Hz; this effected collapse of the high-field transitions* (10,12 and 14,16) of the H-2 resonance. In a subsequent experiment (see Fig. 3C), irradiation of transitions 1,2 resulted in collapse of transitions 9,11 and 13,15**. Inspection of the spin-state assignments shown in Table III indicate that the above experiments are consistent with the possibility that $J_{F,1}$ and $J_{1,2}$ have the same *relative* sign. Since homonuclear decoupling had already indicated that $J_{F,1}$ and $J_{F,2}$ have the same *relative* signs, and we know that *vicinal* ^{1}H–^{1}H couplings are *absolutely positive*, it follows that $J_{F,1}$ and $J_{F,2}$ are also *absolutely positive*. We made no attempt to apply this heteronuclear-decoupling method to other derivatives, and have assumed, in normalizing all of the values given in Table III, that all other couplings follow the pattern observed for compound **2**.

TABLE III

SPIN-STATE ASSIGNMENTS FOR THE ^{19}F, H-1, AND H-2 RESONANCES OF 3,4,6-TRI-*O*-ACETYL-2-BROMO-2-DEOXY-α-D-MANNOPYRANOSYL FLUORIDE (2)

Spin states of neighboring nuclei	*^{19}F*				*H-1*				*H-2*							
	Transition number															
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*13*	*12*	*14*	*15*	*16*
^{19}F					α	α	β	β	α	α	β	α	β	α	β	β
H-1	α	α	β	β					α	β	α	α	β	β	α	β
H-2	α	β	α	β	α	β	α	β								
H-3									α	α	α	β	α	β	β	β

It may be inferred from the above discussion that the signs of *vicinal* ^{19}F–^{1}H couplings are not subject to an angular dependence, at least as far as angles of *ca.* 60 and 180° are concerned. Further studies[17] have shown that the same is true for dihedral angles of *ca.* 0 and 120°. The *positive* sign is consistent with previous determinations of *relative* sign of non-carbohydrate derivatives[18] and with a recent *absolute* determination of sign on[19] $CHCl_2F$ and another on[20] CH_3F. It would still be of interest to determine the sign of a *vicinal* ^{19}F–^{1}H coupling between substituents separated by *ca.* 90°, for which there remains the possibility[21] of a change of sign.

---

*See Fig. 4 for a full assignment of the ^{1}H transition numbers.

**It may be seen from Figs. 3B and 3C that the H-1 transitions are more strongly perturbed in the first experiment (Fig. 3B) than in the second (Fig. 3C); this observation is consistent with the assignment made above for the two selective-irradiation experiments. Consider the situation in these *field-sweep* experiments as the change in magnetic field ($\mathbf{H}_0$) brings $\mathbf{H}_2$ towards the ^{19}F resonance, and $\mathbf{H}_1$ towards the ^{1}H spectrum. In the experiment designed to irradiate transitions 3,4 while H-2 is observed, it is clearly possible for $\mathbf{H}_2$ to perturb the ^{19}F spin-states while the H-1 resonance is being observed. It can be imagined that $\mathbf{H}_1$ reaches the ^{1}H spectrum as $\mathbf{H}_2$ approaches the ^{19}F spectrum, and thus $\mathbf{H}_1$ will detect *all possible* perturbations of the ^{19}F spectrum, including the desired decoupling. However, when $\mathbf{H}_2$ is chosen for the irradiation of transitions 1,2, $\mathbf{H}_1$ has already been swept through the ^{1}H spectrum as far as the H-2 resonance itself, before $\mathbf{H}_2$ has reached the ^{19}F spectrum, and is thus never in position to detect the spurious perturbations induced in the ^{1}H spectrum.

All of the homonuclear determinations of sign described in this and the previous paper[1] have utilized the frequency-sweep method. However, at this time, many carbohydrate laboratories have access only to 60-MHz, *field*-sweep, n.m.d.r. apparatus, and it seemed worth while to demonstrate that relative signs can be determined quite readily with such equipment. The results of field-sweep, homonuclear determinations of sign on fluoride **2** are shown in Fig. 4. Transitions 5–8 correspond to the H-1 resonance, and transitions 9–16 correspond to H-2; the spin states of these resonances have already been ascribed in Table III. It may be seen that irradiation of the higher-field transitions of H-1 (7,8), which correspond to F = β, results in the collapse of the higher-field transitions of H-2 (11,12 and 15,16), which also have F = β (see Fig. 4B). Thus, $J_{F,1}$ and $J_{F,2}$ have the same relative sign. This conclusion is verified in Fig. 4C, where irradiation of the lower-field transitions 5,6 results in a collapse of the lower-field transitions (9,10 and 13,14). The principal disadvantage of this field-sweep method is that there is no indication on the spectrum itself of the precise point of irradiation; thus, considerable care must be exercised in selecting the frequencies of the audio-oscillators used for generating the decoupling field (H_2) in such experiments.

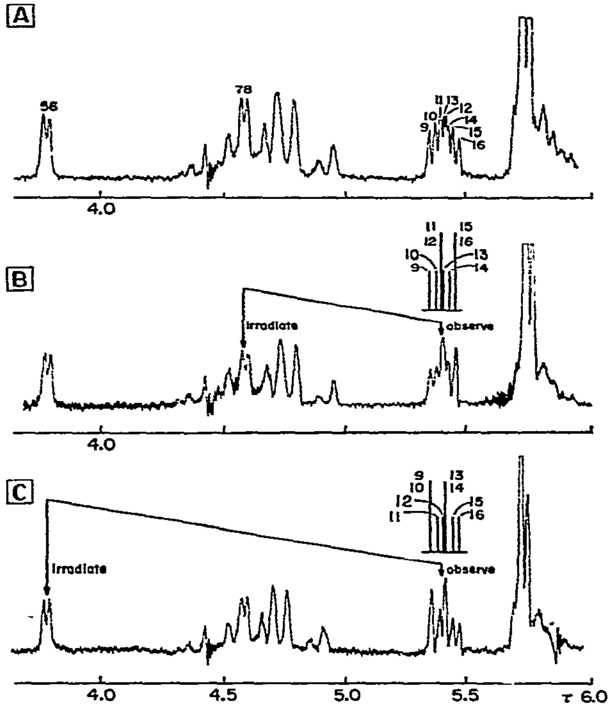


Fig. 4. Partial, 60-MHz p.m.r. spectra of 3,4,6-tri-*O*-acetyl-2-bromo-2-deoxy-α-D-mannopyranosy fluoride (**2**) in chloroform solution: (A) normal spectrum; (B) with simultaneous irradiation o transitions 7,8; and (C) with simultaneous irradiation of transitions 5,6. Both (B) and (C) are field-swept, decoupled spectra.

ACKNOWLEDGMENTS

It is a pleasure to acknowledge financial support of this work by the National Research Council of Canada and the National Cancer Institute of Canada.

REFERENCES

1 L. D. HALL AND J. F. MANVILLE, *Carbohyd. Res.*, 8 (1968) 295.
2 L. D. HALL AND J. F. MANVILLE, *Chem. Ind.* (London), (1967) 468.
3 L. D. HALL AND J. F. MANVILLE, *Chem. Ind.* (London), (1965) 991.
4 L. D. HALL AND J. F. MANVILLE, *Chem. Commun.*, (1968) 158.
5 L. D. HALL, D. J. JONES, J. F. MANVILLE, AND B. WEBSTER, unpublished results.
6 R. J. ABRAHAM AND L. CAVALLI, *Mol. Spectry.*, 9 (1965) 67.
7 K. L. WILLIAMSON, Y. F. LI, F. H. HALL, AND S. SWAGER, *J. Amer. Chem. Soc.*, 88 (1966) 5678
8 R. F. MERRITT AND F. A. JOHNSON, *J. Org. Chem.*, 31 (1966) 1859.
9 L. F. JOHNSON, *Varian Associates Technical Information Bull.*, 3 (1962) 5.
10 R. FREEMAN AND D. H. WHIFFEN, *Proc. Phys. Soc.* (London), 79 (1962) 794.
11 W. A. ANDERSON AND R. FREEMAN, *J. Chem. Phys.*, 37 (1962) 85.
12 R. FREEMAN AND W. A. ANDERSON, *J. Chem. Phys.*, 37 (1962) 2053.
13 P. C. LAUTERBUR AND R. J. KURLAND, *J. Amer. Chem. Soc.*, 84 (1962) 3405.
14 F. A. L. ANET, *J. Amer. Chem. Soc.*, 84 (1962) 3767.
15 A. D. BUCKINGHAM AND K. A. MCLAUCHLAN, *Proc. Chem. Soc.*, (1963) 144; R. E. J. SEARS AND E. L. HANN, *J. Chem. Phys.*, 45 (1966) 2753.
16 S. MEIBOOM AND L. C. SNYDER, *J. Amer. Chem. Soc.*, 89 (1967) 1038.
17 L. D. HALL, D. L. JONES, AND J. F. MANVILLE, *Chem. Ind.* (London), (1967) 1787.
18 D. F. EVANS, S. L. MANATT, AND D. D. ELLEMAN, *J. Amer. Chem. Soc.*, 85 (1963) 238.
19 E. L. MACKOR AND D. MACLEAN, *J. Chem. Phys.*, 44 (1966) 64.
20 R. A. BERNHEIM AND B. J. LAVERY, *J. Amer. Chem. Soc.*, 89 (1967) 1279.
21 M. KARPLUS, *J. Chem. Phys.*, 30 (1959) 11; *J. Amer. Chem. Soc.*, 85 (1963) 2871.

Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

ACID AND ALKALINE HYDROLYSIS OF GLYCOPYRANOSYL FLUORIDES

J. E. G. BARNETT

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

(Received May 13th, 1968)

ABSTRACT

Hydrolyses of α- and β-D-glucopyranosyl, α-D-galactopyranosyl, α-D-xylopyranosyl, and β-L-arabinopyranosyl fluorides with perchloric acid and sodium hydroxide have been investigated over a range of temperatures and concentrations. All acid hydrolyses show pseudo-first-order kinetics and, except for β-D-glucopyranosyl fluoride, a dependence of $\log_{10}$(rate) on H_0 with slopes of -0.76 to -0.85, and have positive entropies of activation, suggesting an A1 mechanism. The relative rates of hydrolysis are similar to those of the corresponding methyl glycopyranosides and suggest the same cyclic carbonium-ion intermediate. β-D-Glucopyranosyl fluoride, in which the hydroxyl at C-2 is trans to the fluorine, has a negative entropy of activation, and hydrolysis may involve an intramolecular A2 mechanism. The alkaline hydrolyses present three different cases. α-D-Xylopyranosyl and β-L-arabinopyranosyl fluorides react initially with pseudo-first-order kinetics to give the free sugar as the only primary product. A plot of $\log_{10}$(rate) against pH gave a slope close to 1.0, and the fluorides appear to react with nucleophilic attack of hydroxyl ion. α-D-Glucopyranosyl and α-D-galactopyranosyl fluorides also give pseudo-first-order kinetics initially, and show a fair dependence of $\log_{10}$(rate) on H_- with slope 1.0 over the range 1.0 to 5.0N. These fluorides give a mixture of free sugar and 1,6-anhydro-β-D sugar as the products, and two simultaneous reactions probably occur, one involving direct nucleophilic attack by hydroxyl ion at C-1, and the other involving equilibrium ionization of the hydroxyl group at C-6 followed by intramolecular attack. β-D-Glucopyranosyl fluoride reacts 5000 times faster than the α-anomer, necessitating the use of second-order kinetics. The product is 1,6-anhydro-β-D-glucose, and the reaction must proceed through a 1,2-epoxide. All of the alkaline hydrolyses studied showed a negative entropy of activation.

INTRODUCTION

It has recently been shown[1] that glycosyl fluorides are substrates for the corresponding glycosidases. Hydrolysis of glycosides by glycosidases probably involves concerted acid and base catalysis[2], and in an attempt further to understand this process and the hydrolysis of glycosyl fluorides, data concerning the rates of reaction and probable mechanism for acid and alkaline hydrolysis were required. Although a considerable amount of qualitative information was known[3], quantitative data existed only for the acid hydrolysis of β-L-arabinopyranosyl fluoride and its 2-bromo-2-deoxy derivative[4]. Investigation of the acid and alkaline hydrolysis of selected glycopyranosyl fluorides was therefore undertaken.

MATERIALS

Glycosyl fluorides were made by the established methods and had the following characteristics: α-D-glucopyranosyl fluoride[5], decomp. 120–130°, $[\alpha]_D^{20}$ +90 ±2° (*c* 1.0, water); β-D-glucopyranosyl fluoride[6], m.p. 91–92° (decomp.), $[\alpha]_D^{20}$ +25 ±3° (*c* 0.2, water); α-D-galactopyranosyl fluoride[1], decomp. 130–135°, $[\alpha]_D^{20}$ +127 ±5° (*c* 2.4, water); β-L-arabinosyl fluoride[4], m.p. 94–96° (decomp.), $[\alpha]_D^{20}$ +175 ±5° (*c* 0.4, water); α-D-xylopyranosyl fluoride[7], m.p. 106–107° (decomp.), $[\alpha]_D^{20}$ +73 ±3° (*c* 0.03, water).

Carbon dioxide-free sodium hydroxide was obtained from British Drug Houses Ltd., Poole, Dorset., as a concentrated, volumetric solution. Alkaline hydrolyses were carried out in carbon dioxide-free solution, and, periodically, stock solutions were assayed for carbon dioxide content by the barium chloride method[8]. Sodium carbonate never accounted for more than 1% of the alkalinity. Perchloric acid was standardized against sodium hydroxide. Deuterium oxide was supplied by British Drug Houses Ltd.

METHODS

Kinetic measurements. — (*a*) *Polarimetric method.* Solutions were pre-incubated at the temperature (maintained to ±0.2°) of the experiment, and portions (5–6 ml) were added to the pre-incubated sugar (100 μmoles). Each solution was thoroughly mixed and placed in a 5-ml, 1-dm, jacketed cell, and the reaction was followed by using the sodium D-line in a Perkin–Elmer 141 polarimeter with potentiometric read-out onto a Smith pen recorder. Whenever possible, readings were discounted for the first 5 min. In the case of β-D-glucopyranosyl fluoride, the reaction in sodium hydroxide could not be followed by using pseudo-first-order conditions, since the reaction was so fast. In this case, the sugar (50 μmoles) was dissolved in water (5 ml) and placed in the polarimeter cell, and N sodium hydroxide (0.1 ml) was added to start the reaction. All kinetics were determined polarimetrically, except those for the acid hydrolysis of β-D-glucopyranosyl fluoride where the rotational change was small.

(*b*) *By release of fluoride ion.* The sugar (5 mg) was dissolved in pre-incubated acid or alkali, and 10–50-μl portions were removed and pipetted into sodium acetate buffer (pH 4.3; 0.2 ml) to stop the reaction. The free fluoride ion was then measured by the method of Belcher, Leonard, and West[9], on one-tenth scale. In the case of β-D-glucopyranosyl fluoride, the tubes were read at 20, rather than 60, min after the addition of alizarin complexone. This method was used for acid hydrolysis of β-D-glucopyranosyl fluoride and for one acid and one alkaline condition on some of the sugars to check that the rates calculated from the polarimetric data corresponded to the release of fluoride.

Chromatography. — The products of each hydrolysis were deionized with either Amberlite IR-120(H^+) resin (alkaline hydrolyses) or Amberlite MB-1 resin (acid hydrolyses), and chromatographed on Whatman No. 1 paper with butyl alcohol–ethanol–water (4:1:5, organic layer) or ethyl acetate–pyridine–water (120:50:40). Sugars were detected by alkaline silver nitrate.

RESULTS

Identification of products. — The only product of acid hydrolysis appeared to be the free sugar. The products of alkaline hydrolysis were more complex.

Spots corresponding to the 1,6-anhydro-β-D sugar (hexoses only) and free sugars were obtained. In most cases, there were also weak spots corresponding to epimeric sugars and the corresponding ketoses, together with a weak, slower spot, tentatively identified as the saccharinic acid. Similar products, with the exception of the 1,6-anhydro sugars, were obtained from the dissolution of the corresponding sugar in alkali. The proportion of free sugar was estimated from the value of the final rotation. The final ratios of free sugar to anhydro sugar at 20° in 0.2N sodium hydroxide were: α-D-glucopyranosyl fluoride, 30:70; α-D-galactopyranosyl fluoride, 25:75; and for β-D-glucopyranosyl fluoride at 10°, with initially 0.02N sodium hydroxide, 20:80.

Kinetic data. — Pseudo-first-order kinetics were obtained in all cases except that of β-D-glucopyranosyl fluoride in alkali, for which good second-order kinetics were obtained. Pseudo-first-order rate-constants (k_1) were calculated either from the expression $\ln(\alpha_t - \alpha_\infty) = k_1 t + C$, or by the method of Swinbourne[10] which does not require a value of the rotation at infinite time. The latter method was used for all alkaline hydrolyses, where the initially formed free sugar isomerized slowly. Either method generally gave the same rate constant.

Generally, determinations of k were not duplicated, but when they were, reproducibility was within 2%. Occasionally, reproducibility was only within 5%. Individual plots used to determine k showed no significant departure from straight lines. There was good agreement, within the accuracy of the latter method (5–10%), between the polarimetric and fluoride ion methods.

The variation of rate of hydrolysis of fluorides with different concentrations of perchloric acid (Table I) or sodium hydroxide (Table II) was determined. Plots of $\log_{10} k_1$ against the Hammett acidity function[11,12] (H_0) gave straight lines for the fluorides tested (Fig. 1). The slopes (m) were: α-D-glucopyranosyl fluoride, 0.76; α-D-galactopyranosyl fluoride, 0.84; α-D-xylopyranosyl fluoride, 0.77. The true rate-constant (k_2) was derived from the pseudo-first-order rate-constant (k_1) by using the relationship $\log_{10} k_2 = \log_{10} k_1 - mH_0$, where H_0 is the Hammett acidity function, ($k_2 = k_1$ when $H_0 = O$). It was assumed that this relationship held at each temperature in calculating the kinetic parameters $\log_{10} A$, E, and S.

TABLE I

VARIATION OF FIRST-ORDER RATE CONSTANT WITH CONCENTRATION OF PERCHLORIC ACID AT 20°

Normality	0.5	1.0	2.0	3.0
pH	0.3	0.0	−0.3	−0.48
H_0	0.3	−0.11	−0.70	−1.13
α-D-Glucopyranosyl fluoride				
k_1, sec^{-1} (× 10^5)	7.36	16.5	35.0	95.2
$\log_{10}k_1+5$	0.867	1.218	1.544	1.979
α-D-Galactopyranosyl fluoride				
k_1, sec^{-1} (× 10^5)	28.3	67.0	188.5	433
$\log_{10}k_1+5$	1.452	1.826	2.275	2.637
α-D-Xylopyranosyl fluoride				
k_1, sec^{-1} (× 10^5)	30.7	63.5	167	377
$\log_{10}k_1+5$	1.487	1.803	2.223	2.576

TABLE II

VARIATION OF FIRST-ORDER RATE CONSTANT WITH CONCENTRATION OF SODIUM HYDROXIDE ION AT 6.5°

Normality	0.2	1.0	2.0	3.0	4.0	5.0
$pH_W+\log_{10}[OH]$	13.47	14.17	14.47	14.65	14.77	14.87
H_-(NaOH)		14.00	14.36	14.64	14.93	15.14
α-D-Glucopyranosyl fluoride						
k_1, sec^{-1} (× 10^5)	1.98	20.7	69.5	136	202	311
$\log_{10}k_1+5$	0.287	1.316	1.842	2.134	2.305	2.494
α-D-Galactopyranosyl fluoride						
k_1, sec^{-1} (× 10^5)	4.49	64.7	206	435	607	1177
$\log_{10}k_1+5$	0.653	1.811	2.314	2.638	2.783	3.071
β-L-Arabinopyranosyl fluoride						
k_1, sec^{-1} (× 10^5)	9.1	69.6	168	227	340	455
$\log_{10}k_1+5$	0.959	1.843	2.224	2.356	2.532	2.658
α-D-Xylopyranosyl fluoride						
k_1, sec^{-1} (× 10^5)	6.94	36.5	55.6	69.7	79.4	90.9
$\log_{10}k_1+5$	0.814	1.562	1.745	1.844	1.900	1.959

The alkaline hydrolysis of β-D-glucopyranosyl fluoride followed second-order kinetics. The rate constant was obtained from the equation $k(a-b)t = \ln b/a + \ln(a-x)/(b-x)$, where a and b are the initial molar concentrations, and x the amount reacted after time t.

At 6.5°, all the other fluorides showed dependence of rate on sodium hydroxide concentration (Fig. 2). A straight-line relationship between $\log_{10}k_1$ and $\log_{10}[OH^-]$ was obtained for each fluoride other than α-D-xylosyl fluoride, where the rate was low at high concentrations of sodium hydroxide. The slope for β-L-arabinopyranosyl fluoride was 1.2, and the initial slope for α-D-xylopyranosyl fluoride was 1.0. The hexosyl fluorides, however, gave a slope of 1.6. Above N sodium hydroxide, these fluorides gave a reasonable straight line of slope 1.0 on plotting[13] $\log_{10}k_1$ against H_-, the alkalinity function[14] for sodium hydroxide at 20° (Fig. 3).

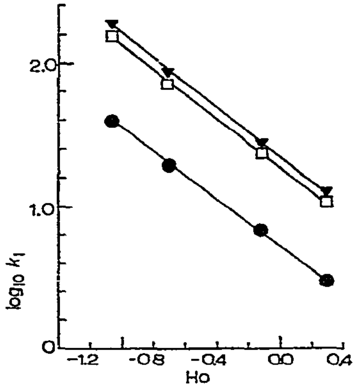
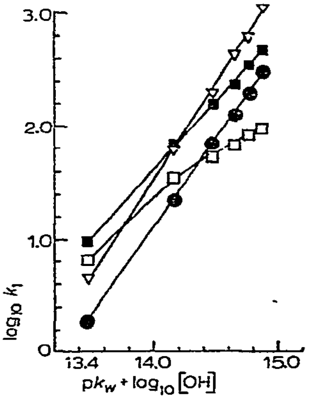


Fig. 1. Plot of $\log_{10}k_1$ at 20° against the Hammett acidity function, H_0, for the perchloric acid hydrolysis of glycopyranosyl fluorides. ▲———▲, α-D-Galactopyranosyl fluoride; □———□, α-D-xylopyranosyl fluoride; ●———●, α-D-glucopyranosyl fluoride.



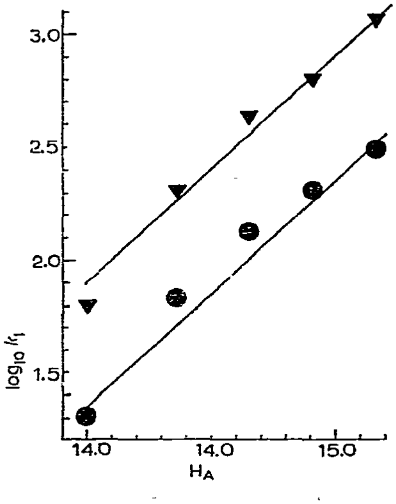


Fig. 2. Plot of $\log_{10}k_1$ at 6.5° against $pk_w+\log_{10}[OH^-]$ for the sodium hydroxide hydrolysis of glycopyranosyl fluorides. △———△, α-D-Galactopyranosyl fluoride; ●———●, α-D-glucopyranosyl fluoride; ■———■, β-L-arabinopyranosyl fluoride; □———□, α-D-xylopyranosyl fluoride.

Fig. 3. Plot of $\log_{10}k_1$ at 6.5° against the alkalinity function, H_-, for aqueous sodium hydroxide at 20°. ▲———▲, α-D-Galactopyranosyl fluoride; ●———●, α-D-glucopyranosyl fluoride. The straight lines are lines of slope 1.0 drawn through the points.

The rates of hydrolysis in 98% deuterium oxide are given in Table V.

TABLE III

PSEUDO-FIRST-ORDER RATE-CONSTANTS AT TEMPERATURES BETWEEN 6.5 AND 44.3° FOR THE ACID OR ALKALINE HYDROLYSIS OF GLYCOPYRANOSYL FLUORIDES

Acid (0.5N perchloric acid)									
Temperature, °C	6.5	15.1	20.0	30.1	40.1	44.3	$\log_{10}A$	E	$\Delta S^{\ddagger}$
$1/t$ °K ($\times 10^3$)	3.575	3.468	3.410	3.297	3.192	3.150			
α-D-Glucopyranosyl fluoride									
k_1, sec^{-1} ($\times 10^5$)	1.28	3.91	7.37[a]	29.8	111	236	14.24	24.2	+5.4
							±0.5	±0.7	±2.3
$\log_{10}k_1+5$	0.107	0.593	0 868	1.475	2.046	2.373			
β-D-Glucopyranosyl fluoride									
k_1, sec^{-1} ($\times 10^5$)	—	—	29.2	88.2	234	—	10.92	19.0	−5.4
							±0.4	±0.5	±1.7
$\log_{10}k_1+5$	—	—	1.465	1.946	2.369	—			
α-D-Galactopyranosyl fluoride									
k_1, sec^{-1} ($\times 10^5$)	—	14.1	27.8	131.8	375.8	739	14.84	24.2	+9.8
							±1.4	±0.9	±7.0
$\log_{10}k_1+5$	—	1.148	1.444	2.120	2.575	2.869			
β-L-Arabinopyranosyl fluoride									
k_1, sec^{-1} ($\times 10^5$)	—	45.3	95.2	380	1175	2487	15.41	24.3	+12.0
							±0.5	±0.7	±2.5
$\log_{10}k_1+5$	—	1.656	1.979	2.579	3.070	3.396			
α-D-Xylopyranosyl fluoride									
k_1, sec^{-1} ($\times 10^5$)	7.0	11.2	30.8	81.3	360	718	15.3	22.2	+11.5
							±0.5	±1.7	±1.8
$\log_{10}k_1+5$	0.845	1.057	1.488	1.910	2.556	2.856			
Alkali (0.2N sodium hydroxide)									
α-D-Glucopyranosyl fluoride									
k_1, sec^{-1} ($\times 10^5$)	1.94	3.85	7.46	26.1	70.3	106	11.38	19.4	−6.4
							±0.5	±0.7	±2.4
$\log_{10}k_1+5$	0.287	0.586	0.872	1.417	1.847	2.026			
α-D-Galactopyranosyl fluoride									
k_1, sec^{-1} ($\times 10^5$)	4.49	10.7	21.5	66.0	171	240	11.64	19.0	−5.2
							±0.3	±0.4	±1.4
$\log_{10}k_1+5$	0.652	1.030	1.332	1.820	2.233	2.380			
β-L-Arabinopyranosyl fluoride									
k_1, sec^{-1} ($\times 10^5$)	9.1	26.4	37.8	126	258	414	10.61	17.6	−10.0
							±0.4	±0.5	±1.8
$\log_{10}k_1+5$	0.959	1.422	1.578	2.102	2.411	2.617			
α-D-Xylopyranosyl fluoride									
k_1, sec^{-1} ($\times 10^5$)	6.94	14.0	23.9	79.9	175	202	9.59	16.7	−14.6
							±0.6	±0.8	±2.9
$\log_{10}k_1+5$	0.841	1.146	1.378	1.903	2.243	2.305			

[a] At 19.6°C.

TABLE IV

SECOND-ORDER RATE-CONSTANTS AT TEMPERATURES BETWEEN 1.6 AND 15.0° FOR β-D-GLUCOPYRANOSYL FLUORIDE

Temperature, °C	1.6	3.6	6.5	10.3	15.0	*log*$_{10}$A	E	$\Delta S^{\ddagger}$
$1/t$°K (× 10^3)	3.639	3.612	3.575	3.526	3.410			
k_2, sec^{-1}	0.70	0.76	0.95	1.43	2.58	10.42	13.25	−10.8
						±0.6	±0.8	±2.9
$\log_{10}k_2+1$	0.845	0.881	0.978	1.155	1.412			

TABLE V

PSEUDO-FIRST-ORDER RATE-CONSTANTS FOR GLYCOPYRANOSYL FLUORIDE HYDROLYSIS BY ALKALI (0.20N SODIUM HYDROXIDE) IN WATER AND DEUTERIUM OXIDE (98%) AT 20°

	$k_1 \times 10^5$, sec^{-1}		*ratio* k_{D_2O}/k_{H_2O}
	water	*deuterium oxide*	
α-D-Glucopyranosyl fluoride	2.0	2.88	1.44
α-D-Galactopyranosyl fluoride	5.7	8.17	1.43
β-L-Arabinopyranosyl fluoride	9.8	12.4	1.26
α-D-Xylopyranosyl fluoride	8.95	10.2	1.14

The activation parameters were calculated by using the data in Tables III and IV from the equation $\ln k_2 = \ln kT/h - E/RT + \Delta S^{\ddagger}/R$, where k_2 is the absolute rate constant calculated from the pseudo-first-order rate-constant k_1 (assuming that the variation with sodium hydroxide concentration at 6.5° held at each temperature), k is the Boltzman constant, h is Planck's constant, and T is the absolute temperature. Plots of $\log_{10}k_2$ against $1/T$ were presumed to be linear, and the gradients and intercepts were determined by the method of least squares. The calculated standard errors for $\log_{10}A$, E, and $\Delta S^{\ddagger}$ are given. The parameters for α-D-glucopyranosyl and α-D-galactopyranosyl fluorides are the sum of two competing reactions.

DISCUSSION

Acid hydrolyses. — Acid-catalysed reactions can proceed by two types of reaction.

$$\left.\begin{array}{l} X + H^+ \rightleftharpoons XH^+ \text{ (equilibrium)} \\ XH^+ \rightarrow Y^+ \text{ (slow)} \\ Y^+ + H_2O \rightarrow \text{Products (fast)} \end{array}\right\} \text{A1}$$

$$\left.\begin{array}{l} X + H^+ \rightleftharpoons XH^+ \text{ (equilibrium)} \\ XH^+ + H_2O \rightarrow \text{Products} \end{array}\right\} \text{A2}$$

Since the A1 mechanism does not involve water in the rate-determining step, the rate should give a linear correlation with the Hammett acidity function which is an experimental measure of the ability of the solution to protonate a base. At high concentrations of acid, this increases more rapidly than the hydrogen ion concentration. In contrast, the rate of reactions following the A2 mechanism, which depends on the activity of water, appears to follow the hydrogen ion concentration, and the rate correlates[15–17] with pH. The two types of reaction have also been distinguished by the value of the entropy of activation[18]. The constrained transition state of the A2 mechanism would be expected to give a low, probably negative, value, and the A1 mechanism a high, probably positive, value.

The dependence of the acid hydrolyses on H_0 rather than on pH agrees with the earlier investigation on β-L-arabinopyranosyl fluoride[4], and, together with the positive entropies of activation, suggests the A1 mechanism. The plots of $\log_{10}k_1$ against H_0 gave slopes of −0.76 to −0.85 rather than 1.0 (Fig. 1). These values are similar to those found for perchloric acid hydrolysis of methyl glycopyranosides[17,19] and other reactions for which the A1 mechanism is suggested[18]. There is no reason to suspect that the mechanism of the acid hydrolysis of 1,2-*cis*-glycopyranosyl fluorides is different from that proposed for the acid hydrolysis of methyl glycopyranosides. This would involve protonation of the fluorine atom, followed by elimination of hydrogen fluoride and formation of a cyclic carbonium ion as the rate-determining step, followed by rapid attack of a water molecule. Much of the evidence for the cyclic nature of the carbonium ion derives from a comparison of the relative rates of hydrolysis of the various methyl glycopyranosides which are largely explained by the relative ease of formation of the half-chair form of such an ion[20,22], although electronic[23,24] effects can be important. The rates relative to glucose for the two series of methyl glycopyranosides and glycopyranosyl fluorides are very similar. The values[22] for methyl glycopyranosides are: α-D-glucose, 1.0; α-D-xylose, 4.5; α-D-galactose, 5.2; and β-L-arabinose, 9.0. The values for glycopyranosyl fluorides are: α-D-glucose, 1.0; α-D-xylose, 4.3; α-D-galactose, 4.4; and β-L-arabinose, 12.7. These ratios seem to confirm the similarity of mechanism.

In contrast, the higher rate of hydrolysis of the β-D-glucopyranosyl fluoride compared with the α-anomer is associated with a considerable decrease in activation energy, together with a decrease in the entropy of activation to −5.4. This suggests that, for this compound, the mechanism may change to involve the trans hydroxyl group at C-2 in a nucleophilic attack, forming an intermediate 1,2-epoxide. A similar change in mechanism to an A2 type has been proposed for the alkyl furanosides, where a drop in entropy of activation compared with the pyranosides is associated with a decrease in activation energy[25].

Alkaline hydrolysis. — The alkaline hydrolyses of the fluorides tested present three different situations. The pentopyranosyl fluorides give the free sugar as the only primary product, whereas α-D-glucopyranosyl and α-D-galactopyranosyl fluorides, in which the hydroxyl group at C-6 is favourably placed for intramolecular attack, give preponderantly the 1,6-anhydro sugar, together with some free sugar. The 1,6-anhydro sugar is also the preponderant product from β-D-glucopyranosyl fluoride, in which the fluorine atom is trans to the hydroxyl group at C-2. Here, a 1,2-epoxide must be formed, which is then attacked by the C-6 hydroxyl group.

The pentosyl fluorides must undergo nucleophilic attack by hydroxyl ion at C-1. The kinetic data support this suggestion, since the plot of $\log_{10}k_1$ against $\mathrm{pK}_w + \log_{10}[\mathrm{OH}^-]$ gives a straight line having slope 1.2 for β-L-arabinosyl fluoride and slope 1.0 for α-D-xylopyranosyl fluoride initially (Fig. 2). The rate at high concentrations of sodium hydroxide falls below this line in the latter case. The difference in behaviour between the two fluorides is difficult to interpret. A possible explanation is that, at higher concentrations, the hydroxyl ions are associated, but that in β-L-arabinopyranosyl fluoride, the axial hydroxyl group at C-4 can form a hydrogen bond to the attacking hydroxyl group. The negative entropies of activation support the $\mathrm{S_N2}$ type of reaction mechanism.

The nature of the preponderant products of alkaline hydrolysis of α-D-glucopyranosyl and α-D-galactopyranosyl fluorides, that is the 1,6-anhydro sugars, shows that the C-6 hydroxyl group must be involved in the attack at C-1. The initial step, therefore, must be the ionization of the hydroxyl group at C-6, and the rate-limiting step either[a] the abstraction of the proton from this hydroxyl group, which might be expected to give a plot of $\log_{10}k_1$ similar to that found for the pentopyranosyl fluorides, since both involve nucleophilic attack by hydroxyl ion, or[b] the attack of the C-6 hydroxyl group on C-1. In the latter case, the rate would be proportional to the effectiveness with which the hydroxyl group was ionized.

$\mathrm{OH^- + XH \rightleftharpoons X^-}$ (equilibrium)
$\mathrm{X^- \rightarrow Y^-}$ (fast)

A plot of $\log_{10}k$ would be expected to correlate[13,14] with the alkalinity function H_-, which increases at a rate greater than $\log_{10}[\mathrm{OH}^-]$, in a similar way to the correlation with H_0 in the A1 mechanism. The correlation of the experimentally determined H_- is fair for both α-D-glucopyranosyl and α-D-galactopyranosyl fluorides over the range 1.0 to 5.0N sodium hydroxide, giving slopes of 1.0 (Fig. 3). The increase in rate from 0.2 to N sodium hydroxide is, however, much greater than expected.

The rates of reaction in deuterium oxide (Table V) show a clear division into two groups, and, in each case, there is an increase in rate. This can be attributed to the greater nucleophilicity or greater basicity of the deutero-oxide, rather than hydroxide, ion in the hydrolysis of the pentosyl or hexosyl fluorides, respectively.

Analysis of the products of the alkaline hydrolysis of the hexopyranosyl fluorides showed the presence of the free sugars. Since the 1,6-anhydro sugars are not rapidly hydrolysed under the conditions used, it is clear that direct attack of hydroxyl ion, as suggested for the pentosyl fluorides, must occur as a competing reaction. Since the formation of 1,6-anhydro sugar follows H_-, which increases rapidly with alkali concentration, whereas the direct attack appears to follow the hydroxyl ion concentration, this explains the previous qualitative observation that the free sugars are formed in dilute alkali and the 1,6-anhydro sugars in strong alkali[3,6,26].

Since the variation of rate with temperature had to be obtained at 0.2N sodium hydroxide, and since the rates at higher concentrations were too rapid, the activation data are the sum of both reactions in which anhydride formation predominates. The entropies of activation were negative for both fluorides, indicating a constrained transition state. The enhanced rate of the galactose derivative over the glucose must reflect the ease of attaining this state which requires conversion of the sugar into the *1C*(D) form, in which glucose has all axial hydroxyl groups and galactose has one equatorial group.

β-D-Glucopyranosyl fluoride reacted at a much greater rate than the other fluorides, and the rate was only measurable under second-order conditions. The effect on rate of high concentrations of alkali could therefore not be measured. Here, also, traces of free sugar were found as well as the 1,6-anhydro sugar, indicating some competition. The greatly enhanced rate (× 5000) of hydrolysis of the fluoride compared with the α-anomer, despite the identity of product and similarity of starting material, is a good example of neighbouring-group participation and provides a good analogy with an enzyme reaction, which can be regarded as an intramolecular reaction. The high reaction rate, together with the overall retention of configuration, indicates that a 1,2-epoxide is first formed. This must facilitate attack by the C-6 hydroxyl group on C-1. The rate-limiting step could be either of these reactions, since the high overall rate made further analysis of the reaction impossible. Since the reaction of α-D-glucopyranosyl fluoride is also intramolecular, but very much slower, it seems possible that the attainment of the half-chair form by the epoxide brings the C-6 hydroxyl group into a more favourable position for attack.

The high, relative rate of this hydrolysis indicates that the 1,2-epoxide, which has been proposed as a possible intermediate in the enzymic hydrolysis of glycosides[27], should not be discounted.

The high rate of alkaline hydrolysis of glycopyranosyl fluorides shows that the mechanism previously proposed for their hydrolysis by glycosidases should be extended to include the possibility of concerted acid and base catalysis.

ACKNOWLEDGMENTS

I thank Mr. R. W. Earney for technical assistance, the Science Research Council for financial support, Mr. R. Plummer for programming the computer for the method of least squares, and Mr. B. T. C. Bailey for suggesting a method for the determination of standard error.

REFERENCES

1 J. E. G. Barnett, W. T. S. Jarvis, and K. A. Munday, *Biochem. J.*, 105 (1967) 669.
2 R. L. Nath and H. N. Rydon, *Biochem. J.*, 57 (1954) 1.
3 F. Micheel and A. Klemer, *Advan. Carbohyd. Chem.*, 16 (1961) 85.
4 P. W. Kent and J. E. G. Barnett, *J. Chem. Soc.*, (1964) 5196.
5 B. Helferich, K. Bäuerlein, and F. Wiegand, *Ann.*, 447 (1926) 27.
6 F. Micheel and A. Klemer, *Ber.*, 85 (1952) 187.
7 F. Micheel, A. Klemer, M. Nolte, H. Noordiek, L. Tork, and H. Westermann, *Ber.*, 90 (1957) 1612.
8 A. I. Vogel, *A Textbook of Quantitative Inorganic Analysis*, 3rd Edn., Longmans, London, 1961, p. 249.

9 R. Belcher, M. A. Leonard, and T. S. West, *J. Chem. Soc.*, (1959) 3577.
10 E. S. Swinbourne, *J. Chem. Soc.*, (1960) 2371.
11 L. P. Hammett, *Physical Organic Chemistry*, McGraw-Hill, New York, N.Y., 1940, p. 237.
12 L. P. Hammett and M. A. Paul, *J. Amer. Chem. Soc.*, 56 (1934) 827.
13 C. H. Rochester, *Quart. Rev.* (London), 20 (1966) 511.
14 G. Schwarzenbach and R. Sulzberger, *Helv. Chim. Acta*, 27 (1944) 348.
15 L. Zucker and L. P. Hammett, *J. Amer. Chem. Soc.*, 61 (1939) 2791.
16 F. A. Long, *Proc. Chem. Soc.*, (1957) 220.
17 C. A. Bunton, T. A. Lewis, D. R. Llewellyn, and C. A. Vernon, *J. Chem. Soc.*, (1955) 4419.
18 F. A. Long, J. G. Pritchard, and F. E. Stafford, *J. Amer. Chem. Soc.*, 79 (1957) 2362.
19 T. E. Timell, *Can. J. Chem.*, 42 (1964) 1456.
20 J. N. BeMiller, *Advan. Carbohyd. Chem.*, 22 (1967) 25.
21 J. T. Edward, *Chem. Ind.* (London), (1955) 1102.
22 M. S. Feather and J. F. Harris, *J. Org. Chem.*, 30 (1965) 153.
23 W. G. Overend, C. W. Rees, and J. S. Sequeira, *J. Chem. Soc.*, (1962) 3429.
24 M. D. Saunders and T. E. Timell, *Carbohyd. Res.*, 6 (1968) 12.
25 B. Capon and D. Thacker, *J. Amer. Chem. Soc.*, 87 (1965) 4199.
26 F. Micheel, A. Klemer, and G. Baum, *Ber.*, 88 (1955) 475.
27 D. E. Koshland, Jr., *Biol. Rev. Cambridge Phil. Soc.*, 28 (1953) 416.

Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

MANUAL AND AUTOMATED SPECTROPHOTOMETRIC DETERMINATIONS OF PERIODATE-RESISTANT CARBOHYDRATE RESIDUES

P. V. PEPLOW AND P. J. SOMERS
Department of Chemistry, The University, Birmingham, 15 (Great Britain)

(Received June 10th, 1968)

ABSTRACT

A spectrophotometric method is described for the direct determination of hexoses and 6-deoxyhexoses during the oxidation of carbohydrates with periodate. The method is illustrated by the measurement of residual sugar in the oxidation of glucosides and the polysaccharide nigeran. An automated procedure is described and employed to monitor the oxidation of maltitol and lactitol with periodate.

INTRODUCTION

Certain sugar residues in carbohydrates can survive oxidation with periodate due to their position of substitution. Any residue containing no vicinal hydroxyl groups (*e.g.*, a 3-*O*- or a 2,4-di-*O*-substituted hexopyranose) will be resistant to oxidation with periodate[1]. In structural studies of polysaccharides, it can be advantageous to allow incomplete oxidation and to determine the rate of disappearance of the sugar units.

Unoxidised sugar residues cannot be determined directly by many of the usual spectrophotometric procedures, due to interference by aldehyde groups in the oxidised material. Similar difficulties may be encountered with the corresponding polyalcohol obtained by reduction, because of the glycolaldehyde fragment released from C-1 and C-2 during acid hydrolysis. Hydrolysis of the polyalcohol, with subsequent separation of the fragments by chromatography, may be employed, although the method is lengthy and not easily made quantitative[2]. Methanolysis of the polyalcohol, followed by removal of glycolaldehyde as the volatile dimethyl acetal[3], can be used to allow direct, colorimetric analysis by, for example, the phenol–sulphuric acid method[4].

This paper describes a method for the direct spectrophotometric determination of hexoses and 6-deoxyhexoses remaining in periodate-oxidation reactions.

EXPERIMENTAL

Determination of optimal concentration of bisulphite. — Aqueous sodium metabisulphite (0.01–1.00M, 0.10 ml) was added to stoppered test-tubes containing periodic acid (0.01M, 0.20 ml). After 5 min, aqueous D-glucose (67 μg/ml, 0.20 ml) was added, followed by a solution of L-cysteine hydrochloride monohydrate (0.07% w/v) in sulphuric acid (86% v/v, 2.5 ml). The solutions were heated for 3 min at 100° and then cooled to room temperature, and the absorbance was determined at 415 nm. A similar experiment was performed with M sodium metabisulphite (0.10 ml) and sodium metaperiodate (0–25mM, 0.20 ml). The results obtained are shown in Table I.

TABLE I

VARIATION OF RECOVERED D-GLUCOSE WITH RELATIVE CONCENTRATION OF BISULPHITE IN THE CYSTEINE–SULPHURIC ACID REACTION

Bisulphite added (μmoles) per 2 μmoles of periodate	100	50	10	5	1	0
Recovered D-glucose (%)	99	99	101	100	42	0
Periodate added (μmoles) per 100 μmoles of bisulphite	5	4	3	2	1	0
Recovered D-glucose (%)	103	100	97	103	98	100

Standardised analytical procedure and calibration. — To aliquots (0–0.4 ml) of periodate-oxidation mixtures containing hexose (0–30 μg) and periodate (0–25mM), M sodium metabisulphite (0.10 ml) was added. After at least 5 min, a solution of L-cysteine hydrochloride monohydrate (0.07% w/v) in sulphuric acid (86% v/v, 2.5 ml) was added, the solutions were heated for 3 min at 100° and then cooled to room temperature, and the absorbance was determined at 415 nm.

In calibration experiments, D-glucose (0–30 μg) or L-rhamnose (0–50 μg) was added after destruction of periodate with bisulphite. Hexoses and 6-deoxyhexoses gave characteristic chromophores having λ_{max} 415 and 400 nm, respectively. Mixtures of hexose and 6-deoxyhexose were resolved by solution of equations involving absorbances at 400 and 415 nm of reference and unknown solutions. Calibration graphs and absorption spectra are shown in Fig. 1.

Glyoxal, glyceraldehyde, glycolaldehyde, glyoxylic acid, and glycolic acid did not give interfering chromophores or depress chromophore development in the analysis procedure, when present in ten-fold molar excess.

Oxidation of glycosides with periodate. — Methyl α-L-rhamnopyranoside solution (1.26mM, 10 ml) was treated with periodic acid (0.01M, 10 ml). The uptake of periodate was determined from the absorption at 222.5 nm, after dilution of an aliquot (0.40 ml) of the oxidation mixture to 25 ml with distilled water. The rhamnose content of the oxidation mixture was determined in aliquots (0.2 ml) by the standardised procedure. The results are presented in Table II.

Methyl α-D-glucopyranoside and methyl α-D-mannopyranoside solutions (152 μg/ml, 10 ml) were treated with neutralised periodic acid (0.015M, pH 7.5, 10 ml). Aliquots (0.20 ml) of the reaction mixtures were withdrawn at 10-min intervals and added to M sodium metabisulphite (0.10 ml), and the glucose and mannose

TABLE II

L-RHAMNOSE CONTENT OF METHYL α-L-RHAMNOPYRANOSIDE DURING OXIDATION WITH PERIODATE

Oxidation time (h)	0	0.25	0.5	0.75	1.0	1.25
L-Rhamnose content (mole) per mole of methyl α-L-rhamnopyranoside	1.00	0.69	0.46	0.32	0.23	0.20
Oxidation time (h)	1.5	1.75	2.0	4.0	7.5	23.0
L-Rhamnose content (mole) per mole of methyl α-L-rhamnopyranoside	0.12	0.10	0.08	0.04	0.02	0.00
Periodate uptake (moles/mole)	—	—	—	1.60	1.64	1.82

contents were then determined as described previously. The results are presented in Table III.

TABLE III

HEXOSE CONTENT OF PERIODATE-OXIDATION MIXTURES OF METHYL HEXOPYRANOSIDES

Oxidation time (min)	0	10	20	30	40	50	60
D-Glucose (mole) per mole of methyl α-D-glucopyranoside	1.00	0.99	0.94	0.95	0.88	0.85	0.83
D-Mannose (mole) per mole of methyl α-D-mannopyranoside	1.00	0.82	0.73	0.57	0.46	0.43	0.33

Oxidation of nigeran. — Nigeran (11 mg) was dissolved in distilled water (50 ml), and the total glucose content was determined in aliquots (0.10 ml) by the cysteine–sulphuric acid method. A portion (10 ml) of the nigeran solution was mixed with periodic acid (0.01M, 12.5 ml) and diluted to 25 ml with distilled water. The intact sugar residues were determined on aliquots (0.20 ml) at intervals by the standard procedure. After 18 h, the glucose content was stationary at 48% by weight of "anhydro glucose", with a periodate consumption of 0.50 mole/mole of "anhydro glucose".

An aliquot (20 ml) of the reaction mixture was neutralised ($BaCO_3$), and reduced by the addition of sodium borohydride (20 mg) during 2 h. Cations were removed by the addition of Dowex-50Wx8 (H^+), and the solution was repeatedly evaporated with methanol *in vacuo*. The residue was hydrolysed with N sulphuric acid (5.0 ml) for 3 h at 98°. The hydrolysate was neutralised ($BaCO_3$), deionised with Dowex-50Wx8 (H^+), and concentrated to 10 ml. An aliquot (5.0 ml) was fractionated by chromatography on Dowex-1x8 (molybdate form) at 50° in water[5]. By comparison with standard mixtures, the glucose to erythritol ratio was found to be 1.00:0.96.

Selective oxidation of maltitol. — (*a*) *With sodium metaperiodate* (0.4mM). Maltitol solution (2.11mM, 1.75 ml) was treated with sodium metaperiodate (4mM, 5.0 ml), and the solution was rapidly diluted to 50 ml with distilled water. Periodate uptake was followed in aliquots (0.20 ml) by determination of the absorption at 222.5 nm after dilution to 1.0 ml.

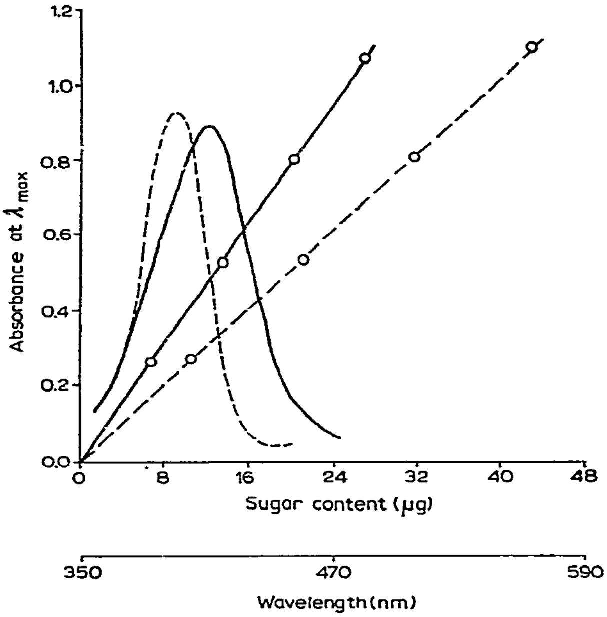


Fig. 1. Calibrations and chromophore absorption spectra for the cysteine–sulphuric acid reaction of hexoses and 6-deoxyhexoses in periodate-oxidation mixtures. – – – –, L-Rhamnose; ———, D-glucose.

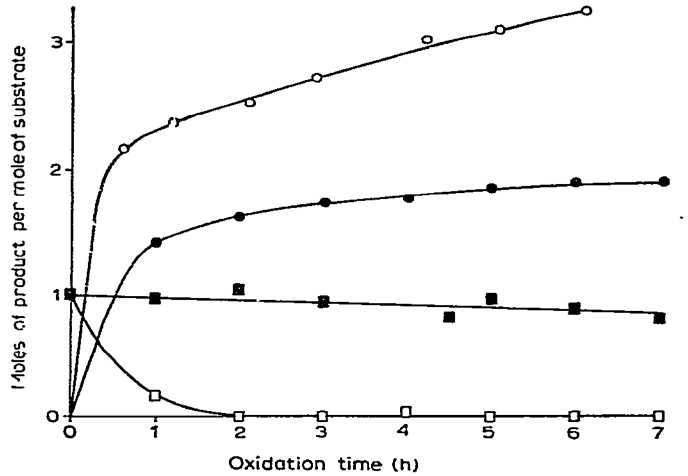


Fig. 2. Oxidation of maltitol with sodium metaperiodate. (*a*) Oxidation with 0.4 mM sodium metaperiodate: —○—, periodate consumption; —●—, formaldehyde production; —■—, glucose unoxidised. (*b*) Oxidation with 20mM sodium metaperiodate: —□—, glucose unoxidised.

The formaldehyde released was determined for aliquots (0.50 ml) by treatment with sodium arsenite solution (0.25M, pH 8.0, 0.50 ml), and, after removal of all aliquots, pentane-2,4-dione (0.02M) in ammonium acetate (1.0M, 1.00 ml). The solutions were heated for 3 min at 98° and cooled to room temperature, and the absorbance was then determined at 415 nm.

The glucose content of the oxidation mixture was determined by the standard procedure using aliquots (0.20 ml) removed at intervals.

(*b*) *Oxidation with sodium metaperiodate* (20mM). — Maltitol solution (2.11mM, 0.35 ml) was treated with sodium metaperiodate (0.10M, 2.0 ml), and the solution was rapidly diluted to 10 ml with distilled water. The production of formaldehyde and the amount of unoxidised glucose were determined, as previously, on aliquots (0.1 ml) of the reaction mixtures. The results are presented in Fig. 2.

Automated analysis of sugar residues resistant to oxidation with periodate.—The automated procedure employed identical reagents to those used in the manual procedure. Technicon Autoanalyzer modular equipment was employed throughout, and a schematic representation is presented in Fig. 3. The periodate oxidation mixture was sampled continuously (0.10 ml/min) and mixed with sodium bisulphite solution (0.05 ml/min) over a period of 6 min. After removal of air, a proportion of the sample was continuously pumped (0.10 ml/min) and mixed with the cysteine–sulphuric acid reagent (0.53 ml/min). After being heated to 95° for 3 min, the reaction stream was cooled, and the absorption was determined at 420 nm.

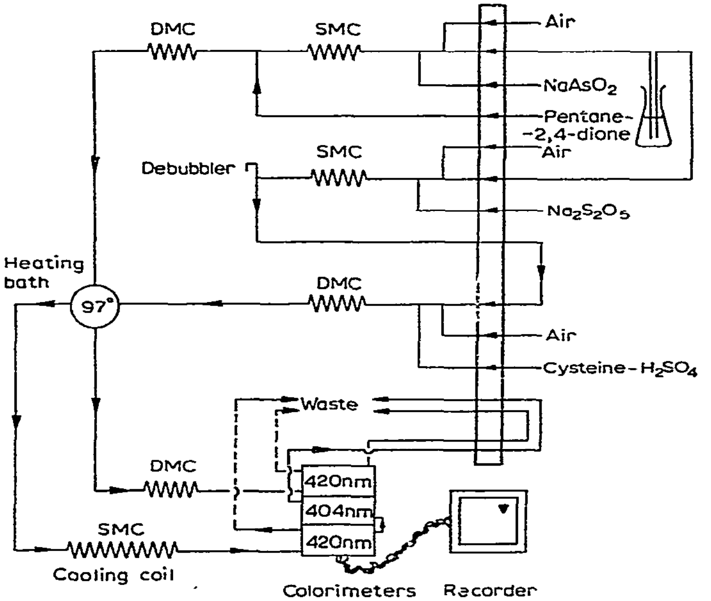


Fig. 3. Schematic representation of automated system for determination of formaldehyde and hexose in periodate-oxidation mixtures. Reagent composition and flow rates: (*a*) Formaldehyde determination; air (0.23 ml/min), sample (0.10 ml/min), $NaAsO_2$ (0.25M, adjusted to pH 8.0 with HCl, 0.16 ml/min), pentane-2,4-dione (0.02M) in ammonium acetate (M) and acetic acid (0.05M, 0.32 ml/min). (*b*) Hexose determination; air (0.10 ml/min), sample (0.10 ml/min), $Na_2S_2O_5$ (M, 0.05 ml/min); recycled sample (0.10 ml/min), air (0.32 ml/min), L-cysteine hydrochloride (0.07% w/v) in sulphuric acid (86% v/v, 0.53 ml/min).

The formaldehyde released during the oxidation was measured by a standard procedure[6] with a modified reagent composition and with omission of the periodate-oxidation stage.

Automated monitoring of formaldehyde production and hexose destruction in the oxidation of maltitol and lactitol. — Maltitol or lactitol solution (2.11mM, 1.75 ml) was diluted to 50 ml with distilled water. The solution was sampled into the automated analysis system at a total rate of 0.20 ml/min. After 25 min, sodium metaperiodate (0.09M, 0.20 ml) was added to the solution, which was continuously mixed with the aid of a magnetic stirrer. The production of formaldehyde and destruction of hexose are shown in Fig. 4.

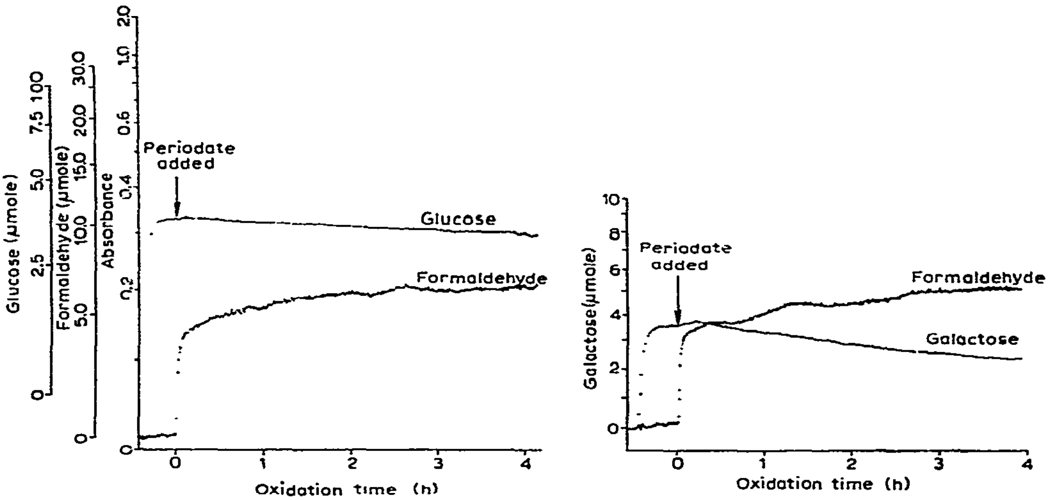


Fig. 4. Automated determination of formaldehyde production and hexose content in the oxidation of lactitol and maltitol with periodate (0.4mM).

DISCUSSION

In polymeric carbohydrates, the positions of glycosidic linkages may render individual monosaccharide residues stable to oxidation by periodate. Thus, a 3-*O*-substituted hexopyranose residue in a polysaccharide is resistant to oxidation, and the hexose may be obtained by acid hydrolysis. Direct measurement, using a simple spectrophotometric method, of intact sugar residues in oxidised polysaccharides can yield information as to the course of the reaction and the structure of the molecule. Direct methods of spectrophotometric analysis are made difficult by the necessity for removal of excess of periodate, in order to avoid further destruction of sugar residues or the chromophores derived therefrom, and by the interference of polyaldehydic products. Periodate and iodate may be removed as the lead salts, but the method is cumbersome and time consuming, particularly with analytical procedures based on sulphuric acid, making the method unsuitable, for example, in rate studies. Destruction of excess of periodate with sodium bisulphite is a suitable alternative. The complete destruction of periodate (0–25mM) by bisulphite was confirmed by the addition of hexose to the reaction mixture. Analysis by a modified cysteine–sulphuric acid method[7] revealed a linear calibration (passing through the origin) for both hexose and 6-deoxyhexose, indicative of complete removal of excess of periodate. Glyoxal, glycolaldehyde, and glyceraldehyde did not give interfering chromophores and did not depress or elevate chromophore development.

Determination of the residual rhamnose in the oxidation of methyl α-L-rhamnopyranoside with periodate (5mM) showed complete loss of the characteristic rhamnose chromophore (λ_{max} 400 nm) after 7.5 h, the periodate consumption at this time being 1.64 moles/mole, rising further to a final value of 1.95. The complete loss of the characteristic chromophore provided further confirmation that the dialdehydic (or internal hemiacetal) products of oxidation do not give rise to interfering chromophores. Since the requirement for chromophore development in the cysteine–sulphuric acid reaction is the formation of 5-(hydroxymethyl)-2-furaldehyde or 5-methyl-2-furaldehyde, cleavage of only one vicinal diol group in the sugar ring is required to preclude chromophore development. Application of this procedure to the oxidation of methyl α-D-glucopyranoside and methyl α-D-mannopyranoside by periodate (7.5mM) at pH 7.5 showed the different rates of oxidation of the two glycosides. The mannopyranose ring, containing *cis* vicinal hydroxyl groups, was 67% oxidised after 1 h, at which time the all-*trans* glucopyranose ring was only 17% oxidised.

Oxidation of the polysaccharide nigeran[8,9] [(1→3)-α-D-glucopyrano-(1→4)-α-D-glucopyranan] with periodic acid (5mM) showed a stationary value of 48% unoxidised "anhydroglucose" residues after 18 h, in agreement with the observed periodate consumption (0.50 mole per "anhydroglucose" residue) and the known structure. Reduction of the resulting polyaldehyde with sodium borohydride, followed by acid hydrolysis, gave glucose and erythritol in the molar ratio of 1.00:0.96, as determined by automated chromatography[5] on Dowex-1x8 (molybdate), in agreement with the proportion of unoxidised glucose determined by the direct analysis method.

The selective oxidation of certain disaccharides and disaccharide alcohols has been studied[10], and the hexopyranose ring is reported to be resistant to oxidation at low concentrations of periodate (0.4mM). The present technique provides a rapid and more definitive method for a direct study of the oxidation of the non-reducing pyranose ring with periodate. Oxidation of maltitol with periodate (0.4mM) showed a rapid, initial uptake of oxidant and release of formaldehyde, with a low rate of destruction of the glucopyranose ring (Fig. 2). In contrast, complete destruction of the non-reducing glucose residue was evident after 4 h when the oxidation was performed with 20mM periodate.

Automation of the technique enabled simultaneous determination of the formaldehyde produced and the sugar remaining in periodate-oxidation mixtures. This provides a convenient method for measurement of these values with time of oxidation, the continuous sampling and analysis ensuring more reproducible results. Application of this technique to the oxidation of maltitol with periodate (0.4mM) revealed results

(Fig. 4) identical with those obtained manually. After the zero-time levels of glucose and formaldehyde had been determined, concentrated periodate was added (to a final concentration of 0.4mM) to the continuously stirred solution, enabling the initial production of formaldehyde to be examined more readily. A comparison of the oxidation of maltitol and lactitol with periodate (0.4mM) demonstrated the effect of introducing a more readily oxidised vicinal *cis*-diol group in the pyranoid ring of the disaccharide alcohol. After oxidation for 4 h, both lactitol and maltitol produced 1.72 moles of formaldehyde per mole, the theoretical value for complete oxidation of the glucitol portion of the molecule being 2.0. After this oxidation, 15% of the D-glucopyranosyl residues in maltitol had been oxidised, whereas 40% of the D-galactopyranosyl residues in lactitol were oxidised as a result of the more rapid oxidation of vicinal *cis*-diols. These results indicate that, although there is a rapid initial oxidation of the glucitol portion of the molecule, oxidation of the pyranoside becomes significant before complete oxidation of the glucitol is achieved, this being a more pronounced effect when the pyranoside contains a vicinal *cis*-diol group.

The method described for direct measurement of intact residues of hexoses and 6-deoxyhexoses in periodate-oxidation mixtures provides a rapid means for the determination of periodate-resistant units in polymeric carbohydrates. The technique is of particular value in the measurement, either manually or automatically, of residual sugar units as a function of time.

ACKNOWLEDGMENTS

We thank Professor M. Stacey, C.B.E., F.R.S., and Dr. S. A. Barker for their interest in this work, the Fentham Trust for the provision of a scholarship (P.V.P.), and R. R. Woodbury for technical assistance financed by the Medical Research Council.

REFERENCES

1 F. Smith and R. Montgomery, *Chemistry of Plant Gums and Mucilages*, Reinhold, New York, 1959, p. 209.
2 M. Abdel-Akher, J. K. Hamilton, R. Montgomery, and F. Smith, *J. Amer. Chem. Soc.*, 74 (1952) 4970.
3 O. P. Bahl, T. L. Hullar, and F. Smith, *J. Org. Chem.*, 29 (1964) 1076.
4 M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith, *Anal. Chem.*, 28 (1956) 350.
5 S. A. Barker, M. J. How, P. V. Peplow, and P. J. Somers, *Anal. Biochem.*, (1968) in press.
6 O. Samuelson and H. Stromberg, *Carbohyd. Res.*, 3 (1966) 89.
7 Z. Dische, L. B. Shettles, and M. Osmos, *Arch. Biochem.*, 22 (1949) 169.
8 S. A. Barker, E. J. Bourne, and M. Stacey, *J. Chem. Soc.*, (1953) 3084.
9 S. A. Barker, E. J. Bourne, D. M. O'Mant, and M. Stacey, *J. Chem. Soc.*, (1957) 2448.
10 M. J. Clancy and W. J. Whelan, *Chem. Ind.* (London), (1959) 673.

Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

IMMUNOCHEMICAL STUDIES ON BLOOD GROUPS

XL. SCISSION OF OLIGOSACCHARIDES BY SODIUM HYDROXIDE IN THE PRESENCE OF SODIUM BOROHYDRIDE: A MODEL FOR THE DEGRADATION OF BLOOD-GROUP SUBSTANCES

KENNETH O. LLOYD* AND ELVIN A. KABAT

Departments of Microbiology and Neurology, College of Physicians and Surgeons, Columbia University and the Neurological Institute, Presbyterian Hospital, New York, New York 10032 (U.S.A)

(Received July 29th, 1968)

ABSTRACT

To assess the competition between reduction and elimination at each step in the peeling of the carbohydrate chains from blood-group A, B, H, and Le^a substances by sodium hydroxide–sodium borohydride, the amount of scission of some model compounds, namely, oligosaccharides containing the various sugar linkages in blood-group substances, was studied. The extent of cleavage was estimated by determining the reduced, eliminated residues by gas chromatography of their per(trimethylsilyl) derivatives. β-D-Gal*p*-(1→3)-D-GNAc (1) and β-D-Gal*p*-(1→3)-D-GalNAc (2) were found to be the most labile of such disaccharides, showing about 50 per cent cleavage. At 4°, reactions were slower, and there was less scission, than at 24°. β-D-GNAc*p*-(1→3)-D-Gal (5) is also labile, but is cleaved to a much smaller extent (7%). β-D-Gal*p*-(1→4)-D-GNAc (3) and β-D-GNAc*p*-(1→6)-D-Gal (4) are stable. The oligosaccharides isolated from blood-group substances are considered in terms of the extent of peeling to be expected from the results obtained with the model compounds. Products from the reducing residues of the disaccharides were observed by gas–liquid and paper chromatography.

INTRODUCTION

Sodium hydroxide–sodium borohydride and the corresponding deuterium compounds have recently been used extensively in studying the structure of glycoproteins and mucopolysaccharides. These reagents yield reduced oligosaccharides from blood-group substances[1-3] and salivary mucins[4]. Alkali has also been used for investigating the nature of the linkage between the carbohydrate and protein portions in these substances[5] and in mucopolysaccharides[6]. In blood-group substances, the release of carbohydrate chains from the protein by alkali is followed by degradation of the chains by a peeling reaction, to produce a series of oligosaccharides of various chain-lengths[2,3,7]. The nature and lengths of the oligosaccharides formed are governed

---

*Present address: Departments of Biochemistry and Dermatology, College of Physicians and Surgeons, Columbia University, New York, New York 10032, U.S.A.

by competition, at each step of the peeling reaction, between reduction to give stable chains and elimination to produce chains that are one sugar residue shorter[7]. In an attempt to understand the pattern of oligosaccharides produced from blood-group substances, the cleavage of some oligosaccharides as model systems for the degradation of blood-group substances has been studied.

After treatment of a disaccharide with sodium hydroxide–sodium borohydride, three products are possible: (i) reduced disaccharide (this is the only product formed from an alkali-stable disaccharide), (ii) the eliminated residue in its reduced form, and (iii) the residue from the reducing end, after modification and reduction. In the present study, the extent of scission of disaccharides was measured by determining the amount of eliminated, reduced product that is formed. Figure 1 illustrates this

$$\begin{tikzcd}
\underset{\mathbf{1}}{\beta\text{-D-Gal}p\text{-}(1\to3)\text{-D-GNAc}} \arrow[r, "BH_4^-"] \arrow[d, "OH^-"] & \beta\text{-D-Gal}p\text{-}(1\to3)\text{-2-acetamido-2-deoxy-D-glucitol} \\
{[\beta\text{-D-Gal}+\text{chromogen}]} \arrow[r, "BH_4^-"] & \text{galactitol}+\text{reduced chromogen}
\end{tikzcd}$$

Fig. 1. Scheme for the action of sodium hydroxide–sodium borohydride on β-D-Gal*p*-(1→3)-D-GNAc (**1**).

reaction for β-D-Gal*p*-(1→3)-D-GNAc (**1**) as an example. The eliminated products (galactitol or 2-acetamido-2-deoxy-D-glucitol) were determined by gas chromatography of their per(trimethylsilyl) ethers[8]. Figure 2 shows a gas chromatogram of a standard mixture of sugars, and a chromatogram given by the products from β-D-Gal*p*-(1→3)-D-GalNAc (**2**). In each case, the presence of the products was confirmed by paper chromatography. That no epimerization of D-galactose (**7**) occurs after elimination and before reduction was established by the identification, by gas chromatography[9,10] of its hexaacetate, of galactitol as the only hexitol (recovery 98%) formed by treating D-galactose with sodium hydroxide–sodium borohydride for one day at 24°.

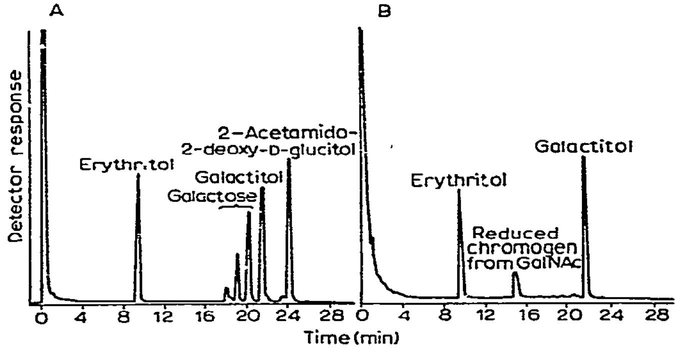


Fig. 2. Gas–liquid chromatogram of sugars and alditols as their TMS derivatives. A: Standard mixture. B: Products from treatment of β-D-Gal*p*-(1→3)-D-GalNAc with NaOH–NaBH$_4$ (erythritol added as reference compound).

Two disaccharides, β-D-Gal*p*-(1→4)-D-GNAc (3) and β-D-GNAc*p*-(1→6)-D-Gal (4) were found to be stable under the conditions used (see Table I); this observation is in agreement with the known stability of such linkages to alkali[11]. The other oligosaccharides were cleaved to various extents, although the scission was not complete because of the presence of borohydride (see Table I). At room temperature, the reactions were complete after 3 h, and the values after 7 days (the period that has been used in the alkaline degradation of blood-group substances) remained essentially unchanged. At 4°, the rates of cleavage were lower, and, at 3 h, only about half of the final degree of scission of compounds **1** and **2** had occurred; both galactitol and galactose could be detected in the reaction mixture at this stage. After 24 h, the reaction was complete, and a value of about 80% of that at room temperature was reached, *i.e.*, there is less scission at 4° than at 24°. Oligosaccharides having (1→3)-2-acetamido-2-deoxy-D-hexose linkages were particularly susceptible to degradation[12], whereas those having (1→3)-D-galactose linkages were considerably more stable.

The determination of the lability of the disaccharides representing the various linkages present in blood-group substances permits estimation of the degree of peeling of the chains and the extent of production of oligosaccharides. Figure 3 shows the structure of the mono-L-fucosyl carbohydrate chains in blood-group H substance, based on the sequence proposed earlier[7], with the lability of the linkages to sodium hydroxide–sodium borohydride (as determined from model compounds) indicated. Two points of termination of the peeling reaction are evident: (i) at the penultimate D-galactose residue, where the (1→6)-D-galactose linkage should be completely stable, thus giving the type 2 determinant intact, and (ii) at the terminal D-galactose residue of the type 1 chain, where the (1→2)-D-galactose linkage should be stable[13]. As the majority of the oligosaccharides isolated from blood-group A, B, H, and Le^a substances are terminated by a reduced (and often degraded) form of the penultimate D-galactose residue, and because high yields of α-L-Fuc*p*-(1→2)-D-galactitol were isolated from A and H substances[3,7,14], it is clear that this prediction has been experimentally verified. Oligosaccharides terminated by other residues should be present to extents proportional to the stability of the corresponding linkages. Thus, approximately half of the chains should be intact and terminated with 2-acetamido-2-deoxy-D-galactitol residues, and about 93% of the remainder should have been stabilized at the next residue, *i.e.*, terminated with galactitol. Fractions representing these long chains were not isolated in a purified form, although they might be present in the large amounts of non-dialyzable fractions and dialyzable fractions of high molecular weight which contain oligosaccharides but which could not be fractionated to give homogeneous products[2,3,7,14]. Similarly, branched oligosaccharides terminated by the penultimate D-galactose residue and having both the type 1 and type 2 chains still attached would be expected to be present in reasonable proportions. Since, in the majority of the chains, these determinants would have one or even two L-fucose residues attached, they would give rise to large fragments, and no oligosaccharides of this structure were isolated from A, B, and H substances. However, an Le^a substance having a very low content of L-fucose gave β-D-Gal*p*-(1→4)-β-D-GNAc*p*-(1→6)-

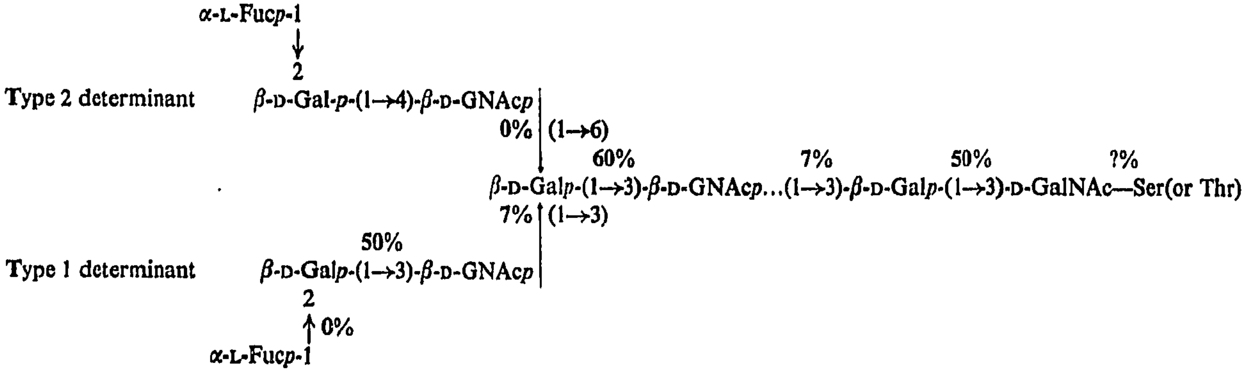


Fig. 3. Proposed structure for the mono-L-fucosyl carbohydrate chains in blood-group H substance, showing the lability of the various linkages to sodium hydroxide–sodium borohydride, as determined from model compounds, when peeling reaches the individual residues. This simplified sequence does not show any of the short side-chains thought to be substituted on the D-GalNAc residue and, possibly, also on the branched D-galactose residue (see ref. 7).

[β-D-Gal*p*-(1→3)-β-D-GNAc*p*-(1→3)]-D-galactitol[7], representing stabilization at this position in the chain.

This scheme, therefore, explains many of the features of the degradation of blood-group substances by sodium hydroxide–sodium borohydride. In addition, it explains: (i) the isolation mainly of oligosaccharides having the type 2 structure, and destruction of most of those having the type 1 structure, and (ii) the conversion of the penultimate D-galactose residue into residues of a 3-hexenetetrol and of a 3-deoxyhexitol by elimination from C-3 of the type 1 chain and of an unknown group from C-4 (see ref. 7 for further details). It is surprising, however, that no α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→3)-2-acetamido-2-deoxy-D-glucitol from the undegraded half of the type 1 chain was detected. Also, although it is not possible to calculate the theoretical yields of the various oligosaccharides (because of uncertainty as to the lengths of chains), it would seem that the high yield of some of the smaller oligosaccharides indicates that the degradation is more extensive than would be expected on the basis of the degree of scission of disaccharides. Possibly, the elimination of a substituent from a reducing sugar is influenced by the presence of other substituents, and highly branched chains, such as those found in blood-group substances, may be particularly susceptible to peeling.

In addition to producing the eliminated, reduced residues from the model compounds, the reactions should also give products from the reducing residues of the oligosaccharides. These were detected by paper chromatography and by g.l.c. Figure 2 shows, for example, such a product from β-D-Gal*p*-(1→3)-D-GalNAc. The peak other than that for galactitol is designated "reduced chromogen", as it is probably related to the chromogens produced in the Morgan–Elson reaction[15]. A related, if not identical, compound was detected by Bray *et al.*[16] with an amino acid analyzer (peak 3) after treating keratosulfate, *N*-acetylchondrosine, and related compounds with sodium hydroxide–sodium borohydride. These workers suggested that "peak 3" arises from reduction and saturation of the chromogen formed by elimination of a substituent on C-3 of 2-acetamido-2-deoxy-D-galactose.

Treatment of the trisaccharide β-D-GNAc*p*-(1→3)-[β-D-GNAc*p*-(1→6)]-D-Gal (6) should produce a disaccharide in which β-D-GNAc is still attached to C-6 of a modified and reduced D-galactose residue. A compound having the expected R_F value was observed by paper chromatography (see Table I). According to a mechanism suggested earlier[7] to account for the degradation of certain residues in the carbohydrate chains of blood-group substances, this modified residue would be expected to be that of a 3-deoxyhexitol. The reaction products do not decolorize potassium permanganate, and the disaccharide is chromatographically different from β-D-GNAc*p*-(1→6)-3-hexenetetrol. These observations support the suggestion that disubstitution on the penultimate D-galactose residue of the carbohydrate chains in blood-group substances does not lead to unsaturated residues at this point (after treatment with sodium hydroxide–sodium borohydride); it is inferred, therefore, that these chains have trisubstituted-branched-point D-galactose residues.

In experiments in which NaOD–$NaBD_4$ was used for producing oligosaccharides from blood-group substances, somewhat larger proportions of nondialyzable products were obtained, indicating that the extent of peeling may be less under these conditions. Another alkaline reagent that has been used is triethylamine[17]. With these reagents, the position of stabilization of the peeling depends only on the lability of the linkages to alkali, and many oligosaccharides terminated by (1→4)-D-GNAc were isolated, as would be predicted. The difference in the types of oligosaccharides produced by the two reagents (sodium hydroxide–sodium borohydride and triethylamine) has been discussed[10].

EXPERIMENTAL

Materials. — Oligosaccharides **4**, **5**, and **6** (see ref. 18) were gifts from Dr. Z. Yosizawa, Tohoku University, Japan. Compound **2** (see ref. 19) was provided by Dr. H. Flowers, Weizmann Institute, Israel. Disaccharides **1** and **3** (see ref. 20) were gifts from Dr. F. Zilliken. The purity of each sample was checked by paper chromatography; **4** contained a trace of free galactose.

Method. — The sample (*ca.* 1 mg) was dissolved in 50 μl of 200 mM sodium hydroxide containing 1% of sodium borohydride at 24° or 4°. At the times indicated in Table I, a portion (10 μl) was removed and added to 10 μl of 4M acetic acid containing a known amount of erythritol (*ca.* 35 μg). The solution was de-ionized with about 100 μl of Dowex 50 (H^+) ion-exchange resin, and borate was removed by addition and evaporation of methanol.

For examination by paper chromatography, the residue was dissolved in water (20 μl) and a portion (5 μl) was chromatographed by the descending method on Schleicher and Schuell No. 589 Green Label paper in 6:4:3 (v/v) butanol–pyridine–water. Compounds were detected with the silver nitrate–sodium hydroxide reagent[21]. The results are given in Table I.

For the quantitative estimation of the galactitol and 2-acetamido-2-deoxy-D-hexitols liberated, the per(trimethylsilyl) ethers of the products were prepared with 20 μl of reagent (1.0 ml of pyridine, 200 μl of hexamethyldisilazane, and 100 μl of chlorotrimethylsilane), as described by Sweeley *et al.*[8]. The separation by g.l.c. was performed as described previously[7], on an SE 30 column (10% on Diatoport S) with a temperature gradient from 150 to 240°, rising at 4°/min. Areas of the peaks were calculated by use of a disc integrator, and the ratio of each to the erythritol added was determined. The absolute amount present was determined by comparison with a standard mixture of sugars. The compounds gave the following detector-responses, relative to erythritol (1.00): galactose (1.15 ± 0.02), galactitol (1.25 ± 0.02), and 2-acetamido-2-deoxy-D-glucitol (0.66 ± 0.04). Figure 2 shows a chromatogram given by these compounds. The amount of scission of an oligosaccharide is given by: (weight of galactitol or 2-acetamido-2-deoxy-D-glucitol formed) × 100/(weight of oligosaccharide in sample). The results are given in Table I.

A portion of the product from the treatment of D-galactose with sodium hydroxide–sodium borohydride for one day at 24° was used for demonstrating that

TABLE I

EFFECT OF SODIUM HYDROXIDE–SODIUM BOROHYDRIDE ON VARIOUS OLIGOSACCHARIDES

Compound	*Scission* (%)						R_{Gal} *of*
	At 24°			*At 4°*			*products identified by paper chromatogarphy*[a]
	3 h	24 h	7 days	3h	24 h	7 days	
	Percent recovery						
β-D-Gal*p*-(1→4)-D-GNAc (3)	0	0	0	0	0	0	0.72 (reduced 3)
β-D-Gal*p*-(1→3)-D-GNAc (1)	60	60	66	20[b]	48	52	0.86 (reduced 1), 1.20 (galactitol), 1.61 (tr.)[c], 1.92 (reduced chromogen)
β-D-Gal*p*-(1→3)-D-GalNAc (2)	51	50	48	24[d]	37	40	0.93 (reduced 2), 1.19 (galactitol), 1.61 (tr.), 1.98 (reduced chromogen)
β-D-GNAc*p*-(1→6)-D-Gal (4)	—	2	—	—	—	—	—
β-D-GNAc*p*-(1→3)-D-Gal (5)	7	7	—	—	—	—	0.89 (reduced 5), 1.62 (2-acetamido-2-deoxy-D-glucitol)
β-D-GNAc*p*-(1→3)-[β-D-GNAc*p*-(1→6)]-D-Gal (6)	—	4	—	—	—	—	0.38 (reduced 6), 0.09[e], 1.61 (2-acetamido-2-deoxy-D-glucitol)
D-Gal (7)	105[f]	95	98	99[g]	96	94	1.20 (galactitol)

[a] In 6:4:3 butanol–pyridine–water after treatment of oligosaccharides for 24 h at 24°. [b] 26% of the product was D-galactose. [c] tr. = trace. [d] 50% of the product was D-galactose. [e] Presumably β-D-GNAc*p*-(1→6)-3-deoxyhexitols. [f] % recovery as galactitol. [g] 22% of the product was D-galactose.

there is no epimerization of D-galactose under these conditions. The product was acetylated with 1:1 (v/v) acetic anhydride–pyridine, and the peracetate was examined by g.l.c. on an ECNSS-M column[9] under conditions[10] that separate all of the hexitol hexaacetates from each other. Galactitol (97%) was identified as the sole product formed.

ACKNOWLEDGMENT

This work was aided by a grant (GB-3675) from the National Science Foundation.

REFERENCES

1 G. Schiffman and E. A. Kabat, *Federation Proc.*, 20 (1961) 67.
2 G. Schiffman, E. A. Kabat, and W. Thompson. *Biochemistry*, 3 (1964) 113.
3 K. O. Lloyd, E. A. Kabat, E. J. Layug, and F. Gruezo, *Biochemistry*, 5 (1966) 1489.
4 D. M. Carlson, *J. Biol. Chem.*, 241 (1966) 2984; R. L. Katzman and E. H. Eylar, *Biochem. Biophys. Res. Commun.*, 23 (1966) 769; D. M. Carlson, *J. Biol. Chem.*, 243 (1968) 616; V. L. N. Murty and M. I. Horowitz, *Carbohyd. Res.*, 6 (1968) 266.
5 V. P. Bhavanandan, E. Buddecke, R. Carubelli, and A. Gottschalk, *Biochem. Biophys. Res. Commun.*, 16 (1964) 353; K. Tanaka, M. Bertolini, and W. Pigman, *ibid.*, 16 (1964) 404; S. Harbon, G. Herman, B. Rossignol, P. Jollès, and H. Clauser, *ibid.*, 17 (1964) 57; E. A. Kabat, E. W. Bassett, K. Pryzwansky, K. O. Lloyd, M. E. Kaplan, and E. J. Layug, *Biochemistry*, 4 (1965) 1632; R. Carubelli, V. P. Bhavanandan, and A. Gottschalk, *Biochim. Biophys. Acta*, 101 (1965) 67; K. Tanaka and W. Pigman, *J. Biol. Chem.*, 204 (1965) PC1487.
6 B. Anderson, P. Hoffman, and K. Meyer, *Biochim. Biophys. Acta*, 74 (1963) 74; *J. Biol. Chem.*, 240 (1965) 156; B. Anderson, N. Seno, P. Sampson, J. G. Riley, P. Hoffman, and K. Meyer, *ibid.*, 239 (1964) PC2716.
7 K. O. Lloyd, E. A. Kabat, and E. Licerio, *Biochemistry*, 7 (1968) 2976.
8 C. C. Sweeley, R. Bentley, M. Makita, and W. W. Wells, *J. Amer. Chem. Soc.*, 85 (1963) 2497.
9 J. S. Sawardeker, J. H. Sloneker, and A. R. Jeanes, *Anal. Chem.*, 37 (1965) 1602.
10 K. O. Lloyd and E. A. Kabat, *Carbohyd. Res.*, 4 (1967) 165.
11 C. E. Ballou, *Advan. Carbohyd. Chem.*, 9 (1954) 91; J. Kenner, *Chem. Ind.* (London), (1955) 727; R. W. Jeanloz and M. Trémège, *Federation Proc.*, 15 (1956) 282; R. L. Whistler and J. N. BeMiller, *Advan. Carbohyd. Chem.*, 13 (1958) 289.
12 R. Kuhn, A. Gauhe, and H. H. Baer, *Chem. Ber.*, 87 (1954) 1138.
13 R. Kuhn, H. H. Baer, and A. Gauhe, *Ann.*, 611 (1958) 242.
14 K. O. Lloyd and E. A. Kabat, *Biochem. Biophys. Res. Commun.*, 16 (1964) 385; K. O. Lloyd, S. Beychok, and E. A. Kabat, *Biochemistry*, 6 (1967) 1448.
15 W. T. J. Morgan and L. A. Elson, *Biochem. J.*, 28 (1934) 988; R. Kuhn and G. Krüger, *Chem. Ber.*, 89 (1956) 1473; 90 (1957) 264.
16 B. A. Bray, R. Lieberman, and K. Meyer, *J. Biol. Chem.*, 242 (1967) 3373.
17 V. P. Rege, T. J. Painter, W. M. Watkins, and W. T. J. Morgan, *Nature*, 203 (1964) 360; 204 (1964) 740; T. J. Painter, W. M. Watkins, and W. T. J. Morgan, *ibid.*, 206 (1965) 594.
18 Z. Yosizawa, *J. Biochem.* (Japan), 51 (1962) 1, 145.
19 H. L. Flowers and D. Shapiro, *J. Org. Chem.*, 30 (1965) 2041.
20 F. Zilliken, P. N. Smith, C. S. Rose, and P. György, *J. Biol. Chem.*, 208 (1954) 299; A. Alessandrini, E. Schmidt, F. Zilliken, and P. György, *ibid.*, 220 (1956) 71.
21 W. E. Trevelyan, D. P. Proctor, and J. S. Harrison, *Nature*, 166 (1950) 144.

Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

THE SYNTHESIS OF BRANCHED AND LINEAR ARABINANS*

N. K. Kochetkov, A. F. Bochkov, and I. G. Yazlovetsky
N. D. Zelinsky Institute of Organic Chemistry, Academy of Sciences of U.S.S.R., Moscow (U.S.S.R.)

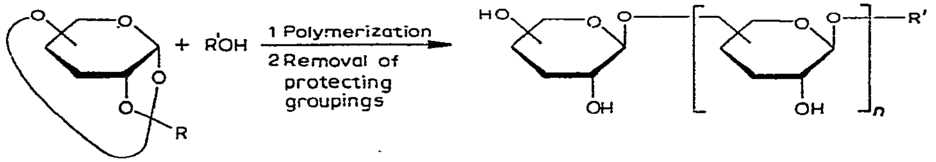
(Received March 6th, 1968; in revised form, April 29th, 1968)

ABSTRACT

The ortho-ester method of glycosylation has been applied to the synthesis of polysaccharides by using the tricyclic ortho-ester β-L-arabinofuranose 1,2,5-orthobenzoate (**1**). The resulting polysaccharide (50% yield) was examined by hydrolytic, periodate-oxidation, and methylation techniques, and identified as a highly branched L-arabinan containing α-(1→5)- [and a few (1→3)-] linkages, with branch points at position 3. Polymerisation of the 3-acetate of ortho ester **1** was similarly accomplished in the presence of an initiator (1,2,3,4-tetra-*O*-acetyl-β-D-glucopyranose) to give an L-arabinan having α-(1→5)-, and a few (1→2)-, linkages; the reducing unit of each chain is a D-glucose residue.

INTRODUCTION

The availability of model polysaccharides of known structure is essential for the solution of many problems in the organic, physical, and biological chemistry of polysaccharides and other carbohydrate-containing polymers. The present-day difficulties associated with the isolation, control of homogeneity, and complete structural analysis of natural polysaccharides suggest chemical synthesis of compounds of predetermined structure as the only reasonable way to obtain such models. Many publications[2–5] have been concerned with polysaccharide synthesis, but, until recently, the synthetic methods afforded only polysaccharides having irregular structures characterized by a random distribution of linkage types, configuration, and ring-size. Directed synthesis of regular polysaccharides has been reported[6–8], but the methods are not general. A general solution of the problem would involve stereospecific glycosylating agents, the polymerization of which could result in the formation of regular polysaccharide chains. The ortho-ester method of glycosylation possesses these attributes[9–10], and provides a promising new approach to this problem. The present paper is concerned with the polymerization of sugar ortho-esters according to the following scheme:



---

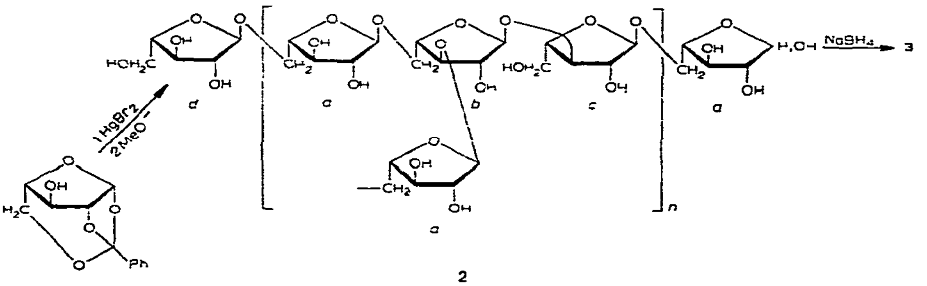
*Some results of these studies have been reported as short communications[1].

The reaction needs an initiator (R'OH) which becomes attached to the reducing end of the nascent polysaccharide chain. The approach has been applied now to the synthesis of branched and linear arabinans.

RESULTS

Synthesis of a branched arabinan (S-1)

The starting monomer in this synthesis was the tricyclic arabinofuranose orthobenzoate[11] **1**. This compound contains a hydroxyl group capable of initiating polymerization to give the branched polymer **2**.



1

2

Polymerization of ortho-ester **1** has been performed under standard conditions[10], *i.e.*, in nitromethane with catalytic amounts of mercuric bromide, but with a somewhat longer reaction period. The polymerization products were subjected to mild, acidic hydrolysis to remove any intermonomer bonds of the ortho-ester type, and then saponified, and purified by gel-filtration on Sephadex G-25. The absence of acyl groupings in the polymer was shown by the absence of a carbonyl band in the i.r. spectrum. The yield of polysaccharide **2** (arabinan S-1) was 50%. The structure generalized by formula **2** was established as follows.

Hydrolysis of arabinan S-1 with 0.1N sulphuric acid or 0.025N oxalic acid (100°, 3 h) caused complete degradation of the polysaccharide, affording only arabinose. The specific rotation (−91°) of arabinan S-1 has the magnitude expected for structure **2**. Hydrolysis with α-L-arabinofuranosidase* caused complete degradation into arabinose; no starting polysaccharide or oligomer fragments could be detected in this hydrolysate. Hence, the polymer is built uniformly of α-L-arabinofuranose residues. Periodate oxidation of arabinan S-1 and of the corresponding polyol **3**, obtained by reduction of polymer **2** with sodium borohydride, proceeds slowly and typically for compounds containing *trans*-glycol groupings on five-membered rings.

---

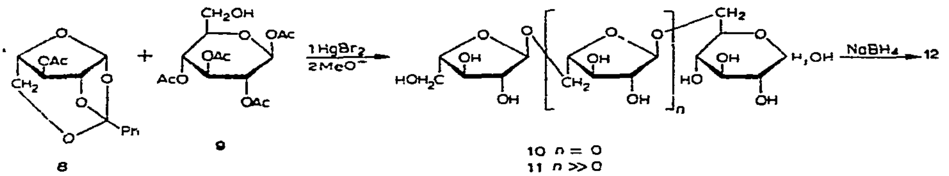
*Enzymic hydrolysis of arabinans S-1 and S-2 (see below) has been performed by Professor Neukom (personal communication) and subsequently reproduced by us with an enzyme preparation generously presented by Professor Neukom.

The limited consumption of periodate suggests the absence of over-oxidation. Oxidation of the polysaccharide with 0.28M sodium metaperiodate or with 0.039M sodium metaperiodate in acetate buffer (pH 5.8) resulted in consumption of 0.69 moles of oxidant per "anhydro-pentose" unit (constant limit), *i.e.*, 30% of the arabinose residues were unoxidised. Oxidation of polyol **3** with 0.05M sodium metaperiodate resulted in consumption of 0.70 mole of periodate per "anhydro-pentose" unit (constant limit). The yield of formaldehyde after oxidation of the polyol under the above conditions was 0.0168 mole per "anhydro-pentose" unit. Hence, arabinan S-1 has a number-average degree of polymerization $\overline{\mathrm{DP}} = 59.5$.

Methylation[12] of polyol **3** and subsequent hydrolysis or methanolysis afforded 2,3,5-tri-*O*-methyl-L-arabinofuranose (**4**), 2,3-di-*O*-methyl-L-arabinose (**5**), 2,5-di-*O*-methyl-L-arabinofuranose (**6**), and 2-*O*-methyl-L-arabinose (**7**). Compounds **4**, **5**, and **7** were chromatographically identical[13] with authentic samples. The methyl glycosides of compounds **4** and **5** were identical with authentic samples in gas–liquid chromatography. 2,5-Di-*O*-methyl-L-arabinofuranose does not react with triphenyltetrazolium chloride[14], and remains at the starting line during electrophoresis in borate buffer. This evidence proves the presence of a methoxyl group at position 2. The non-identity of the compound with 2,3-di-*O*-methyl-L-arabinose leaves two possible structures, *viz.* 2,4- and 2,5-di-*O*-methyl-L-arabinose. The chromatographic behaviour of compound **6** is close to that reported for 2,5-di-*O*-methyl-L-arabinofuranose and significantly different from that of the 2,4-isomer. The position of the methyl group in 2-*O*-methyl-L-arabinose was confirmed by the fact that the ether does not react with triphenyltetrazolium chloride[14]. The ratio of partially methylated arabinoses **5** and **7** in the products of methylation of polyol **3** was determined as follows. The mixture was reduced with sodium borohydride and subjected to preparative, paper chromatography. The bands of 2,3-di-*O*-methyl-L-arabinitol (from **5**) and 2-*O*-methyl-L-arabinitol (from **7**) were eluted, and the compounds were oxidized with periodate. The ratio of the yields of formaldehyde (3:2) corresponds to the ratio of **5**:**7**. Together with the data of periodate oxidation, this result makes possible a determination of the content of all structural elements in arabinan S-1 (see formula **2**): $a+b+c+d = 59.5$; $a/b = 1.5$; $d = b+1$; $a+d = (59.5 \times 0.69) - 1 = 40.05$; hence, $a = 23.43$; $b = 15.62$; $c = 3.83$; and $d = 16.62$. These values lead to the following conclusions: arabinan S-1 is a highly branched polysaccharide having chains of α-L-arabinofuranose residues (39 residues per molecule) linked (1→5); 15–16 of these residues are points of branching through position 3; 3–4 residues per molecule are linked (1→3); and the number of chains is 16–17 per molecule.

The synthesis of linear arabinan (S-2)

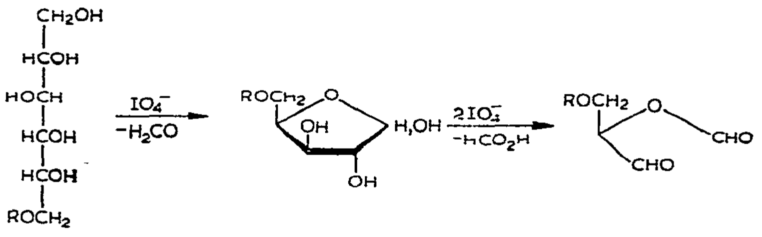
In order to obtain a linear polysaccharide from an ortho ester of type **1**, it was necessary to protect the free hydroxyl group, and to add an alcohol initiator. For this purpose, we effected polymerization of 3-*O*-acetyl-β-L-arabinofuranose[11] 1,2,5-orthobenzoate **8** in the presence of 1,2,3,4-tetra-*O*-acetyl-β-D-glucopyranose (**9**), according to the following scheme:



The polymerization was accomplished in the same way as in the case of **2**. The results of **1**, the reaction depends critically on the monomer: initiator ratio. In the absence of initiator, practically no polymerization occurs. At a molar ratio of **8**:**9** = 1:1, the reaction proceeds rapidly and gives mainly disaccharide **10**, together with a small proportion of higher oligosaccharides. On the other hand, with a starting ratio of **8**:**9** = 20:1, polymerization is slow (overall reaction period, 130 h) and affords polysaccharide **11**, together with some higher oligosaccharides and traces of compound **10**. The polymer fraction (20%), isolated from the mixture by gel-filtration on Sephadex G-25, was named arabinan S-2, and its structure **11** was proved as follows:

Acidic and enzymic hydrolysis of the polysaccharide (accomplished as described for arabinan S-1) proceeded to completion to yield, in both cases, only arabinose and glucose in a ratio of 20:1, and no oligomer derivatives. Acidic hydrolysis of polyol **12**, obtained by borohydride reduction of arabinan S-2, afforded arabinose as the only reducing sugar, glucitol, and traces of arabinitol. The specific rotation (−85°) of arabinan S-2 is consistent with structure **11**. Hence, arabinan S-2 is built uniformly of α-L-arabinofuranose residues. The glucose residue occurs only at the reducing end of the molecule. The traces of arabinitol found in the hydrolysate of polyol **12** suggest that some molecules have arabinose residues at the reducing end. These chains are probably formed by polymerization of starting monomer, with traces of water serving as initiator.

As with arabinan S-1, periodate oxidation of arabinan S-2 proceeded slowly with no over-oxidation. With 0.039M sodium metaperiodate in acetate buffer (pH 5.8), 1.11 moles of oxidant (constant limit) per "anhydro-pentose" unit were consumed; less than 10% of the arabinose residues survived oxidation. Oxidation of polyol **12** with 0.05M unbufferred sodium metaperiodate resulted in consumption of 1.00 mole of periodate per "anhydro-pentose" unit. The yield of formaldehyde was 0.0424 mole per "anhydro-pentose" unit, leading to a $\overline{DP}$ value of 23.6. If it is assumed[15] that a 6-*O*-substituted D-glucitol consumes 3 moles of periodate, the theoretical consumption of periodate by a polymer having this $\overline{DP}$ is 1.085 moles per "anhydro-



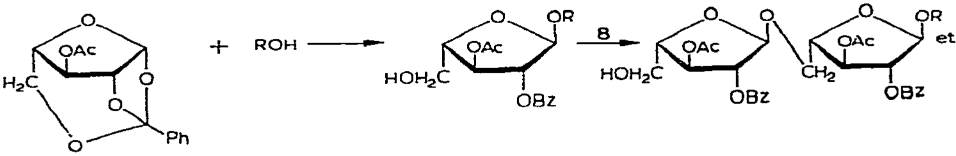
pentose" residue. The experimental value (1.00 mole) corresponds to 8% of unoxidized arabinofuranose units in the polyol **12**.

Methylation of polyol **12** was performed as for arabinan S-1 to give the partially methylated derivatives **4**, **5**, 3,5-di-*O*-methyl-L-arabinofuranose[13] (**13**), and 1,2,3,4,5-penta-*O*-methyl-D-glucitol (**14**). An authentic sample of compound **13** was obtained by a new, simple route from 3,5-di-*O*-benzoyl-β-L-arabinofuranose 1,2-(methyl orthobenzoate)[11], details of which will be published elsewhere. Penta-*O*-methyl-D-glucitol was identical in g.l.c. with an authentic sample obtained by saponification, reduction, methylation, and detritylation of 6-*O*-trityl-β-D-glucopyranose.

The ratio of the products obtained on methanolysis of methylated polyol **12** (determined by g.l.c.) is **4**:**5**:**13**:**14** = 1:16:2:1, in good accord with the periodate-oxidation data. Hence, arabinan S-2 is an unbranched polysaccharide consisting of α-L-arabinose residues mainly (1→5)-linked; 8% of the residues (*ca.* 1.9 residues per macromolecule) are linked (1→2). The reducing termini of the polysaccharide chains are D-glucose residues, and the $\overline{\text{DP}}$ value is 23.6

GENERAL DISCUSSION

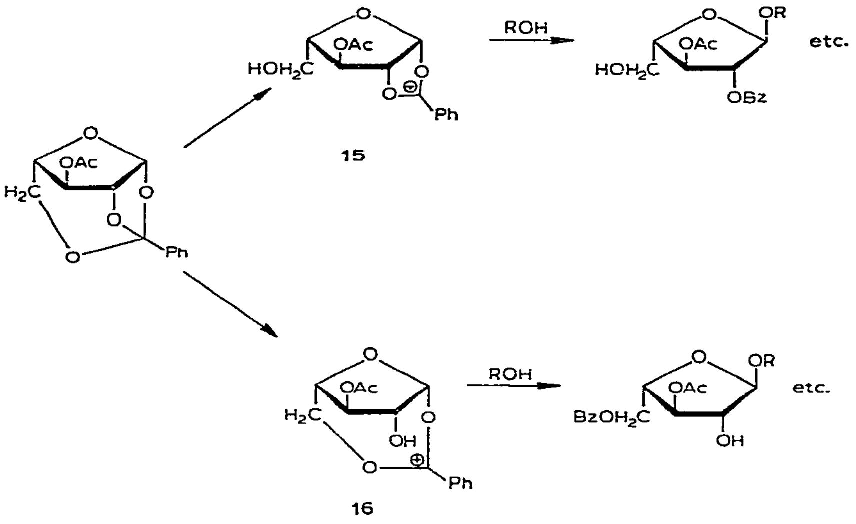
The polymerisation of ortho esters **1** or **8** is a stepwise reaction beginning with glycosylation of the alcohol initiator by a molecule of ortho ester. This process exposes a hydroxyl group, so that further reaction is possible according to the scheme:



8

Hence, the concentration of free hydroxyl groups remains constant and equal to the starting concentration of initiator. This scheme accounts for the effect of initiator concentration on the rate of the process, particularly for the small rate of polymerization of ortho ester **8** in the presence of 5% of alcohol initiator, compared with the polymerization of ortho ester **1** containing a free hydroxyl group. Stepwise elongation of the polysaccharide chain during polymerization of ortho ester **8** is confirmed by the fact that its reaction with one equivalent of tetra-acetate **9** affords disaccharide rather than polymer. Thus, it is possible to control the molecular weight of the products by varying the monomer–initiator ratio.

The formation of *ca.* 8% of (1→2) bonds in the arabinan S-2 may be explained by a glycosylation process involving the seven-membered acyloxonium ion **16**; the usual process involves the five-membered ion **15**. This direction of reaction, although making a minor contribution to the structure of the product, apparently has no analogy in the well-studied glycosylation reaction of ortho esters used in the synthesis of oligosaccharides and other glycosides (*e.g.*, Ref. 10), nor in the polymerization of ortho ester **1**,



Usual process involves the five-membered ion 15

Arabinans S-1 and S-2 are the first examples of synthetic polysaccharides containing only furanose units. Arabinan S-1 is closely similar in structure to naturally occurring, plant arabinans[16]. The specific rotation of arabinan S-1, which contains no other polysaccharide impurities and has glycoside bonds of uniform configuration, may be regarded as characteristic and may be used as a criterion of individuality in the isolation of arabinans from natural sources. Moreover, the difference in the specific rotation between arabinans S-1 and S-2 is close to that calculated for the presence of *ca.* 5% of D-glucose, in spite of the marked difference of the structures, and it may be assumed that the degree of branching has no significant effect on the specific rotation. Hence, the value (−91°) found may be regarded as characteristic of arabinans built of α-L-arabinofuranose residues.

The synthesis of arabinans S-1 and S-2 demonstrates the successful application of the polymerization of tricyclic ortho-esters to polysaccharide synthesis. The relatively high yields and molecular weights of the arabinans obtained demonstrate that polymerization proceeds readily and that the new approach may serve as a basis for the development of methods for synthesis of different polysaccharides.

EXPERIMENTAL

Nitromethane was distilled *in vacuo* (100–200 mm) over urea and subsequently twice over P_2O_5. Chromatography was performed on papers "Goznak", Leningrad "S", and Whatman No. 1, with (*A*) propyl alcohol–ethyl acetate–water (6:1:3); (*B*) ethyl acetate–pyridine–water (10:4:3); (*C*) butyl alcohol–benzene–pyridine–

water (5:1:3:3); (*D*) formic acid–butanone–*tert*-butyl alcohol–water (3:6:8:3); (*E*) butanone saturated with 2% aqueous ammonia; (*F*) ethyl acetate–acetic acid–formic acid–water (18:3:1:4). Electrophoresis on paper was performed in 0.01M $Na_2B_4O_7$ at 900 volts. Gas–liquid chromatography (g.l.c.) was performed with instrument LKh M-5 (Experimental Shop of the Institute of Organic Chemistry, Acad. Sci. of U.S.S.R.) at 168°, with a column of 10% of neopentylglycol succinate on Zeolite 545, and catharometer detection. Neutralization of solutions, unless otherwise stated, was effected with resins KU-2×8 (H^+ or $C_5H_5NH^+$) and Dowex-1x8 (HCO_3^-). For demineralization after reduction with sodium borohydride, the solutions were neutralized with cation resin, and boric acid was removed by evaporation with methanol. Evaporations were conducted *in vacuo* at 40°.

Arabinan S-1 (**2**). — A solution of compound **1** (1.00 g, 4.20 mmoles) in nitromethane (50 ml) was added dropwise during 1 h to a solution of mercuric bromide (72 mg, 0.20 mmole) in nitromethane (10 ml). During the addition, the solvent was distilled off at such a rate that the volume of reaction mixture remained constant (10 ml). The final mixture was refluxed for 22 h. Two drops of pyridine were then added, and the mixture was evaporated to dryness. T.l.c. (silica gel, ethyl acetate) revealed the absence of starting material. The syrupy residue was dissolved in acetone (20 ml), and 0.1N H_2SO_4 (2 ml) was added. After 1 h at room temperature, the acid was neutralized and the solution was evaporated to dryness. The residue was shaken with dry methanol (50 ml) and M methanolic sodium methoxide (5 ml) for 18 h. Water (30 ml) was added, the solution was deionized and evaporated to dryness, and the residue (0.56 g) was eluted with water from a column (18 cm² x 79 cm) of Sephadex G-25 (coarse). The fractions containing a substance having R_{Ara} 0.0–0.2 (System *A*) were combined and evaporated to dryness to give compound **2** (0.28 g, 50%) as a colourless glass, $[\alpha]_D$ −91° (*c* 0.58, water). The i.r. spectrum contained no carbonyl bands. This fraction was used for the structural analysis.

Repeated gel-filtration of the polysaccharide on Sephadex G-50 (coarse) (10 cm² x 65 cm, elution with water) afforded a fraction (117 mg, 21%) having R_{Ara} 0.0–0.1, $[\alpha]_D$ −90° (*c* 0.62, water), and a fraction (128 mg, 25%) having R_{Ara} 0.0–0.2.

Polyol (**3**). — Arabinan S-1 (**2**, 0.17 g), dissolved in 30 ml of water, was treated with sodium borohydride (0.20 g) overnight at room temperature. Deionization and evaporation afforded the polyol **3** (0.16 g).

*6-O-α-*L*-Arabinofuranosyl-*D*-glucose* (**10**). — 1,2,3,4-Tetra-*O*-acetyl-β-D-glucopyranose (**9**) (0.35 g, 1 mmole) and 3-*O*-acetyl-β-L-arabinofuranose 1,2,5-orthobenzoate (**8**) (0.28 g, 1 mmole) were dissolved in dry nitromethane (25 ml), 15 ml of solvent were distilled off, and 0.01M mercuric bromide in benzene (5 ml) was added. Solvent was again distilled off (until the residual volume was 10 ml), and the mixture was refluxed for 4.5 h. Two drops of pyridine were added, the solution was evaporated, and the dried residue was shaken with dry methanol (10 ml) and M methanolic sodium methoxide (1 ml) for 10 h. Water (5 ml) was added, and the solution was deionized and evaporated. Paper chromatography (System *D*) revealed the presence of glucose, arabinose, disaccharide[10] **10** (R_{Ara} 0.50, major component), and also substances of R_{Ara} 0.30 and 0.16.

Arabinan S-2. — Compound **9** (0.35 g, 1.00 mmole) dissolved in nitromethane (15 ml) was treated with 2 ml of a solution of compound **8** (5.56 g, 20.0 mmoles) in nitromethane (39 ml) and 5 ml of 0.01M mercuric bromide in benzene (5 ml), and the solvents were distilled off to a residual volume of 5 ml which was then refluxed with protection from atmospheric moisture. Every 6 h, 2 ml of the solution of compound **8** were added to the boiling solution, and the solvent was distilled off to a constant volume (5 ml) of reaction mixture. After the 8th addition, the volume of the reaction mixture was increased to 7 ml, and after the 14th addition, to 9 ml. After the introduction of the last (20th) portion, the mixture was refluxed for 10 h. The overall reaction period was 130 h. Two drops of pyridine were added, the solution was evaporated to dryness, and the residue was dissolved in acetone (50 ml) containing 5 ml of 0.1N H_2SO_4. After 1 h at 20°, the solution was neutralized and evaporated. The dry syrupy residue was shaken with dry methanol (100 ml) and M methanolic sodium methoxide (10 ml) for 12 h at room temperature. Water (50 ml) was added, the solution was deionized and evaporated, and the residue (2.80 g) was chromatographed on Sephadex G-25 (medium, 12 cm^2 × 38 cm, elution with water). Fractions having R_{Ara} 0.0–0.5 (System *D*) were combined and evaporated; yield, 0.89 g (32%). Rechromatography on Sephadex G-25 (medium, 12 cm^2 × 67 cm, elution with water) afforded compound **11** (0.57 g, 20%), R_{Ara} 0.0–0.3 (System *D*), $[\alpha]_D$ $-85°$ (*c* 0.72, water). The i.r. spectrum contained no carbonyl bands.

Polyol **12**. — Arabinan S-2 (0.22 g) was dissolved in water (50 ml), sodium borohydride (0.02 g) was added, and the mixture was kept for 25 h at room temperature, deionized, and evaporated to dryness. Yield of polyol **12**, 0.22 g.

Hydrolysis of polysaccharides. — (*a*) *Acidic hydrolysis.* Portions (2–4 mg) of polysaccharides were heated in sealed tubes with 0.1N H_2SO_4 (1–2 ml) or 0.025N oxalic acid for 3 h at 100°. Only arabinose was detected in the hydrolysate of arabinan S-1 (paper chromatography, systems *A*, *B*, *C*, and *D*). Arabinose and glucose were detected (ratio 1:20, system *D*, quantitative determination[17]) in the hydrolysate of arabinan S-2. Arabinose was the only reducing sugar detected in the hydrolysate of polyol **12** (by paper chromatography, System *D*), together with glucitol and traces of arabinitol. The amounts of glucitol and arabinitol, estimated by visual, comparative densitometry of chromatograms[18], were *ca.* 5 and *ca.* 1%, respectively.

(*b*) *Enzymic hydrolysis.* Portions (1 mg) of polysaccharides dissolved in 0.2M phosphate–citrate buffer (pH 3.9) were treated with 2 mg of α-L-arabinofuranosidase, and the mixture was incubated for 8 h at 35°. Five volumes of ethanol were then added, the precipitate was removed by centrifugation, the supernatant was deionized and evaporated (starting polysaccharides do not sediment under these conditions). Only arabinose was detected on paper chromatograms in the hydrolysate of arabinan S-1, and glucose and arabinose in that of arabinan S-2. No changes of the polysaccharides occurred in control mixtures with no added enzyme.

Periodate oxidation. — Periodate oxidation of the arabinans and of the corresponding polyols was performed in the dark. Consumption of periodate was estimated by the arsenite method[19], and formaldehyde was determined colorimetrically[20,21]. Aliquots (1 ml) of the oxidizing mixture were taken for the determinations.

(*a*) *Arabinan S-1* (2). For a solution of arabinan S-1 (50.4 mg) in 0.280M sodium metaperiodate (10.00 ml), the consumption of periodate was as follows:

Time (h)	1	2	3	27	70
Consumption of periodate (moles per mole of "anhydro-pentose")	0.56	0.62	0.68	0.69	0.69

For a solution of arabinan S-1 (10.20 mg) in 0.0390M sodium metaperiodate (10.00 ml) in 0.2M acetate buffer (pH 5.8), the oxidant consumption was as follows:

Time (days)	2 h	5	6	8	10	11
Consumption of periodate (moles per mole of "anhydro-pentose")	0.19	0.52	0.55	0.67	0.68	0.69

After completion of oxidation, the reaction mixture was deionized and evaporated. The residue was dissolved in water (2 ml), sodium borohydride (2 mg) was added, and the mixture was left for a day at room temperature, deionized, hydrolyzed in 2 ml of 0.1N H_2SO_4 (100°, 3 h), neutralized with $BaCO_3$, and evaporated. The arabinose content (determined by quantitative, paper chromatography, System *D*, visual densitometry) was 30% of that present in the original polysaccharide.

(*b*) *Polyol* 3. When polyol 3 (10.48 mg) was dissolved in 10.00 ml of 0.05M sodium metaperiodate at 18°, the consumption of oxidant was as follows:

Time (h)	2	7	20	51
Consumption of periodate (moles per mole of "anhydro-pentose")	0.59	0.71	0.69	0.70

For a solution of polyol 3 (11.30 mg) in 10.00 ml of 0.05M sodium metaperiodate at 18°, the yield of formaldehyde was as follows:

Time (h)	2	7	20	51	82	Average
Formaldehyde (moles per mole of "anhydro-pentose") ($\times 10^2$)	1.66	1.58	1.66	1.73	1.73 1.69 1.73	1.68

(*c*) *Arabinan S-2*. For a solution of arabinan S-2 (10.73 mg) in 10.00 ml of 0.039M sodium metaperiodate in 0.2M acetate buffer (pH 5.8) at 20°, the consumption of periodate was as follows:

Time (h)	2	30	52	71	90
Consumption of periodate (moles per mole of "anhydro-pentose")	0.86	1.11	1.10	1.10	1.11

After completion of the reaction, the residue was treated as described above for the oxidation of arabinan S-1. Glycerol and arabinose were detected by paper chromatography in System *D*. Quantitative determination[17] of the latter revealed that 10% of the arabinose survived periodate oxidation.

For a solution of polyol **12** (10.85 mg) in 10.00 ml of 0.05M sodium metaperiodate at 18°, the consumption of oxidant was as follows:

Time (h)	2	7	20	51	82
Consumption of periodate (moles per mole of "anhydro-pentose")	0.85	0.88	1.00	1.00	1.00

Methylation of polyols **3** *and* **12**. — The polyol **3** (50 mg) was dissolved in dry methyl sulphoxide (2 ml), and treated with the methylsulphinyl cation (obtained from 0.13 g of a 50% emulsion of sodium hydride in mineral oil and 1.2 ml of dry methyl sulphoxide) and methyl iodide (2.3 ml). Methylation was completed by Purdie's method with 30 ml of methyl iodide in the presence of silver oxide (0.5 g). Inorganic salts were filtered off, the filtrate was evaporated, and the residue was dissolved in ether (30 ml). The solution was decanted, filtered, washed with water (3 × 10 ml), and evaporated, and the residue was dried over P_2O_5 at 78°/1 mm. The resulting, colourless syrup (42 mg, 69%) showed no i.r. absorption for hydroxyl groups. Methylation of polyol **12** obtained from arabinan S-2 (42.6 mg) was performed similarly, but without the Purdie methylation, to give 37 mg of a slightly yellow syrup (72%); its i.r. spectrum contained no band for hydroxyl groups.

Analysis of the methylation products. — (*a*) *Methanolysis*. The methylated polyol (10–15 mg) was dissolved in 10 ml of 2% methanolic hydrogen chloride, and heated in a sealed tube for 8 h at 100°. The acid and methanol were removed in a desiccator with $CaCl_2$ and KOH (150 mm, 20°). G.l.c. with authentic samples revealed the following products: For arabinan S-1, 2,3,5-tri-*O*-methyl-L-arabinofuranose (**5**) and 2,3-di-*O*-methyl-L-arabinose (**6**); and for arabinan S-2, 2,3,5-tri-*O*-methyl-L-arabinofuranose (**5**), 2,3-di-*O*-methyl-L-arabinose (**6**), 3,5-di-*O*-methyl-L-arabinofuranose (**13**), and 1,2,3,4,5-penta-*O*-methyl-D-glucitol (**14**), in the ratio 1:16:2:1.

(*b*) *Hydrolysis*. The methylated polyol (3–8 mg) was hydrolyzed in 2 ml of 0.5N H_2SO_4 (100°, 6 h). The neutralized hydrolysate was evaporated, and the residue was studied by paper chromatography (Whatman No. 1, System *E*, *F*, and *G*). The following methyl ethers of arabinose were identified:

Arabinan S-1

Derivative of L-*arabinose*	R_X[a]		
	Solvent E	*Solvent* F	*Solvent* G
2,3,5-Tri-*O*-Me-	1.59 (1.48)	1.17 (1.18)	1.10 (1.09)
2,3-Di-*O*-Me-	0.78 (0.79)	0.91 (0.91)	0.92 (0.92)
2,5-Di-*O*-Me-	1.13 (1.11)	1.05 (1.065)	0.98 (—)
2-*O*-Me-	0.26 (0.29)	0.68 (0.65)	0.70 (0.72)

[a] X = 2,3,4-Tri-*O*-methyl-L-arabinose (lit.[13] values in parentheses).

None of the compounds reacted with triphenyltetrazolium chloride. The hydrolysate contained no substances mobile in paper electrophoresis.

Arabinan S-2

Derivative of L-*arabinose*	R_X[a] *Solvent* E	*Solvent* F	*Solvent* G
2,3,5-Tri-*O*-Me-	1.59 (1.48)	1.17 (1.185)	1.10 (1.09)
2,3-Di-*O*-Me-	0.76 (0.79)	0.89 (0.91)	0.93 (0.92)
3,5-Di-*O*-Me-[b]	1.13 (1.13)	1.00 (1.02)	—

[a] x = 2,3,4-Tri-*O*-methyl-L-arabinose (lit.[13] values in parentheses). [b] This component reacted with triphenyltetrazolium chloride.

3,5-Di-*O*-methyl-L-arabinofuranose was the only identified product in the hydrolysate which was mobile in borate electrophoresis.

*Determination of the ratio of 2,3-di- and 2-*O*-methyl-*L*-arabinose in the hydrolysate of methylated polyol* **3**. — The hydrolysate of methylated polyol **3** (1 mg) was dissolved in water (5 ml), and sodium borohydride (15 mg) was added. After 18 h at room temperature, the solution was deionized and evaporated, and the residue was fractionated by preparative chromatography on "Goznak" paper (15 × 50 cm) with butyl alcohol–water (86:14). The bands corresponding to 2-*O*-methyl-L-arabinitol and 2,3-di-*O*-methyl-L-arabinitol were cut into pieces and extracted with equal volumes of aqueous 50% methanol (25 ml). The paper was filtered off, and washed on the filter with 10 ml of 50% methanol. The combined filtrate and washings were evaporated, and the residue was treated with 2 ml of 0.02M sodium periodate (20°, 16 h). The formaldehyde released[20] was as follows:

Methyl ether of L-arabinitol	2,3-di-*O*-Me-	2-*O*-Me-	Ratio of 5:7
Absorbance	0.450	0.300	3.0:2.0

ACKNOWLEDGMENT

The authors gratefully acknowledge the generous gift by Prof. H. Neukom of α-L-arabinofuranosidase.

REFERENCES

1 N. K. Kochetkov, A. J. Khorlin, A. F. Bcchkov, and I. G. Yazlovetsky, *Carbohyd. Res.*, 2 (1966) 84; *Izv. Akad. Nauk SSSR, Otd. Khim. Nauk*, (1966) 385, 1872.
2 Yu. L. Pogosov and Z. A. Rogovin, *Usp. Khim.*, 30 (1961) 1215.
3 J. Klar, *Chemiker Ztg.*, 87 (1963) 731.
4 Midzuko Takasi, *J. Japan. Chem.*, 21 (1967) 145.
5 I. J. Goldstein and T. L. Hullar, *Advan. Carbohyd. Chem.*, 21 (1966) 431.
6 E. Husemann and G. J. M. Müller, *Makromol. Chem.*, 91 (1966) 212.

7 E. R. RÜCKEL AND C. SCHUERCH, *J. Org. Chem.*, 31 (1966) 2233.
8 S. HAQ AND W. J. WHELAN, *J. Chem. Soc.*, (1956) 4543.
9 N. K. KOCHETKOV, A. J. KHORLIN, AND A. F. BOCHKOV, *Tetrahedron Lett.*, (1964) 289.
10 N. K. KOCHETKOV, A. J. KHORLIN, AND A. F. BOCHKOV, *Tetrahedron*, 23 (1967) 693.
11 N. K. KOCHETKOV, A. J. KHORLIN, A. F. BOCHKOV, AND I. G. YAZLOVETSKY, *Izv. Akad. Nauk SSSR, Otd. Khim. Nauk*, (1966) 2030.
12 P. A. SANDFORD AND H. E. CONRAD, *Biochemistry*, 5 (1966) 1508.
13 J. K. N. JONES AND S. C. WILLIAMS, *Can. J. Chem.*, 45 (1967) 275.
14 K. WALLENFELS, *Naturwissenschaften*, 37 (1950) 491.
15 M. CANTLEY, L. HOUGH, AND A. O. PITTET, *J. Chem. Soc.*, (1965) 2527.
16 J. K. N. JONES AND V. TANAKA, *Methods Carbohyd. Chem.*, 5 (1965) 74.
17 S. B. MISRA AND V. K. MOHAN RAO, *J. Sci. Ind. Res.* (India), B.C. 19 (1960) 173.
18 J. K. N. JONES, M. B. PERRY, AND W. SOWA, *Can. J. Chem.*, 41 (1965) 2712.
19 J. R. DYER, *Methods Biochem. Anal.*, 3 (1954) 111.
20 T. NASH, *Biochem. J.*, 55 (1953) 416.
21 V. E. VASKOVSKY *et al.*, *Anal. Biochem.*, in press.

Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

THE SYNTHESIS OF A (1→3)-GLUCAN RELATED TO LAMINARAN

N. K. KOCHETKOV AND A. F. BOCHKOV
N. D. Zelinsky Institute of Organic Chemistry, Academy of Sciences of U.S.S.R., Moscow (U.S.S.R.)

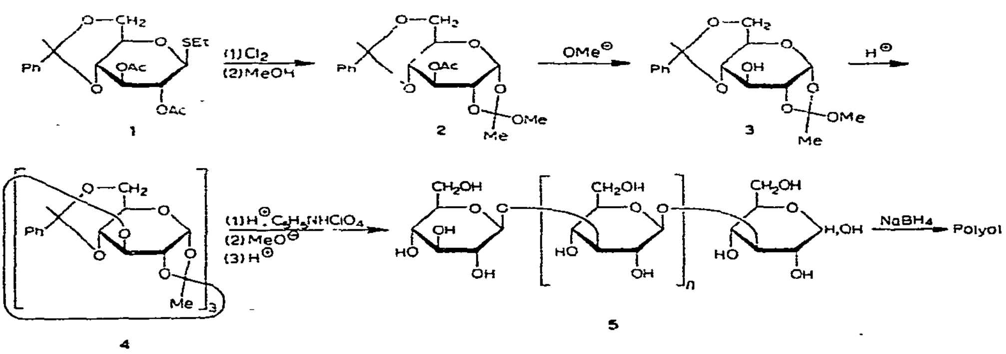
(Received March 6th, 1968; in revised form, April 29th, 1968)

ABSTRACT

Application of the ortho-ester method of glycosylation to an oligomeric ortho-ester derived from 4,6-*O*-benzylidene-α-D-glucopyranose 1,2-(methyl orthoacetate) gave a mixture of saccharides from which a glucan was isolated by chromatography on Sephadex G-25 or by ethanol precipitation. Hydrolytic and periodate-oxidation studies showed that the polysaccharide is essentially a β-(1→3)-linked D-glucan. The glucan has $\overline{\text{DP}}$ 30 and closely resembles the G-chain of insoluble laminaran.

INTRODUCTION

Recently, we proposed two synthetic routes to polysaccharides of predetermined structure based on the ortho ester glycosylation method[1]. These routes involve (*a*) polycondensation of sugar ortho esters containing free hydroxyl groups[2,3], and (*b*) polymerization of sugar ortho esters having only carbohydrate oxygen atoms as substituents in the ortho ester ring-system[4–7]. The present paper is concerned with a new synthesis in which the second approach, now involving polymerization of a macrocyclic, oligomeric, ortho ester *via* a two-stage ortho ester glycosylation[8], is used to synthesise a polysaccharide resembling the laminaran[9–12] of brown algae. Of the group of closely related polysaccharides present in natural laminarans, we have chosen for synthesis the so-called G-chain of insoluble laminaran[10]. The synthesis of polysaccharide **5** was performed according to the following scheme:



DISCUSSION

The first stage of the synthesis was oligomerization of ortho ester **3**, affording the trimeric, macrocyclic, ortho ester[13] **4**. The reported synthesis[14] of ortho ester **2** was not reproducible in our hands, and a more-reliable method, starting with ethyl 2,3-di-*O*-acetyl-4,6-*O*-benzylidene-1-thio-β-D-glucopyranoside[15] (**1**), was devised by using literature methods[16,17].

Polymerization of the macrocyclic, ortho ester **4** was performed under glycosylation conditions described earlier[8]. Removal of the protecting groups after polymerization caused some difficulties. The polymerization product was subjected first to mild hydrolysis with acid to remove surviving ortho ester bonds, and then to saponification to remove acetyl groups. Because of the insolubility of the saponified product in methanol, this stage did not proceed to completion as revealed by i.r. spectroscopy. Hence, after mild hydrolysis with acid to remove benzylidene groups, final removal of the ester groups was performed under acidic conditions[18]. This gave a product, containing no carbonyl or aromatic groups, which appeared to be a mixture of oligomers ranging from disaccharide to polysaccharide. Fractionation of the mixture on Sephadex G-25, or by precipitation with ethanol from water, afforded three fractions: *A* (*ca.* 40–50%), containing traces of glucose and oligosaccharides from di- to hexa-saccharide; *B* (*ca.* 30–40%), containing tetra- to hexasaccharides as major components, and a considerable proportion of a polymeric component; and *C* (13–20%), containing a polysaccharide (glucan S-1).

The yield of polymeric fraction depended on the method of fractionation; an increased yield results in a decrease of the number-average degree of polymerization ($\overline{DP}$), and *vice versa*. The proof of structure **5** for glucan S-1, isolated by precipitation with ethanol from water in a yield of 13%, is based on the following evidence.

Complete hydrolysis of glucan S-1 with acid affords only glucose. Partial hydrolysis, under conditions similar to those applied for partial hydrolysis of laminaran[19], gave a series of reducing oligosaccharides, identical in paper chromatography with oligosaccharides obtained by partial hydrolysis of soluble and insoluble laminarans from *Laminaria japonica* and of laminaran (sample No. 1) described in Ref. 10. The same result was obtained by partial hydrolysis of polyol **6**. Oligosaccharides in fractions *A* and *B* were also identical with the corresponding components of the hydrolysates of natural laminarans (Table I). The regularity of the structure of natural laminaran, and the linear dependence of the R_M values on the degree of polymerization (Fig. 1) suggest that the oligosaccharides constitute the series from laminaribiose to laminariheptaose. Hence, the data of partial hydrolysis suggest that the synthetic polysaccharide is essentially a β-D-(1→3)-linked glucan, and that the oligosaccharides of fractions *A* and *B* are laminaridextrins. Determination of the molecular weight and a more-detailed structural analysis of glucan S-1 were performed by periodate oxidation of the corresponding polyol **6**.

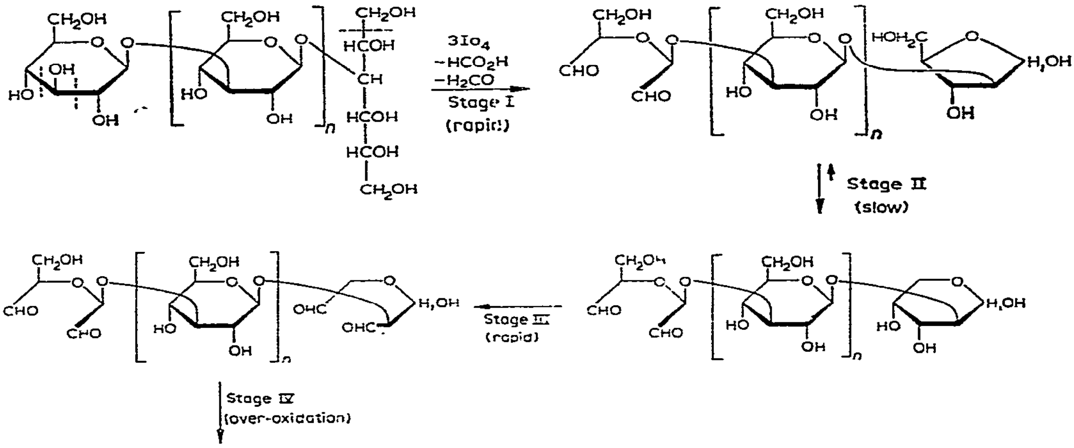
Oxidation of polyol **6** was performed under conditions that permit[10] formation of one molecule of formaldehyde from the terminal 3-*O*-substituted D-glucitol residue, and the results are presented in Fig. 2. The curves for the consumption of periodate and formation of formic acid exhibit characteristic inflexions, indicating that these values reached an intermediate limit after 4–6 h, but subsequently continued to increase. This rather unexpected result was consistently observed in a series of experiments and may be explained by the following scheme:

TABLE I

PAPER CHROMATOGRAPHY OF LAMINARIDEXTRINS

Substrate			*Degree of polymerization*							
			1	*2*	*3*	*4*	*5*	*6*	*7*	*>7*
Solvent C		RG	*1.00*	*0.80*	*0.55*	*0.38*	*0.26*	*0.17*	*0.12*	*<0.12*[a]
	Partial hydrolysis	Insoluble laminaran	+	+	+	+	+	+	+	+
		Soluble laminaran	+	+	+	+	+	+	+	+
		Insoluble laminaran, Sample 1	+	+	+	+	+	+	+	+
		Glucan S-1	+	+	+	+	+	+	?[a]	+
		Polyol VI	+	+	+	+	+	+	?[a]	+
	Fraction *A*		−	+	+	+	+	+	−	−
	Fraction *B*		−	−	+	+	+	?[a]	?[a]	+
	Fraction *C*		−	−	−	−	−	−	−	+
Solvent D		RG	*1.00*	*0.71*	*0.45*	*0.29*	*0.18*	*0.12*	*< 0.12*[a]	
	Partial hydrolysis	Insoluble laminaran	+	+	+	+	+	+	+	
		Soluble laminaran	+	+	+	+	+	+	+	
		Glucan S-1	+	+	+	+	+	?[a]	+	
	Fraction *B*		−	−	±	+	+	?[a]	+	
	Fraction *C*		−	−	−	−	−	−	+	
Solvent E		RG	*1.00*	*0.58*	*0.32*	*0.19*	*0.09*	*< 0.09*[a]		
	Partial hydrolysis	Insoluble laminaran	+	+	+	+	?[a]	+		
		Soluble laminaran	+	+	+	+	?[a]	+		
		Glucan S-1	+	+	+	+	+	+		
	Fraction *B*		−	−	+	+	?[a]	+		

[a]Tailing from origin; no clear spots.



The detection, along with glucose, of traces of arabinose (but not of xylose) in the hydrolysate of the oxidation mixture, after 4–6 h of oxidation, is consistent with this scheme. Obviously, the time interval of the intermediate plateau of periodate

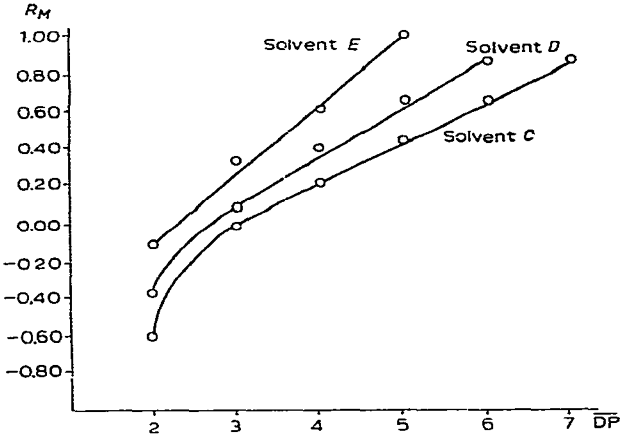


Fig. 1. The dependence of R_M values on the degree of polymerization of laminaridextrins [$R_M = \log(1/R_F - 1)$].

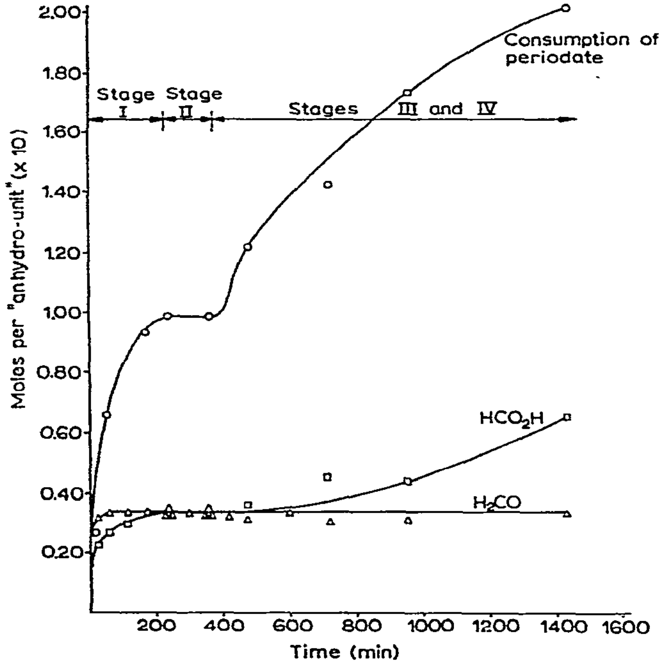


Fig. 2. Periodate oxidation of polyol from glucan S-1 (1.5 mM unbuffered sodium metaperiodate at 16°).

consumption and release of formic acid corresponds to completion of Stage I. The yield of formaldehyde at this plateau was 0.033 molecule per "anhydro-hexose" unit, corresponding to a $\overline{\text{DP}}$ value of 30. At the same period, the yield of formic acid remained at the level of 0.033 molecule, and the consumption of periodate reached 0.099 molecule per "anhydro-hexose" unit. The equal yields of formic acid and formaldehyde suggest that the only source of formic acid during the oxidation is the non-reducing terminus; at the same time, the consumption of periodate is exactly the same as that expected for formation of formaldehyde and formic acid from the α-glycol and the α-triol groupings. Hence, only the terminal residues of the polysaccharide molecule are oxidizable by periodate, and glucan S-1 (within the limits of experimental error) contains no monosaccharide residues bound by other than (1→3) links.

The considerable bulk of evidence on stereospecificity of the ortho-ester glycosylation method[1,4–8] strongly suggests that, in the synthetic polysaccharide, the glycosidic linkages have the β-D configuration. The specific rotation (−9°) of S-1 is the same as that of insoluble laminaran[19], and close to that (−10.3°) of laminaripentaose[20]. The i.r. spectrum of glucan S-1 contains a band at 900 cm^{-1}, corresponding to an axial proton at C-1 in each residue, and no band in the range 820–880 cm^{-1} characteristic of an equatorial proton at C-1. The above data show that polysaccharide S-1 is a regular β-(1-3)-linked D glucan having $\overline{\text{DP}} = 30$ and a structure similar to that of the G-chain of insoluble laminaran.

EXPERIMENTAL

Carbon tetrachloride was distilled from calcium chloride; chloroform from calcium carbonate and 1,2-dichloroethane twice from calcium carbonate and then twice from phosphorus pentaoxide. Thin-layer chromatography (t.l.c.) was performed on neutral alumina (Brockman III) with chloroform–ethyl acetate 95:5 (*A*) and 80:20 (*B*). Paper chromatography was performed on "Goznak" paper with (*C*) butyl alcohol–pyridine–water (6:4:3), (*D*) ethyl acetate–*tert*-butyl alcohol–formic acid–water (6:8:3:3); (*E*) butyl alcohol–acetic acid–water (4:1:5); and (*F*) ethyl acetate–acetic acid–water saturated with boric acid (9:1:1); detection was effected with aniline phthalate. Solutions were evaporated *in vacuo*. Melting points were determined with a Kofler block.

3-O-Acetyl-4,6-O-benzylidene-1,2-O-(1-methoxyethylidene)-α-D-glucopyranose (**2**). — Compound **1** (19.80 g, 50 mmoles) was dissolved with heating in carbon tetrachloride (200 ml), the solution was cooled, and a solution of 5.40 g (76 mmoles) of chlorine in 40 ml of dry chloroform was added (cooling with water, exothermic reaction). The mixture was left for 3 h at room temperature and then evaporated to dryness at <40°. The yellowish crystals were dried *in vacuo* (*ca.* 1 mm, 1 h), and a solution of 20 ml of 2,6-lutidine in 100 ml of dry methanol was added. The suspension was stirred during 3 h and left for 20 h at room temperature (incomplete dissolution at the beginning; crystallization in 1.5–2 h). Light petroleum (150 ml) was added, and the mixture was refrigerated. The product (14.74 g, 80.5%) was collected, and washed with light petroleum. Recrystallization from benzene–ether–light petroleum afforded material having m.p. 140–149°; yield, 55–65%; chromatographically homogenous and identical with an authentic sample (System *A*). It was completely hydrolyzed under the conditions for analytical testing for ortho esters[1].

Ortho ester **4**. — Compound **2** (6.22 g, 17 mmoles) was treated with a mixture of 0.1M methanolic sodium methoxide (15 ml) and methanol (60 ml) for 2 h at 20° (the insoluble suspension dissolved in 20–30 min). Chloroform (300 ml) was added, and the solution was washed with water (3 × 100 ml) and evaporated to dryness, and the residue was thoroughly dried; yield of compound **3**, 5.43 g (98%). The product was dissolved in 80 ml of 1,2-dichloroethane, and the solution was boiled under distillation conditions; fresh solvent was added to the reaction mixture at such a rate that the volume remained constant. After 20–30 ml of solvent had been distilled off, toluene-*p*-sulphonic acid (8.8 mg, 0.051 mmole) was added, and the mixture was boiled under the same conditions. The composition of the mixture was periodically determined by t.l.c. (System *B*). After 45–60* min, a considerable amount of compound **4** had accumulated, at which stage the mixture became a thick, crystalline slurry. The addition of solvent was then stopped, and the mixture was evaporated at atmospheric pressure to about half of the starting volume. Pyridine (0.5 ml) was added, followed by 225 ml of methanol containing 0.5 ml of pyridine. Cooling gave white crystals that were collected, and washed with methanol; yield of compound **4**, 2.07 g (42%), m.p. 278–281°, chromatographically homogeneous (System *B*). The mother liquor contained compound **3** as the major component (t.l.c.), and was evaporated to dryness. The residue was dissolved in chloroform (100 ml), and the solution was washed with water (3 × 35 ml) and evaporated to dryness, and heptane was distilled several times from the residue in order to complete removal of pyridine; yield, 2.96 g (9.2 mmoles). With this residue, the above treatment was repeated (50 ml of 1,2-dichloroethane, 4.8 mg of toluene-*p*-sulphonic acid) yield of **4** was 0.87 g (32,5%). Thus, the overall yield of **4** was 60%.

Repeated crystallization from 1,2-dichloroethane–methanol or chloroform–methanol, in the presence of several drops of pyridine, resulted in almost no loss of material and afforded a preparation[13] having m.p. 283–285°.

Polymerization of ortho ester **4**. — Compound **4** (4.38 g, 5.00 mmoles) and 1,2-dichloroethane (75 ml) were boiled under distillation conditions, with simultaneous addition of fresh solvent at a rate to keep constant the volume of the reaction mixture. After 20–30 ml of solvent had been distilled off, 51.8 mg (0.30 mmole) of toluene-*p*-sulphonic acid and 54.4 mg (0.30 mmole) of pyridinium perchlorate[21] were added, and the mixture was boiled under the same conditions during 15 min, and then refluxed for 23 h. The reaction was monitored by chromatography (System *B*) and was shown to be complete in 15–17 h. The reaction time varied (3–20 h) in a series of experiments. The homogenous mixture was diluted with chloroform (100 ml), and the

---

*The duration of the reaction appeared to depend on the amounts of reagents allowed to react. The beginning of crystallization with starting amounts of compound **3** of 10, 17, 23, and 42 mmoles was after 18, 40, 50, and 100 min, respectively, but this did not change the yield of compound **4**.

solution was washed with water and evaporated to dryness. A solution of the residue in 5 ml of warm 98% acetic acid was evaporated to dryness with toluene, and the residue (thoroughly dried to remove acetic acid) was dissolved in 25 ml of dry chloroform, and treated with dry methanol (25 ml) and 0.1M methanolic sodium methoxide (50 ml). The mixture was left for 75 min at 20°, and the turbid solution was then acidified with 1 ml of acetic acid and evaporated to dryness. The residue was heated with acetic acid (15 ml) and water (10 ml) for 1 h at 100° (insoluble residue had almost completely gone into solution after 20–30 min) and evaporated to dryness. The residue (carbonyl band in i.r. spectrum) was heated at 90–100° with a mixture of 60 ml of *p*-dioxane (peroxide-free) and 60 ml of water. The solution was cooled, 8N sulphuric acid (5 ml) was added, and the mixture was kept for 2 days at room temperature, diluted with water (100 ml), and neutralized with Amberlite IRA-400 (HCO_3^-) resin. The solution was then treated with a mixture of KU-2 (H^+) and Amberlite IRA-400 (HCO_3^-) resins, the resins were filtered off, and the filtrate and washings were evaporated to dryness. A solution of the residue in 20 ml of warm water was centrifuged, the floating organic phase was removed, and the aqueous solution was diluted with ethanol (200 ml) and kept overnight. The resulting precipitate was centrifuged off, washed with ethanol and ether, and dried; yield, 0.50 g. A solution of this material in water (7 ml) was diluted with ethanol (28 ml) and kept for a few hours. The resulting precipitate was collected by centrifugation, washed with ethanol and ether, and dried, to give Fraction *C* (glucan S-1) as a white powder, 0.30 g (13%), $[\alpha]_D$ −9° (*c* 1.26, water). The first and the second supernatants and ethanol washings were combined and evaporated to dryness, the residue was dissolved in water (10 ml), and ethanol (30 ml) and acetone (100 ml) were added. After storage overnight, the precipitate was collected by centrifugation, washed with acetone and ether, and dried, to give Fraction *B* as an almost white powder (0.92 g, 38%). The supernatant and acetone washings were combined and evaporated to give Fraction *A* as a yellow, amorphous residue (1.02 g, 43%). The i.r. spectra of Fractions *A*, *B*, and *C* contained no bands corresponding to carbonyl or aromatic groups.

An analogous result was obtained by fractionation of the mixture by gel-filtration on Sephadex G-25 in 0.1N acetic acid.

Reduction of glucan S-1. — Glucan S-1 (88 mg) in water (5 ml) was treated with sodium borohydride (150 mg) for 1 day at room temperature. The solution was neutralized with KU-2 (H^+) resin and evaporated to dryness. Boric acid was removed from the residue by evaporation with methanol, and the residue was dissolved in 1 ml of water, ethanol (10 ml) was added, and the mixture was left for 3 days. The resulting precipitate was collected by centrifugation, washed with ethanol and ether, and dried, to give polyol **6** (51 mg, 58%).

Fractionation of laminaran of Laminaria japonica. — The laminaran (1.68 g), isolated by Dr. O. S. Chizhov, was dissolved in 25 ml of hot water, and the solution was filtered through cotton and kept for a week at room temperature. The precipitate was collected by centrifugation, washed with ethanol and ether, and dried. Yield of "insoluble" laminaran, 0.06 g. The supernatant and ethanol washings were combined and diluted with ethanol (80 ml), and the precipitate was collected by centrifugation, washed with ethanol and ether, and dried, to yield "soluble" laminaran (1.45 g).

Partial hydrolysis. — Partial hydrolysis of the soluble and insoluble laminarans of *L. japonica*, "Sample No. 1" of insoluble laminaran[10], glucan S-1, and polyol 6 were performed on 1% solutions of polysaccharides (5–10 mg) in 0.1N oxalic acid, which were heated in sealed tubes for 6 h at 100°. The hydrolysates were neutralized with Dowex-2-x8 (HCO_3^-), evaporated, and analysed by paper chromatography, together with Fractions *A* and *B*. The results are presented in Table I.

Periodate oxidation. — Oxidation was performed in the dark. All solutions were prepared with CO_2-free H_2O. The consumption of periodate was determined by titration with 0.01N thiosulphate[22], and formaldehyde by Nash's method[23] in modified form[24]. The yield of formic acid was determined by colorimetry of solutions containing an acid–base indicator*. Polyol 6 (24.40 mg) was oxidized in 50.00 ml of 1.50 mM unbuffered, sodium metaperiodate at 16°. Aliquots (1.00 ml) were removed for the determination of periodate, formic acid, and formaldehyde. The results are presented in Fig. 2.

An aliquot (2 ml) of the reaction mixture was removed after 4.5 h deionized with resins, and evaporated, and the residue was hydrolyzed with N H_2SO_4 (100°, 2 h), neutralized (anion resin), and evaporated. Paper chromatography in System *F* revealed glucose and traces of arabinose; xylose was not detected.

ACKNOWLEDGMENT

The authors thank Professor D. J. Manners and Dr. O. S. Chizhov for the samples of natural laminarans, and Dr. E. D. Sverdlov for useful discussion of the kinetic results.

REFERENCES

1 N. K. Kochetkov, A. Ya. Khorlin, and A. F. Bochkov, *Tetrahedron*, 23 (1967) 693.
2 N. K. Kochetkov, A. F. Bochkov, and I. G. Yazlovetsky, *Carbohyd. Res.*, 5 (1967) 243.
3 N. K. Kochetkov, A. F. Bochkov, I. G. Yazlovetsky, and V. J. Snyatkova, *Izv. Akad. Nauk SSSR, Otd. Khim. Nauk*, (1968) 1802.
4 N. K. Kochetkov, A. Ya. Khorlin, A. F. Bochkov, and I. G. Yazlovetsky, *Carbohyd. Res.*, 2 (1966) 84.
5 A. F. Bochkov, I. G. Yazlovetsky, and N. K. Kochetkov, *Izv. Akad. Nauk SSSR, Otd. Khim. Nauk*, (1968) 1812.
6 N. K. Kochetkov, A. F. Bochkov, and I. G. Yazlovetsky, *Izv. Akad. Nauk SSSR, Otd. Khim. Nauk*, (1968) 1818.
7 N. K. Kochetkov, A. F. Bochkov, and I. G. Yazlovetsky, *Carbohyd. Res.*, in press.
8 A. F. Bochkov, V. I. Snyatkova, and N. K. Kochetkov, *Izv. Akad. Nauk SSSR, Otd. Khim. Nauk*, (1967) 2684.
9 W. D. Annan, E. L. Hirst, and D. J. Manners, *J. Chem. Soc.*, (1965) 220.
10 W. D. Annan, E. L. Hirst, and D. J. Manners, *J. Chem. Soc.*, (1965) 885.
11 M. Fleming and D. J. Manners, *Biochem. J.*, 94 (1965) 17.

---

*Details of this method will be published elsewhere.

12 M. Fleming, E. L. Hirst, and D. J. Manners, *Proc. 5th Intern. Seaweed Symp.*, Halifax, Pergamon, 1966, p. 255.

13 N. K. Kochetkov and A. F. Bochkov, *Tetrahedron Lett.*, (1967) 4669.

14 W. Korytnyc and J. A. Mills: *J. Chem. Soc.*, (1959) 636.

15 A. F. Bochkov, and A. Ch. Jain, *Izv. Akad. Nauk SSSR, Otd. Khim. Nauk*, (1968) 179.

16 F. Weygand and H. Ziemann, *Ann.*, 657 (1962) 179.

17 M. L. Wolfrom and W. Groebke, *J. Org. Chem.*, 28 1(963) 2986.

18 H. O. Bouveng, *Acta Chem. Scand.*, 15 (1961) 96.

19 F. B. Anderson, E. L. Hirst, D. J. Manners, and A. G. Ross, *J. Chem. Soc.*, (1958) 3233.

20 S. Peat, W. J. Whelan, and H. G. Lawley, *J. Chem. Soc.*, (1958) 724.

21 F. Arndt and P. Nachtwey, *Ber.*, 59B (1926) 448.

22 L. M. Likhosherstov and L. E. Brossard, *Khim. Prirodn. Soedin., Akad. Nauk SSSR, Inst. Khim. Prirodn. Soedin.*, (1967) 7.

23 T. Nash, *Biochem. J.*, 55 (1953) 416.

24 V. E. Vaskovsky *et al.*, *Anal. Biochem.*, in press.

Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

REACTIONS OF FURANOSIDE EPOXIDES. THE PREPARATION OF BROMOHYDRINS FROM METHYL 5-*O*-ACETYL-2,3-ANHYDRO-α (AND β)-D-RIBOFURANOSIDE

ELMER J. REIST AND SANDRA L. HOLTON
Life Sciences Research, Stanford Research Institute, Menlo Park, California 94025 (U.S.A.)

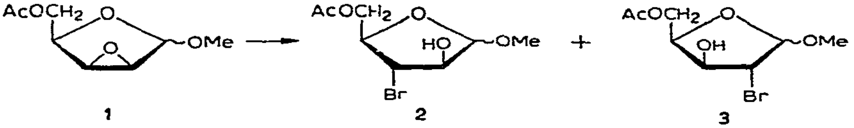
(Received May 6th, 1968; in revised form, June 10th, 1968)

ABSTRACT

The reaction of anhydrous magnesium bromide with methyl 5-*O*-acetyl-2,3-anhydro-α-D-ribofuranoside gives a mixture of methyl 5-*O*-acetyl-2-bromo-2-deoxy-α-D-arabinofuranoside and methyl 5-*O*-acetyl-3-bromo-3-deoxy-α-D-xylofuranoside in which the latter preponderates. With methyl 5-*O*-acetyl-2,3-anhydro-β-D-ribofuranoside, the same reagent gives methyl 5-*O*-acetyl-3-bromo-3-deoxy-β-D-xylofuranoside as the only isolable product. An adjacent methoxyl group shows greater steric inhibition than an adjacent acetoxymethyl group.

RESULTS AND DISCUSSION

A recent publication from these laboratories[1] contained a description of the acid-catalyzed opening of the anhydride ring of the anomeric methyl 5-*O*-acetyl-2,3-anhydro-D-lyxofuranosides (**1**) with anhydrous magnesium bromide to give the bromohydrins **2** and **3**.

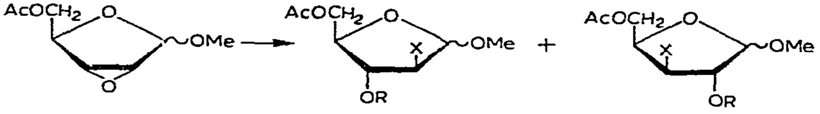


1 2 3

a series = α-D anomers
b series = β-D anomers

It was observed that **1a** gave only the 3-bromohydrin (**2a**) with no detectable amount of 2-bromohydrin (**3a**), a result in accord with the observation that the reaction of 2,3-anhydrofuranosides with nucleophilic reagents generally results in epoxide opening at C-3. On the other hand, the reaction of the β-epoxide (**1b**) with anhydrous magnesium bromide gave predominant opening of the epoxide at C-2, resulting in a ratio of 2-bromohydrin (**3b**) to 3-bromohydrin (**2b**) of 2:1.

In view of the profound effect of the anomeric configuration on the course of reaction with the methyl 2,3-anhydro-D-lyxofuranosides (**1**), it was of interest to examine a similar reaction with the anomeric methyl 5-*O*-acetyl-2,3-anhydro-α(and β)-D-ribofuranosides[2] (**4a** and **b**).



4

5 X = Br, R = H
6 X = Br, R = $SiMe_3$
7 X = Br, R = Ac
8 X = H, R = Ac
9 X = H, R = $CO_2C_6H_4Me$-*p*
10 X = R = H

11 X = Br, R = H
12 X = Br, R = $SiMe_3$
13 X = Br, R = Ac
14 X = H, R = Ac
15 X = R = H

a series = α-D anomers
b series = β-D anomers

The reaction of methyl 5-*O*-acetyl-2,3-anhydro-α-D-ribofuranoside (**4a**) with anhydrous magnesium bromide[3] gave a mixture of two bromohydrins in the ratio of 5:2. Separation was effected by vapor-phase chromatography (v.p.c.) of the trimethylsilyl ethers[4]. De(trimethylsilyl)ation of the separated components gave methyl 5-*O*-acetyl-2-bromo-2-deoxy-α-D-arabinofuranoside (**5a**) and methyl 5-*O*-acetyl-3-bromo-3-deoxy-α-D-xylofuranoside (**11a**) as syrups, which were acetylated to give methyl 3,5-di-*O*-acetyl-2-bromo-2-deoxy-α-D-arabinofuranoside (**7a**) and methyl 2,5-di-*O*-acetyl-3-bromo-3-deoxy-α-D-xylofuranoside (**13a**), respectively, both as analytically pure syrups. The n.m.r. spectrum of the acetylated bromohydrin that preponderated was compatible with structure **7a**, resulting from attack at C-2 of the epoxide. Thus, H-1 gave rise to a singlet, and H-2 to a doublet having a small coupling constant, a result to be expected for the *trans*-*trans* relationship of H-1, H-2, and H-3 that exists in **7a**. This assignment was confirmed by hydrogenolysis of **7a** to give methyl 3,5-di-*O*-acetyl-2-deoxy-α-D-*erythro*-pentofuranoside (**8a**), which proved to be identical in all respects with **8a** prepared from 2-deoxy-D-*erythro*-pentose via the known methyl 2-deoxy-3,5-di-*O*-*p*-toluoyl-α-D-*erythro*-pentofuranoside[5] (**9a**).

The n.m.r. spectrum of the other acetylated bromohydrin was poorly resolved, and could not be used for structural assignment. That the material was a *trans*-bromohydrin, however, was demonstrated when the starting epoxide (**4a**) was regenerated in high yield by treating the bromohydrin with sodium methoxide and acetylating the product. The location of the bromine atom on C-3 was demonstrated by hydrogenolysis of the acetylated bromohydrin **13a** to give methyl 2,5-di-*O*-acetyl-3-deoxy-α-D-*erythro*-pentofuranoside (**14a**). Although a satisfactory analytical sample of **14a** could not be obtained, the n.m.r. spectrum was compatible with the structure proposed*. Conclusive structural identification was obtained by removing

---

*Mechanistically, the 5-*O*-acetyl group can participate in this reaction by way of a 1,3-orthoester ion (i). The products from such participation should be methyl 3-*O*-acetyl-5-bromo-5-deoxy-α-D-xylofuranoside (ii) and methyl 5-*O*-acetyl-3-bromo-3-deoxy-α-D-ribofuranoside (iii). It would be the substituents from **14a**, to give 3-deoxy-D-*erythro*-pentose, characterized as its known (*p*-nitrophenyl)osazone[8].

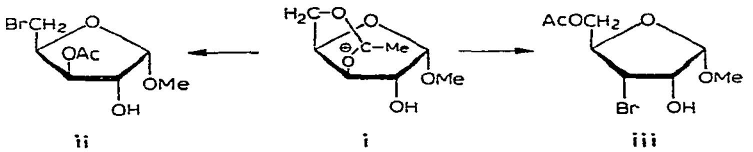
The reaction of magnesium bromide with methyl 5-*O*-acetyl-2,3-anhydro-β-D-ribofuranoside (**4b**), not unexpectedly, was much slower than with the α-D anomer (**4a**). Thus, after a 10-day period of reflux, a significant quantity of unreacted starting material was still present. The product isolated appeared to be homogeneous, and, after acetylation to the diacetate, it gave an n.m.r. spectrum consistent with the steric arrangement present in methyl 2,5-di-*O*-acetyl-3-bromo-3-deoxy-β-D-xylofuranoside (**13b**). This structural assignment was verified by treatment with sodium methoxide, followed by acetylation, whereupon **4b** was renegerated, thus demonstrating the *trans*-bromohydrin structure. The location of the bromine atom on C-3 was shown by hydrogenolysis of **14b** to give methyl 2,5-di-*O*-acetyl-3-deoxy-β-D-*erythro*-pentofuranoside (**15b**) having n.m.r. and i.r. spectra that were in all respects identical with those of an authentic sample of **15b** prepared by an alternative route[8].

It is interesting to compare the results of the reaction of magnesium bromide with methyl 5-*O*-acetyl-2,3-anhydro-α-D-lyxofuranoside (**1a**) and with methyl 5-*O*-acetyl-2,3-anhydro-α-D-ribofuranoside (**4a**). In each case, the preponderant product results from the attack of bromide on the ring carbon atom that is sterically less hindered. When a methoxyl group is responsible for the steric hindrance (as in **1a**) only the 3-bromohydrin (**2a**) results. When the steric bulk is due to the acetoxymethyl group (as in **4a**), the steric control is not so complete, and a mixture of products (namely, **5a** and **11a**) results. The greater steric control of the methoxyl group is again illustrated by the fact that the 3-bromohydrin **11b** is the sole product from the β-D-riboside **4b**. Finally the reaction with the β-D-lyxoside (**1b**), in which there should be no significant steric hindrance, gave predominant attack[1] at C-2.

That the methoxyl group should exhibit a steric effect stronger than that of the acetoxymethyl group is somewhat surprising, as the acetoxymethyl group is the bulkier. It is possible that the dipole interaction that leads to the "anomeric effect" in pyranoses[9] may be responsible for this result. Such an interaction would have a

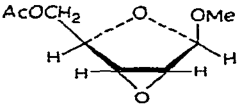
---

expected that the 5-bromoxyloside **ii**, resulting from attack by bromide on the primary carbon should preponderate[6].



Hydrogenolysis of **ii** would give a 5-deoxy-D-xylose derivative, which should be readily identifiable by its n.m.r. spectrum; however, no evidence was obtained for the presence of **ii**. Treatment of **iii** with base would not regenerate the epoxide **4a**. The high recovery of epoxide precludes the presence of any significant proportion of **iii**, and this, in turn, precludes any significant participation by the 5-acetate. These results are in agreement with those of Hughes and Speakman[7], in which treatment of 5-*O*-benzoyl-1,2-*O*-isopropylidene-3-*O*-*p*-tolylsulfonyl-α-D-ribofuranose with sodium benzoate in *N*,*N*-dimethylformamide afforded no evidence for benzoate participation via a 1,3-orthoester ion.

tendency to push the methoxyl group into a pseudo-axial position. The 4-(acetoxymethyl) group (not, itself, affected by any dipole interaction with the ring-oxygen atom) will assume more of a pseudo-equatorial position, within the limits permitted by the twisted furanose ring. Such a conformation would hinder SN2 attack at C-2 by bromide, but would make C-3 more accessible to attack. Examination of the n.m.r. spectrum showed that the coupling constants of **4b** are very similar to those of methyl 2,3-anhydro-β-D-ribofuranoside[10]. Thus, H-1 resonated at τ 5.05 (s), H-2 and H-3 resonated at τ 6.3 ($J_{2,3}$ 3 Hz, $J_{3,4}$ ~1 Hz), and H-4 and H-5 resonated at τ 5.8 (m). Examination of a molecular model of **4b** revealed that the pseudo-equatorial attachment of the 4-(acetoxymethyl) group, and the pseudo-axial bond to the 1-methoxyl group, are compatible with the V_o (envelope) structure (**16**) for **4b** and its resulting small coupling constants $J_{1,2}$ and $J_{3,4}$.



16

EXPERIMENTAL

Vapor-phase chromatograms, both analytical and preparative, were performed with a Wilkens Autoprep 700 gas chromatograph equipped with a 5 ft × 3/8 in stainless-steel column packed with 15% phenyl (diethylamino)succinate on Chromosorb W (60–80 mesh). Helium was used as the carrier gas. The column temperature was 170°, unless otherwise noted. Specific rotations were determined with a Rudolph photoelectric polarimeter. Thin-layer chromatograms were performed with silica gel HF (E. Merck, A.-G., Darmstadt), and spots were detected by means of iodine vapor. Organic solutions were dried with anhydrous magnesium sulfate. N.m.r. spectra were recorded with either a Varian A-60 or HA-100 spectrometer, for solutions in chloroform-*d*, with tetramethylsilane (τ 10.00) as the internal standard.

Methyl 3,5-di-O-acetyl-2-bromo-2-deoxy-α-D-arabinofuranoside (**5a**) *and methyl 2,5-di-O-acetyl-3-bromo-3-deoxy-α-D-xylofuranoside* (**11a**). — A solution of 1.3 g (6.9 mmoles) of methyl 5-*O*-acetyl-2,3-anhydro-α-D-ribofuranoside[2] (**4a**) in 65 ml of 1,2-dimethoxyethane was heated for 4 h at reflux with magnesium bromide prepared from 1.3 g (53.5 mmoles) of magnesium and 7.2 ml (83.5 mmoles) of 1,2-dibromoethane[1]. The solution was cooled, 30 ml of water was added, the pH was adjusted to 2 with 5M hydrochloric acid, and the solution was extracted with two 30-ml portions of chloroform. The chloroform extracts were combined, washed successively with 10 ml of 5% aqueous sodium thiosulfate and 10 ml of water, dried, and evaporated to dryness *in vacuo* to yield 1.57 g (85%) of mixed bromohydrins (**5a** and **11a**) as a yellow oil. V.p.c. of the trimethylsilyl ethers[4] of the crude product showed that the mixture contained two components in the ratio of 5:2.

Treatment of 180 mg of the mixed bromohydrins with methanolic sodium methoxide at room temperature, followed by acetylation of the product, gave an 87% recovery of methyl 2,3-anhydro-5-*O*-acetyl-α-D-ribofuranoside (**4a**), which had an i.r. spectrum identical with that of authentic **4a**. The behavior of this compound on t.l.c. [with 1:3 (v/v) cyclohexane–ether] was identical with that of authentic **4a**, and was significantly different from that of the acetylated bromohydrin **13a**.

To a solution of crude product (**5a** and **11a**) in 5 ml of dry pyridine was added 7.85 ml of hexamethyldisilazane and 800 μl of chlorotrimethylsilane. The mixture was stirred for 5 min, 40 ml of dry benzene was added, and the solution was centrifuged to remove the precipitated salts. The supernatant liquor was purified by means of preparative v.p.c. at 150°, to give the trimethylsilyl ethers **6a** (retention time 2.5 min), and **12a** (retention time 4.0 min), in yields of 0.28 g (14%), and 0.16 g (8%), respectively.

A solution of **6a** in 15 ml of 50% aqueous methanol was heated for 4.5 h at 50°, and evaporated to dryness *in vacuo*; abs. ethanol was added and evaporated off (to remove the last traces of water), yielding 0.22 g of methyl 5-*O*-acetyl-2-bromo-2-deoxy-α-D-arabinofuranoside (**5a**) as a colorless oil.

Acetylation of **5a** with 440 μl of acetic anhydride in 10 ml of dry pyridine gave 0.24 g of methyl 3,5-di-*O*-acetyl-2-bromo-2-deoxy-α-D-arabinofuranoside (**7a**) as a colorless oil; λ_{max}^{film} 5.70, 8.10 μm (acetate -CO_2-). There was no hydroxyl absorption at 2.9 μm. The n.m.r. spectrum showed a singlet at τ 4.81 (H-1), a doublet at τ 5.65 ($J_{2,3}$ 2 Hz, H-2), a quartet at τ 4.76 ($J_{2,3}$ 2 Hz, $J_{3,4}$ 4 Hz, H-3), and multiplets at τ 5.6–5.9 (H-4, H-5).

Anal. Calc. for $C_{10}H_{15}BrO_6$: C, 38.6; H, 4.86. Found: C, 38.8; H, 5.04.

In the same way, 158 mg of methyl 5-*O*-acetyl-3-bromo-3-deoxy-2-*O*-(trimethylsilyl)-α-D-xylofuranoside (**12a**) was de(trimethylsilyl)ated with 50% aqueous methanol and the product was acetylated with acetic anhydride in pyridine, to give 128 mg of methyl 2,5-di-*O*-acetyl-3-bromo-3-deoxy-α-D-xylofuranoside (**13a**); λ_{max}^{film} 5.70, 8.10 μm (acetate -CO_2-). The n.m.r. spectrum of **13a** was poorly resolved, and the signals of the ring protons were divided into two groups, a 2-proton group centered at τ 4.8 (presumably H-1 and H-3), and a 4-proton group centered at τ 5.6 (H-2, H-4, H-5 and H-5').

Anal. Found: C, 38.9; H, 4.58.

*Methyl 3,5-di-O-acetyl-2-deoxy-α-*D-erythro-*pentofuranoside* (**8a**). — *A. From the bromohydrin* **7a**. A solution of 199 mg of methyl 3,5-di-*O*-acetyl-2-bromo-2-deoxy-α-D-arabinofuranoside (**7a**) in 10 ml of 2-methoxyethanol was treated with Norit, and the suspension was filtered. To the filtrate were added 103 mg of 5% palladium-on-carbon and 100 mg of anhydrous sodium acetate, and the mixture was stirred under one atmosphere of hydrogen for 18 h at room temperature, by which time uptake of hydrogen had ceased. The suspension was filtered (Celite pad), the filtrate was evaporated to dryness *in vacuo*, and the residue was partitioned between ether and water (20 ml of each). The ether layer was washed successively with 10 ml of saturated aqueous sodium hydrogen carbonate and 10 ml of water, dried, and evaporated to dryness *in vacuo* to give 89 mg of product (**8a**) as a colorless oil that was homogeneous (R_F 0.5) by t.l.c. with 19:1 chloroform–ethyl acetate as the developing solvent. The n.m.r. spectrum was identical with that of authentic methyl 3,5-di-*O*-acetyl-2-deoxy-*β*-D-*erythro*-pentofuranoside (**8a**) prepared from 2-deoxy-D-*erythro*-pentose (route *B*).

*B. From 2-deoxy-*D-erythro-*pentribose.* To a solution of 1.24 g of methyl 2-deoxy-3,5-di-*O*-(*p*-toluoyl)-*α*-D-*erythro*-pentofuranoside[5] (**9a**) in 35 ml of methanol was added 1 ml of 100 mM methanolic sodium methoxide, and the solution was boiled for 1 h under reflux. The solution was cooled to room temperature, neutralized to pH 6 with Dowex-50 (H^+), the suspension was filtered, and the filtrate was evaporated to dryness *in vacuo*. The residue was partitioned between chloroform and water (20 ml of each). The aqueous layer was evaporated to dryness, to give 400 mg of crude methyl 2-deoxy-*α*-D-*erythro*-pentofuranoside (**10a**) as a colorless syrup, λ_{max}^{film} 2.90 μm (OH); no carbonyl absorption at 5.8 μm.

Acetylation of methyl 2-deoxy-*α*-D-*erythro*-pentofuranoside (**10a**, 400 mg) with 1.28 ml of acetic anhydride in 10 ml of pyridine gave 590 mg of methyl 3,5-di-*O*-acetyl-2-deoxy-*α*-D-*erythro*-pentofuranoside (**8a**) as a colorless syrup, $[\alpha]^{25}$ +56° (*c* 0.87, chloroform); λ_{max}^{film} 5.70, 8.05 μm (acetate -CO_2-); no hydroxyl absorption at 2.9 μm.

Anal. Calc. for $C_{10}H_{16}O_6$: C, 51.7; H, 6.94. Found: C, 51.7; H, 6.89.

*Methyl 2,5-di-*O*-acetyl-3-deoxy-α-*D-erythro-*pentofuranoside* (**14a**). — A solution of 92 mg of methyl 2,5-di-*O*-acetyl-3-bromo-3-deoxy-*α*-D-xylofuranoside (**11a**) in 10 ml of 2-methoxyethanol was treated with Norit, and was then hydrogenated by using 50 mg of 5% palladium-on-carbon and 50 mg of anhydrous sodium acetate as described for the hydrogenation of **7a**. It was necessary to repeat the hydrogenation in order to effect complete debromination; 43 mg of product **12a** was obtained as a colorless oil. T.l.c. with 19:1 chloroform–ethyl acetate showed one main spot at R_F 0.3, as well as a weaker spot at the origin.

Saponification and hydrolysis, followed by preparation of the (*p*-nitrophenyl)-osazone by a procedure described for the *β*-D anomer[6], gave crystalline 3-deoxy-D-*erythro*-pentose (*p*-nitrophenyl)osazone, m.p. 252–254°, identical spectroscopically with a sample prepared by an alternative route[6].

*Methyl 2,5-di-*O*-acetyl-3-bromo-3-deoxy-β-*D*-xylofuranoside* (**13b**) — A solution of 2.0 g of methyl 5-*O*-acetyl-2,3-anhydro-*β*-D-ribofuranoside (**4b**) in 100 ml of dry 1,2-dimethoxyethane was heated for 10 days at 70° with magnesium bromide (prepared from 2.0 g of magnesium and 11 ml of 1,2-dibromoethane). The mixture was processed as described for the *α*-D anomer, to give 1.97 g of crude product as a yellow oil. V.p.c. of the trimethylsilyl ether of the crude product showed that it contained two components, the first of which appeared to be unreacted starting-material.

The trimethylsilyl ether was prepared as described for the *α*-D anomer, and the product was submitted to preparative v.p.c. to give 175 mg of recovered starting-material, followed by 386 mg of methyl 5-*O*-acetyl-3-bromo-3-deoxy-2-*O*-(trimethylsilyl)-*β*-D-xylofuranoside (**12b**).

A solution of **12b** in 5 ml of 50% aqueous methanol was heated for 4.5 h at

55°, and evaporated to dryness *in vacuo*. The last traces of water were removed by the addition and evaporation of 5 ml of abs. ethanol, to give 291 mg of methyl 5-*O*-acetyl-3-bromo-3-deoxy-β-D-xylofuranoside (**11b**). T.l.c. with 1:3 (v/v) cyclohexane–ether showed one main component at R_F 0.65, and several trace contaminants at R_F 0–0.2.

Acetylation of **11b** with 460 μl of acetic anhydride in 5 ml of pyridine gave 232 mg of methyl 2,5-di-*O*-acetyl-3-bromo-3-deoxy-β-D-xylofuranoside (**13b**) as a colorless syrup, $[\alpha]_D^{25}$ +13° (*c* 0.99, chloroform). The n.m.r. spectrum showed a singlet at τ 5.12 (H-1), a singlet at τ 4.70 (H-2), and a multiplet at τ 5.7 (H-3, H-4, and H-5).

Anal. Calc. for $C_{10}H_{15}BrO_6$: C, 38.6; H, 4.86; Br, 25.7. Found: C, 38.7; H, 4.85; Br, 25.8.

Treatment of **13b** (100 mg) with methanolic sodium methoxide, followed by acetylation of the product, gave a 70% yield of methyl 5-*O*-acetyl-2,3-anhydro-β-D-ribofuranoside (**4b**), identical by i.r. spectrum and t.l.c. (1:3 cyclohexane–ether) with authentic **4b**.

In a second preparation, purification of **11b** was accomplished by preparative t.l.c. with 1:3 cyclohexane–ether, to give a 29% yield of bromohydrin **11b**; the product was identical in all respects with the bromohydrin obtained by preparative v.p.c. of the trimethylsilyl ether **12b**.

*Methyl 2,5-di-*O*-acetyl-3-deoxy-β-*D-erythro*-pentofuranoside* (**14b**). — A solution of 156 mg of methyl 2,5-di-*O*-acetyl-3-bromo-3-deoxy-β-D-xylofuranoside (**13b**) in 10 ml of 2-methoxyethanol was hydrogenated, in the presence of 79 mg of anhydrous sodium acetate and 83 mg of 5% palladium-on-carbon, as described for the preparation of **7a**, to give 52 mg (45%) of methyl 2,5-di-*O*-acetyl-3-deoxy-β-D-*erythro*-pentofuranoside (**14b**) as a colorless oil. The n.m.r. spectrum was identical with that of authentic **12b** prepared by an alternative route[6].

ACKNOWLEDGMENT

This investigation was supported by Public Health Service Research Grant GM 11438 from the National Institute of General Medical Sciences.

REFERENCES

1 E. J. Reist and S. L. Holton, *Carbohyd. Res.*, 2 (1966) 181.
2 C. D. Anderson, L. Goodman, and B. R. Baker, *J. Amer. Chem. Soc.*, 80 (1958) 5247.
3 G. N. Richards, L. F. Wiggins, and W. S. Wise, *J. Chem. Soc.*, (1956) 496.
4 C. C. Sweeley, R. Bentley, M. Makita, and W. W. Wells, *J. Amer. Chem. Soc.*, 85 (1963) 2497.
5 D. L. MacDonald and H. G. Fletcher, Jr., *J. Amer. Chem. Soc.*, 84 (1962) 1262.
6 Ö. K. J. Kovács, G. Schneider, L. K. Láng, and J. Apjok, *Tetrahedron*, 23 (1967) 4181.
7 N. A. Hughes and P. R. H. Speakman, *Carbohyd. Res.*, 1 (1966) 341.
8 C. D. Anderson, L. Goodman, and B. R. Baker, *J. Amer. Chem. Soc.*, 81 (1959) 898.
9 E. L. Eliel, N. L. Allinger, S. J. Angyal, and G. A. Morrison, *Conformational Analysis*, Interscience, New York, 1965, p. 375.
10 L. D. Hall, *Chem. Ind.* (London), (1963) 950.

Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

MASSENSPEKTROMETRISCHE UNTERSUCHUNGEN

XIX. MITTEILUNG*. KOMBINATION VON GASCHROMATOGRAPHIE UND MASSENSPEKTROMETRIE ZUR ANALYSE PARTIELL METHYLIERTER ZUCKERDERIVATE. DIE MASSENSPEKTREN VON PARTIELL METHYLIERTEN METHYLGLUCOSIDEN

K. Heyns, K. R. Sperling und H. F. Grützmacher
Institut für Organische Chemie der Universität Hamburg (Deutschland)

(Eingegangen den 5. April, 1968; modifiziert den 7. Juni, 1968)

ABSTRACT

Some partially methylated methyl glucosides were investigated by the gas chromatography–mass spectrometry method. The main properties of their mass spectra can be derived from the fragmentation scheme of permethylated pyranoses by a consideration of the effects of the different substituents. Apart from a shift of the ion masses, due to replacement of methoxyl by hydroxyl groups, the main effect of the substituent is a variation of the inductive stabilisation of the positive charge of the fragments.

Furthermore, the derivatives having methoxyl groups at the 1,2,3-, 1,2,3,6-, 1,3,4-, or 1,3,4,6-positions produce a very characteristic peak at *m/e* 161.

The stereochemistry of the partially methylated methyl glucosides has no significant effect on their mass spectra, but the effect of the position of the substituent is important. The type of substitution can thence be deduced from the mass spectra.

The gas chromatography–mass spectrometry method is much more appropriate for the identification of a mixture of monosaccharides obtained from a permethylated polysaccharide by solvolysis than the gas-chromatographic method alone.

ZUSAMMENFASSUNG

Partiell methylierte Methylglucoside wurden gaschromatographisch-massenspektrometrisch untersucht. Die Haupteigenschaften ihrer Massenspektren sind aus dem Zerfallschema für permethylierte Pyranoside durch Berücksichtigung von Substituenten-Effekten ableitbar. Wesentlichster Substituenten-Effekt ist außer der Verschiebung einzelner Ionenmassen durch Austausch von OCH_3- gegen OH-Gruppen die unterschiedliche induktive Stabilisierung positiver elektrischer Ladungen in den Fragmenten.

Weiterhin entsteht durch eine Umlagerungsreaktion bei den Derivaten mit Methoxygruppen in 2,3-, 2,3,6-, 3,4- und in 3,4,6-Stellung ein sehr charakteristischer Peak der MZ 161.

---

*XVIII. Mitteilung: *Ber.*, 100 (1967) 2664.

Die Eigenschaften der Massenspektren partiell methylierter Methylglycoside werden nur unwesentlich durch deren Stereochemie, maßgeblich jedoch durch die Stellung des Substituenten bestimmt. Der Substitutionstyp wird damit aus den Massenspektren ablesbar.

Dadurch ist die gaschromatographisch-massenspektrometrische Methode wesentlich besser als die gaschromatographische Methode allein geeignet, die Bestandteile eines durch Solvolyse permethylierter Polysaccharide erhältlichen Monosaccharidgemisches zu identifizieren.

EINFÜHRUNG

In neueren Arbeiten stützen sich Strukturbeweise für Polysaccharide überwiegend auf gaschromatographische Retentionsvolumina der durch Permethylierung und Solvolyse gebildeten partiell methylierten Monosaccharide[1,6–8]. Die alleinige Anwendung der Gaschromatographie zur Analyse eines Gemisches partiell methylierter Monosaccharide birgt jedoch erhebliche Schwierigkeiten und Unsicherheiten, denn die Retentionsvolumina der partiell methylierten Derivate verschiedener Zucker liegen dicht beieinander[1–6], sind aber nur innerhalb 5% reproduzierbar[1]. Eine hinreichend sichere Identifizierung ist daher oft nicht einmal durch direkten Vergleich mit Testsubstanzen möglich[1,6]. Eine Verbesserung kann erreicht werden, wenn außer den Retentionsvolumina als weitere stoffspezifische Kenngrößen Spektren der getrennten Substanzen aufgenommen werden. Hier bietet sich eine direkte Kopplung zwischen Gaschromatograph und Massenspektrometer an[10,11]. Die Retentionsvolumina einer großen Anzahl von partiell methylierten Monosacchariden sind bereits tabelliert worden (s.o.). Zusammen mit einer massenspektrometrischen Identifizierung des Substitutionstyps sollte daher trotz der geringen Reproduzierbarkeit der in der Literatur angegebenen Retentionsvolumina eine eindeutige Zuordnung möglich sein.

Bei der Untersuchung permethylierter Methylglycoside der Hexosen und Pentosen hat sich gezeigt, daß die Massenspektren vom sterischen Bau dieser Verbindungen wenig beeinflußt werden. In allen Fällen werden die gleichen Ionen gebildet, kleinere Unterschiede treten nur in den relativen Intensitäten einzelner Ionentypen auf. Dies gilt auch für die Massenspektren der α- und β-Anomeren. Erfolgt die durch Elektronenstoß induzierte Fragmentierung der partiell methylierten Monosaccharide nach den gleichen Mechanismen, wie die der permethylierten Derivate, so ist auch für die Massenspektren dieser Verbindungen nur ein geringer sterischer Effekt zu erwarten. Um die charakteristischen Merkmale der Substitutionstypen zu bestimmen, genügt daher die Untersuchung der verschiedenen Derivate eines einzigen Monosaccharids.

Wegen des sterischen Einflusses, der sich durch geringe Intensitätsverschiebungen der Ionen beim Übergang von einem zum anderen Monosaccharid bemerkbar macht, können für die Identifizierung des Substitutionstyps nur hervorstechende Merkmale, wie Massenverschiebungen und kräftige Intensitätsänderungen der

Ionen in Abhängigkeit von der Stellung der Substituenten herangezogen werden. Dieses Verfahren ist auch zu empfehlen, weil im allgemeinen Massenspektren, die mit der kombinierten gaschromatographisch-massenspektrometrischen Methode aufgenommen werden, weniger gut quantitativ reproduzierbar sind als üblich.

Als „Modellsubstanzen", mit denen der Einfluß der Substituenten am Ring auf die Massenspektren untersucht werden kann, wurden die partiell methylierten Methylglucopyranoside gewählt (Darstellung gemäß[9,12]). Von diesen sind vor allem jene wichtig, mit denen man bei der Solvolyse eines permethylierten Polysaccharids rechnen muß. Das sind die Methyl-tri-*O*-methylglucopyranoside mit jeweils einer freien OH-Gruppe in Stellung 2, 3, 4 oder 6. Darüber hinaus wurden — zur Kontrolle — einige Methyl-di-*O*-methylglucopyranoside und ein Methyl-mono-*O*-methylglucopyranosid dargestellt und untersucht.

VERSUCHSBEDINGUNGEN

(*a*) Alle gaschromatographischen Messungen wurden mit einem Perkin–Elmer-Gaschromatographen Typ F 20 unter folgenden Bedingungen durchgeführt: Säule: 2 m Glas, 4 mm ⌀; Phasen: 5% Äthylenglykolsuccinat auf Chromosorb W; Säulentemperatur: 200°; Einspritzblock-Temperatur: 260°; Durchflußgeschwindigkeit: 25 ml He/min.; Anzeige: FID bzw. Massenspektrometer.

(*b*) Kopplungselement zum Massenspektrometer war eine mit Butandiolsuccinat beschichtete, auf 140–180° beheizte Metallkapillare. Sie wurde an die Austrittsdüse des Flammenionisationsdetektors angeschlossen. Die Verbindung mit dem Massenspektrometer bestand aus einer feinen Glaskapillare, durch die ein bestimmter Anteil des aus dem Gacshromatographen über die Kopplungs-Kapillare austretenden Gasstroms in das Massenspektrometer eingesogen wurde.

(*c*) Die Massenspektren wurden mit einem Atlas CH-4-Gerät unter folgenden Bedingungen aufgenommen: Substanz-Einlaß: Über den „Hochtemperatur-Einlaß" bzw. über den Gaschromatographen; Arbeitstemperatur (in Vorratsbehälter und Ionenquelle): 150–170°. Elektronenenergie: 70 eV. Ionenanzeige: SEV-System; Registriereinheit: Kompensationsschreiber; bei Einlaß über den Gaschromatographen: Lichtpunktschreiber. Registrierzeit: Für den Bereich von 40–250 ME mit dem Kompensationsschreiber *ca.* 20 min, mit dem Lichtpunktschreiber *ca.* 10 sek (bei einer Durchlaufzeit von 30–120 sek für einen gaschromatographischen Peak).

VERSUCHSERGEBNISSE

Die gemessenen Retentionsvolumina wurden auf Methyl-2,3,4,6-tetra-*O*-methyl-α-D-glucopyranosid als inneren Standard bezogen. Die so erhaltenen relativen Retentionsvolumina werden im folgenden R_r genannt (Tab. I).

Bezüglich der Retentionswerte fallen vier, auch chemisch gut unterscheidbare Gruppen sofort auf: Methyl-tetra-*O*-methylglucopyranoside bei $R_r = 0–1$, Methyl-tri-*O*-methylglucopyranoside bei $R_r = 2–3$, Methyl-di-*O*-methylglucopyranoside bei

R_r = 4–7, Methyl-mono-*O*-methylglucopyranoside bei $R_r \geqslant 10$. Die schwerflüchtigen und polaren Methyl-3-*O*-methylglucopyranoside wurden hartnäckig im Gerät festgehalten. Die R_r-Werte wurden deshalb bei erhöhter Einspritzblocktemperatur (320°) gemessen. Die beobachteten Retentionszeiten lagen bei 2.5 Stunden.

TABELLE I

RETENTIONSWERTE DER PARTIELL METHYLIERTEN METHYLGLUCOSIDE

Verbindungen[a]	*Gemessene Werte* R_r *unter folgenden Bedingungen*[b]	
	GC[c] 200°, Glassäule mit EGS[e]	GC–MS[d] 200°, Glassäule mit EGS, Kapillare mit BDS[f]
β-2,3,4,6	0.74	0.77
α-2,3,4,6	1.00	1.00
β-2,3,6	2.20	2.35
α-2,3,6	2.92	3.20
β-2,4,6	2.15	2.75
α-2,3,4	2.38	2.50
α-3,4,6	2.00	2.20
α-2,3	6.86	
α-4,6	4.50	7.10
α-3,4	4.26	6.3
β-3	16.50	
α-3	23.60	

[a] Die einzelnen Methylglucosid-Derivate sind durch jene Stellen bezeichnet, an denen im Molekül außer in Position 1 Methoxygruppen sitzen. [b] Temperaturangaben beziehen sich auf die Säulentemperatur. [c] GC, Gaschromatographie unter Standardbedingungen [d] GC–MS, Gaschromatographie mit der Anordnung Gaschromatograph–Verbindungskapillare–Massenspektrometer [e] EGS, Äthylenglycolsuccinat-Polyester als flüssige Phase. [f] BDS, Butandiolsuccinat-Polyester als flüssige Phase.

Eine Auftrennung von Gemischen partiell methylierter Methylglucopyranoside in die Gruppen der Tri-, der Di- und der Mono-methyläther gelingt stets. Überlappungen der einzelnen gaschromatographischen Peaks treten nur innerhalb dieser Gruppen auf (s.u.). Die Massenspektren der einzelnen isomeren Tri-, Di- oder Mono-methyläther sind aber in so charakteristischer Weise voneinander verschieden, daß selbst bei ungenügender Auftrennung des Gemisches eine eindeutige massenspektrometrische Identifizierung der Komponenten möglich ist. So haben das Methyl-2,3,6-tri-*O*-methylglucopyranosid und das Methyl-2,4,6-tri-*O*-methylglucopyranosid praktisch gleiche Retentionszeiten, ihre Massenspektren unterscheiden sich jedoch besonders stark voneinander. Im Spektrum des ersteren sind auffällig starke Peaks bei den MZ 161, 88 und 75 zu finden, während für das Spektrum des letzteren starke Peaks bei den MZ 102 und 101 charakteristisch sind.

Da die Massenspektren der untersuchten Substanzen verschiedene Hauptpeaks aufweisen, erhält man nur dann vergleichbare Werte, wenn man die Peakintensitäten als Prozent vom Gesamt-Ionenstrom beschreibt.

Die nach der kombinierten gaschromatographisch-massenspektrometrischen

Methode gemessenen Spektren enthielten größere Intensitätsanteile, die nicht aus den untersuchten Substanzen stammten („Untergrund“). Um die Peakintensitäten auf einen möglichst untergrundarmen „Gesamt-Ionenstrom“ zu beziehen, wurden zu seiner Ermittlung nur die relativen Intensitäten folgender Peaks addiert (MZ = Massenzahl): MZ 40–100, sofern ≥5% des Hauptpeaks; MZ 100–160, sofern ≥2.5% des Hauptpeaks; MZ 160, sofern ≥1% des Hauptpeaks. Stärkere Abweichungen wurden im unteren MZ-Bereich (40–60) beobachtet. Da Peaks dieser MZ wenig charakteristisch sind, wurde auf sie bei der vergleichenden Betrachtung verzichtet. Die Meßergebnisse sind in Abb. 1–11 und in Tab. II wiedergegeben.

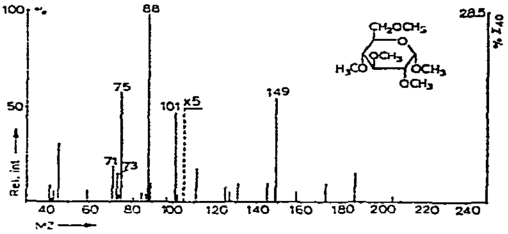


Abb. 1

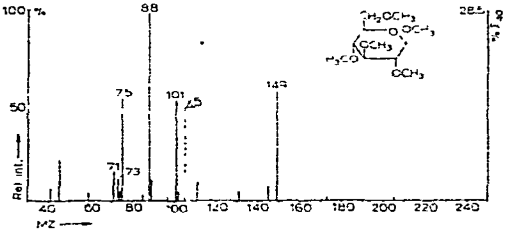


Abb. 2

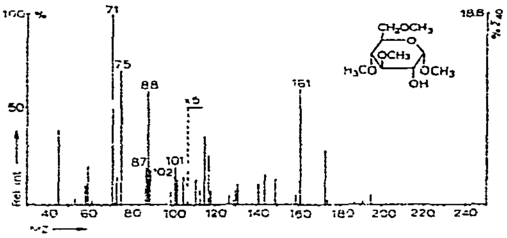


Abb. 3

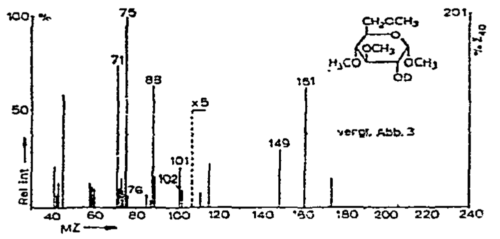


Abb. 3a

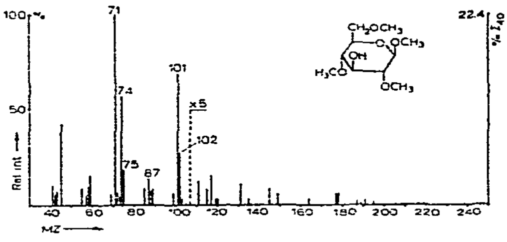


Abb. 4

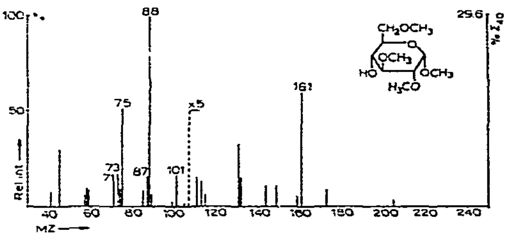


Abb. 5

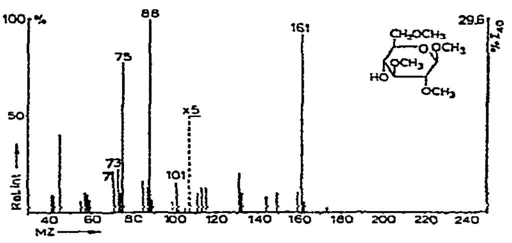


Abb. 6

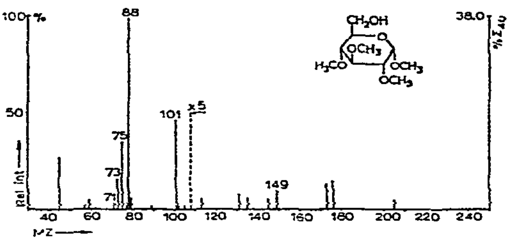


Abb. 7

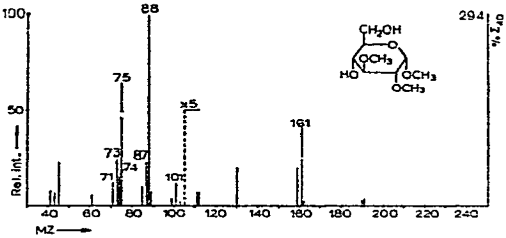


Abb. 8

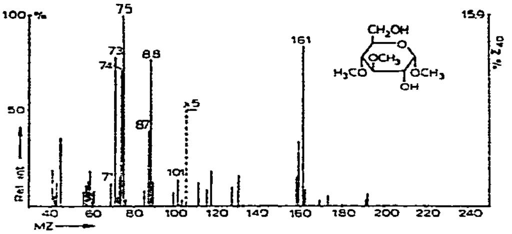


Abb. 9

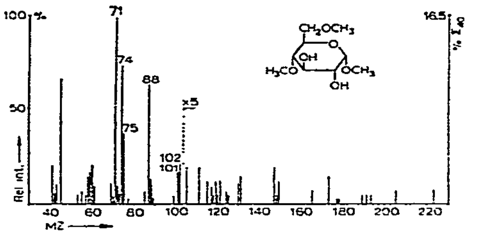


Abb. 10

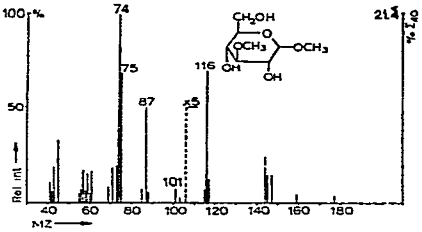


Abb. 11

TABELLE II

GESCHÄTZTE UND GEMESSENE HÖHEN DER CHARAKTERISTISCHEN PEAKS IM MASSENSPEKTRUM EINIGER PARTIELL METHYLIERTER METHYLGLUCOSIDE

Verb.[a]		2,3,4				2,3,6		
MZ	*Ionen*[b]	*Peakhöhe in % Σ_{40}*			*Ionen*[b]	*Peakhöhe in % Σ_{40}*		
		Ber.[c]	*Kor.*[d]	*Gem.*[e]		*Ber.*[c]	*Kor.*[d]	*Gem.*[e]
102		0.6		0.7		0.1		0.3
101	$s_2s_3s_4s_5s_6$	11.5	$\leqslant +6$	17.8	$s_2s_4s_5$	2.3	$\leqslant +6$	5.0
88	$r\,t_1t_2t_3$	29.6	$\leqslant +11$	38.0	$r\,t_1t_2$	25.0	$\leqslant +11$	29.6
87	s_1	2.0	$\geqslant -6$	1.15	$s_1s_3s_6$	11.6	$\geqslant -11$	4.7
75	v_1v_2	16.0		13.3	v_1	13.8	$\leqslant +6$	15.7
74		0.1			t_3	4.6	$\geqslant -6$	2.5
73	$w_1w_2w_3w_4$	4.4		6.1	$w_1w_2w_3$	4.0		5.0
161					j	0	$\leqslant +6$	4.0

Verb.[a]		2,4,6				3,4,6		
MZ	*Ionen*[b]	*Peakhöhe in % Σ_{40}*			*Ionen*[b]	*Peakhöhe in % Σ_{40}*		
		Ber.[c]	*Kor.*[d]	*Gem.*[e]		*Ber.*[c]	*Kor.*[d]	*Gem.*[e]
102	r	1.7	$\leqslant +6$	6.0	r	1.2	$\leqslant +6$	2.2
101	$s_1s_3s_4$	11.1	$\leqslant +6$	15.4	$s_1s_2s_6$	2.7	$\leqslant +6$	3.8
88	t_1	2.9	$\leqslant +1$	1.5	t_2	4.6	$\leqslant +11$	11.2
87	$s_2s_5s_6$	2.4	$\geqslant -1$	3.0	$s_3s_4s_5$	10.9	$\geqslant -11$	3.6
75	v_2	4.4	$\geqslant +1$	4.0	v_1	14.8	$\leqslant +1$	13.1
74	t_2t_3	25.7	$\geqslant -16$	12.8	t_1t_2	24.0	$\geqslant -16$	9.3
73	$w_1w_2w_4$	2.3		0.9	$w_1w_3w_4$	2.6		2.6
161					j	0	$\leqslant +6$	3.6

TABELLE II Fortsetzung

Verb.[a] 2,3 3,4

MZ	*Ionen*[b]	*Peakhöhe in % Σ_{40}*			*Ionen*[b]	*Peakhöhe in % Σ_{40}*		
		Ber.[c]	*Kor.*[d]	*Gem.*[e]		*Ber.*[c]	*Kor.*[d]	*Gem.*[e]
102		0.1						0.1
101	$s_2s_4s_5$	2.4	$\leqslant +6$	3.6	s_2s_6	0.7	$\leqslant +6$	2.3
88	t_1t_2	24.5	$\leqslant +11$	29.5	$r\,t_3$	6.0	$\leqslant +6$	12.4
87	s_3s_6	9.3	$\geqslant -6$	6.8	$s_1s_3s_4s_5$	12.9	$\geqslant -11$	6.2
75	v_1	13.3	$\leqslant +11$	19.2	v_1	14.2	$\leqslant +6$	15.9
74	$r\,t_3$	5.8	$\geqslant -6$	4.5	t_1t_2	24.1	$\geqslant -11$	11.4
73	$s_1w_1w_2w_3$	7.1	$\geqslant -1$	7.1	$w_1w_3w_4$	2.6		2.6
161	j	0	$\leqslant +6$	2.4	j	0	$\leqslant +6$	2.7

Verb.[a] 4,6 3

MZ	*Ionen*[b]	*Peakhöhe in % Σ_{40}*			*Ionen*[b]	*Peakhöhe in % Σ_{40}*		
		Ber.[c]	*Kor.*[d]	*Gem.*[e]		*Ber.*[c]	*Kor.*[d]	*Gem.*[e]
102	r	1.1	$\leqslant +6$	3.6				
101	s_1	2.05	$\leqslant +6$	2.9	s_2	0.3	$\leqslant +6$	1.7
88				2.2				1.3
87	$s_2s_3s_4s_6$	9.75	$\geqslant -1$	10.4	$s_4s_5s_6$	2.4	$\leqslant +6$	10.65
75		1.8		6.0	v_1	13.9	$\leqslant +6$	14.6
74	t_1t_3	7.5	$\leqslant +6$	12.0	$r\,t_1t_2t_3$	30.0	$\geqslant -6$	21.4
73	$s_4s_5w_1$	2.2		0.9	$s_1s_3w_1w_3$	13.1	$\geqslant -11$	4.3
147	j	0	$\leqslant +1$	0.7	j	0	$\leqslant +1$	0.7

---

[a] Die Verbindungen sind hier wie in Tabelle I bezeichnet. [b] Siehe Tabelle III. [c] Peakhöhen in % Σ_{40} berechnet unter der Annahme, daß die Intensität der einzelnen Ionen durch die Substituenten nicht verändert wird. [d] Korrektur unter Berücksichtigung der in Tabelle IV zusammengefaßten Effekte (% Σ_{40}). [e] Meßwerte (% Σ_{40}).

DISKUSSION

Wegen der deutlichen Unterschiede in den Massenspektren von Zuckern mit verschiedenem Methylierungsgrad ist durch Spektrenvergleich die sichere Identifizierung unbekannter Zuckerderivate möglich. Um jedoch von einem Spektrenkatalog der partiell methylierten Derivate der verschiedenen Zucker unabhängig zu sein, ist es vorteilhaft, wenn die Massenspektren der partiell methylierten Methylglucoside in übersichtlicher und eindeutiger Weise von denen der gut untersuchten permethylierten Derivate abgeleitet werden können.

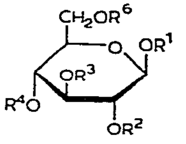
Durch Markierung der einzelnen Gruppen eines permethylierten Zuckers mit Deuterium ist bekannt, aus welchen Fragmentierungs-Reaktionen die verschiedenen Fragment-Ionen entstehen und in welcher Weise sich die einzelnen Gruppen des Zuckermoleküls auf die verschiedenen Ionen verteilen[10,13–15]. Da im

Vergleich zu den permethylierten Derivaten bei partiell methylierten Zuckern bestimmte OCH_3-Gruppen gegen OH-Gruppen ausgetauscht sind, werden auch die Massen bestimmter Ionen um 14 Masseneinheiten kleiner. Entsprechend verschiebt sich die MZ des zugehörigen Peaks. So werden im Massenspektrum permethylierter Methylhexopyranoside die Peaks der MZ 101 und 88 zu etwa 83% aus Ionen gebildet, die den Substituenten des C-Atoms 2 enthalten. Man sollte daher im Massenspektrum des Methyl-3,4,6-tri-*O*-methylglucopyranosids eine Verschiebung der entsprechenden Intensitätsanteile von den MZ 101 und 88 zu den MZ 87 und 74 erwarten. Wie Abb. 3 zeigt, tritt tatsächlich die erwartete Peakverschiebung ein, keineswegs aber im berechneten Ausmaß. Die unbefriedigende Übereinstimmung zwischen gemessenen und berechneten Massenspektren ist für zwei partiell methylierte Methylhexopyranoside bereits von Kochetkov *et al.* beobachtet worden[15]. Dabei benutzten die Autoren allerdings ein unrichtiges Fragmentierungsschema. Obwohl dieses Schema berichtigt wurde, konnten die Beobachtungen aber an dem vorliegenden umfangreichen Material bestätigt werden (s. Tabelle II). Die Substituenten müssen daher außer die Ionenmassen auch den Fragmentierungsprozeß durch weitere Effekte entscheidend beeinflussen. Damit die in dieser Arbeit für partiell methylierte Methylglucopyranoside erhaltenen Ergebnisse auf andere Zucker übertragen werden können, müssen Natur und Ausmaß dieser Substituenteneffekte bekannt sein.

Ausgehend von den Ergebnissen der massenspektrometrischen Untersuchung permethylierter Methyl-pentopyranoside und -hexopyranoside[13–15] wurden daher erneut die Massenspektren der partiell methylierten Derivate eingehend berechnet und durch Vergleich mit den experimentellen Werten die Substituenteneffekte bestimmt. Das soll anhand jener Ionen erläutert werden, die bei den permethylierten Derivaten die MZ 102, 101, 88, 75 und 71 haben. Mit Hilfe dieser intensiven Ionen ist eine eindeutige Identifizierung von Massenspektren partiell methylierter Zucker möglich.

Tab. III enthält eine Liste der genannten Ionen mit ihren MZ und den Anteil in %, welchen sie am Gesamt-Ionenstrom des permethylierten Methylglucosids haben. Tab. II enthält u.a. jene Peakintensitäten, welche für die partiell methylierten Methylglucoside unter der Maßgabe errechnet wurden, daß die elektronenstoßinduzierte Fragmentierung dieser Derivate in gleicher Weise abläuft, und daß sich alle Ionen mit der gleichen Intensität am Gesamt-Ionenstrom beteiligen, wie bei der permethylierten Verbindung. Tab. II enthält weiterhin die entsprechenden Meßergebnisse.

Wichtig für die folgenden Betrachtungen ist, daß die besonders intensiven Fragment-Ionen durch wenige „Hauptspaltungsreaktionen“ (und Umlagerungen) gebildet werden. Diese „Reaktionen“ und die durch sie gebildeten „Hauptfragment-Ionen“ sind in Abb. 12 dargestellt. Das Grundmolekül wird hier beschrieben als:



Anomere werden nicht unterschieden und jedes R kann ein H-Atom oder eine CH_3-Gruppe sein.

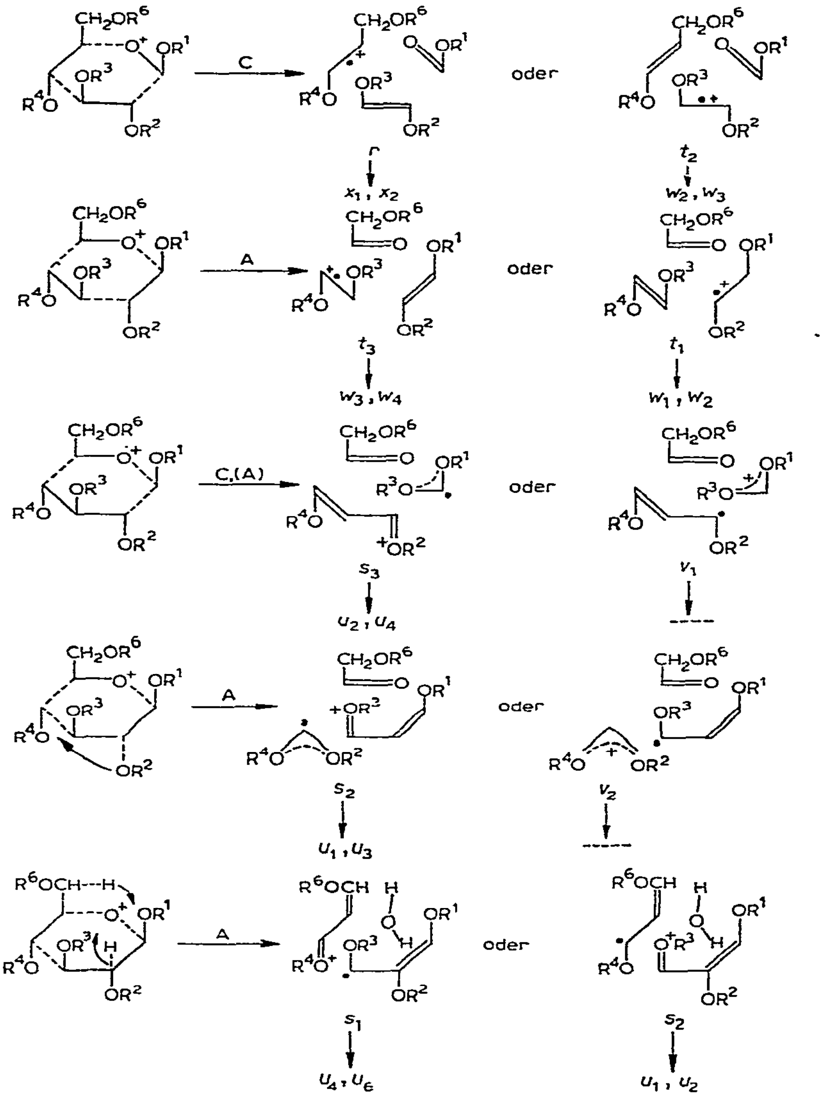


Abb. 12

Verglichen mit dem Spektrum des permethylierten Derivats können folgende Effekte zu Intensitätsverschiebungen einzelner Ionentypen führen:

(*1*) Beim Zerfall des Molekülions in mehrere Bruchstückgruppen konkurrieren die einzelnen Fragmente miteinander um die elektrische Ladung. Da Methoxygruppen positive Ladungen stärker induktiv stabilisieren als Hydroxygruppen, wird die „*Ladungsverteilung*" auf die einzelnen Fragmente durch Substitution von OCH_3

gegen OH geändert. Die Intensität eines Ions nimmt dann zugunsten der Intensität seines Konkurrenten ab.

(*2*) Ein weiterer Einfluß des Substituenten auf die Ionenintensität ist die Benachteiligung primärer Spaltungsreaktionen im Molekülion und damit ganzer Bruchstückgruppen zugunsten anderer. So wird die Primärspaltung *C*, bei der zunächst die positive Ladung am C-2 stabilisiert werden muß (Ion c_1), zurückgedrängt, wenn dort statt OCH_3 die schwächer induktiv-stabilisierende OH Gruppe sitzt.

Da bei den betrachteten Reaktionen im ersten Zerfallsschritt immer eine der

TABELLE III

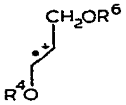
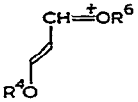
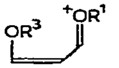
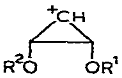
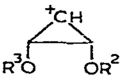
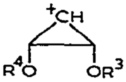
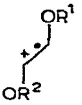
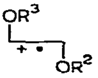
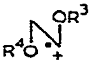
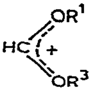
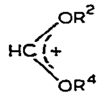
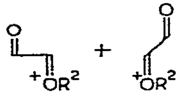
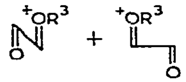
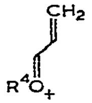
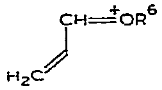
Ionenbezeichnung	*Ionenbau*	*Zerfreihe*	*MZ*	*Peak-Intensität*[a] *in* % Σ_{40}
r		*D*	102, 88, 74	1.1
s_1		*A*	101, 87, 73	2.05
s_2		*A*	101, 87	0.25
s_3		*C*	101, 87, 73	8.85
s_4			101, 87, 73	0.25
s_5		*A, C*	101, 87, 73	1.75
s_6			101, 87, 73	0.40
t_1		*A*	88, 74	2.85
t_2		*C*	88. 74, 60	21.2
t_3		*A*	88, 74, 60	4.5
v_1		*C*	75, (61)	11.6

TABELLE III Fortsetzung

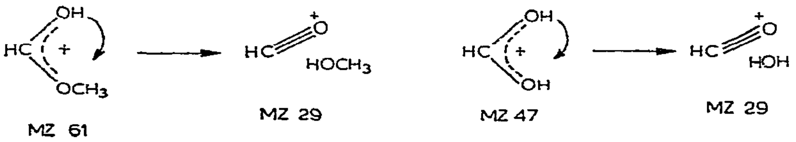
Ionenbezeichnung	*Ionenbau*	*Zerfreihe*	*MZ*	*Peak-Itensität*[a] *in* % Σ_{40}
v_2		*A*	75, (61), (47)	2.15
	Weitere Ionen v, die R^2, R^3 und R^6 enthalten können		75	2.75
w_1		*A*	73	0.20
w_2		*A*, *C*	73, 59	1.75
w_3		*A*, *C*	73, 59	2.0
w_4		*A*	73, 59	0.40
x_1		*D*	71, 57	2.30
x_2		*D*	71, 57	3.10

---

[a] Wenn das betreffende Ion aus Methyl-2,3,4,6-tetra-*O*-methyl-α-D-glucopyranosid entstand.

Bindungen zum C-1 gespalten wird, ist wegen der geringen Reichweite induktiver Effekte nur von R^2 ein Einfluß auf die Primärspaltungsreaktionen zu erwarten. Ähnlich ist der Einfluß der Substitution auf die Sekundärspaltungsreaktionen. Dieser Effekt wird aber durch den unter (*1*) angeführten völlig überdeckt.

(*3*) Die betrachteten Ionen sind Zwischenprodukte in längeren Zerfallsreihen. Ihre Intensität kann sich daher vermindern, wenn durch Substitution von OCH_3- gegen OH-Gruppen die Bereitschaft zur Bildung der Folge-Ionen (Ionenreaktivität) wesentlich vergrößert wird. So können Ionen vom Typ *v*, wenn sie eine oder zwei Hydroxygruppen enthalten, durch Wanderung der beweglichen H-Atome aus den Hydroxygruppen unter Eliminierung von CH_3OH bzw. H_2O weiter zerfallen:



(*4*) Umlagerungsreaktionen durch Wanderung der beweglichen H-Atome aus den Hydroxygruppen können bereits in den Molekülionen partiell methylierter Methylpyranoside auftreten. Da beim permethylierten Derivat entsprechende Reaktionen nicht möglich sind, können dadurch ebenfalls die Intensitätsverhältnisse verändert werden. Schließlich entstehen Ionen des Typs *s* und *r* in den Massenspektren der permethylierten Verbindung durch Wanderung von Methoxygruppen. Da Hydroxygruppen eine andere Wanderungstendenz haben können als Methoxygruppen, sind auch auf diese Weise Intensitätsverschiebungen für jene Ionen möglich.

Die Stärke der obengenannten Einflüsse läßt sich nur schwierig exakt berechnen. So können Intensitätsveränderungen einzelner Ionentypen schwer erkannt werden, weil die einzelnen Peaks meist von vielen Ionen verschiedenen Ursprungs, aber gleicher MZ, gebildet werden. Auch sind an einer Intensitätsverschiebung oft mehrere einander überlagernde Effekte beteiligt. Da diese Überlagerungen nicht einfach durch Faktoren oder additiv zu erfassen sind, entsteht ein schwer durchschaubares Bild vom Ineinandergreifen aller Einflüsse. Dennoch sind Abschätzungen möglich, weil alle übrigen Effekte gering sind gegenüber denen der Ladungsverteilung innerhalb einer Bruchstückgruppe und der Beeinflussung der Primärspaltung[16].

Um zu sicheren Aussagen über die Wirkung der Haupt-Effekte zu gelangen, muß ihre Verfälschung durch die übrigen Effekte ausgeschlossen werden. Dazu müssen alle Einflüsse betrachtet und ihre Größenordnungen bestimmt werden:

(*1*) *Masseverschiebung*. Einige typische Erscheinungen lassen sich schon aus der Masseverschiebung besonders intensiver Ionen vorhersagen. So kann der Peak mit der MZ 102 nur vom Ion *r* mit $R^4 = R^6 = CH_3$ gebildet werden. Entsprechendes gilt für den Peak der MZ 161. Wie später gezeigt wird, können Ionen dieser MZ nur entstehen, wenn eine Hydroxygruppe am C-2 oder C-4 vorhanden ist. Außerdem wird ein Peak mit der MZ 75 nur beobachtet, wenn die Ionen v_1 und v_2 diese Masse haben (R^3 oder R^2 und $R^4 = CH_3$). Alle diese Vorhersagen werden durch die Messungen ausnahmslos bestätigt.

(*2*) *Ionenreaktivität. Ionen des Typs* v. — Wie bereits erwähnt, zerfallen Ionen des Typs *v* bei einer Substitution von OCH_3 durch OH schnell weiter. Dadurch werden die Peaks der MZ 61 und 47 zusätzlich geschwächt. Bei der vergleichenden Betrachtung konnte jedoch auf diese Peaks verzichtet werden.

Ionen des Typs t. — Beim Methyl-4,6-di-*O*-methyl-α-D-glucopyranosid wird der Peak mit der MZ 74 allein von den Ionen t_1 und t_3 gebildet. Die Intensitätssumme dieser Ionen ist daher 12% Σ_{40}. Da wegen der OH-Gruppe in 2-Stellung die *A*-Spaltung gegenüber der *C*-Spaltung begünstigt wird, ist diese Summe etwas größer als bei der permethylierten Verbindung mit 7.4% Σ_{40}; t_1 und t_3 enthalten beide eine Hydroxygruppe (MZ 74). Der Meßwert zeigt daher, daß die Reaktivität der Ionen vom Typ *t* durch Substitution einer OCH_3- gegen eine OH-Gruppe nicht wesentlich geändert wird. Eine entsprechende Betrachtung der Derivate mit Methoxygruppen in 3,4,6- und in 4,6-Stellung ergibt für die Intensitätssumme der Ionen *r* und t_2 Werte von gleicher Größenordnung. Das bedeutet, da *r* beide Male 2 OCH_3-Gruppen (MZ 102), t_2 jedoch einmal eine (MZ 74) und einmal 2 OH-Gruppen (MZ 60) besitzt, daß die Reaktivität von Ionen des Typs *t* auch durch den Ersatz beider OCH_3- durch OH-Gruppen nicht merklich verändert wird.

Ionen des Typs s. — Beim Methyl-2,3-di-*O*-methyl-α-D-glucopyranosid liegt die Intensität des Ions s_3 mit der MZ 87 (1 OH-Gruppe, 1 OCH_3-Gruppe) in der erwarteten Größenordnung (6.5–5.5% Σ_{40}). Sie müßte wesentlich kleiner sein, wenn Ionen dieses Typs durch Substitution von OCH_3- durch OH-Gruppen reaktiver würden.

Entsprechendes gilt auch für die Derivate mit Methoxygruppen in 3,4- und 4,6-Stellung. Bei letzterer werden s_3 und s_2, beide mit einer OH-Gruppe und der MZ 87, in der vermuteten Größenordnung gebildet. Ihre Intensitätssumme ist 10% Σ_{40} gegenüber 9.15% Σ_{40} bei der permethylierten Verbindung.

Ionen des Typs r. — Das Ion *r* bildet sehr bereitwillig die Folge-Ionen x_1 und x_2. Diese Tendenz verstärkt sich noch, wenn in ihm OCH_3- Gruppen durch OH- substituiert sind. *r*-Peaks sind jedoch nur bei der MZ 102 charakteristisch, weil sie bei den MZ 88 und 74 von *t*-Peaks überdeckt werden. Auf die Betrachtung dieser Anteile wird daher im folgenden verzichtet.

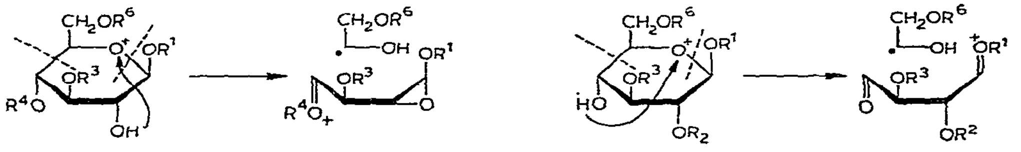
(*3*) *Spezifische Umlagerungs-Reaktionen.* Die Ionen s_2 und s_3 entstehen durch OR-Gruppen-Wanderung. Beim Methyl-4,6-di-*O*-methyl-α-D-glucopyranosid ist die wandernde Gruppe beide Male eine Hydroxygruppe. Die Intensität von s_2 und s_3 ist zusammen 10% Σ_{40} gegenüber 9.15% Σ_{40} bei der permethylierten Verbindung. Daraus muß man schließen, daß die OH-Gruppe mit ähnlicher Bereitwilligkeit wandert wie die OCH_3-Gruppe, die Ladung jedoch bei dem höher methylierten Fragment verbleibt. Einen weiteren Hinweis liefert das Spektrum des Methyl-2,4,6-tri-*O*-methylglucopyranosids. Dort wird der Peak der MZ 101 von s_3 gebildet. Dieses Ion entsteht durch OH-Gruppen-Wanderung. Seine Intensität ist mit 13.5% Σ_{40} so hoch, wie es aus der Ladungsverteilung bei gleichbleibender Umlagerungstendenz zu erwarten ist.

Eine neue Umlagerung wurde bei partiell methylierten Methylglucosiden mit einer Hydroxygruppe am C-2 oder C-4 gefunden. Die Spektren dieser Verbindungen zeigen bei der MZ 161 einen großen Peak. Ein Ion dieser MZ ist nach dem Abbauschema[10,16] nicht möglich, ist aber characteristisch für die Massenspektren permethylierter Methylglucofuranoside. Eine Verunreinigung der Substanzen durch Furanoside ist jedoch durch die Synthese und durch kombinierte gaschromatographisch-massenspektrometrische Untersuchungen ausgeschlossen.

Folgende Hinweise für die Entstehung des Ions MZ 161 konnten gesammelt werden: (*a*) Wegen seiner hohen MZ muß das Ion MZ 161 mindestens 4 Gerüst-Atome enthalten. (*b*) Voraussetzung für die Bildung des Ions ist eine OH-Gruppe am C-2 und OCH_3-Gruppen an C-3 und C-4 oder eine OH-Gruppe an C-4 und OCH_3-Gruppen am C-2 und C-3. (*c*) Der Substituent R^6 hat keinen Einfluß. C-6 mit seinem Substituenten ist daher wahrscheinlich nicht im Ion enthalten. (*d*) Ein Austausch des H-Atoms aus der freien OH-Gruppe in Methyl-3,4,6-tri-*O*-methyl-α-D-glucopyranosid gegen Deuterium verändert weder die MZ noch die Intensität des

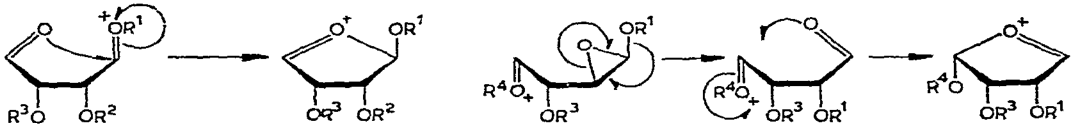
Peaks der MZ 161. Das Ion der MZ 161 wird daher offensichtlich durch eine Wanderung des H-Atoms aus der OH-Gruppe an das neutrale Fragment gebildet.

Ausgehend von diesen Befunden wird für die Bildung der Ionen *j* der MZ 161 eine Wanderung des H-Atoms aus der Hydroxygruppe am C-2 oder C-4 in einem 5-gliedrigen Übergangszustand zum Ringsauerstoff vorgeschlagen. Dieser Wanderung schließt sich die Abspaltung des neutralen Radikals R^6 OCH_2-$\dot{C}$H-OH an:



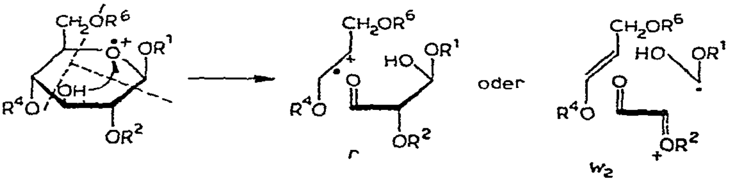
j_1 (MZ$_{\text{max.}}$: 161) j_2 (MZ$_{\text{max}}$: 161)

Die Bildung der Ionen *j* ist mit einer *A*-Spaltung verknüpft. Da die Primärspaltung *A* durch eine OH-Gruppe am C-2 begünstigt werden sollte, ist in diesem Falle eine stärkere Bildung des Ions j_1 zu erwarten. Die Meßwerte stimmen damit überein. Aus der Struktur der Ionen *j* kann keine günstige Stabilisierung der positiven Ladungen abgeleitet werden, welche die große Intensität des entsprechenden Peaks erklären würde. Es ist jedoch möglich, daß eine Isomerisierung zu cyclischen Ionen erfolgt:



j_2 j_3 (MZ$_{\text{max}}$: 161) j_1 j_4 (MZ$_{\text{max}}$: 161)

Die Ionen j_3 und j_4 entsprechen dem bei den permethylierten Furanosiden auftretenden Ion der MZ 161. Beim Ion j_1 muß dazu eine Umlagerung mit Öffnung des Expoxyringes erfolgen. Solche Umlagerungen sind an Cyclopropanen und Epoxiden beobachtet worden[17,18]. Eine H-Wanderung aus der OH-Gruppe in Position 3 führt nicht zu Ionen vom Typ *j*. Offenbar deshalb nicht, weil mit ihr eine Reaktion konkurriert, bei der die H-Wanderung mit einer *C*- oder *D*-Spaltung gekoppelt ist:



Für diesen Zerfallsweg lassen sich in der Tat Anhaltspunkte finden. Der Peak MZ 73, normalerweise 1/5 × so groß wie der Peak MZ 88, ist beim Methyl-2,4,6-tri-*O*-methylglucopyranosid fast genauso groß wie dieser. Auch die Intensität des dem Ion *r* entsprechenden Peaks der MZ 102 ist mit 6% Σ_{40} statt der sonst zu erwartenden 2% Σ_{40} ungewöhnlich groß.

(*4*) *Induktive Effekte.* — Vergleicht man die Massenspektren des Methyl-3,4,6-tri-*O*-methylglucopyranosids mit dem der permethylierten Verbindung, so erkennt man die erwartete Bevorzugung der *A*-Spaltung an der größeren Intensitätssumme der durch sie entstehenden Bruchstücke t_1+t_3 (mindestens 11.2% Σ_{40} gegenüber 7.4% Σ_{40}). Andererseits ist die *C*-Spaltung — wie ebenfalls zu erwarten war — behindert: So ist die Intensitätssumme der Bruchstückgruppe v_1+s_3 höchstens 16% Σ_{40} gegenüber 20.5% Σ_{40} bei der permethylierten Verbindung. Im gleichen Sinne ist die Intensitätssumme der Bruchstückgruppe $r+t_2$ verändert (maximal 11.8% Σ_{40} gegenüber 22.25% Σ_{40} bei der permethylierten Verbindung[16]. Diese Beobachtung läßt sich gut verstehen, wenn man annimmt, daß r und t_2 über den — jetzt behinderten — Reaktionsweg *C* gebildet werden. Daß r mit 2.25% Σ_{40} trotzdem stärker als bei der permethylierten Verbindung (1.1% Σ_{40}) auftritt, wird zwanglos durch die Ladungsverteilung innerhalb der Bruchstückgruppe erklärt. Der Intensitätsgewinn von r geht also zu Lasten der Intensität der Ionen t_2.

Der Einfluß der OH-Gruppe am C-2 auf die Primärspaltungen im Methyl-4,6-di-*O*-methylglucopyranosid-Molekülion muß der gleiche sein wie beim Methyl-3,4,6-tri-*O*-methylglucopyranosid. Das Ladungsgewicht innerhalb der Bruchstückgruppe $r+t_2$ muß jedoch stärker zu r hin verschoben sein, denn während r unverändert bleibt, besitzt t_2 nur noch Hydroxygruppen und kann daher positive elektrische Ladungen besonders schlecht induktiv stabilisieren. Tatsächlich ist der r-Peak mit 3.05% Σ_{40} (gegenüber 2.25% Σ_{40}) weiter erhöht. Die Intensitätssummen der Bruchstückgruppen t_1+t_3 und $r+t_2$ haben sich mit 12.0% Σ_{40} bzw. 7.05% Σ_{40} nicht wesentlich verändert.

In entsprechender Weise lassen sich die Derivate mit Methoxygruppe in 2,3- und in 3,4-Stellung miteinander vergleichen. Bei beiden Verbindungen haben v_1 und s_3 die gleiche Struktur, so daß in der Ionenreaktivität und der Ladungsverteilung keine wesentlichen Unterschiede auftreten können. Folglich sollte beim Methyl-3,4-di-*O*-methylglucopyranosid durch Benachteiligung der Primärspaltung *C* der Gesamt-Ionenstrom-Anteil sowohl von v_1 als auch von s_3 gegenüber der anderen Verbindung herabgesetzt sein. Das ist mit 15% Σ_{40} gegenüber 19.2 Σ_{40} für v_1 und mit 3.5–5% Σ_{40} gegenüber 5.5–6.5% Σ_{40} für s_3 der Fall.

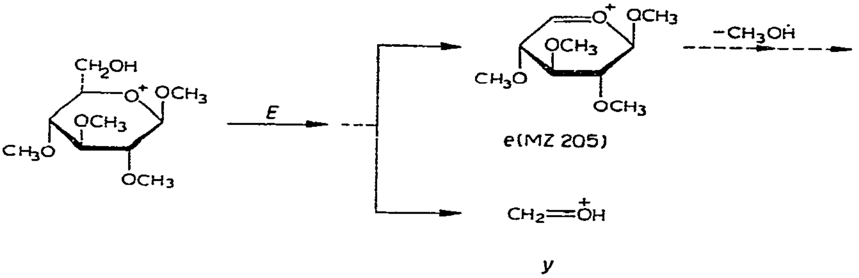
In entsprechender Weise läßt sich die Wirkung der Ladungsverteilung durch Vergleich der Massenspektren vom Methyl-2,3,6-tri-*O*-methylglucopyranosid, vom Methyl-2,4,6-tri-*O*-methylglucopyranosid und vom permethylierten Derivat studieren. Für diese Verbindungen wurden folgende Intensitätswerte in % Σ_{40} ermittelt:

Ion	2, 3, 6	2, 3, 4, 6	2, 4, 6
v_1	15.7 (MZ 75)	11.6 (MZ 75)	– (MZ 61)
s_3	1–3 (MZ 87)	8.9 (MZ 101)	11–13.5 (MZ 101)

Bei keiner der Verbindungen wird der Anteil einer Primärspaltung gegenüber den anderen deutlich verändert. Die Substitution von OCH_3 gegen OH hat auf die Reaktivität von s_3 — wie bereits gezeigt — keinen Einfluß. Somit ist beim Methyl-

2,3,6-tri-*O*-methylglucopyranosid das Absinken der s_3-Intensität gegenüber der permethylierten Verbindung von 8.9 auf 1–3% Σ_{40} zugunsten seines Konkurrenz-Ions v_1 (von 11.6 auf 15.7% Σ_{40} eine Folge der geringeren Ladungsstabilisierung durch die Hydroxygruppe. Ebenso wird beim Methyl-2,4,6-tri-*O*-methylglucopyranosid gegenüber der permethylierten Verbindung ein weiterer Intensitätsanstieg von s_3 beobachtet, weil sich hier die Fähigkeit des Konkurrenz-Ions v_1 zur Ladungsstabilisierung durch die Substitution verringert hat.

Der Einfluß der OH-Gruppe auf die Stabilisierung positiver elektrischer Ladungen wird auch bei anderen als den in Abb. 11 angegebenen Reaktionen beobachtet. So sollte bei der *E*-Spaltung des Methyl-2,3,4-tri-*O*-methylglucopyranosids die Ladung weniger beim Fragment *y* als bei *e* und seinen Folgeprodukten verbleiben.



Tatsächlich sind die entsprechenden Peaks bei 205 und 173 ME im Massenspektrum dieser Verbindung wesentlich stärker als beim permethylierten Derivat[16].

Zusammenfassend lassen sich für den Einfluß, den der Austausch von OCH_3-Gruppen gegen OH-Gruppen auf die Ladungsverteilung und auf die Primärspaltungsreaktionen bei der Fragmentierung des Molekülions hat, folgende Richtwerte angeben: (*a*) Ist in einer Bruchstückgruppe bei einem Ion eine OCH_3-Gruppe durch OH ersetzt, das Konkurrenz-Ion hingegen unsubstituiert, so ist die Intensität des substituierten Ions zugunsten des anderen um *ca.* 50% vermindert. (*b*) Ist in beiden Konkurrenz-Ionen jeweils eine OCH_3-Gruppe durch eine OH-Gruppe substituiert so ändert sich die Ladungsverteilung nicht. (*c*) Enthält ein Fragment 2 OH-Gruppen, sein Konkurrenz-Ion hingegen keine, so wird der Anteil dieses Ions am Gesamt-Ionenstrom um 75% zugunsten des anderen Fragments herabgesetzt. (*d*) Eine OH-Gruppe am C-2 setzt die Ionenbildung durch Primärspaltung *C* um 25% zugunsten der *A*-Spaltung herab.

Außer der Masseverschiebung beeinflussen also nur zwei der Substitutionseffekte die Massenspektren partiell methylierter Methylglucopyranoside maßgeblich: (*1*) Umlagerungsreaktionen, die zu neuen, intensiven Ionen führen (Ionen *j*). (*2*) Induktive Stabilisierung positiver Ladungen durch den Substituenten. Dabei wird hauptsächlich die Ladungsverteilung innerhalb einer Bruchstückgruppe und, bei Änderung des Substituenten am C-2, in geringerem Ausmaß das statistische Gewicht der Primärspaltungsreaktionen verändert.

Alle beobachteten Effekte treten schon in der Gruppe der Tri-*O*-methylderivate auf. Zusätzliche Effekte durch mehrere Hydroxygruppen sind nicht zu

TABELLE IV

SUBSTITUTIONSEFFEKTE BEI DEN HAUPTSPALTUNGSREAKTIONEN

		Verb.[a]															
Ion[b, c]		*2,3,4,6*	*2,3,4*	*2,3,6*	*2,4,6*	*3,4,6*	*2,3*	*2,4*	*3,4*	*2,6*	*3,6*	*4,6*	*2*	*3*	*4*	*6*	—
r	*MZ*	102	88	88	102	102	74	88	88	88	88	102	74	74	88	88	74
	LV	.	—	—	+	+	—	.	.	.	.	++	—	—	+	+	.
	P	.	.	.	.	—	.	.	—	.	—	—	.	—	—	—	—
t_2	*MZ*	88	88	88	74	74	88	74	74	74	74	60	74	74	60	60	60
	LV	.	+	+	—	—	++	.	.	.	.	—	+	+	—	—	.
	P	.	.	.	.	—	.	.	—	.	—	—	.	—	—	—	—
t_1	*MZ*	88	88	88	88	74	88	88	74	88	74	74	88	74	74	74	74
	LV	.	.	+	+	—	+	+	—	++	.	.	++	.	.	+	+
	P	.	.	.	.	+	.	.	+	.	+	+	.	+	+	+	+
t_3	*MZ*	88	88	74	74	88	74	74	88	60	74	74	60	74	76	60	60
	LV	.	.	—	—	+	—	—	+	——	.	.	—	.	.	—	—
	P	.	.	.	.	+	.	.	+	.	+	+	.	+	+	+	+
s_3	*MZ*	101	101	87	101	87	87	101	87	87	73	87	87	73	87	73	73
	LV	.	.	—	+	—	—	+	—	.	——	.	.	——	.	—	—
	P	.	.	.	.	—	.	.	—	.	—	—	.	—	—	—	—
v_1	*MZ*	75	75	75	.	75	75	.	75	.	75	.	.	75	.	.	.
	LV	.	.	+	—	+	+	—	+	.	++	.	.	++	.	+	+
	P	.	.	.	.	—	.	.	—	.	—	—	.	—	—	—	—
s_2	*MZ*	101	101	101	87	101	101	87	101	87	101	87	87	101	87	87	87
	LV	.	.(+)	+(+)	—(—)	+	+(+)	—	+(+)	.	++(+)	.(—)	.(+)	++(++)	.	+	+(+)
	P	.	.	.	.	+	.	.	+	.	+	+	.	+	+	+	+
v_2	*MZ*	75	75	.	75	.	.	75	.	.	.	.	.	.	.	.	.
	LV	.	.	—	+	—	—	+	—	.	—	.	.	—	.	—	—
	P	.	.	.	.	+	.	.	+	.	+	+	.	+	+	+	+
s_1	*MZ*	101	87	87	101	101	73	87	87	87	87	101	73	73	87	87	73
	LV	.	—	—	+	.	——	.	—	.	—	+	—	—	.	.	—
	P	.	.	.	.	+	.	.	+	.	+	+	.	+	+	+	+

---

[a] Die Verbindungen sind hier wie in Tabelle I bezeichnet.

[b] LV = Wirkung induktiver Effekte auf die Ladungsverteilung. In dieser Spalte bedeutet ein "+", daß das betreffende Ion eine Methoxygruppe mehr besitzt als sein Konkurrenz-Ion (Intensitätsvermehrung um 50%). Das Konkurrenz-Ion hat dann ein "–" (Intensitätsverminderung um 50%). Besitzt das betreffende Ion 2 Methoxygruppen mehr als sein Konkurrenz-Ion, so ist es durch ein "++" gekennzeichnet. Seine Intensität vermehrt sich damit um 75–100% gegenüber dem aus der permethylierten Verbindung stammenden Ion gleichen Typs zu Lasten seines Konkurrenz-Ions. Dieses ist dann durch ein "– –" gekennzeichnet. "+(–)" beim Ion s_2 berücksichtigt seine Entstehung als Konkurrenz-Ion zu s_1.

[c] P = Wirkung induktiver Effekte auf die Primärspaltung. In dieser Spalte bedeutet "+" eine Bevorzugung der Primärspaltungsreaktion, durch die das betreffende Ion entsteht. (Intensitätsvermehrung um 25% gegenüber der permethylierten Verbindung). Das gleiche gilt mit umgekehrten Vorzeichen für das Symbol "–".

erwarten, wohl aber stärkere Intensitätsverschiebungen, wenn ein Ion 2 Hydroxygruppen und sein Konkurrenz-Ion 2 Methoxygruppen trägt. Dies trifft für das Methyl-2,3-di-*O*-methylglucopyranosid und seine Isomeren mit Methoxygruppen in 3,4- und in 4,6-Stellung zu. Die beobachteten Intensitätsverschiebungen bei diesen Verbindungen überschritten das erwartete Ausmaß nicht.

Durch induktive Effekte werden Intensitätsänderungen höchstens bis zu 150% der jeweiligen Peakhöhe möglich. Die Peaks der zahlreichen, wenig intensiven Ionen werden deshalb im Spektrum nicht auffallend verändert, bei den sehr intensiven Hauptionen aber entstehen kräftige Intensitätsverschiebungen, deren Richtung man bestimmen und deren Ausmaß man durch Richtwerte abschätzen kann.

Die für alle Hauptionen der partiell methylierten Methylglucoside zu erwartenden Substitutionseffekte auf die Primärspaltung und auf die Ladungsverteilung beschreibt Tab. IV.

Mit Hilfe dieser Angaben kann man jetzt die Richtung und das ungefähre Ausmaß von Intensitätsverschiebungen für die von einzelnen Ionentypen gebildeten Peaks vorhersagen. Ein Vergleich mit den Meßwerten zeigt, daß so eine deutliche Verbesserung erreicht wurde.

Entsprechend den in Tab. II gemachten Angaben ist jedes Derivat durch mehrere charakteristische Merkmale von allen anderen unterscheidbar (s. auch Tab. V).

TABELLE V

CHARAKTERISTISCHE MERKMALE DER MASSENSPEKTREN PARTIELL METHYLIERTER METHYLGLUCOSIDE

	Verb.[a]													
MZ	*2,3,4*	*2,3,6*	*2,4,6*	*3,4,6*	*2,3*	*2,4*	*2,6*	*3,4*	*3,6*	*4,6*	*2*	*3*	*4*	*6*
161	.	0	.	0	0	.	.	0	.	.	.	.	.	.
102	.	.	×	0	.	.	.	.	.	0	.	.	.	.
101	× ×	0	× ×	0	0	× ×	.	0	.	0	.	.	.	.
88	× × ×	× × ×	×	×	× × ×	×	×	× ×	.	.	× ×	.	.	.
87	0	×	0	0	×	0	× ×	×	0	× ×	× ×	×	× ×	0
75	× ×	× ×	0	× ×	× ×	×	0	× ×	× ×	0	0	× ×	.	0
74	.	0	× ×	×	0	× ×	× × ×	× ×	× × ×	× ×	× × ×	× × ×	× ×	×

[a] Die Verbindungen sind hier wie in Tabelle I bezeichnet; . = vernachlössigbar kleiner Peak ($\leqslant 1\ \%\Sigma_{40}$); 0 = kleiner Peak (Größenordnung etwa 1–5 $\%\Sigma_{40}$); × = mittlerer Peak (Größenordnung etwa 5–10 $\%\Sigma_{40}$); × × = großer Peak (Größenordnung etwa 10–20 $\%\Sigma_{40}$); × × × = sehr großer Peak ($\geqslant 20\ \%\Sigma_{40}$).

Da neben den Masseverschiebungen nur die sterisch unabhängigen induktiven Effekte die Massenspektren der partiell methylierten Methylglucopyranoside wesentlich beeinflussen, kennzeichnen die besonderen Merkmale dieser Massenspektren auch den Substitutionstyp aller übrigen partiell methylierten Hexopyranoside. Diese Charakteristika gibt Tab. V wieder. Man kann sie sinngemäß für ähnliche Verbindungen, etwa die Pentopyranoside, ableiten.

LITERATUR

1 C. T. Bishop, *Advan. Carbohyd. Chem.*, 19 (1964) 95.
2 C. T. Bishop, *Methods Biochem. Anal.*, 10 (1962) 1.
3 H. W. Kircher, *Methods Carbohyd. Chem.*, 1 (1962) 13.
4 C. T. Bishop und F. P. Cooper, *Can. J. Chem.*, 38 (1960) 388.
5 G. O. Aspinall, *J. Chem. Soc.*, (1963) 1676.
6 A. M. Stephen, M. Kaplan, G. L. Taylor, und E. C. Leisegang, *Tetrahedron*, 22 (1966) 233.
7 G. A. Adams und C. T. Bishop, *Can. J. Chem.*, 38 (1960) 2380.
8 P. Kooiman und G. A. Adams, *Can. J. Chem.*, 39 (1961) 889.
9 E. J. Bourne und S. Peat, *Advan. Carbohyd. Chem.*, 5 (1950) 145.
10 K. Heyns, H. F. Grützmacher, H. Scharmann, und D. Müller, *Fortschr. Chem. Forsch.*, 5 (1966) 488.
11 C. Brunée, J. Jenckel, und K. Kronenberger, *Z. Anal. Chem.*, 197 (1963) 42.
12 H. O. Bouveng and B. Lindberg, *Methods Carbohyd. Chem.*, 5 (1965) 298.
13 K. Heyns und D. Müller, *Tetrahedron*, 21 (1965) 55.
14 N. K. Kochetkov und O. S. Chizov, *Advan. Carbohyd. Chem.*, 21 (1966) 39.
15 N. K. Kochetkov, N. S. Wulfson, O. S. Chizov und B. M. Zolotarev, *Tetrahedron*, 19 (1963) 2209.
16 K. R. Sperling, Diplomarbeit, Univ. Hamburg, 1967.
17 H. Andier, J. F. Dupin, M. Fetizon, und Y. Hopillard, *Tetrahedron Lett.*, (1966) 2077.
18 P. Brown, J. Kossanyi, und C. Djerassi, *Tetrahedron*, *Suppl.* 8, Pt. 1 (1966) 241.

Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

NITROGEN-CONTAINING CARBOHYDRATE DERIVATIVES

PART XIX*. CONFORMATION AND PROTON SPIN-COUPLING IN METHYL AMINO-4,6-*O*-BENZYLIDENE-DEOXY-α-D-GLYCOSIDES

C. B. BARLOW, E. O. BISHOP, P. R. CAREY, AND R. D. GUTHRIE
School of Molecular Sciences, University of Sussex, Brighton BN1 9QJ (Great Britain)

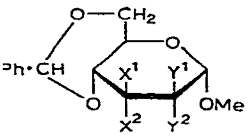
(Received June 4th, 1968)

ABSTRACT

A detailed study of the proton magnetic resonance spectra of the complete series of methyl 2(or 3)-amino-4,6-*O*-benzylidene-2(or 3)-deoxy-α-D-glycosides has shown that they exist in the expected chair conformation in chloroform. Complete analyses of the ring-proton spectra were carried out in several cases. Coupling constants over two to five bonds are discussed.

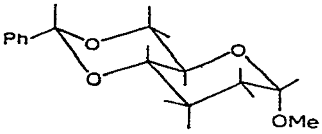
INTRODUCTION

A proton resonance study has been made on an interrelated series of compounds, namely, all eight possible stereoisomers of methyl 4,6-*O*-benzylidene-α-D-glycosides having amino and hydroxyl substituents in the 2(or 3)- and 3(or 2)-positions [formulae **1**–**8**]. The synthesis of these compounds has been described previously[1].



1 X^2=OH , Y^2=NH_2
2 X^2=NH_2 , Y^2=OH
3 X^2=OH , Y^1=NH_2
4 X^2=NH_2 , Y^1=OH
5 X^1= OH , Y^2=NH_2
6 X^1= NH_2 , Y^2=OH
7 X^1= OH , Y^1=NH_2
8 X^1= NH_2 , Y^1=OH

The remaining substituents are H



9

THE RING-PROTON SPECTRA

As far as possible, measurements were conducted in the same solvent (chloroform-*d*). This permitted detailed analyses of four of the spectra [those of **1**–**3** and **5**], but interpretation of first-order features only of the remaining four. Compound **8** gave a well-displayed spectrum in pyridine-d_5, which allowed complete analysis. The ring-proton region of this spectrum is shown in Fig. 1, and experimental and calculated spectra of compound **5** (in chloroform-*d* solution) in Fig. 2. The ring protons (numbered as in the formula, with 6_{eq} = 6 and 6_{ax} = 6′) form an isolated, seven-spin system. Possible coupling to the NH_2 and OH groups was eliminated by addition of

---

*Part XVIII: E. O. BISHOP, R. D. GUTHRIE, AND J. E. LEWIS, *Carbohyd. Res.*, 5 (1967) 477.

one drop of D_2O. Coupling to the benzylidene and methyl protons is believed to be less than 0.2 Hz.

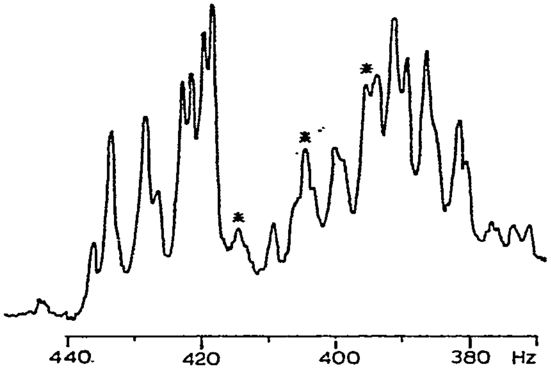


Fig. 1. Ring-proton spectrum of compound 8 in pyridine-d_5. The principal lines of H-4 are marked with an asterisk.

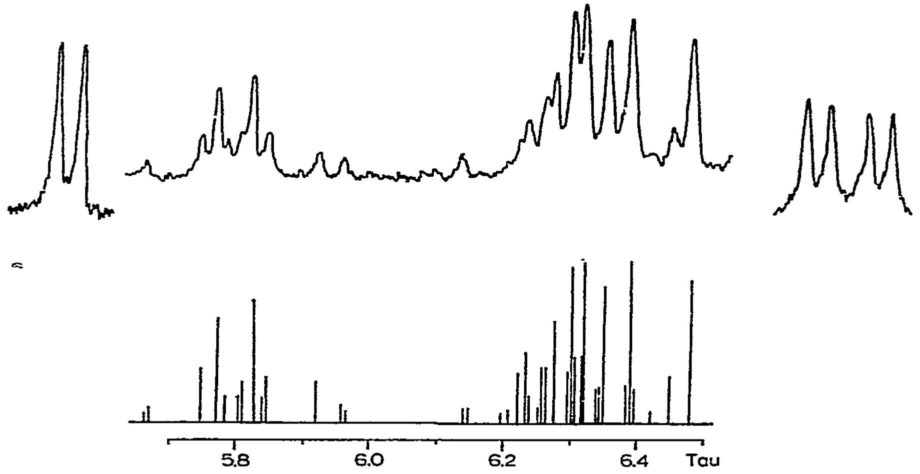


Fig. 2. Experimental and calculated spectrum of compound 5 in chloroform-*d* solution.

Of the ring protons, the easiest assignment to make was that of H-1 which gave a doublet (sometimes with the components scarcely resolved) well displaced to low field in every case. In most cases (see Table I), H-2 could then be identified as a high-field multiplet (2, 3, or 4 lines), by spin decoupling from H-1. The chemical shift (ν_3) of H-3 could then be located approximately by spin decoupling, with the decoupling frequency at ν_2. Tentative line assignment and location of the remaining chemical shifts were helped by two considerations which should be useful in other cases. First, the chemical shifts of the protons on C-5 and C-6 were only slightly affected by changing the stereochemistry at C-2 and C-3, and were similar in all the present compounds. The assumption of a chemical shift sequence H-6, H-5, H-6′ in order of increasing shielding, as reported for similar compounds[2,3], was justified by the subsequent analyses.

TABLE I

CHEMICAL SHIFT (ν, IN τ UNITS) AND SPIN-COUPLING (J, IN HZ) PARAMETERS IN CHLOROFORM-d SOLUTION

	Compound	ν_1	ν_2	ν_3	ν_4	ν_5	ν_6	$\nu_{6'}$	J_{12}	J_{23}	J_{34}	H_{45}	J_{56}	$J_{56'}$	$J_{66'}$	*Benzylidene H*	*OMe*
1	2-amino-*allo*	5.48	7.22	6.06	6.63	6.01	5.72	6.38	3.83	3.24	3.64	10.09	5.09	+10.52	−10.05	4.52	6.63
2	3-amino-*allo*	5.32	6.31	6.18	6.51	6.11	5.67	6.26	4.24	3.35	3.70	9.05	4.34	10.00	−9.36	4.44	6.62
3	2-amino-*altro*	5.49	6.75	6.09	5.69	6.18	5.75	6.24	[a]	2.5	4.4	10.0	~5	~10	~−10	4.48	6.68
4	3-amino-*altro*	5.44	6.12	6.78	—	—	5.72	—	—	—	—	—	—	—	—	4.43	6.70
5	2-amino-*gluco*	5.41	7.33	6.35	6.59	6.32	5.81	6.37	3.60	9.20	9.28	9.21	4.86	10.87	−10.56	4.50	6.68
6	3-amino-*gluco*	5.32	—	—	—	—	5.76	—	~3.5	—	—	—	—	—	—	4.55	6.60
7	2-amino-*manno*	5.39	—	—	—	—	—	—	[a]	—	—	—	—	—	—	4.5	6.72
8[b]	3-amino-*manno*	(5.08)	(5.80)	(6.63)	(6.00)	(6.09)	(5.72)	(6.20)	(1.40)	(3.20)	(9.76)	(9.38)	(4.24)	(10.53)	(−9.63)	—	—
		5.35	—	6.81	—	—	5.77	6.37	[a]	—	—	—	—	—	—	4.50	6.65

[a] $\leqslant$ 1.4 Hz.

[b] Bracketted values are for pyridine-d_5, and should strictly not be quoted as τ values.

The second consideration arises whenever there is a ring proton (X) widely displaced in chemical shift from all the others. Its resonance usually consists of a nearly first-order multiplet of up to four lines only, since no ring proton except H-5 has more than two proton neighbours (A, B) on adjacent carbon atoms (H-1 has only one), and longer range couplings are too small to give resolvable line splitting in this situation. An abnormal pattern with extra splittings may occasionally be produced by virtual coupling[3,4], but is not observed in the present series. Component spacings within the first-order X multiplet are usually duplicated closely within the A and B multiplets, even when the latter lie within the complex ring-proton region and are essentially mixed transitions. This is because protons A and B are usually each coupled strongly to no more than one other proton within the complex region. By picking out certain of the A and B lines in this way, one can obtain an initial rough estimate of ν_A and ν_B. Any ambiguities can often be resolved by spin decoupling from X. In Fig. 1, for example, the principal lines of H-4 (starred) duplicate the spacing in the high-field H-3 multiplet. This assignment was confirmed by spin decoupling from H-3, but it would have been difficult to interpret the decoupled spectrum in detail without prior knowledge of the possibilities.

Full analyses were carried out by using the computer program LAOCOON II*, as described in an earlier paper[3]. Compounds **1** and **2** were treated exactly as seven-spin systems. In compounds **3** and **5**, the wide displacement in chemical shift of H-1 from H-2 and the other ring protons allowed treatment as a six-spin system, interaction with H-1 being considered as first order. Paucity of well-resolved lines between τ 6.1 and 6.4 in the spectrum of compound **3** has limited the accuracy of certain parameters (see Table I), but their general validity is confirmed by double resonance and the excellent fit obtained for the rest of the spectrum. The spectrum of compound **8** in pyridine-d_5 could be treated as a five-spin system, since interactions of H-1, H-2, and H-3 are all close to first order. Conformational changes being excluded, the coupling constants should be very similar to those in chloroform-*d* solution[5].

CHEMICAL SHIFTS

Available values are given in Table I, together with coupling constants over two and three bonds. The benzylidene and methoxyl proton resonances appeared in characteristic positions. Of the sugar-ring protons, the chemical shifts of H-6, H-5, and H-6′ fell within narrow ranges (τ 5.67–5.81, 6.01–6.32, and 6.24–6.38, respectively, in chloroform-*d* solution), in keeping with the assumption made earlier. The low-field shift (τ 5.32–5.49) of H-1 is also highly characteristic for glycosides having the methoxyl group axial. The wide range of shifts for H-2 and H-3 reflects the variety of relative configurations at these two centres and demonstrates the well-known, high-field displacement of an axial proton as compared to an equatorial proton

---

*Typically 37 transitions are assigned to 28 lines for the iterative part of the program, the final iteration having a root mean square error of 0.069. Couplings over six bonds were set to zero. The probable error for computed *J*'s in Table I are ±0.1 Hz.

in an otherwise similar environment[5]; compare, for example, ν_2 for the 2-NH_2 derivatives **3** (*altro*, H-2eq) and **1** (*allo*, H-2ax) and ν_3 for the 2-NH_2 derivatives **5** (*gluco*, H-3ax) and **1** (*allo*, H-3eq). The low τ value for H-4 in compound **3**, as compared to those in compounds **1**, **2**, and **5**, appears to be a long-range anisotropy effect due to the axial 2-substituent, but this is conjectural in the absence of data from the other compounds.

STEREOCHEMISTRY OF THE SYSTEM

Coxon[4] has made a study of a series of derivatives of methyl 4,6-*O*-benzylidene-α-D-aldohexopyranosides, including a number of amino-deoxy derivatives, but only one of the compounds used in the present study, namely the 2-amino-2-deoxy-*altro* derivative. Our assignments agree with his for this compound. By analysis of the $J_{1,2}$, $J_{2,3}$, and $J_{3,4}$ values in his compounds, Coxon concluded that compounds of this class having the *allo*, *altro*, *gluco*, and *manno* configurations all exist in the basic conformation **9**.

The coupling constants obtained for the series of compounds **1–8** (Table I) are consistent only with the compounds' being in the same conformation.

COUPLING CONSTANTS

(*a*) *Over two bonds.* — The geminal coupling constant $J_{6,6'}$ has been shown elsewhere[3] to be negative, and in this series of compounds lies between −9.3 and −10.6 Hz.

(*b*) *Over three bonds.* — Coxon has discussed the factors that may be responsible for the general observation that $J_{ee} < J_{ae}$. The compounds of the present study support his observations. For the H-1eq–H-2eq systems **3**, **4**, **7**, and **8**, $J_{1,2}$ = 0–1.4 Hz, whereas for the H-1eq–H-2ax systems **1**, **2**, **5**, and **6**, $J_{1,2}$ = 3.5–4.2 Hz. For the H-2–H-3 and H-3–H-4 couplings, the same difference is seen; $J_{1,2}$ (eq, eq) for compound **3** is 2.5 Hz, whereas several $J_{ax,eq}$ values are in the range 3.2–4.4 Hz. Thus, $J_{ax,eq}$ values fall within the same general range for all couplings, whereas $J_{eq,eq}$ for H-1–H-2 is markedly smaller even than that for H-2–H-3.

Many values of $J_{ax,ax}$ couplings were observed, and they fell in the range 9.2–10.9 Hz.

(*c*) *Over four bonds* (*cf.*, ref. 6). — Generally, it is possible to determine the relative importance of genuine long-range coupling and virtual coupling only by a complete analysis of the system under study (although partial virtual coupling may be easily recognised in an isolated multiplet, since some lines are completely unaffected[3]). Therefore, only those values confirmed by iterative analysis are given in Table II.

Axial–axial coupling constants found over four bonds are in the range −0.3 to −1.24 Hz, and axial–equatorial coupling constants are from +0.3 to −0.53 Hz. Both ranges are in fair agreement with those predicted by the empirical equations of

Bystrov and Stepanyants[7]. Similarly, these workers predicted positive equatorial–equatorial coupling constants of > 1 Hz, and this has been subsequently confirmed by first-order analysis and spin tickling[6]. In this paper, the only long-range equatorial–equatorial interactions are between H-1 and H-3. Because it is rather small *and* H-1 is chemically shifted far from the other sugar-ring protons, $J_{1,3}$ merely causes line broadening instead of resolvable splitting. Furthermore, the *mean* positions of both the H-1 and H-3 resonances are very insensitive to $J_{1,3}$, so that it cannot be found either by inspection or by complete, iterative analysis. In compound **3**, however, a strong decoupling field at ν_2 caused the H-1 resonance to assume a poorly resolved doublet structure of separation 0.75 ±0.25 Hz. Since $J_{1,5}$ is believed to be less than 0.2 Hz, one can conclude in this case that $0.5 < J_{1,3}$ (eq, eq) < 1.0 Hz.

TABLE II

PROTON–PROTON COUPLING CONSTANTS ACROSS FOUR BONDS[a]

Compound	**1**	**2**	**3**	**5**	**8**
$J_{4\,6'}$ (ax, ax)	−0.84 ±0.15	−0.57 ±0.13	small and negative	−1.24 ±0.17	−0.47 ±0.23
$J_{4\,6}$ (ax, eq)	−0.20 ±0.08	+0.06 ±0.15		−0.12 ±0.10	−0.33 ±0.13
$J_{3\,5}$ (ax, ax)				−0.30 ±0.13	
$J_{3\,5}$ (ax, eq)		−0.53 ±0.11			−0.04 ±0.2
$J_{2\,4}$ (ax, ax)				−0.33 ±0.15	
$J_{2\,4}$ (ax, eq)		+0.30 ±0.17			+0.03 ±0.2

[a] Quoted errors are probable errors.

(*d*) *Over five bonds.* — Since only two compounds were analysed as seven-spin systems, information on coupling over five bonds is limited. Of the interactions included in the analyses, the only values significantly different from zero are $J_{3,6} = -0.22 \pm 0.1$ Hz in compound **5**, and $J_{2,5} = +0.26 \pm 0.1$ Hz in compound **1**.

EXPERIMENTAL

The proton resonance spectra of compounds **1**–**8** were recorded at 100 MHz (Varian HA-100 instrument) for nearly saturated solutions in chloroform-*d* and in pyridine-d_5. Chemical shifts and line positions were measured relative to 1% tetramethylsilane (TMS) as internal standard, and those in chloroform-*d* are given throughout in p.p.m. relative to TMS = 10.000 and referred to as τ values. (This is not strictly correct, since the τ-scale refers to infinite dilution in carbon tetrachloride, but the approximation is expected to be reasonable.) Chemical shifts in pyridine-d_5 should not be quoted as τ values. As water was not rigorously excluded, the resonance positions of the solute hydroxyl and amino groups are not very significant and are not recorded. They occur in the range τ 7–8 in most cases.

The compounds were prepared as described previously[1].

REFERENCES

1 C. B. Barlow and R. D. Guthrie, *J. Chem. Soc. (C)*, (1967) 1194.
2 M. Sharma and R. K. Brown, *Can. J. Chem.*, 44 (1966) 2825.
3 C. B. Barlow, E. O. Bishop, P. R. Carey, R. D. Guthrie, M. A. Jensen, and J. E. Lewis, *Tetrahedron*, (1968), 4517.
4 B. Coxon, *Tetrahedron*, 21 (1965) 3481.
5 J. W. Emsley, J. Feeney, and L. H. Sutcliffe, *High Resolution Nuclear Magnetic Resonance Spectroscopy*, Pergamon, London, 1965.
6 L. D. Hall, J. F. Manville, and A. Tracey, *Carbohyd. Res.*, 4 (1967) 514.
7 V. F. Bystrov and A. V. Stepanyants, *J. Mol. Spectr.*, 21 (1966) 241.

Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

STUDIES ON CARBOHYDRATE-METABOLISING ENZYMES

PART XX*. SWEET-CORN DEBRANCHING ENZYMES

D. J. Manners and Karen L. Rowe**

Department of Brewing and Biochemistry, Heriot-Watt University, Edinburgh (Great Britain)

(Received May 16th, 1968)

ABSTRACT

Extracts of sweet corn contain a mixture of debranching enzymes which have been separated to give a typical R-enzyme (which acts only on amylopectin) and an isoamylase (which acts on both glycogen and amylopectin). The action of isoamylase on phytoglycogen gives a mixture of maltosaccharides and a residual polysaccharide which had a glycogen rather than an amylopectin-type structure. This suggests that, *in vivo*, amylopectin is not formed by the enzymic debranching of a glycogen precursor. Sweet-corn extracts also contain a third α-(1→6)-glucosidase which hydrolyses pullulan.

INTRODUCTION

During studies on the purification of sweet-corn branching enzymes[1], certain protein fractions caused an increase in the iodine-staining power of glycogen and amylopectin, rather than the expected decrease. The action of debranching enzymes such as broad-bean R-enzyme (amylopectin 6-glucanohydrolase)[2] or yeast isoamylase (glycogen 6-glucanohydrolase)[3] results in an increase in the iodine-staining power of their substrates, arising from the hydrolysis of the outermost α-(1→6)-D-glucosidic inter-chain linkages. It seemed probable, therefore, that the sweet-corn extracts contained a mixture of both branching and debranching enzymes. The latter activity has now been investigated and, as reported in a preliminary communication[4], shown to be due to a mixture of two α-(1→6)-glucosidases, one of which can act on amylopectin but not on glycogen, and the other which will hydrolyse the inter-chain linkages in both polysaccharides. In accord with current nomenclature, these activities will be referred to as R-enzyme and isoamylase, respectively.

MATERIALS AND METHODS

Materials. — The following polysaccharides were laboratory samples: pullulan, rabbit-liver glycogen, sockeye-salmon liver glycogen, *Trichomonas foetus* glycogen,

---

*Part XIX: see Ref. 1.

**Present address: Department of Pathology, University of British Columbia, Vancouver 8, Canada.

phytoglycogen A and B, potato amylopectin, waxy sorghum starch; average chain-lengths and β-amylolysis limits had been determined by standard methods. β-Limit dextrins were prepared by incubation of the polysaccharide with Wallerstein β-amylase (50 units[5]/mg of polysaccharide) until the expected percentage conversion into maltose had been obtained (usually 48–72 h); the enzyme was inactivated by heating, the digest was centrifuged and dialysed for three days against running tap-water, and the β-dextrin was isolated by freeze-drying.

Enzyme extracts were prepared from defatted flour of *Zea mays* (var. Golden Bantam)[1].

For germination studies, mature grains of *Zea mays* were steeped in water for 4 h at 20°, and then washed in 1% sodium hypochlorite solution and in distilled water. The washed grains were then germinated on Whatman No. 1 paper in petri-dishes at 30°. During germination, *ca.* 75 grains were removed at various times, ground in a chilled mortar, and extracted with water (the pH of each homogenate being noted), and the extract was centrifuged (1,000 *g* for 20 min) to give a protein solution which was dialysed against running tap-water for 24 h at 2°.

Analytical methods. — The methods were those used in the previous paper[1]. Descending paper chromatograms were prepared with (*A*) propyl alcohol–ethyl acetate–water[6] (14:2:7) or (*B*) ethyl acetate–pyridine–water (10:4:3) as solvents. The carbohydrase activity of protein fractions was examined by using digests containing substrate [5 mg in 0.3 ml of 0.05M sodium citrate (pH 6.0)] and enzyme (5 mg in 0.2 ml of water) which were incubated at 37° and analysed by paper chromatography with solvent *B*.

The successive and combined action of a debranching enzyme and β-amylase was examined in digests containing polysaccharide (4 mg), freeze-dried enzyme preparation (15 mg), and citrate buffer (pH 6.2) in a total volume of 5 ml. After 24 h at 37°, the digests were halved. One part was heated (100° for 3 min) and cooled, the pH was adjusted to 4.8 with acetate buffer, and β-amylase (250 units) was added. β-Amylase was also added directly to the other half, and samples (0.2 ml) were withdrawn after incubation with β-amylase for 24, 48, and 72 h for the estimation (Nelson–Somogyi[7]) of the liberated maltose. The concentrations of the polysaccharides were determined by a phenol–sulphuric acid method[8].

Electrophoresis. — A Beckman Spinco Model CP continuous-electrophoresis cell was used at a constant voltage (600–900 volts) for the fractionation of protein material. Up to 8 racks of 32 tubes were collected. In certain experiments, smaller scale fractionations were carried out with a high-voltage Pherograph Type 65 apparatus. These separations were carried out in 0.001M citrate buffer (pH 6.0) with Whatman 3 MM paper (8 × 40 cm) at 1500 volts and 70 mamp for 1 h at 3°. The paper was then dried at room temperature and divided into strips (8 × 2 cm) which were numbered from the datum line. Each strip was immersed in 5 ml of 0.01M citrate buffer (pH 6.0) for 30 min at 37°, and the resulting protein solutions were then assayed.

Assay of debranching enzymes. — Enzyme activity was assayed by measurement of the increase in iodine-staining power of potato amylopectin (at 550 nm) and rabbit-liver glycogen (at 470 nm), although the relation between this change and the number of inter-chain linkages hydrolysed is not known. The method therefore provides a comparative, rather than absolute, measure of activity. Standard digests contained polysaccharide (2 mg), buffer (1 ml), and enzyme solution (2 ml), and samples (1 ml) were withdrawn after incubation times of 0 and 2 h at 37°, and added to standard iodine reagent (5 ml), and the absorbances were measured. In digests containing between 0 and 1.80 mg of protein, there was a linear relationship between the enzyme concentration and the percentage increase in the iodine stain of glycogen and amylopectin, the slope of the line being slightly greater for glycogen than for amylopectin. For example, with 0.90 mg of protein, the percentage increases were 6.5 and 5.8%, respectively, and with 1.80 mg of protein, 12.7 and 12.0%, respectively. (The standard iodine reagent consisted of 20 ml of 0.2% iodine in 2.0% aqueous potassium iodide and 6 ml of 6N hydrochloric acid diluted with water to 400 ml).

For the assay of electrophoresis or column fractions, 1-ml samples were added directly to polysaccharide (2 mg) in an appropriate buffer (2 ml). After incubation for 2 h, samples (1 ml) were added to the iodine reagent (20 ml for glycogen; 10 ml for amylopectin and amylose). For the latter, absorbances at 630 nm were measured.

RESULTS

An extract of sweet corn was fractionated with ammonium sulphate as described previously[1]. The distribution of certain enzymic activities and the composition of the fractions are given in Table I.

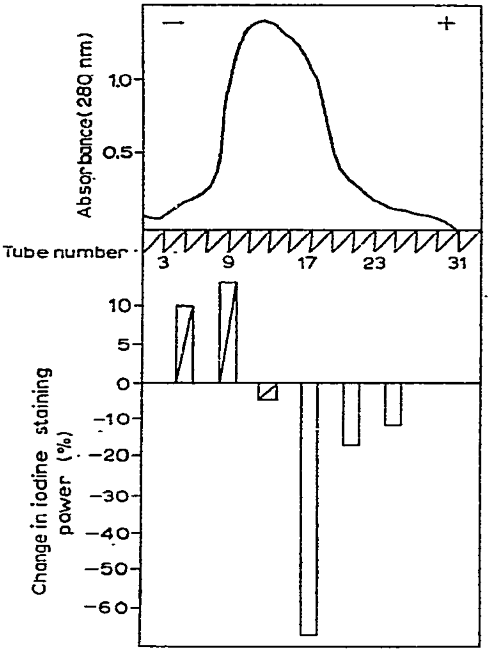
TABLE I

FRACTIONATION OF SWEET-CORN EXTRACT WITH AMMONIUM SULPHATE

Fraction	1	2	3	4
Percentage of ammonium sulphate (w/v)	0–22	23–32	33–35	56–70
Weight (g)	2.3	0.8	64.0	0.6
Protein (%)	35.7	10.7	27.3	14.1
Polysaccharide (%)	30.1	5.3	25.3	2.1
α-Amylase + branching activity[a]	0.091	0.059	0.135	0.120
α-Amylase activity[a]	0.005	0.026	0.040	0.063
Branching activity[a]	0.086	0.033	0.095	0.057
Relative carbohydrase activity[b]				
Maltase	+ +	+ +	+ +	+
Maltotriase	+ +	+ +	+ +	−
Limit dextrinase	+ +	−	+ +	−
Pullulanase	+	−	+	−

[a] Determined by the method of Krisman[9]. [b] Relative activity indicated by: −, nil; +, weak; + +, substantial.

A solution of fraction 3 (4.0 g containing 1.1 g of protein) in water (20 ml) was dialysed at 2° against running tap-water for 3 h and against 0.01M sodium citrate buffer (pH 6.3) for 16 h. The dialysed solution was subjected to continuous electrophoresis at 600 volts and 70 mamp during 42 h with 0.01M sodium citrate buffer (pH 6.3). The distribution of protein and enzymic activity is shown in Fig. 1. Tubes from individual racks showing similar activities were combined, made 0.01M with respect to citrate buffer (pH 6.0), and freeze-dried. The debranching-enzyme fraction (the contents of tubes 3–10 from several racks, yield 3.1 g) contained 18.3% of protein and 18.5% of polysaccharide; the branching-enzyme fraction (the contents of tubes 17–20, yield 0.6 g) contained 20.5% of protein and 15.0% of polysaccharide. These two fractions contained 64% of the total protein applied to the electrophoresis curtain.



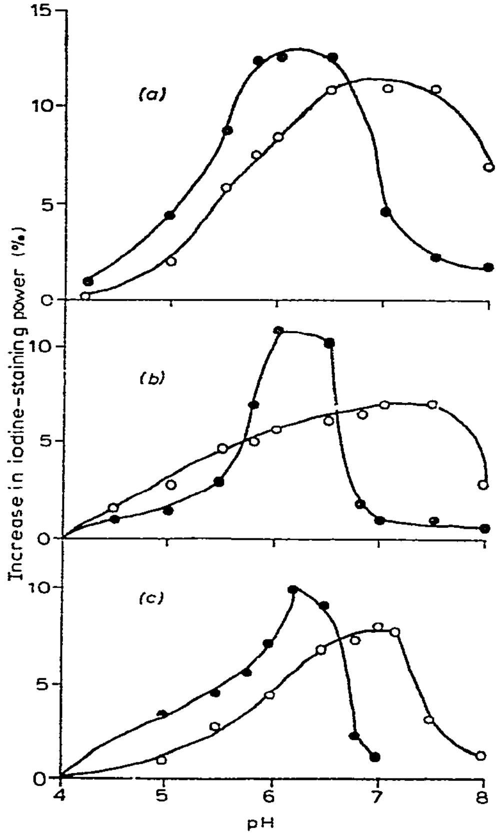


Fig. 1. Distribution of protein (absorbance at 280 nm) and enzymes during the continuous electrophoresis of sweet-corn fraction 3. The substrates were glycogen ▨ and amylopectin ☐, and the changes in iodine-staining power were measured at 470 and 550 nm, respectively. The results from tubes 5–6, 9–10, 13–14, 17–18, 21–22, and 25–26 of the second rack are shown.

Fig. 2. Effect of pH on debranching-enzyme activities (*a*) of the debranching-enzyme fraction, (*b*) of isoamylase and R-enzyme separated by gel filtration, and (*c*) of isoamylase and R-enzyme separated by ultracentrifugation followed by continuous electrophoresis. The substrates were glycogen, ●—●; and amylopectin, ○—○.

PROPERTIES OF THE DEBRANCHING-ENZYME FRACTION

Qualitative examination showed the presence of limit dextrinase activity and small amounts of pullulanase and maltase activity in the debranching-enzyme fraction. The latter had no effect on the iodine-staining power of amylose, and was therefore free of α-amylase and branching enzymes.

The effect of temperature on enzymic activity was examined in digests containing enzyme preparation (10 mg) and polysaccharide (2 mg) in 3 ml of 0.05M citrate buffer (pH 6.0). As shown in Table II, on incubation for 1 h, the optimum temperature with glycogen as the substrate was *ca.* 30°, whereas, with amylopectin, the optimum temperature range was *ca.* 35°.

TABLE II

EFFECT OF TEMPERATURE ON DEBRANCHING-ENZYME ACTIVITIES[a]

Temperature (degrees)	18	20	25	30	35	40	50	60
Glycogen	0	3	7	13	11	9	7	5
Amylopectin	0	1	9	13	14	13	8	0

[a] Results are expressed as the percentage increase in iodine-staining power at 470 and 550 nm, respectively.

The effect of pH (using phosphate–citrate buffers) on the activity towards glycogen and amylopectin, as shown in Fig. 2(*a*), is different, the optimum pH being about 6.0 and 7.0, respectively. However, on preincubation for 30 h at 37° in 0.01M Tris–hydrochloric acid buffer, the activity towards amylopectin was inhibited by *ca.* 50% at pH 6.0 and was completely inhibited at pH 8.0. By contrast, the activity towards glycogen was not affected at pH 6.0, and was still appreciable at pH 8.0 (see Table III).

TABLE III

EFFECT OF TRIS BUFFER ON DEBRANCHING-ENZYME ACTIVITIES[a]

Time of incubation (h)	0.5	1.0	1.5	2.0	4.0	6.0	10.0
Glycogen							
Control	4	10	11	13	13	13	13
Tris, pH 6.0	5	6	12	13	14	14	14
Tris, pH 8.0	3	3	4	4	5	5	5
Amylopectin							
Control	6	8	9	10	12	13	13
Tris, pH 6.0	1	4	5	5	6	6	6
Tris, pH 8.0	0	0	0	0	0	0	0

[a] Results are expressed as the percentage increase in iodine-staining power at 470 and 550 nm, respectively.

The effect of various concentrations of mercuric chloride, ammonium molybdate, and sodium borate on the activity towards glycogen and amylopectin is shown in

Table IV. These salts, which are already known as inhibitors of yeast isoamylase[3], R-enzyme[10], and Q-enzyme[11], have different effects on the two activities.

TABLE IV

EFFECT OF SALTS ON DEBRANCHING-ENZYME ACTIVITIES[a]

Salt (mM)	*Inhibition* (%)	
	Glycogen substrate	*Amylopectin substrate*
Mercuric chloride		
0.005	70	75
0.05	76	89
5	100	100
Ammonium molybdate		
0.2	22	72
2	40	84
200	100	93
Sodium borate		
1	0	0
2	0	7
4	+5	6
8	+5	7

[a] Results are based on the increase in iodine-staining power after incubation for 1 h at 37°, relative to control digests containing water.

The increase in the iodine-staining power of glycogen and amylopectin was accompanied by an increase in the β-amylolysis limit. As expected, the combined action of the sweet-corn preparation and β-amylase resulted in virtually complete degradation (see Table V), whilst their successive action caused the β-amylolysis limits of glycogen to increase by 7–13%, and those of amylopectin or its β-dextrin to rise by 12 or 83%.

TABLE V

EFFECT OF DEBRANCHING-ENZYME PREPARATION ON THE β-AMYLOLYSIS LIMITS OF POLYSACCHARIDES

Substrate	*Average chain-length*	*β-Amylolysis limit* (%)		
		Original	*Successive action*[a]	*Combined action*[b]
Rabbit-liver glycogen	13	54	67	99
Sockeye-salmon liver glycogen	12	48	55	95
Trichomonas foetus glycogen	15	61	70	97
Phytoglycogen A	11	52	65	98
Phytoglycogen B	9	41	54	98
Potato amylopectin	22	61	73	99
Waxy-sorghum amylopectin β-dextrin	11	0	83	100

[a] Measured after 72-h incubation with β-amylase. The results after incubation for 24 and 48 h were either the same or differed by less than 2%, showing that β-amylolysis was virtually completed within 24 h. [b] Measured after 72 h.

SEPARATION OF SWEET-CORN R-ENZYME AND ISOAMYLASE

Since the results reported in Fig. 2(*a*) and Tables II–IV show the presence of two different debranching enzymes, attempts were made to separate the two activities by various protein-fractionation techniques.

Gel-filtration studies. — A solution of fraction 3 (4.5 g in 40 ml of water) was centrifuged at 105,000 *g* for 2.5 h at 5° to yield a particulate phytoglycogen pellet and a clear solution of protein which was subjected to continuous electrophoresis at 700 volts and 40 mamp over 48 h at 2°. The action of the electrophoretic fractions on glycogen at pH 6.2, amylopectin at pH 7.2, and amylose was examined, and fractions 1–14 from eight racks of tubes were combined, and concentrated by freeze-drying to 15 ml.

A sample (equivalent to 6.7 mg of protein) was passed through a column (2.4 × 34 cm) of Sephadex G-200 which had been equilibrated against 0.2M sodium citrate buffer (pH 6.2) which was 1% with respect to sodium chloride, and fractions (5 ml) were collected and assayed [Fig. 3(*a*)]. Activity towards glycogen was present in fractions 1–28, with maximum activity in fraction 14; with amylopectin, activity was present in fractions 1–28, with maximum activity in fraction 22. Accordingly, fractions 1–16 were combined, the volume was reduced to 20 ml (containing 2.7 mg of protein), and the solution was again applied to the column of Sephadex G-200.

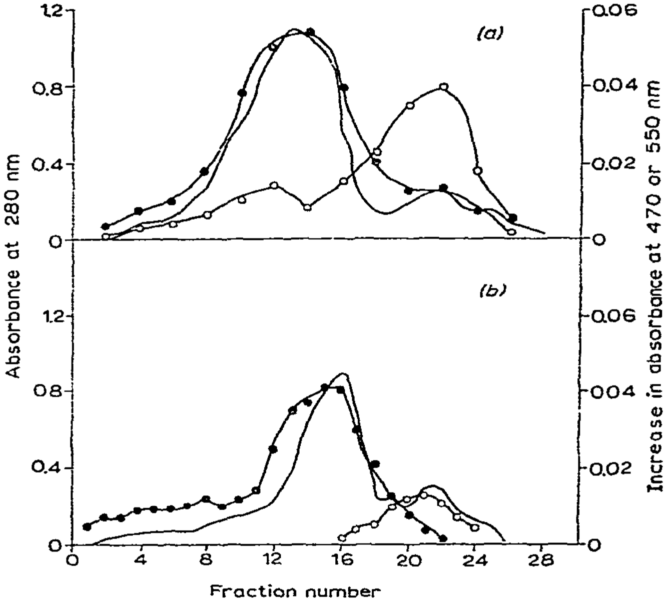


Fig. 3. Gel filtration of sweet-corn debranching enzymes on Sephadex G-200. Fractions (5 ml) were collected and assayed against glycogen (●—●) or amylopectin(○—○). The debranching-enzyme fraction was applied to the column, and elution gave diagram (*a*). Fractions 1–16 were combined, and the isoamylase was separated from R-enzyme by gel filtration as in diagram (*b*). The distribution of protein (absorbance at 280 nm) is shown by the continuous lines.

The results of the enzyme assays of the column fractions are shown in Fig. 3(*b*). Fractions 1–16 and 17–24 [see Fig. 3(*b*)] were combined to give solutions of isoamylase and R-enzyme, respectively. The effect of pH (using citrate–phosphate buffers) on the action of isoamylase on glycogen and of R-enzyme on amylopectin is shown in Fig. 2(*b*) and confirm the previous conclusion that the two enzymes differ significantly in optimum pH.

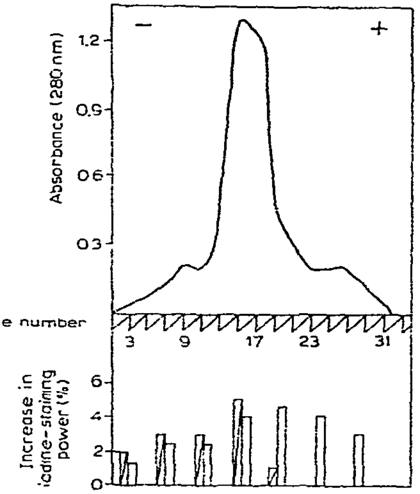
The isoamylase was incubated with various polysaccharides at pH 6.3 for 24 h; this caused the following increases in β-amylolysis limit to occur: rabbit-liver glycogen from 54 to 65%; horse-muscle glycogen from 47 to 57%; malted-barley amylopectin from 51 to 60%; and potato amylopectin from 61 to 69%. These results confirm the presence, in *Zea mays*, of a debranching enzyme which can act on both amylopectin and glycogen.

Ultracentrifugation and electrophoresis studies. — A solution of fraction 3 [4.0 g in 100 ml of 0.02M citrate buffer (pH 6.3) which was 7 mM with respect to EDTA] was centrifuged at 105,000 *g* for 2.5 h at 5°, and the phytoglycogen pellet was collected, resuspended in citrate buffer, and again subjected to ultracentrifugation. The procedure was repeated. The three supernatant solutions and a suspension of the pellet in citrate buffer were stored at −15° until they had been assayed. Previous studies in this[1] and other laboratories[12] have shown that branching-enzyme activity is freely soluble and is present in the supernatant solutions obtained on ultracentrifugation. With each successive extraction of the pellet, the branching enzyme and α-amylase activities of the supernatant solutions decreased, so that, after the third extraction, the solution, in fact, caused an increase in the iodine-staining power of glycogen and amylopectin during incubation for 24 h.

The phytoglycogen pellet was homogenised in 0.1M citrate buffer (pH 6.3), and, after centrifugation at 1,000 *g* for 20 min at 4°, the opalescent solution (55 ml containing 190 mg of protein) was submitted to continuous electrophoresis at 800 volts and 40 mamp for 48 h at 2°. The distribution of enzyme activity in the electrophoresis samples is shown in Fig. 4. Tubes 3–17 contained isoamylase, and there was a clear separation from R-enzyme (tubes 21–27). The contents of the appropriate tubes were combined and freeze-dried; the yields of isoamylase (75 mg of protein) and R-enzyme (31 mg of protein) represent a recovery of 56% of the total protein which was applied to the electrophoresis curtain.

In view of the different effects of various salts on the original debranching-enzyme preparation (Table IV), their effects on the purified isoamylase and R-enzyme were examined. The results (Table VI) show a marked difference between the susceptibility of the two enzymes to ammonium molybdate. EDTA (0.07–7.0 mM) and *p*-chloromercuribenzoate (0.01 mM) had no effect on either enzyme activity. The effect of Tris buffer on the two debranching enzymes is shown in Fig. 5, and the results provide a further means of distinguishing between isoamylase and R-enzyme.

The effect of the purified enzymes on the β-amylolysis limits of various branched α-glucans was measured. Isoamylase increased the β-amylolysis limit of rabbit-liver glycogen from 54 to 65%, of phytoglycogen A from 52 to 64%, and of potato amylopectin from 61 to 70%. By contrast, R-enzyme had a similar action on potato amylopectin, but had no effect on either the β-amylolysis limit or iodine-staining power of rabbit-liver glycogen or phytoglycogen A.



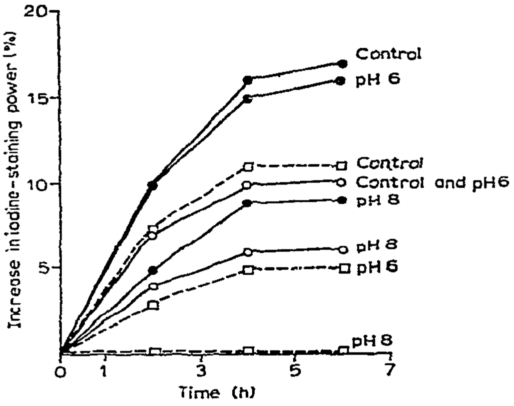


Fig. 4. Separation of isoamylase and R-enzyme by continuous electrophoresis. The substrates were glycogen ▨ and amylopectin ☐, respectively. The distribution of protein is shown by the continuous line.

Fig. 5. Effect of Tris buffer at pH 6 and 8 on the activity of debranching enzymes. Key: isoamylase acting on glycogen, (●—●); isoamylase acting on amylopectin, (○—○); R-enzyme acting on amylopectin, (☐----☐).

TABLE VI

EFFECT OF INHIBITORS ON SWEET-CORN ISOAMYLASE AND R-ENZYME

Inhibitor (mM)	*Isoamylase*[a]	*R-enzyme*[a]
Mercuric chloride		
0.005	35	28
0.05	57	57
0.5	63	91
5.0	93	100
Ammonium molybdate		
0.2	5	19
2.0	5	56
20	7	83
200	7	100

[a] Results are expressed as percentage inhibition based on iodine-staining assays after incubation for 2 h at 37°, relative to control digests containing water.

Action of sweet-corn isoamylase on phytoglycogen. — Although the available evidence suggested that the debranching action of isoamylase involved the hydrolysis of the outer inter-chain linkages of glycogen, experimental confirmation of this was required. The action of amylo-1,6-glucosidase on glycogen may involve the transfer of D-glucose residues from A-chains (side chains) to B-chains (main chains) with a resultant increase in iodine-staining power and β-amylolysis limit, but without the hydrolysis of the outermost α-(1→6)-D-glucosidic linkages[13].

A phytoglycogen pellet was homogenised in buffer and centrifuged at 1,000 *g* as described previously, and the opalescent solution (45 ml containing 189 mg of protein) was subjected to continuous electrophoresis. Appropriate fractions were collected, assayed, and combined to give isoamylase (88 mg of protein) and R-enzyme (43 mg of protein) representing 69% of the total protein. Each enzyme solution was concentrated to 5 ml by freeze-drying, and then submitted to gel filtration on a column (8 × 70 cm) of Sephadex G-200 fitted to a LKB Model 4900A Recycling Chromatography apparatus. The enzyme solutions were cycled through the column three times, using 0.01M citrate buffer (pH 6.0) as eluent, and samples (3 ml) were collected with a LKB Model 7000 UltraRac fraction collector, assayed, and combined as appropriate. With isoamylase, a symmetrical peak showing the same relative activity towards glycogen and amylopectin was obtained. With R-enzyme, a symmetrical peak showing marked activity towards amylopectin, but having no action on glycogen, was observed. The combined solutions were finally freeze-dried, to give an isoamylase preparation (355 mg; protein content, 20.0%) and R-enzyme (259 mg; protein content, 12.5%). The recovery of protein from the Sephadex column was 81 and 74%, respectively.

Neither enzyme preparation had any action on amylose, maltose, maltotriose, α-limit dextrins, or pullulan. There was no transferase activity with maltotriose, and, on prolonged incubation, neither enzyme preparation underwent autolysis with the production of reducing sugars.

Phytoglycogen A (200 mg) and isoamylase (100 mg) in 0.005M citrate buffer (pH 6.3) (40 ml) were incubated for 72 h at 37°. The iodine-staining power of a sample (0.5 ml) increased by 12, 20, 25, and 32% after 24, 36, 48, and 72 h, respectively. The digest was then heated (10 min at 100°), cooled, and centrifuged (40,000 *g* for 20 min), and further isoamylase (100 mg) was added to the supernatant solution. During the next 36 h, there was no further increase in iodine-staining power. The enzyme was inactivated by heating, denatured protein was removed by centrifugation at 40,000 *g*, and the solution was concentrated to *ca.* 3 ml and applied to a column (3 × 37 cm) of Sephadex G-50. With water as eluent, fractions (3 ml) were collected, and assayed for carbohydrate by using the phenol–sulphuric acid reagent. Fractions 8–24 contained polysaccharide (isodextrin, 145 mg) and fractions 28–52 contained a mixture of oligosaccharides (maltosaccharides, 40 mg) which were isolated by freeze-drying.

The maltosaccharides were freed from citrate and other ions by use of a BTL chromatographic desalting-apparatus, and shown by paper chromatography in solvent *A* to be a mixture of maltosaccharides having *DP* 2–7, with a trace of malto-octaose.

The isodextrin had a β-amylolysis limit of 62% and an average chain-length of

12.5 D-glucose residues (by α-amylolysis), and the iodine complex had λ_{max} 445 nm and O.D.$_{max}$ 0.66. The corresponding properties of the original phytoglycogen A are: β-amylolysis limit, 52%; average chain-length, 11.1 D-glucose residues; iodine complex, λ_{max} 440 nm and O.D.$_{max}$ 0.50.

The iodine-staining properties of the β-limit dextrins of phytoglycogen-A isodextrin and amylopectin (waxy sorghum starch) are compared in Fig. 6. Since the β-dextrin of the isodextrin had λ_{max} 440 nm and O.D.$_{max}$ 0.39 in water, and λ_{max} 430 nm and O.D.$_{max}$ 0.51 in half-saturated ammonium sulphate solution, it clearly shows a typical glycogen-type spectrum. The corresponding figures for amylopectin β-dextrin are λ_{max} 535 nm and O.D.$_{max}$ 0.64 in water, and λ_{max} 530 nm and O.D.$_{max}$ 0.73 in ammonium sulphate solution.

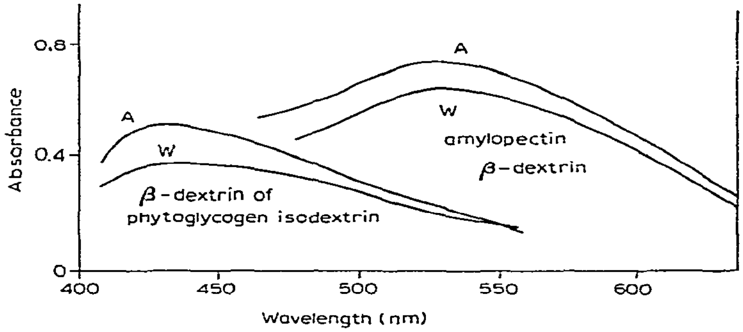


Fig. 6. Light-absorption curves of polysaccharide-iodine complexes; W, in aqueous solution; A, in presence of half-saturated ammonium sulphate.

CHANGES IN ENZYMIC ACTIVITY DURING THE GERMINATION OF SWEET CORN

Extracts which had been prepared from sweet corn, after germination for various periods, were made 70% saturated by the addition of solid ammonium sulphate, and the precipitated protein was collected by centrifugation (1,000 *g* for 20 min) and dissolved in water, and the solution was dialysed for 48 h against running tap-water and freeze-dried. (All operations were carried out at 2°.) The composition of the extracts is given in Table VII. The diffusable products of polysaccharide degradation and proteolysis were removed by the dialysis during the preparation of the extracts.

Each enzyme preparation (50 mg) was subjected to high-voltage electrophoresis, and aliquots of the extracts of each electrophoretic, paper strip were assayed against glycogen and amylopectin. Typical results are represented diagrammatically in Fig. 7. Although the high-voltage electrophoresis method did not separate the mixture of R-enzyme and isoamylase from each other, or the mixture of branching enzymes (Q-enzyme and amylopectin-branching glycosyltransferase), it provides clear evidence of the changes in enzymic activity which accompany germination.

TABLE VII

PROPERTIES OF EXTRACTS FROM GERMINATED SWEET-CORN

Germination time (h)	*pH of homogenate*	*Wt. of extract (g)*	*Protein-N content (%)*	*Polysaccharide content (%)*	*Debranching activity*[a]	
					Glycogen	*Amylopectin*
0	6.9	0.20	10.2	84	11	3
18	6.8	0.19	11.4	78	11	2
24	6.8	0.17	13.1	69	12	4
36	6.5	0.19	10.1	43	19	3
48	6.0	0.13	9.5	37	25	3
72	5.3	0.16	8.4	25	7	1
96	5.1	0.13	7.1	20	7	0
120	4.9	0.11	6.0	8	6	0
144	4.5	0.12	4.0	8	0	0

[a] Maximum percentage increase in iodine-staining power.

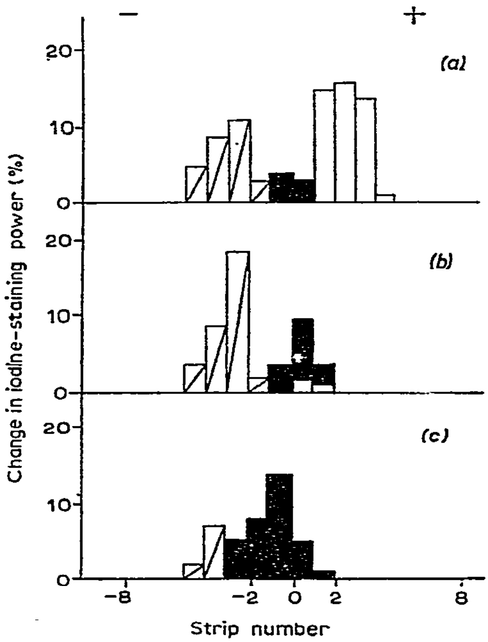


Fig. 7. Distribution of enzymes on high-voltage electrophoresis of sweet-corn extracts at various stages of germination (*a*) initially, (*b*) after 36 h, (*c*) after 96 h. Enzyme activities are shown: branching enzymes, □; debranching enzymes, ▨; α-amylase, ■; and are expressed in terms of the changes in iodine-staining power of glycogen and amylopectin. Each strip was 2 cm in width.

DISCUSSION

As shown in Fig. 7, ungerminated sweet-corn contains substantial amounts of branching and debranching enzymes, and a small amount of α-amylase. During germination, there is a rapid degradation of polysaccharide (more than 75% being broken down during 96 h), which is caused by an initial increase in debranching-enzyme activity, followed by a marked increase in α-amylolytic activity. During germination, branching-enzyme activity is diminished.

The continuous electrophoresis of an ammonium sulphate fraction of an extract of ungerminated sweet-corn revealed a cathodic migration of an enzymic activity which produced an increase in the iodine-staining power of glycogen and amylopectin. The enzyme preparation caused an increase in the β-amylolysis limit of both glycogens and amylopectins (Table V), and these results are the first demonstration of a plant enzyme which can hydrolyse the inter-chain linkages in glycogen. This electrophoretic preparation also showed maltase, limit-dextrinase, and pullulanase activities. Examination of the effect of pH, temperature, and various salts on the enzyme preparation (Tables II–IV) showed that two debranching enzymes were present, which were separated by gel filtration (Fig. 3) or by ultracentrifugation and continuous electrophoresis (Fig. 4).

The amylopectin-debranching enzyme (R-enzyme) was free of Q-enzyme, α-amylase, maltase, maltotriase, and pullulanase; the recent suggestion by Griffin and Erlander[14] that R-enzyme is a physical anomaly which acts on amylopectin because of the existence of limit dextrinase and α-amylase impurities is completely incompatible with our experimental observations. Sweet-corn R-enzyme, like that from broad beans[15] and malted barley[16], has no significant effect on glycogens of normal chain-length.

The action of sweet-corn isoamylase on phytoglycogen causes the liberation of maltosaccharides having *DP* 2–7, and the formation of an isodextrin which consists of the B-chains of the original polysaccharide. The separation of the maltosaccharides from the isodextrin provides definite evidence that debranching by α-(1→6)-glucosidase action had taken place, and that the observed changes in β-amylolysis limit and iodine-staining power were not due to the enzymic transfer of D-glucose residues from A-chains to B-chains. This latter process has been demonstrated with muscle[13] and yeast[17] debranching-enzyme preparations.

The residual isodextrin is still a glycogen-type polysaccharide, as shown by the degree of branching and by the iodine-staining properties of the isodextrin and the corresponding β-limit dextrin (Fig. 6). Erlander[18,19] has proposed that starch synthesis involves a glycogen-precursor mechanism in which glycogen is synthesised from sucrose *via* adenosine 5-(D-glucosyl pyrophosphate) (ADPG) and is then converted into amylopectin by a debranching enzyme. The liberated A-chains are combined together to form amylose. A major difficulty with this theory was that a plant enzyme which could debranch glycogen was not known prior to the present work. The enzymic debranching of glycogen to produce amylopectin would require a very selective attack on the α-(1→6)-D-glucosidic inter-chain linkages, in such a way that debranching occurred throughout the whole glycogen molecule, causing an increase in the interior chain-length (*i.e.*, the number of D-glucose residues between branch points) from *ca.* 3 to 6. The present results show this does not occur; enzyme action is confined to the removal of the outermost side-chains (A-chains), and the interior of the molecule is unaltered. As expected, the enzyme protein (whose molecular weight is presumably of the order of 10^4–10^5) is unable to penetrate into the interior of the phytoglycogen macromolecule which has a molecular weight of 10^6–10^7. The inability of other debranching enzymes (*eg.*, yeast isoamylase[20] and pullulanase[21]) to effect the complete debranching of glycogen is also attributed to similar steric effects. In contrast to the recent suggestion of Erlander and McGuire[22], we conclude that sweet-corn isoamylase is not involved in the enzymic conversion of phytoglycogen into amylopectin. Further comments on the glycogen-precursor theory have been given elsewhere[23]; it is relevant to emphasise here that starch synthesis according to this theory does not require the action of Q-enzyme[1] which occurs with other starch-metabolising enzymes in sweet corn. It seems more logical to suggest that the known starch-metabolising enzymes are involved in the synthesis of starch and phytoglycogen by separate pathways, rather than to ignore their occurrence and to propose instead the existence of other, hypothetical enzymes whose mode of action is very different from that of any known enzymes.

Extracts of sweet corn contains in addition to R-enzyme and isoamylase, a third α-(1→6)-glucosidase named pullulanase. The presence of this enzyme in sweet corn was first reported by Taylor and Whelan[24] who suggested that it was presumably akin to R-enzyme. The present results show that they are distinct enzymes, since, after gel filtration, the purified R-enzyme had no action on pullulan. Isoamylase likewise is a separate enzyme.

ACKNOWLEDGMENTS

The authors are indebted to the Science Research Council and the Royal Society for equipment grants, and to the University of Edinburgh for a postgraduate scholarship (to K.L.R.).

REFERENCES

1 D. J. Manners, J. J. M. Rowe, and K. L. Rowe, *Carbohyd. Res.*, 8 (1968) 72.
2 P. N. Hobson, W. J. Whelan, and S. Peat, *J. Chem. Soc.*, (1951) 1451.
3 Z. H. Gunja, D. J. Manners, and Khin Maung, *Biochem. J.*, 81 (1961) 392.
4 K. L. Rowe, Ph. D. Thesis, University of Edinburgh, 1967; D. J. Manners and K. L. Rowe, *Arch. Biochem. Biophys.*, 119 (1967) 585.
5 P. N. Hobson, W. J. Whelan, and S. Peat, *J. Chem. Soc.*, (1950) 3566.
6 J. D. Commerford, G. T. Van Duzee, and B. L. Scallett, *Cereal Chem.*, 40 (1963) 482.
7 N. Nelson, *J. Biol. Chem.*, 153 (1944) 375; M. Somogyi, *ibid.*, 195 (1952) 19.
8 M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith, *Anal. Chem.*, 28 (1956) 350.
9 C. R. Krisman, *Biochim. Biophys. Acta*, 65 (1962) 307.
10 W. J. Whelan, in *Encyclopedia of Plant Physiology*, Part. VI, W. Ruhland (Ed.), Springer-Verlag, Berlin, 1958, p. 154.
11 G. A. Gilbert and A. J. Swallow, *J. Chem. Soc.*, (1949) 2849.
12 N. Lavintman, *Arch. Biochem. Biophys.*, 116 (1966) 1.
13 D. H. Brown and B. I. Brown, *Methods Enzymol.*, 8 (1966) 515.
14 H. L. Griffin and S. R. Erlander, unpublished work cited in ref. 22.

15 S. Peat, W. J. Whelan, P. N. Hobson, and G. J. Thomas. *J. Chem. Soc.*, (1954) 4440; I. D. Fleming and D. J. Manners, *Chem. Ind.* (London), (1958) 831.
16 D. J. Manners and K. L. Sparra, *J. Inst. Brewing*, 72 (1966) 360.
17 E. Y. C. Lee, L. D. Nielsen, and E. H. Fischer, *Arch. Biochem. Biophys.*, 121 (1967) 245.
18 S. R. Erlander, *Enzymologia*, 19 (1958) 273.
19 S. R. Erlander and H. L. Griffin *Stärke*, 19 (1967) 34.
20 G. N. Bathgate and D. J. Manners, *Biochem. J.*, 107 (1968).
21 M. Abdullah, B. J. Catley, E. Y. C. Lee, J. Robyt, K. Wallenfels, and W. J. Whelan, *Cereal Chem.*, 43 (1966) 111.
22 S. R. Erlander and J. P. McGuire, *Stärke*, 19 (1967) 402.
23 D. J. Manners, *Advan. Carbohyd. Chem.*, 17 (1962) 371.
24 P. M. Taylor and W. J. Whelan, *Biochem. J.*, 95 (1965) 26P.

Note

A 1:1 adduct of methyl α-D-glucopyranoside and potassium hydrogen carbonate*

During an investigation of a reaction of methyl α-D-glucopyranoside (**1**) carried out in potassium hydroxide solution, a substance was isolated which apparently has not been reported previously[1]. It could be isolated when the basic solution was treated with carbon dioxide.

The colorless crystalline solid (**2**) was recrystallized from 95% ethanol to m.p. 192–194° (with decomposition). Titrimetric analysis of the compound indicated an equivalent weight of 294.3, corresponding to the formula $C_7H_{14}O_6 \cdot KHCO_3$, a 1:1 adduct of methyl α-D-glucopyranoside and potassium hydrogen carbonate. The elemental analysis was also consistent with this formula. A comparison of the i.r. spectra of **2** and **1** showed many similarities, but they were clearly not identical. Acetylation of **2** produced methyl 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranoside, identical with an authentic sample. A 2:1 adduct of amylose and potassium hydrogen carbonate has been reported by Senti and Witnauer[2].

N.m.r. spectra of the adduct (**2**) and **1** were obtained in formamide and in methyl sulfoxide-d_6 (see Table I). In formamide solution, the hydroxyl–proton signals of **1** are centered at τ 4.82, whereas those of the exchangeable hydrogens of **2** (four hydroxyl protons and the hydrogen carbonate proton) are masked by the solvent. A comparison of the spectra of **1** and **2** in methyl sulfoxide-d_6 showed that the hydroxyl-proton signals of **2** are shifted upfield[3,4] by 0.5 p.p.m. Integration of the spectrum of **2** in methyl sulfoxide-d_6 before and after the addition of D_2O showed that another exchangeable proton resonates in the τ 6.38–6.98 region. It is presumed that this resonance is that of the hydrogen carbonate proton, because it has approximately the same chemical shift as the hydrogen carbonate proton in methyl sulfoxide-d_6 solution. The adduct (**2**) can be recovered unchanged from formamide and from methyl sulfoxide-d_6 by addition of a less polar solvent.

Synthesis of **2** was accomplished either by treating a potassium hydroxide solution containing **1** with carbon dioxide, or by bringing together equimolar amounts of **1** and potassium hydrogen carbonate in water.

EXPERIMENTAL

Melting points are uncorrected. I.r. spectra were recorded on a Perkin–Elmer Infracord spectrometer, Model 137. N.m.r. spectra were measured on a Varian

---

*The University of Arizona, Agricultural Experiment Station, Paper No. 1360.

TABLE I

N.M.R. SPECTRAL DATA FOR METHYL α-D-GLUCOPYRANOSIDE AND THE METHYL α-D-GLUCOPYRANOSIDE–POTASSIUM HYDROGEN CARBONATE ADDUCT[a]

Compound	*2-OH, 3-OH, 4-OH, 6-OH*	*H-1*	*Hydrogen carbonate proton*	*Ring protons*	*OMe*
1[b]	4.82	5.51d (2.6)		6.37–7.02	6.84
2[b]		5.57d (3.1)		6.44–7.16	7.00
$KHCO_3$[b]			6.03		
1[c]	5.15–5.61	5.43d (3.4)		6.15–6.90	6.71
2[c]	5.78–6.18	5.48	~6.9	6.38–6.98	6.76
$KHCO_3$[c]			6.71		

[a]Chemical shifts are given on the τ scale, relative to Me_4Si (Me_2SO-d_6); d, doublet; J values (Hz) are given in parentheses. [b]In $HCONH_2$. [c]In Me_2SO-d_6.

Associates HA-100 spectrometer (100 MHz), and tetramethylsilane (τ = 10.00) was used as the internal standard. Methyl sulfoxide-d_6 was obtained from Merck, Sharp and Dohme, Ltd., Canada, and was used without purification. All peaks assigned to hydroxyl groups were confirmed by exchange with deuterium oxide. The elemental analysis was performed by Schwarzkopf Microanalytical Laboratory, Woodside, New York.

Formation of methyl α-D-glucopyranoside–potassium hydrogen carbonate adduct (**2**) *by carbonation.* — A mixture of 5.0 g (90 mmoles) of potassium hydroxide, 50 g (250 mmoles) of methyl α-D-glucopyranoside, and 50 ml of water was warmed to give a clear solution. The solution was cooled to room temperature, and carbon dioxide gas was bubbled through it until no further change in pH occurred. The solution was evaporated to dryness on a rotary evaporator, and the residue was extracted with five 100-ml portions of hot 95% ethanol. The extracts were combined, and kept overnight at room temperature, and the crystalline material that separated was collected by filtration. This crude product, m.p. 191–194° (dec.), was recrystallized from 95% ethanol to give 22.5 g (85%) of colorless, crystalline product; m.p. 192–194° dec., ν_{max}^{KBr} 3600–3200 vs, 2900 m, 1440 m, 1420 m, 1360 m, 1330 m, 1280 sh, 1190 w, 1145 m, 1110 m, 1075 sh, 1050 vs, 1025 vs, 1010 sh, 990 sh, 918 sh, 900 w, 835 w, and 755 w cm^{-1}. Methyl α-D-glucopyranoside gave ν_{max}^{KBr} 3600–3200 vs, 2900 m, 1450 w, 1420 w, 1390 sh, 1360 w, 1330 w, 1280 sh, 1260 w, 1220 w, 1190 m, 1140 ms, 1110 ms, 1100 ms, 1070 sh, 1060 sh, 1055 vs, 1045 vs, 990 ms, 900 w, 845 w, and 745 w cm^{-1}. (Both spectra were calibrated by means of the polystyrene band at 1601 cm^{-1}.)

Anal. Calc. for $C_8H_{15}KO_9$: C, 32.65; H, 5.15, ash (K), 13.29. Found: C, 32.49; H, 5.10, ash (K), 13.06.

Formation of methyl α-D-glucopyranoside–potassium hydrogen carbonate adduct (**2**) *from the component compounds.* — To a solution of 20 g (200 mmoles) of potassium hydrogen carbonate in 50 ml of water was added 39 g (200 mmoles) of methyl α-D-glucopyranoside. The mixture was warmed, and the resulting solution was evaporated to dryness on a rotary evaporator. The residue was extracted with nine 100-ml portions of 95% ethanol, and the extracts were combined, and kept overnight at room temperature. The crude product that separated was recrystallized from 95% ethanol to give 49 g (83%) of colorless crystals, m.p. 192–194° (dec.).

*Formation of methyl 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranoside by acetylation of the adduct* (**2**). — Acetylation of 2.9 g (10 mmoles) of **2** was accomplished according to a published procedure[5]. The product, yield 2.35 g (65%), was identical in all respects with an authentic sample of the tetraacetate.

ACKNOWLEDGMENTS

This work was done under contract with the U.S. Department of Agriculture, and authorized by the Research and Marketing Act. The contract was supervised by the Northern Utilization Research and Development Division, Agricultural Research Service.

Department of Agricultural Biochemistry,
University of Arizona,
Tucson, Arizona 85721 (U. S. A.)

J. W. BERRY
B. I. MAYALL
J. T. MARVEL
A. J. DEUTSCHMAN, JR.

REFERENCES

1 J. A. RENDLEMAN, JR., *Advan. Carbohyd. Chem.*, 21 (1966) 216.
2 F. R. SENTI AND L. P. WITNAUER, *J. Polymer Sci.*, 9 (1952) 115.
3 B. CASU, M. REGGIANNI, G. GALLO, AND A. VIGEVANI, *Tetrahedron Lett.*, (1965) 2253.
4 J. T. MARVEL, S. K. SEN, J. W. BERRY, AND A. J. DEUTSCHMAN, JR., *Carbohyd. Res.*, 8 (1968) 148.
5 G. N. BOLLENBACK, *Methods Carbohyd. Chem.*, 2 (1963) 327.

(Received June 7th, 1968)

Preliminary communication

Synthesis of 2-deoxy-2-methylamino-D-gulose, a component of streptothricin-like antibiotics

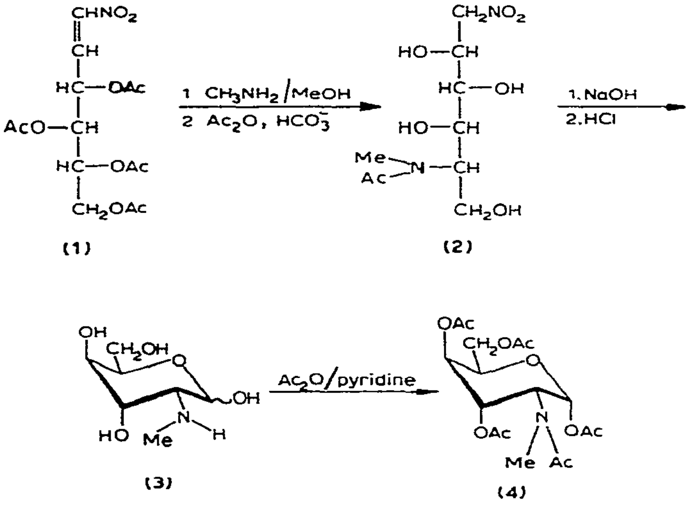
The present communication describes the synthesis of 2-deoxy-2-methylamino-D-gulose, reported to be a component of the streptothricin-like antibiotic, LL-AC 541[1], and possibly of the similar antibiotic BD-12 isolated in our laboratory[2].

Addition of methylamine to tetra-*O*-acetyl-1-nitro-1-hexene-D-*xylo*-3,4,5,6-tetrol (**1**) synthesized by the method of Sowden and Fischer[3] from D-xylose and nitromethane gave a syrupy mixture presumably consisting of 5,6-dideoxy-5-methylacetamido-6-nitro-L-glucitol (**2**) in preponderant proportion and of the corresponding D-iditol derivative[4]. On treatment with conc. hydrochloric acid (Nef reaction)[5], the sodium salt of the above mixture gave a crude 2-deoxy-2-methylaminohexose, which was chromatographed on Dowex-50 (H$^+$) with 0.2M hydrochloric acid as developing agent. The main fractions showing a positive Elson–Morgan test and containing a single component, as shown by paper chromatography, were evaporated to give a 2-deoxy-2-methylaminohexose hydrochloride (yield 25%). This was repeatedly recrystallized from methanol–ethanol to give pure 2-deoxy-2-methylamino-D-gulose hydrochloride (**3**) as white needles, m.p. 181° (dec.); $[\alpha]_D^{26}$ +38.5° (after 5 min) → -28° (after 20 h) (*c* 1, water); R_F 0.72 on Whatman No. 1 paper in ethyl acetate–acetic acid–pyridine–water (5:5:1:3)[6].

Anal. Calc. for $C_7H_{15}NO_5 \cdot HCl$: C, 36.61; H, 7.02; N, 6.10. Found: C, 36.72; H, 7.13; N, 6.49.

The pentaacetate (**4**) was obtained from **3** with pyridine–acetic anhydride; m.p. 175–176°, $[\alpha]_D^{21}$ +72° (*c* 1, chloroform).

Anal. Calc. for $C_{17}H_{25}NO_{10}$: C, 50.62; H, 6.25; N, 3.47. Found: C, 50.64; H, 6.41; N, 3.15.



The structure of 3 was established by positive Tollens and Elson–Morgan tests and by a negative ninhydrin test indicating the *N*-substitution of the 2-amino-2-deoxyaldose.

The n.m.r. spectra were recorded with a JEOL-JNM-C-60 spectrometer at 60 MHz and are expressed as p.p.m. from an external tetramethylsilane reference (δ-scale) for deuterium oxide solution or from an internal tetramethylsilane reference for deuterated chloroform solution. The n.m.r. signal of 3 at δ 2.98 (3 H, singlet) in deuterium oxide solution confirmed the presence of the *N*-methyl group. The configuration of C-3, C-4 and C-5 is unequivocal, because 3 was derived from D-xylose. In a freshly prepared deuterium oxide solution of 3 at 26°, a pair of doublets corresponding to a total of one proton (intensity ratio, 3:2) at δ 5.58 (J 3.0 Hz) and δ 5.22 (J 8.0 Hz) and a triplet and a quartet corresponding to a total of one proton (intensity ratio 3.3:2) at δ 3.63 (J 3.0 and 3.0 Hz) and δ 3.30 (J 3.0 and 8.0 Hz) were observed. After 4 h, both intensity ratios had changed to 1:3 owing to mutarotation.

On the basis of Lemieux[1] and Stevens' work[7], the n.m.r. signals of 3 were assigned as follows: doublet at δ 5.58 to H-1-α with $J^{\alpha}_{1,2}$ 3.0 Hz; doublet at δ 5.22 to H-1-β with $J^{\beta}_{1,2}$ 8.0 Hz; triplet at δ 3.63 to H-2-α with $J^{\alpha}_{1,2} = J^{\alpha}_{2,3}$ 3.0 Hz; quartet at δ 3.30 to H-2-β with $J^{\beta}_{1,2}$ 8.0 and $J^{\beta}_{2,3}$ 3.0 Hz. These assignments were confirmed by spin-decoupling experiments.

The n.m.r. signal at δ 6.13 (doublet, H-1, J 3.0 Hz) of 4 indicated that it was the α-pentaacetate. Signals at δ 5.50 (1 H, triplet, J 3.0 and 3.0 Hz) and 5.25 (1 H, triplet, J 3.0 and 3.0 Hz) were attributed to H-2 and H-3 respectively. The large coupling constant between H-1-β and H-2-β ($J_{1,2}$ 8 Hz) indicated an axial orientation of H-2. Therefore, the small coupling constant between H-2 and H-3 ($J_{2,3}$ 3 Hz) suggested an equatorial orientation of H-3. thus indicating a *cis* relationship between C-2 and C-3, in agreement with the *gulo* configuration.

2-Deoxy-2-methylamino-D-gulose was a major product of the present reaction. No other Elson–Morgan positive substances, except 3 could be isolated from the crude reaction product, although some could be detected on paper chromatogram.

The R_F value and g.l.c. pattern of the per(trimethylsilyl)derivative of 3 were identical with those of the 2-deoxy-2-methylaminohexose isolated from the antibiotic BD-12.

The procedure described in this communication is different from that briefly reported by Noorzad *et al.*[8]. Direct comparison of the 2-deoxy-2-methylamino-D-gulose reported in this communication with that synthesized by Noorzad *et al.*[8] showed both compounds to be identical.

The authors thank Dr. T. Okuda, manager of this laboratory, for his encouragement, Dr. K. Kotera and his collaborators of the Analytical Center of this Company for the instrumental and elementary analyses and Dr. H. K. Zimmerman for a sample of synthetic 2-deoxy-2-methylamino-D-gulose.

Microbial Chemistry Research Laboratory,
Tanabe Seiyaku Co. Ltd.,
Toda-shi, Saitama-ken, Japan

Y. ITO
Y. OHASHI
T. MIYAGISHIMA

REFERENCES

1 D. B. Borders, W. K. Hausman, E. R. Wetzel, and E. L. Patterson, *Tetrahedron Lett.*, (1967) 4187.
2 Y. Ito, Y. Ohashi, Y. Sakurai, M. Sakurazawa, S. Awataguchi and T. Okuda, *J. Antibiotics*, 21 (1968) 307.
3 J. C. Sowden and H. O. L. Fischer, *J. Amer. Chem. Soc.*, 69 (1947) 1048.
4 *cf.* J. C. Sowden and M. L. Oftedahl, *J. Org. Chem.*, 26 (1961) 2153.
5 J. C. Sowden and H. O. L. Fischer, *J. Amer. Chem. Soc.*, 66 (1944) 1312.
6 F. G. Fischer and H. J. Nebel, *Z. Physiol. Chem.*, 302 (1955) 10.
7 R. U. Lemieux and J. D. Stevens, *Canad. J. Chem.*, 44 (1966) 249.
8 H. Noorzad, H. K. Zimmerman, and P. H. Gross, *Abstr. Papers Amer. Chem. Soc. Meeting*, 155 (1968) 18C.

(Received September 30th, 1968; in revised form, November 18th, 1968)

Announcement

The *Carbohydrate Discussion Group* (A Chemical Society Subject Group) is holding a meeting at the University College of North Wales at Bangor from March 31st to April 2nd, 1969. The meeting will include a small symposium on polysaccharide chemistry.

For further details of this meeting, please write to Dr. N. A. Hughes, Department of Organic Chemistry, The University, Newcastle upon Tyne NE1 7RU.

Book review

Rodd's Chemistry of Carbon Compounds, Volume IF, Carbohydrate Chemistry: edited by S. Coffey. Elsevier, Amsterdam, 1967, xvi + 780 pp. £14. 15s. (£13. 5s. subscription price).

This volume of the second edition of Rodd's Chemistry of Carbon Compounds can be considered not only as part of a series, but also in isolation as a text-book of carbohydrate chemistry. It is divided into three sections: polyols (66 pages) by L. Hough and A. C. Richardson, monosaccharides and their derivatives (529 pages) by the same authors, and oligosaccharides, polysaccharides, and related compounds (119 pages) by G. O. Aspinall, E. Percival, D. A. Rees, and M. Rennie; there is a subject index but no author index. The contents of this volume show the extent of the advances and developments in carbohydrate chemistry in the fifteen years since the first edition was published.

The authors are to be congratulated on producing an excellent, up-to-date account of carbohydrate chemistry; many 1967 references are included. Modern concepts are stressed, and conformational and mechanistic aspects of many reactions are discussed. Very good accounts are given of the application of physical methods; the section on the uses of nuclear magnetic resonance is exceptionally good and is an ideal introduction for anyone wishing to learn about the application of this powerful tool to carbohydrate chemistry.

The text is relatively free from errors. The most serious found by the Reviewer was the incorrect pyrazoline structure shown on page 440 for the product from the acetylation of D-mannose phenylhydrazone. The correct structure for this product (D-*arabino*-3,4,5,6-tetra-acetoxy-1-arylazo-*trans*-hex-1-ene) was established in 1962. In the section on the general methods of structural study of polysaccharides (p. 666 ff.), acetolysis is not mentioned. On page 139, tetramethylsilane is described as "a toxic substance", an error which it is hoped has not alarmed too many n.m.r. spectroscopists; on page 90, there is a grossly incorrect formula for L-glucose.

The main criticism of this book must fall on the Subject Index, which is not compiled using the usual conventions. Compounds are not listed under the parent sugar, as, for example, D-glucofuranose, di-*O*-isopropylidene-, but appear under the first letter of the name; hence, the above compound is listed under "D".

It is a great pity that the prices of more and more essential reference books put them beyond the reach of potential purchasers. Here is an excellent text that ought to be in the hands not only of professional carbohydrate chemists (and it will be too expensive for many) but also of every graduate student as well.

University of Sussex R. D. Guthrie

Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

ENOLIZATION OF ALDOSULOSE DERIVATIVES. 4-*O*-ACETYL-1,6-ANHYDRO-2,3-*O*-ISOPROPYLIDENE-β-D-*threo*-HEX-3-ENOPYRANOSE, THE 3,4-ENEDIOL ACETATE OF A FUSED-RING *keto* SUGAR, AND FORMATION OF A BRANCHED-CHAIN SUGAR DERIVATIVE*†

D. Horton and E. K. Just
Department of Chemistry, The Ohio State University, Columbus, Ohio 43210 (U.S.A.)

(Received July 26th, 1968)

ABSTRACT

1,6-Anhydro-2,3-*O*-isopropylidene-β-D-*lyxo*-hexopyranos-4-ulose (**1**), a *keto* sugar having a [3.2.1] bicyclic ring-system, is converted by acetic anhydride–triethylamine at room temperature into a crystalline dimer (**5**), a branched-chain sugar derivative apparently formed by attack of C-3 of the 3,4-enediolate anion of a molecule of **1** on the carbonyl group of a second molecule of **1**. Under more vigorous conditions, the reagent converts the dimer **5** into the crystalline 3,4-enediol acetate (**2**) of the ketone **1**; no 4,5-enediol acetate is formed because of steric reasons (Bredt rule). The enediol acetate **2** is hydrolyzed readily to the parent ketone **1**, and is converted by ethanolic base into the crystalline, hydrated ketone **3**. Reduction of **2** with borohydride gives 1,6-anhydro-2,3-*O*-isopropylidene-β-D-talopyranose (**6**). The n.m.r. spectrum of the enediol acetate **2**, analyzed with the use of spin decoupling, shows a long-range coupling between H-2 and the *exo* proton at H-6.

INTRODUCTION

Previous papers from this laboratory have reported the synthesis of 1,6-anhydro-β-D-talopyranose[2,3], the last 1,6-anhydro-β-D-hexopyranose to be characterized. 1,6-Anhydro-2,3-*O*-isopropylidene-β-D-mannopyranose (**4**) was oxidized to the corresponding 4-ketone[4] (1,6-anhydro-2,3-*O*-isopropylidene-β-D-*lyxo*-hexopyranos-4-ulose, **1**), the latter was reduced stereospecifically to the D-*talo* analog (**6**) of **4**, and the *O*-isopropylidene group was cleaved from **6**. Optical rotatory correlations among the 1,6-anhydro-β-D-hexopyranoses have been made[5], and aminated analogs have been synthesized[6]. It was shown[2] that H-3 of the ketone **1** is exchanged readily by solvent protons in aqueous base, whereas H-5, which is at the bridgehead of a [3.2.1] bicyclic system, is not exchanged. This type of exchange reaction, in similar

---

*Supported by the National Institutes of Health, Public Health Service, Department of Health, Education, and Welfare, Bethesda, Maryland 20014; Grant No. GM-11976-04 (The Ohio State University Research Foundation Project 1820).

†For a preliminary report, see ref. 1.

fused-ring systems, was suggested[2] as a stereospecific method for the synthesis of specifically deuterated or tritiated sugars, and also as a route to branched-chain systems. Although it was considered that planarity at C-5 (the bridgehead of the [3.2.1] bicyclic system) would be sterically impossible in **1**, the observed exchange of H-3 clearly demonstrates that there is no such restriction at C-3, although C-3 is at the bridgehead of a [4.3.0] bicyclic system formed by the cyclic acetal group fused to the pyranose ring.

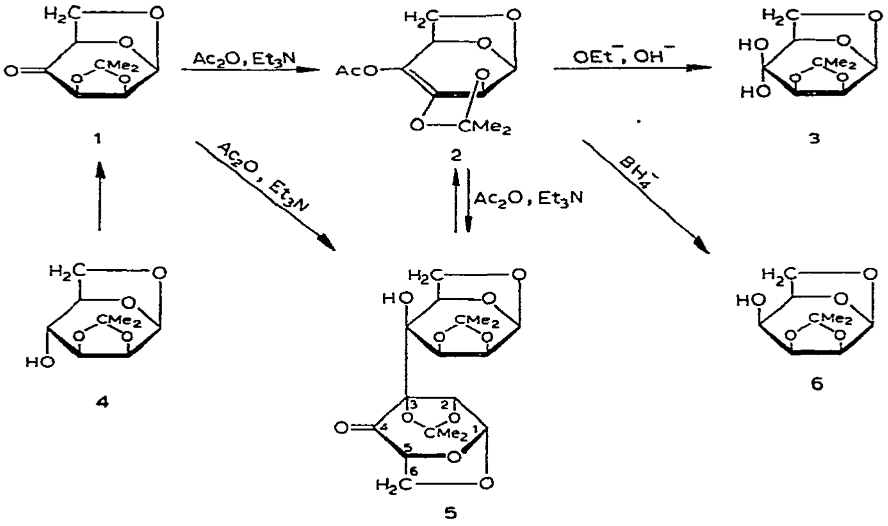
Inspection of molecular (Dreiding) models indicated that the 3,4-enediol of the ketone **1** is, indeed, sterically feasible. The object of the present work was to prepare and characterize a stable derivative of this enediol, in order to provide independent proof that a double bond can be formed at the bridgehead of this [4.3.0] bicyclic system. In the case of a 1,2-cyclic acetal of a *furanose* sugar, it has been supposed[7] that a double bond at the bridgehead of a [3.3.0] bicyclic system is sterically impossible.

Enediol acetates of *keto* sugars have received very little study. Now that fully protected sugars having free carbonyl groups are readily accessible[4], acyl derivatives of their enolic forms offer attractive possibilities for synthesis of novel sugars, including branched-chain sugars, by stereospecific addition reactions at the carbon atom adjacent to the original ketone group.

DISCUSSION

Oxidation of the alcohol **4** to the ketone **1** can be accomplished in high yield[4] by use of either methyl sulfoxide–acetic anhydride[8] or ruthenium tetraoxide[9]. The latter oxidant is the more convenient for preparing small quantities of very pure ketone, but losses of the expensive reagent are a disadvantage. A modified procedure used in the present work gave pure ketone **1** in 85% yield, and permitted quantitative recovery of the reagent. Ruthenium dioxide was oxidized to the tetraoxide by aqueous sodium periodate *in the presence of* carbon tetrachloride[10]; in this way, the reducing substances present in reagent-grade carbon tetrachloride were decomposed before the solution of oxidant was separated, and therefore they could not interfere with the oxidation. At the end of the oxidation, the excess of the oxidant was reduced with isopropyl alcohol, and ruthenium dioxide was recovered quantitatively.

Treatment of the ketone **1** with acetic anhydride and triethylamine for 1 day at room temperature led to almost complete conversion of the starting ketone into a new product, which was isolated crystalline, m.p. 175–176.5°. This product had the same empirical formula as the starting ketone **1**, and was subsequently shown to be a dimer (**5**). When the ketone **1** was heated for 3 days at 50° in acetic anhydride–triethylamine, the dimer **5** was still a major component. However, a second product, having a chromatographic mobility greater than that of **5**, observed as a minor component in the reaction mixture from which **5** had been isolated, was now present in greater proportion. This faster-moving component was isolated crystalline (m.p. 129–130°) in 19% yield by sublimation from the reaction mixture, and it was shown to be the 3,4-enediol acetate (**2**) of **1**. Treatment of the pure dimer **5** with acetic anhydride–triethylamine for 3 days at 70° led also to a mixture of **5** and the enediol acetate **2**; the latter was obtained in 33% yield. The dimer **5** is evidently the kinetic product of the reaction, and it is subsequently converted into the enediol acetate **2**; the latter is probably in equilibrium with **5**.



The initial product (**5**), having m.p. 175–176.5°, showed i.r. spectral absorption for a carbonyl group and a hydroxyl group. The hydroxyl group was inferred to be tertiary, because it had not been acetylated by the acetic anhydride. The n.m.r. spectrum showed that acetyl groups were absent, and the carbonyl absorption in the i.r. spectrum was therefore assigned to a ketonic group. Although the elemental analysis was consistent with the formula $C_9H_{12}O_5$, the n.m.r. spectrum in chloroform-*d* showed a sharp singlet at τ 6.47, one twenty-fourth of the total proton integral, which could be assigned to the proton of the tertiary hydroxyl group because the signal disappeared when the sample was deuterated. Assuming that one hydroxyl group is present per molecule, the dimeric formula $C_{18}H_{24}O_{10}$ is indicated. The n.m.r. spectrum showed the presence of two isopropylidene acetal groups and two one-proton doublets at low field in the region anticipated for anomeric protons in 1,6-anhydrohexose derivatives. These data, and the fact that the dimer can be converted into the enediol acetate **2**, indicate that the dimer is an adduct (**5**) that is formed through attack by C-3 of the 3,4-enediolate anion of one molecule of **1** on C-4 of a second molecule of **1**, in a base-catalyzed, reversible, Claisen type of reaction (see Fig. 1). The fact that the ketone **1** is attacked by even small nucleophiles to give products having the D-*talo* configuration, exclusively[3,6], provides very strong indication that the branched-chain sugar moiety of **5** has the D-*talo* configuration, although this structure has not been proved by classical degradative methods.

The anomeric-proton signal at lowest field (τ 4.21) for **5** is a sharp doublet showing a spacing of 3.8 Hz. This signal, which has a chemical shift similar to that of H-1 in the parent ketone **1**, was assigned to the anomeric proton* (H-1′) of the ketonic moiety of **5**, because a second sharp doublet (at τ 5.16), having identical spacing and related to the signal at τ 4.21 as a typical AB pattern, could be assigned

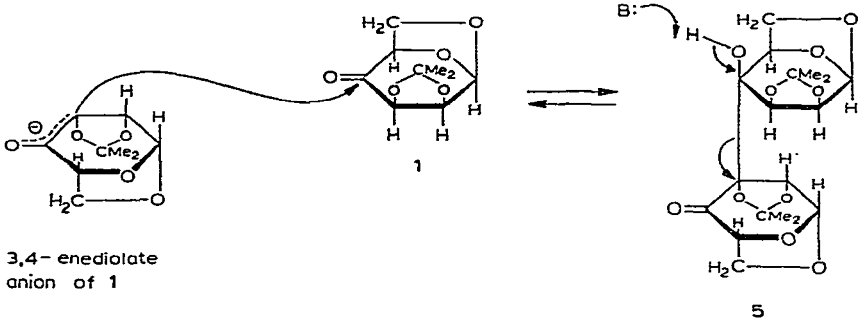


Fig. 1. The reversible, base-catalyzed condensation of the 3,4-enediolate anion of ketone **1** with **1** to give the dimer **5**. The reverse process probably proceeds by initial abstraction of the proton on the 4-hydroxyl group of **5**, because H-3 on the branched-chain moiety is not in the favored geometry for E2 elimination.

to a contiguous proton at C-2′ in a structure having no proton at C-3′. The other anomeric-proton signal, at τ 4.76, was somewhat broadened, and was assigned to H-1 in the branched-chain moiety; the line-broadening is probably caused by long-range or virtual coupling[4,11] of H-1 with H-3 in this moiety.

The product having m.p. 129–130°, obtained by prolonged treatment of the ketone **1** with acetic anhydride–triethylamine, had the empirical formula $C_{11}H_{14}O_6$ anticipated for an enediol acetate of **1**, and the i.r. spectrum showed absorptions at 5.63 and 5.71 μm, suggesting that a conjugated, unsaturated ester was present. The n.m.r. spectrum verified that one acetyl group and one *O*-isopropylidene group

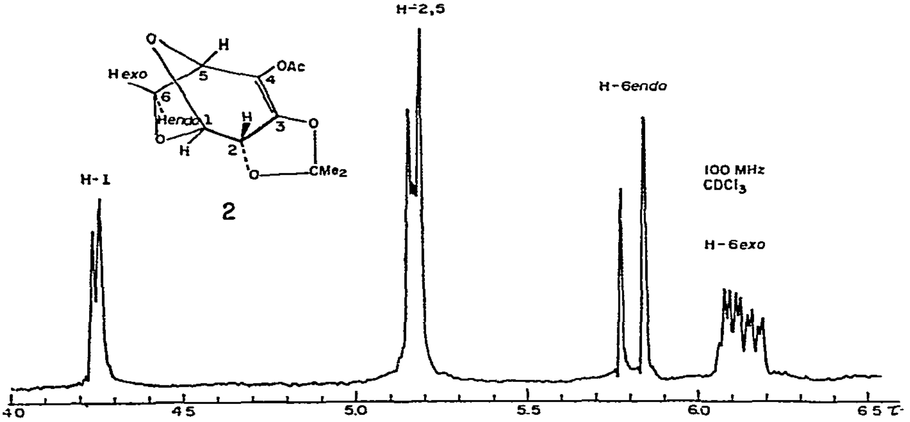


Fig. 2. The low-field portion of the 100-MHz spectrum of the enediol acetate **2** in chloroform-*d*.

---

*Primed numbers refer to the positions on the ketonic moiety of 5; unprimed numbers refer to positions on the branched-chain moiety.

were present, and the low-field portion of the spectrum (see Fig. 2) entirely confirmed the independent chemical evidence that showed the structure to be the 3,4-enediol acetate (**2**) of the ketone **1**.

The multiplicity observed (see Fig. 2) for the signals at τ 5.81 and 6.13 (methylene protons[12]) indicated that a proton was present at C-5 of compound **2**. The spacing of 6.9 Hz in the doublet at τ 5.81 was also present in the octet at τ 6.13. Irradiation of the τ 5.81 doublet caused perturbation of the octet, and the assignment of the two signals to the protons at C-6 was thus confirmed. The doublet at τ 5.81 was assigned to the *endo* proton at C-6; its dihedral angle with H-5 is ~90°, so that the $J_{5,6endo}$ coupling is approximately zero[13]. The H-6*exo* signal at τ 6.13 showed, in addition to the $J_{6exo,6endo}$ coupling, spacings of 3.1 Hz and 1.2 Hz. The former spacing was assigned to $J_{5,6exo}$ coupling, and the latter to a long-range coupling. Since irradiation of the H-6*exo* signal caused collapse of the H-6*endo* signal, but did not perturb the H-1 doublet at τ 4.25, the spacing of 1.2 Hz was assigned to long-range coupling of H-6*exo* with H-2. The remaining signal, a narrow, 2-proton multiplet near τ 5.18, was assigned to H-2 and H-5. The signal was perturbed by irradiating either the H-1 or H-6*exo* signal, and irradiation at τ 5.18 caused the H-1 signal to collapse to a singlet and the H-6*exo* signal to collapse to a doublet having the same spacing as the H-6*endo* signal.

Under anhydrous conditions, the enediol acetate **2** appeared to be stable indefinitely, but, when it was stored without exclusion of moisture, it decomposed over a period of about 2 weeks to give the original ketone **1**.

Attempts to prepare the enediol acetate **2** by treating the ketone **1** with acetic anhydride and piperidine, or with isopropenyl acetate and an acidic catalyst, did not give the desired product (**2**).

Treatment of the enediol acetate **2** with ethanolic potassium hydroxide for 3 h at room temperature gave a crystalline product, m.p. 94–98°, that appeared to be the hydrate (**3**) of the ketone **1**; it showed strong absorption for hydroxyl groups in its i.r. spectrum and, on sublimation, it was converted quantitatively into the ketone **1**.

Reduction of the enediol acetate **2** with ethanolic sodium borohydride was complete in 15 min at room temperature, and the product was 1,6-anhydro-2,3-*O*-isopropylidene-β-D-talopyranose (**6**). None of the 4-epimer of **6** was detected, indicating that the reduction of **2** to **6** was stereospecific. The rapidity of the reduction suggests that it does not proceed by way of the ketone **1**.

Further work is in progress on **2**, **5**, and related systems, to assess the utility of enol acetates and of such branched-chain structures as **5** in syntheses leading to various modified sugars of biological interest.

EXPERIMENTAL

General. — Solutions were evaporated below 50° under diminished pressure. Melting points were determined with a Thomas–Hoover "Unimelt" apparatus and are uncorrected. I.r. spectra were measured with a Perkin–Elmer Model 137 "Infracord" i.r. spectrophotometer. N.m.r. spectra were measured at 60 and 100 MHz with Varian A-60 and HA-100 n.m.r. spectrometers, respectively. Spin-decoupling experiments were performed with the HA-100 instrument. Chemical shifts are given on the τ scale. Unless otherwise stated, spectra were measured at ~30° with solutions (~10%) in chloroform-*d*, with tetramethylsilane (τ = 10.00) as the internal standard. Spectra were analyzed on a first-order basis. Microanalyses were performed by W. N. Rond. X-Ray powder diffraction data give interplanar spacings, Å, for CuKα radiation. Relative intensities were estimated visually: m, moderate; s, strong; v, very; w, weak. The strongest lines are numbered (1, strongest), and double numbers indicate approximately equal intensities. The camera diameter was 114.59 mm. T.l.c. was effected with 250-μm layers of Silica Gel G (E. Merck, Darmstadt, Germany), activated at 110°, as the adsorbent, and 3:1 chloroform–ether as the developer, and indication was effected with sulfuric acid.

*Preparation of 1,6-anhydro-2,3-O-isopropylidene-β-*D-lyxo*-hexopyranos-4-ulose* (**1**). — The following modification of the original procedure[4] was judged to be the most convenient preparative route to the ketone **2**, because of its simplicity; a high yield of pure product is obtained, and the ruthenium dioxide is recovered. A mixture of ruthenium dioxide (5 g), carbon tetrachloride (250 ml), and 10% aqueous sodium periodate (100 ml) was stirred for 0.5 h at 0°. The organic phase was separated, and the aqueous solution was extracted with two 250-ml portions of carbon tetrachloride. The three organic extracts were combined and added, without being dried, to a slurry of 1,6-anhydro-2,3-*O*-isopropylidene-*β*-D-mannopyranose[4,14] (**4**, 4.0 g) in carbon tetrachloride (100 ml) at 0°. The mixture was stirred for 2–3 h at 0° and for 4–5 h at room temperature, and isopropyl alcohol was then added dropwise to decompose the excess of ruthenium tetraoxide; the latter could be detected by its characteristic odor. Filtration of the suspension gave, in almost quantitative yield, ruthenium dioxide, which could be re-used. Evaporation of the filtrate gave the pure ketone **1**, yield 3.40 g (85%), having physical properties in agreement with those reported by Horton and Jewell[4].

*4-O-Acetyl-1,6-anhydro-2,3-O-isopropylidene-β-*D-threo*-hex-3-enopyranose* (**2**). — A solution of the ketone **1** (1.0 g) in a mixture of acetic anhydride (20 ml) and triethylamine (5 ml) was kept for 3 days at 50° under a reflux condenser; t.l.c. then showed that all of the starting material (R_F 0.30) had disappeared, and had been replaced by major components having R_F 0.74 and 0.64, together with minor components having R_F 0.45, 0.32, and 0.17. The dark-brown solution was evaporated at 40°/0.2 torr to a syrup that was extracted with three 50-ml portions of ethyl ether. The extracts were combined and evaporated, and the resultant syrup was kept in a sublimation apparatus for 12 h at 60°/0.2 torr. The yellow sublimate was dissolved in carbon tetrachloride, petroleum ether (b.p. 30–60°) was added to incipient turbidity, and the solution was refrigerated, giving the enediol acetate **2** as white needles; yield 0.23 g (19%), m.p. 129–130°, $[\alpha]_D^{20}$ $-209 \pm 2°$ (*c* 0.4, chloroform); R_F 0.74; λ_{max}^{KBr} 5.63, 5.71 (C=C–C=O), 7.25 μm (CMe$_2$); n.m.r. data (100 MHz, see Fig. 1): τ 4.25 (1-proton doublet, $J_{1,2}$ 2.1 Hz, H-1), τ 5.18 (2-proton multiplet, width between outer peaks ~3.3 Hz, H-2,5), τ 5.81 (1-proton doublet, $J_{6exo,6endo}$ 6.9 Hz, $J_{5,6endo}$ 0 Hz, H-6*endo*) τ 6.13 (1-proton octet, $J_{5,6exo}$ 3.1 Hz, $J_{2,6exo}$ 1.2 Hz, H-6*exo*), τ 7.82 (3-proton singlet, Ac), τ 8.43, 8.49 (3-proton singlets, CMe_2); in benzene-d_6 at 100 MHz: τ 4.42 (1-proton doublet, $J_{1,2}$ 2.0 Hz, H-1), τ 5.37–5.42 (2-proton multiplet, H-2,5), τ 5.94 (1-proton doublet, $J_{6exo,6endo}$ 6.9 Hz, $J_{5,6endo}$ 0 Hz, H-6*endo*), τ 6.46 (1-proton octet, $J_{5,6exo}$ 3.0 Hz, $J_{2,6exo}$ 1.3 Hz, H-6*exo*), τ 8.35 (3-proton singlet, Ac), τ 8.59, 8.83 (3-proton singlets, CMe_2); X-ray powder diffraction data: 9.49 s (3), 8.33 vs (1,1), 6.75 m, 5.77 m, 5.39 w, 5.11 vs (1,1), 4.84 vs (1,1), 4.52 m, 4.09 broad s (2), 3.74 w, 3.63 m, 3.36 m, 3.29 m, 3.03 s, 2.91 w, and 2.82 m.

Anal. Calc. for $C_{11}H_{14}O_6$: C, 54.54; H, 5.82. Found: C, 54.54; H, 5.69.

Compound **2** is soluble in acetone, benzene, carbon tetrachloride, chloroform, ethanol, and ethyl ether. It sublimes at 60°/0.2 torr. When kept without desiccation, the enediol acetate **2** decomposed slowly to give the starting ketone **1**, which was obtained in almost quantitative yield from a sample of **2** that had been exposed to the atmosphere for 2 weeks.

Treatment of the ketone **1** *with other acetylating reagents. — A. With acetic anhydride–piperidine.* A solution of the ketone **1** (0.5 g) in acetic anhydride (20 ml) and piperidine (5 ml) was kept for 3 days at 50° under a reflux condenser. T.l.c. of the product, after processing as described for the preparation of **2**, indicated that the enediol acetate **2** was absent.

B. With isopropenyl acetate and an acid catalyst. Treatment of the ketone **1** with isopropenyl acetate and catalytic amounts of either sulfuric acid (conditions of Engel[15]) or *p*-toluenesulfonic acid hydrate (conditions of Moffett and Weisblat[16]), followed by neutralization with solid sodium hydrogen carbonate, evaporation, and extraction of the residue with ether, gave products containing unreacted **1** and a number of additional products (as revealed by t.l.c.), but no product having the chromatographic characteristics of the enediol acetate **2**.

*1,6-Anhydro-4-*C*-(1,6-anhydro-2,3-*O*-isopropylidene-β-*D-lyxo*-hexopyranos-4-ulose-3-yl)-2,3-*O*-isopropylidene-β-*D*-talopyranose* (**5**). — A solution of the ketone **1** (0.5 g) in freshly distilled acetic anhydride (20 ml) and triethylamine (5 ml) was kept in the dark at 25°. T.l.c. of the solution after 1 day showed the presence of a major component having R_F 0.64, together with minor components having R_F 0.74 (**2**), 0.45, and 0.30 (**1**). The solution was evaporated at 40°/0.2 torr to a syrup, which was extracted with three 50-ml portions of ethyl ether. The extracts were combined, and treated with decolorizing carbon; the suspension was filtered, and the filtrate was evaporated to a syrup which was dissolved in ethyl ether. Petroleum ether (b.p. 30–60°) was added to incipient turbidity, and the solution was refrigerated to give **5** as dense, white prisms; yield 0.20 g (40%), m.p. 175–176.5°, $[\alpha]_D^{20}$ −9.5 ±2° (*c* 0.3, chloroform); R_F 0.64; λ_{max}^{KBr} 2.90 (OH), 5.73 (C=O), 7.26 μm (CMe_2); n.m.r. data (60 MHz): τ 4.21 (1-proton, sharp doublet, $J_{1',2'}$ 3.8 Hz, H-1′), τ 4.76 (1-proton, broadened doublet, $J_{1,2}$ 3.0 Hz, H-1), τ 5.16 (1-proton doublet, H-2′), τ 5.21–5.39 (3-proton multiplet), 5.68 (1-proton doublet of narrow multiplets, principal spacing 7.9 Hz), 5.96–6.38 (4-proton multiplet; H-2,3,5,6,5′,6′), τ 6.47 (1-proton singlet, disappears on deuteration, OH), 8.43, 8.47, 8.60 (3-, 3-, and 6-proton singlets, CMe_2); in benzene-d_6 at 100 MHz: τ 4.49 (1-proton, sharp doublet, $J_{1',2'}$, 3.8 Hz, H-1'), τ 4.84 (1-proton doublet, $J_{1,2}$ ~3 Hz, H-1), τ 5.32 (1-proton doublet, H-2'), τ 4.83–4.92 (1-proton doublet of narrow multiplets), 5.54–5.67 (2-proton, 5-peak multiplet), 5.77–5.92 (2-proton, 7-peak multiplet), 6.39–6.57 (2-proton, 6-peak multiplet; H-2,3,5,6,5',6', excluding an H-6*exo* signal on one moiety), τ 6.58 (1-proton singlet, disappears on deuteration, OH), τ 6.75 (1-proton quartet, $J_{6exo,6endo}$ 7.7 Hz, $J_{5,6exo}$ 4.7 Hz, H-6*exo* of one moiety), τ 8.38, 8.44, 8.49, 8.58 (3-proton singlets, CMe_2); X-ray powder diffraction data: 12.44 s (2,2), 11.12 s (2,2), 9.40 m, 7.40 w, 6.36 s (3), 5.98 vs (1), 5.50 m, 5.03 m, 4.66 m, 4.29 m, 3.86 w, 3.56 w, and 3.22 m.

Anal. Calc. for $C_{18}H_{24}O_{10}$: C, 53.99; H, 6.04. Found: C, 53.90; H, 6.26.

T.l.c. of the mother liquors showed that they still contained a large proportion of **5**, but further direct crystallization of the product was impeded by the side-products present.

Conversion of the dimer **5** *into the enediol acetate* **2**. — A solution of the dimer **5** (10 mg) in acetic anhydride (5 ml) and triethylamine (0.5 ml) was kept for 1 day at room temperature. T.l.c. showed that no significant conversion of **5** into **2** had occurred. The solution was next kept for 3 days at 70°; t.l.c. then indicated the presence of products very similar, both in distribution and relative intensities, to those observed in the reaction used for the preparation of **2**. The reaction mixture was processed as described for the preparation of **2**, to give 4 mg (33%) of enediol acetate **2**, m.p. 128–130°, R_F 0.74, identical with an authentic sample of **2** by mixed m.p. and i.r. spectral comparison.

Base-catalyzed ethanolysis of the enediol ester **2**. — To a solution of **2** (50 mg) in abs. ethanol was added 1 drop of 500 mM ethanolic potassium hydroxide, and the solution was kept at room temperature. Additional portions of base (10 drops total) were added during 2.5 h. The starting material (R_F 0.74) had disappeared after 3 h, and had been replaced by a single product having R_F 0.41. The solution was diluted with chloroform (200 ml), washed successively with aqueous ammonium chloride and water, dried (sodium sulfate), and evaporated to a syrup which was dissolved in ether (3 ml); petroleum ether (b.p. 30–60°) was added to incipient turbidity, and the solution was kept at 0°, to give the hydrated ketone **3** as dense prisms; yield 12.4 mg, m.p. 94–98° (resolidified on cooling and then melted at 82°); R_F 0.41; λ_{max}^{KBr} 2.95 s (OH), 5.74 vw (C=O), 7.26 μm (CMe_2). Sublimation of **3** at 70°/0.2 torr gave the ketone **1**; yield 11 mg (27%), m.p. 82–83°, undepressed on admixture with an authentic sample of **1**. The i.r. spectrum of this product was identical with that of an authentic sample of **1**.

Reduction of the enediol acetate **2** *with borohydride to 1,6-anhydro-2,3-O-isopropylidene-β-*D*-talopyranose* (**6**). — To a solution of the enediol acetate **2** (50 mg) in abs. ethanol (10 ml) was added a solution of sodium borohydride (100 mg) in abs. ethanol (5 ml). The starting material (R_F 0.74) had all disappeared after 15 min at room temperature, and had been replaced by a product having R_F 0.50. The solution was immediately diluted with chloroform (200 ml), successively washed with cold, aqueous ammonium chloride and water, dried (sodium sulfate), and evaporated to give a crystalline residue which was chromatographically homogeneous. It was recrystallized from chloroform–petroleum ether (b.p. 30–60°) to give **6** as plates; yield 20 mg (48%), m.p. 106–107° (lit.[3] m.p. 108.5–109°). The product was identical with an authentic sample[3] of **6** by mixed m.p. and comparative i.r. spectra. The mother liquors from the reaction contained only **6** and none of the 4-epimer (**4**), as shown by g.l.c. of the trimethylsilylated product under the conditions described by Horton and Jewell[3] for differentiation of **4** and **6**.

ACKNOWLEDGMENTS

The authors thank J. S. Jeweli for preliminary experimental work on this problem, R. H. Bell and P. L. Durette for the 60-MHz n.m.r. spectra, J. H. Lauterbach for the 100-MHz n.m.r. spectra and spin-decoupling experiments, and J. K. Thomson for assistance with the g.l.c. analyses.

REFERENCES

1 D. Horton and E. K. Just, *Abstracts Papers Amer. Chem. Soc. Meeting*, 156 (1968) CARB 15.
2 D. Horton and J. S. Jewell, *Carbohyd. Res.*, 3 (1966) 255.
3 D. Horton and J. S. Jewell, *Carbohyd. Res.*, 5 (1967) 149.
4 D. Horton and J. S. Jewell, *Carbohyd. Res.*, 2 (1966) 251.
5 D. Horton and J. D. Wander, *J. Org. Chem.*, 32 (1967) 3780.
6 A. K. Chatterjee, D. Horton, J. S. Jewell, and Kerstin D. Philips, *Carbohyd. Res.*, 7 (1968) 173.
7 W. Meyer zu Reckendorf, *Angew. Chem.*, 79 (1967) 151.
8 J. D. Albright and L. Goldman, *J. Amer. Chem. Soc.*, 87 (1965) 4212.
9 L. M. Berkowitz and P. N. Rylander, *J. Amer. Chem. Soc.*, 80 (1958) 6682; P. J. Beynon, P. M. Collins, D. Gardiner, and W. G. Overend, *Carbohyd. Res.*, 6 (1968) 431.
10 H. Nakata, *Tetrahedron*, 19 (1963) 1959.
11 J. I. Musher and E. J. Corey, *Tetrahedron*, 18 (1962) 791; R. H. Bible, Jr., *Interpretation of NMR Spectra*, Plenum Press, New York, 1965, p. 92.
12 C. V. Holland, D. Horton, Martha J. Miller, and N. S. Bhacca, *J. Org. Chem.*, 32 (1967) 3077.
13 M. Karplus, *J. Chem. Phys.*, 30 (1959) 11; *J. Amer. Chem. Soc.*, 85 (1963) 2870.
14 A. E. Knauf, R. M. Hann, and C. S. Hudson, *J. Amer. Chem. Soc.*, 63 (1941) 1447.
15 C. R. Engel, *J. Amer. Chem. Soc.*, 82 (1960) 3201.
16 R. B. Moffett and D. I. Weisblat, *J. Amer. Chem. Soc.*, 74 (1952) 2183.

Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

THE FORMATION OF EPOXIDES FROM SUBSTITUTED HEXITOLS

M. JARMAN AND W. C. J. ROSS

Chester Beatty Research Institute, Institute of Cancer Research: Royal Cancer Hospital, Fulham Road, London S.W. 3 (Great Britain)

(Received July 5th, 1968)

ABSTRACT

1,6-Dibromo-1,6-dideoxygalactitol (2), 1-*O*-methanesulphonyl-D-mannitol (5), and 3,4-di-*O*-methanesulphonyl-D-mannitol (8) were respectively converted at nearly neutral pH into 1,2:5,6-dianhydrogalactitol (4), 1,2-anhydro-D-mannitol (6), and 2,3:4,5-dianhydro-D-iditol (9). Strongly alkaline conditions yielded 2,3:4,5-dianhydro-L-iditol (7) from 1,6-di-*O*-methanesulphonyl-D-mannitol (1). The structures of compounds **4**, **6**, **7**, and **9** were confirmed by p.m.r. spectroscopy, and by their reactivity towards thiosulphate and iodide ions.

The conversion of 1,2:5,6-dianhydro-D-mannitol (3) into 1,6-dideoxy-1,6-diiodo-D-mannitol, 1,6-dibromo-1,6-dideoxy-D-mannitol, and 1,6-di-*O*-benzoyl-D-mannitol, and 1,2:5,6-dianhydrogalactitol into 1,6-dideoxy-1,6-diiodogalactitol is described.

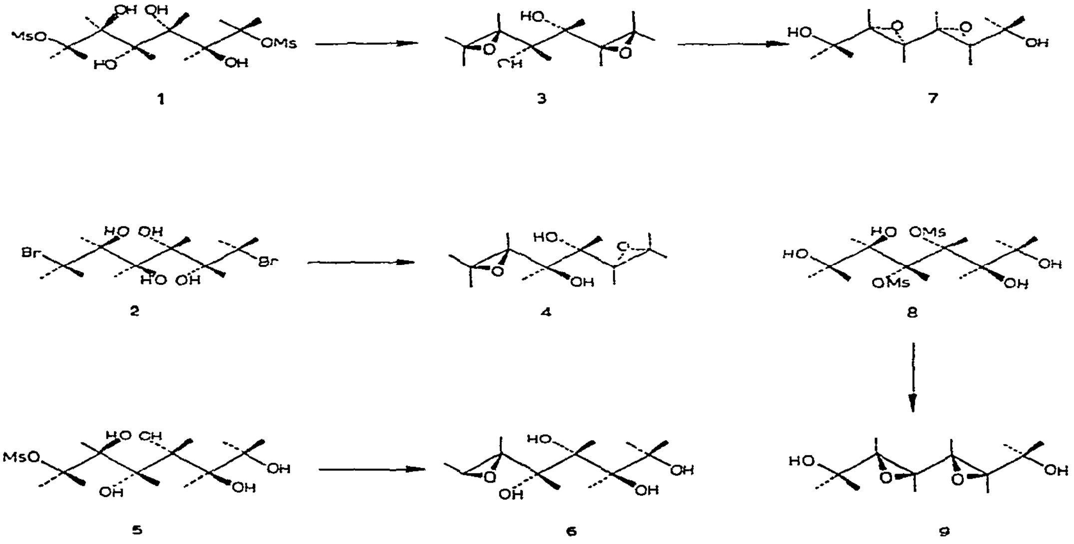
INTRODUCTION

Until recently[1], anhydrohexitols containing the oxirane ring have been isolated only as derivatives[2]. However, their intermediacy in the formation of larger anhydro rings[3] and their existence in solution[4] have been postulated. The principle of the preparative method leading to 1,2:5,6-dianhydro-D-mannitol[1], namely very mild basic treatment of a suitably substituted hexitol, now promises to be generally applicable. Three further epoxide derivatives of hexitols have been prepared by this method, and the utility of the terminal diepoxides in the synthesis of terminally substituted hexitol derivatives is indicated.

RESULTS AND DISCUSSION

The general procedure applied to the synthesis from substituted hexitols of anhydrohexitols containing the oxirane ring consisted in continuously titrating the appropriate *O*-methanesulphonyl, *O*-toluene-*p*-sulphonyl, or bromo derivative in aqueous solution or suspension with N sodium hydroxide, keeping the solution close to pH 8. When no further alkali was consumed, the solution was added dropwise to a stirred suspension of anhydrous sodium carbonate in ethyl acetate. Water was thus removed, and the anhydrohexitol was crystallised from the concentrated ethyl acetate solution. Thus, 1,6-di-*O*-methanesulphonyl-D-mannitol (1), and 1,6-dibromo-1,6-dideoxygalactitol[5] (2) gave crystalline 1,2:5,6-dianhydro-D-mannitol (3) and 1,2:5,6-

dianhydrogalactitol (4) in over 30% yield. These compounds had previously been obtained as their 3,4-*O*-isopropylidene derivatives[6,7], but the acid lability of the oxirane ring precluded selective cleavage of the isopropylidene group.



Similarly, 1-*O*-toluene-*p*-sulphonyl-D-mannitol and 1-*O*-methanesulphonyl-D-mannitol (5), prepared by acidic hydrolysis of their 3,4:5,6-di-*O*-isopropylidene derivatives, were converted into 1,2-anhydro-D-mannitol (6). This compound, isolated in 8–10% yield, was previously known only as its 3,4:5,6-di-*O*-isopropylidene derivative[8].

The nearly quantitative reactivity of terminal oxirane rings towards thiosulphate[9] and iodide[1] ions provided a method for estimating epoxide content. The liberated hydroxyl ion was continuously titrated with standard acid. The epoxide contents of the solutions obtained by the mild, basic treatment of compounds **1**[1], **2**, and **5** were found to be much higher than implied by the isolated yield of epoxide. This loss was attributable to incomplete extraction into ethyl acetate, but was unavoidable, since isolation procedures involving concentration of the aqueous solutions invariably caused extensive cleavage of the oxirane rings.

The reality of oxirane ring formation from terminally substituted hexitol derivatives was disputed by Institoris *et al.*[10]. In cases where the formation of the less strained five- or six-membered rings was possible, they claimed that these would form preferentially. Hence, the 67% conversion in solution of the methanesulphonate **5** into the epoxide **6** appears remarkable, since five ring-sizes (three- to seven-membered) could theoretically result. A closer examination of the problem of hydroxyl-ion catalysed anhydro-ring formation reveals the importance of distinguishing between the rate of formation of the ring and its stability to the reaction conditions. Thus, the rate constants k ($\mathrm{l.mole^{-1}.min^{-1}}$) for the formation in aqueous sodium hydroxide at 30° of ethylene oxide, tetrahydrofuran, and tetrahydropyran from the appropriate, terminally substituted halohydrins were[11], respectively, 1.13, 0.172, and 0.0007, indicating that oxirane ring formation was kinetically preferred. Owing to the relatively greater degree of ionisation of the hydroxyl group adjacent to the electron-withdrawing leaving group, this preference for hydroxyl-ion catalysed oxirane ring formation would be enhanced at a less alkaline pH. The direct competition of oxirane ring formation with both tetrahydrofuran and tetrahydropyran ring formation has been investigated by studying the products of hydroxyl-ion catalysed ring-closure in various mono-*O*-toluene-*p*-sulphonylpentanetriols[12]. The products, where cyclic, were invariably derivatives of tetrahydrofuran and tetrahydropyran, but their stereochemistry was often consistent with the initial formation of an oxirane derivative, followed by a rearrangement involving attack of the oxirane ring by the remaining hydroxyl group. Again, tetrahydropyran ring formation was kinetically least favoured. It is clear from these studies that the present use of a nearly neutral pH for oxirane ring formation is the major factor permitting the isolation of the epoxides, rather than their rearrangement products.

The lability of the terminal oxirane ring was further indicated by the isolation of a different type of rearrangement product. When the dimethanesulphonate **1** reacted with hydroxyl ion at a higher pH (not less than 12.6) than was employed for the preparation of the terminal diepoxide **3**, a new dianhydrohexitol was isolated. Its reaction with sodium thiosulphate gave 74% of the theoretical yield of hydroxyl ion from a diepoxide. The corresponding yield from the terminal diepoxide **3** was 93.5%. Comparison with the hydroxyl-ion yields[13] from 1,2:5,6-diepoxyhexane (98%) and its non-terminal analogue 2,3:4,5-diepoxyhexane (75%) strongly implied that the new product was a non-terminal diepoxide, an interpretation consistent with the finding of only four oxirane-ring CH protons in its p.m.r. spectrum. The initial production of the terminal diepoxide **3**, followed by hydroxyl-ion catalysed migration of the oxirane rings to the non-terminal position, would involve inversion of configuration at C-2 and C-5, and the formation of a non-terminal diepoxide, *viz.*, 2,3:4,5-dianhydro-L-iditol (**7**). Base-catalysed oxirane ring migration has been invoked to explain the formation in aqueous sodium hydroxide from 1-*O*-toluene-*p*-sulphonyl-*myo*-inositol of 1,2-anhydro-*myo*-inositol, whereas apparently milder basic treatment gives 1,2-anhydro-(±)-inositol[14].

The structure of the new diepoxide was confirmed by comparison with its supposed enantiomer. 3,4-Di-*O*-methanesulphonyl-D-mannitol (**8**) was prepared in two stages from 1,2:5,6-di-*O*-isopropylidene-D-mannitol. Hydroxyl-ion catalysed ring-closure at nearly neutral pH gave 2,3:4,5-dianhydro-D-iditol* (**9**). The D-iditol configuration arose from the general observation that the direct formation of anhydro rings from sulphonic esters proceeds with inversion of configuration at the carbon atoms carrying the leaving groups[15], in this case C-3 and C-4. That the compound

---

*Since this paper was submitted, a preparation of 3,4-di-*O*-methanesulphonyl-D-mannitol and its conversion into 2,3:4,5-dianhydro-D-iditol have been described [R. S. TIPSON AND A. COHEN, *Carbohyd. Res.*, 7 (1968) 240].

assigned the structure 7 was an enantiomer of compound 9 was shown by the coincidence of their physical properties (m.p., i.r. and p.m.r. spectra, and paper-chromatographic characteristics), with the exception that their specific rotations, though almost equal in magnitude, were opposite in sign.

The p.m.r. spectra (Table I) of the new anhydrohexitols were consistent with the structures assigned. Oxirane ring protons characteristically resonate at higher field (τ 6.5–7.3) than those adjacent to the oxygen atom of five- or six-membered anhydro rings, for which the characteristic range[16] is τ 6.3–6.7. In addition, the presence of five- or six-membered rings is excluded by their unreactivity towards thiosulphate ions. Oxetane rings react with thiosulphate ion[17], but are excluded by the absence in the p.m.r. spectrum of 1,3-anhydro-D-glucitol[18] of signals above τ 6.5.

TABLE I

THE 60-MHZ P.M.R. DATA FOR HEXITOL EPOXIDES

Compound	*Solvent*	*Chemical shift* (τ)		
		H(1,6)	*H(2,5)*	*H(3,4)*
1,2:5,6-Dianhydro-D-mannitol (3)	D_2O	6.94–7.22	6.60–6.92	6.20–6.43
1,2:5,6-Dianhydrogalactitol (4)	D_2O	6.92–7.23	6.58–6.85	6.26–6.50
1,2-Anhydro-D-mannitol (6)	D_2O	7.00–7.16	6.60–6.90	6.20–6.43
2,3:4,5-Dianhydro-L-iditol[a] (7)	Acetone-d_6 + 10% D_2O	6.06–6.69[b]	6.78–7.00	7.04–7.19

[a]The spectrum of 2,3:4,5-dianhydro-D-iditol (9) was identical. [b]The OH signal at τ 6.20 was distinguished from others in this region by its downfield shift relative to those of *ca.* 0.2 p.p.m. on raising the D_2O content to 20%. The spectrum (in D_2O) of 1,3-anhydro-D-glucitol prepared[18] from 1,3-anhydro-6-*O*-benzyl-2,4-*O*-benzylidene-D-glucitol showed no signals at higher field than τ 6.5.

The 60 MHz p.m.r. spectrum of the anhydrohexitols showed the required signal intensities in the oxirane ring-proton region, equivalent to six protons for the terminal diepoxides 3 and 4, three protons for the monoepoxide 6, and four protons for the non-terminal diepoxides 7 and 9. In each case, three distinct absorption regions were present (Table I). The lowest field signals were attributed to CH protons in the acyclic portion of the molecules, and the two higher field regions to oxirane ring protons. In the spectra of the terminal epoxides 3, 4, and 6, the oxirane ring-proton signals at higher and lower field (intensity ratio, 2:1) were respectively assigned to the methylene and to the methine protons. The higher and the lower field signals for the non-terminal diepoxides 7 and 9 could not be assigned on the basis of their intensity ratio, since the signal intensities were equal. However, a similar assignment of the lower field signals to H-2 and H-5 was favoured, since a similar chemical shift was observed in the spectrum of compound 3 in the same solvent for the environmentally similar methine protons[1].

Although the individual signals were too complex to permit further unequivocal elucidation, the foregoing examination of the p.m.r. spectra of the epoxides, considered with their chemical reactions, sufficed to prove their structures.

The high reactivity of the terminal diepoxides 3 and 4 towards anions was exploited in the preparation of various terminally di-substituted hexitol derivatives. Thus, treatment of aqueous solutions of compounds 3 or 4 with sodium iodide and acidic titration of the liberated hydroxyl ion gave 1,6-dideoxy-1,6-diiodo-D-mannitol and its galactitol analogue. The analogous reaction of compound 3 with lithium bromide, the solubility of which permitted an optimal concentration of the less nucleophilic bromide ion, gave 1,6-dibromo-1,6-dideoxy-D-mannitol, an interesting reversal of the conversion[1] of the latter compound into the diepoxide 3. The reaction of compound 3 with sodium benzoate and benzoic acid gave 1,6-di-*O*-benzoyl-D-mannitol.

This reactivity towards anions may be highly relevant to the toxic and tumour-inhibitory properties of terminally substituted diepoxides. Preliminary results on the biological activity of the mannitol derivative 3 have been reported elsewhere[1].

EXPERIMENTAL

P.m.r. spectra were measured on *ca.* 10% w/v solutions with a Perkin–Elmer R10 spectrometer, operating at 60 MHz. *tert*-Butyl alcohol (in D_2O solutions) and tetramethylsilane (in acetone-d_6 solutions) were used as internal standards; *ca.* 10% v/v of D_2O was added to acetone-d_6 solutions to exchange hydroxyl protons.

Ascending paper chromatography was conducted on Whatman No. 1 paper in butyl alcohol–water (86:14). Spots were detected by three spray tests: periodate–benzidine for *cis*-diol groups; aqueous sodium iodide–phenolphthalein, with which epoxides gave pink spots owing to the liberation of hydroxyl ion in the presence of iodide ion; and potassium permanganate in acetone, which gave yellow spots against a purple background. The last reagent gave a positive reaction with 1,4:3,6-dianhydro-D-mannitol (R_F 0.35) which lacks both epoxide and *cis*-diol groupings, as well as with all compounds having such groupings.

Thin-layer chromatography (t.l.c.) was conducted on microscope slides coated with Merck Kieselgel G. Spots were detected by spraying with sulphuric acid, followed by heating at 150°.

All melting points are corrected.

1,2:5,6-Dianhydrogalactitol. — A stirred suspension of 1,6-dibromo-1,6-dideoxygalactitol[5] (1.23 g, 0.004 mole) in water (5 ml) at 35–40° was continuously titrated with N sodium hydroxide, with phenolphthalein as internal indicator, keeping the solution just pink, until no more acidity had developed; 6.4 ml of alkali was added*, corresponding to a development of 80% of the theoretical acidity. The

---

*Addition of potassium iodide (2 g) at this stage, and titration of the liberated alkali at 35–40° with N hydrochloric acid, using phenolphthalein as internal indicator, and keeping the solution just pink, allowed an estimate of the epoxide content of the solution. The hydroxyl ion liberated corresponded to 5.6 ml of acid. Hence, 70% of the starting material was converted into epoxide. Simultaneous separation of crude 1,6-dideoxy-1,6-diiodogalactitol (1.02 g) occurred (Found: I, 58.30. $C_6H_{12}I_2O_4$ calc.: I, 63.14%). Owing to its insolubility in common crystallisation solvents, the pure compound was best prepared from crystalline 1,2:5,6-dianhydrogalactitol.

solution was added dropwise to a stirred suspension of anhydrous sodium carbonate (30 g) in ethyl acetate (150 ml). The filtered solution was concentrated under diminished pressure below 30° to 25 ml, redried over anhydrous magnesium sulphate, and concentrated to 5 ml. The title compound separated as colourless plates (0.21 g, 36%), m.p. 96.5–98.5°, $[\alpha]_D^{25}$ +2° (*c* 1.25, water) (Found: C, 49.19; H, 6.75. $C_6H_{10}O_4$ calc.: C, 49.28, H, 6.89%).

Paper chromatography showed a single spot, R_F 0.39, which gave a positive reaction with *cis*-diol reagents and with epoxide reagents.

*1,2:5,6-Dianhydro-*D*-mannitol*[1] (3). — By essentially the above procedure, 1,6-di-*O*-methanesulphonyl-D-mannitol was converted into diepoxide 3 (31%), m.p. 64–66°, $[\alpha]_D$ +40° (*c* 1.25, water) (Found: C, 49.45; H, 6.97. $C_6H_{10}O_4$ calc.: C, 49.28; H, 6.89%).

*1-O-Toluene-p-sulphonyl-*D*-mannitol.* — To an ice-cooled solution of 1,2:3,4-di-*O*-isopropylidene-D-mannitol[21] (5 g, 0.019 mole) in dry benzene (7 ml) was added dropwise a solution of toluene-*p*-sulphonyl chloride (3.8 g, 0.020 mole) in dry pyridine (7 ml) with stirring during 1 h. After 16 h at 5°, the solution was evaporated below 30° under diminished pressure. The residue was partitioned between chloroform (25 ml) and water (25 ml). The organic phase was washed (dilute hydrochloric acid, water, and saturated, aqueous sodium hydrogen carbonate) and evaporated. The residue was dissolved in 1:5 water–acetic acid (50 ml). After 18 h at room temperature, the solution was evaporated below 30° under diminished pressure. Co-evaporation with toluene, followed by crystallisation of the solid residue from ethanol (50 ml), yielded the title compound (3.0 g, 47%) as colourless prisms, m.p. 130–131° (Found: C, 46.22; H, 5.98; S, 9.69. $C_{13}H_{20}O_8S$ calc.: C, 46.37; H, 5.99; S, 9.53%).

*1-O-Methanesulphonyl-*D*-mannitol.* — 1,2:3,4-Di-*O*-isopropylidene-D-mannitol (5 g, 0.019 mole) was treated with methanesulphonyl chloride (2.29 g, 0.020 mole) by the foregoing procedure. Identical treatment of the intermediate gave the title compound (1.75 g, 35%) as colourless needles from ethanol (50 ml), m.p. 133–135°, $[\alpha]_D^{25}$ +3° (*c* 2.5, water) (Found: C, 32.57; H, 6.01; S, 12.00. $C_7H_{16}O_8S$ calc.: C, 32.45; H, 6.20; S, 12.31%).

*1,2-Anhydro-*D*-mannitol.* — A stirred suspension of 1-*O*-toluene-*p*-sulphonyl-D-mannitol (1.01 g, 0.003 mole) in water (4 ml) at 35–40° was continuously titrated with N sodium hydroxide, with phenolphthalein as internal indicator, keeping the solution just pink, until no more acidity had developed; 2.65 ml of alkali was added*, corresponding to development of 88% of the theoretical acidity. The solution was

---

*Addition of potassium iodide (2 g) at this stage, and titration of the liberated alkali at 35–40° with N hydrochloric acid, using phenolphthalein as internal indicator and keeping the solution just pink, allowed an estimate of the epoxide content of the solution. The liberated hydroxyl ion corresponded to 1.30 ml of acid. Hence 43% of the starting material was converted into epoxide. The title compound was similarly isolated (0.026 g, 8%) from a titrated solution of 1-*O*-methanesulphonyl-D-mannitol (0.52 g, 0.002 mole) in water (1 ml); 1.8 ml of alkali was consumed, corresponding to development of 90% of the theoretical acidity. Addition of potassium iodide at this stage, as above, showed 67% conversion of the starting material into epoxide.

added dropwise to a stirred suspension of anhydrous sodium carbonate (20 g) in ethyl acetate (100 ml). After further drying over anhydrous calcium sulphate ("Drierite"), the filtered solution was concentrated under diminished pressure below 30° to 5 ml. The title compound separated as colourless needles (0.050 g, 10%), m.p. 102–104°, $[\alpha]_D^{25}$ $-16°$ (c 2.5, water) (Found: C, 43.83; H, 7.19. $C_6H_{12}O_5$ calc.: C, 43.89; H, 7.37%).

Paper chromatography showed a single spot, R_F 0.20, which gave a positive reaction with *cis*-diol reagents and with epoxide reagents.

*2,3:4,5-Dianhydro-*L*-iditol.* — A solution of 1,6-di-*O*-methanesulphonyl-D-mannitol (6.65 g, 0.0197 mole) in N sodium hydroxide (35 ml) was added dropwise after 15 min (pH 12.6) to a stirred suspension of anhydrous sodium carbonate (100 g) in ethyl acetate (500 ml). The filtered solution was concentrated below 30° under diminished pressure to 100 ml, re-dried with anhydrous magnesium sulphate, and evaporated to 5 ml. The title compound separated as colourless, rectangular prisms (0.678 g, 24%), m.p. 98–99°, $[\alpha]_D^{25}$ $-72°$ (c 1.25, water) (Found: C, 49.06; H, 6.74. $C_6H_{10}O_4$ calc.: C, 49.28; H, 6.89).

Paper chromatography showed a single spot, R_F 0.39, which gave no reaction with *cis*-diol reagents, but a positive reaction with epoxide reagents.

Treatment with sodium thiosulphate. — (*a*) *2,3:4,5-Dianhydro-*L*-iditol.* To a solution of the diepoxide (0.073 g, 0.0005 mole) in water (10 ml) at 50–55° was added sodium thiosulphate (5 g). The liberated alkali was continuously titrated with 0.1N hydrochloric acid, using phenolphthalein as internal indicator and keeping the solution just pink. The total hydroxyl ion liberated (in 3 h) consumed 7.40 ml of acid, corresponding to development of 74% of the theoretical alkalinity for a diepoxide.

(*b*) *1,2:5,6-Dianhydro-*D*-mannitol.* — To a solution of the diepoxide (0.146 g, 0.001 mole) in water (20 ml) at 35–40° was added sodium thiosulphate (5 g). Titration of the liberated alkali was conducted as in (*a*); 18.7 ml of acid was required in 30 min, corresponding to a development of 93.5% of the theoretical alkalinity for a diepoxide.

(*c*) *Larger ring anhydrohexitols.* — 1,4-Anhydro-D-mannitol[22], 1,5-anhydro-D-glucitol, and 1,4:3,6-dianhydro-D-mannitol failed to react with thiosulphate ions under either of the above conditions.

*3,4-Di-O-methanesulphonyl-*D*-mannitol.* — 1,2:5,6-Di-*O*-isopropylidene-3,4-di-*O*-methanesulphonyl-D-mannitol[23] (0.25 g, 0.006 mole) was dissolved at room temperature in a mixture of chloroform (1 ml) and 5M HCl in methanol (1 ml). The reaction, monitored by t.l.c. (methanol–chloroform, 3:25), was complete within 5 min. The solution was evaporated below 30° under diminished pressure, and the residue, after standing *in vacuo* overnight over potassium hydroxide, was crystallised from hot ethanol (1.5 ml) to give the title compound (0.13 g, 64%) as colourless needles, m.p. 110–112°, $[\alpha]_D^{25}$ $+27°$ (c 1.3, water) (Found: C, 28.28; H, 5.35; S, 18.89. $C_8H_{18}O_{10}S_2$ calc.: C, 28.40; H, 5.36; S, 18.95%).

*2,3:4,5-Dianhydro-*D*-iditol.* — A stirred solution of 3,4-di-*O*-methanesulphonyl-D-mannitol (0.13 g, 0.39 mole) in water (0.5 ml) at 35–40° was continuously titrated with N sodium hydroxide, with phenolphthalein as internal indicator, and keeping the solution just pink, until no more acidity developed; 0.76 ml of alkali was added, corresponding to the development of 97.5% of the theoretical acidity. The solution was added dropwise to a stirred suspension of anhydrous sodium carbonate (3 g) in ethyl acetate (25 ml). The filtered solution was evaporated below 30° under diminished pressure to a solid residue. Crystallisation from ethyl acetate (5 ml) gave the title compound (0.032 g, 57%) as colourless plates, m.p. 98–99°, $[\alpha]_D^{25}$ +74° (*c* 1.0, water) Found: C, 49.60; H, 6.85. $C_6H_{10}O_4$ calc.: C, 49.28; H, 6.89. Paper chromatography showed a single spot, R_F 0.39, which gave no reaction with *cis*-diol reagents, but a positive reaction with epoxide reagents.

*1,6-Dideoxy-1,6-diiodo-*D*-mannitol.* — To a solution of 1,2:5,6-dianhydro-D-mannitol (0.585 g, 0.004 mole) in water (5 ml) at 35–40° was added potassium iodide (2 g). Continuous acidic titration of the liberated hydroxyl ion, using phenolphthalein as internal indicator, required 6.0 ml of N hydrochloric acid, corresponding to the development of 75% of the theoretical alkalinity. There was simultaneous separation of the title compound as a white solid (0.75 g, 47%), m.p. 165° (decomp.); lit.[6] m.p. 165–167° (decomp.) (Found: C, 17.7; H, 3.16; I, 63.05. $C_6H_{12}I_2O_4$ calc.: C, 17.9; H, 3.01; I, 63.14%).

1,6-Dideoxy-1,6-diiodogalactitol. — To a solution of 1,2:5,6-dianhydrogalactitol (0.0625 g, 0.00043 mole) in water (5 ml) was added potassium iodide (1 g). Acidic titration (as above) required 0.66 ml of N hydrochloric acid, corresponding to development of 77% of the theoretical alkalinity. There was simultaneous separation of the highly insoluble, title compound (0.11 g, 64%), a white solid that decomposed at 181° (Found: C, 18.16; H, 3.12; I, 63.01. $C_6H_{12}I_2O_4$ calc.: C, 17.93; H, 3.01; I 63.14%).

A stirred mixture of 1,6-di-*O*-methanesulphonyl-D-mannitol (1.33 g, 0.039 mole) and water (1 ml) at 35–40° was continuously titrated with N sodium hydroxide, with phenolphthalein as internal indicator, keeping the solution just pink, until no more alkalinity developed; 7.2 ml of alkali was added, corresponding to the development of 90% of the theoretical acidity. Such a solution of 1,2:5,6-dianhydro-D-mannitol was used for the following two preparations.

*1,6-Dibromo-1,6-dideoxy-*D*-mannitol.* — Anhydrous lithium bromide (15 g) was added portionwise to the solution maintained at 35–40°. Continuous acidic titration to consume the liberated hydroxyl ion required 6 ml of N hydrochloric acid. Simultaneous separation of the title compound occurred as a white solid (0.525 g, 43%), m.p. 173–175° (decomp.); lit.[5] m.p. 174–176° (decomp.) (Found: C, 23.41; H, 4.30; Br, 51.70. $C_6H_{12}Br_2O_4$ calc.: C, 23.40; H, 3.95; Br, 51.88%).

*1,6-Di-*O*-benzoyl-*D*-mannitol.* — To the stirred diepoxide solution at 35–40° was added benzoic acid (0.98 g, 0.008 mole) and sodium benzoate (9 g). Complete dissolution within 3 h was followed by overnight precipitation of the title compound (0.475 g, 32%) as colourless needles, m.p. 173–175°, $[\alpha]_D^{25}$ +13° (*c* 2.0, pyridine), unchanged by recrystallisation from ethanol; lit.[24] m.p. 182°, $[\alpha]_D^{20}$ +15.9° in pyridine (Found: C, 61.42; H, 6.06. $C_{20}H_{22}O_8$ calc.: C, 61.53; H, 5.68%).

ACKNOWLEDGMENTS

The work was carried out during the tenure (by M. J.) of the William Shepherd Fellowship of the Chester Beatty Research Institute, and was supported by grants to the Institute from the Medical Research Council and the British Empire Cancer Campaign for Research, and by the U.S. Public Health Service through the National Cancer Institute. We thank Professor A. B. Foster for gifts of 1,5-anhydro-D-glucitol and 1,4:3,6-dianhydro-D-mannitol, and Dr. T. Radford for a gift of 1,3-anhydro-6-*O*-benzyl-2,4-*O*-benzylidene-D-glucitol.

REFERENCES

1 M. Jarman and W. C. J. Ross, *Chem. Ind.* (London), (1967) 1789.
2 L. F. Wiggins, *Advan. Carbohyd. Chem.*, 5 (1950) 191.
3 J. Kuszmann, personal communication.
4 W. Davis and W. C. J. Ross, *Biochem. Pharmacol.*, 12 (1963) 915.
5 L. Institoris, I. P. Horvath, and E. Csanyi, *Arzneimittel-Forsch.*, 17 (1967) 149.
6 L. F. Wiggins, *J. Chem. Soc.*, (1946) 384.
7 L. Vargha and E. Kasztreiner, *Ber.*, 92 (1959) 2506.
8 L. F. Wiggins, *J. Chem. Soc.*, (1946) 388.
9 W. C. J. Ross, *J. Chem. Soc.*, (1950) 2257.
10 A. David, G. Horvath, I. P. Horvath, L. Institoris, A. Neszmelyi, and L. Radics, *Experientia*, (1967) 512.
11 B. Capon, *Quart. Rev.* (London), 18 (1964) 45.
12 F. C. Hartman and R. Baker, *J. Org. Chem.*, 29 (1964) 875.
13 J. L. Everett and G. A. R. Kon, *J. Chem. Soc.*, (1950) 3131.
14 S. J. Angyal, V. Bender, and J. H. Curtin, *J. Chem. Soc.* (*C*), (1966) 798.
15 F. H. Newth, *Quart. Rev.* (London), 13 (1959) 30.
16 N. S. Bhacca and D. H. Williams, *Applications of NMR Spectroscopy in Organic Chemistry*, Holden–Day, San Francisco, 1964, p. 99.
17 S. Searles, *J. Amer. Chem. Soc.*, 73 (1951) 4515.
18 E. Haslam and T. Radford, *Carbohyd. Res.*, 2 (1966) 301.
19 N. S. Bhacca and D. H. Williams, *ref. 16*, p. 46.
20 E. L. Snyder, *J. Amer. Chem. Soc.*, 88 (1966) 1155.
21 L. F. Wiggins, *J. Chem. Soc.*, (1946) 13.
22 L. F. Wiggins, *J. Chem. Soc.*, (1945) 4.
23 P. Bladon and L. N. Owen, *J. Chem. Soc.*, (1950) 598.
24 P. Brigl and H. Grüner, *Ber.*, 65 (1932) 641.

Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

ADDITION REACTIONS OF METHYL 4,6-*O*-BENZYLIDENE-2,3-DIDEOXY-α-D-*erythro*-HEX-2-ENOPYRANOSIDE*†

ESTER L. ALBANO‡, D. HORTON**, AND J. H. LAUTERBACH
Department of Chemistry, The Ohio State University, Columbus, Ohio 43210 (U.S.A.)
(Received, August 30th, 1968)

ABSTRACT

The title alkene (**1**) reacts with bromine in methanol, in the presence of silver acetate, to give a high yield of a crystalline 2,3-dibromide (**2**) having the D-*altro* configuration. Removal of the *O*-benzylidene group from **2** followed by acetylation gave the corresponding 4,6-diacetate **5**, and treatment of **2** with *N*-bromosuccinimide gave methyl 4-*O*-benzoyl-2,3,6-tribromo-2,3,6-trideoxy-α-D-altropyranoside (**6**). Strong base converts the dibromide **2** into methyl 4,6-*O*-benzylidene-2-bromo-2,3-dideoxy-α-D-*threo*-hex-3-enopyranoside (**3**); the bromine atom at C-2 is not removed. Acetyl hypobromite reacts with the alkene **1** to give a mixture of two adducts, namely, methyl 2-*O*-acetyl-4,6-*O*-benzylidene-3-bromo-3-deoxy-α-D-altropyranoside (**7**) and methyl 3-*O*-acetyl-4,6-*O*-benzylidene-2-bromo-2-deoxy-α-D-glucopyranoside (**8**), with the former preponderating; treatment of either **7** or **8** with base gives methyl 2,3-anhydro-4,6-*O*-benzylidene-α-D-mannopyranoside (**10**). *N*-Bromosuccinimide converts **7** into the 6-bromo 4-benzoate analog **9**. Treatment of alkene **1** with diiodomethane and zinc–copper couple gives the cyclopropyl derivative **4**, but **1** is quite inert toward "dichlorocarbene", "ethoxycarbonylcarbene", and "ethoxycarbonylnitrene". The structures assigned to the products **2**–**10** were confirmed by 100-MHz n.m.r. spectral studies and by spin-decoupling experiments.

RESULTS AND DISCUSSION

An earlier paper in this series[4] described the generation of alkene unsaturation between C-2 and C-3 in a methyl 4,6-*O*-benzylidenehexopyranoside skeleton, from a 2,3-*cis*-diol, a 2,3-*trans*-diol, a 2,3-epoxide, and a 2,3-epithio precursor; these methods provide potential routes for introducing alkene unsaturation into various

---

*Part IX in the series Synthesis and Reactions of Unsaturated Sugars. For Part VIII, see ref. 1. For preliminary reports of part of this work, see refs. 2 and 3.

†Supported, in part, by the Agricultural Research Service, U. S. Department of Agriculture, Grants No. 12-14-100-7208(71) and 12-14-100-9201(71) (O.S.U.R.F. Projects 1827 and 2573) administered by the Northern Utilization Research and Development Division, Peoria, Illinois.

‡Stauffer Chemical Co., Fellow, October, 1967–June, 1968.

**To whom inquiries should be addressed.

protected sugars and polysaccharides. It was shown that methyl 4,6-*O*-benzylidene-2,3-dideoxy-α-D-*erythro*-hex-2-enopyranoside (**1**), which is stable to base, is extremely labile to aqueous or alcoholic acid; these acidic reagents cause conversion of **1** into 2-(D-*glycero*-1,2-dihydroxyethyl)furan[4] and, thence, into other furan derivatives[5,6]. The present paper describes the reactions of the alkene sugar derivative **1** with, respectively, bromine, acetyl hypobromite, various carbenoid reagents, a "nitrene", and nitrosyl chloride. The reactions provide a convenient method for introducing bromine atoms at secondary positions by stereospecific processes, and thereby offer procedures for the deoxygenation of sugar derivatives at specific carbon atoms.

Treatment of the alkene **1** with bromine in carbon tetrachloride gave a complex, intractable mixture of products. A similar result was noted by Christensen and Goodman[7]. However, when bromine was added to **1** in methanol in the presence of silver acetate, a crystalline product was isolated in high yield. The elemental analysis of the product showed that it was an adduct of **1** with two bromine atoms, and not the product that might have been anticipated from a Prévost type of reaction[8]. The n.m.r. spectrum of the adduct showed that a monosubstituted benzene ring and a benzylic proton were still present, indicating that bromine had added across the double bond between C-2 and C-3. Four possible, isomeric adducts can be formulated, but, since ionic additions of bromine to alkenes are considered[9] to proceed through bromonium-ion intermediates, neither of the two *cis*-adducts is probable, and consideration of the Fürst–Plattner rule[10] suggests that the diaxial adduct (**2**), having the D-*altro* configuration, would be favored. The n.m.r. spectral data for the adduct in benzene-d_6 (see Tables I–III) are fully consistent with the structure **2** having the D-*altro* configuration, and cannot be reconciled with the three other possible configurations (D-*allo*- D-*gluco*, or D-*manno*); further supporting data were obtained from transformation products derived from **2**.

TABLE I

CHEMICAL SHIFTS OF ACETOXY, BENZOYL, BENZYL, CYCLOPROPYL, AND PHENYL PROTONS

Compound	*Solvent*	*Chemical shifts (τ) from 100-MHz spectra*					
		Ph	*Bz*	*PhCH*	*OMe*	*OAc*	*Cyclopropyl*
2	C_6D_6	2.82		4.67	7.10		
3	$CDCl_3$	2.56		4.45	6.50		
4	$CDCl_3$	2.65		4.44	6.67		8.30–8.90
5	C_6D_6				6.93	8.25	
6	$CDCl_3$		1.90, 2.49		6.52		
7	C_6D_6	2.77		4.55	6.95	8.38	
8	C_6D_6	2.87		4.77	7.06	8.25	
9	$CDCl_3$		1.90, 2.43		6.50	7.81	
10	$CDCl_3$	2.70		4.44	6.57		

The H-1 signal in **2** appeared at τ 5.31 as a narrow multiplet showing $J_{1,2}$ 1.2 Hz and $J_{1,3}$ 0.8 Hz. The small magnitude of $J_{1,2}$ indicates[11] that H-2 is equatorially

TABLE II

CHEMICAL SHIFTS OF RING PROTONS

Compound	Solvent	Chemical shifts (τ) from 100-MHz spectra[a]						
		H-1	H-2	H-3	H-4	H-5	H-6	H-6′
2	C_6D_6	5.31 s[b]	5.67 q	5.50 m	5.95 q	5.63	5.90	6.45
3	$CDCl_3$	5.18 s[b]	5.86 m	3.58 m		[c]	[c]	6.21
4	$CDCl_3$	5.05 d			5.66–6.00 m and 6.20–6.50 m			
5	C_6D_6	5.20 s[b]	5.63 m	5.13 t	4.64 q	[c]	[c]	[c]
6	$CDCl_3$	4.92 s[b]	5.41 m	5.05 t	4.54 q	[c]	[c]	[c]
7	C_6D_6	5.41 s[b]	4.59 d[b]	5.75 t	6.25 q	[c]	[c]	6.43
8	C_6D_6	5.50 d	6.28 q	4.13 t	6.08 t	[c]	[c]	[c]
9	$CDCl_3$	5.24 s[b]	4.68 d[b]	5.40 t	4.77 q	5.50	6.30	6.43
10	$CDCl_3$	5.11 s	[c]	6.86 d	5.75 d	6.15–6.54 m		

[a]First-order values are given. Peak multiplicities: d, doublet; m, multiplet; q, quartet; s, singlet; t, triplet. [b]Broadened. [c]Not determined, because of second-order effects.

TABLE III

FIRST-ORDER COUPLING-CONSTANTS FOR RING PROTONS

Compound	Solvent	Coupling constants (Hz) from 100-MHz spectra							
		$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6e}$	$J_{5,6a}$	$J_{6a,6e}$	$J_{1,3}$
2	C_6D_6	1.2	2.2	3.6	9.1	5.0	10.2	10.2	0.8
3	$CDCl_3$	1	1.5			[a]	[a]	[a]	
4	$CDCl_3$	5.0							
5	C_6D_6	<1	3.5	3.5	8.0	[a]	[a]	[a]	
6	$CDCl_3$	<1	3.0	3.8	9.5	[a]	[a]	[a]	
7	C_6D_6	0.5	2.5	3.5	9.0	[a]	10.2	10.2	
8	C_6D_6	3.5	10.5	9.5	9.5	[a]	[a]	[a]	
9	$CDCl_3$	<1	3.7	4.0	9.0	3.7	6.0	11.5	
10	$CDCl_3$	0	3.5	0	6.0	[a]	[a]	[a]	

[a]Not measured, because of second-order effects.

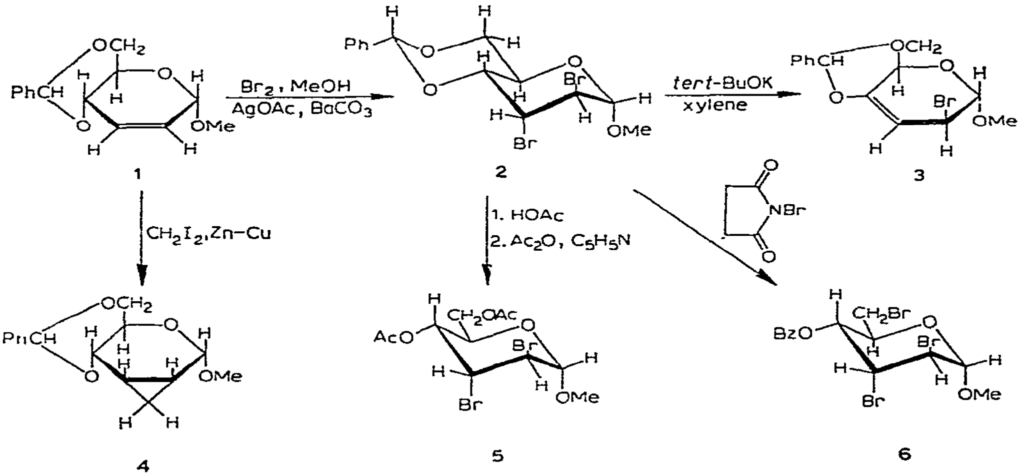
attached, and the long-range $J_{1,3}$ coupling observed suggests that H-3 is also equatorially attached (H-1 and H-3 in a W-arrangement[12]). The H-2 signal was observed as a quartet at τ 5.67, and the H-3 signal as a narrow multiplet at τ 5.50, and the $J_{2,3}$ and $J_{3,4}$ couplings observed were small (2.2 and 3.6 Hz, respectively), showing that there is a *gauche* relationship between H-2 and H-3, and between H-3 and H-4. The H-4 signal (quartet at τ 5.95) showed, in addition to the $J_{3,4}$ coupling, a wide spacing (9.1 Hz) for the $J_{4,5}$ coupling. The couplings observed confirm that H-1, H-2, and H-3 are all equatorial; they fall close to the ranges reported by Coxon[11] for a series of methyl 4,6-*O*-benzylidene-α-D-altropyranoside derivatives. Coxon[11] has shown that the values of the $J_{1,2}$, $J_{2,3}$, and $J_{3,4}$ coupling-constants provide clear differentiation between methyl 4,6-*O*-benzylidene-α-D-hexopyranosides having the D-*allo*, D-*altro*, D-*gluco*, and D-*manno* configurations.

The signal assignments for the spectrum of **2**, and of all other new compounds reported in this paper, were verified by systematic, spin-decoupling studies—by first irradiating the H-1 signal to identify the H-2 signal, and then irradiating the H-2 signal to locate the H-3 signal, and so on for the other protons on the ring.

The crystalline dibromide **2** was obtained in 70% yield when the bromination was conducted in the presence of silver acetate, and it was also obtained, in lower yield, when silver acetate was omitted or when sodium chloride was used instead of silver acetate.

Removal of the *O*-benzylidene group from **2** followed by acetylation gave syrupy methyl 4,6-di-*O*-acetyl-2,3-dibromo-2,3-dideoxy-α-D-altropyranoside (**5**), whose n.m.r. spectrum in chloroform-*d* showed $J_{1,2}$, $J_{2,3}$, and $J_{3,4}$ couplings of <1, 3.5, and 3.5 Hz respectively. The H-1, H-2, and H-3 signals (at τ 5.20, 5.63, and 5.13, respectively) had chemical shifts close to those observed for **2**, but the H-4 signal showed the large downfield shift (~1.3 p.p.m.) anticipated[13], and was observed as a sharp quartet at τ 4.64. Any possibility that second-order effects in the spectrum of **2** might have caused substantial differences between the true $J_{2,3}$, $J_{3,4}$, and $J_{4,5}$ couplings and the observed first-order spacings is excluded by the data for **5**.

Treatment of the dibromide **2** with *N*-bromosuccinimide in refluxing carbon tetrachloride by the general procedure of Hanessian[14] gave crystalline methyl 4-*O*-benzoyl-2,3,6-tribromo-2,3,6-trideoxy-α-D-altropyranoside (**6**), whose n.m.r. spectrum in chloroform-*d* resembled that of **5**, with the H-4 signal as a low-field quartet (τ 4.54); the $J_{1,2}$, $J_{2,3}$, $J_{3,4}$, and $J_{4,5}$ coupling-constants were closely similar to those of **2** and **5**.



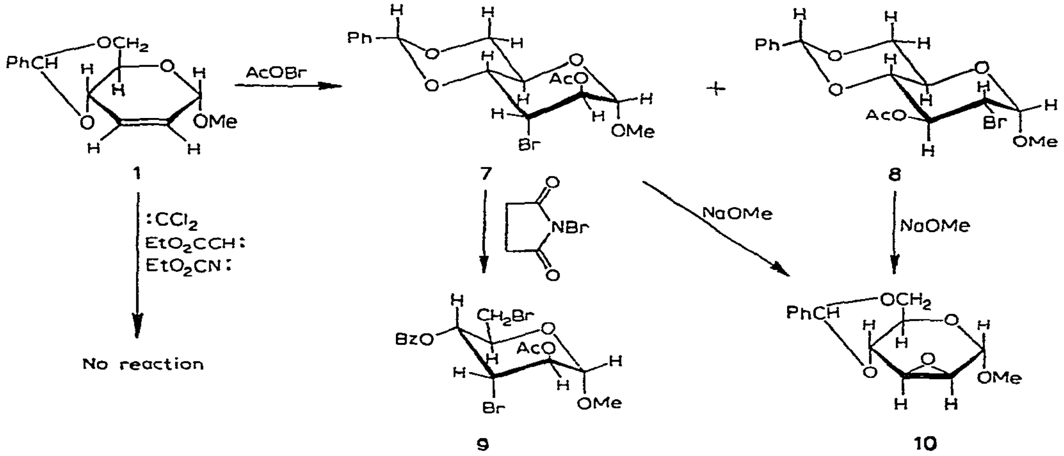
The dibromide **2** was treated with an excess of potassium *tert*-butoxide in refluxing xylene, to give a 90% yield of a crystalline product resulting from the elimination of the elements of hydrogen bromide from **2**; there was no evidence for removal of the second bromine atom, although the reaction conditions were severe. Assuming that the reaction involves *trans*-elimination, the product may be formulated as methyl 4,6-*O*-benzylidene-2-bromo-2,3-dideoxy-α-D-*threo*-hex-3-enopyranoside (**3**). In chloroform-*d*, the H-1 signal of **3** was observed at τ 5.18 as a broadened singlet ($J_{1,2}$ ~1 Hz), and a narrow multiplet at τ 5.86 was identified as the H-2 signal because it collapsed to a doublet of spacing 1.5 Hz ($J_{2,3}$) when the H-1 signal was irradiated. A narrow multiplet in the vinyl-proton region (τ 3.58) was assigned to H-3. Irradiation of the H-2 signal caused the H-1 signal to collapse to a sharp singlet, and the H-3 signal to become a broadened singlet, indicating a small coupling between H-3 and a proton at C-5 or C-6. The protons at C-5 and C-6 gave rise to an ABC type of system[15] that was not amenable to simple analysis. These data are not compatible with conceivable alternative formulations in which the double bond is between C-1 and C-2 and the bromine atom is at C-3, or having a C-2–C-3 double bond and the bromine atom at C-2 or C-3. The structure of **3** was not, however, established independently by degradative experiments.

The failure of **3** to undergo 1,2-elimination is probably attributable to electronic factors, and not to the position of the bromine atom at C-2 being *cis* to H-1. Abstraction of H-1 by base would be especially difficult, because of the presence of two oxygen atoms attached to C-1.

A solution of the alkene **1** in carbon tetrachloride was allowed to react at 0° with acetyl hypobromite[16]. Two isomeric adducts were obtained, and these were separated by fractional recrystallization to give methyl 2-*O*-acetyl-4,6-*O*-benzylidene-3-bromo-3-deoxy-α-D-altropyranoside (**7**) in 77% yield and methyl 3-*O*-acetyl-4,6-*O*-benzylidene-2-bromo-2-deoxy-α-D-glucopyranoside (**8**) in 6% yield. Although there are eight possible structures for an adduct of acetyl hypobromite with the double bond of **1**, the values of the $J_{1,2}$, $J_{2,3}$, and $J_{3,4}$ coupling-constants clearly established that the major adduct has the D-*altro* configuration (diaxial addition) and the minor adduct has the D-*gluco* configuration (diequatorial addition). The low-field location of the H-2 signal in **7** showed that the acetoxy group was at C-2 and the bromine atom at C-3; and, similarly, the acetoxy group in **8** was located at C-3, because the H-3 triplet was at lowest field. The structure of **8** was readily recognized from the spectrum, even before decoupling. Additional support for the configuration assigned to **7** was obtained by treating **7** with *N*-bromosuccinimide to give methyl 2-*O*-acetyl-4-*O*-benzoyl-3,6-dibromo-3,6-dideoxy-α-D-altropyranoside (**9**). The n.m.r. spectrum of **9** resembled that of **7** in the region for ring protons, except that the H-4 signal was shifted downfield by ~1.5 p.p.m., where it appeared as a quartet having the $J_{3,4}$ (4.0 Hz) and $J_{4,5}$ (9.0 Hz) couplings; the H-1 signal was a broadened singlet, that of H-2 was a narrow doublet, and that of H-3 was a narrow triplet.

Independent, chemical verification of the structures assigned to **7** and **8** was provided by the fact that both compounds react with base to give methyl 2,3-anhydro-4,6-*O*-benzylidene-α-D-mannopyranoside (**10**). Of the eight isomeric structures possible for an adduct of acetyl hypobromite and **1**, only **7** and **8** have the oxygen atom *trans* to the vicinal bromine atom, and on the necessary side of the ring for the reaction to give the D-*manno* epoxide (**10**).

The diaxial adduct 7 was the preponderant product, although a higher yield (32%) of the diequatorial adduct 8 was obtained in an experiment performed at 4–6°. As the adducts were not well resolved by chromatography, and had to be separated by recrystallization, the yields isolated are not necessarily reliable indications of the actual distribution of products.



The mechanism of the addition of acetyl hypobromite to **1** was not established. In the steroid field, such additions have been rationalized[16] in terms of an intermediate bromonium ion, formed by attack of Br^+ on the less-hindered side of the double bond, which then suffers *trans*-attack by acetate ion. In both of the observed products (**7** and **8**), the bromine atom is situated below the plane of the ring, and the acetoxy group is *trans* to the bromine atom.

The addition of acetyl hypobromite to **1** provides a useful route, under non-acidic conditions, to *trans*-bromohydrins from the unsaturated sugar. The direct addition of hypohalous acid to **1** is precluded by the extreme acid-lability[4–6] of **1**. The reaction of the acetylated bromohydrin with base furnishes a two-step, stereospecific conversion of the alkene **1** into the D-*manno* epoxide **10** that may be a useful general reaction for converting unsaturated sugars into epoxides under nonacidic conditions. The fact that *trans* acetylated bromohydrins can be readily reconverted into the parent alkenes by use of zinc–copper couple[16] suggests that such adducts as **7** and **8** may be useful for temporary protection of an alkene group during a synthetic sequence.

The alkene **1** was found to be very unreactive toward reagents of the carbenoid and nitrene type[17]. It was recovered unchanged after treatment under forcing conditions with dichlorocarbene[18] (from ethyl trichloroacetate and strong base), ethoxycarbonylcarbene[19] (from ethyl diazoacetate and copper(II) sulfate), and ethoxycarbonylnitrene[20] (from ethyl azidoformate). The behavior of the alkene **1** with dichlorocarbene is in contrast to the behavior of unsaturated sugars of the vinyl ether type, which react to give dichlorocyclopropyl derivatives[21]. However, treatment of **1** with diiodomethane and zinc–copper couple (Simmons–Smith reaction[22]) gave a crystalline, cyclopropyl derivative in 20% yield. The low yield of cyclopropyl derivative in this reaction, even when conducted under forcing conditions, illustrates the low reactivity of **1** toward carbenoid reagents; in contrast, unsaturated sugars having a terminal alkene group, react readily under the Simmons–Smith conditions to give cyclopropyl derivatives in high yield[1,2].

The known directive influence[22] of neighboring oxygen atoms in the Simmons–Smith reaction would suggest that insertion of methylene, by way of iodomethylzinc iodide, should take place from below the plane of the ring, to give methyl 4,6-*O*-benzylidene-2,3-dideoxy-2,3-*C*-methylene-α-D-allopyranoside (**4**). This structure is supported by the observed $J_{1,2}$ coupling of 5 Hz in the n.m.r. spectrum. Hough, Hall, and coworkers[23] have shown that 2,3-epimino, 2,3-epoxy, and 2,3-epithio derivatives of methyl 4,6-*O*-benzylidene-α-D-allopyranose show $J_{1,2}$ values of 2.5–4.5 Hz, whereas the D-*manno* analogs show $J_{1,2}$ of 0 Hz. The structure assigned to **4** has not been verified by degradative methods. An unidentified, iodine-containing side-product accompanied **4** in the reaction mixture.

Nitrosyl chloride, a reagent that reacts readily with unsaturated sugars of the enol-ether type[24,25], reacted with the alkene **1** below room temperature, but the reaction appeared to be readily reversible, because the product decomposed to regenerate the starting alkene **1** when isolation was attempted.

The foregoing reactions of the alkene **1** with various electrophiles show that, for a carbohydrate matrix, the additions proceed with a high degree of stereoselectivity. Such reactions can be useful in synthetic carbohydrate chemistry for introducing novel functionality at secondary and primary positions in a sugar molecule. Since the configurations of the products can be readily determined, the reactions also offer, by suitable degradations, routes to a wide range of optically active, halogenated hydrocarbons, alcohols, and acids, of established stereochemistry.

EXPERIMENTAL

General. — Evaporations were performed under diminished pressure below 40°. Melting points were determined with a Thomas–Hoover "Unimelt" apparatus. I.r. spectra were measured with a Perkin–Elmer Model 137 "Infracord" i.r. spectrophotometer. N.m.r. spectra were recorded at 100 MHz with a Varian HA-100 n.m.r. spectrometer, and chemical shifts refer to an internal standard of tetramethylsilane (τ = 10.00); signal assignments were verified by spin decoupling. Microanalyses were made by W. N. Rond. X-Ray powder diffraction data give interplanar spacings, Å, for CuKα radiation. The camera diameter was 114.59 mm. Relative intensities were estimated visually: m, moderate; s, strong; v, very; w, weak. The strongest lines are numbered (1, strongest); double numbers indicate approximately equal intensities. T.l.c. was performed with Silica Gel G (E. Merck, Darmstadt, Germany), activated at 120°, as the adsorbent, and sulfuric acid as the indicator. An adsorbent thickness of 250 μm was used for analytical t.l.c., and a 1-mm thickness for preparative t.l.c.

Column chromatography was performed, unless otherwise stated, with Silica Gel Davison (60–200 mesh, Davison Division of the W. R. Grace Co., Baltimore, Md.) as the adsorbent, with 1 g of the mixture to be separated per 100 g of adsorbent, and the components were eluted from the columns with the solvents indicated.

*Methyl 4,6-O-benzylidene-2,3-dibromo-2,3-dideoxy-α-*D*-altropyranoside* (**2**). — To a solution of methyl 4,6-*O*-benzylidene-2,3-dideoxy-α-D-*erythro*-hex-2-enopyranoside[4] (**1**, 3.00 g, 12 mmoles) in dry methanol (300 ml) was added silver acetate (2.88 g, 18 mmoles) and barium carbonate (10 g). The resulting mixture was cooled to 0°, and to it was added dropwise, with stirring, a solution of bromine (1.08 ml, 18 mmoles) in dry methanol (100 ml). The mixture was stirred for 16 h at room temperature, and filtered, and the filtrate was evaporated. The resultant syrup was dissolved in carbon tetrachloride, and the solution was washed successively with 20-ml portions of 5% aqueous sodium hydrogen sulfite and water, dried (magnesium sulfate), and evaporated; the resultant syrup was purified by column chromatography on silica gel with benzene as eluant to give the dibromide **2** as a chromatographically homogeneous syrup that crystallized spontaneously; yield 3.45 g (70%), m.p. 79–81°, $[\alpha]_D^{20}$ +72.5 ±1° (*c* 0.7, chloroform); R_F 0.74 (benzene); λ_{max}^{KBr} 13.3, 14.4 μm (phenyl); X-ray powder diffraction data: 11.25 s (2,2), 9.11 s (2,2), 7.72 m, 7.27 vw, 6.72 vw, 5.66 m, 5.34 m, 5.02 vw, 4.70 w, 4.48 m, 4.27 vw, 4.09 vs (1), and 3.67 s (3).

Anal. Calc. for $C_{14}H_{16}Br_2O_4$: C, 41.20; H, 3.95; Br, 39.16. Found: C, 41.45; H, 3.95; Br, 38.96.

The preparation was repeated, once without the use of silver acetate, and once with an equimolar amount of sodium chloride instead of silver acetate. In each case, the dibromide **2** was obtained, but the yields were diminished to 32 and 28%, respectively.

Direct addition of bromine (0.36 ml, 6 mmoles) to the unsaturated sugar **1** (1.00 g, 4 mmoles) in carbon tetrachloride (20 ml) at 0° gave a product, isolated after 5 h at 30°, that was shown by t.l.c. (benzene) to contain at least 6 components, one of which corresponded to the dibromide **2**, but **2** could not be crystallized from the mixture.

*Methyl 4,6-O-benzylidene-2-bromo-2,3-dideoxy-α-*D-threo-*hex-3-enopyranoside* (**3**). — A mixture of the dibromide **2** (306 mg, 750 μmoles) and potassium *tert*-butoxide (168 mg, 1.5 mmoles) in dry xylene (50 ml) was refluxed for 16 h under nitrogen. The mixture was filtered, the filtrate was evaporated, and a solution of the residue in dichloromethane was purified by passage through a column (20 × 100 mm) of neutral, Woelm alumina. Evaporation of the effluent gave crystalline **3**; yield 220 mg (90%), m.p. (after recrystallization from chloroform–petroleum ether to give long needles) 187–189°, $[\alpha]_D^{20}$ +77 ±1° (*c* 1, chloroform); R_F 0.36 (dichloromethane); λ_{max}^{KBr} 6.07 (C=C), 13.3, 14.4 μm (phenyl); X-ray powder diffraction data: 12.84 m, 11.15 w, 6.24 s (3), 5.62 w, 4.99 w, 4.67 w, 4.41 w, 4.14 vs (2), 3.92 vs (1), 3.70 m, 3.55 vw, 3.43 m, 3.30 vw, 3.09 vw, and 2.93 m.

Anal. Calc. for $C_{14}H_{15}BrO_4$: C, 51.39; H, 4.62; Br, 24.42. Found: C, 51.56; H, 4.55; Br, 24.74.

*Methyl 4,6-di-O-acetyl-2,3-dibromo-2,3-dideoxy-α-*D*-altropyranoside* (**5**). —

A solution of the dibromide **2** (750 mg, 1.84 mmoles) in acetic acid (15 ml) was heated in a boiling-water bath, water (10 ml) was added dropwise, and the mixture was then heated for 30 min. The resultant solution was evaporated at 50°, and toluene was several times added to and evaporated from the residue. The residue was treated with dry pyridine (5 ml) and acetic anhydride (3 ml) for 18 h at ~30°. The solution was poured over ice, and, after 30 min, the mixture was extracted with two 50-ml portions of dichloromethane. The extracts were combined, dried (magnesium sulfate), and evaporated; toluene was added to and evaporated from the residue to remove pyridine, and the product was purified by column chromatography on silica gel with 20:1 dichloromethane–ether as eluant. The diacetate **5** was obtained as a chromatographically homogeneous syrup; yield 642 mg (86%), $[\alpha]_D^{23}$ +67.0 ±1° (c 1, chloroform); R_F 0.34 (chloroform); λ_{max}^{film} 5.72 μm (OAc).

Anal. Calc. for $C_{11}H_{16}Br_2O_6$: C, 32.69; H, 3.99; Br, 39.56. Found: C, 32.72; H, 3.95; Br, 39.86.

*Methyl 4-O-benzoyl-2,3,6-tribromo-2,3,6-trideoxy-α-*D*-altropyranoside* (**6**). — To a suspension of *N*-bromosuccinimide (485 mg, 2.7 mmoles) in dry carbon tetrachloride (30 ml) was added the dibromide **2** (1.00 g, 2.4 mmoles) followed by barium carbonate (1.0 g). The mixture was refluxed for 2 h, cooled to 0°, and filtered. The filtrate was washed successively with 10-ml portions of 5% aqueous sodium hydrogen sulfite and water, dried (magnesium sulfate), and evaporated to a syrup. The product was purified by chromatography on a column of silica gel with 1:2 carbon tetrachloride–benzene as eluant, to give **6** as a chromatographically homogeneous syrup; yield 650 mg (54%), crystallized from isopropyl alcohol, m.p. 99–100°, $[\alpha]_D^{23}$ +56.5 ±1° (c 1, chloroform); R_F 0.62 (benzene); λ_{max}^{KBr} 5.80 (OBz), 13.8, 14.2 μm (phenyl); X-ray powder diffraction data: 10.71 w, 9.40 vs (1), 7.70 vw, 6.58 m, 6.00 m, 5.61 m, 5.32 m, 4.59 m, 4.31 m, 4.09 m, and 3.85 s (2).

Anal. Calc. for $C_{14}H_{15}Br_3O_4$: C, 34.52; H, 3.10; Br, 49.23. Found: C, 34.69; H, 3.12; Br, 49.37.

*Methyl 2-O-acetyl-4,6-O-benzylidene-3-bromo-3-deoxy-α-*D*-altropyranoside* (**7**) *and methyl 3-O-acetyl-4,6-O-benzylidene-2-bromo-2-deoxy-α-*D*-glucopyranoside* (**8**). — An 0.1M solution of acetyl hypobromite[16] was prepared as follows. A suspension of silver acetate (4.00 g, 24 mmoles) in carbon tetrachloride (160 ml) was stirred at 0°, and a solution of bromine (1.00 ml, 19 mmoles) in carbon tetrachloride (20 ml) was added dropwise during 30 min. After an additional 1.5 h, the stirring was stopped, and the solution was decanted from the silver bromide that had settled.

A solution of the unsaturated sugar **1** (3.00 g, 12 mmoles) in carbon tetrachloride (200 ml) was cooled to 0° under nitrogen. Barium carbonate (10 g) and 0.1M acetyl hypobromite in carbon tetrachloride (240 ml, 24 mmoles) were added, and the mixture was stirred for 6 h at 0°. Aqueous sodium hydrogen sulfite (5%, 30 ml) was shaken with the solution, solids were filtered off, and the organic phase was washed with two 30-ml portions of water, dried (magnesium sulfate), and evaporated. The resultant syrup was purified on a column of silica gel with dichloromethane as eluant, to remove a small amount of starting material **1** and some colored side-products that remained at the top of the column. The product appeared to be chromatographically homogeneous, but was shown by fractional recrystallization to be a mixture of two isomeric adducts. Crystallization of the product from carbon tetrachloride–petroleum ether gave the D-*gluco* adduct **8** as needles; yield 300 mg (6%), m.p. 171–173°, $[\alpha]_D^{20}$ +89.0 ±1° (*c* 1, chloroform); R_F 0.23 (chloroform); λ_{max}^{KBr} 5.82 (OAc), 13.1, 14.4 µm (phenyl); X-ray powder diffraction data: 12.89 vw, 11.59 vw, 9.58 w, 8.11 m, 6.55 vw, 6.20 vw, 5.79 vw, 4.96 vs (1), 4.74 w, 4.49 s (2), 4.07 m, 3.81 vw, 3.59 m, 3.27 vw, and 3.15 m.

Anal. Calc. for $C_{16}H_{19}BrO_6$: C, 49.62; H, 4.94; Br, 20.64. Found: C, 49.64; H, 5.13; Br, 20.53.

The second adduct (the D-*altro* derivative, **7**) was crystallized from chloroform–petroleum ether to give **7**, yield 3.30 g (77%), having m.p. 101–103°, $[\alpha]_D^{20}$ +80.5 ±1° (*c* 1, chloroform); R_F 0.22 (chloroform); λ_{max}^{KBr} 5.74 (OAc), 13.2, 14.3 µm (phenyl); X-ray powder diffraction data: 12.58 vw, 10.25 vw, 8.79 vw, 7.54 m, 7.21 m, 6.55 w, 6.14 vw, 5.94 m, 5.53 m, 4.92 m, 4.77 vs (1), 4.54 w, 4.03 s (2), 3.87 m, 3.71 m, and 3.58 m.

Anal. Calc. for $C_{16}H_{19}BrO_6$: C, 49.62; H, 4.94; Br, 20.64. Found: C, 49.59; H, 5.19; Br, 20.86.

Repetition of the reaction, but for 16 h at 4–6°, gave the D-*gluco* isomer **8** in 32% yield and the D-*altro* isomer **7** in 13% yield.

Methyl 2-O-acetyl-4-O-benzoyl-3,6-dibromo-3,6-dideoxy-α-D-altropyranoside (**9**). — The D-*altro* adduct (**7**, 820 mg, 21 mmoles) was treated with *N*-bromosuccinimide (415 mg, 23 mmoles) by the procedure used for conversion of **2** into **6**. The resultant syrup was purified on a column of silica gel, with 100:1 dichloromethane–ether as eluant, to give **9** as a chromatographically homogeneous syrup; yield 400 mg (41%). Crystallization of **9** from isopropyl alcohol gave **9** as rosettes, m.p. 92–94°, $[\alpha]_D^{23}$ +42.0 ±1° (*c* 1, chloroform); R_F 0.19 (chloroform); λ_{max}^{KBr} 5.80 (OAc, OBz), 13.9, 14.6 µm (phenyl); X-ray powder diffraction data: 11.94 vs (1), 7.67 m, 6.99 vw, 6.12 m, 5.70 m, 5.12 s (2), 4.61 s (3), 4.06 m, 3.85 vw, 3.73 w, and 3.55 m.

Anal. Calc. for $C_{16}H_{18}Br_2O_6$: C, 41.22; H, 3.89; Br, 34.29. Found: C, 41.28; H, 3.71; Br, 34.53.

Conversion of the adducts 7 *and* 8 *into methyl 2,3-anhydro-4,6-O-benzylidene-α-D-mannopyranoside* (**10**). — Sodium (120 mg, 5.2 mmoles) was dissolved in dry methanol (15 ml), the D-*altro* adduct **7** (1.00 g, 2.6 mmoles) was added, and the mixture was refluxed for 4 h. Needles of the epoxide **10** separated as the solution was cooled; yield 548 mg (81%), m.p. (after recrystallization from ethanol) 144–146°, $[\alpha]_D^{21}$ +106° (*c* 1.5, chloroform) [lit.[26] m.p. 145–147°, $[\alpha]_D^{15}$ +107° (*c* 1.6, chloroform)]; R_F 0.19 (dichloromethane); λ_{max}^{KBr} 13.3, 14.4 µm (phenyl); X-ray powder diffraction data: 8.56 s (3), 7.74 w, 6.02 vs (1), 4.40 w, 4.20 w, 4.00 s (2), 3.63 m, 3.54 vw, and 3.26 w.

Anal. Calc. for $C_{14}H_{16}O_5$: C, 63.62; H, 6.10. Found: C, 63.74; H, 6.05.

The product was identical with an authentic sample of **10** by t.l.c., mixed m.p., i.r. and n.m.r. spectra, and X-ray powder diffraction pattern.

The experiment was repeated, but with the D-*gluco* adduct **8** as the starting material. The product (yield 53%, m.p. 144–145°) was the D-*manno* epoxide **10**, as shown by mixed m.p., t.l.c., i.r. and n.m.r. spectra, and X-ray powder diffraction pattern, in comparison with those of authentic **10**.

*Methyl 4,6-*O*-benzylidene-2,3-*C*-methylene-2,3-dideoxy-α-*D*-allopyranoside* (**4**). — Zinc–copper couple, prepared as previously described[1,27] from granular zinc (2.6 g) and copper(II) acetate monohydrate (50 mg) was placed, together with dry ether (50 ml), in a 250-ml, round-bottomed, 3-necked flask fitted with a reflux condenser, an addition funnel, and a magnetic stirrer. Diiodomethane (3.2 ml, 40 mmoles) was added, and reaction was initiated by gentle heating until small bubbles were generated from the couple. The mixture was boiled for 30 min under reflux, and a solution of the unsaturated sugar **1** (1.00 g, 4 mmoles) in dry ether (50 ml) was then added dropwise during 30 min. Heating was continued for a further 72 h, the solution was cooled, and saturated aqueous ammonium chloride (30 ml) was added to precipitate inorganic salts. The mixture was filtered, the layers of the filtrate were separated, and the aqueous layer was extracted with two 50-ml portions of ether. The ethereal solutions were combined, washed successively with 30-ml portions of saturated, aqueous potassium carbonate and saturated, aqueous sodium chloride, dried (magnesium sulfate), and evaporated to a syrup. T.l.c. of the latter with benzene as developer showed major components having R_F 0.71, 0.15, and 0.10. Resolution of the mixture by preparative t.l.c.[28], with benzene as the developer, gave the component having R_F 0.71 as a crystalline, unidentified product, yield 161 mg, m.p. (after recrystallization from Skellysolve C) 81–83°. The components having R_F 0.15 and 0.10 were incompletely resolved, and were subjected to a second separation by preparative t.l.c., but with 6:1 benzene–ether as the developer, to give the starting material **1**, yield 100 mg, R_F 0.15 (benzene), and the crystalline cyclopropyl derivative **4**, yield 170 mg (20% based on unrecovered **1**), m.p. (after recrystallization from Skellysolve C) 126–128°; R_F 0.10 (benzene), 0.78 (1:1 benzene–ether); $\lambda_{\max}^{\mathrm{KBr}}$ 3.25 (cyclopropyl[29] C–H), 13.2, 14.3 μm (phenyl); X-ray powder diffraction data: 13.08 w, 9.98 w, 8.32 vs (1), 6.69 vw, 5.69 w, 5.37 m, 4.62 w, 4.29 s (2), 4.13 w, 3.90 m, 3.63 m, and 3.36 m.

Anal. Calc. for $C_{15}H_{18}O_4$: C, 68.73; H, 6.91. Found: C, 68.77; H, 6.89.

The side product (*Anal.* Found: C, 60.65; H, 5.28; I, 19.66) showed n.m.r. absorptions (chloroform-*d*) at τ ~2.5 (phenyl), 3.62, 4.35, 4.55, 4.91, 5.12, 5.50–5.82, 6.09–6.50, and 7.0; no intense singlet for an OMe group was present.

Attempted reaction of alkene **1** *with carbenes and nitrenes. — A. With dichloromethylene.* A vigorously stirred slurry of **1** (1.00 g, 4 mmoles) and sodium methoxide (0.80 g, 16 mmoles) in dry hexane (100 ml) was treated with ethyl trichloroacetate (1.52 g, 8 mmoles) at 0°, and the mixture was stirred for 18 h at 30°. Water was then added, the layers were separated, the aqueous phase was extracted with ether, and the organic solutions were combined, dried (magnesium sulfate), and evaporated. The crystalline residue (800 mg) was chromatographically homogeneous, and identical with the starting material (**1**) by t.l.c. and i.r. spectrum. The experiment was repeated, but with heating for 18 h under reflux; unchanged starting-material was again recovered.

B. With ethyl diazoacetate. To a stirred mixture of **1** (200 mg, 800 μmoles) and anhydrous copper(II) sulfate (400 mg) in iso-octane (40 ml) was added ethyl diazoacetate (365 mg, 0.33 ml, 3.2 mmoles) during 1 h at 30°; nitrogen was evolved. The mixture was refluxed for 24 h, and then filtered. Evaporation of the filtrate gave a residue identical with **1** by i.r. spectrum and t.l.c.

C. With ethyl azidoformate. A mixture of **1** (248 mg, 1 mmole) and ethyl azidoformate (115 mg, 1 mmole) in purified heptane was refluxed for 96 h under nitrogen. Evaporation of the solution gave a residue indistinguishable from **1** by i.r. spectrum. T.l.c. (6:1 benzene–ether) showed **1** and traces of colored, nonmigrating products. Additional ethyl azidoformate was added, and the experiment was repeated, but unchanged **1** was again recovered.

Cyclohexene, instead of the alkene **1**, was subjected to the conditions of the foregoing experiments A, B, and C, and, in each case, the expected adduct was obtained.

Reaction of alkene **1** *with nitrosyl chloride.* — Streams of gaseous nitrosyl chloride and nitrogen were passed simultaneously through a mixture of **1** (1.00 g, 4 mmoles) and barium carbonate (5 g) in carbon tetrachloride for 2 h at 0°. The color of the solution turned greenish blue and then brown. T.l.c. (ether) showed starting material (R_F 0.9) and a product having R_F 0.35 in the approximate intensity ratio of 1:4. The solution was concentrated at 25° to remove excess nitrosyl chloride, the solids were filtered off, and the filtrate was evaporated to give a white solid that was indistinguishable from **1** by t l.c. and by i.r. and n.m.r. spectra. In a second experiment, the reaction was monitored by t.l.c. during the isolation procedure, and it was observed that, during the evaporation stages, the product having R_F 0.35 was decomposed to give starting material **1**.

ACKNOWLEDGMENTS

The authors thank Professor L. D. Hall for measuring the n.m.r. spectrum of cyclopropyl derivative **4**, and Dr. T. Tsuchiya for experimental advice.

REFERENCES

1 D. Horton and C. G. Tindall, Jr., *Carbohyd. Res.*, 8 (1968) 328.
2 Ester L. Albano, D. Horton, and C. G. Tindall, Jr., *Abstracts Papers Amer. Chem. Soc. Meeting*, 153 (1967) c13; Ester L. Albano, D. Horton, and T. Tsuchiya, *ibid.*, 155 (1968) c29.
3 Ester L. Albano, D. Horton, and J. H. Lauterbach, *Chem. Commun.*, (1968) 357.
4 Ester L. Albano, D. Horton, and T. Tsuchiya, *Carbohyd. Res.*, 2 (1966) 349.
5 D. Horton and T. Tsuchiya, *Carbohyd. Res.*, 3 (1966) 257.
6 D. Horton and T. Tsuchiya, *Chem. Ind.* (London), (1966) 2011.
7 J. E. Christensen and L. Goodman, *J. Amer. Chem. Soc.*, 83 (1961) 3827.
8 C. Prévost, *Compt. Rend.*, 198 (1934) 2264.
9 I. Roberts and G. E. Kimball, Jr., *J. Amer. Chem. Soc.*, 59 (1937) 947; P. B. De La Mare and R. Bolton, *Electrophilic Additions to Unsaturated Systems*, Elsevier, Amsterdam, 1966, Chapter 7.
10 A. Fürst and P. A. Plattner, *Abstracts Papers 12th Intern. Congr. Pure Appl. Chem., New York*, (1951) 409.

11 B. Coxon, *Tetrahedron*, 21 (1965) 3481.
12 L. D. Hall and L. Hough, *Proc. Chem. Soc.*, (1962) 382; K. Heyns, J. Weyer, and H. Paulsen, *Chem. Ber.*, 98 (1965) 327; D. Horton and J. S. Jewell, *Carbohyd. Res.*, 5 (1967) 149.
13 D. Horton and H. S. Prihar, *Carbohyd. Res.*, 4 (1967) 115.
14 S. Hanessian, *Carbohyd. Res.*, 2 (1966) 86.
15 J. A. Pople, W. G. Schneider, and H. J. Bernstein, *High Resolution Nuclear Magnetic Resonance*, McGraw–Hill Book Co., Inc., New York, N. Y., 1959, p. 132.
16 S. G. Levine and M. E. Wall, *J. Amer. Chem. Soc.*, 81 (1959) 2826.
17 J. I. Cadogan and M. J. Perkins, in *The Chemistry of Alkenes* (S. Patai, Ed.), Wiley–Interscience, New York, 1964, Ch. 9.
18 W. E. Parham and E. E. Schweizer, *J. Org. Chem.*, 24 (1959) 1733.
19 P. S. Skell and R. M. Etter, *Proc. Chem. Soc.*, (1961) 443.
20 W. Lwowski and T. Mattingly, Jr., *J. Amer. Chem. Soc.*, 87 (1965) 1947.
21 J. S. Brimacombe, M. E. Evans, E. J. Forbes, and J. M. Webber, *Carbohyd. Res.*, 4 (1967) 239.
22 H. E. Simmons and R. D. Smith, *J. Amer. Chem. Soc.*, 80 (1958) 5323; 81 (1959) 4256; W. G. Dauben and G. H. Berezin, *ibid.*, 85 (1963) 468.
23 D. H. Buss, L. Hough, L. D. Hall, and J. F. Manville, *Tetrahedron*, 21 (1965) 69.
24 W. J. Serfontein, J. H. Jordaan, and J. White, *Tetrahedron Lett.*, (1964) 1069.
25 R. U. Lemieux, T. L. Nagabhushan, and I. K. O'Neill, *Tetrahedron Lett.*, (1964) 1909; R. U. Lemieux and T. L. Nagabhushan, *ibid.*, (1965) 2143.
26 L. F. Wiggins, *Methods Carbohyd. Chem.*, 2 (1963) 189.
27 E. LeGoff, *J. Org. Chem.*, 29 (1964) 2048.
28 D. Horton and T. Tsuchiya, *Carbohyd. Res.*, 5 (1967) 426.
29 K. Nakanishi, *Infrared Absorption Spectroscopy*, Holden–Day, Inc., San Francisco, 1963, p. 21.

Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

TRANSFORMATIONS OF SUGARS IN ALKALINE SOLUTIONS*

H. S. ISBELL**, H. L. FRUSH, C. W. R. WADE, AND C. E. HUNTER
Division of Analytical Chemistry, Institute for Materials Research, National Bureau of Standards, Washington, D.C. 20234 (U.S.A.)

(Received July 15th, 1968; in revised form, August 31st, 1968)

ABSTRACT

A comparative study has been made of the rates of enolization of sugars in alkaline, tritiated water by measurement of their rates of tritium uptake; the rates for two inososes were found to be more than 1000 times the rate for D-glucose. The low rates of reaction of the sugars in comparison with those for the inososes suggest that one of the factors controlling the rate of enolization is the concentration of an intermediate carbonyl form of the sugar. To account for differences in the behavior of epimeric sugars in alkaline solution and the well-known differences between the rates of mutarotation and of exchange of ^{18}O of an aldose-*1*-^{18}O with ^{16}O of water, it is suggested that the mutarotation reaction proceeds through pseudo-acyclic intermediates that possess some characteristics of the respective ring-forms. This new concept, applied to the interpretation of enolization reactions, leads to the possibility of formation of *cis*- and *trans*-enediols, the proportions of which may differ from sugar to sugar. Enolization experiments were conducted for a variety of sugars in a 0.5M solution of sodium carbonate in water-*t* at 25°. The rates of tritium uptake, based on the rate for D-glucose as unity, range from 0.5 to 4.8 for aldohexoses, 0.5 to 10.7 for aldoheptoses, 4.1 to 28 for aldopentoses, 2.6 to 25 for various ketoses, 0.3 to 0.8 for some 6-deoxy sugars, and 90 to 155 for some 2-deoxy sugars. The comparative rates found for the disaccharides studied range from 2.0 to 240; of these, turanose was the most reactive sugar.

INTRODUCTION

The rearrangements that take place when a reducing sugar is dissolved in water containing a base catalyst have engaged the attention of carbohydrate chemists ever since discovery of these reactions by Lobry de Bruyn and Alberda van Ekenstein[1]. Nef showed that, in alkaline solution, a reducing sugar may yield more than 100 products[2]. After arduous investigation, he concluded that the complex mixtures arise from a series of enolization, fragmentation, and benzylic acid-type reactions.

---

*Presented, in part, before the Division of Carbohydrate Chemistry at the 155th National Meeting of the American Chemical Society, San Francisco, California, April, 1968.
**Present address: Chemistry Department, American University, Washington, D.C. 20016.

Quantitative evaluation of this early work is not feasible, first, because data were not obtained for the initial stages of the reactions, and, second, because the reactions were not terminated by equilibrium conditions but by consumption of the base catalyst by the saccharinic acids formed. Subsequently, many workers have investigated the reactions of sugars in alkaline solutions[3–9]. The use of isotopic tracers (^{14}C, ^{3}H, and ^{2}H) has proved particularly fruitful in the study of mechanisms and the quantitative evaluation of products[10–16]. For better understanding of the reactions, it is necessary to examine the changes in the early stages of the process and to follow each step in detail.

This paper describes a method for evaluating the primary rate of enolization, and gives the results obtained with a large group of sugars. To account for differences in the rearrangement products from epimeric sugars, the concept of pseudo-acyclic intermediates is advanced; these intermediates may lead to enediol mixtures characteristic of the parent sugar. The rates of enolization provide an insight into the comparative reactivity of the various sugars and their tendency to proceed to rearrangement products.

DISCUSSION

Pseudo-acyclic intermediates in mutarotation reactions

Presumably, the alkaline rearrangement of a sugar begins with the formation of an acyclic intermediate by the mutarotation reaction, followed by the formation of an enediol. Prior investigations have indicated that the mutarotation reaction takes place primarily through acyclic intermediates by acid- and base-catalyzed mechanisms[16–20]. When a sugar is dissolved in water, an equilibrium is established, under suitable conditions, among the ionic and solvated forms of the pyranose, furanose, and acyclic modifications of the sugar. The subsequent behavior of the sugar depends in large measure on the position of this equilibrium and the rates at which the modifications are interconverted.

A single acyclic form will not account for certain observations made concerning mutarotation. Thus, when D-glucose-*1*-^{18}O is dissolved in water, the exchange of the ^{18}O with the ^{16}O of the water is much slower than the mutarotation reaction[14,21]. Hence, the carbonyl group is not sufficiently free to become completely and reversibly hydrated during the mutarotation reaction.

The suggestion is therefore advanced that, in the mutarotation reaction, the sugar ring may be opened momentarily, forming a pseudo-acyclic intermediate that has a conformation similar to that of the parent sugar. This intermediate passes through characteristic transitions states to pyranoses, furanoses, and other products. The processes are depicted in Figs. 1 and 2, in which the pseudo-acyclic intermediates are designated $\mu_{\alpha p}$, $\mu_{\beta p}$, $\mu_{\alpha f}$, and $\mu_{\beta f}$. In forming the β-pyranose from the α-pyranose (see Fig. 1, curve 1, and Fig. 2), the intermediate $\mu_{\alpha p}$, formed by rupture of the pyranose ring in transition state A, may pass to transition state B by rotation of the anomeric carbon atom through 60°; from this point, B may either revert to the α anomer, or proceed to the β anomer through the second pseudo-acyclic intermediate $\mu_{\beta p}$ and the

$$\begin{tikzcd}
\alpha\text{-pyranose} \arrow[r, leftrightharpoons] & \mu_{\alpha p} \arrow[r, leftrightharpoons] \arrow[d, leftrightarrows] & \mu_{\beta p} \arrow[r, leftrightharpoons] \arrow[d, leftrightarrows] & \beta\text{-pyranose} \\
\alpha\text{-furanose} \arrow[r, leftrightharpoons] & \mu_{\alpha f} \arrow[r, leftrightharpoons] \arrow[d] & \mu_{\beta f} \arrow[r, leftrightharpoons] \arrow[dl] & \beta\text{-furanose} \\
 & \text{Equilibrium with other acyclic and ring modifications} & &
\end{tikzcd}$$

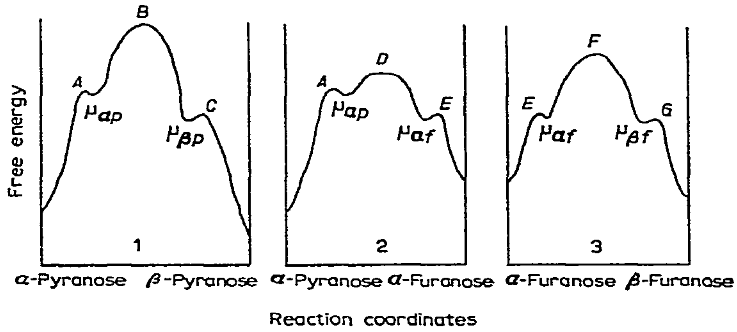


Fig. 1. Hypothetical role of pseudo-acyclic intermediates in mutarotation reactions. The curves qualitatively represent the changes in free energy of a sugar in which the β-pyranose is more stable than the α-pyranose (curve 1). The pyranose–furanose interconversion (curve 2) is faster than either the α–β-pyranose anomerization (curve 1) or the α–β-furanose anomerization (curve 3). The relative stabilities of the isomers are represented in the decreasing order: β-pyranose > α-pyranose > β-furanose > α-furanose.

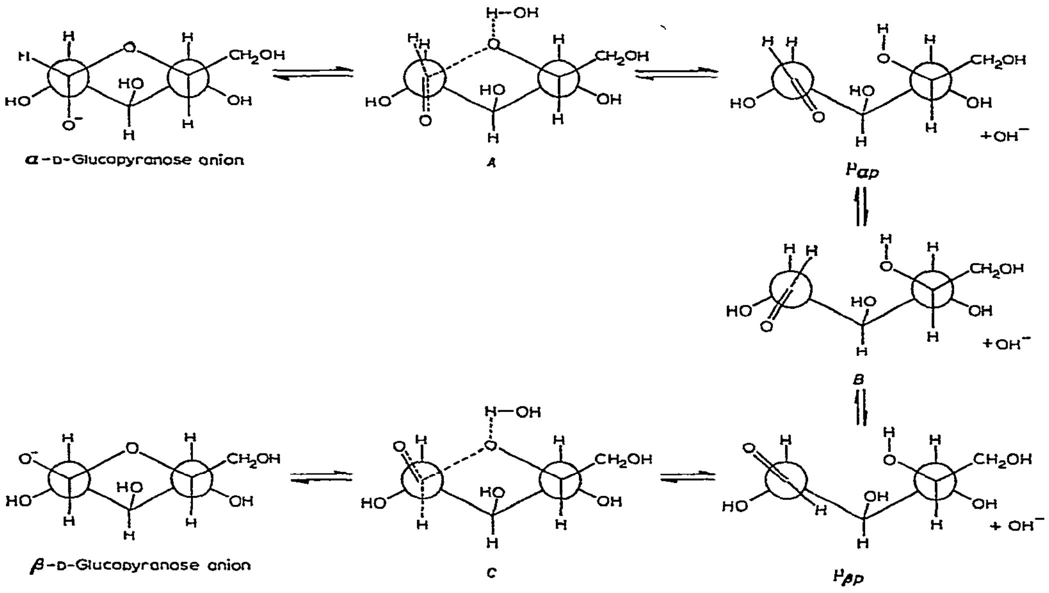


Fig. 2. Hypothetical intermediates in the interconversion of α- and β-D-glucopyranose in alkaline solutions. *A*, *B*, and *C* represent the transitions states of Fig. 1, curve 1.

transition state *C*. The reverse process, starting with the β anomer may yield, in turn, transition state *C*, $\mu_{\beta p}$, transition state *B*, $\mu_{\alpha p}$, transition state *A*, and, finally, the α anomer. The energy barrier between the pseudo-acyclic intermediates $\mu_{\alpha p}$ and $\mu_{\beta p}$ arises from eclipsing of the groups of C-2 by the groups of C-1, when C-1 is turned about the C-1–C-2 axis.

A similar mechanism would apply to furanose anomerizations (see Fig. 1, curve 3). In forming a furanose form from a pyranose form (see Fig. 1, curve 2), the pseudo-acyclic intermediate derived from a pyranose form may pass through a transition state *D* in which the carbon skeleton is so altered that the oxygen atom on C-4 approaches the anomeric carbon atom. This may lead to the pseudo-acyclic intermediate that ultimately gives the furanose forms.

The transformations depicted in Fig. 2 are for the mutarotation of D-glucopyranose in alkaline solutions. Although acid-catalyzed reactions follow somewhat different mechanisms, it is suggested that these, also, involve pseudo-acyclic intermediates. In an enzyme-catalyzed reaction, the enzyme may aid in retaining the conformation of the pseudo-acyclic intermediates.

Pseudo-acyclic intermediates in rearrangement reactions

The pseudo-acyclic intermediates may also function in the formation of the enediols, and may account, in a qualitative way, for certain puzzling differences in the rearrangement products from epimeric sugars. If the 1,2-enediols from D-glucose, D-mannose, and D-fructose were identical, the rates of conversion of any two of the sugars into the third would be proportional to the rates of enolization of those two. However, quantitative study of the conversion products at low temperature (5–7°) has shown that this is not the case[22]. The differences may arise from the presence of *cis* and *trans* isomers of the enediols.

Previously, Topper and Stetten[11] had postulated the formation of a *trans*-enediol from D-glucose, a *cis*-enediol from D-mannose, and interconversion of the two aldoses through D-fructose. However, Sowden and Schaffer[12b] failed to confirm this postulate.

By way of the pseudo-acyclic intermediates postulated here, and removal of the hydrogen atom on C-2 through enolization, α-D-glucopyranose should form a *cis*-enediol, and β-D-mannopyranose should yield the same enediol; similarly, β-D-glucopyranose and α-D-mannopyranose should yield the same *trans*-enediol (Fig. 3). With a ketose, the pseudo-acyclic intermediate may enolize between C-1 and C-2, and between C-2 and C-3 (Fig. 4). The principal enolization (at least in the case of D-fructose) is between C-1 and C-2. Because of the presence of two hydrogen atoms at C-1, the enolization of a single anomer would result in both *cis*- and *trans*-1,2-enediols. Thus, both an aldose and a ketose may yield mixtures of *cis*- and *trans*-enediols, the proportions of which differ from sugar to sugar.

The fate of the enediol in the reaction mixture depends on several competing reactions. Addition of a proton to the double bond of the enediol yields the epimeric sugars and the related ketose. This reaction is reversible, and, in water-*t*, results in hydrogen–tritium exchange. *beta*-Elimination reactions also occur; in these, such a group as -OH, -OR, or -OAc, attached to the carbon atom adjacent to the enolic group, is eliminated by a shift of electrons from the double bond. Formation of the resulting unsaturated sugar does not involve incorporation of hydrogen (or tritium) into the sugar structure, but subsequent reactions may. The relative importance of the de-enolization and *beta*-elimination reactions varies widely. Substances having good leaving-groups adjacent to the enediol group undergo *beta*-elimination reactions rapidly.

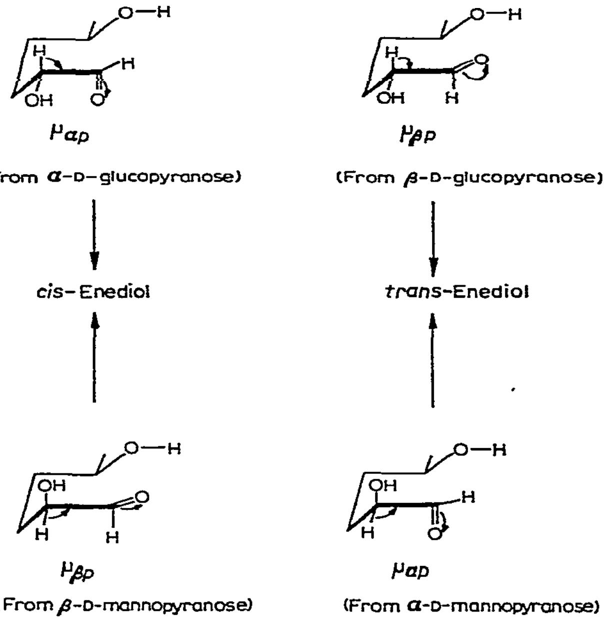


Fig. 3. Suggested formation of *cis*- and *trans*-enediols from pseudo-acyclic intermediates of two epimeric aldohexopyranoses. The hydroxyl groups have been omitted from C-3, -4, and -6 for clarity.

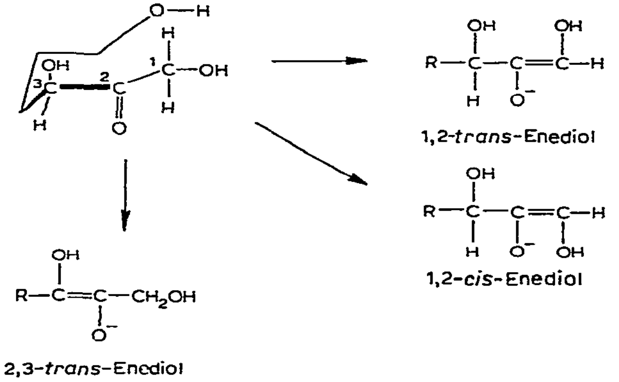
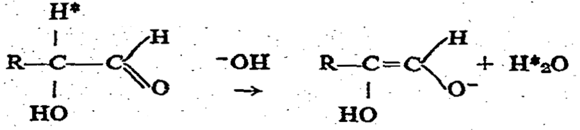


Fig. 4. Suggested formation of *cis*- and *trans*-enediols from pseudo-acyclic intermediates of 2-ketoses. The hydroxyl groups have been omitted from carbon atoms other than C-1, -2, and -3 for clarity.

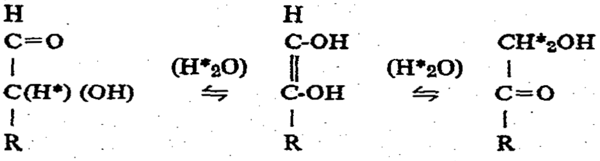
Evaluation of the rate of enolization by hydrogen–tritium exchange

The rate of enolization may be obtained from the rate at which hydrogen is released to the solvent (water) from a sugar labeled with tritium at the *alpha* carbon atom (eq. *1*). The procedure is specific for the enolization step, and is not affected by the subsequent reactions of the enediol. However, it involves a rather large isotope-effect, and it requires use of tritium-labeled sugars that are difficult to prepare.



(*1*)

For the comparative study of the enolization of a large group of sugars, we have developed a method that depends on exchange of the active hydrogen atom of a nonradioactive sugar with tritium of water-*t*. After removal of the base catalyst, water-*t*, and oxygen-bound tritium, the radioactivity of the nonvolatile residue was measured, and the extent of tritium uptake was calculated from the specific activity of the original water-*t*. The uptake of tritium depends on the rate at which the enediol is formed and converted into all products containing tritium. With most sugars, the principal reaction is the reversible enolization of eq. *2*, resulting in the incorporation of tritium into the two epimeric aldoses and the corresponding ketose. By these reversible reactions, tritium may be introduced onto both C-1 and C-2 of an aldose; however, in the early stages of the reaction, labeling of an aldose is principally at C-2.



$$\begin{array}{c} \mathrm{H} \\ \mathrm{C{=}O} \\ | \\ \mathrm{C(H^*)\,(OH)} \\ | \\ \mathrm{R} \end{array} \overset{(\mathrm{H^*_2O})}{\rightleftharpoons} \begin{array}{c} \mathrm{H} \\ \mathrm{C\text{-}OH} \\ \| \\ \mathrm{C\text{-}OH} \\ | \\ \mathrm{R} \end{array} \overset{(\mathrm{H^*_2O})}{\rightleftharpoons} \begin{array}{c} \mathrm{CH^*_2OH} \\ | \\ \mathrm{C{=}O} \\ | \\ \mathrm{R} \end{array}$$

(*2*)

Some results for the tritium uptake of D-glucose at 25° are given in Table I. The rate of enolization of D-glucose is surprisingly low. For D-glucose and certain

TABLE I

TRITIUM UPTAKE BY D-GLUCOSE[a] IN ALKALINE WATER-*t*[b]

Reaction time (h)	*Rate of uptake of H* (equivalents per mole of sugar per h)*	*Extent of reaction[c] (%)*
4	0.000460	0.18
7	0.000463	0.32
28	0.000496	1.39

[a]Conditions: A 0.2M solution of D-glucose in 0.5M sodium carbonate in water-*t* (H^*_2O) at 25°. [b]The pH of a solution prepared from 0.5M sodium carbonate in ordinary distilled water and the same proportion of D-glucose was 11.1. [c]Assuming 100% to represent incorporation of one equivalent of labeled hydrogen (H*) per mole of D-glucose.

other sugars, the rate of tritium uptake increases slightly with time; at present, we cannot account for this increase.

Table II gives comparative values for the tritium uptake by a wide variety of sugars. It is of interest to compare the values for certain sugars with those for the two inososes, compounds related to the sugars, but having a *keto* structure and no oxygen atom in the ring. Since the inososes are cyclic ketones, the carbonyl group is not bound in a hemiacetal structure, and enolization does not depend on liberation of the carbonyl group by the mutarotation reaction. Reversible enolization can take place between C-1 and C-2, and between C-1 and C-6, with incorporation of two atoms of hydrogen. In only two h, 0.9 atom of hydrogen per molecule of DL-*epi*-inosose and 1.4 atoms per molecule of *myo*-inosose-2 were incorporated. Under the experimental conditions used, the tritium uptake per hour for *myo*-inosose-2 was over 1400 times that for D-glucose. The rapidity of the enolization of the inososes is in accord with the well-known rapid enolization of sugar derivatives having a free carbonyl group. Thus, Anet[38] found that penta-*O*-methyl-*aldehydo*-D-glucose is completely converted, in 20 min, into 3-deoxy-2,4,5,6-tetra-*O*-methyl-D-*erythro*-hex-*trans*-2-enose (presumably by enolization and *beta*-elimination).

Sugars of like configuration in the hexose and heptose series react at somewhat similar rates. In the hexose series, altrose and talose react most rapidly. The pentoses as a group, react much more rapidly than the hexoses, 6-deoxyhexoses, and heptoses. Apparently, a large group attached to C-5 of these aldopyranose structures lessens the tendency of the sugar to undergo enolization.

The 2-deoxy sugars react at surprisingly high rates that, presumably, are associated with higher proportions of the acyclic intermediates. The higher rates are in accord with the observation that 2-deoxyaldoses give stronger tests for aldehyde with Schiff reagent than do the corresponding (2-hydroxy) aldoses[39]. Because the compounds react rapidly under the conditions selected for study, the values obtained may represent not only the primary enolization but also further reactions, such as rearrangement and fragmentation. The reactions of 2-deoxy sugars are being studied further.

In general, the rates of hydrogen–tritium exchange for the ketoses are appreciably higher than those for the aldoses. Hydrogen–tritium exchange in the ketoses can take place through either of the two primary hydrogen atoms at C-1, or the secondary hydrogen atom at C-3. Work by Sowden and Schaffer[12b] on enolization in deuterium oxide showed that the enolization of D-fructose occurs in large measure through the hydrogen atoms at C-1. However, enolization of a ketose may readily take place through the hydrogen atoms at both C-1 and C-3, as, for example, in the conversion of D-galactose into both D-tagatose and D-sorbose (formerly ψ-tagatose[40]) and the conversion of D-tagatose into D-sorbose[22]. The hexuloses react more rapidly than the heptuloses, but less rapidly than the ketodisaccharides lactulose and turanose (see Table III). The high uptake of tritium by ketoses may arise, in part, from the products of reverse aldol reaction. Cleavage of a ketohexose yields glyceraldehyde and its enediol. The fragments take up tritium from tritiated water directly by reversible

TABLE II

RATES OF TRITIUM UPTAKE FOR MONOSACCHARIDES IN ALKALINE WATER-*t*

Sugar	*Rate of uptake of H^* (equivalents per mole of sugar per h)*	*Relative rate*[a]	*Source of sugar, or ref. for preparative method*
	Inososes		
DL-*epi*-Inosose	0.464	1,000	23[b]
myo-Inosose-2	0.676	1,470	24[b]
	Aldohexoses		
D-Mannose	0.00024	0.5	[c]
D-Allose	0.00035	0.8	25, 26
D-Galactose	0.00043	0.9	[c]
D-Glucose	0.00046	1.0	[c]
D-Altrose	0.00108	2.3	25, 26
D-Talose	0.00220	4.8	25, 26
	Aldoheptoses		
D-*glycero*-L-*manno*-Heptose	0.00022	0.5	27, 28
D-*glycero*-D-*galacto*-Heptose	0.00031	0.7	29
D-*glycero*-D-*gulo*-Heptose	0.00034	0.7	27, 30
D-*glycero*-L-*galacto*-Heptose	0.00045	1.0	27, 31, 32
D-*glycero*-L-*gluco*-Heptose	0.00057	1.2	27, 33
D-*glycero*-L-*talo*-Heptose	0.00288	6.3	32
D-*glycero*-D-*ido*-Heptose	0.00495	10.7	34
	Aldopentoses		
D-Arabinose	0.0019	4.1	[c]
D-Xylose	0.0032	7.0	[c]
D-Lyxose	0.0041	8.9	[c]
D-Ribose	0.0131	28.0	[c]
	2-Ketoses		
L-Sorbose (L-*xylo*-hexulose)	0.0043	9.3	[c]
D-Fructose (D-*arabino*-hexulose)	0.0049	10.7	[c]
D-Tagatose (D-*lyxo*-hexulose)	0.0115	25.0	35
D-*gluco*-Heptulose	0.0012	2.6	36
D-*manno*-Heptulose	0.0032	7.0	37
	6-Deoxy sugars		
L-Rhamnose	0.00014	0.3	[c]
L-Fucose	0.00037	0.8	[c]
	2-Deoxy sugars		
2-Deoxy-D-ribose (2-deoxy-D-*erythro*-pentose)	0.042	90.0	[c]
2-Deoxy-D-glucose (2-deoxy-D-*arabino*-hexose)	0.072	155.0	[c]

[a] Rates relative to rate of 1.0 for D-glucose. Conditions: A 0.2M solution of the sugar in 0.5M sodium carbonate in water-*t* (H^*_2O) at 25°. The pH range of the solutions of the various sugars was 11.0 to 11.2. [b] Sample supplied by A. J. Fatiadi of this Bureau. [c] Commercial sugar, recrystallized; purity verified by chromatography and optical rotation.

enolization, or indirectly by formation of lactic acid through *beta*-elimination and rearrangement. Recently, this type of reaction has been shown to occur readily with D-fructose 6-phosphate[41] in alkaline solution.

The rates of tritium uptake for disaccharides of various types are given in Table III. The 4- and 6-*O*-D-glucopyranosyl-D-glucoses react 2 to 2.6 times as rapidly as D-glucose. The rates for the other disaccharides are considerably higher, and bear a higher ratio to the rate for the corresponding, reducing monosaccharide. Thus, the rate for the enolization of neolactose (4-*O*-D-galactopyranosyl-D-altrose) is nearly 4 times that for D-altrose. The 3-*O*-D-glycopyranosyl-D-arabinoses are 8 to 12 times as reactive as unsubstituted D-arabinose, and lactulose (4-*O*-D-galactopyranosyl-D-fructose) is about 3.5 times as reactive as D-fructose.

TABLE III

RATES OF TRITIUM UPTAKE FOR DISACCHARIDES IN ALKALINE WATER-t

Sugar	*Rate of uptake of H^* (equivalents per mole of sugar per h)*	*Relative rate*[a]	*Reducing moiety*	*Relative rate*[a] *for reducing moiety*	*Source of sugar, or ref. for preparative method*
4-*O*-*β*-D-Galactopyranosyl-D-glucose (lactose)	0.00091	2.0	D-glucose	1.0	[b]
4-*O*-*α*-D-Glucopyranosyl-D-glucose (maltose)	0.00110	2.4	D-glucose	1.0	[b]
4-*O*-*β*-D-Glucopyranosyl-D-glucose (cellobiose)	0.00120	2.6	D-glucose	1.0	[b]
6-*O*-*α*-D-Galactopyranosyl-D-glucose (melibiose)	0.00091	2.0	D-glucose	1.0	[b]
6-*O*-*β*-D-Glucopyranosyl-D-glucose (gentiobiose)	0.00097	2.1	D-glucose	1.0	[b]
4-*O*-*β*-D-Galactopyranosyl-D-altrose (neolactose)	0.0040	8.7	D-altrose	2.3	42
3-*O*-*β*-D-Galactopyranosyl-D-arabinose	0.0139	30.0	D-arabinose	4.1	43
3-*O*-*α*-D-Glucopyranosyl-D-arabinose	0.0228	50.0	D-arabinose	4.1	44
4-*O*-*β*-D-Galactopyranosyl-D-fructose (lactulose)	0.0169	37.0	D-fructose	10.7	45
3-*O*-*α*-D-Glucopyranosyl-D-fructose (turanose)	0.1105	240.0	D-fructose	10.7	25 (p. 480)

[a] Rates relative to rate of 1.0 for D-glucose. Conditions: An 0.2M solution of the sugar in 0.5M sodium carbonate in water-t (H^*_2O) at 25°. The pH range of the solutions of the various sugars was 11.0 to 11.2. [b] Commercial sugar, recrystallized; purity verified by chromatography and optical rotation.

Aside from the inososes, the most reactive compound studied is the disaccharide turanose (3-*O*-D-glucopyranosyl-D-fructose), which is about 23 times as reactive as D-fructose. Turanose differs from the more common disaccharides in that it is peculiarly susceptible to hydrolysis[46], as are other 3-substituted hexoses[8]. Its unusually high sensitivity to alkaline hydrolysis can be accounted for by its high rate of enolization, as shown by the tritium uptake, and by the fact that, in the 1,2-enediol, the D-glucosyl group at C-3 is *beta* to the enolic hydroxyl group at C-1. Presumably, the alkaline cleavage of turanose (**1**, Fig. 5) yields the enol (**2**) of 3-deoxy-D-*erythro*-hexosulose (**3**) and D-glucose. Formation of this enol from the 1,2-enediol of **1** does not involve incorporation of hydrogen, but the subsequent ketonization step (to **3**) does. Thus, the hypothetical 3-deoxy-D-*erythro*-hexosulose (**3**) derived from turanose in water-*t* should become labeled with tritium at C-1 (from the reversible 1,2-enolization) and at C-3 (from the ketonization of **2**). Also, the metasaccharinic acid (**4**), formed by subsequent rearrangement, should become labeled at C-2 and C-3, but, so far as is known to the authors, this subject has not yet been investigated.

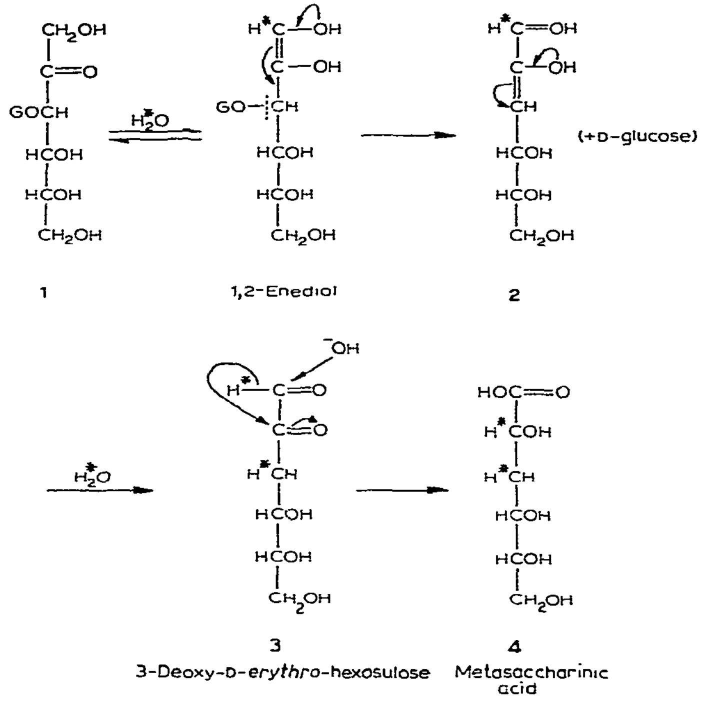


Fig. 5. Hypothetical course of the hydrolysis of turanose in alkaline solution. (G is α-D-glucopyranosyl.)

In the foregoing discussion, it has been assumed that the reactions take place through enolization, with incorporation of tritium from the environment by the reverse reaction. However, evidence is accumulating that some related transformations of sugars may follow other paths. Thus, the conversion of D-fructose-*1-t* 6-phosphate into D-glucose-*2-t* 6-phosphate by phosphoglucose isomerase takes place in water with loss (to the water, by exchange) of only part of the tritium, and direct transfer of the remainder to C-2 of the product[15]. The direct transfer (which predominates at low temperatures) was interpreted in terms of an enediol mechanism in which a basic group associated with the enzyme removes the hydrogen or tritium atom on

C-1 of the substrate and transfers it to C-2 of the product. It was recently reported that D-glucose 3-phosphate, in slightly alkaline deuterium oxide, yields 3-deoxy-D-*erythro*-hexosulose without incorporation of deuterium[47]. The authors explained this important observation as being the result of an intramolecular shift of a hydride ion from C-2 to C-3.

EXPERIMENTAL

Small, glass vials containing 100-μmole quantities of a sugar were sealed with rubber caps, and a hypodermic needle was inserted in each cap. The vials were placed in a desiccator, which was then alternately evacuated and filled with oxygen-free nitrogen. Evacuation and filling with nitrogen were successively repeated several times.

Anhydrous sodium carbonate (265 mg) was weighed into a 10-ml vial; this was closed with a rubber cap, and filled with nitrogen as described above.

Water-*t* (9.97 mCi/ml) was placed in a round-bottomed flask having a standard-taper neck and a rubber-capped side-arm. The flask was connected through a stopper and stopcock to a vacuum pump (protected by a trap at low temperature), and also to a source of oxygen-free nitrogen. While the contents were swirled by hand, the flask was alternately evacuated and filled with nitrogen; the process was repeated several times.

With the flask open to the nitrogen source, water-*t* was withdrawn by needle through the cap of the side-arm into a micrometer buret, and 5 ml was injected into the vial (with attached vent-needle) containing sodium carbonate under nitrogen. After dissolution of the salt, the resulting solution of sodium carbonate in water-*t* was withdrawn by needle into another micrometer buret, for use in the enolization measurements. (The molarity of a solution prepared with ordinary distilled water and the same proportion of sodium carbonate was 0.5, and the pH was 11.56.)

A 0.5-ml quantity of the oxygen-free, 0.5M solution of sodium carbonate in water-*t* was added to each of ten nitrogen-filled vials containing 100 μmoles of a sugar. (Each set included two vials containing sucrose, which was used as a blank.) The vent needles were withdrawn, and the vials were placed in a bath at 25° for periods ranging from 2 to 7 h. After a suitable reaction-time, each vial was opened, and 1 ml of water-*t* (from the lot used in preparing the sodium carbonate solution) was added, followed by 0.5 g of vacuum-dried, strong cation-exchange resin (H^+ form). After evolution of carbon dioxide had ceased, the vial was re-capped and kept in a shaker for 10 min. The suspension was then filtered under vacuum into a 100-ml, round-bottomed flask, and the resin and vial were thoroughly washed with distilled water. The filtrate and washings were combined and freeze-dried, the residue was dissolved in water, and the solution was transferred to a standard-taper test-tube. In order to remove labile tritium, the ten solutions were simultaneously evaporated in a commercial, rotary, vacuum distillation apparatus and repeatedly re-dissolved and re-evaporated. Finally, each residue was thoroughly dried and assayed for radioactivity as described next. All operations were conducted in an efficient hood. Water-*t* vapors were carefully trapped, and the resulting liquid was ultimately discarded in the approved manner.

The tritium uptake was calculated from the radioactivity of the sample and the specific radioactivity of the water-*t*. In each experiment, the sample, prepared as described above, was dissolved in 1 ml of water, and two 100-μl aliquots (each 10 μmoles) were assayed for radioactivity in a liquid scintillation counter. The sucrose samples were similarly analyzed, and the relatively small count (usually 3–4 counts per second) was deducted as a blank. All samples were counted to a probable statistical error of less than 1% (10,000 counts). Each value given in the Tables is the average of at least three experiments. The following are data from a typical determination of tritium uptake (for D-fructose): net counts per second per 10 μmoles of sugar 201.0 (A); counts per second per 10 μequivalents of H* (calculated from analysis of original H^*_2O) 9,950 (B); uptake of H* (equivalents per mole of sugar) 0.0202 (A/B): duration of reaction 4.1 h; rate of uptake of H* (equivalents per mole of sugar per h) 0.0049.

REFERENCES

1 C. A. Lobry de Bruyn and W. Alberda van Ekenstein, *Rec. Trav. Chim.*, 14 (1895) 203; 15 (1896) 92; 16 (1897) 257, 262, 274, 282; 18 (1899) 147.
2 J. U. Nef, *Ann.*, 357 (1907) 214; 376 (1910) 1; 403 (1913) 204.
3 M. L. Wolfrom and W. L. Lewis, *J. Amer. Chem. Soc.*, 50 (1928) 837; R. D. Greene and W. L. Lewis, *ibid.*, 50 (1928) 2813.
4 M. Fedoroňko and K. Linek, *Collect. Czech. Chem. Commun.*, 32 (1967) 2177.
5 H. S. Isbell, *J. Res. Nat. Bur. Stand.*, 32 (1944) 45.
6 J. C. Sowden, *Advan. Carbohyd. Chem.*, 12 (1957) 35.
7 J. C. Speck, Jr., *Advan. Carbohyd. Chem.*, 13 (1958) 63.
8 R. L. Whistler and J. N. BeMiller, *Advan. Carbohyd. Chem.*, 13 (1958) 289.
9 R. U. Lemieux, in P. De Mayo (Ed.), *Molecular Rearrangements*, Part 2, Interscience Publishers, New York, 1964, pp. 743-753.
10 H. Fredenhagen and K. F. Bonhoeffer, *Z. Physik. Chem.*, A181 (1938) 392.
11 Y. J. Topper and D. Stetten, *J. Biol. Chem.*, 189 (1951) 191.
12 J. C. Sowden and R. Schaffer, (a) *J. Amer. Chem. Soc.*, 74 (1952) 499; (b) 74 (1952) 505.
13 Y. J. Topper, *J. Biol. Chem.*, 225 (1957) 419.
14 R. Bentley and D. S. Bhate, *J. Biol. Chem.*, 235 (1960) 1225.
15 I. A. Rose and E. L. O'Connell, *J. Biol. Chem.*, 236 (1961) 3086.
16 H. S. Isbell and C. W. R. Wade, *J. Res. Nat. Bur. Stand.*, 71A (1967) 137.
17 C. S. Hudson and J. K. Dale, *J. Amer. Chem. Soc.*, 39 (1917) 320.
18 T. M. Lowry and G. F. Smith, *Rapports sur les Hydrates de Carbone, Conf. Intern. Union Chem. 10th, Liège*, 1930, p. 79.
19 H. Fredenhagen and K. F. Bonhoeffer, *Z. Physik. Chem.*, A179 (1937) 135.
20 H. S. Isbell and H. L. Frush, *J. Res. Nat. Bur. Stand.*, 46 (1951) 132.
21 D. Rittenberg and C. Graff, *J. Amer. Chem. Soc.*, 80 (1958) 3370.
22 H. S. Isbell and Ruth Ann Verell, Unpublished work.
23 T. Posternak, *Methods Carbohyd. Chem.*, 1 (1962) 289.
24 G. E. McCasland, *Methods Carbohyd. Chem.*, 1 (1962) 291.
25 F. J. Bates and Associates, *Polarimetry, Saccharimetry and the Sugars*, NBS Circ. C440 (1942).
26 H. L. Frush and H. S. Isbell, *J. Res. Nat. Bur. Stand.*, 54 (1955) 267.
27 H. S. Isbell, *J. Res. Nat. Bur. Stand.*, 18 (1937) 505.
28 R. M. Hann, A. T. Merrill, and C. S. Hudson, *J. Amer. Chem. Soc.*, 57 (1935) 2100.
29 H. S. Isbell, *J. Res. Nat. Bur. Stand.*, 20 (1938) 97.
30 N. K. Richtmyer, *Methods Carbohyd. Chem.*, 1 (1962) 160.

31 F. B. LaForge, *J. Biol. Chem.*, 41 (1920) 251.
32 H. S. Isbell, *J. Res. Nat. Bur. Stand.*, 19 (1937) 639.
33 R. M. Hann and C. S. Hudson, *J. Amer. Chem. Soc.*, 59 (1937) 548.
34 H. S. Isbell, *J. Amer. Chem. Soc.*, 56 (1934) 2739.
35 E. L. Totten and H. Lardy, *Methods Carbohyd. Chem.*, 1 (1962) 155.
36 W. C. Austin, *J. Amer. Chem. Soc.*, 52 (1930) 2106.
37 E. M. Montgomery and C. S. Hudson, *J. Amer. Chem. Soc.*, 61 (1939) 1654.
38 E. F. L. J. Anet, *Carbohyd. Res.*, 3 (1966) 251.
39 W. G. Overend, *J. Chem. Soc.*, (1950) 2769; W. G. Overend and M. Stacey, *Advan. Carbohyd. Chem.*, 8 (1953) 62.
40 C. A. Lobry de Bruyn and W. Alberda van Ekenstein, *Rec. Trav. Chim.*, 19 (1900) 5.
41 C. Degani and M. Halmann, *J. Amer. Chem. Soc.*, 90 (1968) 1313.
42 N. K. Richtmyer and C. S. Hudson, *J. Amer. Chem. Soc.*, 57 (1935) 1716.
43 H. S. Isbell and H. L. Frush, *J. Res. Nat. Bur. Stand.*, 50 (1953) 133.
44 J. D. Moyer and H. S. Isbell, *Abstracts Papers Amer. Chem. Soc. Meeting*, 126 (1954) 24D.
45 E. M. Montgomery and C. S. Hudson, *J. Amer. Chem. Soc.*, 52 (1930) 2101.
46 H. S. Isbell, *J. Res. Nat. Bur. Stand.*, 26 (1941) 35.
47 G. Fodor and J. P. Sachetto, *Tetrahedron Lett.*, (1968) 401.

Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

NATURE OF A COLORED BY-PRODUCT FOUND IN CRUDE INOSOSE PHENYLOSAZONES*

ALEXANDER J. FATIADI

Division of Analytical Chemistry, Institute for Materials Research, National Bureau of Standards, Washington, D. C. 20234 (U.S.A.)

(Received July 11th, 1968; in revised form, August 31st, 1968)

ABSTRACT

Long treatment (6 to 14 days) of mono- or di-ketoinositols with phenylhydrazine at room temperature produces, in addition to the corresponding osazone, a red enolic by-product of known structure, namely, DL-*xylo*-2-oxo-1,3-bis(phenylhydrazono)-cyclohexane-4,5,6-triol. Thus, the oxidation mixture from D-inositol affords the red product in 22% yield; the yields of red by-product from other stereoisomers are as follows: from L-inositol (24%), from *myo*-inositol (15%), and from *epi*-inositol (8%). Treatment of mono- or di-ketoquebrachitol with phenylhydrazine gives a new, red enolic compound, L-*xylo*-4-*O*-methyl-2-oxo-1,3-bis(phenylhydrazono)cyclohexane-4,5,6-triol in 3% yield; mono- or di-ketopinitol gives D-*xylo*-5-*O*-methyl-2-oxo-1,3-bis(phenylhydrazono)cyclohexane-4,5,6-triol in 5% yield. The possible reaction pathways for their formation are discussed.

INTRODUCTION

The preparation of the phenylosazones of D- and L-*myo*-inosose-1**, *myo*-inosose-2, and DL-*epi*-inosose-2 by bromine oxidation of the corresponding inositols, followed by treatment with phenylhydrazine, was recently reported[1]. Processing of the crude products has now revealed the presence, in common, of a red, enolic impurity which has been identified as a compound having a known structure[2,3], namely, DL-*xylo*-2-oxo-1,3-bis(phenylhydrazono)cyclohexane-4,5,6-triol (**5**⇄**5a**), first prepared[4] from *myo*-inositol by oxidation with nitric acid, enolization of the product, and treatment with phenylhydrazine.

One possible way by which compound **5a** could be formed from D- or L-inositol is as follows. Extensive oxidation of, for example, D-inositol (**1**), either by *Acetobacter suboxydans*[5] or by aqueous sodium hypobromite[1], produces diketoinositol **2** (not isolated in crystalline form) and, in the presence of a base, the latter enolizes to the

---

*Part IV. Methods in Inositol Chemistry. For Part III, see A. J. FATIADI, Ref. 1.

**The nomenclature used in this paper for this and other inositol derivatives is based on the system of H. G. FLETCHER, JR., L. ANDERSON, AND H. A. LARDY [*J. Org. Chem.*, 12 (1951) 1238; see also G. POST AND L. ANDERSON, *J. Amer. Chem. Soc.*, 84 (1962) 471].

known[4] intermediate **3**; conversion of **2** into **3** follows the general pattern of enolization of isomeric diketoinositols[4].

As reported[4], treatment of **3** with phenylhydrazine and aqueous acetic acid provides **5a** directly, indicating oxidation of **3** to triketoinositol **4**, and the latter, as known[4], condenses with phenylhydrazine in the cold to give **5a** in high yield It is, however, possible that conversion of **3** into **4** in the presence of phenylhydrazine (base) and oxygen (air) may involve a radical intermediate[6]; search for evidence in support of this possibility is in progress. Similar processing of crude inosose phenylosazones from L-quebrachitol (1-*O*-methyl-*levo*-inositol, **9**) or D-pinitol (5-*O*-methyl-*dextro*-inositol, **10**), prepared as described previously[1], revealed the presence of another colored enolic impurity of type **5a** in each, with the structures shown in **6** and **7**, respectively; these compounds were isolated with difficulty in low yields.

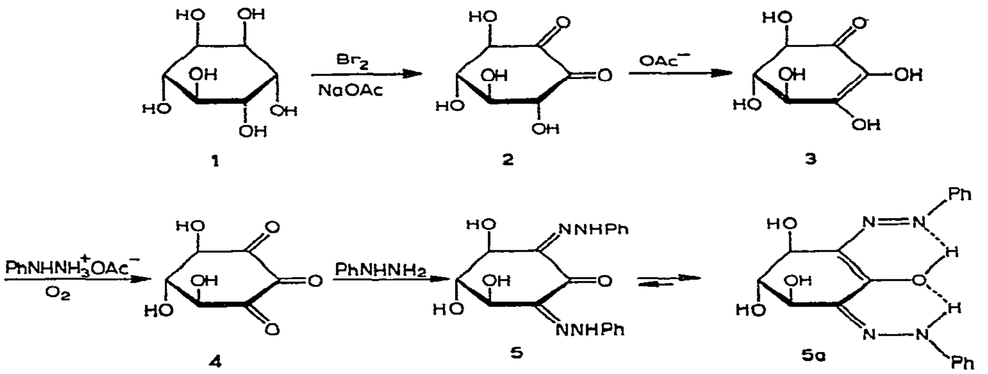
RESULTS AND DISCUSSION

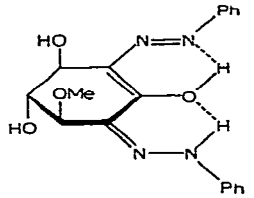
The identity of the red impurities invariably formed during the preparation of inosose phenylhydrazones, or during the conversion of these into the osazones, is a problem of long standing.

In the present study, a solution of an inosose or a diketoinositol in aqueous acetic acid was treated with an excess of phenylhydrazine, and the mixture was exposed to air for 6–14 days. The purpose of this procedure was to obtain an "over-oxidized", crude inosose phenylosazone containing a maximal proportion of the red by-product. Application of heat (water bath at 60–90°) did not raise the yield of the by-product. In general, formation of the red by-product, particularly that from an inosose or inosose phenylhydrazone, is a rather slow process; the highest yield of the by-product has been isolated from reaction mixtures kept at room temperature.

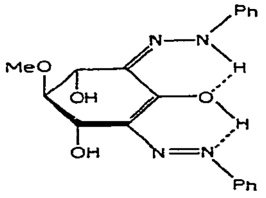
Isolation of compounds **5a**, **6**, or **7** from crude inosose phenylosazones indicates that the inositols examined, having one axial group (or two) in the *ortho* or *meta* positions [*myo* (one); D-, L-, or their monomethyl ethers (two, *ortho*), or *epi* (two, *meta*)], regardless of stereochemical factors favoring oxidation[8] of their axial hydroxyl groups[8], are all converted into triketoinositols having the thermodynamically stable *xylo* configurations. From this, it may be predicted *a priori* that *muco*-inositol should give a stable triketoinositol having the *xylo* configuration, because of the presence of three consecutive, axial hydroxyl groups and a suitable configuration of the remaining hydroxyl groups. It would be of interest to extend this study to other isomeric inositols, for example, *neo*- (two axial hydroxyl groups in *para* position) or *allo*-, *cis*-, or *muco*-inositols (each having three axial hydroxyl groups), in order to (*1*) correlate the relationship between kinetic and thermodynamic control during the oxidation or enolization, and (*2*) determine the stability of triketoinositols formed from inositols having other than the *xylo* configuration.

As pointed out previously[4], compound **3** is the enantiomorph of DL-*xylo*-pentahydroxy-2-cyclohexen-1-one. In nonionic derivatives formed from this enolic acid (3), the configuration is DL-*xylo* (racemic, unless only one enantiomorph is formed), depending on the position of the carbonyl group (C-1 or C-3). Consequently, derivatives from 3 (or its enantiomorph), for example, enolic 1,3-bis(phenylhydrazone)

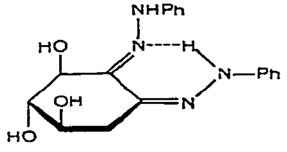




6



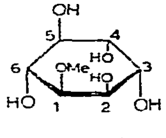
7



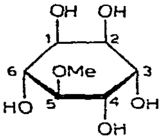
8

5a, have a dissymmetric structure also. Starting from optically active D-inositol (1) (or L-inositol), formation of an optically active 1,3-bis(phenylhydrazone) of type 5a would be expected; however, the red by-product obtained shows no optical activity, and must therefore be racemic. The only logical explanation for formation of DL-1,3-bis(phenylhydrazone) 5a is that simultaneous enolization of the diketoinositol 2 toward C-3 and C-6 occurs. Periodate oxidation of 5a confirms the proposed structure; consumption of two moles of periodic acid per mole of 5a, as known[2], yields a dialdehyde which, on cyclization and dehydration, gives a pyridazine derivative whose structure has been established[2]. Thus, characterization of DL-*xylo*-2-oxo-1,3-bis(phenylhydrazono)cyclohexane-4,5,6-triol (5a and its enantiomorph) as a product from D-inositol or its stereoisomers (*myo*-, *epi*-, or L-inositol, which yield the same product) has now been fully established.

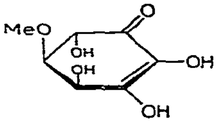
Detailed examination of thin-layer chromatograms of crude inosose phenylosazones from L-quebrachitol (9) and D-pinitol (10) revealed the presence of at least six colored components (see Experimental). The presence of these by-products indicates occurrence of at least two competitive enolization reactions that can yield two different *O*-methylbis(phenylhydrazones), and this accounts for two fast-moving, orange-red components observed on the chromatograms. The third material (slow-moving, cherry-red spot) has also been found in crude *O*-methylinosose phenylosazones. This compound has been identified as **5a** (direct isolation); it apparently arises from scission of the *O*-methyl group, following the condensation with phenylhydrazine of the corresponding inosose from D- or L-inositol. The yellow spots on the chromatogram (in increasing order of their mobility) are found to be derived from (*a*) either D- or L-*myo*-inosose-1 phenylosazone, apparently attributable to scission of the *O*-methyl group (direct isolation and comparison), (*b*) the parent *O*-methylinosose phenylosazones (direct comparison), or (*c*) an unknown osazone (probably an elimination product).



L-Quebrachitol (9)



D-Pinitol (10)



11

Careful examination of u.v. and visible spectra of crude inosose phenylosazones or their column-separated fractions revealed only a trace absorption above 510 nm, thus excluding the presence of conjugated phenylazo components of high molecular weight[3,9].

If it is assumed that oxidation of D-pinitol (**10**) occurs at C-2 and C-3, enolization may proceed either toward C-4 or C-1, and either of the resulting enols can be converted into bis(phenylhydrazones) of type **5a** with retention of the *O*-methyl group. In the first case (enolization toward C-4, attachment of phenylhydrazono groups at C-2 and C-4), the compound will be identical, even to the extent of being the same enantiomorph, with the one (**6**) from L-quebrachitol (**9**). In the second case (enolization toward C-1, attachment of phenylhydrazono groups at C-3 and C-1), the product will be the position isomer, *xylo*-5-*O*-methyl-1,3-bis(phenylhydrazone) (**7**). Since the structure is dissymmetric, it should be designated as D-*xylo*-5-*O*-methyl-2-oxo-1,3-bis(phenylhydrazone) (**7**). It has now been found that enolization of the oxidation product from D-pinitol proceeds mainly toward C-1, to give compound **7** having a positive optical rotation. However, the thin-layer chromatogram (see Experimental) definitely shows the presence of a small proportion of a second component having an R_F value matching that of the position isomer L-*xylo*-4-*O*-methyl-2-oxo-1,3-bis(phenylhydrazone) (**6**), thus confirming two-way enolization as predicted for D-pinitol.

Enolization of the oxidation product from L-quebrachitol (**9**) (assuming that oxidation occurs at C-3 and C-4) toward C-2 (attachment of phenylhydrazono groups at C-2 and C-3), is expected to give as the major product (also dissymmetric) L-*xylo*-4-*O*-methyl-2-oxo-1,3-bis(phenylhydrazone) (**6**). This compound has actually been isolated, and has been found to have a negative optical rotation. However, enolization of the oxidation product from L-quebrachitol (**9**) toward C-5 should theoretically give an enolic intermediate **11**, having the *lyxo* configuration. In fact, the position isomer D-*xylo*-5-*O*-methyl-2-oxo-1,3-bis(phenylhydrazone) (**7**) (attachment of phenylhydrazono groups at C-4 and C-5 in **9**) has been isolated by the use of preparative t.l.c. Formation of the more stable *xylo* isomer **7** can be envisaged as occurring by Walden inversion at C-4 of compound **11**. Further confirmation of the structure of compounds **6** and **7** has been obtained from periodate oxidation; one mole of compound **6** consumed one mole of periodic acid, whereas compound **7** showed practically no consumption of periodic acid.

Interestingly, prolonged treatment of an aqueous solution of a diketoquercitol [obtained after hypobromite or *Acetobacter suboxydans* oxidation of (+)-*proto*-quercitol*] with phenylhydrazine yielded a yellow phenylosazone **8** [n.m.r. spectrum in Me_2SO-d_6, τ -2.60 (N-H chelated), -1.00 (N-H, nonchelated)], almost free from colored by-products. This observation indicates the stability of the *xylo* configuration in the diketoquercitol, also. It appears, therefore, that enolization of diketones derived from cyclohexanepentols and having the *xylo* configuration is less probable than enolization of those from inositols.

Formation of colored by-products arising during the preparation of sugar phenylosazones can reportedly[11] be suppressed by the addition of sodium hydrogen sulfite; however, the reagent has no effect on crude inosose phenylosazones, indicating that the colored by-products have a different character. Formation of the red by-product was, however, indeed suppressed by treatment of the crude inosose phenylosazone with a warm, aqueous ethanolic solution of sodium hydrosulfite[1] ($Na_2S_2O_4$); this reaction will be discussed in a later communication.

EXPERIMENTAL

N.m.r. spectra were recorded with a Varian A-60 spectrometer, with tetramethylsilane (τ = 10.00) as the internal standard. I.r. spectra were recorded with a Perkin–Elmer Model 257 i.r. grating spectrophotometer**, and u.v. spectra with a Beckman DK-2 spectrophotometer**. T.l.c. was performed on activated silica gel G or alumina (5 × 20 or 20 × 20 cm plates, 250 μm thick; 1 mm thick for preparative t.l.c.) for 60 min. A t.l.c. tank of appropriate size was covered on the inside (about 75% of its surface) with Whatman No. 1 paper saturated with the solvent used, in order to maintain homogeneity (solvent saturation) within the vessel; this technique helped considerably in providing reproducible R_F values. If solvent-saturated paper was not present in the developing vessel, inconsistent R_F values were obtained. In all cases, freshly prepared solvent mixtures were used. Column chromatography was performed on Florisil** (100–200 mesh, Floridan Co., Philadelphia, Pennsylvania) or on neutral alumina. Separations on columns were monitored by t.l.c. on microscope slides. Analyses were made by Rolf A. Paulson and William Schmidt of the Microchemical Analysis Section, N.B.S.

---

*(+)-*proto*-Quercitol is named (+)-1-deoxy-*muco*-inositol by the Fletcher–Anderson–Lardy system.
**Certain commercial instruments or materials are mentioned in this paper; this does not imply recommendation or endorsement by the National Bureau of Standards.

Preparation of DL-xylo-*2-oxo-1,3-bis(phenylhydrazono)cyclohexane-4,5,6-triol* (**5a**) *from* D-*inositol.* — An ice-cold solution of 6 g (33 mmoles) of D-inositol (**1**) in water (50 ml) containing sodium acetate trihydrate (14 g) was oxidized with bromine (8 g) according to a published procedure[1]. After 24 h, sodium acetate trihydrate (4 g) and acetic acid (18 ml) were added, and the excess of bromine was removed by bubbling in an ethylene–air mixture. The clear solution was diluted with an equal volume of water and then with methanol (400 ml), and phenylhydrazine (10 ml) was added. The mixture was kept for 14 days at room temperature with occasional stirring; the dark-red solid was collected, washed with water, and dried in the air. The yield of crude phenylosazone, containing **5a**, was 4.5 g.

Alternatively, a solution of 3 g of D-inositol (**1**) in water (150 ml) containing yeast extract (900 mg) and D-glucitol (150 mg) was oxidized with *Acetobacter suboxydans* for 7 days at 30°. The reaction mixture was then treated with saturated, aqueous lead acetate solution (2 ml) and Celite (10 g), and stirred vigorously for 30 min. The suspension was filtered through a layer of Celite and charcoal, and the clear filtrate was diluted with water (300 ml), glacial acetic acid (10 ml), and methanol (200 ml). Phenylhydrazine (6 ml) was added, and the mixture was kept for 14 days at room temperature. The yield of dark-red solid phenylosazone containing **5a** was 2.5 g.

T.l.c. for 60 min [with 4:1:1 (v/v) benzene–acetic acid–butanone (solvent *A*)] of the crude product from both oxidation procedures revealed the presence of the same component at R_F 0.44 ±0.02*, identical with **5a**; the intensity of the red spot, which is not fluorescent, slowly weakens on exposure to light. The mixture also gives a yellow spot at R_F 0.14 ±0.01 (identical with that for L-inosose-1 phenylosazone) and contains unknown, colored impurities, which give a lilac spot at R_F 0.84 ±0.02, an olive-orange spot at R_F 0.71 ±0.02, a yellow spot at R_F 0.59 ±0.01, and a yellow spot at R_F 0.50 ±0.01.

A solution of the crude product (1 g, from the bromine oxidation) in ethyl acetate (60 ml) was introduced onto a column of Florisil (4 × 45 cm) and eluted with 1:1 (v/v) heptane–ethyl acetate. The cherry-red zone (which followed a faster-moving, brown-red zone) was collected, and evaporated to dryness, to give crude **5a**, yield 220 mg (22%). A sample recrystallized from methanol gave dark-red needles, m.p. 183–185° (shrinking at 177°); t.l.c. with 2:1:1 (v/v) benzene–acetic acid–butanone (solvent *B*), R_F 0.66 ±0.02; $\lambda_{\max}^{\mathrm{MeOH}}$ 251 (ε ~19,250); 292 (sh) (ε ~6,300), and 482 nm (ε ~34,800); n.m.r. data (Me_2SO-d_6); τ −4.42 and −4.33 (chelated N–H), τ −1.35 and −0.92 (nonchelated N–H); $\nu_{\max}^{\mathrm{KCl}}$ 3400 s (OH, NH), 1600 s (phenyl ring), 1572 m (N=N), 1432 m (N=N), 1547 (N–N, bending), and 1160 cm^{-1} (Ph–N)[7]; fingerprint region: 1510 sh, 1500 s, 1462 s, 1395 w, 1340 w, 1310 w, 1220 s, 1100 m, 1090 m, 1068 s, 1030 w, 992 m, 960 w, 890 m, 855 m, 828 m, 780 m, 756 s, 700 s, and 672 w cm^{-1}. The compound was identical with an authentic sample[3,4,7] of **5a**.

---

*Values reported are averages of four determinations with the mean deviations indicated.

Processing of an identical, crude product obtained by oxidation with *Acetobacter suboxydans* gave **5a** in 18% yield. Similarly, a crude product obtained from L-inositol (bromine oxidation, followed by treatment with phenylhydrazine for 14 days) gave **5a** in 24% yield; *myo*-inositol (crude DL-*myo*-inosose-1 phenylosazone, 14 days) gave **5a** in 16% yield, and *epi*-inositol (crude DL-*epi*-inosose-2 phenylosazone, 12 days) gave **5a** in 8% yield.

Consumption of periodic acid[5]. — The 1,3-bis(phenylhydrazone) **5a** (35.5 mg, 10 mmoles) in 85% aqueous methanol (97 ml) was treated with aqueous periodic acid (3 ml of 536.2 mM) 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; 2.79 ml of the titrant (100.3 mM) was consumed; the average consumption of periodic acid per mole of **5a** was 2.10 moles.

Preparation of D-xylo-*5-O-methyl-2-oxo-1,3-bis(phenylhydrazono)cyclohexane-4,5,6-triol* (**7**). — A precooled (5°) solution of 6 g (31 mmoles) of D-pinitol (5-*O*-methyl-*dextro*-inositol) (**10**) and sodium acetate trihydrate (14 g) in 50 ml of water was treated with bromine (8 g) by a published procedure[1]. The solution was then diluted with water (1 liter) and acetic acid (18 ml), treated with phenylhydrazine (10 ml), and kept for 7 days at room temperature with occasional stirring; the yield of dark-red solid (containing phenylosazone and **7** was 4.3 g. T.l.c. (solvent *A*) of the crude product showed the following components: a lilac spot at R_F 0.79 ±0.02 (unknown), an olive-orange spot at R_F 0.73 ±0.02 (unknown), an orange-red spot at R_F 0.64 ±0.02 (identical with that for **7**), an orange-red spot at R_F 0.58 ±0.01 (identical with that for **6**); a cherry-red spot at R_F 0.45 ±0.02 (identical with that for **5a**), a yellow spot at R_F 0.25 ±0.01 (identical with that for D-pinitol osazone), and a yellow spot at R_F 0.14 ±0.01 (identical with that for L-inosose-1 phenylosazone). The corresponding R_F values of the crude product in solvent *B* were: 0.85 ±0.02; 0.83 ±0.01; 0.76 ±0.02; 0.72 ±0.03; 0.67 ±0.02; 0.54 ±0.01; and 0.44 ±0.01.

A filtered solution of the crude product (1 g) in ethyl acetate (50 ml) was introduced onto a column of Florisil (4 × 45 cm), and eluted with 1:1 (v/v) heptane–ethyl acetate. A dark-red zone was collected in three fractions: a forerun (25 ml, discarded), the main fraction, and a slower-moving third fraction (C). The second fraction was concentrated to about 30 ml, introduced onto a column of neutral alumina (3 × 40 cm), and eluted with 4:1 (v/v) benzene–methanol. The dark-red zone was resolved into two bands: a faster-moving component (A) and a slower-moving component (B). The solution of component A was evaporated to dryness, and the residue was recrystallized from 2:1 (v/v) benzene–methanol, and then from methanol, to give small, dark-red crystals of **7**; yield 50 mg (5%), m.p. 214–216° (dec., shrinks at 198°), $[\alpha]_D^{22}$ +288 ±20° [*c* 0.005, 1:1 (v/v) ethanol–*p*-dioxane]; R_F 0.64 ±0.02 (solvent *A*), R_F 0.76 ±0.02 (solvent *B*); λ_{max}^{MeOH} 250 (ε ~18,200), 288 sh (ε ~5,800), and 480 nm (ε ~33,500); n.m.r. data (Me_2SO-d_6): τ −2.75 and −2.48 (chelated N–H), τ −0.80 and −0.62 (nonchelated N–H); ν_{max}^{KCl} 3400 (OH, NH), 1650 w (C=N), 1600 s (phenyl ring), 1570 m (N=N), 1438 w (N=N), 1538 w (N–H, bending), 1168 (Ph–N)[7], and a quartet at 1120 s, 1090 s, 1070 s, and 1060 m cm^{-1} (CH_2-O-

$CH_2)^{10}$; fingerprint region: 1510 w, 1500 s, 1460 s, 1360 w, 1320 w, 1310 w, 1290 w, 1212 s, 1030 s, 960 m, 900 w, 870 w, 850 w, 825 s, 800 w, 755 s, 695 s, and 650 cm^{-1}; uptake of periodic acid 0.18 mole per mole of 7.

Anal. Calc. for $C_{19}H_{20}N_4O_4$: C, 61.94; H, 5.47; N, 15.21. Found: C, 61.88; H, 5.60; N, 15.25.

Evaporation of the fraction containing component B, gave an orange-red solid which, on recrystallization from benzene–methanol gave red plates; yield 15 mg (1.5%); R_F (solvent *A*) 0.59 ±0.01; the u.v. and i.r. spectra were identical with those of the isomeric bis(phenylhydrazone) **6**. A smaller volume (5 ml) of a solution of components A and B was successfully separated by preparative t.l.c. on alumina (20 × 20 cm) with 4:1 (v/v) benzene–methanol to give *ca.* 1.5 mg. of bis(phenylhydrazone) **6** and 2.8 mg of **7**. The original third fraction (C) was chromatographed on alumina with 1:1 (v/v) benzene–methanol. Concentration of the eluate to a small volume (0.75 ml) and storage for 72 h at room temperature gave red needles of **5a**; yield 2 mg (0.2%).

L-xylo-*4-O-Methyl-2-oxo-1,3-bis(phenylhydrazono)cyclohexane-4,5,6-triol* (**6**). — A precooled solution of 6 g (31 mmoles) of L-quebrachitol (1-*O*-methyl-*levo*-inositol) (**9**) in water was oxidized with bromine, and treated with phenylhydrazine for 7 days by the procedure described for compound **7**. The yield of cherry-red solid (containing phenylosazone and compound **6**) was 4.5 g. T.l.c. (solvent *A*) of the crude product showed a lilac spot at R_F 0.81 ±0.02 (unknown), an olive-orange spot at R_F 0.76 ±0.02 (unknown), an orange-red spot at R_F 0.66 ±0.02 (identical with that for **7**), an orange-red spot at R_F 0.59 ±0.01 (identical with that for **6**), a cherry-red spot at R_F 0.44 ±0.02 (identical with that for **5a**), a yellow spot at R_F 0.23 ±0.01 (identical with that for L-quebrachitol phenylosazone), and a yellow spot at R_F 0.13 ±0.02 (identical with that for D-inosose-1 phenylosazone). The corresponding R_F values of the crude product in solvent *B* were: 0.86 ±0.02, 0.83 ±0.02, 0.77 ±0.01, 0.72 ±0.02, 0.66 ±0.02, 0.59 ±0.02, and 0.43 ±0.02. The crude mixture also showed a yellow spot at R_F 0.32 ±0.02 (solvent *A*, unknown osazone) which was not detectable by use of solvent *B*.

The crude material (1 g) was chromatographed on Florisil [1:1 (v/v) heptane–ethyl acetate] and neutral alumina [4:1 (v/v) benzene–methanol] by the procedure described for compound 7. A dark-red zone was collected in three fractions. The second fraction was chromatographed on alumina, and was separated into a faster-moving component (A) and a slower-moving component (B). The solution of B was evaporated to dryness, to give an orange-red powder which, on successive recrystallization from 2:1 (v/v) benzene–methanol and methanol gave lustrous, cherry-red plates of **6**; yield 30 mg (3%), m.p. 158–160°, $[\alpha]_D^{22}$ −372 ±20° [*c* 0.005, 1:1 (v/v) ethanol-*p*-dioxane]; R_F 0.58 ±0.01 (solvent *A*); R_F 0.72 ±0.03 (solvent *B*); λ_{max}^{MeOH} 251 (ε ~20,000), 290 sh (ε ~7,100) and 478 nm (ε ~33,000); n.m.r. data (in Me_2SO-d_6): τ −3.00 and −2.82 (chelated N–H), τ 1.00— and −0.76 (nonchelated N–H); ν_{max}^{KCl} (cm^{-1}) 3360 s (OH, NH), 1650 w (C=N), 1562 m (N=N), 1436 w (N=N), 1548 (N–H, bending), 1160 s (Ph–N)^7, triplet at 1095 w, 1090 m, and 1065 m cm^{-1} (CH_2-O-$CH_2)^{10}$; fingerprint region: 1610 w, 1492 s, 1460 m, 1330 sh, 1300 sh, 1250 s, 1210 s, 1030 w, 995 sh, 890 m, 850 w, 830 w, 750 s, and 695 s cm^{-1}; uptake of periodic acid, 1.2 moles per mole of **6**.

Anal. Calc. for $C_{19}H_{20}N_4O_4$: C, 61.94; H, 5.47; N, 15.21. Found: C, 61.83; H, 5.55; N, 15.18.

Evaporation of the fraction containing component A gave an orange-red powder which, on recrystallization from benzene–methanol, gave a micro-crystalline powder; yield 28 mg (2.8%); R_F (solvent *A*) 0.64 ±0.02; the u.v. and i.r. spectra were identical with those of the isomeric bis(phenylhydrazone) **7**. The original third fraction was purified by column chromatography (alumina) to give red prisms of **5a**; yield 8 mg (0.8%).

ACKNOWLEDGMENT

The author thanks one of the referees for useful comments regarding the bis(phenylhydrazones) of D-pinitol and L-quebrachitol.

REFERENCES

1 A. J. Fatiadi, *Carbohyd. Res.*, 8 (1968) 135.
2 H. S. Isbell and A. J. Fatiadi, *Carbohyd. Res.*, 2 (1966) 204.
3 A. J. Fatiadi and H. S. Isbell, *Carbohyd. Res.*, 5 (1967) 302.
4 A. J. Fatiadi and H. S. Isbell, *J. Res. Nat. Bur. Stand.*, 68A (1964) 287.
5 B. Magasanik and E. Chargaff, *J. Biol. Chem.*, 174 (1948) 173.
6 G. A. Russell, A. J. Moye, and K. Nagpal, *J. Amer. Chem. Soc.*, 84 (1962) 4154.
7 A. J. Fatiadi, *J. Res. Nat. Bur. Stand.*, 71A (1967) 277.
8 T. Posternak, *The Cyclitols*, Holden–Day, San Francisco, California, 1965, pp. 152–154; S. J. Angyal and L. Anderson, *Advan. Carbohyd. Chem.*, 14 (1959) 148.
9 H. Zollinger, *Azo and Diazo Chemistry of Aliphatic and Aromatic Compounds*, Interscience Publishers, Inc., New York, 1961, pp. 314–318; R. J. W. Cremlyn, *J. Chem. Soc.*, (1964) 6235.
10 L. J. Bellamy, *The Infra-red Spectra of Complex Molecules*, 2nd ed., John Wiley and Sons, Inc., New York, 1958, p. 116.
11 R. H. Hamilton, *J. Amer. Chem. Soc.*, 56 (1934) 487.

Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

THE ACTION OF BROMINE ON TETRA-*O*-ACETYL-1-*S*-ACETYL-1-THIO-*β*-D-GLUCOPYRANOSE. FORMATION AND DECOMPOSITION OF TETRA-*O*-ACETYL-*β*-D-GLUCOPYRANOSYLSULFENYL BROMIDE*†

R. H. BELL AND D. HORTON**
Department of Chemistry, The Ohio State University, Columbus, Ohio 43210 (U.S.A.)

(Received August 15th, 1968)

ABSTRACT

Treatment of either tetra-*O*-acetyl-1-*S*-acetyl-1-thio-*β*-D-glucopyranose (**1**) or *tert*-butyl tetra-*O*-acetyl-1-thio-*β*-D-glucopyranoside (**6**) with a >3-molar excess of bromine in carbon tetrachloride or pure chloroform, at −10° for 1-3 min, gives tetra-*O*-acetyl-*β*-D-glucopyranosylsulfenyl bromide (**2**) in quantitative yield; prolonged exposure to bromine at room temperature converts the product **2** into tetra-*O*-acetyl-*α*-D-glucopyranosyl bromide (**3**). Slow addition of bromine to the 1-thioglycoside **6** in carbon tetrachloride, or bromination of the thiolacetate **1** in reagent-grade chloroform, gives bis(tetra-*O*-acetyl-*β*-D-glucopyranosyl) disulfide (**4**), also obtainable by treating the sulfenyl bromide **2** with dry ethanol or with the 1-thioglycoside **6**. Oxidation of the disulfide **4** with *m*-chloroperoxybenzoic acid gives a mono-oxide derivative **5**, also obtainable by treating the sulfenyl bromide **2** with water or 95% ethanol. The disulfide **4** and its mono-oxide **5** were both converted into the bromide **3** by prolonged exposure to bromine at room temperature.

RESULTS AND DISCUSSION

It has been shown in earlier work from this laboratory that brief treatment of tetra-*O*-acetyl-1-*S*-acetyl-1-thio-*β*-D-glucopyranose (**1**) with an excess of bromine in carbon tetrachloride gives the crystalline, unstable tetra-*O*-acetyl-*β*-D-glucopyranosylsulfenyl bromide[1] (**2**). The bivalent sulfur atom in this derivative displays electrophilic character and can attack various types of electron-rich centers[2-4]. The present report describes a detailed study of reactions leading to the sulfenyl bromide **2** and to its subsequent decomposition by the reagent or by various solvents. A convenient procedure for preparing **2** in quantity is also described.

Preparation of the sulfenyl bromide **2** by bromination of the thiolacetate **1** in

---

*Preliminary report, R. H. BELL AND D. HORTON, *Abstracts Papers Amer. Chem. Soc. Meeting*, 156 (1968) CARB 8; see also, refs. 1–4.

†Supported, in part, by the Agricultural Research Service, U. S. Department of Agriculture, Grants No. 12-14-100-7208(71) and 12-14-100-9201(71) (O.S.U.R.F. Projects 1827 and 2573) administered by the Northern Utilization Research and Development Division, Peoria, Illinois.

**To whom inquiries should be addressed.

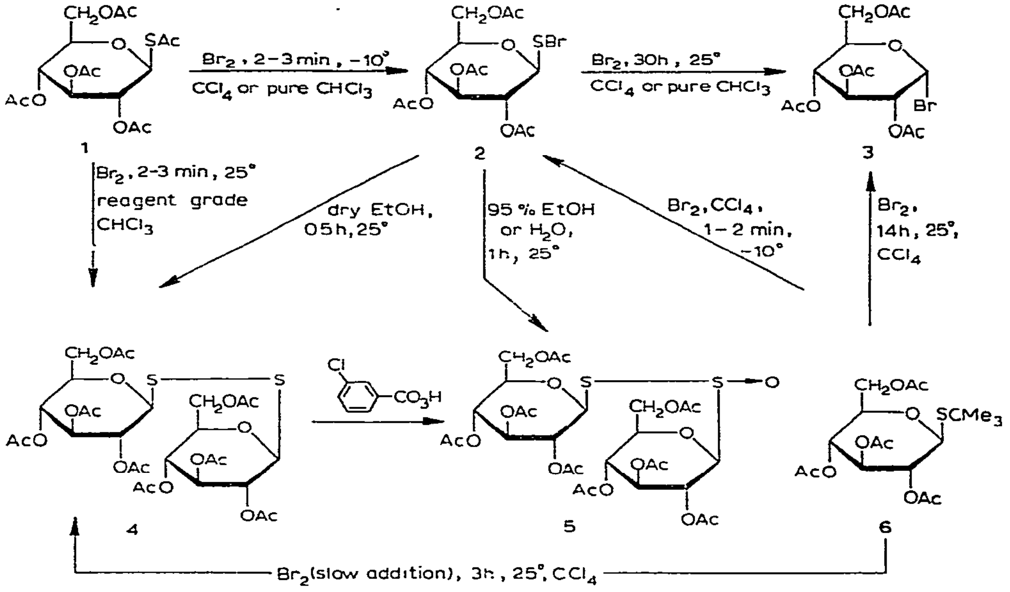
carbon tetrachloride for 5 min at room temperature, as described in the original report[1], gives crystalline product when the reaction is performed on a scale of 250–500 mg, but erratic results are obtained when the scale of the preparation is increased, or other solvents are used; side-products appear to be formed concurrently with **2**, and decomposition of **2** may take place during attempted isolation. In order to establish optimal conditions for the preparation of **2**, and to determine the nature of any side-products, the course of the reaction of **1** with bromine was followed by n.m.r. spectroscopy. The n.m.r. technique was chosen in preference to thin-layer chromatography (t.l.c.) for monitoring the reaction, because the highly reactive sulfenyl bromide **2** would almost certainly undergo decomposition in contact with the chromatographic adsorbent. Detailed n.m.r. spectral correlations of acetylated 1-thioaldopyranoses[5] and acetylated glycosyl halides[6] have been made in this laboratory, and the presence of either of these species in the reaction mixture could be detected readily by the presence of characteristic spectral signals.

Treatment of the thiolacetate **1** in carbon tetrachloride at ~40° with a 3-molar excess of bromine caused complete cleavage of the *S*-acetyl group from **1** within 1 min, as shown by the disappearance of the 3-proton singlet at τ 7.59 (SAc)[5] observed in the spectrum of **1**, and the appearance of a signal corresponding to acetyl bromide at τ 7.19. Tetra-*O*-acetyl-α-D-glucopyranosyl bromide (**3**) was definitely absent from the reaction mixture at this stage, because no low-field doublet (for H-1 of **3**) was observed in the spectrum. The spectrum was consistent with the product's being the sulfenyl bromide **2**, and this supposition is supported by the data from elemental analysis and the reactivity of the product isolated, which clearly establish that it is **2** and not bis(tetra-*O*-acetyl-β-D-glucopyranosyl) disulfide (**4**). The n.m.r. spectra of the sulfenyl bromide **2** and the disulfide **4** differ only in minor details, and in neither case can the H-1 signal be observed clearly to the low-field side of the "envelope" of signals for the methine protons, because the sulfur atom at C-1 exerts a much smaller deshielding effect than an oxygen or halogen atom[5].

When the reaction mixture was kept at 40°, the initial product **2** began to decompose after 3–4 min, with the formation of the glycosyl bromide **3**, and conversion of **2** into **3** was complete in 1 hour, as shown by the n.m.r. spectrum.

For the conversion of **1** into **2**, at least a 3-molar excess of bromine, added in one portion, was required. When smaller proportions of bromine were used, starting material (**1**) could still be detected, even after several minutes of reaction.

A convenient method for the preparation of the sulfenyl bromide **2** on a 50–100 millimolar scale, giving a quantitative yield of crystalline product, consisted in conducting the bromination in carbon tetrachloride for 2–3 min at −10° and evaporating the solvent and excess reagent rapidly at 30° with a vacuum pump. The product was analytically pure without recrystallization, and could be kept for an extended time at the temperature of liquid nitrogen. The compound could be recovered from solutions in nonhydroxylic solvents, but was rapidly decomposed by traces of moisture or hydroxylic solvents; solutions prepared for optical rotatory measurements became more levorotatory on storage, probably because of moisturecatalyzed decomposition. Although the sulfenyl bromide **2** and the disulfide **4** (a product of decomposition of **2**) can be differentiated from each other by the pattern of acetyl-group signals in their n.m.r. spectra, a more convenient method for distinguishing **2** from **4** was by i.r. spectroscopy (see Fig. 1).



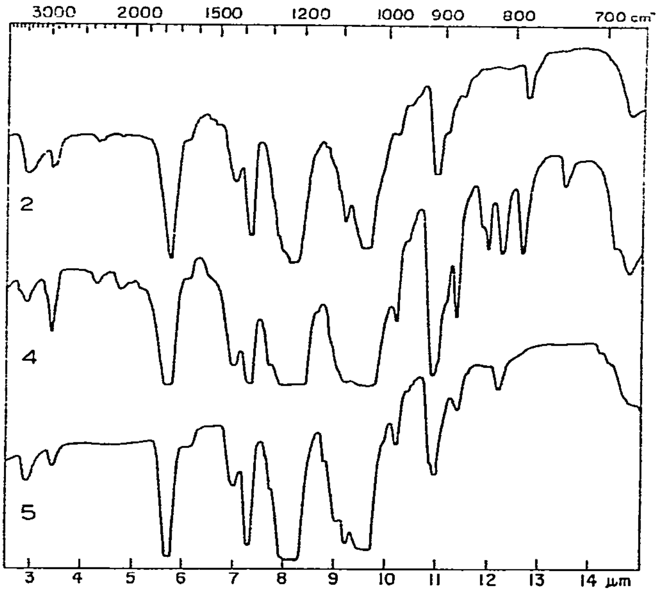


Fig. 1. The i.r. spectra (KBr pellet) of tetra-O-acetyl-β-D-glucopyranosylsulfenyl bromide (**2**), bis(tetra-O-acetyl-β-D-glucopyranosyl) disulfide (**4**), and bis(tetra-O-acetyl-β-D-glucopyranosyl) disulfide mono-oxide (**5**).

Quantitative conversion of the thiolacetate 1 into crystalline 2 could be effected under identical conditions by use of pure chloroform (free from water and ethanol) as the solvent; the product was identified as 2 by physical data and by treatment with cyclohexene to give the crystalline adduct, *trans*-2-bromo-1-(tetra-*O*-acetyl-β-D-glucopyranosylthio)cyclohexane[3,4]. Another route to the sulfenyl bromide 2 involves treatment of *tert*-butyl tetra-*O*-acetyl-1-thio-β-D-glucopyranoside (6) in carbon tetrachloride with a large excess of bromine for 1–2 min at −10°; the reaction gives crystalline 2 in quantitative yield. The product was characterized by physical data and by treatment with benzenethiol to give phenyl tetra-*O*-acetyl-β-D-glucopyranosyl disulfide[3,4].

Treatment of the thiolacetate 1, the sulfenyl bromide 2, or the 1-thioglycoside 6 in carbon tetrachloride with a large excess of bromine for an extended period (30–60 h) at room temperature gave the glycosyl bromide 3, as indicated by n.m.r. spectroscopy and confirmed by isolation of 3.

The course of the reaction of the thiolacetate 1 with a large excess of bromine at −10° was changed completely when reagent-grade chloroform (containing 0.75% of ethanol and, possibly, some water) was used as the solvent. After 2 min of reaction, there was formed a product, isolated crystalline in 79% yield, that was identical with a sample of bis(tetra-*O*-acetyl-β-D-glucopyranosyl) disulfide (4) that had been prepared from 1 by *S*-deacetylation followed by oxidative dimerization. The same product was also formed in high yield when the 1-thioglycoside 6 in carbon tetrachloride was treated dropwise with bromine during 3 h. It may be supposed that, in the latter reaction, bromine reacts with 6 to give the sulfenyl bromide 2, which then reacts with excess 1-thioglycoside 6 to give the disulfide 4 and *tert*-butyl bromide. Such a reaction

$$\mathbf{2}+\mathbf{6} \rightarrow \mathbf{4}+\mathrm{Me_3CBr}$$

would be facilitated by the ease of heterolysis of the C-1–S bond in 6 during attack of the electrophilic sulfur atom of 2 on the nucleophilic sulfur atom of 6. Support for this mechanism is provided by the observation that, in carbon tetrachloride, compound 2 reacts with 6 to give the disulfide 4 in high yield, and a volatile *tert*-butyl derivative is produced.

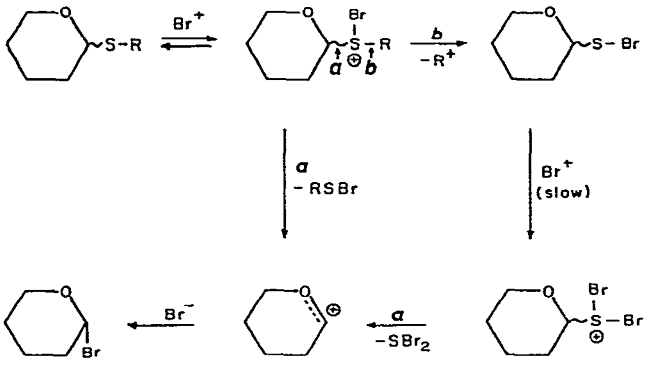
The sulfenyl bromide 2 was decomposed rapidly by dry ethanol, and the major product of reaction after 0.5 h at 25° was the disulfide 4; none of the sulfenyl bromide 2 could be detected. Water, also, caused rapid decomposition of the sulfenyl bromide 2, but in this case the principal crystalline product, isolated in 59% yield, was a compound having the empirical formula $C_{28}H_{38}O_{19}S_2$, corresponding to a mono-oxide of the disulfide 4; compound 4 was obtained concurrently, in 6% yield. The mono-oxide migrated more slowly than 4 on t.l.c., and was absent from the product of reaction of 2 with dry ethanol. Separation of the mono-oxide from the disulfide 4 could be effected readily, because of the extremely low solubility of the former in cold ethanol. The n.m.r. spectra of 4 and the mono-oxide were very closely similar, and differed noticeably only in the pattern of the acetyl-group signals at high resolution; the two compounds were readily differentiated, however, by their i.r. spectra (see Fig. 1) and X-ray powder diffraction patterns.

Oxidation of the disulfide **4** with *m*-chloroperoxybenzoic acid gave a mono-oxide derivative identical with that obtained by treating **2** with water. An excess of the oxidant did not appear to cause oxidation to a more highly oxygenated derivative. Treatment of the sulfenyl bromide **2** with 95% ethanol gave a mixture of the mono-oxide and the disulfide **4**.

The mono-oxide may be formulated either as a sulfenic anhydride (R–S–O–S–R) or as a thiolsulfinate [R–S–S(O)–R]; the formulation R–O–S–S–R is excluded by the n.m.r. data. By analogy with work on simple compounds of related structure[7], the thiolsulfinate structure (**5**) may be considered the more probable, although this supposition has not been proved rigorously. A compound having structure **5** should be capable of existence in two diastereoisomeric forms. In various preparations of the mono-oxide, the crude product had a melting point much lower than that of the product after recrystallization from hot ethanol. Possibly, the crude product was a different structural isomer of the mono-oxide, or a mixture of diastereoisomers[7] of **5**.

Bromination of either the disulfide **4** or the mono-oxide **5** in carbon tetrachloride for 1 day at 35° gave the bromide **3** in high yield. The disulfide **4** was not decomposed under the brief conditions of reaction used for preparing the sulfenyl bromide **2** from **1** or **6**, and only slight conversion of **4** into **3** was observed in the experiment in which the 1-thioglycoside **6** was converted into **4** by slow addition of bromine during 3 h at 25°.

Formation of the sulfenyl bromide **2** from the *tert*-butyl thioglucoside **6**, as well as from the thiolacetate **1**, lends support to the mechanistic rationale (see Scheme I) originally proposed[1] for the formation of **2**, based on the heterolysis of an intermediate bromosulfonium ion at point *b*, if R^+ is a cation more stable than the glycosyl cation that would result from scission at point *a*. The fact that the reaction requires more than one molecule of bromine per molecule suggests that the actual transition-state may involve an additional molecule of bromine that interacts with the bromosulfonium ion. Conversion of the sulfenyl bromide **2** into the bromide **3** is, predictably,

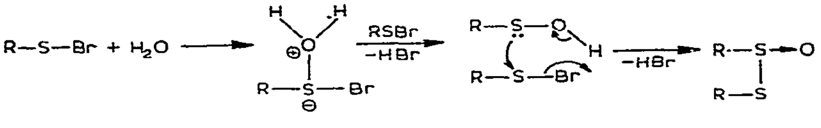


Scheme I

a slower process than formation of **2**, because the sulfur atom in **2** would be expected to be much less susceptible to attack by (positive) bromine than the sulfur atom in **1** or **6**.

The glycosylsulfenyl bromide **2** appears to be intermediate in stability between the extremely reactive alkylsulfenyl bromides[8] and the stable arylsulfenyl bromides[8].

A plausible route for formation of the mono-oxide **5** in the reaction of **2** with water would involve hydrolysis of one molecule of **2** to the sulfenic acid, followed



by reaction of the latter with a second molecule of **2** to give the thiolsulfinate. In the reaction with ethanol, it is conceivable that the observed disulfide **4** may arise by the reaction of a molecule of an ethyl sulfenate with a molecule of the sulfenyl bromide, with formation of ethyl hypobromite by sulfur–oxygen bond-cleavage, since the

$$\text{R–S–Br} + \text{EtOH} \xrightarrow[-\text{HBr}]{} \text{R–S–OEt} \xrightarrow[\text{R–S–Br}]{} \text{R–S–S–R} + \text{EtOBr}$$

Et–O bond would not be susceptible to abstraction of the Et group by bromide ion. These rationalizations have not been proved experimentally.

Reactions of the sulfenyl bromide **2** with thiols, amines, and alkenes are described in the accompanying paper[4].

EXPERIMENTAL

General. — Evaporations were performed under diminished pressure. Strict precautions were taken to prevent ingress of moisture in all of the bromination experiments. Specific rotations were measured in a 2-dm tube. Melting points were determined with a Thomas–Hoover Unimelt apparatus. I.r. spectra were recorded with a Perkin–Elmer "Infracord" Model 137 i.r. spectrophotometer, unless specified otherwise. N.m.r. spectra were recorded at 60 or 100 MHz with Varian A-60 or HA-100 n.m.r. spectrometers; chemical shifts refer to an internal standard of tetramethylsilane ($\tau = 10.00$). Ultraviolet spectra were recorded with a Bausch and Lomb "Spectronic 505" recording spectrometer. Microanalyses were made by W. N. Rond. X-Ray powder diffraction data give interplanar spacings, Å, for CuKα radiation. The camera diameter was 114.59 mm. Relative intensities were estimated visually: m, moderate; s, strong; v, very; w, weak. The strongest lines are numbered (1, strongest); double numbers indicate approximately equal intensities. T.l.c. was performed with Silica Gel G (E. Merck, Darmstadt, Germany), activated at 120°, as the adsorbent, 3:1 (v/v) dichloromethane–ether as the developer, and sulfuric acid as the indicator. Column chromatography was performed with Silica Gel Davison (60–200 mesh, Davison Division of the W. R. Grace Co., Baltimore, Md.), activated at 120°, as the adsorbent, with 100 g of adsorbent per g of the mixture to be separated. Columns were packed by allowing a slurry of the adsorbent in the eluent (3:1 dichloromethane–ether) to settle under gravity, and elution was effected without application of pressure or suction. The reagent-grade chloroform (J. T. Baker Chemical Co., Phillipsburg, N. J.) used was stated to contain 0.75% of ethanol. Purified chloroform, free from ethanol and water, was obtained by passing reagent-grade chloroform through a column of neutral, Woelm alumina immediately before use. The petroleum ether used was a fraction having b.p. 60–110°.

*N.m.r. spectral study of the reaction of tetra-*O*-acetyl-1-*S*-acetyl-1-thio-β-*D*-glucopyranose* (**1**) *in carbon tetrachloride with bromine.* — A suspension of the thiolacetate[9] **1** (126 mg) in carbon tetrachloride (1 ml) was prepared in an n.m.r. sample tube, and a small drop of tetramethylsilane was added. The spectrum at 60 MHz showed a 3-proton singlet at τ 7.59 (SAc), peaks (12 protons) at τ 7.92, 7.98, and 8.00 (OAc), and no signals below τ 4.5. From a stock solution of bromine (15.60 g; 5.0 ml) in carbon tetrachloride (20 ml) a 250-μl aliquot (corresponding to 3.1 moles of bromine per mole of **1**) was added by means of a syringe to the suspension of **1** at ~40°. The suspended material dissolved rapidly. A scan of the n.m.r. spectrum was initiated 60 sec after addition of the bromine, and was completed 100 sec afterwards. The spectrum showed no signals below τ 4.5, but the SAc signal had disappeared completely and a 3-proton singlet at τ 7.19 had appeared (acetyl bromide in carbon tetrachloride shows its proton signal at τ 7.19). A 12-proton multiplet was observed at τ ~7.9 (OAc). The spectrum was rescanned 200 sec after the addition of the bromine; this scan, completed 100 sec later, resembled the previous one, except that a low-intensity doublet at τ 3.33, having $J_{1,2}$ 4.0 Hz (H-1 of the glycosyl bromide **3**), was present. The latter signal increased in intensity with time, and, after 1 h, it had an integrated intensity of one proton (relative to the 12-proton signal at τ ~7.9). At this time, the spectrum was identical with that of the glycosyl bromide **3** in carbon tetrachloride, except for the additional 3-proton singlet at τ 7.19 (AcBr). The reaction solution was evaporated to dryness, and the residue was dissolved in carbon tetrachloride. The n.m.r. spectrum of the solution was unchanged, except that the signal at τ 7.19 had disappeared.

When the experiment was performed with a 1.5-molar excess of bromine, the spectrum indicated that some starting material **1** was present, even after 10–20 min of reaction.

*Preparation of tetra-*O*-acetyl-β-*D*-glucopyranosylsulfenyl bromide* (**2**). — *A. From* **1** *in carbon tetrachloride.* To a suspension of tetra-*O*-acetyl-1-*S*-acetyl-1-thio-β-D-glucopyranose[9] (**1**, 20.00 g, 49.2 mmoles) in anhydrous carbon tetrachloride (200 ml) cooled to about −10° was added a solution of bromine (9.0 ml, 176 mmoles) in carbon tetrachloride (40 ml) and the mixture was stirred for 2–3 min at about −10°. Volatile materials were then rapidly removed on a rotary evaporator at ~30°/< 10 torr, to give **2** as a pale-yellow, crystalline residue, yield 22.27 g (102%), m.p. 102–104°, $[\alpha]_D^{15}$ −66.7 ±1° (c 2.1, tetrahydrofuran) changing to −114 ±1° after 14 h; λ_{max}^{KBr}

(see Fig. 1) 5.72 (OAc), 11.20 μm (axial H at C-1), SAc absent[9]; n.m.r. data (100 MHz, chloroform-*d*): τ 4.55–5.02 (3-proton multiplet), 5.35 (1-proton multiplet) (H-1,2,3,4), τ 5.63–5.99 (2-proton multiplet, H-6), τ 6.14 (1-proton multiplet, H-5)[5], τ 7.90, 7.92, 7.97, 8.00 (12 protons, acetyls).

Anal. Calc. for $C_{14}H_{19}BrO_9S$: C, 37.93; H, 4.32; Br, 18.03; S, 7.23. Found: C, 37.99; H, 4.39; Br, 17.52; S, 7.51.

The product gave an X-ray powder diffraction pattern identical with that previously reported[1]. Rapid recrystallization of the product from carbon tetrachloride gave an almost quantitative recovery of **2**, having physical constants essentially identical with those of the material first isolated. T.l.c. of **2** showed a principal component having R_F 0.45 and minor components having R_F 0.76, 0.71, and 0.29, although none of these components could actually be attributed to **2** itself, because of the probability that **2** undergoes decomposition on the adsorbent.

Compound **2** prepared by the above procedure was used in all preparations described in this paper, unless stated otherwise.

B. From **1** *in purified chloroform.* A solution of the thiolacetate **1** (2.048 g, 5.04 mmoles) in purified chloroform (50 ml) was cooled to −10°, and a solution of bromine (1.5 ml, 29 mmoles) in purified chloroform (8.5 ml) was added. The mixture was kept for 1 min at −10°, and then evaporated rapidly at 30° to give a pale-yellow, crystalline residue; yield 2.153 g (96%), m.p. 103–105°, $[\alpha]_D^{19}$ −74 ±1° (*c* 2.2, chloroform), becoming more levorotatory after several hours. By mixed m.p., i.r. and n.m.r. spectra, and X-ray powder diffraction data, the product was identical with the sulfenyl bromide **2** prepared by procedure *A*.

A suspension of **2** (prepared in purified chloroform from 2.13 g of the thiolacetate **1**) in carbon tetrachloride (50 ml) was treated with cyclohexene (1 ml) in carbon tetrachloride (9 ml), and the mixture was kept for 1 h at 25°; it was then washed successively with water (100 ml), saturated aqueous sodium hydrogen carbonate solution (100 ml), and water (100 ml), dried (magnesium sulfate), and evaporated. The residual syrup was crystallized from ether–petroleum ether to give colorless, stout needles, yield 1.532 g (56%), m.p. 82–88°. Purification of the product by column chromatography gave pure *trans*-2-bromo-1-(tetra-*O*-acetyl-*β*-D-glucopyranosylthio)cyclohexane, m.p. 88–89° (identical with an authentic sample[3,4] by mixed m.p. and comparative i.r. and n.m.r. spectra), together with a small amount of the disulfide derivative **4**.

C. From tert-*butyl tetra-*O*-acetyl-1-thio-β-D-glucopyranoside*[5,10] (**6**) *in carbon tetrachloride.* To a solution of the 1-thioglycoside **6** (516 mg, 1.23 mmoles) in carbon tetrachloride (20 ml) at −10° was added bromine (0.75 ml, 15 mmoles); after 1.5 min at −10°, the mixture was rapidly evaporated at 30° to give the sulfenyl bromide **2** as a yellow, crystalline solid, yield 548 mg (101%), m.p. 97–99°. By comparative i.r. and n.m.r. spectra, and by X-ray powder diffraction data, this product was identical with **2** prepared by methods *A* or *B*.

To a suspension of the product (463 mg) in carbon tetrachloride (40 ml) was added a solution of benzenethiol (0.5 g) in carbon tetrachloride (9.5 ml), and the mixture was kept for 1 h at 25°. The resulting solution was washed successively with water, saturated aqueous sodium hydrogen carbonate, and water, dried (magnesium sulfate), and evaporated to dryness, and the residue was crystallized from ethanol–petroleum ether to give phenyl tetra-*O*-acetyl-β-D-glucopyranosyl disulfide[3,4], yield 200 mg (34%), m.p. 123–125°, identical with an authentic sample[3,4] by mixed m.p., comparative i.r. and n.m.r. spectra, and X-ray powder diffraction data[4].

Bromination of thio derivatives **1**, **2**, *and* **6** *for an extended time.* — *A. Bromination of thioacetate* **1**. To a suspension of the thioacetate **1** (2.33 g, 5.73 mmoles) in carbon tetrachloride (60 ml) was added bromine (2 ml, ~39 mmoles) in carbon tetrachloride (8 ml), and the mixture was kept for 30 h at 25° and then evaporated to dryness. The product was crystallized from ether–petroleum ether to give tetra-*O*-acetyl-α-D-glucopyranosyl bromide (**3**), yield 2.09 g (89%), m.p. 87–88°, indistinguishable from an authentic sample of **3** by mixed m.p., and by i.r. and n.m.r. spectra. T.l.c. of the mother liquors showed a single component, having chromatographic characteristics indistinguishable from those of **3**.

The preceding experiment was repeated, but with purified chloroform as the solvent. The results were identical with those given for the reaction in carbon tetrachloride, and the crystalline bromide **3** was isolated in 90% yield.

B. Bromination of sulfenyl bromide **2**. A suspension of **2** (2.62 mmoles, prepared from 1.065 g of thiolacetate **1**) in carbon tetrachloride (50 ml) was treated with bromine (2 ml, ~39 mmoles) in carbon tetrachloride (8 ml), and the mixture was kept for 30 h at 25°. The resultant, orange solution was evaporated to a syrup that was crystallized from ether–petroleum ether to give the bromide **3**, yield 492 mg (46%), m.p. 87–88°, indistinguishable from authentic **3** by mixed m.p. and by i.r. and n.m.r. spectra. T.l.c. of the mother liquors showed the presence of a major component having the mobility of **3**, and a minor component having R_F 0.45.

C. Bromination of 1-thioglycoside **6**. To a suspension of the 1-thioglycoside **6** (534 mg, 1.27 mmoles) in carbon tetrachloride (20 ml) was added bromine (1.5 ml, 29 mmoles). The resultant, clear solution was kept for 60 h at 25°, and evaporated to dryness; a solution of the resultant syrup in dichloromethane was washed with aqueous sodium hydrogen carbonate, dried (magnesium sulfate), decolorized with carbon, and evaporated to a syrup. Crystallization of the syrup from ether–petroleum ether gave the bromide **3**, yield 167 mg (32%), m.p. 82–85°, identical with an authentic sample of **3** by t.l.c. and i.r. and n.m.r. spectra. T.l.c. of the mother liquors indicated that **3** was the principal component; a minor component had R_F 0.46.

When the reaction was followed by n.m.r. spectroscopy at ~40°, the 9-proton signal at τ 8.61 for the *tert*-butyl group of **6** was absent 1 min after the addition of bromine, and the spectrum showed no signal below τ 4.5. A 9-proton signal at τ 8.19 was observed. After 14 h, the spectrum showed a 1-proton doublet at 3.27, $J_{1,2}$ 4.1 Hz, and was closely similar to that of the bromide **3**, except for the presence of the 9-proton singlet at τ 8.19.

*Preparation of bis(tetra-*O*-acetyl-β-D-glucopyranosyl) disulfide* (**4**). — The general procedure of Richtmyer, Carr, and Hudson[11] was used, except that the starting material employed was tetra-*O*-acetyl-1-*S*-acetyl-1-thio-β-D-glucopyranose (**1**, 10.05 g) instead of tetra-*O*-acetyl-β-D-glucopyranosyl ethylxanthate. The product was obtained as colorless needles, yield 8.014 g (89%), having m.p. 140–141°, $[\alpha]_D^{21}$ $-157 \pm 0.5°$ (*c* 5.5, chloroform) [lit.[11] m.p. 142–143°, $[\alpha]_D^{20}$ $-156°$ (chloroform)]; R_F 0.44; λ_{max}^{KBr} (see Fig. 1) 5.76 (OAc), 11.17 μm (axial H at C-1); λ_{max}^{EtOH} 250 (ε 450), 206 nm (900); n.m.r. data (100 MHz, chloroform-*d*): τ 4.61–5.01 (3-proton multiplet), 5.28–5.37 (1-proton multiplet) (H-1,2,3,4), τ 5.55–5.87 (8-peak multiplet, H-6), τ 6.20 (H-5), τ 8.85, 8.88, 8.96, and 8.98 (3-proton singlets, acetyls); X-ray powder diffraction data: 13.00 vs (1), 9.74 vw, 7.94 m, 7.44 vw, 6.98 w, 5.72 s (3), 5.02 m, 4.72 m, 4.08 s (2), 3.89 m, 3.54 w.

Anal. Calc. for $C_{28}H_{38}O_{18}S_2$: C, 46.28; H, 5.27; S, 8.82. Found: C, 46.22; H, 5.00; S, 8.64.

Formation of the disulfide **4** *by bromination of thiolacetate* **1** *in reagent-grade chloroform.* — A solution of **1** (1.16 g, 2.85 mmoles) in reagent-grade chloroform (8.5 ml) was cooled to about −10°, and a solution of bromine (1.5 ml, 29 mmoles) in reagent-grade chloroform (8.5 ml) was added. After 2 min at −10°, the solution was rapidly evaporated at 30°. Crystallization of the resultant syrup from ether–petroleum ether, and recrystallization from the same solvent mixture, gave the disulfide **4**, yield 0.82 g (79%), m.p. 142–143°, identical by mixed m.p., t.l.c., i.r. and n.m.r. spectra, and X-ray powder diffraction pattern with an authentic sample of **4**.

T.l.c. of the mother liquors revealed a principal component having the mobility of **4**, and traces of two additional components having the mobilities of the bromide **3** and the starting material **1**. In a repetition of the experiment, the yield of crystalline **4** was 77%.

Formation of the disulfide **4** *by slow bromination of the 1-thioglycoside* **6**. — Bromine (0.35 ml, ~7 mmoles) was added dropwise during 3 h to a solution of **6** (544 mg, 1.29 mmoles) in carbon tetrachloride (30 ml), and the reaction was monitored by t.l.c. At the end of the 3-h period, only a trace of **6** remained, and the major component corresponded to the disulfide **4**; a trace of a product having R_F 0.39 was also present. Evaporation of the solution, and crystallization of the residue from ether–petroleum ether, gave the disulfide **4**, yield 358 mg (76%), m.p. (one recrystallization) 140–141°, identical with authentic **4** by mixed m.p., i.r. and n.m.r. spectra, and X-ray powder diffraction pattern.

Fractional recrystallization of the mother liquors gave 13.4 mg (2.5%) of the starting material **6**.

Reaction of the sulfenyl bromide **2** *with 1-thioglycoside* **6** *to give the disulfide* **4**.— A suspension of **2** (1.151 g, 2.60 mmoles) in carbon tetrachloride (50 ml) was mixed with a solution of **6** (1.060 g 2.52 mmoles) in carbon tetrachloride (20 ml) and the mixture was shaken for 16 h at room temperature. The resultant, clear solution was washed successively with aqueous sodium hydrogen carbonate and water, dried (magnesium sulfate), and evaporated to dryness. Crystallization of the residue from ethanol–petroleum ether gave the disulfide **4**; yield (in three crops) 1.314 g (70%), identical with an authentic sample by t.l.c., mixed m.p., i.r. and n.m.r. spectra, and X-ray powder diffraction pattern.

T.l.c. of the mother liquors showed a major component corresponding to **4**, and a minor component corresponding to **6**.

An aliquot of the reaction mixture after 16 h was examined by n.m.r. spectroscopy at 60 MHz. The 9-proton singlet at τ 8.61 ($SCMe_3$) present in the spectrum of the starting 1-thioglycoside **6** had diminished to low intensity, and a sharp singlet at τ 8.19, corresponding to *tert*-butyl bromide formed, was observed. The reaction mixture was evaporated, and the residue was dissolved in carbon tetrachloride. The signal at τ 8.19 (Me_3CBr) was no longer observable in the n.m.r. spectrum of this solution.

Reaction of the sulfenyl bromide **2** *with dry ethanol to give the disulfide* **4**. — Dry ethanol (50 ml) was added to the sulfenyl bromide **2** (prepared from 1.90 g of **1**), and the yellow reaction-mixture was kept for 20–30 min at room temperature; white needles were then present, and t.l.c. indicated the presence of a single component, R_F 0.53, corresponding to the disulfide **4**. No component having R_F 0.39 (corresponding to the oxide **5**) was detected. To the reaction mixture was added 50 ml of saturated aqueous sodium hydrogen carbonate solution, and the mixture was concentrated to remove ethanol. The resulting aqueous suspension was extracted with two 50 ml portions of dichloromethane, and the extracts were combined, washed with water, dried (magnesium sulfate), and evaporated. Crystallization of the residue from ethanol gave the disulfide **4**, yield 847 mg (50%), m.p. (after recrystallization from ethanol) 141–142°, identical with authentic **4** by t.l.c. and X-ray powder diffraction pattern.

T.l.c. of the mother liquors showed a major component having the mobility of the disulfide **4**, and traces of components having R_F 0.77 and 0.12. No component corresponding to the oxide **5** (R_F 0.39) was present.

The experiment was repeated with sulfenyl bromide **2** (from 2.32 g of **1**) in dry ethanol (50 ml). After 20 min, the white needles of **4** that had formed were filtered off; yield 548 mg (26%), m.p. 142–143°. The filtrate was collected in a flask containing α-toluenethiol (1 ml) in dry ethanol (10 ml). After 10 min at 25°, t.l.c. of the mixture showed no component having the mobility (R_F 0.88) of benzyl tetra-*O*-acetyl-β-D-glucopyranosyl disulfide[3,4], indicating that the sulfenyl bromide **2** had been completely decomposed by ethanol within 20 min.

Bis(tetra-O-acetyl-β-D-glucopyranosyl) disulfide mono-oxide (**5**) *by oxidation of disulfide* **4** *with* m-*chloroperoxybenzoic acid.* — A solution of the disulfide **4** (264 mg, 360 μmoles) and *m*-chloroperoxybenzoic acid (49 mg, 80% pure by g.l.c., 230 μmoles of oxidant) in chloroform (50 ml) was kept at 25°. After 40 min, t.l.c. indicated the presence of two components, R_F 0.51 and 0.39, in approximately equal amounts; no change was noted after 4 h. Additional oxidant (~50 mg, 230 μmoles) was added, and after 1 h, t.l.c. indicated no significant change in the mixture; it was therefore washed successively with aqueous sodium hydrogen carbonate solution (50 ml) and water (50 ml), dried (magnesium sulfate), and evaporated to dryness, and the residue was triturated with ethanol (10 ml), whereupon the mono-oxide **5** crystallized as colorless, microscopic needles, yield 110 mg (41%), m.p. 150–151°. Recrystallization from hot ethanol gave pure **5**, m.p. 152.5–153°, $[\alpha]_D^{22}$ −52.1 ±1° (c 2.5 chloroform); R_F 0.39; λ_{max}^{KBr} (see Fig. 1) 5.72 μm (OAc); n.m.r. data (100 MHz, chloroform-d): τ 4.36–5.18 (multiplets, H-1,2,3,4), τ ~5.76 (multiplet, H-6), τ ~6.17 (multiplet, H-5), τ 7.90, 7.95, 7.96, and 7.98 (singlets, acetyls); X-ray powder diffraction data: 11.86 s (2,2), 10.45 s (2,2), 7.68 s (2,2), 5.43 s (3,3), 5.07 m, 4.95 s (3,3), 4.67 vw, 4.44 vs (1), 4.29 w.

The product **5** had extremely low solubility in cold ethanol, in contrast to the disulfide **4**, which was moderately soluble; the two compounds were readily separable because of this difference.

Reaction of the sulfenyl bromide **2** *with water to give the disulfide mono-oxide* **5**. — A suspension of **2** (prepared from 1.043 g of **1**) in carbon tetrachloride (40 ml) was shaken with water (3 ml) for 40 min at 25°. The solvents were evaporated from the resultant white suspension, the residue was dissolved in dichloromethane (100 ml), and the solution was washed successively with saturated aqueous sodium hydrogen carbonate solution and water, dried (magnesium sulfate), and evaporated to a syrup. Addition of ethanol to the syrup caused immediate crystallization, to give the oxide **5**, yield 560 mg (59%), m.p. 151°, identical with an authentic sample by t.l.c., i.r. and n.m.r. spectra, and X-ray powder diffraction data.

T.l.c. of the mother liquors showed that essentially all of the oxide **5** had been removed by crystallization, but a component having the mobility (R_F 0.51) of the disulfide **4** was present. Column-chromatographic fractionation of the mother liquors gave the disulfide **4**, yield 60 mg (6%), m.p. 140°, identical with an authentic sample by t.l.c., i.r. and n.m.r. spectra, and X-ray powder diffraction pattern.

Reaction of the sulfenyl bromide **2** *with 95% ethanol to give the disulfide* **4** *and the mono-oxide* **5**. — To the sulfenyl bromide **2** (prepared from 2.317 g of **1**) was added 95% ethanol (40 ml) at room temperature. The mixture became clear after a few min, and then became turbid and deposited white, fluffy needles. After 8 h, the reaction mixture was processed as in the preceding experiment, to give the mono-oxide **5**, yield 1.239 g (59%), m.p. (after recrystallization from hot ethanol) 151–152°, identical with an authentic sample by mixed m.p., i.r. and n.m.r. spectra, and X-ray powder diffraction pattern.

The mother liquors from the reaction contained a single component, chromatographically identical with the disulfide **4**; t.l.c. of the initial reaction-product indicated that **4** and **5** were present in approximately equal amounts.

The foregoing experiment and the preceding one were repeated several times, either by the procedure described or by directly filtering off the product that crystallized from the reaction mixture. Frequently, the ethanol-insoluble product initially obtained had a melting point lower than that of the material obtained after recrystallization from hot ethanol; values of 101–103°, 111–113°, 113–114°, 132–134°, and 138–139° were observed in different experiments. In each instance, the product was free from the disulfide **4** (t.l.c.), and after refluxing with ethanol, recrystallization gave the oxide **5** having m.p. 152.5–153°, with recoveries of ~60%. A dimorph of **5**, having m.p. 157–158°, was also encountered.

Bromination of the disulfide **4** *to give the bromide* **3**. — To a suspension of **4** (196 mg, 270 μmoles) in carbon tetrachloride (30 ml) was added bromine (1.5 ml, ~29 mmoles), and the resultant solution was kept for 23 h at ~35°. The solution was evaporated to dryness at 30°, carbon tetrachloride was added to, and evaporated from, the residue, and the latter was dissolved in dry chloroform-*d*. The n.m.r. spectrum of the solution was identical with that[6] of the bromide **3**, and the integrated intensities of the H-1 signal (τ 3.25, $J_{1,2}$ 4.0 Hz) and of the acetyl-group signals were in the ratio 1:12. T.l.c. of the solution showed a principal component (R_F 0.80) chromatographically indistinguishable from **3**; very minor side-products, having R_F 0.42, 0.31, 0.29, 0.20, and 0.1, were also present.

Bromination of the mono-oxide **5** *to give the bromide* **3**. — The mono-oxide **5** (133 mg, 180 μmoles) was treated with bromine (1.5 ml, ~29 mmoles) by exactly the procedure used in the preceding experiment, and identical results were obtained.

ACKNOWLEDGMENTS

The authors thank C. V. Holland, Martha J. Miller, and W. N. Turner for preliminary experimental work, and J. H. Lauterbach for recording the 100-MHz n.m.r. spectra.

REFERENCES

1 D. Horton, M. L. Wolfrom, and H. G. Garg, *J. Org. Chem.*, 28 (1963) 2992.

2 C. V. Holland, D. Horton, Martha J. Miller, and W. N. Turner, *Abstracts Papers Amer. Chem. Soc. Meeting*, 149 (1965) 1D.

3 D. Horton and Martha J. Miller, *Carbohyd. Res.*, 1 (1965) 335.

4 R. H. Bell, D. Horton, and Martha J. Miller, *Carbohyd. Res.*, 9 (1969) 201.

5 C. V. Holland, D. Horton, Martha J. Miller, and N. S. Bhacca, *J. Org. Chem.*, 32 (1967) 3077.

6 D. Horton and W. N. Turner, *J. Org. Chem.*, 30 (1965) 3387.

7 E. Vinkler and F. Klivényi, *Acta Chim. Acad. Sci. Hung.*, 11 (1957) 15; 22 (1960) 345; J. L. Kice and G. B. Large, *Tetrahedron Lett.*, (1965) 3537.

8 N. Kharasch, Z. S. Ariyan, and A. J. Havlik, *Quart. Rept. Sulfur Chem.*, 1 (1966) 93; *Proceedings of the Intra-Science Symposium on Sulfenyl Compounds, November 30, 1967* (N. Kharasch, Ed.), *ibid.*, 2 (1967) 289–380.

9 D. Horton and M. L. Wolfrom, *J. Org. Chem.*, 27 (1962) 1794.

10 H. B. Wood, Jr., B. Coxon, H. W. Diehl, and H. G. Fletcher, Jr., *J. Org. Chem.*, 29 (1964) 461.

11 N. K. Richtmyer, C. J. Carr, and C. S. Hudson, *J. Amer. Chem. Soc.*, 65 (1943) 1477.

Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

REACTIONS OF TETRA-*O*-ACETYL-*β*-D-GLUCOPYRANOSYLSULFENYL BROMIDE*†

R. H. BELL, D. HORTON**, AND MARTHA J. MILLER‡
Department of Chemistry, The Ohio State University, Columbus, Ohio 43210 (U.S.A.)

(Received August 19th, 1968)

ABSTRACT

Tetra-*O*-acetyl-*β*-D-glucopyranosylsulfenyl bromide (**2**) has a bivalent sulfur atom that exhibits electrophilic character. With such sulfur nucleophiles as benzenethiol and *α*-toluenethiol, the sulfenyl bromide **2** reacts to give mixed disulfides (**5** and **6**, respectively) and, with such arylamines as aniline or *o*-chloroaniline, the sulfur atom of **2** attacks the nucleophilic nitrogen atom to give sulfenamides (**7** and **8**, respectively). Addition of **2** to the double bond of cyclohexene gives *trans*-2-bromo-1-(tetra-*O*-acetyl-*β*-D-glucopyranosylthio)cyclohexane (**3**); the 3′,3′,6′,6′-tetradeuterated analog was prepared by use of cyclohexene-*3,3,6,6*-d_4. With *N,N*-dimethylaniline, the sulfenyl bromide **2** reacts by attack at the *para* position to give *p*-(dimethylamino)phenyl tetra-*O*-acetyl-1-thio-*β*-D-glucopyranoside (**1**), although a concurrent, major reaction-pathway gives bis(tetra-*O*-acetyl-*β*-D-glucopyranosyl) disulfide (**4**). With phenol and various enolizable ketones, the sulfenyl bromide **2** reacts to give the disulfide **4** in high yield. The structures assigned to the products were confirmed by n.m.r. spectroscopy. Such glycosylsulfenyl halides as **2** thus offer a potential route to a wide range of thio sugar derivatives by reactions with thiols, amines, and alkenes.

RESULTS AND DISCUSSION

The accompanying paper[1] describes reactions leading to the formation of tetra-*O*-acetyl-*β*-D-glucopyranosylsulfenyl bromide (**2**), including a convenient preparative method for obtaining **2** in quantities of 50–100 millimoles. At the time when the sulfenyl bromide **2** was first described[2], the suggestion was made that it might be useful in various types of synthetic reaction based on attack by an electrophilic, bivalent sulfur atom on a site of high electron-density. Experimental support for

---

*Preliminary report, C. V. HOLLAND, D. HORTON, MARTHA J. MILLER, AND W. N. TURNER, *Abstracts Papers Amer. Chem. Soc. Meeting*, 149 (1965) 1D; see also, preceding paper (ref. 1) and refs. 1–3.

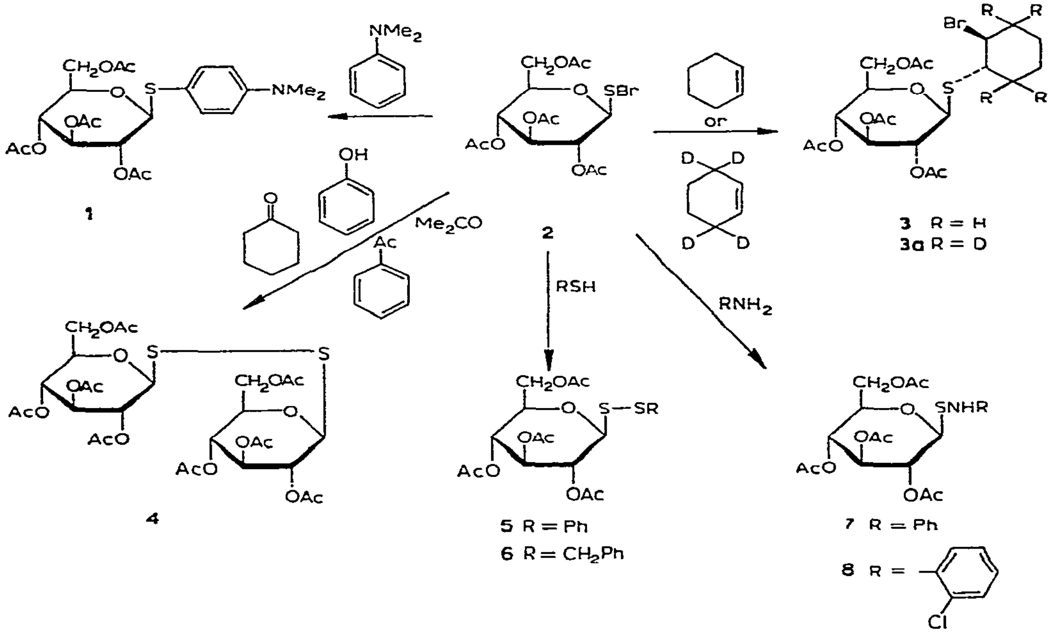
†Supported, in part, by the Agricultural Research Service, U.S. Department of Agriculture, Grants No. 12-14-100-7208(71) and 12-14-9201(71) (O.S.U.R.F. Projects 1827 and 2573) administered by the Northern Utilization Research and Development Division, Peoria, Illinois.

**To whom inquiries should be addressed.

‡Undergraduate Research Participant, 1964–1965.

these predictions has been the subject of a brief report[3]. The present paper records detailed studies of the reaction of the sulfenyl bromide **2** with various thiols, aromatic amines, an alkene, an aromatic system activated for electrophilic attack, various ketones, and phenol.

Reaction of **2** *with thiols.* — Treatment of the sulfenyl bromide **2** with an excess of benzenethiol in carbon tetrachloride for 1 h at room temperature gave the glucosyl phenyl disulfide **5** in high yield. Similar treatment of **2** with α-toluenethiol gave the benzyl analog (**6**), also in high yield. The structures of **5** and **6** are established by the elemental analyses, spectral data, and optical rotatory data recorded in the Experimental section. The n.m.r. spectra of **5** and **6** at 100 MHz in chloroform-*d* (see Tables I and II) showed the H-1, 2, 3, and 4 signals as multiplets perturbed by second-order effects, as anticipated[4] for this type of system.



The facility with which **2** reacts with thiols to give such disulfides as **5** and **6** makes the reaction a useful one for identifying the reactive sulfenyl halide **2** (and related analogs) by "trapping" it as a stable derivative[1,3].

Addition of **2** *to cyclohexene.* — The sulfenyl bromide **2** reacted readily with cyclohexene to give a high yield of a crystalline product whose elemental analysis showed it to be an adduct of the two reactants. A minor side-product was bis(tetra-*O*-acetyl-β-D-glucopyranosyl) disulfide (**4**). The adduct was formulated as *trans*-2-bromo-1-(tetra-*O*-acetyl-β-D-glucopyranosylthio)cyclohexane (**3**) by analogy with the established[5] *trans* mode of addition of simple sulfenyl halides to alkenes; further support for this configuration was provided by n.m.r. spectral data.

A (±) pair of enantiomorphic products will result from *trans*-addition of a non-dissymmetric sulfenyl halide to a cyclic alkene. *trans*-Addition of an optically active sulfenyl halide, such as **2**, to cyclohexene will generate a pair of products that are related as diastereoisomers. The relative proportions of the two diastereoisomers formed will depend on the magnitude of the asymmetric inductive effect[6] in the reaction. Strong asymmetric induction would cause one diastereoisomer to preponderate; if the effect is weak, the two diastereoisomers will be formed in almost equal amounts.

On recrystallization from ether–petroleum ether, the adduct **3** did not behave as a mixture separable by fractional recrystallization; this observation indicated that **3** was either a single diastereoisomer or a cocrystallized mixture of the two diastereoisomers as a molecular complex or solid solution. Consideration of molecular models afforded no evidence suggesting strong asymmetric induction in the addition of **2** to cyclohexene, and, since the yield of crystalline product was high (66%), the occurrence of a cocrystallized mixture of diastereoisomers was suspected. Strong supporting evidence for this supposition was provided by n.m.r. spectroscopy.

The 100-MHz n.m.r. spectrum of the adduct **3** in chloroform-*d* (see Tables I and II) shows the signals of H-1,2,3, and 4 of the sugar moiety as a multiplet (τ 4.67–5.38), the H-6 signals at τ 5.83, and the H-5 signal at τ 6.27; these assignments follow from detailed studies already reported[4] for acetylated 1-thio-β-D-glucopyranose derivatives. A broad, one-proton multiplet, with bands centered at τ 5.41 and 5.63, may be assigned to H-2′ of the cyclohexane moiety*; this is the spectral region where the proton of a C–CHBr–C system in a 6-membered ring is observed[7]. Another one-proton multiplet, at τ 6.68, is assigned to H-1′ by analogy with observations[8] on the proton of a C–CHSR–C group in a 6-membered ring-system. The signals of the methylene protons in the cyclohexane moiety are observed as a complex "envelope" at high field.

Detailed analysis of the H-1′ and H-2′ signals was not possible, because these protons show, in addition to vicinal ($J_{1',2'}$) coupling, further couplings to their adjacent methylene groups (at C-6′ and C-3′, respectively). To clarify the n.m.r. spectral interpretation, the adduct (**3a**) of **2** with cyclohexene-*3,3,6,6-d$_4$* was prepared. In this product, the signals of H-1′ and H-2′ were observed unperturbed by strong couplings to the vicinal (CD_2) groups at C-6′ and C-3′, respectively, because H–D couplings[9] are only 15.4% of the magnitude of the corresponding H–H couplings.

In chloroform-*d*, the H-2′ signal of **3a** was observed, not as one doublet (showing the $J_{1',2'}$ coupling), but as *two* doublets, at τ 5.43 (splitting, 5.0 Hz) and at τ 5.65 (splitting, 5.5 Hz), of approximately equal intensity. The splittings were confirmed to arise from spin-coupling ($J_{1',2'}$), because they were of the same magnitude in the 60-MHz spectrum. The separation of the doublets was 22 Hz in the 100-MHz spectrum, and 13 Hz in the 60-MHz spectrum; as these separations are proportional to the spectrometer frequency, the spacing between the two doublets is a true chemical-shift, and not the result of signal multiplicity[10]. In addition, the separation of the two

---

*Primed numbers refer to the cyclohexane moiety.

doublets was changed by changing the solvent; in acetone-d_6 at 100 MHz, the separation was 14 Hz.

The appearance of *two*, independent H-2′ signals provides strong indication that compound **3a** (and therefore **3**) is a 1:1 mixture of two closely related, molecular species, presumably the diastereoisomeric *trans*-adducts. Complementary evidence is provided by analysis of the H-1′ signal, and by spin-decoupling experiments on the H-1′ and H-2′ resonances. In chloroform-*d* at 100 MHz, the H-1′ signal of **3a** is observed as two overlapping doublets, at τ 6.69 (splitting 5.0 Hz) and τ 6.72 (splitting 5.5 Hz); the splittings are the same in the 60-MHz spectrum, and arise, therefore, from spin coupling ($J_{1',2'}$), whereas the separation of the doublets is a chemical-shift effect, because it is diminished from ~3 Hz (at 100 MHz) to ~2 Hz in the 60-MHz spectrum. In acetone-d_6 at 100 MHz, only one doublet (spacing, ~5.5 Hz) is observed for H-1′, indicating that, in this solvent, the chemical shift of H-1′ is the same for both species present in **3a**.

Spin-decoupling experiments (see Fig. 1) were performed at 100 MHz with solutions of **3a** in chloroform-*d* and also in acetone-d_6. In each case, irradiation of the H-1′ signals caused collapse of both H-2′ doublets into singlets. Irradiation of the low-field, H-2′ doublet did not perturb the higher-field, H-2′ doublet, but one of the H-1′ doublets collapsed to a singlet in the chloroform-*d* spectrum. As the H-1′ doublets coincided in acetone-d_6, the effect was observed by the appearance of a central singlet flanked by a doublet, to give an apparent triplet. Irradiation of the higher-field, H-2′ doublet did not perturb the low-field, H-2′ doublet, but collapse of the corresponding H-1′ doublet took place.

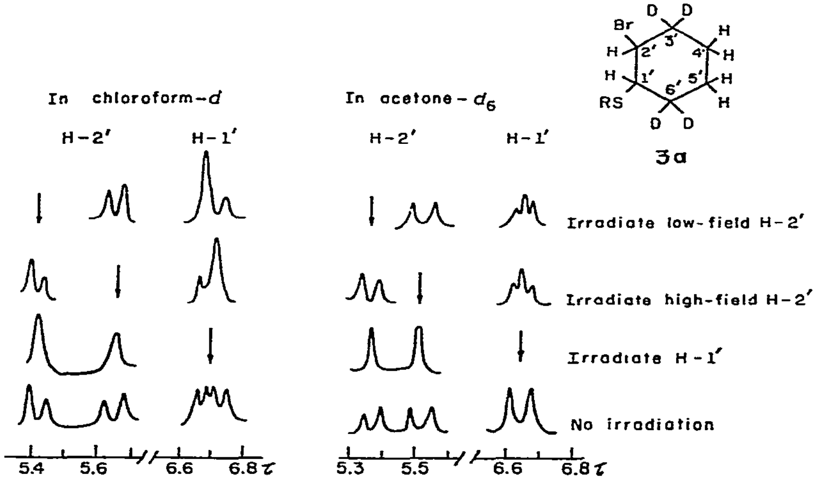


Fig. 1. Spin decoupling of the H-1′ and H-2′ signals in the 100-MHz, n.m.r. spectra of 2-bromo-(tetra-*O*-acetyl-β-D-glucopyranosylthio)cyclohexane-*3′,3′,6′,6′*-d_4 (3a) in chloroform-*d* and acetone-d_6.

The foregoing data establish that **3** (and **3a**) contain approximately equal amounts of two species that are differentiable by n.m.r. spectroscopy. The possibility that hindered rotation is involved is considered remote, because the H-1′ and H-2′ signals in the spectrum of **3** in hexachloroacetone[11] showed no indication of collapse as the temperature was raised from 20 to 105°.

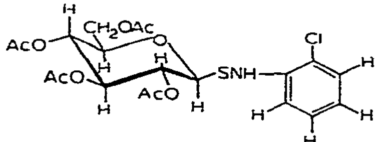
The observed $J_{1',2'}$ couplings (5.0 and 5.5 Hz) accord with formulation of the two forms of **3a** (and therefore **3**) as the diastereoisomeric *trans*-adducts. Had the adducts been *cis*, the $J_{1',2'}$ coupling would have been only 3–3.5 Hz, because of the *gauche* relationship of H-1′ and H-2′ (axial–equatorial or equatorial–axial) in either chair conformation of the cyclohexane ring. In the *trans*-adduct, the favored chair conformer of the cyclohexane moiety (equatorial S and Br) would have H-1′ and H-2′ antiparallel, giving a coupling of ~8 Hz, and this value would be diminished by any substantial contribution from the less-favored conformer, having H-1′ and H-2′ diequatorial ($J_{1',2'}$ 1-2 Hz), in rapid equilibrium[12] with the favored form.

Formulation of **3** as a diastereoisomeric mixture of *trans*-adducts has not been verified independently by chemical degradations.

Reaction of **2** *with amines.* — The sulfenyl bromide **2** reacted readily with aniline to give the crystalline sulfenamide **7** in 82% yield. *o*-Chloroaniline reacted similarly to give the corresponding sulfenamide (**8**). In each case, a trace of the disulfide **4** accompanied the sulfenamide. The elemental analyses of the products established that they had been formed by condensation of one molecule of the amine with one molecule of sulfenyl bromide, with the loss of a molecule of hydrogen bromide. This evidence does not prove the sulfenamide structure, because attack of **2** on the aryl ring-positions cannot be excluded. The n.m.r. spectra provided clear confirmation of the sulfenamide structure, because, in each compound, a one-proton singlet was observed (at τ 4.76 for **7** and at τ 4.24 for **8**, with chloroform-*d* as solvent) that could be assigned to the NH proton of a sulfenamide, because it was exchanged slowly when the sample was deuterated. Furthermore, in the region for aryl protons, compound **7** showed five protons, and **8** showed four protons, thus proving that substitution on the aryl nucleus had not taken place.

The n.m.r. spectra for **7** (see Fig. 2) and **8** were closely similar, and were analyzed completely for the ring protons on the sugar moiety by first-order inspection (see Tables I and II). The assignments were verified by spin decoupling. A striking feature of the spectra is the exceptionally high field-position of the H-1 signal, which is upfield of the H-2, H-3, and H-4 signals and is close to the H-6 signals. Because the H-1 signal is shifted away from the H-2, H-3, and H-4 signals, it is possible to analyze the latter signals readily. In most of the acetylated derivatives of 1-thio-β-D-glucopyranose, the proximity of the H-1 signal to those of H-2, H-3, and H-4 makes detailed spectral analysis difficult[4]. It has not been established whether the unusual shielding of H-1 in **7** and **8** is an inductive effect of the sulfenamide group, or whether it is the effect of the location of H-1 in the shielding region of the π-cloud above or below the aromatic ring.

Reaction of **2** *with an activated aryl derivative.* — The sulfenyl bromide **2** was allowed to react in a carbon tetrachloride medium with two molar equivalents of *N*,*N*-dimethylaniline. The latter was selected as an activated, aromatic molecule that readily undergoes substitution, principally at the *para* position, by electrophilic reagents. The major product isolated was, however, the disulfide[13] **4** (yield 62%), although the anticipated product, *p*-(dimethylamino)phenyl tetra-*O*-acetyl-1-thio-β-D-glucopyranoside (**1**), was formed simultaneously, in low yield. The structure of the product **1** was readily apparent from the data of elemental analysis and n.m.r. spectroscopy; the spectrum was amenable to first-order analysis of the carbohydrate portion, and the assignments (see Tables I and II) were confirmed by spin decoupling. Chemical confirmation of the structure of **1** was obtained by independent synthesis through coupling of tetra-*O*-acetyl-α-D-glucopyranosyl bromide with *p*-dimethylaminobenzenethiol, as first described by Montgomery, Richtmyer, and Hudson[14]. The procedure was improved by preparing the thiol from *p*-(dimethylamino)phenyl thiocyanate by reduction with lithium aluminum hydride instead of tin and acid.



8

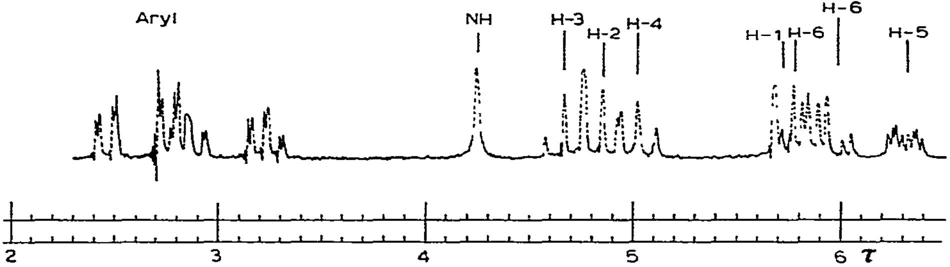
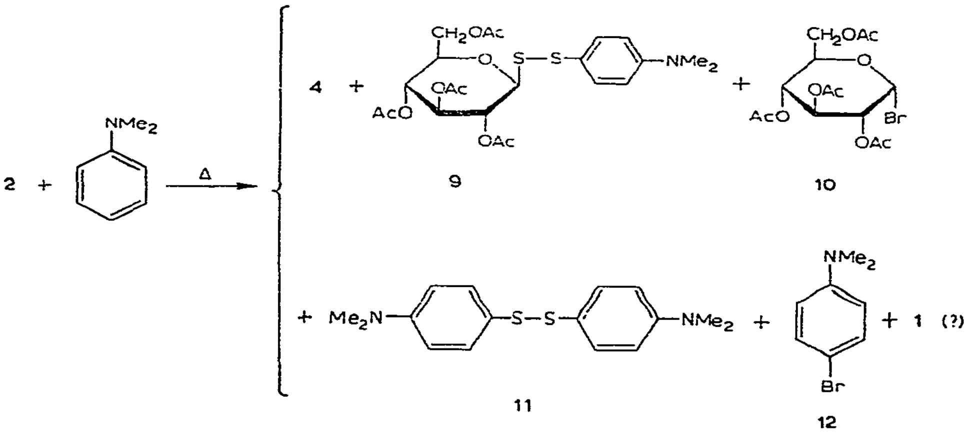


Fig. 2. The low-field portion of the 100-MHz, n.m.r. spectrum of 2-chloro-1-(tetra-*O*-acetyl-β-D-glucopyranosylsulfenamido)benzene (**8**) in chloroform-*d*.



A complex series of secondary reactions ensued if, after the sulfenyl bromide **2** had been treated with two molar proportions of *N*,*N*-dimethylaniline, the reaction

TABLE I

CHEMICAL-SHIFT DATA FOR 1-THIO-β-D-GLUCOPYRANOSE DERIVATIVES[a]

Compound	*Chemical shifts* (τ) *and signal multiplicities*[b]								*Aryl protons* (*integral*)	*Other protons*
	H-1	*H-2*	*H-3*	*H-4*	*H-5*	*H-6a*	*H-6b*	*OAc* (*integral*)		
1[c]	5.51d	5.13t	4.80t	5.02t	6.34m	5.75q	5.87q	7.89, 7.91, 7.99, 8.01	2.58, 2.67, 2.72, 3.32, 3.40(4)	7.00(6) (NMe_2)
3	4.67—5.38(4)				6.27m	5.83m		7.89, 7.99(6), 8.02	5.41m, 5.63m (H-2')[d] 6.68m (H-1')[d]	7.55—8.65(8) [$(CH_2)]_4$
3a	4.67—5.38(4)				6.27m	5.83m		7.90, 7.97, 7.98, 8.02	5.43d, 5.65d (H-2')[d], 6.69d, 6.72d (H-1')[d]	8.22—8.65(4) [$(CH_2)_2$]
3a[e]	4.65—5.21(4)				5.71—6.16			7.99, 8.03(6), 8.08	5.37d, 5.51d (H-2')[d], 6.64d (H-1')[d]	8.27—8.65(4) [$(CH_2)_2$]
5	4.63—5.00(3) and 5.29—5.50(1)				6.26o	5.81q	5.95q	7.97(6), 7.98, 8.00	2.33—2.47 and 2.61—2.80(5)	
6	4.58—4.99(3) and 5.42—5.58(1)				6.29o	5.70q	5.97q	7.92, 7.95, 7.96, 7.98	2.70(5)	5.97(2) (SCH_3)
7[c]	5.72d	4.85t	4.68t	5.02t	6.31o	5.73q	5.95q	7.86, 7.99(6), 8.08	2.74—3.27(5)	4.76(1) (NH)[f]
8[c]	5.72d	4.86t	4.68t	5.01t	6.31o	5.78q	5.98q	7.84, 7.98(6), 8.08	2.40—2.50, 2.70—2.96, 3.15—3.30(4)	4.24(1) (NH)[f]
9	4.69—4.98(3) and 5.32—5.43(1)				6.26m	5.72q	5.90q	7.92, 7.97, 8.00, 8.04	2.47, 2.56, 2.74, 3.38, 3.47(4)	7.04(6) (NMe_2)

[a] Data taken, unless otherwise stated, from 100-MHz spectra measured in chloroform-*d*. [b] Signals are singlets unless a range or multiplicity (d, doublet; m, multiplet; o, octet; q, quartet; t, triplet) is given. [c] Assignments verified by spin decoupling. [d] Protons on cyclohexyl moiety. [e] In acetone-d_6. [f] Signal disappears after deuteration for 3 days, or after shaking the solution with D_2O and a trace of tributylamine[22] for 1 min.

TABLE II

FIRST-ORDER COUPLING-CONSTANTS FOR 1-THIO-β-D-GLUCOPYRANOSE DERIVATIVES[a]

Compound	*Coupling constants in Hz*[b]						
	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6a}$	$J_{5,6b}$	$J_{6a,6b}$
1	9.5	9.5	8.8	9.3	3	4.5	13
3[c]	*d*	*d*	*d*	*d*	*d*	*d*	*d*
3a[c]	*d*	*d*	*d*	*d*	*d*	*d*	*d*
3a[c,e]	*d*	*d*	*d*	*d*	*d*	*d*	*d*
5	*d*	*d*	*d*	9	4.0	2	12
6	*d*	*d*	*d*	9	4.5	2	12
7	9.4	9.5	9.5	9.6	2.7	4.2	12.2
8	9.3	9.5	9.3	9.6	2.5	4.1	12.2
9	*d*	*d*	*d*	*d*	4.5	2.6	12.3

[a]The data refer to 100-MHz spectra and solutions in chloroform-*d*. [b]H-6a and H-6b are the protons on C-6 of the sugar moiety resonating at lower and higher field, respectively. [c]See Fig. 1 and Discussion for details of coupling of H-1′ and H-2′. [d]Not measured because of second-order effects. [e]In acetone-d_6.

mixture (presumably containing the product **1**; *N*,*N*-dimethylaniline hydrobromide; probably, the disulfide **4**; and, possibly, other products) was heated for 20 min at a relatively high temperature (105°). Fractionation of the reaction mixture gave, in addition to bis(tetra-*O*-acetyl-β-D-glucopyranosyl) disulfide (**4**), *p*-bromo-*N*,*N*-dimethylaniline (**12**), bis(*p*-dimethylaminophenyl) disulfide (**11**), tetra-*O*-acetyl-α-D-glucopyranosyl bromide (**10**), and a product closely resembling the adduct **1** in its n.m.r. spectral data, but containing an additional sulfur atom; it was formulated as *p*-(dimethylamino)phenyl tetra-*O*-acetyl-β-D-glucopyranosyl disulfide (**9**). These products probably arise by a series of metathetical reactions between primary reaction products, and free-radical bromination of the amine by **2** is a possible route to **12**. The factors controlling the distribution of these products were not investigated.

Reaction of **2** *with ketones and phenol.* — To determine whether the sulfenyl bromide **2** would react with enolizable ketones by attack of sulfur at the α-position to the carbonyl group, separate experiments were conducted with **2** and acetophenone, acetone, and cyclohexanone, respectively. In each case, the reaction gave bis(tetra-*O*-acetyl-β-D-glucopyranosyl) disulfide (**4**) in high yield. Possibly, free-radical bromination of the ketones provides a more favored reaction-pathway than electrophilic attack by sulfur on the enolic forms of the ketones. The disulfide **4** was also obtained in high yield in the reaction between the sulfenyl bromide **2** and phenol, and no product of attack on the aryl ring by sulfur was detected.

EXPERIMENTAL

General. — The general procedures used in this paper are the same as those given in the preceding paper[1].

*Phenyl tetra-*O*-acetyl-β-*D*-glucopyranosyl disulfide* (5). — To a suspension of tetra-*O*-acetyl-β-D-glucopyranosylsulfenyl bromide (2) that had been prepared[1] from tetra-*O*-acetyl-1-*S*-acetyl-1-thio-β-D-glucopyranose (2.08 g, 5.1 mmoles) in carbon tetrachloride (60 ml) was added benzenethiol (1 ml, 9.8 mmoles) in carbon tetrachloride (9 ml), and the mixture was kept, with occasional swirling, for 1 h at room temperature. The resultant, clear solution was washed successively with water (100 ml), aqueous sodium hydrogen carbonate (100 ml), and water, dried (magnesium sulfate), and evaporated to give a crystalline residue. Recrystallization from ethanol gave **5** as needles; yield 1.85 g (63%), m.p. 123–124°, $[\alpha]_D^{15}$ −241 ±1° (*c* 1.9, chloroform); R_F 0.87; λ_{max}^{KBr} 5.72 (OAc), 6.32, 6.77, 6.96 (aryl), and 9.12 μm (aryl C–S); λ_{max}^{EtOH} 287 (ε 3,300) (shoulder), 274 (3,800), 236 (10,300), and 207 nm (12,300); n.m.r. data, see Tables I and II; X-ray powder diffraction data: 13.91 vw, 10.91 vs (1), 9.46 w, 8.93 w, 6.93 vw, 7.14 vw, 6.37 vw, 5.87 vw, 5.50 s (3,3), 5.26 s (2), 4.98 m, 4.78 s (3,3), 4.43 w, 4.20 m, 3.95 m, 3.72 m, and 3.62 m. The product was homogeneous by t.l.c.

Anal. Calc. for $C_{20}H_{24}O_9S_2$: C, 50.83; H, 5.12; S, 13.57. Found: C, 50.53; H, 5.12; S, 13.40.

The mother liquors contained a major and a minor component, having the chromatographic characteristics of **5** and the disulfide **4**, respectively.

*Benzyl tetra-*O*-acetyl-β-*D*-glucopyranosyl disulfide* (6). — The procedure of the foregoing experiment was used, but with α-toluenethiol (1 ml, 8.4 mmoles) instead of benzenethiol. The crude product, obtained as an oil, crystallized spontaneously. Recrystallization from ethanol–petroleum ether gave **6** as white, fluffy needles, yield 1.68 g (68%), m.p. 117–118° (a form having m.p. 94–95° was also encountered[3]), $[\alpha]_D^{21}$ −185 ±1° (*c* 2,3, chloroform); R_F 0.88; λ_{max}^{KBr} 5.72 (OAc), 6.71, and 6.90 μm (aryl); λ_{max}^{EtOH} 271 (ε 2,200), 220 (11,000) (shoulder), and 208 nm (14,000); n.m.r. data, see Tables I and II; X-ray powder diffraction data: 12.66 m, 11.26 m, 10.25 w, 9.28 s (2), 7.34 w, 6.59 vvw, 5.48 vs (1,1), 4.96 vs (1,1), and 4.68 vs (1,1).

Anal. Calc. for $C_{21}H_{26}O_9S_2$: C, 51.84; H, 5.39; S, 13.18. Found: C, 51.99; H, 5.29; S, 13.28.

T.l.c. of the mother liquors revealed a major component having the same mobility as **6**, together with a trace of a product having the chromatographic characteristics of the disulfide **4**.

trans-*2-Bromo-1-(tetra-*O*-acetyl-β-*D*-glucopyranosylthio)cyclohexane* (3). — A suspension of the sulfenyl bromide **2** (22.27 g, 50.2 mmoles, prepared[1] from 20.00 g of tetra-*O*-acetyl-1-*S*-acetyl-1-thio-β-D-glucopyranose) in carbon tetrachloride (200 ml) was shaken for 1 h at room temperature with a solution of cyclohexene (5.1 ml, 50.3 mmoles) in carbon tetrachloride (10 ml). The resulting, clear solution was washed successively with water (100 ml), saturated aqueous sodium hydrogen carbonate (100 ml), and water, dried (magnesium sulfate), and evaporated to a syrup. The syrup was dissolved in the minimal volume of ether, and petroleum ether was added to incipient turbidity. The product **3** had a tendency to separate as an oil, but, by careful manipulation, it could be obtained as colorless, stout needles; yield (in 5 crops) 15.0 g (57%, raised to 66% by chromatographic isolation of further **3** from the mother liquors), m.p. 86–87° (87–88° after one recrystallization), $[\alpha]_D^{22}$ $-22.0 \pm 1.0°$ (*c* 2.8, chloroform); R_F 0.88 (chromatographically homogeneous); λ_{max}^{KBr} (Perkin–Elmer Model 337 spectrometer) 5.70, 5.76 (OAc), 11.17 (axial H at C-1), 13.58, and 13.84 μm (equatorial[15] C–Br); λ_{max}^{EtOH} 274 (ε 70) (shoulder), 242 (290) (shoulder), 224 (1025) (shoulder), and 207 nm (1300); n.m.r. data, see Tables I and II; X-ray powder diffraction data: 10.19 m, 7.01 m, 6.22 m, 5.29 vs (1), 5.00 s (3), 4.72 vw, 4.45 vw, 4.22 s (2,2), 4.05 vw, 3.90 w, 3.75 vw, 3.56 vw, 3.44 s (2,2), 3.24 w, 3.10 w, 3.01 vw, and 2.86 w.

Anal. Calc. for $C_{20}H_{29}BrO_9S$: C, 45.72; H, 5.56; Br, 15.21; S, 6.10. Found: C, 45.88; H, 5.26; Br, 14.99; S, 6.36.

The mother liquors were combined and evaporated. T.l.c. of the residue indicated the presence of two components, having the characteristics of **3** and the disulfide **4**. Column-chromatographic fractionation gave **3** as the faster-moving component, obtained crystalline from ether–petroleum ether, yield 2.018 g (9%), m.p. 82–85°. The slower-moving component was crystallized from ether–petroleum ether to give bis(tetra-*O*-acetyl-β-D-glucopyranosyl) disulfide (**4**); yield 303 mg (1.7%), m.p. (after one recrystallization) 139–140°, identical with an authentic sample by mixed m.p., elemental analysis, and comparative i.r. spectra.

When the experiment was conducted on a 2-gram scale, the yield of **3** isolated by direct crystallization was 73%.

trans-*2-Bromo-1-(tetra-*O*-acetyl-β-D-glucopyranosylthio)cyclohexane*-3,3,6,6-d_4 (**3a**). — This compound was prepared by the route used for **3**, but with 489 mg of the sulfenyl bromide **2** and 96 mg of cyclohexene-*3,3,6,6-d_4* (Ref. 16), and the deuterated derivative **3a** was obtained, m.p. and mixed m.p. with **3**, 85–86°; n.m.r. data: see Tables I and II and Fig. 1.

Tetra-O-acetyl-β-D-glucopyranosylsulfenamidobenzene (**7**). — To a suspension of the sulfenyl bromide (3.24 g, 7.3 mmoles) in carbon tetrachloride (40 ml) was added aniline (1.4 ml, 15.4 mmoles), and the mixture was kept for 1 h at room temperature. Water (100 ml) was added to the cloudy, yellow, reaction mixture, and the mixture was shaken. The organic layer was separated, and treated by the procedure used in the preparation of compound **5**. The resultant syrup was dissolved in ether (50 ml), and the solution was decolorized with carbon, and concentrated, and petroleum ether was added to opalescence. Refrigeration of the solution gave **7** as colorless, fine needles; yield 2.76 g (83%), m.p. (after one recrystallization) 116–117°, $[\alpha]_D^{21}$ $-330 \pm 2°$ (*c* 2.4, chloroform); R_F 0.85 (homogeneous); λ_{max}^{KBr} 5.72 (OAc), 6.23, and 6.70 μm (aryl); λ_{max}^{EtOH} 288 (ε 2,000) (shoulder), 244 (10,300), and 206 nm (13,900); n.m.r. data, see Tables I and II; X-ray powder diffraction data: 12.53 vvw, 10.13 vs (1), 7.16 w, 6.40 w, 5.69 w, 5.31 s (2), 5.14 m, 4.49 m, 4.27 m, 3.96 w, 3.67 m, and 3.52 m.

Anal. Calc. for $C_{20}H_{25}NO_9S$: C, 52.73; H, 5.53; N, 3.08; S, 7.04. Found: C, 52.78; H, 5.45; N, 3.28; S, 6.85.

T.l.c. of the mother liquors indicated the presence of **7** and of a minor component having the mobility of the disulfide **4**.

2-Chloro-1-(tetra-O-acetyl-β-D-glucopyranosylsulfenamido)benzene (**8**). — To a suspension of **2** (5.11 g, 11.5 mmoles) in carbon tetrachloride (70 ml) was added *o*-chloroaniline (3 ml, 28.4 mmoles) in carbon tetrachloride (7 ml). A white solid separated at once. After 1 h at room temperature, the suspension was filtered to remove the insoluble amine salt, and the filtrate was treated by the procedure used for compound **5**. The resultant syrup was kept for 12 h at 30° under vacuum to remove traces of solvent, and was then dissolved in anhydrous ether (10 ml). Petroleum ether was added to opalescence, a crystal nucleus was added, and the solution was refrigerated for 18 h to give **8** as colorless needles; yield 2.36 g (42%), m.p. (after one recrystallization) 84–85°, $[\alpha]_D^{21}$ $-311 \pm 2°$ (*c* 3.9, chloroform); R_F 0.87; λ_{max}^{KBr} 5.72 (OAc), 6.25, 6.80 (aryl), and 7.34 μm (secondary arylamine); λ_{max}^{EtOH} 295 (ε 5,000) (shoulder), 276 (8,000) (shoulder), 244 (14,000), and 210 nm (25,000); n.m.r. data, see Fig. 2 and Tables I and II; X-ray powder diffraction data: 12.58 w, 9.99 m, 8.15 m, 6.63 m, 6.28 m, 5.91 w, 5.51 vw, 5.28 w, 5.00 vw, 4.77 vs (1,1), 4.29 vs (1,1), 4.01 s (2), 3.70 m, 3.35 w, and 3.16 m.

Anal. Calc. for $C_{20}H_{24}ClNO_9S$: C, 49.03; H, 4.94; Cl, 7.24; N, 2.86; S, 6.55. Found: C, 48.91; H, 4.94; Cl, 7.34; N, 3.10, S, 6.66.

T.l.c. of the mother liquors revealed a major component corresponding to **8**, and a trace of a product chromatographically indistinguishable from the disulfide **4**.

p-(*Dimethylamino*)*phenyl tetra*-O-*acetyl-1-thio-β*-D-*glucopyranoside* (**1**). — *A. From sulfenyl bromide* **2** *and* N,N-*dimethylaniline*. To a suspension of the sulfenyl bromide **2** (2.972 g, 6.7 mmoles) in carbon tetrachloride (50 ml) was added *N*,*N*-dimethylaniline (2 ml, 15.8 mmoles); the mixture turned green. After 1 h at room temperature, the mixture was evaporated (bath temperature <43°), and the resultant syrup was dissolved in dichloromethane (100 ml). The solution was treated by the procedure used in the preparation of compound **5**. The resultant, green syrup was dissolved in ether, and petroleum ether was added to opalescence. Several crops of crystals were collected; the first three (total yield 1.522 g, 62%, m.p. 126–127°) were recrystallized to give pure bis(tetra-*O*-acetyl-β-D-glucopyranosyl) disulfide **4** (1.348 g), m.p. 141–142°, indistinguishable from an authentic sample[1] by t.l.c., i.r. spectra, and X-ray powder diffraction pattern. The fourth and fifth crystal fractions, yield 206 mg, were shown by t.l.c. to contain **4** as a minor component, together with a principal component (**1**) having R_F 0.81. Recrystallization (twice) from ethanol gave **1** as colorless needles, m.p. 150°, $[\alpha]_D^{20}$ $-54.7 \pm 2°$ (*c* 0.9, chloroform) (lit.[14] m.p. 150–151°, $[\alpha]_D^{20}$ $-47°$ in chloroform); R_F 0.81; λ_{max}^{KBr} 5.72 (OAc), 6.23 and 6.68 μm (aryl); λ_{max}^{EtOH} 276 (ε 12,500) and 208 nm (16,900); n.m.r. data, see Tables I and II; X-ray powder diffraction data: 15.63 vw, 12.71 vw, 10.58 vs (1,1), 8.48 vs (1,1), 6.96 m, 5.60 m, 5.27 m, 5.00 m, 4.74 w, 4.51 w, 4.39 s (2,2), 4.23 s (2,2), and 4.04 w.

Anal. Calc. for $C_{22}H_{29}NO_9S$: C, 54.64; H, 6.05; N, 2.90; S, 6.63. Found: C, 54.87; H, 6.11; N, 3.21; S, 6.96.

B. From tetra-O-*acetyl-α*-D-*glucopyranosyl bromide and* p-*dimethylaminobenzenethiol*. The procedure of Montgomery, Richtmyer, and Hudson[14] was modified. *p*-Dimethylaminophenyl thiocyanate[17] (18 g, 101 mmoles) in dry ether (200 ml) was added with stirring to a suspension of lithium aluminium hydride (4.0 g, 105 mmoles) in dry ether (200 ml) that was maintained at 0°, and stirring was continued for 45 min. The mixture was processed by the general procedure[18] used for the reduction of nitriles with lithium aluminum hydride. The resultant, ethereal solution was decanted from the gray-white residue, and evaporated to give *p*-dimethylaminobenzenethiol (9.0 g, 59 mmoles). The latter was not further purified, because of the ease with which it undergoes oxidative dimerization, and it was condensed immediately with tetra-*O*-acetyl-α-D-glucopyranosyl bromide (20 g 48.6 mmoles) by the general method of Purves[19]. Evaporation of the yellow, turbid reaction-mixture gave a yellow syrup, which was partitioned between dichloromethane (100 ml) and water (100 ml). The turbid mixture was filtered, and the organic phase was separated, dried (magnesium sulfate), and evaporated to give a yellow syrup. Crystallization of the syrup from ether–petroleum ether gave **1**; yield 5.2 g (22%), m.p. (after recrystallization) 149–150°, identical by mixed m.p., i.r. spectrum, and X-ray powder diffraction pattern with **1** prepared by method *A*.

Reaction of sulfenyl bromide **2** *with* N,N-*dimethylaniline to give* p-*(dimethylamino)phenyl tetra-*O*-acetyl-β-*D*-glucopyranosyl disulfide* (**9**). — To a suspension of **2** (3.086 g, 7.0 mmoles) in carbon tetrachloride (50 ml) was added *N,N*-dimethylaniline (2 ml, 15.8 mmoles), and the mixture was kept for 0.5 h at 30°. The resultant solution was evaporated to a syrup at ~90°, the syrup was dissolved in dichloromethane (100 ml), and the solution was processed as described for compound **5**. The syrup obtained showed major components having R_F 0.88 and 0.51 (t.l.c.). The mixture was subjected to column chromatography. The first fractions eluted from the column were evaporated to a syrup that was distilled at 180° (bath)/0.15 torr to give *p*-bromo-*N,N*-dimethylaniline (**12**); yield 0.168 g, m.p. 52–54° (lit.[20] m.p. 54.7°), R_F 0.99 (detected very slowly by sulfuric acid).

Anal. Calc. for $C_8H_{10}BrN$: C, 48.02; H, 4.89; Br, 39.82; N, 7.00. Found: C, 47.85; H, 4.89; Br, 39.94; N, 7.09.

The product was identical with an authentic sample of **12** by mixed m.p. and by i.r. and n.m.r. spectra.

Continued elution of the column gave a chromatographically homogeneous component, R_F 0.88, that crystallized from ethanol to give the disulfide **9** as pale-yellow needles; yield 307 mg (8.5%), m.p. (after one recrystallization) 156–157°, $[\alpha]_D^{22}$ $-324 \pm 3°$ (*c* 1.6, chloroform); R_F 0.9; λ_{max}^{KBr} 5.72 (OAc), 6.25, and 6.65 μm (aryl); λ_{max}^{EtOH} 311 (ε 11,500), 268 (8,800), and 208 nm (12,700); n.m.r. data, see Tables I and II; X-ray powder diffraction data: 16.50 vvw, 15.22 vvw, 10.84 vs (1), 8.48 s (3,3) 7.76 w, 6.76 vvw, 6.05 w, 5.74 vvw, 5.40 m, 5.09 m, 4.80 s (2), 4.41 w, 4.22 w, 4.06 s, (3,3), 3.78 s (3,3), and 3.68 m.

Anal. Calc. for $C_{22}H_{29}NO_9S_2$: C, 51.25; H, 5.67; N, 2.72; S, 12.44. Found: C, 51.06; H, 5.78; N, 2.97; S, 12.13.

Continued elution of the column gave, first, a mixture of components having R_F 0.88 and 0.50, and then the component having R_F 0.50, alone; from the latter fractions, the crystalline disulfide **4** was obtained, yield 1.11 g (44%), m.p. (after recrystallization from ethanol) 141–142°, identical with authentic **4** by mixed m.p., i.r. and n.m.r. spectra, and X-ray powder diffraction pattern.

A second experiment was performed under similar conditions, except that 5.0 g (11.3 mmoles) of **2** and 5 ml (39.4 mmoles) of *N*,*N*-dimethylaniline was used, and the initial syrupy product was heated for ~20 min at 105°/0.2 torr. Column-chromatographic fractionation of the product gave, in the early fractions, bis(*p*-dimethylaminophenyl) disulfide (**11**), isolated crystalline from ether; yield 566 mg, m.p. 117.5–118.5° (lit.[21] m.p. 118°).

Anal. Calc. for $C_{16}H_{20}N_2S_2$: C, 63.11; H, 6.62; N, 9.20; S, 21.06. Found: C, 63.18; H, 6.56; N, 9.20; S, 20.98.

Further elution of the column gave fractions that contained tetra-*O*-acetyl-α-D-glucopyranosyl bromide (**10**), yield 2.155 g (46%), identical with an authentic sample by i.r. and n.m.r. spectra.

The next compound to be eluted from the column was the disulfide **9**, yield 324 mg (6%), identical with the product obtained in the preparation already described. Subsequent fractions yielded the disulfide **4**, yield 446 mg (5%), m.p. 141–142°.

In a third experiment, the reaction between **2** (1.17 g) and 2 equivalents of *N*,*N*-dimethylaniline was allowed to proceed for 60 h at 30°, and the product was isolated without heating it above 40°. Fractionation gave the monosulfide **1** (188 mg, 15%), and the disulfide **4** (579 mg, 60%).

Reaction of sulfenyl bromide **2** *with* (*a*) *acetophenone*, (*b*) *acetone*, (*c*) *cyclohexanone*, *and* (*d*) *phenol*. — Suspensions of the sulfenyl bromide **2** in carbon tetrachloride were treated, in separate experiments, with the following ketones or enols, in the molar proportions indicated (relative to 0.1 mole of **2**): acetophenone (1.0 mole) acetone (4.4 moles), cyclohexanone (1.0 mole), and phenol (1.1 moles). The reaction mixtures were kept for 1–3 h at room temperature, and then processed by the general procedures used for isolating coupling products formed from **2**. In each of the four experiments, the principal product, isolated crystalline in 70%, 63%, 76%, and 75% yield, respectively, was bis(tetra-*O*-acetyl-β-D-glucopyranosyl) disulfide (**4**), m.p. 142–143°, identical with an authentic sample by mixed m.p., i.r. spectrum, and X-ray powder diffraction data.

ACKNOWLEDGMENTS

The authors thank Dr. Saul Wolfe of Queen's University (Kingston, Ontario) for a sample of cyclohexene-*3,3,6,6-d*$_4$, and Dr. L. D. Hall (University of British Columbia) and J. H. Lauterbach of this laboratory for 100-MHz, n.m.r. spectral measurements.

REFERENCES

1 R. H. BELL AND D. HORTON, *Carbohyd. Res.*, 9 (1969) 187.
2 D. HORTON, M. L. WOLFROM, AND H. G. GARG, *J. Org. Chem.*, 28 (1963) 2992.
3 D. HORTON AND MARTHA J. MILLER, *Carbohyd. Res.*, 1 (1965) 335.

4 C. V. Holland, D. Horton, Martha J. Miller, and N. S. Bhacca, *J. Org. Chem.*, 32 (1967) 3077.

5 N. Kharasch and A. J. Havlik, *J. Amer. Chem. Soc.*, 75 (1953) 3734; N. R. Slobodkin and N. Kharasch, *ibid.*, 82 (1960) 5837; W. H. Mueller and P. E. Butler, *ibid.*, 88 (1966) 2866; G. H. Schmid and V. M. Csizmadia, *Can. J. Chem.*, 44 (1966) 1338.

6 E. L. Eliel, *Stereochemistry of Carbon Compounds*, McGraw–Hill Book Co., New York, 1962, pp. 68–83.

7 Ester L. Albano, D. Horton, and J. H. Lauterbach, *Chem. Commun.*, (1968) 357.

8 A. E. El Ashmawy, D. Horton, Ligaya G. Magbanua, and J. M. J. Tronchet, *Carbohyd. Res.*, 6 (1968) 299.

9 R. H. Bible, *Interpretation of NMR Spectra*, Plenum Press, New York, 1965, p. 105.

10 J. A. Pople, W. G. Schneider, and H. J. Bernstein, *High-resolution Nuclear Magnetic Resonance*, McGraw–Hill Book Co., New York, 1959, p. 97.

11 D. Horton and H. S. Prihar, *Carbohyd. Res.*, 4 (1967) 115.

12 N. S. Bhacca and D. Horton, *J. Amer. Chem. Soc.*, 89 (1967) 5993.

13 N. K. Richtmyer, C. J. Carr, and C. S. Hudson, *J. Amer. Chem. Soc.*, 65 (1943) 1477.

14 E. M. Montgomery, N. K. Richtmyer, and C. S. Hudson, *J. Org. Chem.*, 11 (1946) 301.

15 K. Kozima, K. Sakashita, and S. Maeda, *J. Amer. Chem. Soc.*, 76 (1954) 1965; L. J. Bellamy, *The Infra-red Spectra of Complex Molecules*, Methuen, London, 1958, pp. 331–332.

16 S. Wolfe and P. G. C. Campbell, *Can. J. Chem.*, 43 (1965) 1184.

17 R. Q. Brewster and W. Schroeder, *Org. Syn. Coll. Vol.*, 2 (1943) 574.

18 L. H. Amundsen and L. S. Nelson, *J. Amer. Chem. Soc.*, 73 (1951) 242.

19 C. B. Purves, *J. Amer. Chem. Soc.*, 51 (1929) 3619.

20 A. Weber, *Ber.*, 8 (1875) 714; R. J. B. Marsden and L. E. Sutton, *J. Chem. Soc.*, (1936) 599.

21 V. Merz and W. Weith, *Ber.*, 19 (1886) 1570.

22 A. E. El Ashmawy and D. Horton, *Carbohyd. Res.*, 3 (1966) 191.

Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

OXAZOLINE SYNTHESIS OF 1,2-*trans*-2-ACETAMIDO-2-DEOXYGLYCOSIDES

S. E. ZURABYAN, T. P. VOLOSYUK, AND A. J. KHORLIN
Institute for Chemistry of Natural Products, U.S.S.R. Academy of Sciences, Moscow (U.S.S.R.)

(Received June 14th, 1968; in revised form, July 27th, 1968)

ABSTRACT

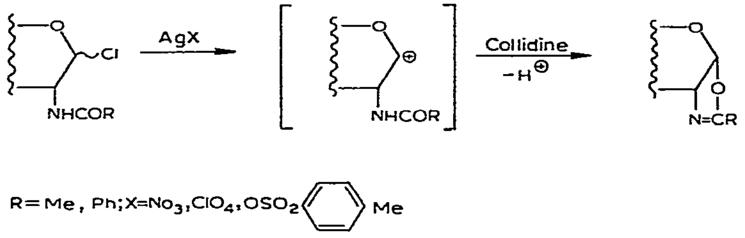
The glycosylating activity of 2-methyl-glyco[1′,2′:4,5]-2-oxazolines has been investigated in connection with the synthesis of 1,2-*trans*-2-acetamido-2-deoxyglycosides. Treatment of 2-methyl-4,5-(3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyrano)-2-oxazoline (**1**) and 2-methyl-4,5-[4-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-3,6-di-*O*-acetyl-2-deoxy-α-D-glucopyrano]-2-oxazoline (**2**) with *p*-nitrophenyl 2-acetamido-3,4-di-*O*-acetyl- (**3**) and *p*-nitrophenyl 2-acetamido-3-*O*-acetyl-2-deoxy-β-D-glucopyranoside (**4**) in the presence of catalytic amounts of toluene-*p*-sulphonic acid gives *p*-nitrophenyl 2-acetamido-6-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-3,4-di-*O*-acetyl-2-deoxy-β-D-glucopyranoside (**7**), *p*-nitrophenyl 2-acetamido-6-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-3-*O*-acetyl-2-deoxy-β-D-glucopyranoside (**9**), and *p*-nitrophenyl *O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→4)-*O*-(2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→6)-2-acetamido-3,4-di-*O*-acetyl-2-deoxy-β-D-glucopyranoside (**11**) in yields of up to 50%.

INTRODUCTION

Amongst the methods for the synthesis of 1,2-*trans*-2-amino-2-deoxyglycosides[1-13], that based upon the reaction of 2-substituted glyco[1′,2′:4,5]-2-oxazolines with an aglycon is distinguished by its simplicity and efficiency[10-13]. The oxazoline method, however, has been confined to the synthesis of 1,2-*trans*-2-benzamido-2-deoxyglucosides, because the only readily available reagents were 2-phenyl-gluco[1′,2′:4,5]-2-oxazolines[10-15]. Although 2-methyl-4,5-(3,4,6-tri-*O*-acetyl-2-deoxy-α-D-galactopyrano)- and 2-methyl-4,5-(3,4,6-tri-*O*-acetyl-2-deoxy-β-D-mannopyrano)-2-oxazolines have been described recently as by-products of the acetylation of 2-acetamido-2-deoxy-D-galactose and the D-*manno* analogue[16], such reagents are still very inaccessible.

A general route for the synthesis of 2-substituted glyco[1′,2′:4,5]-2-oxazolines (including the 2-methyl derivatives) is shown on the following page.

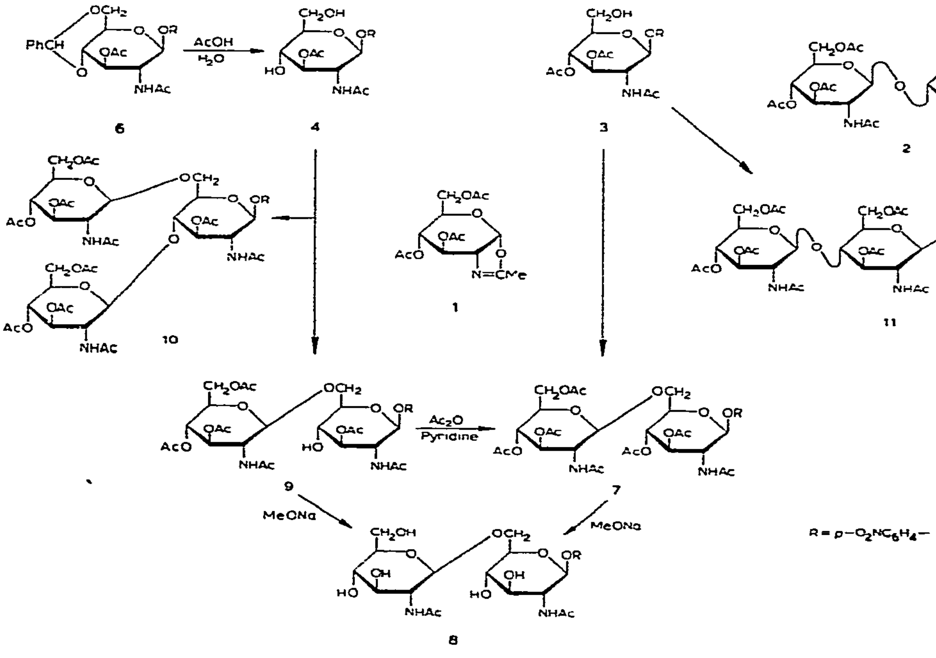
This paper describes glycosylation by 2-methyl-glyco[1′,2′:4,5]-2-oxazoline in the synthesis of 1,2-*trans*-2-acetamido-2-deoxy-D-glycosides. These compounds are of interest, since, in all known cases, the 2-amino-2-deoxy sugar residues of naturally occurring carbohydrates are *N*-acetylated, and because 2-acetamido-2-deoxy-D-glycosides are analogues of glycosidase (*i.e.*, lysozyme) substrates.



R = Me, Ph; X = NO_3, ClO_4, $OSO_2C_6H_4Me$

RESULTS AND DISCUSSION

The glycosylating capability of 2-methyl-4,5-(3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyrano)-2-oxazoline (**1**) and 2-methyl-4,5-[4-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-3,6-di-*O*-acetyl-2-deoxy-α-D-glucopyrano]-2-oxazoline (**2**) was investigated by examination of their reactions with *p*-nitrophenyl 2-acetamido-3,4-di-*O*-acetyl-2-deoxy-[6] (**3**) and *p*-nitrophenyl 2-acetamido-3-*O*-acetyl-2-deoxy-β-D-glucopyranosides (**4**). Glycoside **4** was obtained by treatment of *p*-nitrophenyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranoside[18] (**5**) with acetic anhydride in pyridine, followed by removal of the benzylidene group from the resulting 3-acetate **6** by treatment with 60% aqueous acetic acid for 30 min at 100°, which also caused some glycoside hydrolysis.



On heating a solution of the oxazoline **1** in dry toluene–nitromethane with the alcohol **3** in the presence of a catalytic amount of toluene-*p*-sulphonic acid, *p*-nitrophenyl 2-acetamido-6-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-3,4-di-*O*-acetyl-2-deoxy-β-D-glucopyranoside (**7**) was obtained in 49% yield; the Koenigs–Knorr reaction gave[6] a 2.4% yield of compound **7**. The β-D-configuration of the (1→6)-glycosidic bond in compound **7** was established from the n.m.r. spectrum (H-1 doublet at δ 5.40 p.p.m., $J_{1,2}$ 9.0 Hz), and also by comparison of the observed and calculated molecular rotation values. Saponification (Zemplén) of the acetate **7** yielded the glycoside **8**.

Glycosylation of the diol **4** with the oxazoline **1** under the above conditions gave 46% of *p*-nitrophenyl 2-acetamido-6-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-3-*O*-acetyl-2-deoxy-β-D-glucopyranoside (**9**). The structure of compound **9** was proved by its conversion into acetate **7**, and by *O*-deacetylation to give glycoside **8**. Paper chromatography of the reaction mixture indicated also the formation of the branched trisaccharide, *p*-nitrophenyl 2-acetamido-4,6-di-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-3-*O*-acetyl-2-deoxy-β-D-glucopyranoside (**10**). The synthesis of disaccharide **9** shows that 2-methyl-gluco[1′,2′:4,5]-2-oxazolines can be used for selective glycosylation of a primary hydroxyl group, thus providing a means for obtaining starting materials for the synthesis of hetero-oligosaccharide derivatives containing amino-sugar residues.

Under the above conditions, the glycosylating activity of the oxazoline **2** is similar to that of the analogue **1**. Condensation of oxazoline **2** with the partially protected glycoside **3** gave *p*-nitrophenyl *O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→4)-*O*-(2-acetamido-3,6-di-*O*-acetyl-β-D-glucopyranosyl)-(1→6)-2-acetamido-3,4-di-*O*-acetyl-2-deoxy-β-D-glucopyranoside (**11**) in 30% yield. The acetate **11** was saponified by Zemplén's procedure to give the trisaccharide glycoside **12**. The β-D-configuration of the (1→6)-linkage in the trisaccharide derivatives **11** and **12** was indicated by comparison of the observed and calculated molecular rotations.

Thus, it appears that 2-methyl-glyco[1′,2′:4,5]-2-oxazolines may serve generally as convenient starting materials for the synthesis of 1,2-*trans*-2-acetamido-2-deoxyglycosides. Moreover, the synthesis of the trisaccharide derivative **12** reveals an advantage of the oxazoline method, in that an oligosaccharide chain may be lengthened by two 2-acetamido-2-deoxy-D-glycopyranose residues in a single step. The formation of trisaccharide derivative **10** indicates that 2-methyl-glyco[1′,2′:4,5]-2-oxazolines are able to glycosylate secondary hydroxyl groups, although more drastic conditions are required for these reactions. The method is being studied further.

EXPERIMENTAL

Melting points were measured on a Kofler apparatus and are corrected. Optical rotations were determined with a Hilger M-142 polarimeter. Silica gel KSK (100–

150 mesh for columns and 150 mesh with 5% gypsum for t.l.c.) was used for chromatography. The ratio of weight of adsorbent: weight of substance was 50–100: 1. Detection was effected with ammonia vapour or conc. sulphuric acid. Paper chromatography was performed on Filtrak Niederschlag FN3 paper with chloroform–methanol (9:1, *A*; and 19:1, *B*) and butyl alcohol–ethanol–watei (5:2:5, *C*). Evaporations were performed at 35–40° *in vacuo*. N.m.r. spectra were obtained with a JEOL JNM-4H-100 spectrometer with tetramethylsilane as internal reference and methyl sulphoxide as solvent.

p-*Nitrophenyl 2-acetamido-3-*O*-acetyl-4,6-*O*-benzylidene-2-deoxy-β-*D*-glucopyranoside* (**6**). — Compound **5** {2.36 g, m.p. 241.5–242° (decomp.), $[\alpha]_D^{20}$ −32° (pyridine)}[18] was acetylated with pyridine (45 ml) and acetic anhydride (21 ml) in the usual way to give the title compound (2.15 g, 83%), m.p. 274–276° (decomp., from *N,N*-dimethylformamide–methanol), $[\alpha]_D^{20}$ −2° (*c* 1.2, *N,N*-dimethylformamide), R_F 0.67 (t.l.c., solvent *A*) (Found: C, 58.2; H, 5.3: N, 5.9. $C_{23}H_{24}N_2O_9$ calc.: C, 58.5; H, 5.3; N, 5.9%).

p-*Nitrophenyl 2-acetamido-3-*O*-acetyl-2-deoxy-β-*D*-glucopyranoside* (**4**). — A suspension of compound **6** (6 g) in 60% aqueous acetic acid (180 ml) was stirred for 30 min at 100°. The solution was then evaporated, benzaldehyde was removed from the residue by codistillation with water, and traces of acetic acid were removed by codistillation with toluene. Compound **4** was isolated by elution of the residue (4.9 g) from silica gel, using chloroform–methanol with an increase in the methanol content from 5–20%. The fractions were monitored by t.l.c. (solvent *A*). The fractions containing the substance with R_F 0.25–0.28 were combined and evaporated to give material (2.58 g, 53%), m.p. 200–201° (decomp., from ethyl acetate–light petroleum), $[\alpha]_D^{20}$ −17° (*c* 1.45, methanol) (Found: C, 50.0; H, 5 3; N, 7.2. $C_{16}H_{20}N_2O_9$ calc.: C, 50.0; H, 5.2; N, 7.3%).

On heating compound **4** (44 mg) with anhydrous zinc chloride (40 mg) and benzaldehyde (0.5 ml), the benzylidene derivative **6** (26 mg) was obtained having m.p. and mixed m.p. 272° (decomp.).

p-*Nitrophenyl 2-acetamido-6-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-*D*-glucopyranosyl)-3,4-di-*O*-acetyl-2-deoxy-β-*D*-glucopyranoside* (**7**). — To a mixture of compounds **1** {2.7 g, $[\alpha]_D^{20}$ +10° (chloroform)}[17] and **3** {1.03 g, m.p. 235–236° (decomp.)}[6] in dry toluene (15 ml), toluene-*p*-sulphonic acid (10 mg) was added. Dry nitromethane (7.5 ml) was added to the boiling suspension, and the heating was continued for 45 min at 120–130°. The product was collected, washed with toluene and ether, and recrystallised from methanol to give the title compound (0.95 g, 49%), m.p. 258.5–260° (decomp.), $[\alpha]_D$ −27°, $[M]_D$ −204° (*c* 0.42, chloroform), R_F 0.55 (t.l.c., solvent *A*); the sum of the $[M]_D$ values of 2-acetamido-2-deoxy-β-D-glucose tetra-acetate[19] and *p*-nitrophenyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside[20] is −209°; n.m.r. data: H-1 doublet, $\delta = 5.40$ p.p.m. ($J_{1,2}$ 9.0 Hz); (Found: C, 51.15; H, 5.5; N, 5.9. $C_{32}H_{41}N_3O_{18}$ calc.: C, 50.9; H, 5.5; N, 5.6%). For the hydrate of compound 7, Osawa[6] recorded m.p. 257–258° (decomp.), $[\alpha]_D^{23}$ −43° (chloroform).

p-Nitrophenyl 2-acetamido-6-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-β-D-glucopyranoside (**8**). — The acetate **7** (0.5 g) was saponified in dry methanol (10 ml) with M methanolic sodium methoxide (0.5 ml). The product was collected, washed with methanol, and recrystallised from 90% methanol to give the title compound (275 mg, 74%), m.p. 218–219°, $[\alpha]_D^{20}$ −43° (*c* 0.49, 70% methanol in water) (Found: C, 48.1; H, 5.7; N, 7.6. $C_{22}H_{31}N_3O_{13}$ calc.: C, 48.4; H, 5.7; N, 7.7%). The substance was homogeneous in paper chromatography (R_{GluNAc} 1.44, solvent *C*). For the dihydrate of compound **8**, Osawa reported[6] m.p. 220–221° (decomp., softening at 210°) and $[\alpha]_D^{25}$ −55° (70% methanol in water).

p-Nitrophenyl 2-acetamido-6-O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)-3-O-acetyl-2-deoxy-β-D-glucopyranoside (**9**). — A suspension of compounds **1** (1.61 g) and **4** (0.47 g) and a catalytic amount of toluene-*p*-sulphonic acid in a mixture of toluene (6 ml) and nitromethane (4.5 ml) was heated for 40 min at 120–130°. The product was then collected, washed with nitromethane–toluene and ether to give chromatographically homogeneous compound **9** (0.46 g, 46%), m.p. 245–246° (decomp.) (from methanol), $[\alpha]_D^{20}$ −25° (*c* 0.8, methanol), R_F 0.20 (t.l.c., solvent *B*) (Found: C, 50.4; H, 5.5; N, 5.8. $C_{30}H_{39}N_3O_{17}$ calc.: C, 50.5; H, 5.5; N, 5.9%).

With acetic anhydride and pyridine in the usual manner, compound **9** was converted into the acetate **7**, m.p. and mixed m.p. 258–259° (decomp.). Saponification of compound **9** with sodium methoxide in methanol afforded compound **8**, m.p. 205° (decomp.), chromatographically identical with authentic material.

The mother liquor remaining after the isolation of compound **9** was treated with 2 drops of pyridine and evaporated to dryness. The excess of compound **1** was extracted with ether, and the residue was treated with charcoal in methanol. The filtered solution was treated with acetic anhydride and pyridine to give a product which was eluted from silica gel with chloroform–methanol (0→5% methanol). The fractions containing the substance with R_F 0.15–0.17 (t.l.c., solvent *B*) were combined and evaporated, and the residue (46 mg) was saponified, and analysed by paper chromatography. In addition to compound **8**, a substance with R_{GluNAc} 1.03 was detected, which was, presumably, *p*-nitrophenyl 2-acetamido-4,6-di-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-β-D-glucopyranoside.

p-Nitrophenyl O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→4)-O-2-acetamido-3,6-di-O-acetyl-2-deoxy-β-D-glucopyranosyl-(1→6)-2-acetamido-3,4-di-O-acetyl-2-deoxy-β-D-glucopyranoside (**11**). — A mixture of the oxazoline[17] (**2**) {1.27 g, m.p. 189–190°, $[\alpha]_D^{20}$ −8° (chloroform)}, compound **3** (1.2 g), and a catalytic amount of toluene-*p*-sulphonic acid was heated for 30 min at 130–140° in dry nitromethane (10 ml) and toluene (8 ml). The product was collected, washed with nitromethane–toluene (30 ml, 1:2) and ether to give chromatographically homogeneous compound **11** (0.65 g, 30% calculated with respect to the oxazoline **2**), m.p. 289–290° (decomp.) (from methanol), $[\alpha]_D^{20}$ −31°, $[M]_D$ −323° (*c* 0.55, methanol–nitromethane, 1:1), R_F 0.44 (t.l.c., solvent *A*); the sum of the $[M]_D$ values of methyl di-*N*-acetyl-βchitobioside penta-acetate[21] and *p*-nitrophenyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-*β*-D-glucopyranoside[20] is −494° (Found: C, 50.7; H, 5.6; N, 5.3. $C_{44}H_{58}N_4O_{25}$ calc.: C, 50.7; H, 5.6; N, 5.4%).

p-*Nitrophenyl* O-*(2-acetamido-2-deoxy-β-*D*-glucopyranosyl)-(1→4)-*O*-(2-acetamido-2-deoxy-β-*D*-glucopyranosyl)-(1→6)-2-acetamido-2-deoxy-β-*D*-glucopyranoside* (**12**) — A suspension of compound **11** (0.6 g) in dry methanol (80 ml) and M methanolic sodium methoxide (0.8 ml) was heated until dissolution was complete. The mixture was then maintained at 5° for 2 days, and the product was collected, and washed with methanol to yield chromatographically homogeneous compound **12** (0.36 g, 80%), m.p. 239–241° (decomp.) (from aqueous methanol), $[\alpha]_D^{20}$ −47.5°, $[M]_D$ −371° (*c* 1.19, water), R_{GluNAc} 1.13 (solvent *C*); the sum of the $[M]_D$ values of methyl di-*N*-acetyl-*β*-chito-bioside[21] and *p*-nitrophenyl 2-acetamido-2-deoxy-*β*-D-glucopyranoside[20] is −218° (Found: C, 45.6; H, 6.1; N, 6.9. $C_{30}H_{44}N_4O_{18}.2H_2O$ calc.: C, 45.9; H, 6.2; N, 7.1%).

REFERENCES

1 R. Kuhn and W. Kirschenlohr, *Ber.*, 87 (1954) 384.
2 Y. Inouye, K. Onodera, S. Kitaoka, and H. Ochiai, *J. Amer. Chem. Soc.*, 79 (1957) 4218.
3 H. M. Flowers and R. W. Jeanloz, *J. Org. Chem.*, 28 (1963) 1564, 2983.
4 P. F. Lloyd and G. P. Roberts, *J. Chem. Soc.*, (1965) 6910.
5 F. E. Hardy, *J. Chem. Soc.*, (1965) 375.
6 T. Osawa, *Carbohyd. Res.*, 1 (1966) 435.
7 R. G. Strachan, W. V. Ruyle, T. Y. Shen, and R. Hirschmann, *J. Org. Chem.*, 31 (1966) 507.
8 K. Heyns, R. Harrison, and H. Paulsen, *Ber.*, 100 (1967) 271.
9 D. Shapiro, A. J. Acher, and E. S. Rachaman, *J. Org. Chem.*, 32 (1967) 3767.
10 F. Micheel and H. Köchling, *Ber.*, 90 (1957) 1597; 91 (1958) 673.
11 F. Micheel and E. Drescher, *Ber.*, 91 (1958) 670.
12 B. Lindberg and H. Agback, *Acta Chem. Scand.*, 18 (1964) 185.
13 R. Gigg and C. D. Warren, *J. Chem. Soc.*, (1965) 1351.
14 S. Konstas, I. Photaki, and L. Zervas, *Ber.*, 92 (1959) 1288.
15 F. Micheel and H. Petersen, *Ber.*, 92 (1959) 298.
16 N. Pravdić, T. D. Inch, and H. G. Fletcher, Jr., *J. Org. Chem.*, 32 (1967) 1815.
17 A. J. Khorlin, M. L. Shulman, S. E. Zurabyan, I. M. Privalova, and Y. L. Kopaevich, *Izv. Akad. Nauk SSSR, Ser. Khim.*, (1968) 227, 2094.
18 R. W. Jeanloz, E. Walker, and P. Sinaÿ, *Carbohyd. Res.*, 6 (1968) 184.
19 D. Horton, *J. Org. Chem.*, 29 (1964) 1776.
20 D. H. Leaback and P. G. Walker, *J. Chem. Soc.*, (1957) 4754.
21 U. Zehavi, and R. W. Jeanloz, *Carbohyd. Res.*, 6 (1968) 129.

Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

BIOSYNTHESIS OF THE β-D-GLUCAN OF *Sclerotium rolfsii* SACC. DIRECTION OF CHAIN PROPAGATION AND THE INSERTION OF THE BRANCH RESIDUES*

K. K. BATRA**, J. H. NORDIN†, AND S. KIRKWOOD

Department of Biochemistry, College of Biological Sciences, University of Minnesota, St. Paul, Minnesota 55101 (U. S. A.)

(Received September 3rd, 1968)

ABSTRACT

The polysaccharide produced by the mold *Sclerotium rolfsii* Sacc. lends itself to studies of *in vivo* biosynthesis, since means are available to degrade pulse-labeled polysaccharide and determine the specific activity of all of the types of D-glucose residue present in its structure. Studies of this kind show, by direct measurement of the specific activities of the reducing and nonreducing terminals, that the direction of chain elongation is toward the nonreducing terminal. They show further that the interbranch, branch-point, and branch D-glucose residues are inserted into the structure of the molecule at about the same point in time, and that the D-glucose residues go intact into the structure, with no rearrangement of the carbon chain. The significance of these observations to the route of biosynthesis of the polysaccharide is discussed.

INTRODUCTION

Because of the availability of specific means of chemical degradation, the β-D-glucan of *S. rolfsii* lends itself to the study of certain aspects of its *in vivo* biosynthesis. This glucan consists of a backbone of β-D-(1→3)-linked D-glucopyranosyl residues bearing single β-D-(1→6)-linked D-glucopyranosyl residues as branches that are attached, on the average, to every third D-glucose residue in the main chain[1]. The availability of a highly purified D-glucanase that quantitatively degrades the polymer to a mixture of D-glucose and gentiobiose[2], coupled with the use of the Smith-degradation[1], permits the determination of the specific activities of all of the different types of D-glucose residue present in pulse-labeled polysaccharide. These residue types are: D-glucose residues at the reducing and nonreducing terminals, D-glucose residues between the branch points (interbranch D-glucose), branch-point D-glucose,

---

*This is Paper No. 6665, Scientific Series, Minnesota Agricultural Experiment Station.

**Present address: Department of Biochemistry, University of California at Berkeley, Berkeley, California 94720, U.S.A.

†Present address: Department of Biochemistry, University of Massachusetts, Amherst, Massachusetts 01003, U. S. A.

and side-chain D-glucose. It is also worthy of note that it is possible to measure, sequentially, the specific activities of the D-glucose residues in the main chain, proceeding from the nonreducing terminal toward the interior of the polysaccharide.

Thus, it is possible to pulse-label polysaccharide *in vivo* by feeding uniformly labeled D-glucose to *S. rolfsii*, and, through measurement of the relative specific activities of the different types of D-glucose, to determine both the direction of chain propagation and the relative time of insertion of the branch and the main-chain D-glucose residues. It is the purpose of the present article to describe these methods of degradation, and present specific activity data obtained from pulse-labeled material.

RESULTS AND DISCUSSION

Degradation methods. — The procedure used for measuring the specific activities of the reducing and nonreducing terminal residues involved repeated Smith-degradation (periodate oxidation, borohydride reduction, and mild hydrolysis with acid[1]). When the polysaccharide is submitted to Smith-degradation, all of the side-chain residues and the nonreducing terminal residues are removed, and the reducing terminal is converted into a D-arabinitol residue. A second Smith-degradation on this material converts this D-arabinitol into a stably bound glycerol residue, and liberates glycerol from the nonreducing, terminal D-glucose residue. The specific activity of the glycerol liberated is the same, on a molar basis, as that of the nonreducing terminal of the polysaccharide, if uniformly labeled D-glucose is used in the experiment. Repeated application of the Smith-degradation technique will yield glycerol from the nonreducing terminal newly established, but the other end of the polysaccharide molecule is not affected. By this means may be measured, in a sequential manner, the specific activities of the D-glucose residues in the main chain, proceeding from the nonreducing terminal toward the interior of the polysaccharide. The specific activity of the reducing terminal can be determined by hydrolyzing, under more strenuous conditions, the material resulting from a complete Smith-degradation; this liberates the glycerol situated at the reducing terminal end, and the specific activity of this, on a molar basis, is the same as that of the D-glucose residue that originally occupied the reducing terminal position.

The procedure used for determining the specific activities of the branch, branch-point, and interbranch D-glucose residues involved hydrolysis of the polysaccharide with the exo-β-D-(1→3)-glucanase[2] of *Basidiomycete* QM806. This enzyme has been shown to convert the polysaccharide quantitatively into D-glucose and gentiobiose. The D-glucose arises from the interbranch D-glucose residues, and it can readily be isolated and its specific activity determined. The reducing moiety of the gentiobiose arises from the branch-point D-glucose residues, and the nonreducing moiety comes from the branches. The gentiobiose is reduced with sodium borohydride, the product is hydrolyzed, and the D-glucose in the hydrolyzate is separated from the D-glucitol by electrophoresis; the specific activity of each compound is then determined.

Preliminary studies on the uptake of labeled D-*glucose.* — In order to find out if labeled D-glucose is readily taken up by the organism and incorporated intact into the polysaccharide, the fungus was grown for 44 h on D-glucose-*1*-^{14}C and on D-glucose-*6*-^{14}C, respectively. The labeled polysaccharide was then isolated, the average specific activity of the D-glucose residues in it was determined, and the distribution of the specific activity and the location of the label in three of the D-glucose fractions already described (interbranch, branch-point, and branch D-glucose) were determined. It was found that D-glucose is readily taken up and incorporated into the polysaccharide, and that the specific activities of the three types of D-glucose are the same, within experimental error, after 44 h. Each of the types of D-glucose was degraded, essentially by the procedure of Bevill *et al.*[3], to determine the location of the label. It was found that, when D-glucose-*1*-^{14}C is fed, all three D-glucose fractions are labeled at C-1, and there is no significant label at the other five carbon atoms. Similarly, when D-glucose-*6*-^{14}C is fed, all three D-glucose fractions are labeled solely at C-6. These results established that D-glucose is taken up from the medium and incorporated into the polysaccharide without alteration of the carbon chain.

Direction of chain propagation. — The direction of propagation of the chain during the biosynthesis of a polysaccharide (*i.e.*, toward the nonreducing or reducing terminal) is a matter of some importance. In the past, it has been assumed that all polysaccharide chains are propagated in the direction of the nonreducing terminal, but there is little evidence to support this as a general phenomenon. In fact, Robbins *et al.*[4] have recently produced convincing evidence that the polysaccharide that constitutes the surface O-antigen of *Salmonella newington* is elaborated toward the reducing terminal. Furthermore, Ebert and Patat and their co-workers claim that dextransucrase and levansucrase elaborate their polysaccharides toward the reducing terminal through an insertion type of mechanism[5–7]. Their evidence is based on kinetic studies, and is not convincing to the present authors, but it must be admitted that the reaction mechanism they propose is chemically feasible. On the other hand, Hehre has established that the amylosucrase from *Neisseria perflava* elaborates its polysaccharide toward the nonreducing terminal[8], and Dedonder and his co-workers claim that the levansucrase of *Bacillus subtilis* does the same[9–11].

The *S. rolfsii* polysaccharide is particularly suitable for studies on the direction of chain elongation because, in contrast to previous studies of this type on other polysaccharides, it is possible to determine the specific activities of the reducing and nonreducing terminals directly. The results of such studies on a sample of polysaccharide labeled with a 2-minute pulse of uniformly labeled D-glucose are shown in Table I. From the reasoning given, it is evident that, if the D-glucose pulse is sufficiently short to result in asymmetric labeling of the two chain-terminals, the direction of propagation can be determined by comparing the specific activities of the terminals. If the nonreducing terminal has the higher specific activity, the direction of propagation is toward that end. If the reverse is true, the direction of propagation is toward the reducing terminal. From examination of Table I, it is evident that the direction of propagation is toward the nonreducing terminal, as its specific activity is more than 20 times that of the reducing terminal.

TABLE I

DIRECTION OF CHAIN PROPAGATION

Substance counted	*Specific activity of the glucose* ($d^a.min^{-1}.\mu mol^{-1}$)
Polysaccharide resulting from the first Smith-degradation	788
Nonreducing terminal of the above polysaccharide	3070
Polysaccharide resulting from the second Smith-degradation	764
Nonreducing terminal from the above polysaccharide	2930
Glucose from the reducing terminal	140

[a] d = disintegrations.

Insertion of the side chains. — A number of possible mechanisms for the biosynthesis of a polysaccharide of the *S. rolfsii* type may be envisaged. A trans-D-glucosidase could transfer D-glucosyl residues from a donor to a growing β-D-(1→3)-linked chain. This chain could then be acted upon by another trans-D-glucosidase that would transfer single D-glucosyl residues from a donor to the 6-hydroxyl group of residues in the chain. Should this type of synthesis be involved, it would be expected that, after a pulse-label of sufficiently short duration, the side-chain D-glucose residues would have a significantly higher specific activity than those in the main chain, since the incoming, labeled D-glucose would be transferred as branches to pre-existing unlabeled chains. Certain other schemes of synthesis are equally plausible. The biosynthesis could involve the combined action of one trans-D-glucosidase building up the β-D-(1→3)-backbone from a donor and another that transfers single D-glucosyl residues from the nonreducing terminal to O-6 of the penultimate, D-glucosyl residue. Such a mechanism would result in a product in which the various types of D-glucosyl residues in the pulse-labeled polysaccharide all have the same specific activity, as they all enter the same region of the polysaccharide at about the same time. A biosynthesis that involves the synthesis of a tetrasaccharide donor, followed by a trans-D-glycosylase that transfers these tetrasaccharide units into the growing chain, could also result in uniform specific activity in pulse-labeled material.

Sclerotium rolfsii appears to elaborate its polymer on the surface of the mycelium, and then to extrude it into the medium. An appreciable proportion of the polymer cannot be washed off the mycelium, but it can be obtained by boiling the mycelia, disrupting them in a sonifier, and then washing the disrupted cells with water. As would be expected, during pulse experiments, the polymer on the cell surface (referred to as "cellular polysaccharide") becomes labeled sooner than the polysaccharide in the medium (referred to as "extracellular polysaccharide"). This is shown in Table II, from which it is evident that significant labeling of the cellular polysaccharide can be obtained at 30 sec, at which time there is no detectable activity in the extracellular polysaccharide. The distribution of specific activity among the various types of D-glucose in both cellular and extracellular polysaccharide is shown in Table III. It is evident that, even at the shortest pulse-time that will provide significant labeling of the polysaccharide, the different types of D-glucose residue all have the same specific activity. This result excludes a mechanism involving synthesis of the backbone followed by later insertion of the side branches, and clearly establishes that, in any given region in the polysaccharide, the interbranch, branch-point, and branch residues are inserted at about the same moment in time.

TABLE II

INCREASE OF SPECIFIC ACTIVITY WITH TIME IN THE CELLULAR AND EXTRACELLULAR POLYSACCHARIDES

Time of exposure to labeled D-glucose (sec)	*Specific activity ($d^a.min^{-1}.\mu mol^{-1}$)*	
	Cellular polysaccharide	*Extracellular polysaccharide*
30	491±27[b]	0
900	5,920	1,820
1,800	7,100	3,950
3,600	20,200	9,750
7,200	24,700	22,700
72,000	49,400	48,200

[a] d = disintegrations.

[b] This is the statistical variation in the count of this sample; the statistical variation for all other samples is negligible.

TABLE III

DISTRIBUTION OF SPECIFIC ACTIVITY IN THE CELLULAR AND EXTRACELLULAR POLYSACCHARIDES

Time of exposure to labeled D-glucose (sec)	*Specific activity ($d^a.min^{-1}.\mu mol^{-1}$ of glucose)*				
	Whole polysaccharide	*Interbranch D-glucose*	*Gentiobiose*	*Branch unit D-glucose*	*Branch-point D-glucose*
Cellular polysaccharide					
30	491 ±27[b]	439 ±17[b]	424 ±26[b]	450 ±32[b]	429 ±24[b]
900	5,920	5,450	5,400	5,320	5,800
3,600	20,200	19,300	18,000	19,200	19,300
Extracellular polysaccharide					
30	0	0	0	0	0
900	1,825	1,800	1,770	1,750	1,820
1,800	3,950	3,850	3,720	3,890	3,750

[a] d = disintegrations.

[b] This is the statistical variation in the counts of these samples; the statistical variation for all other samples is negligible.

EXPERIMENTAL

General methods. — Measurements of radioactivity were made with a liquid scintillation counter, Nuclear Chicago Corporation Model 724. All evaporations were conducted in a rotary evaporator under vacuum at 40°. De-ionization was accomplished by passing solutions through columns of Amberlite IR-120 (H^+) and Amberlite IR-45 (OH^-) ion-exchange resins, unless otherwise indicated. Reducing power was measured by the Nelson–Somogyi method[12], and carbohydrate spots on paper chromatograms and electrophoretograms were visibilized by the procedure of Trevelyan *et al.*[13]. Paper chromatography was performed by the descending method, with one of two solvents: (*A*) 7:1:2 (v/v) propyl alcohol–ethyl acetate–water, or (*B*) 7:1:2 (v/v) pyridine–ethyl acetate–water. Centrifugation was conducted at 4° and 35,000 *g*. Formaldehyde liberated by periodate oxidation was determined by the chromotropic acid procedure[14]. Periodate was determined by the method of Aspinall and Ferrier[17], and total glucose by the phenol–sulfuric method[16]. Whenever a compound was determined in the eluate of a chromatogram spot, an appropriate correction was made for the blank for the paper.

Growth of Sclerotium rolfsii. — The organism was maintained on Bacto malt agar (Difco Laboratories), and was grown in liquid culture, in Erlenmeyer flasks, on a rotary shaker (200 cycles/min) at room temperature (26–27°). This aeration of submerged cultures was necessary for both maximum growth and polymer production. The medium had the following composition (g/100 ml), unless otherwise noted; D-glucose, 4.0; $NaNO_3$, 0.2; $MgSO_4 \cdot 7H_2O$, 0.15; KH_2PO_4, 0.1; Bacto yeast extract, 0.1. *S. rolfsii* produces sclerotia (vegetative fruiting-bodies), and all mycelial preparations used in this work were produced by inoculating the liquid medium (15 ml in a 125-ml Erlenmeyer flask) with a few mature sclerotia. After the sclerotia had germinated, the medium was homogenized under sterile conditions in a Servall Omnimixer for 1–2 min, and then grown for another 24 h, at which time, by plating, the culture was checked for contamination. This procedure provided a highly disperse preparation of mycelia that could be used for labeling experiments or for the further propagation of vegetative cells.

Procedure for producing labeled polysaccharide. — In the experiments in which D-glucose-*1*-^{14}C and D-glucose-*6*-^{14}C were used, the labeled sugar (50 μCi, specific activity 150 mCi/mmol) was added under sterile conditions to the contents of a 125-ml flask prepared as described, and the mixture was shaken for 44 h. Cells were removed by centrifugation, and the polysaccharide was precipitated by the addition of ethanol (5 vol). The polysaccharide (20–40 mg) was dissolved in 100 ml of water, and the solution was dialyzed against distilled water for 48 h. The precipitation with ethanol and the dialysis were then repeated, and the polymer was precipitated with ethanol, and dissolved in water for further manipulation. The specific activity of the polysaccharide remained constant after both dialyses, and, as a further check on freedom from contamination with labeled small molecules, it was subjected to electrophoresis in borate buffer, pH 9.2, at 600 volts. The polysaccharide is not mobile in this system, but glucose and glucose esters move to the anode. No radioactivity could be detected, other than at the origin.

Polysaccharide used in the study of the direction of chain propagation was prepared by suspending 92 g (wet weight) of 64-h-old mycelia in 200 ml of growth medium (D-glucose omitted), shaking for 15 min, and then adding 500 μCi of uniformly labeled D-glucose-^{14}C (150 mCi/mmol). Shaking was continued for 2 min, and the preparation was heated for 15 min at 100°. The mycelia were centrifuged, the residual mat was mixed with 100 ml of water in an omnimixer at low speed, and the preparation was recentrifuged. This washing procedure was repeated twice more, the original extract and washings were combined, and the polysaccharide was precipitated by the addition of ethanol. The precipitate was washed with three 50-ml portions of 80% ethanol, and reprecipitated twice more from 400 ml of water with ethanol. It was dissolved in 800 ml of water, and the solution was dialyzed for 24 h at 4°, and stored for future use.

When material pulse-labeled for a short time was desired, the level of D-glucose in the growth medium was lessened from 4.0 to 0.5%. The organisms were grown in a 500-ml Erlenmeyer flask containing 100 ml of growth medium which was inoculated with 20 ml of homogenized inoculum prepared as already described. The culture was shaken for 48 h, and then uniformly labeled D-glucose was added under sterile conditions (100 μCi, specific activity 150 mCi/mmol). The flask was re-placed on the shaker, and 15-ml aliquots of the growing culture were removed at the elapse of the time intervals indicated. The samples were chilled in stainless-steel centrifuge-tubes kept at −10°, and were centrifuged to separate the mycelia from the medium; the mycelia were stored at −10° pending further use. The polymer in the medium was precipitated by addition of ethanol (4 ml per ml), removed by centrifugation, and dissolved in 30 ml of *N,N*-dimethylformamide; the solution was dialyzed for 24 h at 4° against 1:19 (v/v) *N,N*-dimethylformamide–water, and then against water for 72 h. This procedure completely removed labeled D-glucose that had been added to unlabeled polysaccharide. The polysaccharide was precipitated from the final dialysis medium by the addition of ethanol.

Cellular polysaccharide was prepared from the mycelium by thawing the mycelial preparation, heating it in 7 ml of water for 5 min at 100°, cooling to 4°, and disrupting the cells in a Branson Sonifier for 2 min at 6.4 amp. (Microscopic examination showed that the mycelia are completely disrupted by this treatment.) The samples were centrifuged, and the residue was washed with 5 ml of water. The supernatant liquor and washings were combined, and the polysaccharide was precipitated by the addition of 40 ml of ethanol, collected by centrifugation, and dialyzed as described for the extracellular polysaccharide.

Enzymic hydrolysis of the polysaccharide, and isolation of gentiobiose and D-*glucose*. — Polysaccharide (1–2.5 mg) was dissolved in 10 ml of 50 mM acetate buffer, pH 4.8, containing 20 units of highly purified exo-β-D-(1→3)-glucanase, and the mixture was incubated for 36 h at 37°. The enzyme had a specific activity of 260 units/mg; the unitage and assay have been described previously[2]. Determination of the reducing power liberated showed that enzymic hydrolysis was complete after 36 h. The mixture was then heated in boiling water, de-ionized, concentrated, and chromatographed on Whatman No. 1 paper with solvent *A* for 30 h. Only two radioactive products appeared on the chromatograms, namely, gentiobiose and glucose, and each was isolated by eluting the proper area on the paper.

Determination of the specific activities of interbranch, branch-point, and branch D-*glucose.* — The specific activity of the glucose eluted from the chromatograms was found by counting the solution and determining the amount of glucose present by use of the Nelson–Somogyi procedure[12].

The gentiobiose (0.2–0.4 mg) was dissolved in 1 ml of water, and sodium borohydride was added in two 1-mg portions, 2 h apart. The mixture was kept overnight at room temperature, made acid with 500 mM acetic acid, and passed through a column of Amberlite IR-120 (H^+) ion-exchange resin to remove sodium ions. The eluate from the column was evaporated to dryness, and the borate was removed as methyl borate by repeated addition and evaporation of methanol. The resulting gentiobiitol was dissolved in 2 ml of 500 mM sulfuric acid, and the solution was heated for 12 h at 100°, cooled, and de-ionized. It was concentrated to a small volume, applied to Whatman No. 1 paper, and electrophoresed for 2.5 h at 600 volts with 100 mM sodium molybdate, pH 5.0, as the electrolyte. The glucose and glucitol spots were located, and were eluted from the paper without prior removal of the molybdate. In order to remove the molybdate, each sample was adjusted to pH 10.0 with 500 mM sodium hydroxide, and successively passed through columns of Dowex AG-1 x2 (Cl^-) and Amberlite IR-120 (H^+) ion-exchange resins. The samples were then counted, and the concentration of glucose was determined by the Nelson–Somogyi method[12] and that of glucitol by periodate oxidation followed by determination, with chromotropic acid, of the formaldehyde released[14]. The specific activity of each sample could then be calculated.

Determination of the specific activity of the reducing and nonreducing terminals by Smith-degradation. — The polysaccharide (360 mg) was treated in the dark with 15 mM periodic acid (1 liter) for 117 h at 4°. The consumption of periodate was followed by the method of Aspinall and Ferrier[15]; it became constant after 50 h (380 mmol per D-glucose residue). The excess of periodate was decomposed by addition of the calculated amount of ethylene glycol, and the solution was concentrated to 400 ml and dialyzed for three days against several changes of water.

To the dialyzed solution (460 ml) was added a solution of sodium borohydride (32 mg) in 10 ml of water. The reduction was allowed to proceed for 14 h at room temperature with occasional shaking. Hydrochloric acid (50 mM) was added to neutrality, and the solution was concentrated to 200 ml.

Partial hydrolysis with acid was accomplished by adding hydrochloric acid to pH 1.0 and keeping the solution overnight at 25°. The resulting polymer was centrifuged off, and washed with 80% ethanol until the washings were free from radioactivity. The supernatant liquor and washings were combined, and de-ionized, and glycerol (the only material in the supernatant liquor that gave a silver spot by the Trevelyan procedure[13]) was separated by paper chromatography with solvent *B*, eluted from the paper, and counted. The glycerol was determined by the method of Lambert and Neish[14], and its specific activity was calculated. This Smith-degradation procedure was repeated twice more, and the glycerol liberated was isolated each time and its specific activity determined. In each case, glycerol was the only Tollens-positive material in the supernatant liquor.

After the third Smith-degradation, the residual polysaccharide (188 mg), which contained no free glycerol, was dissolved in 2 ml of 90% formic acid, 500 mM sulfuric acid (25 ml) was added, and the mixture was heated under reflux for 14 h in a boiling-water bath. The solution was cooled, and made alkaline with sodium hydroxide, and a stream of air was bubbled through the solution for 2 h at reflux temperature. During this time, the reducing power of the solution fell to a negligible value. The solution was then de-ionized and concentrated, and the material was chromatographed in solvent *B*. Glycerol was the only Tollens-positive material detected, and its specific activity was determined as already described.

ACKNOWLEDGMENT

This investigation was supported by Grant GM09239, National Institutes of Health.

REFERENCES

1 J. Johnson, Jr., S. Kirkwood, A. Misaki, T. E. Nelson, J. V. Scaletti, and F. Smith, *Chem. Ind.* (London), (1963) 820.
2 F. I. Huotari, T. E. Nelson, F. Smith, and S. Kirkwood, *J. Biol. Chem.*, 243 (1968) 952.
3 R. D. Bevill, E. A. Hill, F. Smith, and S. Kirkwood, *Can. J. Chem.*, 43 (1965) 1577.
4 P. W. Robbins, D. Bray, M. Dankert, and A. Wright, *Science*, 158 (1967) 1536.
5 K. H. Ebert and F. Patat, *Z. Naturforsch.*, 17B (1962) 738.
6 K. H. Ebert and H. Sticker, *Z. Naturforsch.*, 19B (1964) 287.
7 F. Patat, *Chimia*, 18 (1964) 233.
8 E. J. Hehre, *Proc. IUPAC Intern. Sympos. Macromol. Chem.* (*Tokyo-Kyoto, 1966*), 1 (1966) 178; *J. Polymer Sci., C*, 23 (1968) 239.
9 G. Rapoport and R. Dedonder, *Bull. Soc. Chim. Biol.*, 48 (1966) 1311.
10 G. Rapoport, R. Dionne, E. Toulouse, and R. Dedonder, *Bull. Soc. Chim. Biol.*, 48 (1966) 1323.
11 G. Rapoport and R. Dedonder, *Bull. Soc. Chim. Biol.*, 48 (1966) 1349.
12 M. Somogyi, *J. Biol. Chem.*, 195 (1952) 19.
13 W. E. Trevelyan, D. P. Procter, and J. S. Harrison, *Nature*, 166 (1950) 444.
14 M. Lambert and A. C. Neish, *Can. J. Res.*, 28B (1950) 83.
15 G. O. Aspinall and R. J. Ferrier, *Chem. Ind.* (London), (1957) 1216.
16 M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith, *Anal. Chem.*, 28 (1956) 350.

Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

THE ACETONATION OF D-TALOSE

J. S. BRIMACOMBE AND (MISS) P. A. GENT
Chemistry Department, The University, Birmingham 15 (Great Britain)

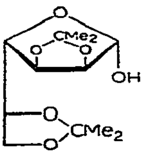
(Received July 16th, 1968)

ABSTRACT

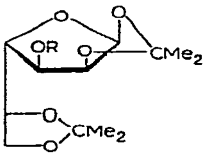
The acetonation of α-D-talopyranose in the presence of anhydrous copper(II) sulphate and sulphuric acid has been re-examined, and the major products have been shown to be 2,3:5,6-di-*O*-isopropylidene-α-D-talofuranose (**1**, 28%) and 1,2:5,6-di-*O*-isopropylidene-β-D-talofuranose (**2**, 10%). These structures were allocated primarily on the basis of mass spectrometry and nuclear magnetic resonance spectroscopy. Oxidation of diacetal **1** with acetic anhydride in methyl sulphoxide gave 2,3:5,6-di-*O*-isopropylidene-D-talono-1,4-lactone (**4**). 1,2:5,6-Di-*O*-isopropylidene-3-*O*-toluene-*p*-sulphonyl-β-D-talofuranose (**3**) was obtained on sulphonylation of diacetal **2**.

INTRODUCTION AND DISCUSSION

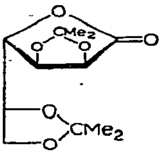
Bimolecular nucleophilic displacement of *endo*-sulphonyloxy groups attached to trioxabicyclo[3.3.0]octane ring systems has afforded stereospecific syntheses of a number of 3-amino-3-deoxy[1,2] and 3-deoxy-3-fluoro sugars[3,4] which are often difficult to prepare by other methods. Such displacements have been reported with 1,2:5,6-di-*O*-isopropylidene-3-*O*-toluene-*p*-sulphonyl-α-D-allofuranose[1,3] and 1,2:5,6-di-*O*-isopropylidene-3-*O*-toluene-*p*-sulphonyl-α-D-gulofuranose[2,4]. With a view to extending these studies, we sought to prepare 1,2:5,6-di-*O*-isopropylidene-3-*O*-toluene-*p*-sulphonyl-β-D-talofuranose (**3**) which contains an *endo*-sulphonyloxy group that should, by analogy, undergo ready displacement.



1



2 R = H
3 R = $SO_2 \cdot C_6H_4Me$



4

Acetonation of D-talose, in the presence of anhydrous copper(II) sulphate and concentrated sulphuric acid, was reported[5] to yield a mixture of 2,3:5,6-di-*O*-isopropylidene-D-talofuranose (**1**) and 1,2:5,6-di-*O*-isopropylidene-β-D-talofuranose (**2**).

Diacetal **1** was not isolated, and its presence was indicated by the isolation of 2,3:5,6-di-*O*-isopropylidene-D-talono-1,4-lactone (**4**) following oxidation of the products of acetonation with alkaline permanganate. The structure of diacetal **2** was founded on its recovery after oxidation, which implied the absence of a free, primary hydroxyl group. Clearly, a more definitive allocation of structure is required in this case, and it was thought that modern spectroscopic methods might be applied advantageously to this problem.

Although new preparations of D-talose have been reported[6,7] recently, hydroxylation of D-galactal[8] with perbenzoic acid appeared to be most amenable to large-scale work. This procedure gives a mixture of D-talose and D-galactose that is separated by extraction of D-talose into boiling methanol, in which D-galactose is relatively insoluble. In our hands, complete separation of the two hexoses was not achieved by the extraction procedure, although the methanol-soluble material was enriched in D-talose. Chromatographically pure D-talose was obtained either by peracetylation of the hexose mixture, followed by fractional crystallisation of the derived penta-*O*-acetyl-α-D-talopyranose and deacetylation, or, more efficiently, by using the counter-current extraction procedure described in the Experimental section.

Acetonation[5] of α-D-talopyranose was shown (t.l.c.) to give two major products and a small proportion of a third component. Chromatography on silica gel afforded the major components, which were designated as *A* and *B* in order of their elution from the column. Compound *A* (28%) was further purified by distillation, and compound *B* (10%) was obtained in crystalline form. Elemental analyses showed that they were both diacetals, and this was also clearly indicated[9] by the presence of a prominent peak at *m/e* 245 (M − 15) in their mass spectra (see Figs. 1 and 2).

Mass spectrometry provides[9] an elegant means of distinguishing between di-*O*-isopropylidenealdohexopyranoses and their furanose counterparts. The most characteristic fragmentation process of di-*O*-isopropylidenealdohexofuranoses is scission of the C-4–C-5 bond, which gives rise[9] to an ion of *m/e* 101. Peaks at *m/e* 101 were present in the mass spectra of both *A* and *B* (Figs. 1 and 2), which are thus identified as talofuranose diacetals. Although the mass spectra of 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose and 2,3:5,6-di-*O*-isopropylidene-α-D-mannofuranose are closely similar, the 2,3:5,6-diacetal has a more intense peak at *m/e* 243 (M − 17), due[9] to the attachment of the hydroxyl group to C-1 rather than to C-3. This difference was also apparent* in the spectra of diacetals *A* and *B*, which were tentatively assigned the 2,3:5,6- and 1,2:5,6-arrangements, respectively, on this basis.

These assignments were supported by n.m.r. spectroscopy (deuteriochloroform) of the diacetals by comparison of the H-1 couplings. The H-1 signals in both spectra were shifted well downfield, and, for diacetal *A*, H-1 appeared as a doublet (*J* 9 Hz) centred at τ 4.59, which collapsed to a singlet upon the addition of deuterium oxide. This treatment also removed a doublet (*J* 9 Hz) centred at τ 5.64, which can be

---

*This is seen more clearly from the original mass spectra, where peak amplifications of fourteen- and fifty-fold are recorded, than from Figs. 1 and 2.

ascribed to the hydroxylic proton. The n.m.r. data are readily reconciled with structure **1**, where strong coupling is observed initially between the hydroxylic proton[10] and H-1. Exchange of the hydroxylic proton for deuterium removed this coupling. The resulting appearance of H-1 as a singlet ($J < 0.5$ Hz) signified a *trans*-arrangement[11] of the protons at C-1 and C-2. The H-1 signal for diacetal *B* was observed as a doublet (J 4 Hz), centred at τ 4.16, which was unaffected by the addition of deuterium oxide. The size of the H-1–H-2 coupling is compatible[11] with a *cis*-arrangement of these protons, as required by structure **2**. The combined spectroscopic evidence supports the structures 2,3:5,6-di-*O*-isopropylidene-α-D-talofuranose (**1**) for *A* and 1,2:5,6-di-*O*-isopropylidene-β-D-talofuranose (**2**) for *B*.

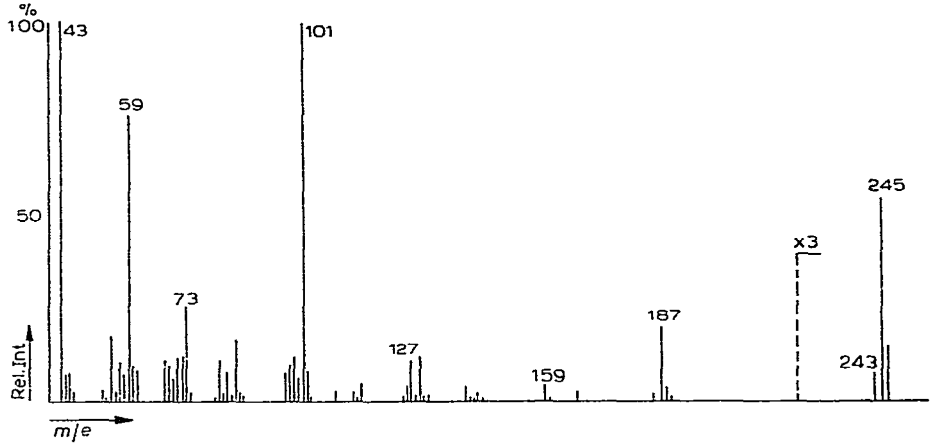


Fig. 1. Mass spectrum of diacetal *A* (2,3:5,6-di-*O*-isopropylidene-α-D-talofuranose).

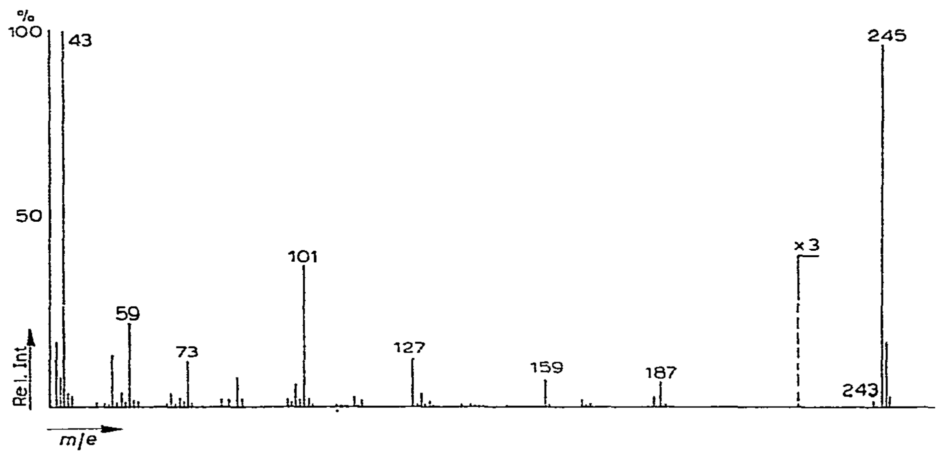


Fig. 2. Mass spectrum of diacetal *B* (1,2:5,6-di-*O*-isopropylidene-β-D-talofuranose).

Confirmation of the structure of diacetal **1** was afforded by its conversion into the known[5] 2,3:5,6-di-*O*-isopropylidene-D-talono-1,4-lactone (**4**) on oxidation with acetic anhydride–methyl sulphoxide[12]. 1,2:5,6-Di-*O*-isopropylidene-β-D-talofuranose (2) gave a crystalline sulphonate (3) on treatment with toluene-*p*-sulphonyl chloride in pyridine.

Mills[13] predicted, from conformational principles, that the 2,3:5,6-diacetal 1 would preponderate at equilibrium on acetonation of D-talose, and our findings substantiate his arguments. The proportion of diacetal 2 found in this investigation is significantly less than that (~ 34%) obtained in a comparable experiment by Bosshard[5], whose material was of uncertain purity.

2,3:5,6-Di-*O*-isopropylidenealdohexoses are currently of interest, since they offer a means, by way of the chloro derivatives, of synthesising nucleosides containing these sugars[14].

EXPERIMENTAL

Thin-layer chromatography (t.l.c.) was performed on Kieselgel G, and detection was effected with the vanillin–sulphuric acid reagent[15]. Infrared spectra were obtained on a Perkin–Elmer 257 spectrometer, and n.m.r. spectra were obtained with a Perkin–Elmer R-10 spectrometer for *ca.* 10% solutions in deuteriochloroform with tetramethylsilane as internal reference. Mass spectra were measured with an A.E.I. MS9 spectrometer (ionising potential 58 ev), using a direct insertion technique.

Preparation of α-D-talopyranose. — A mixture of D-talose and D-galactose was prepared by hydroxylation of D-galactal as described in the literature[8]. The proportion of D-talose in the mixture was increased by a single extraction with boiling methanol, followed by filtration and evaporation. This mixture was used in the purification procedures described below. Repeated treatments with boiling methanol failed to give D-talose in chromatographically pure form.

(*a*) *Acetylation.* The mixture (2 g) in pyridine (48 ml) containing acetic anhydride (50 ml) was stirred for 17.5 h at room temperature, and t.l.c. (ethyl acetate–acetone, 1:1) then indicated that the reaction was complete. The solution was poured into ice–water (300 ml), the aqueous solution was extracted with chloroform (3 × 500 ml), and the combined extracts were washed with water (3 × 500 ml) and dried ($CaCl_2$). Removal of the solvent afforded a syrup which crystallised (0.58 g) from ethanol. Recrystallisation from chloroform–ethanol gave penta-*O*-acetyl-α-D-talopyranose (0.11 g), m.p. 106–107°; lit., m.p. 106.5–107° (ref. 16), m.p. 104–105° (ref. 7). Additional quantities (totalling 0.67 g) of the pentaacetate were obtained from the mother liquors by fractional crystallisation.

The pentaacetate (0.6 g) was deacetylated by using the Zemplén procedure, and, on complete reaction, the solution was stirred with a slight excess of Amberlite IR-120(H^+) resin until it was neutral. The filtered solution was concentrated, and the syrupy residue was crystallised from an equal quantity of methanol to give α-D-talopyranose (0.14 g), m.p. 129–130°; lit.[8], m.p. 133–134°. This material was shown to be pure by paper chromatography (ethyl acetate–pyridine–water, 40:11:6).

(*b*) *Countercurrent extraction.* D-Talose and D-galactose had R_F values of 0.13

and 0.07, respectively, in butyl alcohol saturated with water, and this solvent system was the most suitable of those examined for use with a Gallenkamp countercurrent apparatus. A typical run was conducted as follows.

The mixture (2 g) was dissolved in the lower phase of water saturated with butyl alcohol (60 ml), and the solution was distributed in the first six tubes of the countercurrent apparatus. Five hundred transfers were then carried out automatically by using an upper phase of butyl alcohol saturated with water, with shaking and settling times of 3 min each; the volumes of the upper and lower phases employed were 20 ml and 10 ml, respectively. Combination and evaporation of the appropriate fractions from the lower phase (as indicated by paper chromatography) gave a syrup which was crystallised from an equal volume of methanol at 0°. Two recrystallisations gave α-D-talopyranose (1.1 g), m.p. 128–129°, which was shown to be free from D-galactose by paper chromatography.

Acetonation of α-D-talopyranose. — Crystalline D-talose (1 g) in dry acetone (25 ml) containing conc. sulphuric acid (0.05–0.1 ml) and anhydrous copper sulphate (2.5 g) was shaken for 48 h at room temperature, after which time t.l.c. (ethyl acetate–chloroform, 2:1) showed the presence of two major components, together with a small proportion of a slower-moving component and unreacted hexose. Solid material was filtered off and washed thoroughly with acetone, and the filtrate was neutralised with a few drops of conc. ammonia and filtered. Removal of the solvent left a syrupy residue (*ca.* 1 g) which was chromatographed on silica gel (elution with ethyl acetate–hexane, 1:1) to give, in order of elution, 2,3:5,6-di-*O*-isopropylidene-α-D-talofuranose (**1**) (0.41 g, 28%), b.p. 150–160° (bath)/0.1–0.2 mm, $[\alpha]_D$ +2 ±1° (*c* 2, chloroform), ν_{max} 1375 (CMe_2) and 3400 cm^{-1} (OH) (Found: C, 55.8; H, 8.1. $C_{12}H_{20}O_6$ calc.: C, 55.4; H, 7.7%); and 1,2:5,6-di-*O*-isopropylidene-β-D-talofuranose (**2**) (0.14 g, 10%), m.p. 85–86° (from ether–light petroleum, b.p. 40–60°), $[\alpha]_D$ −25 ±1° (*c* 0.47, chloroform), ν_{max} 1375 (CMe_2) and 3465 cm^{-1} (OH) (Found: C, 55.2; H, 7.4. $C_{12}H_{20}O_6$ calc.: C, 55.4; H, 7.7%). Bosshard[5] reported $[\alpha]_D$ −25.2° (*c* 3.1, acetone) for a syrupy material of uncertain purity.

The n.m.r. spectra showed the following salient features: diacetal **1**, τ 4.59 (1-proton doublet, *J* 9 Hz, H-1); 5.64 (1-proton doublet, *J* 9 Hz OH); 8.53, 8.62, and 8.68 (12 protons, integrated ratio *ca.* 2:1:1, 2 CMe_2); addition of deuterium oxide resulted in the collapse of the H-1 doublet at τ 4.59 to a singlet and the disappearance of the doublet at τ 5.64; diacetal **2**, τ 4.16 (1-proton doublet, $J_{1,2}$ 4 Hz, H-1); 8.43, 8.59, and 8.63 (12 protons, integrated ratio *ca.* 1:1:2, 2 Me_2C).

2,3:5,6-Di-O-isopropylidene-D-talono-1,4-lactone (**4**). — A solution of the diacetal **1** (0.11 g) in methyl sulphoxide (8 ml) and acetic anhydride (1 ml) was stirred for 19 h at room temperature, whereupon t.l.c. (ethyl acetate–hexane, 1:1) revealed the formation of a single product. Most of the solvent was evaporated off at low pressure (*ca.* 0.5 mm), the residue was dissolved in chloroform (25 ml), and the organic layer was washed with water (5 × 15 ml) and dried ($MgSO_4$). Removal of the solvent afforded a crystalline residue (0.1 g) which was recrystallised from light petroleum (b.p. 100–120°) to give the lactone **4**, m.p. 129–130°, $[\alpha]_D$ +19 ±2° (*c* 0.49, acetone),

ν_{max} 1770 (γ-lactone) and 1380 cm^{-1} (CMe_2); lit.[5], m.p. 129–130°, $[\alpha]_D$ +17 ±2° (*c* 1.85, acetone).

*1,2:5,6-Di-*O*-isopropylidene-3-*O*-toluene-*p*-sulphonyl-β-*D*-talofuranose* (3). — Treatment of the diacetal **2** with toluene-*p*-sulphonyl chloride in pyridine, in the usual way, gave the sulphonate **3** (73%), m.p. 136–137° (from aqueous ethanol), $[\alpha]_D$ −84.5° (*c* 0.6, chloroform) (Found: C, 54.75; H, 6.2; S, 7.9. $C_{19}H_{26}O_8S$ calc.: C, 55.1; H, 6.3; S, 7.7%).

ACKNOWLEDGMENTS

The authors thank Professor M. Stacey, C.B.E., F.R.S., for his interest, Dr. E. F. Mooney for measuring the n.m.r. spectra, and Dr. J. R. Majer for obtaining the mass spectra. The award of a studentship (P.A.G.) by the Science Research Council is gratefully acknowledged.

REFERENCES

1 W. Meyer zu Reckendorf, *Angew. Chem.*, 78 (1966) 1023; J. S. Brimacombe, J. G. H. Bryan, A. Husain, M. Stacey, and M. S. Tolley, *Carbohyd. Res.*, 3 (1967) 318; D. T. Williams and J. K. N. Jones, *Can. J. Chem.*, 45 (1967) 8.
2 J. S. Brimacombe, P. A. Gent, and M. Stacey, *J. Chem. Soc. (C)*, (1968) 567.
3 K. W. Buck, A. B. Foster, R. Hems, and J. M. Webber, *Carbohyd. Res.*, 3 (1966) 137; 5 (1967) 292.
4 J. S. Brimacombe, A. B. Foster, R. Hems, and L. D. Hall, *Carbohyd. Res.*, 8 (1968) 249.
5 W. Bosshard, *Helv. Chim. Acta*, 18 (1935) 482.
6 D. Horton and J. S. Jewell, *Carbohyd. Res.*, 5 (1967) 149.
7 H. Paulsen, F. G. Espinosa, and W. P. Trautwein, *Ber.*, 101 (1968) 186.
8 R. S. Tipson and H. S. Isbell, *Methods Carbohyd. Chem.*, 1 (1962) 157.
9 D. C. De Jongh and K. Biemann, *J. Amer. Chem. Soc.*, 86 (1964) 67.
10 N. L. Bauld and Y. S. Rim. *J. Org. Chem.*, 33 (1968) 1303.
11 K. L. Rinehart, Jr., W. S. Chilton, M. Hichens, and W. Von Phillipsborn, *J. Amer. Chem. Soc.*, 84 (1962) 3216; J. E. Christensen and L. Goodman, *J. Org. Chem.*, 28 (1963) 2995; R. U. Lemieux and R. Nagarajan, *Can. J. Chem.*, 42 (1964) 1270.
12 J. D. Albright and L. Goldman, *J. Amer. Chem. Soc.*, 87 (1965) 4214; D. Horton and J. S. Jewell, *Carbohyd. Res.*, 2 (1966) 251.
13 J. A. Mills, *Advan. Carbohyd. Chem.*, 10 (1955) 1.
14 L. M. Lerner and P. Kohn, *J. Org. Chem.*, 31 (1966) 339; L. M. Lerner, B. D. Kohn, and P. Kohn, *ibid.*, 33 (1968) 1780.
15 *Chromatography*, E. Merck A. G., Darmstadt, 2nd edn., p. 30.
16 W. W. Pigman and H. S. Isbell, *J. Res. Natl. Bur. Std.*, 19 (1937) 189.

Note

Structural studies on the O-specific side-chains of the cell-wall lipopolysaccharide from *Salmonella typhimurium* LT2

In a previous publication[1], we reported on structural studies of the O-specific side-chains of the cell-wall lipopolysaccharide from *Salmonella typhimurium* 395 MS, and a detailed structure for these side chains was proposed. This structure differred in certain respects from the general structure suggested previously for group B *Salmonella* lipopolysaccharides[2,3]. This indicates either that there are considerable structural variations within the group B *Salmonella* lipopolysaccharides or that the previously suggested structure has to be revised. The structural investigation of different lipopolysaccharides from this group is therefore a matter of some interest, and we now report on such studies on the lipopolysaccharide from *Salmonella typhimurium* LT2.

The lipopolysaccharide was isolated from bacterial cell-walls, and the presence of O factors 4, 5, and 12 in the bacteria and the lipopolysaccharide were established as previously described[1]. The chemical studies were also performed by the methods employed in the investigation of the 395 MS lipopolysaccharide[1] and will only be described briefly here.

A hydrolysate of the lipopolysaccharide contained D-glucose, D-galactose, D-mannose, L-rhamnose, and abequose, in the relative molar proportions 14:26:21:20:20. The presence of *O*-acetyl groups was demonstrated by i.r. and n.m.r. [in $(CD_3)_2SO$] spectroscopy.

The lipopolysaccharide was fully methylated and hydrolysed, the methylated sugars were converted into alditol acetates, and the mixture was analysed by g.l.c.[4] and mass spectrometry[5]. The results are summarised in Fig. 1 and in Table I, which also gives the results from the methylation analysis of the 395 MS lipopolysaccharide. 2,3-Di-*O*-methyl-L-rhamnose and 2,3,4,6-tetra-*O*-methyl-D-glucose did not separate on the ECNSS-column, but were well separated on a polyphenyl ether column.

The percentage of 2,3,4,6-tetra-*O*-methyl-D-glucose is higher for the LT2 lipopolysaccharide (9.7%) than for the 395 MS lipopolysaccharide (1.8%), the difference accounting for the higher percentage of D-glucose residues in the former (14%) than in the latter (5%). The terminal D-glucose residues in the repeating units of the side chains are linked to the 4-position of the D-galactose residues, and a corresponding increase in the percentage of 2,6-di-*O*-methyl-D-galactose and a decrease in the percentage of 2,4,6-tri-*O*-methyl-D-galactose are also observed. 2,6-Di-*O*-methyl-D-galactose was only tentatively identified in the previous study. The substitution pattern was evident from the mass spectrum, and the T-value was the same as that given by an authentic derivative. The retention times of the corresponding D-glucose and D-mannose derivatives differ from that of the galactose derivative, thus identifying the sugar unambiguously.

TABLE I

METHYL ETHERS FROM THE HYDROLYSATE OF METHYLATED LIPOPOLYSACCHARIDE

Sugars	*Peak*	T[a]	*Mol %*[b]	
			LT2	*395 MS*
2,4-Di-*O*-methylabequose	A	0.32	3.8	7.0
2,3-Di-*O*-methyl-L-rhamnose	B	0.98	19.5	21.4
2,3,4,6-Tetra-*O*-methyl-D-glucose	B	1.00	9.7	1.8
2,3,4,6-Tetra-*O*-methyl-D-galactose	C	1.25	1.6	1.3
2,4,6-Tri-*O*-methyl-D-mannose	D	2.08	2.7	2.1
2,4,6-Tri-*O*-methyl-D-galactose	E	2.26	10.7	20.9
3,4,6-Tri-*O*-methyl-D-hexose	F	2.50	1.3	—
4,6-Di-*O*-methyl-D-mannose	G	3.29	18.8	21.2
2,6-Di-*O*-methyl-D-galactose	H	3.62	9.7	1.8
3,6-Di-*O*-methyl-D-galactose	I	4.30	2.0	1.1
2,4-Di-*O*-methyl-D-glucose	K	5.10	2.0	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 mol % of the methylated sugars are given relative to that of 2,3-di-*O*-methyl-L-rhamnose, which it is assumed represents the mol % of L-rhamnose in the original lipopolysaccharide.

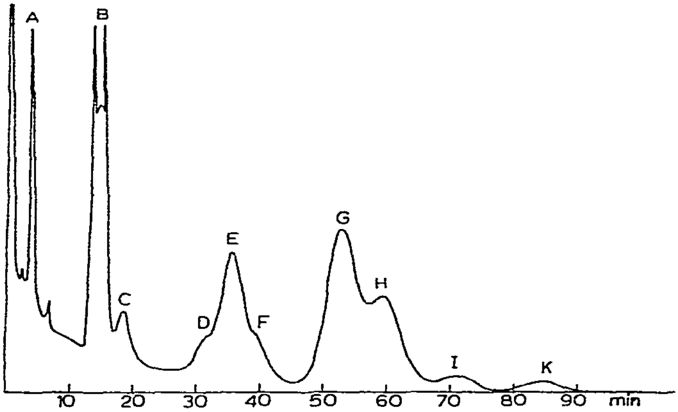


Fig. 1. G.l.c. separation of methylated sugars, as their alditol acetates, obtained from the hydrolysate of the fully methylated lipopolysaccharide.

The substance in peak F is, according to its mass spectrum, derived from a 3,4,6-tri-*O*-methylhexose. The retention time (T 2.50) differs from that of the D-mannose derivative (T 1.95). Rerefence samples of the corresponding D-glucose and

D-galactose derivatives were not available. This methyl ether was not detected in the methylation analysis of the 395 MS lipopolysaccharide, where the component was probably hidden in the tail of the large peak of the 2,4,6-tri-*O*-methyl-D-galactose derivative.

The component in peak I, previously identified as a 3,6-di-*O*-methylhexose derivative, has now been identified as the 3,6-di-*O*-methyl-D-galactose derivative.

The *T*-values of some D-galactose and D-mannose derivatives, for which reference samples were not available, were determined as follows. Methyl β-D-galactopyranoside and methyl α-D-mannopyranoside, respectively, were partially methylated with the Purdie reagent, the reaction mixtures were converted into alditol acetates and fractionated by g.l.c., and the components were identified by mass spectrometry.

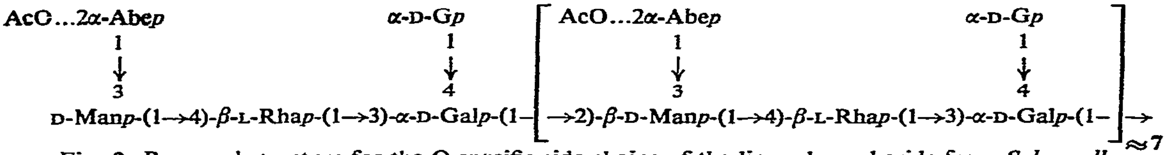
The molar percentages of D-glucose, D-galactose, D-mannose, L-rhamnose, and abequose, calculated from the methylation analysis and referred to the percentage of L-rhamnose found in the sugar analysis, are 12:24:22:20:4, respectively. The agreement between these values and the sugar analysis is good, except for abequose. It seems probable that a considerable proportion of the volatile 2,4-di-*O*-methylabequose and derivatives was lost during concentration of the solutions.

Part of the lipopolysaccharide was subjected to a mild, acid treatment, in order to hydrolyse all of the abequosidic linkages. A methylation analysis was then performed on the remaining polymeric and oligomeric material. As with the 395 MS lipopolysaccharide, it was found that virtually all of the 4,6-di-*O*-methyl-D-mannose had disappeared, being replaced by 3,4,6-tri-*O*-methyl-D-mannose.

To locate the *O*-acetyl groups, the free hydroxyl groups in the lipopolysaccharide were protected as acetals by treatment with methyl vinyl ether, the *O*-acetyl groups were removed under alkaline conditions, and the product was methylated and hydrolysed. Analysis of the mixture of sugars obtained, as their alditol acetates, revealed the presence of D-glucose, D-galactose, D-mannose, and L-rhamnose, in the same relative proportions as in the original lipopolysaccharide. About 50% of the abequose had disappeared and was replaced by 2-*O*-methylabequose.

The lipopolysaccharide, in 0.05M sulphuric acid, was kept at 80°, and the change in optical rotation was followed. A rapid decrease from $[\alpha]_{5780}$ $+74 \rightarrow +42°$ was observed over 4 h, followed by a slower decrease to +24° over 20 hours. This demonstrates that the abequose residues are α-D linked and that the L-rhamnose is β-linked.

The present results show that the O-antigenic side-chains of the *S. typhimurium* LT2 lipopolysaccharide have the same general structure (Fig. 2) as was found for the 395 MS lipopolysaccharide. There are, however, some differences between the two polysaccharides. In the LT2 lipopolysaccharide, about 50% of the D-galactose residues in the side chains carry α-D-glucopyranose residues in the 4-position, whereas, in the 395 MS lipopolysaccharide, the proportion is considerably lower. Furthermore, in the former, only about 50% of the abequose residues are acetylated in the 2-position, whereas, in the latter, all of the abequose residues are substituted. The average number of repeating units in the side chains in the former is approximately 8 and in the latter approximately 1:1, as estimated from the percentages of 2,4,6-tri- and 4,6-di-*O*-methyl-D-mannose.



$$\overset{\substack{\text{AcO}\ldots2\alpha\text{-Abe}p\\ 1\\ \downarrow\\ 3}}{\text{D-Man}p}\text{-}(1\to4)\text{-}\beta\text{-L-Rha}p\text{-}(1\to3)\text{-}\overset{\substack{\alpha\text{-D-G}p\\ 1\\ \downarrow\\ 4}}{\alpha\text{-D-Gal}p}\text{-}(1-\left[\to2)\text{-}\beta\text{-}\overset{\substack{\text{AcO}\ldots2\alpha\text{-Abe}p\\ 1\\ \downarrow\\ 3}}{\text{D-Man}p}\text{-}(1\to4)\text{-}\beta\text{-L-Rha}p\text{-}(1\to3)\text{-}\overset{\substack{\alpha\text{-D-G}p\\ 1\\ \downarrow\\ 4}}{\alpha\text{-D-Gal}p}\text{-}(1-\right]_{\approx7}\to$$

Fig. 2. Proposed structure for the O-specific side-chains of the lipopolysaccharide from *Salmonella typhimurium* LT2.

The minor components identified in the methylation analysis, 2,3,4,6-tetra-*O*-methyl-D-galactose, 3,4,6-tri-*O*-methylhexose, 3,6-di-*O*-methyl-D-galactose, and 2,4-di-*O*-methyl-D-glucose are probably derived from the basal core of the lipopolysaccharide. The presence of 2,3,4,6-tetra-*O*-methyl-D-galactose, 2,4-di-*O*-methyl-D-glucose, and a tri-*O*-methylhexose is consistent with general ideas on the structure of this part of the molecule[2,3], but the presence of 3,6-di-*O*-methyl-D-galactose is not. The structural significance of these findings is, however, uncertain, and structural studies on the basal core should preferably be performed with the R-mutants, which lack the O-specific side-chains.

EXPERIMENTAL

The bacteria were killed by irradiation with ^{60}Co γ-rays (12000 rad/min for 1 h) before disintegration, but, in all other respects, the experimental methods used were identical with those used in the investigation of the *Salmonella typhimurium* 395 MS lipopolysaccharide[1].

ACKNOWLEDGMENTS

We are indebted to Pharmacia AB for a fellowship to one of us (S. S.), to Professor Gunnar Widmark, Department of Analytical Chemistry, Stockholm University, for placing the mass spectrometry facilities at our disposal, to Professor Lars Ehrenberg, Department of Radiobiology, Stockholm University, for the opportunity of using the γ-radiation source, and to Dr. Björn Lüning, Department of Organic Chemistry, Stockholm University, for recording the n.m.r. spectra. The skilled technical assistance of Miss Mary Andersson and Miss Berit Lindholm is also gratefully acknowledged. This work was supported by grants from the Swedish Medical Research Council (No. B68-4OX-656-03), from Emil and Wera Cornells Stiftelse and The Swedish Natural Science Research Council.

Institutionen för organisk kemi, C. G. HELLERQVIST
Stockholms Universitet, B. LINDBERG
Stockholm (Sweden) S. SVENSSON
Bakteriologiska institutionen, Karolinska Institutet, T. HOLME
Stockholm (Sweden)
Statens bakteriologiska laboratorium, A. A. LINDBERG
Stockholm (Sweden)

REFERENCES

1 C. G. Hellerqvist, B. Lindberg, S. Svensson, T. Holme, and A. A. Lindberg, *Carbohyd. Res.*, 8 (1968) 43.
2 O. Lüderitz and O. Westphal, *Angew. Chem.*, 3 (1966) 172.
3 O. Lüderitz, A. M. Staub, and O. Westphal, *Bacteriol. Rev.*, 30 (1966) 1801.
4 H. Björndal, B. Lindberg, and S. Svensson, *Acta Chem. Scand.*, 21 (1967) 1801.
5 H. Björndal, B. Lindberg, and S. Svensson, *Carbohyd. Res.*, 5 (1967) 433.

(Received May 17th, 1968)

Note

Formation of a difficultly soluble saccharate of lactulose with calcium hydroxide

The formation of compounds ("saccharates") of D-fructose and the sugars of the sucrose group with barium, calcium, and strontium hydroxides is well known. The formation of saccharates has been used for isolating these sugars from mixtures containing aldoses, and also from biological materials[1-3]; however, it has not been applied with reducing oligosaccharides containing ketose moieties.

In this study, it is shown that, among the alkaline earth hydroxides, that of calcium gives with lactulose (4-*O*-β-D-galactopyranosyl-D-fructose) a difficultly soluble saccharate which may be used for partial isolation of lactulose from mixtures.

EXPERIMENTAL

Materials. — The alkaline earth hydroxides were purchased from the Kanto Chemical Co., Inc. (Tokyo) as type-guaranteed reagents and were used without further purification. Crystalline lactulose was prepared according to the method of Montgomery and Hudson[4], modified in that ion exchangers were used to remove lactobionic acid and inorganic salts. Lactose (Koso Chemical Co., Ltd., Tokyo) was purified by recrystallization.

Determination of lactulose. — To 10 ml of a 2.0–2.5% solution of lactulose in each of a number of 15–16 × 150 mm test tubes was added a weighed amount of one of the various alkaline earth hydroxides. The samples were mixed and kept in an ice–water bath with frequent agitation for 1 h at 0–4°, and the samples were then filtered. For determination of lactulose remaining in the filtrates, the cysteine–carbazole–sulfuric acid reaction[5] was applied to 1 ml of diluted filtrate as described previously[6]. A 1:500 dilution of the filtrates was suitable for the measurements.

Paper chromatography of the reaction mixtures. — Circular paper chromatography was performed according to the procedures in the previous paper[7] with 4:1:2 butyl alcohol–acetic acid–water as the developer. Spots were detected by aniline hydrogen phthalate[8] and Dimedon[9] spray reagents. Calcium was determined volumetrically with sodium (ethylenedinitrilo)tetraacetate, with calcein as the indicator[10].

Formation of saccharates of lactulose with alkaline earth hydroxides. — Equal aliquots of a 2.5% solution of lactulose were respectively treated with barium, calcium, and strontium hydroxides at 0–4°. The hydroxides dissolved rapidly on vigorous agitation. The proportions of the different alkaline earth hydroxides added are given in Table I. It is evident from the diminished content of lactulose in the filtrate that the formation of a difficultly soluble lactulose compound occurs with calcium hydroxide, but not with barium or strontium hydroxides. In most cases, the calcium hydroxide–lactulose complex formed suddenly as a pasty or flocculent precipitate within 3–5 min after addition of the calcium hydroxide.

TABLE I

EFFECT OF THREE ALKALINE EARTH HYDROXIDES ON THE PRECIPITATION OF SACCHARATES OF LACTULOSE

Alkaline earth hydroxide	*Wt. added (mg)*	*Molar ratio of hydroxide: lactulose*	*Lactulose remaining in the filtrate*[a] *(%)*
Calcium hydroxide	51	0.99	89.2
	102	1.96	32.5
	153	2.95	26.7
Barium hydroxide	440	2.00	98.5
Strontium hydroxide	372	2.00	99.0

[a]The percentage of the initial concentration (2.5%) of lactulose in the starting solution.

After the addition of 105 mg of calcium hydroxide to 10 ml of 2.5% lactulose solution, the mixture was centrifuged at 5000 *g* for 10 min at 0°, and immediately afterwards both the supernatant liquor and the precipitate were analyzed. The precipitate was suspended in a small volume of water, and then 200 μl of concentrated hydrochloric acid was added dropwise to dissolve the solid completely. The temperature of the suspension during mixing, and subsequently, did not exceed that of the ice bath. (Uncontrolled heating would cause degradation of lactulose.) Calcium and lactulose were determined: 18.6 and 80.8 mg in the supernatant liquor, and 38.2 and 162.5 mg in the precipitate, respectively. Thus, the molar ratio of calcium hydroxide to lactulose in the precipitate was 2.02:1. As the calcium hydroxide dissolved to form an almost clear solution before precipitation occurred, the ratio should fairly represent the composition of the calcium hydroxide compound of lactulose ("calcium lactulosate").

Precipitation of calcium lactulosate in the presence of lactose. — Solutions were prepared containing 20 mg of lactulose per ml in aqueous solutions containing graded concentrations of lactose. To each of these preparations was added 300 mg of calcium hydroxide at 0–4°, and each mixture was then assayed for lactulose. In Fig. 1, the optical absorbance at 560 nm, after color development in the reaction mixture, is plotted as a function of the concentration of lactose. Lactulose remaining in the filtrate of the treated mixture increases with increasing concentration of lactose

Concentrations as low as 4.5% of lactose were found to depress the precipitation of the lactulosate significantly.

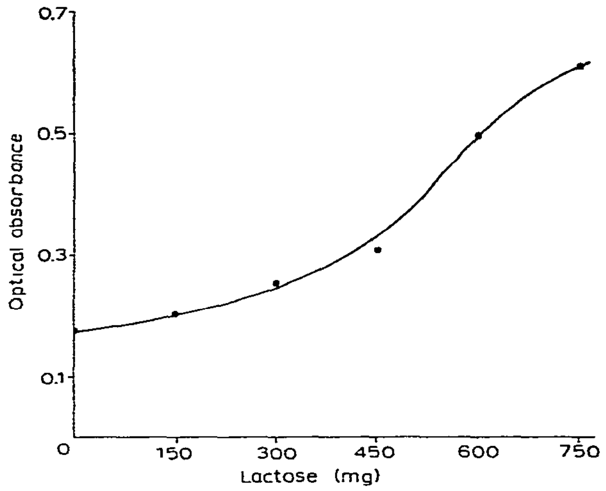


Fig. 1. Effect of lactose on the precipitation of calcium lactulosate. The concentration of lactulose was 200 mg/ml before the treatment with calcium hydroxide. The optical absorbance is a measure of the lactulose in the filtrate (not precipitated by calcium hydroxide).

Table II presents the data obtained from the samples to which had been added different weights of calcium hydroxide (10 ml of the 2.0% lactulose solution containing 3.0% of lactose). The lactulose remaining in solution is decreased markedly with increased weight of calcium hydroxide added, in the range of 105–400 mg.

TABLE II

PRECIPITATION OF LACTULOSE IN LACTOSE–LACTULOSE MIXTURES CONTAINING VARIOUS PROPORTIONS OF CALCIUM HYDROXIDE[a]

Calcium hydroxide (mg)	*Lactulose remaining in the filtrate*	
	Optical absorbance[b]	*%*[c]
105	0.530	97.0
200	0.325	57.0
400	0.185	32.5
600	0.170	29.8

[a]Assays were performed as described in the text, except that the amounts of calcium hydroxide were varied as indicated. [b]See legend to Fig. 1. [c]Based upon the initial lactulose content, as shown in Table I.

Regeneration of lactulose from the precipitated calcium hydroxide complex. — Samples (50 ml) of 2.5% lactulose solution (with or without 1 g of added lactose) were treated with 1.5 g of calcium hydroxide. To prevent the formation of a pasty precipitate, it was found necessary to add the reactants alternately in small portions, with mixing.

At completion of the precipitation, the complexes were filtered off by suction, and washed with three 15-ml portions of chilled lime-water. The filter cakes were then suspended in chilled water, and the suspensions were carbonated at 2–6° by addition of a small amount of chopped Dry Ice. After the calcium carbonate formed had been filtered off and washed, the filtrates were evaporated to dryness *in vacuo*. No indication of a carbohydrate other than lactulose (R_F 0.45) was found in the concentrates. The yields and optical rotations of the anhydrous products were 0.8 g, $[\alpha]_D^{19}$ $-50°$ (equilibrium, *c* 3, water) for the sample without lactose, and 0.7 g, $[\alpha]_D^{19}$ $-49°$ (*c* 2, water) for the sample with lactose. The $[\alpha]_D^{20}$ of anhydrous lactulose is $-51°$.

Essentially the same results were obtained by replacing the 1 g of lactose with 0.5 g of D-glucose or D-galactose. When the concentration of lactulose was below 1%, formation of the precipitate did not proceed satisfactorily. If the precipitate was kept for a longer time prior to carbonation, gradual decomposition of lactulose could be detected. Even in samples kept for a day in a refrigerator (0 ±4°), color changes were observed.

DISCUSSION

The difficultly soluble saccharate of D-fructose has been shown to contain 1 atom of calcium per molecule[1]. Evidence has now been presented which indicates that one molecule of calcium lactulosate contains two atoms of calcium. The weight ratio (0.235) of lactulose to calcium in the lactulosate coincides with the calculated ratio (0.234), assuming that the formula is $C_{12}H_{22}O_{11}\cdot 2Ca(OH)_2$. Thus, it would appear that the precipitate is dicalcium lactulosate. In this connection, it may be of interest that saccharates of sucrose, raffinose, and stachyose, containing two moles of alkaline earth hydroxides per molecule, have been isolated[11,12].

Since lactose, like other sugars and polyhydric alcohols, forms a soluble complex with calcium ion[13–16], the decreasing precipitation of lactulosate in the presence of lactose might be due to the chelation of part of the calcium hydroxide with the lactose. It would seem, therefore, that the increased solubility of calcium lactulosate in the presence of lactose may be reversed by addition of an excess of calcium hydroxide (above the concentration range required for formation of a chelate with lactose). The changes in solubility of the calcium lactulosate are in general accord with the findings of earlier investigators, who observed an increased solubility of calcium D-fructosate proportional to the concentration of D-glucose in solution[1].

These techniques may be used for achieving partial separation of lactulose from lactose or other aldoses in solution.

Laboratory of Animal Products Technology,
College of Agriculture,
Tohoku University,
Sendai (Japan)

SUSUMU ADACHI

REFERENCES

1 R. F. Jackson, C. G. Silsbee, and M. G. Proffitt, *Sci. Papers Bur. Std.*, 20 (1926) 587.
2 W. Z. Hassid and C. E. Ballou, in *The Carbohydrates: Chemistry, Biochemistry, Physiology* (W. Pigman, Ed.), Academic Press, New York, 1957, p. 478.
3 T. Yamane, in *Sugar Handbook* (E. Hamaguchi and Y. Sakurai, Eds.), Asakura, Tokyo, 1964, p. 13.
4 E. M. Montgomery and C. S. Hudson, *J. Amer. Chem. Soc.*, 52 (1930) 2101.
5 Z. Dische and E. Borenfreund, *J. Biol. Chem.*, 192 (1951) 583.
6 S. Adachi, *Anal. Biochem.*, 12 (1965) 137.
7 S. Adachi and H. Sugawara, *Arch. Biochem. Biophys.*, 100 (1963) 468.
8 S. M. Partridge, *Nature*, 164 (1949) 443.
9 S. Adachi, *Anal. Biochem.*, 9 (1964) 224.
10 H. A. Ntailianas and R. M. Whitney, *J. Dairy Sci.*, 47 (1964) 19.
11 J. E. Mackenzie and J. P. Quin, *J. Chem. Soc.*, (1929) 951.
12 J. A. Rendleman, Jr., *Advan. Carbohyd. Chem.*, 21 (1966) 209.
13 P. Charley and P. Saltman, *Science*, 139 (1963) 1205.
14 J. E. Hodge, E. C. Nelson, and B. F. Moy, *J. Agr. Food Chem.*, 11 (1963) 126.
15 M. Srinivasan, P. N. Achuta Murthy, A. Sreenivasan, and V. Subrahmanyan, *Food Technol.* 17 (1963) 112.
16 A. van Kreveld, *Neth. Milk Dairy J.*, 17 (1963) 313.

(Received May 9th, 1968; in revised form, June 6th, 1968)

Note

The degree of polymerisation of xylan in the cell wall of the green seaweed *Penicillus dumetosus*

The cell walls of several siphoneous, green algae are known to be constituted principally of a xylan[1—3]. Chemical investigations of the xylans of some of these species suggest that only β-(1→3)-linked D-xylose residues may be present[1,3]. The seaweed under discussion here does not appear to have been examined extensively by chemical means, but studies of several "xylan" weeds, including *Penicillus* species, by physical techniques (including X-ray diffraction analysis) have established that xylan chains occur bonded together helically in partly crystalline microfibrils[2,4]. The latter observation, especially, suggests that the xylan chains themselves may occur as reasonably long molecules in the cell walls. This is true of cellulose which has a similar biological function to the algal xylans. However, available estimates of the degrees of polymerisation (*DP*) of the xylans from several species favour[1] both number and weight averages in the range 42–67. These results suggest that the lengths of the native xylans are both uniform and short (200–300 Å). The earlier workers did not comment on the magnitudes of the measured *DP* values, but, since the isolation procedures included treatments with hot acidic and alkaline solutions, it seems probable that these are too low.

To obtain further information, a more detailed study of the xylan from *Penicillus dumetosus* has been undertaken. This has involved the preparation of a fully nitrated xylan derivative which can be made in quantitative yields from the cell-wall fibre. This derivative is readily soluble in ethyl acetate and other organic liquids, and so is suitable for molecular weight studies. Some preliminary results obtained by the technique of osmometry are given here.

EXPERIMENTAL AND RESULTS

The fibrous cell-wall material was obtained by successively extracting fresh fronds (from a deep-frozen supply) with aqueous solutions of butyl alcohol (saturated), sodium lauryl sulphate (0.3% w/v), and urea (50% w/v) several times for short periods (30 sec) in a Waring Blendor. The residue was suspended overnight in aqueous EDTA (2% w/v), and then dialysed against running water (4 days), centrifuged, and washed with methanol and ether. The insoluble residue so obtained (*ca.* 20% of the dry plant) consisted of carbohydrate (*ca.* 75%, determined after acid hydrolysis[5]) and protein (*ca.* 12%), the remainder being most probably bound water and residual inorganic substances. (The native xylan is known to include 10–15% of bound water in its structure even after extensive drying over phosphorus pentoxide[4].) The carbohydrate component consisted of xylose (94%) and glucose (6%), as determined by a gas–liquid chromatographic procedure after acid hydrolysis and conversion of the reducing monosaccharides into the alditol acetates.

Xylan nitrates were prepared by suspending the fibrous cell-wall (0.1 g) for 1 h in a nitration mixture (10 ml) containing 40.4 g of phosphorus pentoxide per 100 g of 90% fuming nitric acid (sp. gr. 1.483 at 20°)[6]. In this way, nitrations were carried out at temperatures ranging from −40 to +17°. The crude, nitrated products were precipitated in ice–water and washed well with cold water. They were stabilised by treatment with water (3 × 100 ml for 2 min) at 90°, and finally washed with methanol and ether. The products (*ca.* 0.13 g) were suspended in ethyl acetate (10 ml) and agitated gently for 1 h before centrifuging (4000 *g* for 15 min). The supernatants were concentrated to 5 ml and added to light petroleum (100 ml). The purified xylan nitrates were obtained as fibrous, white solids after drying *in vacuo* for 1 h at 50°. Nitrogen contents were determined by a modified Kjeldahl procedure[7] (theoretical nitrogen content for a di-nitrated xylan is 12.6%). Portions of the nitrated xylans were denitrated with ammonium hydrosulphide solution[8]. The reconstituted xylans (80% recovery) contained 90–94% of carbohydrate, consisting of xylose and glucose in the same proportions as in the original xylan.

Osmotic pressure measurements were obtained at 30° in ethyl acetate solution by means of a Hewlett–Packard model 503 osmometer. Stable solution pressures were usually obtained within 5–10 min, and no permeation of the membrane by solute was observed.

The results (Table I, Fig. 1) show that the average *DP* values of xylan chains in *Penicillus dumetosus* (and presumably other species) are considerably greater than hitherto indicated in the literature. The values of *DP* obtained depend markedly on the temperature of the nitration reaction, due presumably to the increasing degradation of chains with rising reaction temperature, although at temperatures below −20°, a limiting value of DP_N appears to be obtained. This value provides a minimum estimate of about 1000 for the average *DP* of the native xylan.

TABLE I

Nitration temperature (°)	*Yield of xylan nitrate*[a] (%)	*N* (%)	DP_N
+17	95	12.4	125 ±13
+ 2	94	12.3	450 ±45
−10	89	12.4	700 ±70
−20	80	12.5	950 ±95
−40	30	—	840 ±84

[a] Calculated on cell-wall xylan content of 75%.

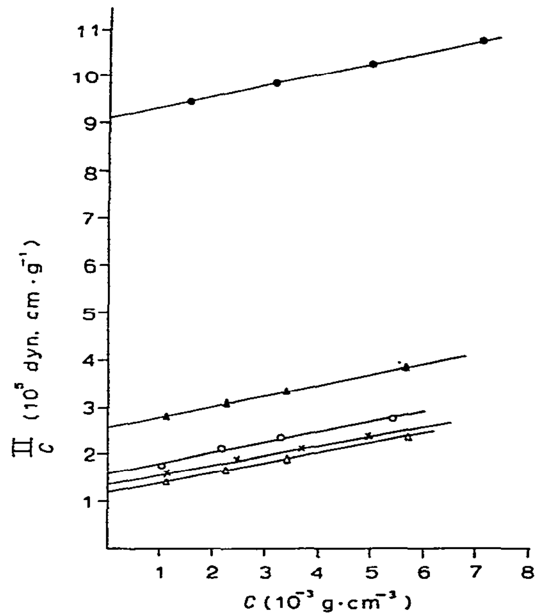


Fig. 1. Plots of π/c v concentration: ●—●, +17°; ▲—▲, +2°; ○—○, −10°; △—△, −20°; ×—×, −40°.

Work is in progress to complement these results by investigating this polymer system with other physical techniques.

ACKNOWLEDGMENT

I thank Dr. P. B. Tomlinson, Fairchild Tropical Garden, Florida, U.S.A., for the supply of *Penicillus dumetosus*, and Professor R. D. Preston, F.R.S., for his interest.

The Astbury Department of Biophysics, W. MACKIE
The University,
Leeds 2 (Great Britain)

REFERENCES

1 Y. IRIKI, T. SUZUKI, K. NISIZAWA, AND T. MIWA, *Nature*, 187 (1960) 82.
2 E. FREI AND R. D. PRESTON, *Proc. Roy. Soc.* (London), *Ser. B*, 160 (1964) 293.
3 I. M. MACKIE AND E. PERCIVAL, *J. Chem. Soc.*, (1959) 1151.
4 E. D. T. ATKINS, K. D. PARKER, AND R. D. PRESTON, *Proc. Roy. Soc.* (London), *Ser. B*, in press.
5 M. DUBOIS, K. A. GILLES, J. K. HAMILTON, P. A. REBERS, AND F. SMITH, *Anal. Chem.*, 28 (1956) 350.
6 W. J. ALEXANDER AND R. L. MITCHELL, *Anal. Chem.*, 21 (1949) 1497.
7 T. E. TIMELL AND C. B. PURVES, *Svensk Papperstid.*, 54 (1951) 328.
8 T. E. TIMELL, *Pulp Paper Mag. Can.*, 56 (1955) 104.

(Received June 22nd, 1968)

Note

Déplacement nucléophile d'un *p*-toluènesulfonate *exo* sur hétérocycle furanose — Accès au 3-amino-3-désoxy-D-ribose

Dans un récent travail[1], nous avons montré que l'assistance d'un groupe hydroxyle était susceptible de favoriser le déplacement, par des agents nucléophiles, de sulfonates stériquement encombrés.

Nous rapportons l'application de cette méthode à la préparation du 3-amino-3-désoxy-D-ribose. On sait que ce composé est un constituant de l'antibiotique puromycine.

Deux méthodes d'accès au 3-amino-3-désoxy-D-ribose ont été décrites par Baker et coll.[2]. Elles font toutes deux intervenir l'ouverture d'un cycle époxyde par l'ammoniac et conduisent de ce fait à des mélanges d'isomères qui rendent laborieuse la préparation de cet aminopentose.

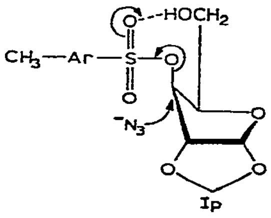
Nous sommes partis d'un dérivé aisément accessible, le 1,2-*O*-isopropylidène-3-*O*-*p*-tolylsulfonyl-D-xylofuranose **1** obtenu en une étape en partant du D-xylose.

L'action de l'azothydrate de sodium sur ce composé **1** en solution dans la *N'*, *N"*, *N'''*-hexaméthylphosphoramide conduit avec un rendement de 16% au dérivé 3-azido **2** de configuration *ribo*.

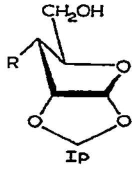
Le spectre infra-rouge de ce composé montre bien l'absorption à 2100 cm^{-1} caractéristique du groupement azide. Le spectre de masse montre des fragmentations à M-15, M-31 et M-74 correspondant au clivage du groupement *O*-isopropylidène[3] et de l'alcool primaire exocyclique en C-5.

Le spectre de r.m.n. est très proche de celui obtenu pour le 3-désoxy-1,2-*O*-isopropylidène-3-thiocyano-D-ribofuranose[1]. Outre le doublet à δ 1,46 ppm (6 H) caractéristique du radical isopropylidène, on remarque un doublet à δ 5,78 ppm dû à l'hydrogène anomérique couplé avec le proton en α; $J_{1,2}$ 4 Hz. L'hydrogène en C-2 donne un triplet à δ 4,71 ppm; $J_{1,2}$ 4,5 Hz, $J_{2,3}$ 4,5 Hz. Le proton en position 3, déplacé par l'effet inductif du groupement azide, se retrouve à δ 3,53 ppm sous forme d'un quadruplet; $J_{2,3}$ 4,5 Hz et $J_{3,4}$ 10 Hz. Le proton en C-4 se manifeste par un doublet de triplets à δ 4,13 ppm; $J_{3,4}$ 10 Hz et $J_{4,5}$ 5 Hz.

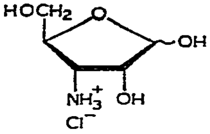
Ces résultats confirment l'obtention du 3-azido-3-désoxy-1,2-*O*-isopropylidène-D-ribofuranose. Il est probable que la formation de ce composé partant d'un sulfonate de configuration *xylo* fait intervenir un mécanisme identique à celui que nous avons proposé pour la formation du thiocyanate correspondant[1]. En effet, l'action de l'azothydrate de sodium sur le 5-désoxy-1,2-*O*-isopropylidène-3-*O*-*p*-tolylsulfonyl-D-xylofuranose ne conduit pas dans les mêmes conditions expérimentales à un déplacement appréciable du sulfonate en C-3. La formation probable d'une liaison hydrogène entre l'un des atomes d'oxygène du sulfonate et le groupe hydroxyle en C-5 serait donc, comme nous l'avons proposé précédemment[1], à l'origine d'un déplacement des charges négatives sur le sulfonate, favorisant ainsi l'établissement d'un état de transition plus propice à l'attaque nucléophile sur C-3.



1



2 R= N_3
3 R = NH_2
4 R= NH—$C_6H_3(NO_2)_2$



5

L'hydrogénation catalytique de l'azide **2** en présence de platine d'Adams conduit avec un rendement quantitatif au 3-amino-3-désoxy-1,2-*O*-isopropylidène-D-ribofuranose (**3**) isolé sous forme de son dérivé 2,4-dinitrophényle **4**.

L'action de l'acide chlorhydrique dilué sur le dérivé aminé **3** conduit au 3-amino-3-désoxy-D-ribose **5** isolé sous forme de son chlorhydrate et identique au composé décrit par Baker et coll.[2].

PARTIE EXPÉRIMENTALE

Méthodes générales. — Les spectres de r.m.n. ont été mesurés dans $CDCl_3$ à la fréquence de 60 MHz sur appareil Varian A-60 par M^{me} L. Alais. Les déplacements chimiques sont mesurés en δ (ppm) à partir de la raie du tétraméthylsilane prise comme zéro de référence.

Le spectre de masse a été mesuré sur un appareil Atlas CH_4 sous la direction de M. B. C. Das.

Les microanalyses élémentaires ont été réalisées sous la direction de M^{lle} Müller du Laboratoire Central de Micro-analyse du C.N.R.S.

Les pouvoirs rotatoires ont été déterminés à l'aide du Quick polarimètre de Roussel et Jouan.

Les points de fusion ont été mesurés sous microscope sur platine de Leitz et sont corrigés.

*1,2-O-Isopropylidène-3-O-p-tolylsulfonyl-*D*-xylofuranose* (**1**). — Le 1,2-*O*-isopropylidène-D-xylofuranose[4] (14,5 g) est dissous dans de la pyridine anhydre (55 ml) et additionné de chlorure de triphénylcarbinol (21,3 g, 1 équiv. molaire) préalablement recristallisé. On laisse 48 h à température ambiante. On ajoute alors le chlorure de *p*-toluènesulfonyle (17,3 g, 1,2 équiv. molaire) dissous dans la pyridine (60 ml) et on laisse de nouveau 24 h à température ambiante. Le mélange total est alors versé dans l'eau et la solution aqueuse extraite à plusieurs reprises par le chloroforme. Les solutions chloroformiques réunies sont lavées par l'hydrogénosulfate de potassium à 5% jusqu'à élimination complète de la pyridine, puis par l'hydrogénocarbonate de sodium en solution aqueuse saturée, enfin par l'eau. Après séchage par le sulfate de sodium, la solution chloroformique est amenée à sec et le résidu dissous dans du méthanol (300 ml) contenant 0,16% (v/v) d'acide chlorhydrique commercial. On suit l'hydrolyse du radical triphénylméthyle par chromatographie sur plaque de gel de silice. Au bout de 3,5 h d'agitation à température ambiante, la réaction est complète. L'acide chlorhydrique est éliminé par le carbonate d'argent et la solution méthanolique, après filtration et évaporation, conduit à une huile qui est placée sur colonne de gel de silice équilibrée dans le benzène. Par élution avec benzène–éther (3:1), on obtient 15,5 g de produit identique au composé primitivement décrit[1].

*3-Azido-3-désoxy-1,2-O-isopropylidène-*D*-ribofuranose* (**2**). — Le *p*-toluènesulfonate **1** (9,44 g) est dissous dans la *N'*, *N''*, *N'''*-hexaméthylphosphoramide (50 ml) et additionné d'azothydrate de sodium (10,65 g, 6 équiv. molaires). On garde pendant 4 jours à 110°. Le solvant est alors évaporé sous vide; le résidu, repris par le chloroforme, est lavé par une solution contenant 20% de chlorure de sodium. La solution chloroformique, séchée avec le sulfate de sodium, est réduite sous vide. L'huile obtenue est purifiée par chromatographie sur colonne de gel de silice. Par élution avec benzène–éther (3:1), on obtient 700 mg de produit; $eb_{0,4}$ 105–110°; n_D^{23} 1,4812; $[\alpha]_D^{26}$ +126,7° (*c* 1,42, $CHCl_3$).

Anal. Calc. pour $C_8H_{13}N_3O_4$: C, 44,64; H, 6,09; N, 19,53. Trouvé: C, 44,71; H, 6,06; N, 19,49.

*3-Désoxy-3-(2,4-dinitrophénylamino)-1,2-O-isopropylidène-*D*-ribofuranose* (**4**). — L'azide **2** (930 mg) est dissous dans l'éthanol et hydrogéné à pression ordinaire pendant 4 h en présence d'oxyde de platine d'Adams (280 mg). Une partie (168 mg) du composé aminé ainsi obtenu sous forme d'une huile est dissoute dans l'éthanol (5 ml) et additionnée de 2,4-dinitrofluorobenzène (300 mg) dissous dans l'éthanol (5 ml). Après addition de carbonate de calcium (2 g), on laisse 24 h à température ambiante, avec agitation. L'extraction, réalisée de la manière habituelle, permet d'isoler une huile (250 mg) qui cristallise spontanément et qui est recristallisée dans un mélange benzène–éther de pétrole. P.f. 146–147°; $[\alpha]_D^{28}$ +86° (*c* 1,25, $CHCl_3$).

Anal. Calc. pour $C_{14}H_{17}N_3O_8$: C, 47,32; H, 4,82; N, 11,83. Trouvé : C, 47,60; H, 4,72; N, 11,62.

*3-Amino-3-désoxy-*D*-ribose chlorhydrate* (**5**). — Le dérivé aminé **3** (270 mg) est dissous dans l'acide chlorhydrique aqueux à 1% (10 ml) et la solution est chauffée à reflux pendant 6.5 h. Une chromatographie sur papier indique que l'hydrolyse est alors totale : alcool butylique–acide acétique–eau (4:1:5); papier Whatman n° 1; R_F du dérivé **3** 0,57; R_F du dérivé **5** 0,15; révélation à la ninhydrine pour **3** et **5** et au periodate–benzidine pour **5**. On amène à sec à une température ne dépassant pas 50°. Le chlorhydrate de 3-amino-3-désoxy-D-ribose est recristallisé dans un mélange eau–méthanol contenant 1% d'acide chlorhydrique; p.f. 164–165°; $[\alpha]_D^{26}$ $-38,4 \rightarrow -25,6°$ (10 min. *c* 1,17; H_2O).

Anal. Calc. pour $C_5H_{12}NO_4Cl$: C, 32,35; H, 6,47; N, 7,54. Trouvé : C, 32,42; H, 6,67; N, 7,57.

REMERCIEMENTS

Cette étude a bénéficié d'une subvention du Comité de Biologie Moléculaire (Délégation Générale à la Recherche Scientifique et Technique auprès du Premier Ministre), convention de recherche n° 66 00 308. Nous remercions M. le professeur E. Lederer de l'intérêt qu'il a porté à ce travail.

Institut de Chimie des
Substances Naturelles,
C.N.R.S.,
91, Gif-sur-Yvette (France)

J. DEFAYE
A. M. MIQUEL

BIBLIOGRAPHIE

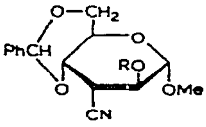
1 J. DEFAYE ET J. HILDESHEIM, *Carbohyd. Res.*, 4 (1967) 145.
2 B. R. BAKER ET R. E. SCHAUB, *J. Org. Chem.*, 19 (1954) 646; B. R. BAKER, R. E. SCHAUB ET J. H. WILLIAMS, *J. Amer. Chem. Soc.*, 77 (1955) 7.
3 D. C. DEJONGH ET K. BIEMANN, *J. Amer. Chem. Soc.*, 86 (1964) 67.
4 P. A. LEVENE ET A. L. RAYMOND, *J. Biol. Chem.*, 102 (1933) 317.

(Reçu le 1er juillet 1968)

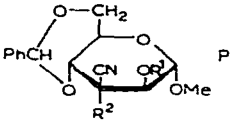
Preliminary communication

Methyl 4,6-*O*-benzylidene-3-cyano 2,3-dideoxy-α-D-*erythro*-hex-2-enopyranoside and its addition reactions

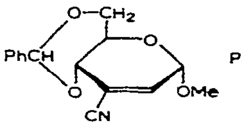
Recently[1], a method of synthesis of such cyano sugars as compound **1** was described, as was the epimerisation **1** → **4** in the presence of dilute, methanolic sodium methoxide. The structure of compound **1** was established by an X-ray crystallographic study on its 2-*O*-*p*-bromobenzenesulphonyl (brosyl) derivative, which showed that there was considerable deformation of the pyranose ring due to interaction between the *syn*-axial cyano and methoxyl groups. It was of interest, therefore, to study the structure of compound **4**, and the preparation of its 2-*O*-brosyl derivative **5** was undertaken.



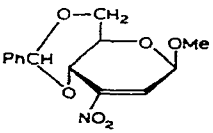
1 R = H
2 R = Ts
3 R = Ac



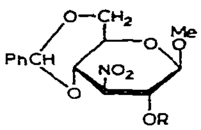
4 R¹=H=R²
5 R¹=Bs, R²=H
6 R¹=Me, R²=H
7 R¹=Me, R²=D



8



9



10 R = H
11 R = Ac

The product, however, did not have the expected structure **5**, but was shown to be the cyano-olefin **8**; m.p. 214.5–215.5°; $[\alpha]_D^{26}$ +159° (*c* 0.94, chloroform); H-2, τ 3.55; M^+ 273.099, $C_{15}H_{15}NO_4$ calc.: 273.1001; which must have arisen *via* prior sulphonylation to give compound **5**, having H-3 and the C-2 ester group antiparallel, which then underwent facile elimination. The altroside **2** (and its brosyl analogue) do not have the required stereochemistry for this type of elimination. That these derivatives can be prepared from compound **1** in pyridine solution shows that epimerisation about the cyano group does not occur in that solvent.

Compound **8** should undergo addition reactions and lead to a variety of branched-chain sugar derivatives. It is of interest to compare its reactions with those of the much-studied[2-4], analogous nitro-olefin **9**. Many reagents, including alcohols and amines, have been added across the double bond of compound **9**, which may also be prepared *in situ* from the precursors **10** and **11**.

Treatment of cyano-olefin **8** with methanolic sodium methoxide gave the 3-cyano-3-deoxy-2-*O*-methyl-mannoside **6** {m.p. 141–141.5°, $[\alpha]_D^{26}$ -13.6° (*c* 0.84, chloroform)}, which was characterised by n.m.r. spectroscopy ($J_{1,2}$ 1 Hz, $J_{2,3}$ 3 Hz, $J_{3,4}$ 11 Hz). Treatment of compound **8** with sodium methoxide in deuteromethanol gave the deutero compound **7**; the quartet at τ 6.7, assigned to H-3, in the n.m.r. spectrum of compound **6** disappeared in the conversion **6** → **7**. Compound **6** was also obtained by prolonged attack of methanolic sodium methoxide on the 2-*O*-toluene-*p*-sulphonyl-altroside **2**. The same reagents with the 2-*O*-acetyl-altroside **3** caused only deacetylation and epimerisation to the mannose derivative **4** (*cf.* ref.3). The methanol–toluene–alumina reagent system (used by Baer[3] on the hydroxy derivative **10**) with compound **2** gave only the cyano-olefin **8**. Treatment of compound **2** with diethylamine–tetrahydrofuran (which, with compound **11**, gave[3] an addition product *via* the nitro-olefin) again gave only the cyano-olefin **8** (90%); this is the best route found so far to compound **8**.

The above differences in the reactivity of compounds **8** and **9** reflect the different abilities of the cyano and nitro groups to activate the double bond. It is of further interest to note that additions to the nitro-olefin system were carried out[2-4] on a β-D anomer (*i.e.* **9**) and gave addition products having the *gluco* configuration, whereas addition to the cyano-olefin was with an α-D anomer (*i.e.* **8**) and gave a product having the *manno* configuration. These results show that the attacking group approaches from the least-hindered side to give a product having a *trans*-1,2-configuration. Since the additions are carried out under basic conditions, the group at C-3 (either nitro or cyano) adopts the more-stable, equatorial position.

We thank ICI (Pharmaceuticals Division) for financial support.

School of Molecular Sciences,
University of Sussex,
Brighton, BN1 9PJ (Great Britain)

B. E. DAVISON
R. D. GUTHRIE

REFERENCES

1 B. E. Davison, R. D. Guthrie, and A. T. McPhail, *Chem. Commun.*, (1968) 1273.
2 H. H. Baer and T. Neilson, *J. Org. Chem.*, 32 (1967) 1068.
3 H. H. Baer, T. Neilson, and W. Rank, *Can. J. Chem.*, 45 (1967) 991.
4 H. H. Baer and F. Kienzle, *J. Org. Chem.*, 32 (1967) 3169.

(Received November 1st, 1968; in revised form, November 30th, 1968)

Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

PREPARATION AND STABILITY OF EXO-AMYLOLYTIC ENZYMES CHEMICALLY COUPLED TO MICROCRYSTALLINE CELLULOSE

S. A. BARKER, P. J. SOMERS,
Department of Chemistry, The University, Birmingham 15 (Great Britain)

AND R. EPTON
Department of Applied Science, Wolverhampton College of Technology, Wolverhampton (Great Britain)

(Received June 22nd, 1968; in revised form, July 25th, 1968)

ABSTRACT

The preparation of active, water-insoluble derivatives of β- and γ-amylase by chemical coupling with a diazotised 3-(*p*-aminophenoxy)-2-hydroxypropyl ether of cellulose, and a water-insoluble derivative of β-amylase by coupling with a 2-hydroxy-3-(*p*-isothiocyanatophenoxy)propyl ether of cellulose is described. The activity retained on insolubilisation is considerably higher than that commonly retained on water-insolubilisation of hydrolytic enzymes active against macromolecular substrates. It is suggested that this is connected with the exo-hydrolytic character of β- and γ-amylase. The insoluble enzymes are relatively more resistant to heat denaturation than the free enzymes in solution.

INTRODUCTION

We have previously described the insolubilisation of α-amylase by chemical reaction with derivatives of microcrystalline cellulose[1]. The choice of microcrystalline cellulose was motivated by the need for a dense, hydrophilic carrier, available in fine particulate form. Low enzyme-to-carrier ratios were employed, in an attempt to avoid over-crowding of the enzyme molecules on the carrier surface, thus minimising the serious steric effects encountered with water-insoluble enzymes active against macromolecular substrates. Although the enzyme preparations having lowest enzyme-to-carrier ratios had the highest activities, maximal activity was still only 6% of that of the free enzyme in aqueous solution. To date, of the hydrolytic enzymes coupled to insoluble carriers with retention of activity, most have been of the endo-hydrolase type. It may be that reactions involving such enzymes are more susceptible to steric effects than are the corresponding reactions in which the enzymes attack the polymeric substrate from the chain ends. It seemed logical therefore to extend our investigations to the exo-amylolytic enzymes β- and γ-amylase.

EXPERIMENTAL

Materials. — The 3-(*p*-aminophenoxy)-2-hydroxypropyl ether hydrochloride of cellulose (20.7 μequiv. of ether linkage/g) was prepared as previously described[1].

Crystalline β-amylase (sweet potato) was purchased from the Sigma Chemical Company, London, England, and a partially purified solution of γ-amylase (*A. niger*) was a gift from the Lord Rank Research Laboratories, High Wycombe, Buckinghamshire, England.

Further purification of γ-amylase. — After exhaustive dialysis against acetate buffer (0.02M, pH 4.5), the γ-amylase solution was diluted with an equal volume of ethanol (96%) cooled to 0–2° and treated with starch according to the method of Cameron[2].

Water-insoluble β-amylase derivatives. — (*a*) *Obtained by coupling β-amylase with the 3-*(p-*aminophenoxy*)*-2-hydroxypropyl ether of cellulose activated with thiophosgene.* The cellulose ether hydrochloride (100 mg) was placed in a stoppered test-tube and magnetically stirred into a slurry with phosphate buffer (3.5M, pH 6.8, 0.5 ml). Thiophosgene solution (10%, 0.2 ml) in carbon tetrachloride was added, stirring was continued for 20 min, and a further aliquot (0.2 ml) of thiophosgene solution was then added. After a further 20 min, acetone (15 ml) was added, and the solid 2-hydroxy-3-(*p*-isothiocyanatophenoxy)propyl ether was recovered by centrifugation. The washing cycle was repeated twice with sodium hydrogen carbonate solution (0.5M, 15 ml) and twice with borate buffer (0.05M, pH 8.6, 15 ml). After decantation of the final washings, a solution of β-amylase (5 mg) in borate buffer (0.05M, pH 8.6, 1 ml) was added, and coupling was allowed to proceed with gentle magnetic stirring for 48 h at 0–5°. The water-insoluble β-amylase derivative was subjected to five cycles of alternate washing with acetate buffer (0.02M, pH 4.8, 15 ml) and a solution of sodium chloride (M, 15 ml) in the same buffer. After two further washings with acetate buffer (15 ml) and final decantation of the washings, the β-amylase derivative was re-suspended in the same buffer (10 ml).

(*b*) *Obtained by coupling β-amylase with the 3-*(p-*aminophenoxy*)*-2-hydroxypropyl ether of cellulose activated with nitrous acid.* The cellulose ether hydrochloride (100 mg) was stirred magnetically at 0° with hydrochloric acid (N, 5 ml). Aqueous sodium nitrite (2%, 4 ml), precooled to 0°, was added, and stirring was continued for 15 min. The diazotised cellulose ether was washed four times with phosphate buffer (0.075M, pH 7.6–7.7, 15 ml) at 0°. After decantation of the final washings, a solution of β-amylase (5 mg) in phosphate buffer (0.075M, pH 7.6–7.7, 1 ml) was added, and coupling was allowed to proceed with gentle magnetic stirring for 48 h at 0–5°. A solution of phenol (0.01%, 5 ml) in saturated, aqueous sodium acetate at 0° was then added. After a further 15 min, the water-insoluble β-amylase derivative was recovered by centrifugation. The supernatant was discarded, and the derivative was subjected to the washing procedure described for the β-amylase derivative prepared by isothiocyanato coupling, and suspended in acetate buffer (10 ml).

The effectiveness of the washing procedure was checked on a control sample of the cellulose ether hydrochloride (100 mg) which had been stirred with a solution of β-amylase (5 mg) in phosphate buffer (0.075M, pH 7.6–7.7, 1 ml) for 48 h at 0–5°.

Water-insoluble γ-amylase derivatives. — (*a*) *Obtained by coupling γ-amylase with the 3-*(p-*aminophenoxy*)*-2-hydroxypropyl ether of cellulose activated with thio-*

phosgene. The coupling was performed as described for the corresponding β-amylase derivative. The washing procedure adopted for the water-insoluble γ-amylase derivative was also similar to that used for the β-amylase derivative, except that acetate buffer (0.02M, pH 4.5) was employed. After decantation of the final washings, the γ-amylase derivative was suspended in the same buffer (10 ml).

(*b*) *Obtained by coupling γ-amylase with the 3-(p-aminophenoxy)-2-hydroxypropyl ether of cellulose activated with nitrous acid.* The coupling was performed as for the corresponding β-amylase derivative. The washing procedure and preparation of the final suspension of the water-insoluble γ-amylase derivative were similar to those used for the water-insoluble γ-amylase derivative prepared by isothiocyanato coupling. The effectiveness of the washing procedure was checked by means of a control experiment similar to that described for β-amylase.

Determination of bound enzymes. — The water-insoluble enzyme derivatives were hydrolysed with 6N hydrochloric acid for 18 h at 110°, and the hydrolysates were neutralised with 6N sodium hydroxide and then subjected to ninhydrin assay[3]. The original enzyme concentration in the hydrolysates was estimated by reference to standard graphs relating absorbance (570 nm) to known amounts of a hydrolysate of the free enzyme. A control assay was performed on the neutralised hydrolysate of the 3-(*p*-aminophenoxy)-2-hydroxypropyl ether of cellulose which demonstrated that the carrier did not affect the ninhydrin assay.

Determination of amylolytic activity. — β-Amylase activity was determined by measuring the initial rate of hydrolysis of a solution of soluble starch (1%) in acetate buffer (0.02M, pH 4.8) at 20°. γ-Amylase activity was similarly determined against soluble starch (1%) in acetate buffer (0.02M, pH 4.5) at 45°. The same rate of magnetic stirring was employed for all determinations.

The initial rate of hydrolysis was determined by following the rate of formation of reducing sugar by assay of aliquots of the digests with the dinitrosalicylate reagent of Bernfeld[4]. The absorbance changes (520 nm) thus recorded were interpreted in terms of reducing sugar by means of standard graphs for maltose (β-amylase) or D-glucose (γ-amylase).

Stability of water-insoluble β- and γ-amylase to storage in suspension in aqueous buffer. — After storage at 0–5° for three months, the activity of the preparations was redetermined, and the percentage of the original activity remaining was calculated.

Stability of water-insoluble β- and γ-amylase to heat denaturation. — A suspension of the β-amylase derivative prepared by the diazonium salt technique was maintained at 40° in acetate buffer (0.02M, pH 4.8) for 7 days, and its activity determined at intervals (see Fig. 1) against magnetically stirred starch solution, as described previously. A control incubation was performed in which the water-insoluble β-amylase suspension was replaced by a solution of free β-amylase in acetate buffer (0.02M, pH 4.8). The percentage of the original activities remaining after various time intervals was then calculated. The experiment was repeated at 50° over an incubation period of 4 days. The stability of γ-amylase (see Fig. 2) was determined by means of similar

TABLE I

PROTEIN INCORPORATED[a] AND ENZYME ACTIVITY IN THE WATER-INSOLUBLE AMYLASE PREPARATIONS

Enzyme	*Enzyme units[b]/mg of free protein*	*Method of protein binding*	*Bound protein (mg/100 mg of derivative)*	*Enzyme units/mg of bound protein*	*Activity (%) retained by enzyme after coupling*
β-Amylase	488.7	isothiocyanato-coupling	1.75	123.2	25.1
		diazo-coupling	4.24	81.4	16.6
γ-Amylase	52.15	isothiocyanato-coupling	0.96	Inactive	Inactive
		diazo-coupling	1.25	9.27	17.7
α-Amylase[c]	87.5	isothiocyanato-coupling	1.42	3.58	4.09
		diazo-coupling	1.09	3.99	5.30

[a] In the control experiments to test the efficiency of the washing procedure in removing physically attached protein, the cellulose carrier had, in the case of β-amylase, a residual protein content of 0.27 mg/100 mg of carrier and, in the case of γ-amylase, a residual protein content of 0.19 mg/100 mg of carrier. [b] One amylase unit was taken as that which liberated reducing sugar equivalent to 1 μmole of maltose at 20° (α- and β-amylase) or 1 μmole of D-glucose at 45° (γ-amylase) in 1 min. [c] Results taken from previous paper[1].

experiments performed over an incubation period of 4 days at 50° and 60° and over an incubation period of 2 days at 70°.

DISCUSSION

The level of amylolytic activity retained by β- and γ-amylase on coupling with the diazotised 3-(*p*-aminophenoxy)-2-hydroxypropyl ether of cellulose, and by β-amylase on coupling with the 2-hydroxy-3-(*p*-isothiocyanatophenoxy)propyl ether of cellulose, is several times higher than that reported[1] for the corresponding derivatives of α-amylase (see Table I). It seems probable that the relatively high activities exhibited by water-insoluble β- and γ-amylase reflect the mode of action of these enzymes. Whereas α-amylase hydrolyses the intracatenary linkages of amylopectin, producing maltose and maltodextrins, β- and γ-amylase effect hydrolysis from the non-reducing chain-ends, with the liberation of maltose and D-glucose, respectively. Step-wise hydrolysis from the chain ends should be much less susceptible to steric interference from the cellulose carrier.

The relatively high levels of activity retained by water-insoluble β- and γ-amylase are also well in excess of the activities retained towards macromolecular substrates on water-insolubilisation of nearly all hydrolytic enzymes previously reported. With

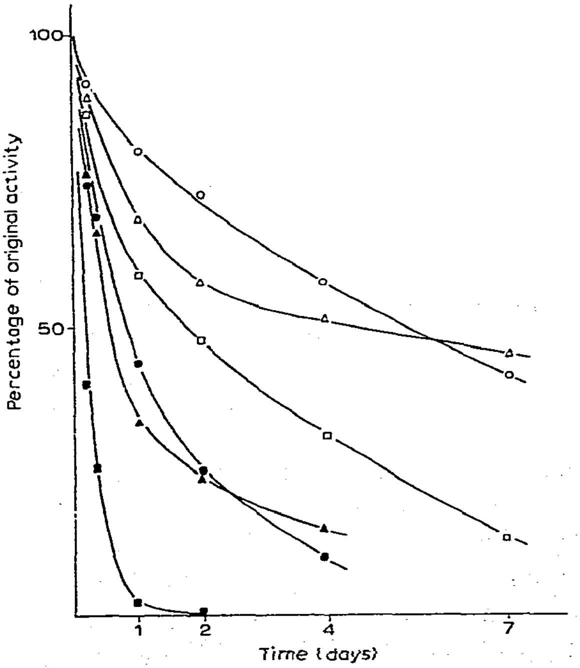


Fig. 1. Heat denaturation of soluble, isothiocyanato-coupled, and diazo-coupled β-amylase on incubation in acetate buffer. ○ — ○, water-insoluble, diazo-coupled β-amylase at 40°; △—△, water-insoluble, isothiocyanato-coupled β-amylase at 40°; □—□, soluble β-amylase at 40°; ●—●, water-insoluble, diazo-coupled β-amylase at 50°; ▲—▲, water-insoluble, isothiocyanato-coupled β-amylase at 50°; ■—■, soluble β-amylase at 50°.

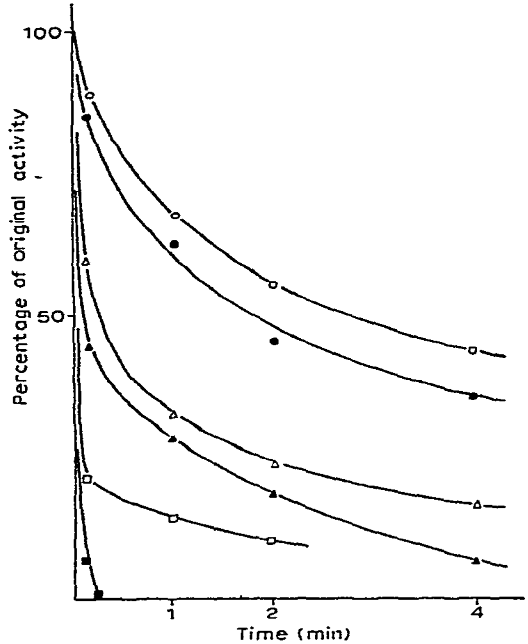


Fig. 2. Heat denaturation of soluble and diazo-coupled γ-amylase on incubation in acetate buffer. ○—○, water-insoluble γ-amylase at 50°; ●—●, soluble γ-amylase at 50°; △—△, water-insoluble γ-amylase at 60°; ▲—▲, soluble γ-amylase at 60°; □—□, water-insoluble γ-amylase at 70°; ■—■, soluble γ-amylase at 70°.

the spectacular exception of papain[5–8], the activity of such surface-bound enzymes towards macromolecules[9,10] never exceeds more than about 5% of the free solution activity.

The lack of activity of the isothiocyanato-coupled γ-amylase preparation demonstrates the importance of investigating covalent binding of a given enzyme by more than one type of linkage. Coupling *via* the isothiocyanato group, which involves reaction with proteins at the α-amino chain-ends and the ε-amino group of lysine[9], must either damage the active site in γ-amylase or cause it to be attached in such a way that the active site is completely hindered.

It is interesting to note that Axen and Porath[10] have prepared enzymically inactive, water-insoluble β-amylase derivatives by chemical reaction with the 2-hydroxy-3-(*p*-isothiocyanatophenoxy)propyl ether of crosslinked dextran (Sephadex). Loss of activity was explained in terms of exclusion of the substrate from the interior of the three-dimensional matrix in which the enzyme was buried. The high retention of enzyme activity in the isothiocyanato-coupled β-amylase preparation, in which the enzyme is dispersed on the carrier surface, seems to support the conclusion of these workers.

The activity retained after storage for 3 months in acetate buffer by diazo-coupled β-amylase (100%), isothiocyanato-coupled β-amylase (89%), and diazo-coupled γ-amylase (100%), was a considerable improvement on the diazo-coupled α-amylase (62% retention of activity over 4 months) previously reported.

Investigation of the rate of heat denaturation of free, isothiocyanato-, and diazo-coupled β- and γ-amylase (Figs. 1 and 2) at different temperatures revealed that the water-insoluble derivatives were more stable in all cases. Two points emerged from these experiments. First, the difference in stability between the soluble and water-insoluble enzymes was much more marked at higher temperatures, and second, some of the chemically bound enzyme molecules are much more stable to heat denaturation than others. This was particularly marked with γ-amylase, the water-insoluble form of which lost over 75% of its activity during incubation for three hours at 70° and was thereafter relatively stable. These observations may be consistent with the binding of some enzyme molecules by more covalent linkages than others. Support of the enzyme at several points by covalent linkages may effectively help in preserving its tertiary structure against disruptive thermal vibrations.

ACKNOWLEDGMENTS

We thank Professor M. Stacey, C.B.E., F.R.S., for his interest in this work, and Ranks Hovis McDougall Ltd. are thanked for financial assistance (R.E.).

REFERENCES

1 S. A. Barker, P. J. Somers, and R. Epton, *Carbohyd. Res.*, 8 (1968) 491.
2 M. P. Cameron, *Ciba Found. Symp.*, Churchill, London, 1967, p. 177.
3 S. Moore and W. H. Stein, *J. Biol. Chem.*, 176 (1948) 367.
4 P. Bernfeld, *Methods Enzymol.*, 1 (1955) 149.
5 J. J. Cebra, D. Givol, H. I. Silman, and E. Katchalski, *J. Biol. Chem.*, 236 (1961) 1720.
6 G. Manecke and G. Günzel, *Makromol. Chem.*, 51 (1962) 199.
7 G. Manecke and H. J. Förster, *Makromol. Chem.*, 91 (1966) 136.
8 G. Manecke and G. Günzel, *Naturwissenschaften*, 54 (1967) 531.
9 R. Axen and J. Porath, *Acta Chem. Scand.*, 18 (1964) 2193.
10 R. Axen and J. Porath, *Nature*, 210 (1966) 367.

Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

SULPHATED POLYSACCHARIDES OF THE GRATELOUPIACEAE FAMILY

PART III[1]. A POLYSACCHARIDE FROM *Phyllymenia cornea*

J. R. NUNN AND H. PAROLIS
Chemistry Department, Rhodes University, Grahamstown (South Africa)

(Received June 24th, 1968; in revised form, July 24th, 1968)

ABSTRACT

Phyllymenia cornea, a red seaweed of the Grateloupiaceae yielded a sulphated polysaccharide phyllymenan, which, on acid hydrolysis, gave D-galactose, 2-*O*-methyl-D-galactose, 4-*O*-methyl-L-galactose, 6-*O*-methylgalactose, and xylose. Alkali treatment of phyllymenan indicated that all of the sulphate units were alkali-stable. Periodate-oxidation studies on phyllymenan, and on both partially and fully desulphated phyllymenan, indicated that no α-glycol groups were formed during desulphation. Partial hydrolysis of phyllymenan resulted in the isolation and characterisation of 4-*O*-β-D-galactopyranosyl-D-galactose and 4-*O*-β-D-galactopyranosyl-2-*O*-methyl-D-galactose.

INTRODUCTION

The family Grateloupiaceae of the Rhodophyceae consists of ten genera[2] comprising some hundred species. The sulphated polysaccharides elaborated by this family of seaweeds have received little attention, and only in the case of the sulphated polysaccharide (aeodan) of *Aeodes orbitosa*[1,3] has any detailed investigation been made. Apart from this, Araki *et al.*[4] have reported the isolation of DL-galactose, 3,6-anhydro-D-galactose, 2-*O*-methyl-L-galactose, 4-*O*-methyl-D-galactose, D-xylose, and 2-*O*-methyl-3,6-anhydro-L-galactose from the sulphated polysaccharide of *Grateloupia elliptica*. In this paper, we report the results of studies on the sulphated polysaccharide (phyllymenan) extracted from *Phyllymenia cornea*.

RESULTS AND DISCUSSION

Phyllymenia cornea is fairly easily recognised by its broad, bright-red, wrinkled fronds. The weed, found in fair quantity in certain areas along the east coast of Southern Africa, grows mainly in deep water and is best collected from the drift after spring tide.

Exhaustive extraction of fresh, wet *Phyllymenia cornea* with hot water, followed by centrifugation, and precipitation of the mucilage into ethanol, afforded a sulphated polysaccharide, phyllymenan. Purification of phyllymenan was effected by dissolution in water, centrifugation, and precipitation into ethanol. Phyllymenan was extracted and purified from three batches of seaweed (see Table I). The weed was found growing at Kowie Point (*ca.* 120 miles east of Port Elizabeth), but not at Palmiet (*ca.* 130 miles east of Port Elizabeth) where it was collected from the drift. All three samples of phyllymenan failed to precipitate from aqueous solution when mixed with potassium chloride solution, and in this respect it resembles aeodan[3] and the sulphated polysaccharide of *Grateloupia elliptica*[4]. Two of the three samples of phyllymenan had identical specific rotations. The lower specific rotation of the third sample can probably be attributed to contamination of the polysaccharide with protein (N, 0.48%). All three samples of phyllymenan contained similar proportions of ester sulphate, and, like aeodan[1,3], contained galactose, 2-*O*-methyl-D-galactose, and 6-*O*-methyl-D-galactose, together with smaller proportions of xylose, 4-*O*-methyl-L-galactose, and 3,6-anhydrogalactose.

TABLE I

POLYSACCHARIDES EXTRACTED FROM *Phyllymenia cornea*

Date of collection	*15-7-1966*	*22-8-1966*	*15-1-1967 to 26-1-1967*
Place	Palmiet	Kowie Point	Palmiet
Weight of *Phyllymenia* (g)	320	200	2,400
Weight of polysaccharide (g)	32	10	240
$[\alpha]_D^{18}$ (degrees)	+81.2 (*c* 0.64)	+81.1 (*c* 0.74)	+63.3 (*c* 0.49)
N (%)	0.00	0.00	0.48
OCH_3 (%)	5.09	4.30	4.98
SO_4^{2-} (%)	18.09	19.28	19.62
$NaSO_3^-$ [*A*] (%)	19.41	20.68	21.05
3,6-Anhydrogalactose[18] (%)	0.52	0.40	0.56
Galactose[5] [*B*] (%)	43.40	45.22	42.20
Monomethylgalactose [*C*] (%)	28.88	24.4	28.27
Sulphated ash (%)	8.24	14.78	15.91
Molar ratios of *A*:*B*:*C*	19:27:16	20:28:14	20:26:16

The quantity of galactose (calc. as $C_6H_{10}O_5$) present in each sample of phyllymenan (see Table I) was determined by the Somogyi micromethod[5], after hydrolysis, and separation by paper chromatography. The quantity of mono-*O*-methylgalactoses (calc. as $C_7H_{12}O_5$) present in each sample of phyllymenan, calculated from the methoxyl values, was found to vary from 24 to 29%. In this respect, phyllymenan differs from aeodan[3] which contained *ca.* 10% of mono-*O*-methylgalactoses. 2-*O*-Methyl-D-galactose was found to be the preponderant mono-*O*-methylgalactose present in each sample of phyllymenan. The ratio of hexose to sulphate in the three samples of phyllymenan was *ca.* 42:20 (*cf. ca.* 42:27 for aeodan[3]). All three samples of phyllymenan contained *ca.* 0.5% of 3,6-anhydrogalactose. It is interesting to note that the polysaccharide of *Grateloupia elliptica*[4] contained 8% of 3,6-anhydro-D-galactose.

The products of hydrolysis of a sample of phyllymenan were separated on a cellulose column, using half saturated butyl alcohol, to give, in crystalline form, galactose, 2-*O*-methyl-D-galactose, and 4-*O*-methyl-L-galactose. Galactose was characterised by conversion into mucic acid. In addition, xylose and 6-*O*-methylgalactose were detected in the mixed fractions, but it was not possible to separate pure samples of these sugars by paper chromatography. However, a fraction containing galactose and xylose and one containing 2-*O*-methylgalactose and 6-*O*-methylgalactose were obtained when the partial hydrolysate of phyllymenan was eluted from a charcoal–Celite column, and it was possible to separate pure samples of xylose and 6-*O*-methylgalactose from these two fractions (see later). No glycerol was detected in the hydrolysates of any of the samples of phyllymenan (*cf.* aeodan[3]).

A sample of phyllymenan was examined in the ultracentrifuge in phosphate buffer, and only a single peak was observed even after 188 min of centrifugation. This peak was not as sharp as that given by aeodan[3]. Nevertheless, the sample appeared to be homogeneous. The infrared spectrum of phyllymenan exhibited the general absorption band for ester sulphate at 1240 cm^{-1}, but did not show any well-defined bands[6] for axial, equatorial, or primary ester sulphate (800–860 cm^{-1}).

Treatment of phyllymenan with alkali in the presence of borohydride[7] (to minimize end-group degradation) resulted in an 89% yield of polysaccharide containing 17.67% of sulphate. Although a reduction in sulphate content of 1.6% occurred during this treatment, there was no increase in the content of 3,6-anhydrogalactose[8]. Hence, phyllymenan differs from aeodan[3] in not containing any (1→2)- or (1→4)-linked galactose 6(or 3)-sulphate residues. Hydrolysis of the alkali-modified phyllymenan, followed by paper chromatography, revealed the presence of galactose, 2-*O*-methylgalactose, 6-*O*-methylgalactose, 4-*O*-methylgalactose (trace), and xylose (trace). The small decrease in sulphate content which occurred during alkali treatment of phyllymenan was probably the result of O–S cleavage of alkali-stable groups.

Complete desulphation of the polysaccharide was achieved by shaking at room temperature for 48 h with 0.15M methanolic hydrogen chloride[9]. This treatment gave a 86% yield of desulphated polysaccharide. Paper-chromatographic examination of a hydrolysate of the non-reducing, methanol-soluble material revealed that galactose, 6-*O*-methylgalactose, and traces of 2-*O*- and 4-*O*-methylgalactose were removed during the desulphation process. Less than 10% of the sugar units present in phyllymenan were removed during the desulphation process. Treatment of phyllymenan with 0.1M methanolic hydrogen chloride for 48 and 72 h at room temperature decreased the sulphate content of the polysaccharide from 19.28% to 8% and 4.7%, respectively.

It is well known that polysaccharides containing uronosyl linkages are resistant to hydrolysis and undergo very little degradation when desulphated with methanolic hydrogen chloride[9]. No uronic acid was detected in any of the samples of phyllymenan, and hence the stability of the glycosidic linkages to cleavage in phyllymenan can probably be ascribed to the shielding effect of the large number of methoxyl groups present in the macromolecule.

Oxidation of phyllymenan with periodate ceased after 72 h at room temperature (Tables II and III) when 0.121 (0.153) mole of periodate had been consumed per C_6-anhydro-unit (sulphate-free anhydrohexose unit). In the case of desulphated and partially desulphated phyllymenan, the reduction of periodate ceased after 24 h at room temperature. The desulphated phyllymenan had then consumed 0.161 (0.161) mole per C_6-anhydro-unit, whereas partially desulphated phyllymenan had consumed 0.149 (0.157) mole per C_6-anhydro-unit.

TABLE II

PERIODATE REDUCED (MOLE) PER C_6-ANHYDRO-UNIT

Time (h)	*5*	*24*	*48*	*72*	*96*
Phyllymenan	0.067	0.092	0.111	0.121	0.121
Desulphated phyllymenan	0.150	0.161	0.161	0.161	0.161
Partially desulphated phyllymenan (SO_4^{2-}, 4.7%)	0.116	0.149	0.149	0.149	0.149

TABLE III

PERIODATE REDUCED (MOLE) PER SULPHATE-FREE ANHYDROHEXOSE UNIT

Time (h)	*5*	*24*	*48*	*72*	*96*
Phyllymenan	0.085	0.116	0.140	0.153	0.153
Desulphated phyllymenan	0.150	0.161	0.161	0.161	0.161
Partially desulphated phyllymenan (SO_4^{2-}, 4.7%)	0.122	0.157	0.157	0.157	0.157

The accurate determination of the true reduction of periodate for every sugar residue in a sulphated heteropolysaccharide is complex, and the reduction of periodate in terms of a "C_6-anhydro-unit" is usually chosen for simplicity and is calculated on the assumption that the polysaccharide is a homohexan. Such results are of value for comparison with those for substances of similar composition. However, the results for sulphated polysaccharides expressed in this manner cannot be used for direct comparison with those for similar desulphated polysaccharides. If, on the other hand, allowance is made for the different sulphate content of these polysaccharides, and the results of periodate reduction are expressed on a sulphate-free basis, *viz.*, per sulphate-free anhydrohexose unit, then direct comparisons become more valid (see Table III). The low consumption of periodate by desulphated phyllymenan suggests the presence of either (*a*) a large proportion of (1→3) links, or (*b*) units containing other glycosidic links, but carrying methoxyl groups and/or branches in such positions as to render these units immune to periodate, or (*c*) a mixture of (*a*) and (*b*).

The reduction of periodate (calculated on a sulphate-free basis) (Table III) by phyllymenan, desulphated phyllymenan, and partially desulphated phyllymenan was approximately the same in each case. This indicates that desulphation of phyllymenan does not result in the production of any new α-glycol groups. These results, together with the fact that all of the sulphate groups in phyllymenan are alkali stable, indicate that only those units which are substituted in position 3 carry sulphate.

Partial hydrolysis of phyllymenan with acid, followed by separation of the neutral products on a charcoal–Celite column, yielded four monosaccharide and several oligosaccharide fractions. Since xylose and 6-*O*-methylgalactose had only been tentatively identified by paper chromatography, the opportunity was taken, while isolating the oligosaccharides, of isolating and characterising these two sugars. The major saccharides isolated were 4-*O*-β-D-galactopyranosyl-D-galactose (**1**) and 4-*O*-β-D-galactopyranosyl-2-*O*-methyl-D-galactose (**2**). The latter compound gave galactose and 2-*O*-methylgalactose on hydrolysis, and galactose as the only reducing sugar after borohydride reduction and hydrolysis. Hence, 2-*O*-methyl-D-galactose occupied the reducing end of the molecule. This was confirmed when the disaccharide failed to react with the triphenyltetrazolium chloride spray. G.l.c. of the methanolysed, methylated disaccharide revealed the presence of methyl 2,3,4,6-tetra-*O*-methylgalactoside and methyl 2,3,6-tri-*O*-methylgalactoside in the molar ratio 1.0:1.08. In addition to the above two disaccharides, smaller amounts of a galactosylgalactose (**3**) and a second methylated oligosaccharide (**4**) were isolated from the partial hydrolysate. The disaccharide **3** gave galactose and unchanged material on hydrolysis, and its infrared spectrum was different from that given by 4-*O*-β-D-galactopyranosyl-D-galactose. It seems likely that **3** is composed of D- and L-galactose or two molecules of L-galactose. The possibility that it is in the furanose form is ruled out by its resistance to acid hydrolysis. The methylated saccharide **4** was obtained as a syrup that gave 2-*O*-methylgalactose, 6-*O*-methylgalactose, and unchanged material on partial hydrolysis with acid. Reduction of this saccharide with borohydride, followed by paper chromatography of a hydrolysate of the product, revealed a major spot having the mobility of 6-*O*-methylgalactose, and a very faint spot having the mobility of galactose. The minute trace of galactose most probably resulted from demethylation of some of the 6-*O*-methylgalactose during the reduction procedure. Examination of the methanolysed, methylated saccharide by g.l.c. revealed peaks due to methyl 2,3,4,6-tetra-*O*-methylgalactoside and methyl 2,3,6-tri-*O*-methylgalactoside. The peaks at T 5.33 and 5.82 were abnormally large, and, since the T values of methyl 2,4,6-tri-*O*-methylgalactoside coincide with these two T values, it was concluded that the abnormality was due to the presence of some methyl 2,4,6-tri-*O*-methylgalactoside. Calculations (based on the assumption that the area under the peak at T 3.85 equals 100% of methyl 2,3,6-tri-*O*-methylgalactoside) indicated that the methyl 2,4,6-tri-*O*-methylgalactoside constituted *ca.* 23% of the methanolysate. The molar ratios of methyl 2,3,4,6-tetra-*O*-methylgalactoside, methyl 2,3,6-tri-*O*-methylgalactoside, and methyl 2,4,6-tri-*O*-methylgalactoside in the methanolysate were thus 1.31:0.76:0.60. These results suggest that this saccharide is a mixture of 2-*O*-methyl-3-*O*-(6-*O*-methyl)-D-galactopyranosyl)-D-galactose and 2-*O*-methyl-4-*O*-(6-*O*-methyl-D-galactopyranosyl-D-galactose. On the other hand, paper chromatography of the oligosaccharide indicated a single compound.

There is thus clear evidence that the macromolecule contains a high proportion of 4-*O*-β-D-galactopyranosyl-2-*O*-methyl-D-galactose residues and a lesser proportion of 4-*O*-β-D-galactopyranosyl-D-galactose residues. There is also tentative evidence for the presence of 2-*O*-methyl-3-*O*-(6-*O*-methyl-D-galactopyranosyl)-D-galactose residues and 2-*O*-methyl-4-*O*-(6-*O*-methyl-D-galactopyranosyl)-D-galactose residues in small proportions. It seems, furthermore, that L-galactose, 3,6-anhydrogalactose, and xylose could be minor constituents.

EXPERIMENTAL

Unless otherwise stated, concentration of solutions was carried out at 40°/20 mm, and specific rotations were measured in water. Paper chromatography was carried out with Whatman No. 1 filter paper. The following solvent systems were used: (1) ethyl acetate–acetic acid–formic acid–water (18:3:1:4), (2) butyl alcohol–pyridine–water (9:2:2), (3) butyl alcohol–ethanol–water (40:11:9), and (4) methyl ethyl ketone saturated with water containing 1% of conc. ammonia. *p*-Anisidine hydrochloride[10], periodate–benzidine[11], aniline–diphenylamine–phosphoric acid[12], and 20% sulphuric acid in ethanol are sprays *a*, *b*, *c*, and *d*, respectively. R_{Gal} values refer to rates of movement relative to that of galactose. Thin-layer chromatography (t.l.c.) was carried out on glass plates coated with silica gel G containing calcium sulphate as binder, employing methyl ethyl ketone–water (85:7) as solvent. R_{TMG} values of methylated sugars refer to the rates of travel relative to that of tetra-*O*-methyl-D-galactose on thin-layer plates. Gas–liquid chromatography (g.l.c.) was carried out on a Beckman GC-2A chromatograph equipped with a flame-ionization detector. The stationary liquid phase, ethylene glycol succinate (14%) and polyethylene glycol (0.1%), was supported on Chromosorb W (80–100 mesh) and maintained at an operating temperature of 170°. Retention times (*T*) are relative to that of methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucopyranoside. Infrared spectra were recorded on a Beckman IR-8 spectrophotometer using KBr discs.

Extraction and purification of polysaccharide. — Wet *Phyllymenia cornea* was mixed with hot water, and acetic acid was added to pH 3. The mixture was heated (0.75 h) with constant stirring until the weed had disintegrated, and the solution was then strained through muslin and centrifuged while still hot, yielding a clear mucilage. The residual weed was extracted a second and a third time, and the combined extracts were set aside for 24 h at 4°, when a colloidal precipitate appeared which was removed by centrifugation. Precipitation into ethanol (5 vol.) afforded a fibrous, white product which was collected, washed with ether, and dried. Purification of the polysaccharide was effected by dissolution in water, centrifugation of the solution, and precipitation into ethanol (5 vol.). The recovered polysaccharide was washed with ether, and dried *in vacuo* at 45°. The properties of the polysaccharides isolated from three samples of *Phyllymenia* are compared in Table I.

Chromatographic examination (solvents 1, 2, 3, and 4) of the neutralised acid hydrolysate of each of the polysaccharides revealed the presence of galactose (major), 2-*O*-methylgalactose (major), 6-*O*-methylgalactose, 4-*O*-methylgalactose (trace), and xylose (trace) with sprays *a* and *b*. The polysaccharide failed to precipitate from solution when mixed with potassium chloride solution.

Separation and characterisation of the components of the polysaccharide. — Polysaccharide (16 g; $[\alpha]_D^{16}$ +81.2°; SO_4^{2-}, 18.09%) was heated on a boiling-water bath with N sulphuric acid (75 ml) for 16 h. The hydrolysate was neutralised with barium carbonate, deionised with Amberlite IR-120 (H^+) and IR-4B (OH^-) resins, and evaporated to a thick, mobile syrup (9.5 g) which was applied to a cellulose column (47 × 5.4 cm). The column was eluted with butyl alcohol–water (95:5), and fractions (*ca.* 50 ml) were collected. On the basis of paper chromatography, recombination into four fractions was effected.

Fraction I. The syrup (0.34 g) contained the degradation products of the hydrolysis.

Fraction II. The syrup (2.3 g) was shown by paper chromatography (solvent 3) to be a mixture of 2-*O*-methylgalactose (major sugar, R_{Gal} 2.1) and 6-*O*-methylgalactose (R_{Gal} 1.85). An aqueous solution of the syrup was decolourised with charcoal, filtered, and evaporated to dryness, and the residue was crystallised from ethanol–ethyl acetate. Recrystallisation from the same solvent gave 2-*O*-methyl-D-galactose, m.p. and mixed m.p. 146–148°, $[\alpha]_D^{17}$ +56.6 (2 min)→ +86.6° (*c* 0.6). Oldham and Bell[13] reported m.p. 147–149°, $[\alpha]_D$ +53→+82.6°. The infrared spectrum of this sugar was identical with that of authentic 2-*O*-methyl-D-galactose, but different from those of 6-*O*-methyl- and 4-*O*-methyl-galactose.

Fraction III. The syrup (0.73 g) was shown by paper chromatography (solvents 1, 3, and 4) to contain 6-*O*-methylgalactose (major sugar), xylose, 2-*O*-methylgalactose, and 4-*O*-methylgalactose. An aqueous solution of the syrup was decolourised with charcoal, filtered, and evaporated to dryness, and the residue was crystallised from methanol to give needles (5 mg), m.p. 202–207°, $[\alpha]_D^{16}$ −53.4 (2 min)→−83.5° (*c* 0.34). This sugar moved with the mobility of authentic 4-*O*-methylgalactose in solvents 1, 2, 3, and 4. Demethylation[10] with hydrobromic acid, followed by paper chromatography, revealed the presence of galactose and unchanged material. The sugar showed no depression in m.p. when mixed with 4-*O*-methyl-L-galactose[14], but, with authentic 4-*O*-methyl-D-galactose[3], the m.p. was depressed to 186–192°. The infrared spectra of the D and L sugars were identical.

Fraction IV. The crystalline solid (2.1 g), after several recrystallisations from ethanol, had $[\alpha]_D^{16}$ +80° (*c* 1.0), m.p. and mixed m.p. 166–167° with authentic D-galactose. Oxidation with nitric acid–water (1:1) yielded mucic acid, m.p. and mixed m.p. 212–213°.

Action of alkali on the polysaccharide. — The polysaccharide (1.57 g; $[\alpha]_D^{16}$

$+81.1°$; SO_4^{2-}, 19.28%) in water (200 ml) containing sodium borohydride[7] (0.5 g) was set aside for 48 h at room temperature. Sodium hydroxide (20 g) and sodium borohydride (1.5 g) were then added, and the solution was heated for 9 h at 80°, with additions of sodium borohydride (0.5 g) every hour. After dialysis against frequently changed distilled water, the solution was concentrated and freeze-dried to a white foam (1.4 g; 89%). It had $[\alpha]_D^{16}$ $+43.5°$ (c 0.83) (Found: 3,6-anhydrogalactose[8], 0.67; SO_4^{2-}, 17.67%). Chromatography of an acid hydrolysate (solvents 3 and 4) revealed the presence of galactose, 2-*O*-methylgalactose, 4-*O*-methylgalactose (trace), 6-*O*-methylgalactose, and xylose (trace).

Desulphation of the polysaccharide[9]. — The polysaccharide (1 g; $[\alpha]_D^{16}$ $+81.1°$; SO_4^{2-}, 19.28%) was shaken with 0.15M methanolic hydrogen chloride (75 ml) for 48 h at room temperature. The undissolved material was filtered off and washed with methanol (3 × 30 ml). The filtrate and washings were neutralised with silver carbonate, and concentrated to a non-reducing syrup (350 mg). Paper chromatography of an acid hydrolysate of the syrup showed the presence of galactose (major sugar), 6-*O*-methylgalactose (minor sugar), and traces of 2-*O*- and 4-*O*-methylgalactose.

The insoluble material was dissolved in water (100 ml) and dialysed (3 days) against distilled water. After concentration, the polysaccharide (0.69 g) was isolated by freeze-drying. It had $[\alpha]_D^{17}$ $+90°$ (c 0.8) (Found: SO_4^{2-}, 0.0%). Paper chromatography of an acid hydrolysate revealed the presence of galactose, 2-*O*-methylgalactose, and traces of 4-*O*- and 6-*O*-methylgalactose.

In another experiment, the polysaccharide (1 g; $[\alpha]_D^{16}$ $+81.1°$; SO_4^{2-}, 19.28%) was shaken with 0.1M methanolic hydrogen chloride (75 ml) for 48 h to give a 74% yield of desulphated polysaccharide, $[\alpha]_D^{17}$ $+95°$ (c 0.61) (Found: SO_4^{2-}, 8%).

In a third experiment, the polysaccharide (1 g; $[\alpha]_D^{16}$ $+81.1°$; SO_4^{2-}, 19.28%), after being shaken for 72 h with 0.1M methanolic hydrogen chloride, gave a 70% yield of desulphated polysaccharide (Found: SO_4^{2-}, 4.7%). Paper chromatography of an acid hydrolysate of this material revealed the presence of galactose, 2-*O*-methylgalactose, and traces of 4-*O*- and 6-*O*-methylgalactose, and paper chromatography of a hydrolysate of the derived methyl glycosides showed the presence of galactose, 6-*O*-methylgalactose, and minute traces of 2-*O*- and 4-*O*-methylgalactoses.

In a fourth experiment, the polysaccharide ($[\alpha]_D^{16}$ $+63.3°$; SO_4^{2-}, 19.62%) gave a 67% yield of polysaccharide after a single (48 h) treatment with 0.15M methanolic hydrogen chloride (Found: SO_4^{2-}, 12.3%).

Periodate oxidation of the polysaccharide. — The polysaccharide (28.9 mg; $[\alpha]_D^{16}$ $+81.1°$; SO_4^{2-}, 19.28%), desulphated polysaccharide (29 mg; $[\alpha]_D^{17}$ $+90°$; SO_4^{2-}, 0%), and partially desulphated polysaccharide (25 mg; SO_4^{2-}, 4.7%) were dissolved separately in water (5 ml), and 0.298M sodium metaperiodate (5 ml) was added to each solution. The solutions were set aside at room temperature in the dark, and, at intervals, the periodate consumption was determined[15] on aliquots (0.10 ml) (Tables II and III). The oxopolysaccharides, isolated from these solutions by freeze-drying after dialysis, contained galactose, 6-*O*-methylgalactose, 2-*O*-methylgalactose.

and 4-*O*-methylgalactose (trace).

Quantitative estimation of galactose in the polysaccharide. — This was determined as described for aeodan[3]; see Table I for results.

Examination of the polysaccharide in the ultracentrifuge. — The polysaccharide ($[\alpha]_D^{16}$ +81.1°; SO_4^{2-}, 19.28%) was examined at a concentration of 0.5% in 0.1M sodium phosphate buffer at an ultracentrifuge speed of 56,000 r.p.m.

Partial hydrolysis of the polysaccharide. — Hydrolysis with N acid for 1 h proved to be satisfactory for the production of some oligosaccharides, whereas others were produced in greater concentration when the hydrolysis was carried out with 0.75N acid for 0.75 h.

The polysaccharide (20 g; $[\alpha]_D^{16}$ +63.3°; SO_4^{2-}, 19.62%) was heated on a boiling-water bath with 0.75N sulphuric acid (130 ml) for 0.75 h. Ethanol (240 ml) was added, and the precipitated polysaccharide (0.2 g) was removed by filtration. The filtrate was concentrated (150 ml) under diminished pressure, neutralised with barium carbonate, deionised, and evaporated to a mobile syrup (2.8 g). In a second hydrolysis experiment, the polysaccharide (20 g) was heated with N sulphuric acid for 1 h, and the mixture of neutral sugars (3.8 g) was isolated as above. The syrups were combined, dissolved in the minimum quantity of water, and applied to a charcoal–Celite column (1:1; 5.4 × 60 cm). Monosaccharides were eluted with water, and oligosaccharides with aqueous alcohol of increasing strength. Fractions (*ca.* 50 ml) were combined on the basis of paper chromatography.

Fraction I. The syrup (600 mg) contained galactose (major sugar) and xylose. Separation of this mixture of Whatman 3MM paper (solvent 1), followed by extraction of the appropriate portions of the papers, yielded D-galactose (300 mg), m.p. and mixed m.p. 165–167°, $[\alpha]_D^{16}$ +76.9° (*c* 1.56), and D-xylose (15 mg), m.p. and mixed m.p.[16] 145–146°, $[\alpha]_D^{16}$ +19.0° (*c* 0.55).

Fraction II. The syrup (350 mg) contained (paper chromatography using solvents 1, 3, and 4) galactose, 4-*O*-methylgalactose, and 2-*O*-methylgalactose.

Fraction III. The syrup (260 mg) contained 2-*O*-methylgalactose (major sugar) and 4-*O*-methylgalactose. 2-*O*-Methylgalactose, m.p. 148–150°, was obtained when this fraction was mixed with ethanol–ethyl acetate.

Fraction IV. The syrup (244 mg) was a mixture of 2-*O*-methylgalactose and 6-*O*-methylgalactose. The 2-*O*-methylgalactose readily crystallised from ethanol–ethyl acetate; m.p. 148–150°. The mother liquor was concentrated to a syrup and separated on Whatman 3MM paper (solvent 2). The portions of the papers corresponding to 6-*O*-methylgalactose were extracted with water, and the extracts were evaporated to dryness, yielding a solid which, after recrystallisation from ethanol, had $[\alpha]_D^{16}$ +76.5°, m.p. 118–120° alone and in admixture with authentic 6-*O*-methyl-D-galactose. Nunn and von Holdt[17] reported m.p. 122–123°.

Fraction V. The syrup (90 mg), eluted with 4–10% aqueous ethanol (3.0 l), was a mixture of three oligosaccharides, R_{Gal} 0.21, 0.3, and 0.45 (solvent 1).

Fraction VI. The syrup (50 mg), eluted with 4–10% aqueous ethanol (1.5 l), was a mixture of two oligosaccharides, R_{Gal} 0.21 and 0.3 (solvent 1). Repeated separation on Whatman No. 1 paper (solvent 1) gave a chromatographically pure syrup (R_{Gal} 0.3), which crystallised from aqueous methanol to give material (4 mg) having m.p. 236–238°, $[\alpha]_D^{16}$ $-51.9°$ (*c* 0.24). Paper chromatography (solvent 1) of a partial, acid hydrolysate revealed the presence of galactose and the original material. It gave a red colour with triphenyltetrazolium chloride and a blue colour with spray *c*. The infrared spectrum was different from that for 4-*O*-β-D-galactopyranosyl-D-galactose.

Fraction VII. The syrup (294 mg), eluted with 10% aqueous ethanol (1.7 l), was a mixture of two sugars, R_{Gal} 0.33 and 0.41 (trace). The main component crystallised readily from aqueous ethanol and, after recrystallisation from the same solvent, had $[\alpha]_D^{16}$ $+65°$ (*c* 0.6), m.p. 206–208° alone and in admixture with 4-*O*-β-D-galactopyranosyl-D-galactose. It gave galactose on hydrolysis, was readily detected as a red spot with the triphenyltetrazolium chloride spray, and gave a grey–blue colour with spray *c*. The disaccharide (10 mg) dissolved in *N*,*N*-dimethylformamide (2 ml) was treated with methyl iodide (2 ml) and dry silver oxide (2 g). This was followed by three treatments with Purdie's reagents, and methylation was then found to be complete (t.l.c.). The methylated product was hydrolysed (N sulphuric acid) and examined by t.l.c. (spray *c*). Spots having the mobilities of 2,3,4,6-tetra-*O*-methylgalactose (blue–green; R_{TMG} 1) and 2,3,6-tri-*O*-methylgalactose (grey; R_{TMG} 0.86) were observed.

Fraction VIII. The syrup (155 mg), eluted with 10–15% aqueous alcohol (1 litre), consisted mainly of two sugars, R_{Gal} 0.33 and 0.78, together with traces of two other sugars, R_{Gal} 0.41 and 0.49 (solvent 1).

Fraction IX. The syrup (650 mg), eluted with 10–15% aqueous ethanol (6.7 l), contained a sugar having R_{Gal} 0.78, and minute traces of three other sugars, R_{Gal} 0.18, 0.33, and 0.49 (solvent 1). This fraction readily crystallised when triturated with methanol and, after recrystallisation from the same solvent, had m.p. 213–214°, $[\alpha]_D^{16}$ $+87.6$ (4 min)$\rightarrow$ $+70.1°$ (*c* 0.69). It gave a yellow–green colour with spray *c* and failed to react with the triphenyltetrazolium chloride spray. Paper chromatography of a partial, acid hydrolysate revealed the presence of galactose, 2-*O*-methylgalactose, and the original material, and complete hydrolysis gave galactose and 2-*O*-methylgalactose in approximately equal amounts. To the sugar (5 mg) in water (2 ml) was added sodium borohydride (5 mg). After 2 h, the solution was treated with Amberlite IR-120 (H^+) resin and evaporated, and methanol was distilled from the residue to remove borate. Hydrolysis of the non-reducing syrup, followed by paper chromatography (solvent 1), revealed the presence of galactose. A portion (10 mg) in *N*,*N*-dimethylformamide (2 ml) was treated with methyl iodide (2 ml) and dry silver oxide (2 g) to give a partially methylated product which, after three treatments with Purdie's reagents[18], was found to be completely methylated (t.l.c., spray *d*). The methylated product was refluxed with 2% methanolic hydrogen chloride for 12 h, and the derived methyl glycosides were examined by g.l.c. Peaks corresponding to 2,3,4,6-tetra-*O*-methylgalactose (*T* 2.00) and 2,3,6-tri-*O*-methylgalactose (*T* 3.86, 4.72, 5.35, and 5.90), in the molar ratio 1.0:1.08, were observed. This disaccharide is probably 4-*O*-β-D-galactopyranosyl-2-*O*-methyl-D-galactose. The β-D configuration is assumed from the specific rotation of the compound.

Fraction X. The syrup (260 mg), eluted with 15% aqueous ethanol (7 l), contained at least four oligosaccharides, R_{Gal} 0.14, 0.22, 0.78, and 1 (solvent 1). This fraction was not further investigated.

Fraction XI. The syrup (240 mg), eluted with 15–50% aqueous ethanol (2 l), consisted of a fast-moving oligosaccharide (R_{Gal} 1.86) and at least three other oligosaccharides (R_{Gal} 0.1–0.22) (solvent 1). Separation on Whatman No. 1 paper (solvent 1), followed by extraction of the appropriate portions of the papers with methanol, afforded a chromatographically pure syrup (35 mg), $[\alpha]_D^{16}$ +55.5° (*c* 0.69, R_{Gal} 1.86 (solvent 1), 1.67 (solvent 2), and 1.55 (solvent 3); it gave a yellow–green colour with spray *c*, but failed to react with the triphenyltetrazolium chloride spray. Partial, acid hydrolysis, followed by paper chromatography (solvents 2 and 3), revealed the presence of 2-*O*-methylgalactose, 6-*O*-methylgalactose, and starting material, Reduction of the saccharide, followed by paper chromatography of the hydrolysate of the non-reducing syrup, revealed the presence of 6-*O*-methylgalactose and a minute trace of galactose. A portion of the oligosaccharide (10 mg) was methylated and methanolysed, and the derived methyl glycosides were examined by g.l.c. Peaks corresponding to 2,3,4,6-tetra-*O*-methylgalactose (*T* 1.98), 2,3,6-tri-*O*-methylgalactose (*T* 3.85, 4.38, 5.33, and 5.82), and 2,4,6-tri-*O*-methylgalactose (*T* 5.33 and 5.82), in the approximate molar ratios of 1.31:0.76:0.60, were observed. In addition, a small peak due to 2,3,4-tri-*O*-methylgalactose (*T* 9.30) was observed. This sugar is considered to have arisen from demethylation of some of the 2,3,4,6-tetra-*O*-methylgalactose during methanolysis. The above evidence suggests that this "saccharide" is a mixture of 2-*O*-methyl-4-*O*-(6-*O*-methyl-D-galactopyranosyl)-D-galactose and 2-*O*-methyl-3-*O*-(6-*O*-methyl-D-galactopyranosyl)-D-galactose. However, paper chromatography of the oligosaccharide (solvents 1–4) always revealed a single, discrete spot.

The eluate after Fraction XI revealed an almost continuous streak with no discrete spots. Hence, no further fractions were collected.

ACKNOWLEDGMENTS

We are indebted to Dr. M. Pocock for identifying *Phyllymenia cornea*, and to Prof. A. M. Stephen for allowing the gas chromatography to be carried out by Mr. G. R. Woolard in his laboratory. The ultracentrifuge examination and the microanalyses were performed by the National Chemical Research Laboratory, Pretoria.

REFERENCES

1 Part II: J. R. NUNN AND H. PAROLIS, *Carbohyd. Res.*, 8 (1968) 363.
2 H. KYLIN, *Die Gattungen der Rhodophyceen*, C. W. K. Gleerups, Lund, 1956, p. 213.
3 J. R. NUNN AND H. PAROLIS, *Carbohyd. Res.*, 6 (1968) 1.

4 S. Hirase, C. Araki, and K. Watanabe, *Bull. Chem. Soc. Japan*, 40 (1967) 1445.
5 M. Somogyi, *J. Biol. Chem.*, 195 (1952) 19.
6 A. G. Lloyd and K. S. Dodgson, *Biochim. Biophys. Acta*, 46 (1961) 116; A. G. Lloyd, K. S. Dodgson, R. G. Price, and F. A. Rose, *ibid.*, 46 (1961) 108.
7 D. A. Rees, *J. Chem. Soc.*, (1961) 5168.
8 W. Yaphe, *Anal. Chem.*, 32 (1960) 1327.
9 J. P. McKinnell and E. Percival, *J. Chem. Soc.*, (1962) 3141.
10 L. Hough, J. K. N. Jones, and W. H. Wadman, *J. Chem. Soc.*, (1950) 1702.
11 J. A. Cifonelli and F. Smith, *Anal. Chem.*, 26 (1954) 1132.
12 S. Schwimmer and A. Bevenue, *Science*, 123 (1956) 543.
13 J. W. H. Oldham and D. J. Bell, *J. Amer. Chem. Soc.*, 60 (1938) 323.
14 A. J. R. Allsobrook, J. R. Nunn, and H. Parolis, unpublished results.
15 G. O. Aspinall and R. J. Ferrier, *Chem. Ind.* (London), (1957) 1216.
16 E. Percival and J. K. Wold, *J. Chem. Soc.*, (1963) 5459.
17 J. R. Nunn and M. M. von Holdt, *J. Amer. Chem. Soc.*, 77 (1955) 2551.
18 T. Purdie and J. C. Irvine, *J. Chem. Soc.*, 83 (1903) 1021.

Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

THE ACID HYDROLYSIS OF PHENYL β-D-XYLOPYRANOSIDES

F. VAN WIJNENDAELE AND C. K. DE BRUYNE
H.I.K.W., Lab. Anorg. Scheikunde A, State University, Ghent (Belgium)

(Received July 12th, 1968)

ABSTRACT

Twenty-eight substituted phenyl *β*-D-xylopyranosides were hydrolysed in hydrochloric acid. Rate coefficients and kinetic parameters were determined. Application of the Hammett–Zucker, the Bunnett, and the entropy criteria indicate a unimolecular (A-1) mechanism. The linear relation between activation entropy and enthalpy, proved by the Exner method, indicates that some of the xylosides may be hydrolysed *via* a different mechanism. *ortho*-Substituents have a rather complex influence on the reaction.

INTRODUCTION

In a previous paper[1], we described the acid-catalysed hydrolysis of alkyl *β*-D-xylopyranosides. The present investigation is concerned with the influence of substituents on the rate parameters of the hydrochloric acid-catalysed hydrolysis of phenyl *β*-D-xylopyranosides.

The accepted mechanism, first suggested by Edward[2], is analogous to the A-1 mechanism for the hydrolysis of acetals[3–4]. The slow, rate-limiting step involves unimolecular heterolysis of the glycoside conjugate acid to form a cyclic carbonium–oxonium ion, which then reacts with water.

Similar investigations have been carried out for substituted phenyl *α*-D-glucopyranosides[5], phenyl *β*-D-glucopyranosides[6,7], and phenyl *β*-D-glucopyranosiduronic acids[7]. In these studies, the authors accepted the A-1 mechanism with fission of the glucosyl-oxygen bond[8], but without ring-opening. They showed that, for the *β*-D-series, electron-releasing substituents facilitate the reaction. The Hammett reaction constant has a low value because the substituents have an influence on both the formation of the conjugate acid and its subsequent heterolysis, but affect these two processes in opposing manners, thus partially cancelling each other.

RESULTS AND DISCUSSION

Twenty-eight phenyl *β*-D-xylopyranosides were hydrolysed in 0.1M aqueous hydrochloric acid at different temperatures. Rate coefficients, energy and entropy of activation, and estimated standard deviations are presented in Table I. All of the reactions are first order and ln k is a linear function of $1/T$.

TABLE I

RATE COEFFICIENTS AND KINETIC PARAMETERS FOR THE HYDROLYSIS OF PHENYL β-D-XYLOPYRANOSIDES IN 0.1M HYDROCHLORIC ACID

No.	*Substituent*	*10^5k_1 (sec^{-1})* 60°	70°	80°	*E* kcal.mole^{-1}	*$\Delta S^‡$ (60°)* cal.deg^{-1}.mole^{-1}	*$\Delta G^‡$ (60°)* kcal.mole^{-1}
1	None	2.59 ±0.05	9.50	32.5	29.6 ±0.5	+11.6 ±1.5	25.08
2	*p*-Chloro	2.21 ±0.03	8.00	26.9	29.2 ±0.6	+10.0 ±1.5	25.19
3	*m*-Chloro	2.19 ±0.07	7.64	25.3	28.6 ±0.3	+8.3 ±1.5	25.16
4	*o*-Chloro	4.44 ±0.03	14.9	46.3	27.4 ±0.2	+6.1 ±1.0	24.70
5	*p*-Methyl	2.40 ±0.04	8.51	27.9	28.7 ±0.4	+9.0 ±1.0	25.12
6	*m*-Methyl	2.96 ±0.10	10.7	36.3	29.3 ±0.6	+10.0 ±1.7	25.00
7	*o*-Methyl	2.21 ±0.03	7.98	26.9	29.2 ±0.2	+9.6 ±1.0	25.17
8	*p*-Nitro	1.31 ±0.03	4.34	14.7	28.3 ±0.3	+6.3 ±1.0	25.51
9	*m*-Nitro	1.47 ±0.01	4.93	15.6	27.6 ±0.3	+4.4 ±1.0	25.50
10	*o*-Nitro	4.90 ±0.06	14.6	39.9	24.7 ±0.2	−1.7 ±1.0	24.62
11	*p*-Chloro-*m*-methyl	2.21 ±0.04	8.08	27.3	29.4 ±0.3	+10.7 ±1.0	25.19
12	2,4-Dimethyl	2.18 ±0.13	8.28	29.1	30.3 ±0.7	+13.8 ±2.0	25.18
13	3,4-Dimethyl	2.36 ±0.14	9.09	32.6	30.7 ±1.2	+14.7 ±4.0	25.14
14	2,6-Dimethyl	7.95 ±0.30	30.2	106	30.3 ±0.9	+16.0 ±3.0	24.33
15	2,3,5-Trimethyl	1.50 ±0.01	6.83	28.7	34.5 ±0.3	+25.1 ±1.0	25.44
16	2,4-Dichloro	3.26 ±0.18	11.4	37.3	28.5 ±1.2	+8.7 ±3.0	24.92
17	*p*-Amino	0.99 ±0.03	3.40	10.9	28.0 ±0.6	+4.8 ±2.0	25.70
18	*o*-Amino	0.17 ±0.01	0.57	1.8	27.5 ±0.8	−0.2 ±2.3	26.86
19	*m*-Amino	0.82 ±0.07	2.92	9.8	29.1 ±0.2	+7.7 ±0.5	25.90
20	*p*-Acetamido	2.85 ±0.05	10.4	35.5	29.5 ±0.7	+11.4 ±2.0	25.01
21	*m*-Acetamido	3.25 ±0.12	10.5	31.6	26.6 ±0.9	+3.2 ±2.0	24.93
22	*o*-Acetamido	2.61 ±0.03	9.66	31.7	29.2 ±0.2	+10.5 ±1.0	25.06
23	*p*-Methoxy	1.93 ±0.02	7.43	26.7	30.7 ±0.3	+14.3 ±0.8	25.27
24	*o*-Methoxy	7.50 ±0.07	24.8	76.8	27.2 ±0.2	+6.6 ±0.6	24.37
25	*p*-Ethoxy	2.09 ±0.05	8.31	30.7	31.4 ±0.4	+16.5 ±1.0	25.22
26	*p*-Benzyloxy	2.08 ±0.07	7.49	25.1	29.1 ±0.4	+9.7 ±1.2	25.22
27	*p*-Bromo	1.94 ±0.04	7.15	24.6	29.7 ±0.3	+11.3 ±1.0	25.26
28	*m*-Bromo	2.05 ±0.06	6.92	21.7	27.6 ±0.4	+5.1 ±1.3	25.22

Influence of the acid concentration. — If the rate-limiting step involves the unimolecular heterolysis of the conjugate acid to a glycosyl carbonium ion (A-1), a linear relation between the logarithm of the rate coefficient and the Hammett acidity function should exist[9]. For a number of phenyl β-D-xylosides, the pseudo-first-order rate coefficient k_1 was determined at constant temperature and various concentrations of hydrochloric acid (Tables II and III).

In all cases, log k_1 showed a linear dependence on H_0, but the requirement of unit slope was only approximately fulfilled. A least-squares fit of the data yields the values given in Table IV, where b represents the slope, s_b the estimated standard deviation on b, $s_{y/x}$ the standard error of the estimate, n the number of points, and

TABLE II

INFLUENCE OF THE ACID CONCENTRATION ON THE RATE COEFFICIENT (COLORIMETRIC DETERMINATIONS)

$10^5 k_1$ sec^{-1}

HCl (M)		0.1	0.25	0.30	0.50	0.75	1.0	1.5
H_0^a		+0.98	+0.55	+0.45	+0.20	−0.03	−0.20	−0.47
Substituent	*Temp. (degrees)*							
None	60	2.59	—	8.26	15.3	—	40.0	65.8
p-Chloro	60	2.19	—	7.39	13.3	—	32.1	57.6
p-Methyl	60	2.70	—	9.00	15.4	—	38.0	66.8
p-Chloro-*m*-methyl	60.2	2.23	4.77	—	13.3	20.7	32.8	—
p-Nitro	45	0.18	0.465	—	0.98	1.76	2.45	—
o-Nitro	60	4.20	—	—	23.6	—	55.7	164[b]
p-Ethoxy	60	2.09	6.18	—	13.6	20.9	32.0	—
p-Benzyloxy	59.9	2.11	5.36	—	12.5	—	28.8	—
2,4-Dimethyl	65	4.33	9.07	—	25.4	—	62.5	—
3,4-Dimethyl	65	4.71	12.4	—	27.3	—	69.9	—

[a] From Ref. 13. [b] In 2M HCl ($H_0 = -0.69$).

TABLE III

INFLUENCE OF THE ACID CONCENTRATION ON THE RATE COEFFICIENT (POLARIMETRIC DETERMINATIONS)

$10^5 k_1$ (sec^{-1}) at 45°

HCl (M)	1	2	3	4	5
H_0^a	−0.20	−0.69	−1.05	−1.40	−1.76
Substituent					
None	4.59	14.4	36.3	79.4	176
p-Methyl	4.89	14.3	37.0	81.6	186
p-Chloro	3.93	12.3	30.2	65.9	137
p-Nitro	2.45	7.5	18.1	37.6	79
o-Nitro	8.75	26.6	62.7	119	—
m-Acetamido	4.33	12.4	30.7	65.2	142
p-Ethoxy	4.31	13.4	34.1	73.9	168

[a] From Ref. 13.

a the intercept of the function $\log 10^5 k_1 = a + bH_0$, as calculated by regression analysis. For the parameter b, however, a t-test indicates that, in most cases, the deviation from unity is not significant.

According to Bunnett[10], a better criterion is a plot of $\log k_1 + H_0$ *versus* log (activity water) ($\log a_{H_2O}$). The slope of the resulting straight line defines the new parameter w. However, if this is done for the phenyl β-D-xylosides, all of the plots are curved and the slopes are dependent on the acid concentration. It is thus impossible to calculate the w parameters (a rough graphical estimate is given in Table IV). In all cases where the slope b of the Hammett–Zucker plot is greater than unity, the sign of w is negative and thus in accordance with a unimolecular mechanism. In the other cases, *w* is positive, which should lead to the conclusion that the hydrolysis proceeds by an S_N2 mechanism entailing a nucleophilic attack of water on the conjugate acid. This change of mechanism is highly improbable. Moreover, if the sign of *w* is determined by the value of *b*, whose deviation from unity is statistically not significant, the sign itself is determined by the random variations, and the positive *w*-values cannot invalidate the conclusion from the Zucker–Hammett criterion. The only possible conclusion is that, in all cases, *w approaches* zero, suggesting the same A-1 mechanism for all of the phenyl β-D-xylosides. For alkyl β-D-xylosides[1], all *b*-slopes are greater than unity and all *w*-values negative, but, in these cases, the deviations from unity are statistically significant.

TABLE IV

SLOPES OF THE ZUCKER–HAMMETT PLOTS; *w* PARAMETER

Substituent	*Method*	$-b$	$s_{y/x}$	s_b	n	a	w
None	C[a]	0.985	0.023	0.020	5	0.376	+0.5
	P[b]	1.021	0.018	0.015	5	0.464	−2.0
p-Methyl	P	1.022	0.020	0.017	5	0.476	−0.2
p-Chloro	C	0.980	0.009	0.008	5	0.308	+0.8
	P	0.997	0.023	0.019	5	0.407	+0.1
p-Chloro-*m*-methyl	C	1.00	0.048	0.051	5	0.298	−0.2
p-Ethoxy	C	0.991	0.025	0.026	5	0.311	+0.4
	P	1.025	0.017	0.014	5	0.433	−0.2
p-Nitro	C + P	0.975	0.016	0.006	10	0.204	+0.3
2,4-Dimethyl	C	0.982	0.002	0.002	4	0.601	+1.0
3,4-Dimethyl	C	0.987	0.008	0.009	4	0.638	+0.7
p-Benzyloxy	C	0.969	0.015	0.017	4	0.273	+1.7
o-Nitro	C	0.952	0.006	0.004	4	0.558	+2.0
m-Acetamido	P	0.980	0.018	0.015	5	0.437	+0.2

[a]Colorimetric. [b]Polarimetric.

When $\log k_1 - \log [\mathrm{HCl}]$ is plotted *versus* $\log a_{H_2O}$, the slope of this line defines the parameter w^*. According to Bunnett[10], water acts as a nucleophile in the rate-determining step if $w^* < -2$. When the values of k_1 determined in the interval 1 to 5M hydrochloric acid (Table III) are used, the plots are approximately linear and the w^*-parameter takes the value −6 to −7 for all xylosides. If the k_1-values determined at lower acid concentration (Table II) are used, the points are scattered, and an exact determination of w^* is impossible. A rough estimation, however, gives a constant value of *ca.* −10 for all of the xylosides. Hence, in all cases, w^* indicates an S_N2 mechanism, in contrast to the *w*-parameter, the Hammett criterion, and the entropy criterion.

Thus, it seems that, for the hydrolysis of glycosides, the *w* and w^* parameters reflect more than a simple hydration change in the transition of the conjugate acid to an activated complex.

Isokinetic relationship. — In this series of similar reactions, a linear relation between the activation enthalpy and entropy was proved by plotting two values of log k_1, obtained at two different temperatures, against each other according to the Exner[11] method. Using the k_1 values from Table I, a plot of log 10^6k_1 (80°) *versus* log 10^6k_1 (60°) gives a linear relation. Regression analysis yields the equation:

log 10^6k (80°) = 1.102 + 0.982 log 10^6k (60°), with slope $b = 0.982 \pm 0.050$, isokinetic temperature $\beta = -155$°K, $T_2/T_1 = 0.943$, $n = 28$, the correlation coefficient $r = 0.977$, and the standard error of the estimate $s_{y/x} = 0.067$.

If the values of the derivatives 10, 15, and 18, which deviate slightly from the calculated line, are omitted, one finds the equation:

log 10^6k(80°) = 1.10 + 0.983 log 10^6k(60°), with $b = 0.983 \pm 0.042$, $\beta = -138$°K, $r = 0.983$, $s_{y/x} = 0.045$, and $n = 25$.

A *t*-test indicates that, in both cases, the slope b does not deviate significantly from unity. Since this deviation, however, causes a large uncertainty in the β-value, the calculations were repeated using log 10^6k at 70°. This yields:

log 10^6k(70°) *versus* log 10^6k(60°): $b = 0.99 \pm 0.02$, $\beta = -95$°K, and log 10^6k(80°) *versus* log 10^6(70°): $b = 1.00 \pm 0.02$, $\beta = 0$°K. The most probable b-value approaches unity, and hence β approaches zero. In the Exner classification, this represents case 2, with E constant and a rate controlled mainly by changes of $\Delta S^\ddagger$, which means that purely electronic effects of the substituents are of minor importance. If, on the other hand, the β-value is not zero, but negative, and $1 > b > T_2/T_1$, this represents case 4 or the reverse of compensation.

The same reaction series was analyzed by the Leffler[12] method in which E is plotted *versus* $\Delta S^\ddagger$. Regression analysis yields the equation (60°):

E(kcal) = 26.0 + 0.317$\Delta S^\ddagger$, with $\beta = 317$°K, $s_\beta = 6$°K, $s_{y/x} = 0.488$, $r = 0.964$, and $n = 28$.

In this case, the isokinetic temperature is different from the value in the Exner plot and approaches the mean temperature (343°K) of the experiment. Since log k is approximately constant, this β-value probably represents an "error slope". With the exception of the 2,3,5-trimethylphenyl and possibly the *o*-nitrophenyl and *o*-aminophenyl derivatives, all points fit the calculated Exner isokinetic line, which suggests that these compounds are hydrolyzed by the same A-1 mechanism. Even normal *ortho* effects do not deviate the points from the line.

Alkyl β-D-xylopyranosides[1] reacted in a somewhat different way. In contrast to the phenyl analogues, both activation parameters were variable, but in the sense that their effects partially compensated each other. The Leffler plot was quite real and resulted in an isokinetic temperature of the same order as found in the Exner plot. Moreover, the β-value was positive and higher than the temperature of the experiment.

Free-energy relationship. — Graphical analysis indicates that several points deviate strongly in a log 10^6k_1(60°) *versus* σ plot. The values for the compounds 4, 7, 10, 12, 14, 15, 16, 17, 18, 19, 22, and 24 were thus omitted in the calculations.

For the remaining points, regression analysis yields the equation:

$\log 10^6 k_1 (60°) = 1.36 - 0.146\,\sigma$, with reaction constant $\varrho = -0.146 \pm 0.052$, $s_{y/x} = 0.084$, $r = -0.6$, and $n = 16$.

Using this equation, $\log 10^6 k_1$ was recalculated for all derivatives and compared with the observed values (Table V). In this calculation, the σ-values used were the Hammett[14] substituent constant σ for *para* and *meta* groups, the Taft[14] polar constant σ^*) for *ortho*-substituted benzene derivatives (for *ortho* groups, the additive constants ($\Sigma\sigma$) for poly-substituted derivatives, and σ^- from Hine[15] for amino groups (as NH_3^+). However, these last values are uncertain since they are particularly solvent-dependent.

If one considers a deviation greater than 0.1 log-units to be significant, the exceptions are *ortho*-substituted compounds. The fact that the reaction constant ϱ is small for these xylosides proves that polar effects are unimportant, probably because the opposing effects on protonation and heterolysis will tend to cancel out. An electron-attracting group will lower the electron density of the glycosyl oxygen-atom and thus the concentration of the conjugate acid. On the other hand, the heterolysis step will be favored, and the rate should thus increase. Since the overall rate decreases, the effect on protonation seems dominant. This rather unpredicable, but small overall, effect partially explains the fact that polar factors, even for the best-fitting points, account for only 60% of the variance in rate. For a series of phenyl β-D-glucosides, Nath and Rydon[6] calculated $\varrho = -0.66$, and Semke *et al.*[7] $\varrho = -0.48$. For phenyl β-D-glucuronides, Semke[7] found $\varrho = -0.09$. For these compounds too, the rate is thus diminished by a lowering of the electron density of the glycosyl group.

In this reaction series, $\Delta S^\ddagger$ is not constant. The change of the rate constants by the substituents is not a precise function of the changes in potential energy. The fact that the Hammett equation seems to apply for most substituents should mean that, in these cases, the enthalpy is indeed a linear function of the entropy, and hence that the isokinetic relationship is real.

According to Exner and Leffler, the reaction constant ϱ becomes zero at the isokinetic temperature and changes its sign when surpassing this temperature. If the β-value, as obtained from the Leffler plot (317°K), is real, it approaches the temperature of the experiment (333°K) and hence ϱ approaches zero. In this case, neither the sign nor the value of ϱ should have a mechanistic meaning. If the β-value from the Exner plot (0°K) is real, then ϱ is significant. The experimentally attainable temperature will always be far above this isokinetic temperature.

Influence of ortho *substituents.* — The effect of *ortho* substituents on the reaction parameters seems very complex, yet the isokinetic relationship indicates the same mechanism for all, except three, derivatives.

The normal polar effect of an *ortho* substituent should parallel the effect of the same group in the *para* position. This seems to be the case for *o*-methyl, 2,4-dimethyl, and *o*-acetamido substituents. If one uses the Taft polar substituent constants, these derivatives do not deviate from the Hammett plot (Table V), and neither the rate nor the activation parameters are changed significantly. This also indicates that steric effects play a minor role. In fact, because of the equatorial position of the aglycon and the unimolecular nature of the rate-limiting heterolysis of the protonated reactant, steric hindrance factors in the transition state of the A-1 mechanism are unlikely.

TABLE V

LINEAR FREE-ENERGY RELATIONSHIP

Substituent	σ	*log* 10^6k_1		Δ*log* 10^6k_1
		observed	*calculated*	
None	0.00	1.412	1.361	+0.051
p-Chloro	+0.277	1.344	1.328	+0.016
m-Chloro	+0.373	1.340	1.307	+0.033
o-Chloro	+0.20	1.647	1.332	+0.315
p-Methyl	−0.170	1.380	1.386	−0.006
m-Methyl	−0.069	1.471	1.371	+0.100
o-Methyl	−0.17	1.344	1.386	−0.042
p-Nitro	+1.27	1.117	1.176	−0.059
m-Nitro	+0.710	1.167	1.258	−0.091
o-Nitro	+1.22	1.690	1.183	+0.507
p-Chloro-*m*-methyl	+0.158	1.344	1.338	+0.006
2,4-Dimethyl	−0.34	1.338	1.411	−0.073
3,4-Dimethyl	−0.239	1.373	1.396	−0.023
2,6-Dimethyl	−0.34	1.900	1.411	+0.489
2,3,5-Trimethyl	−0.308	1.176	1.406	−0.230
2,4-Dichloro	+0.427	1.513	1.298	+0.215
p-Amino	+0.60	0.996	1.273	−0.277
o-Amino	—	0.230	—	—
m-Amino	+0.86	0.911	0.912	−0.001
p-Acetamido	0.00	1.454	1.361	+0.093
m-Acetamido	+0.21	1.512	1.331	+0.181
o-Acetamido	0.00	1.417	1.361	+0.056
p-Methoxy	−0.268	1.286	1.400	−0.114
o-Methoxy	−0.39	1.875	1.418	+0.457
p-Ethoxy	−0.24	1.320	1.396	−0.076
p-Benzyloxy	−0.03[a]	1.318	1.364	−0.046
p-Bromo	+0.232	1.288	1.327	−0.039
m-Bromo	+0.391	1.312	1.304	+0.008

[a] From Ref. 16.

These factors, however, can influence the initial state of the molecule. In the case of the 2,6-dimethyl derivative, the enhanced rate is probably due to a decrease of the entropy in the initial conformation, the activation energy remaining constant. A second *o*-methyl group, in contrast to the same group in the *meta* (3,4-dimethyl) or *para* position (2,4-dimethyl), prevents the rotation of the aglycon. When only one *o*-methyl substituent is present, this is not the case, and the rate is not changed significantly.

The other *ortho* groups have a more complex influence, and neither polar nor steric factors alone can explain the rate changes. These derivatives deviate significantly from the Hammett plot; the effect of the *ortho* group does not parallel the effect of the same group in the *para* position, *e.g.*, a nitro group increases the rate in the *ortho*, but decreases it in the *para* position; a methoxyl group ($\sigma^* = -0.39$) and a chloro group ($\sigma^* = +0.20$) both increase the rate.

Even if one considers the possibility that some of the *ortho* substituents interfere sterically or by hydrogen bonding with the glycon, so that the *p*-orbitals of the substituent and of the benzene ring are prevented from becoming parallel to each other, thus inhibiting delocalisation through the π-electrons, the remaining inductive effect[16] (σ_1) cannot explain the differences in rate. In one case, the *o*-methoxyl group, inhibition of the resonance changes[16] the sign of σ ($\sigma_1 = +0.33$) and *can* thus explain why this substituent has the same effect as the chloro group, but even then the point still deviates from the Hammett plot.

From the values of Tables I and V, it follows that *ortho* groups capable of hydrogen bonding with the hydroxyl group at C-2 increase the rate by lowering the energy of activation. The entropy change becomes less favorable. The hydrogen bond influences the electron density around the glycosyl-oxygen atom more than is accounted for by the σ-value, but because of the two opposing effects on protonation and heterolysis, the overall effect on the rate remains speculative. Although this explanation cannot be ruled out, it becomes improbable if the influence on protonation is dominant (ϱ is negative), because hydrogen bonding should then decrease the rate. Hydrogen bonding may also result in a decrease in entropy of the initial state of the molecule, caused by restriction imposed upon rotation of the aglycon. Even the conformation of the glycon may be changed, either by steric strain imposed by bulky groups or by hydrogen bonding. These factors may alter the change of molecular order on passing to the transition state and/or the energy necessary to reach a half-chair conformation. Probably several of these effects co-operate, and for the moment it seems impossible to separate them.

Three of the phenyl β-D-xylosides show exceptional activation parameters. The 2,3,5-trimethylphenyl derivative has a high activation energy and entropy. For the *o*-nitrophenyl and *o*-aminophenyl compounds, both parameters are small. It is difficult to imagine that the high entropy in the case of the 2,3,5-trimethylphenyl xyloside is a result of a decrease in entropy of the initial conformation because none of the dimethylphenyl xylosides show this effect. It is also noteworthy that these compounds do not fit the isokinetic relationship. This, together with the unusual value of $\Delta S^{\ddagger}$, suggests a change in mechanism. One possibility is the open-chain mechanism, with protonation of the ring-oxygen atom (in this case owing to the base-weakening effect of the strong electron-withdrawing substituents on the glycosidic oxygen), and heterolysis with ring opening. Since, in this case, the aglycon remains attached to the glycosyl oxygen-atom in the heterolysis step, steric effects can play a dominant role and markedly influence $\Delta S^{\ddagger}$. In the open-chain mechanism, electron-withdrawing groups impede both protonation and heterolysis, and the rate should thus decrease. This is, however, not the case, and in the present state of knowledge this explanation must remain speculative.

EXPERIMENTAL

The synthesis of the substituted phenyl β-D-xylopyranosides was performed as described previously[17–19]. The hydrolyses were carried out in 10-ml, glass-stoppered tubes, immersed in a thermostat bath (accurate to within 0.05°). Exactly measured portions of the xyloside solution (0.001M) in hydrochloric acid were transferred into the tubes. These were stoppered and placed simultaneously in the thermostat bath. When they had reached thermostat temperature, the first tube was withdrawn and this time taken as zero. After measured intervals, the other tubes were withdrawn, cooled, and neutralized with the calculated amount of sodium hydroxide. The concentration of the phenol was then determined colorimetrically. For nitrophenol, the yellow color developed in alkali was measured with a Beckman C colorimeter at 400 nm (Jenaer Schott IL/PIL filter). For the other phenols, the concentration was determined by the method of Folin and Ciocalteu[20], the blue colours being measured with a Klett–Summerson colorimeter with filter 42 (400–465 nm). For each phenol, a calibration curve had to be constructed, using the absorption of known concentrations of the phenol in similar conditions.

The first-order rate coefficients ($1n\ k$, sec^{-1}) were calculated from least-squares, straight-line fits of the usual log plots: $\log S_t = \log S_0 - kt$, which were always linear.

The polarimetric measurements, carried out with a Perkin–Elmer Model 141 photoelectric polarimeter, and the calculations of the thermodynamic activation functions with their estimated standard deviation, were performed as described previously[1].

ACKNOWLEDGMENTS

We thank Prof. Dr. L. Massart for his interest in this work, and Miss J. De Lat for technical assistance.

REFERENCES

1 C. K. De Bruyne and F. Van Wijnendaele, *Carbohyd. Res.*, 6 (1968) 367.
2 J. T. Edward, *Chem. Ind.* (London), (1955) 1102.
3 J. M. O'Gorman and H. J. Lucas, *J. Amer. Chem. Soc.*, 72 (1950) 5489.
4 A. M. Wenthe and E. H. Cordes, *J. Amer. Chem. Soc.*, 87 (1965) 3173.
5 A. N. Hall, S. Hollingshead, and H. N. Rydon, *J. Chem. Soc.*, (1961) 4290.
6 R. L. Nath and H. N. Rydon, *Biochem. J.*, 57 (1954).
7 L. K. Semke, N. S. Thompson, and D. G. Williams, *J. Org. Chem.*, 29 (1964) 1041.
8 C. Armour, C. A. Bunton, S. Patai, L. H. Selman, and C. A. Vernon, *J. Chem. Soc.*, (1961) 412.
9 L. Zucker and L. P. Hammett, *J. Amer. Chem. Soc.*, 61 (1939) 2791.
10 J. F. Bunnett, *J. Amer. Chem. Soc.*, 83 (1961) 4956, 4968, 4973, 4978.
11 O. Exner, *Collection Czech. Chem. Commun.*, 29 (1964) 1094.
12 J. E. Leffler, *J. Org. Chem.*, 20 (1955) 1202.

13 M. A. Paul and F. A. Long, *Chem. Rev.*, 57 (1957) 1.
14 J. Hine, *Physical Organic Chemistry*, McGraw-Hill, New York, 1962, pp. 87 and 98.
15 J. Hine, *J. Amer. Chem. Soc.*, 82 (1960) 4877.
16 O. Exner, *Collection Czech. Chem. Commun.*, 31 (1966) 65.
17 C. K. De Bruyne, H. Versele, and M. Claeyssens, *Nature*, 205 (1965) 900.
18 C. K. De Bruyne and H. Vervoort, *Nature*, 211 (1966) 1292.
19 C. K. De Bruyne and F. Van Wijnendaele, *Carbohyd. Res.*, 4 (1967) 102.
20 O. Folin and V. Ciocalteu, *J. Biol. Chem.*, 73 (1927) 627.

Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

NUCLEOPHILIC DISPLACEMENT REACTIONS IN CARBOHYDRATES

PART IX*. THE SOLVOLYSIS OF METHYL 6-*O*-METHANESULPHONYL-2,3-DI-*O*-METHYL-β-D-GALACTOPYRANOSIDE: A METHOXY-GROUP PARTICIPATION**

J. S. BRIMACOMBE AND O. A. CHING
Chemistry Department, The University, Birmingham 15 (Great Britain)

(Received July 26th, 1968)

ABSTRACT

The solvolysis of methyl 6-*O*-methanesulphonyl-2,3-di-*O*-methyl-β-D-galactopyranoside (**8**) in boiling 50% aqueous methanol, in the presence of sodium acetate, afforded three products which were identified as methyl 3,6-anhydro-2-*O*-methyl-β-D-galactopyranoside (**9**), methyl 2,3,6-tri-*O*-methyl-β-D-galactopyranoside (**12**), and methyl 2,3-di-*O*-methyl-β-D-galactopyranoside (**7**). Anhydro-sugar **9** is considered to result from attack of solvent on an oxonium-ion intermediate **14**, formed as a consequence of methoxy-group participation in displacement of the sulphonic ester group, while compounds **7** and **12** may result either from solvent attack upon the oxonium ion or from a solvent-assisted displacement of the sulphonic ester group.

INTRODUCTION

Investigations by Winstein and his co-workers[1,2] and others[3] have established that methoxy-group participation in solvolytic displacement reactions is substantial for the MeO-5 and MeO-6† cases. For example, both *p*-bromobenzenesulphonates **1** and **2** undergo anchimerically assisted solvolyses in ethanol, *via* the cyclic oxonium ion **3**, to give a mixture of compounds **4** and **5**, together with a small proportion of 2-methyltetrahydrofuran (**6**). Examples of methoxy-group participation reported in the carbohydrate field have all involved the migration of a methoxy group that was part of an acetal system. Thus, a small proportion of 1,3,4,6-tetra-*O*-acetyl-2-*O*-methyl-D-glucopyranose was formed on brominolysis[4] of methyl 3,4,6-tri-*O*-acetyl-2-deoxy-2-iodo-α-D-mannopyranoside in acetic acid, and methyl 5-*O*-*p*-bromobenzenesulphonyl-6-deoxy-2,3-*O*-isopropylidene-β-L-allofuranoside gave 6-deoxy-2,3-*O*-isopropylidene-5-*O*-methyl-D-talofuranose on treatment with sodium hydroxide in aqueous *p*-dioxane[5]. More recently, 1-*O*-benzoyl-2,3,5-tri-*O*-benzyl-4-*O*-methyl-L-lyxose methyl hemiacetal was obtained from a benzoate displacement reaction on 2,3,5-tri-*O*-benzyl-4-*O*-toluene-*p*-sulphonyl-D-ribose dimethyl acetal as a consequence

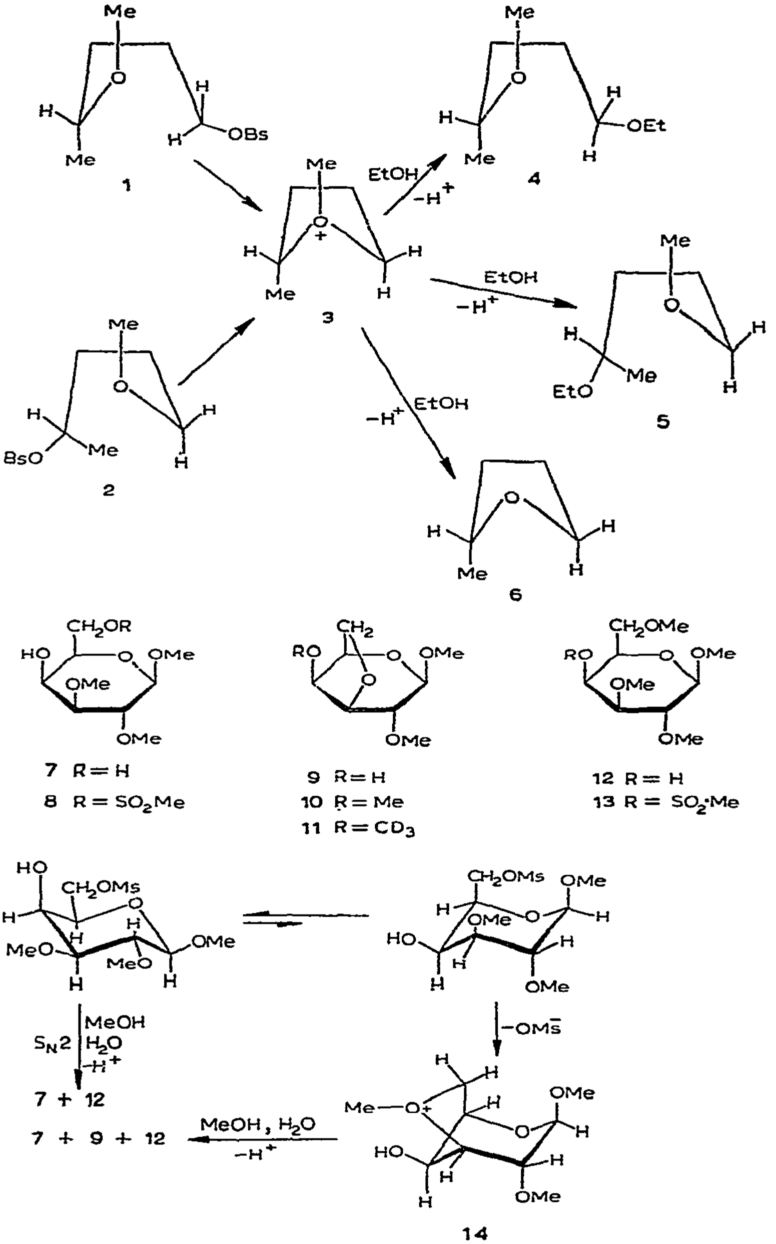
---

*Part VIII: J. S. Brimacombe and F. Hunedy, *J. Chem. Soc. (C)*, in press.

**Preliminary communication: *Chem. Commun.*, (1968) 781.

†In describing nucleophilic participation, it is frequently convenient to use the symbol "G-*n*", where G is the participating group and *n* the size of the ring formed in the transition state.

of methoxy-group participation and migration[6]. Benzyloxy-group participations are also known[7,8], and, pertinent to the subsequent discussion, methyl 2,3-di-*O*-benzyl-6-*O*-methanesulphonyl-β-D-galactopyranoside was readily solvolysed in boiling, aqueous methanol to give[7] methyl 3,6-anhydro-2-*O*-benzyl-β-D-galactopyranoside



Scheme: possible mechanisms for the formation of products from the solvolysis of methanesulphonate 8.

In view of the participation obtained in the latter case, it was of interest to see if the methylated analogue **8** would undergo an assisted solvolysis. Mechanistic rationalisations invoking a participation mechanism should preferably be based on both kinetic and product data[3]. Kinetic data are not readily obtained with carbohydrate sulphonates containing acid-labile groups, because of the need to neutralise the sulphonic acid released. In these cases, a participation mechanism can often be inferred from the isolation of products (for example, compounds **5** and **6** from ethanolysis of the aliphatic sulphonate **1**) which would not be expected from an unassisted reaction.

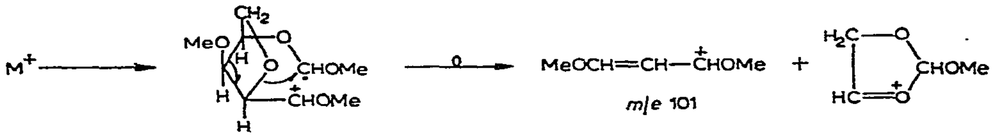
DISCUSSION

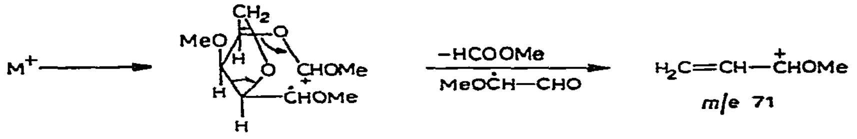
The title sulphonate **8** was prepared by graded, acidic hydrolysis of methyl 4,6-*O*-benzylidene-2,3-di-*O*-methyl-β-D-galactopyranoside followed by selective methanesulphonylation of the primary hydroxyl group of the resulting diol **7**. On solvolysis in boiling 50% aqueous methanol, in the presence of sodium acetate, methanesulphonate **8** was slowly transformed into three products, and the solvolysis was terminated when t.l.c. indicated the virtual disappearance of starting material; this required approximately 95 h compared with a time of 17 h for the complete solvolysis of methyl 2,3-di-*O*-benzyl-6-*O*-methanesulphonyl-β-D-galactopyranoside under comparable conditions[7]. The products of the solvolysis were separated by chromatography on silica gel and were identified as methyl 3,6-anhydro-2-*O*-methyl-β-D-galactopyranoside (**9**), methyl 2,3,6-tri-*O*-methyl-β-D-galactopyranoside (**12**), and methyl 2,3-di-*O*-methyl-β-D-galactopyranoside (**7**) in the following way.

The n.m.r. spectra of compounds **7** and **12** revealed the presence of three and four methoxyl groups, respectively, and both gave D-galactose (chromatographic identification) as the only reducing sugar on demethylation[9]. Williams and Jeanloz[10] have recently prepared compound **12** by partial methylation of diol **7** with methyl iodide and silver oxide. Our compound was identical with an authentic specimen prepared by this method, and both compounds were converted into the same crystalline 4-methanesulphonate **13**. The t.l.c. properties and the n.m.r. and infrared spectra of the dimethylgalactoside were identical with those of diol **7**, and this structure was confirmed by its conversion into the crystalline methanesulphonate **8**.

Elemental analyses of the third product, which was eluted first from the column, were in reasonable agreement with the molecular formula $C_8H_{14}O_5$, and the appearance of a top mass peak at *m/e* 159 (M−31) in its mass spectrum was consistent with this molecular formula. N.m.r. spectroscopy confirmed the loss of the sulphonic ester group, and, more significantly, the spectrum contained resonances attributable to the presence of only two methoxy groups. From this evidence and by analogy with the solvolysis of methyl 2,3-di-*O*-benzyl-6-*O*-methanesulphonyl-β-D-galactopyranoside[7], it is reasonable to assign the structure methyl 3,6-anhydro-2-*O*-methyl-β-D-galactopyranoside (**9**) to this compound. Methylation of the hydroxyl group gave a product formulated as methyl 3,6-anhydro-2,4-di-*O*-methyl-β-D-galactopyranoside (**10**), which was identical, with a sample prepared by acid-catalysed anomerisation[11] of methyl 3,6-anhydro-2,4-di-*O*-methyl-α-D-galactopyranoside. Both materials had melting points (72–74°) somewhat lower than that (83–84°) recorded in the literature[11], although their optical rotations were in close agreement with reported values; repeated recrystallisation and sublimation did not alter the melting points. The compounds appeared to be homogeneous by t.l.c., and the n.m.r. spectra were identical and compatible with the structure assigned.

This discrepancy between the two sets of melting points is not readily accounted for, and an unequivocal identification of anhydro-sugar **9** is essential to subsequent mechanistic considerations. In order to verify the proposed structure, the permethylated anhydro-sugar **10** and the trideuteriomethylated analogue **11** were examined by mass spectrometry. In addition to a prominent peak at *m/e* 173 (M−31), the mass spectrum of anhydro-sugar **10** contained prominent peaks at *m/e* 101 and 71, which arise by the fragmentations shown. These fragmentation patterns have been substantiated[12] for other permethylated 3,6-anhydrohexopyranosides, and the base peak at *m/e* 71 is characteristic of these sugars; additional mass-spectroscopic evidence for structure **10** is reported in the Experimental section. It can be seen that the ions of *m/e* 101 and 71 contain carbon atoms 2–4 and 4–6 of the parent sugar, respectively. In the mass spectrum of the trideuteriomethylated analogue **11**, comparable fragments appeared at *m/e* 176, 104, and 74. This fragmentation pattern is entirely consistent with structure **11**, and, thus, the accumulated evidence supports structure **9** for the product from the solvolysis.





There is ample analogy with other systems[1–3] to indicate that anhydro-sugar **9** results from nucleophilic attack on a bicyclic, oxonium-ion intermediate **14**, presumably formed by a methoxy-assisted solvolysis of the sulphonate **8** (see Scheme). Compounds **7** and **12** may have arisen either from a solvent-assisted displacement or from attack of solvent at C-6 of the oxonium-ion intermediate **14**. Although the direct displacement of carbohydrate sulphonates is known with the more nucleophilic hydroxide[13] and methoxide[14] ions, there is little information on their behaviour under conditions comparable to those used in the present investigation. However we have recently observed[15] that methyl 6-*O*-methanesulphonyl-2,3-di-*O*-methyl-α-D-allopyranoside undergoes a solvent-assisted displacement under comparable conditions. The relative extents of the two possible pathways leading to compounds **7** and **12** will depend on the driving force provided by methoxy-group participation, on which we have no information.

Allred and Winstein[1d] have attributed the extents of Me-O and methylene C-O bond cleavage with five- and six-membered, cyclic oxonium-ions to differences in ring strain and to steric hindrance at the O-methylene carbon atoms. These factors will presumably control the formation of products from oxonium ion **14**, which can be considered as a fused five- and seven-membered ring system. It is likely that oxonium ion **14** will adopt a *1C* conformation of the pyranoid ring, for reasons which we have suggested[7] previously and which are discussed[16] for structurally related 3,6-anhydro-sugars. It is noteworthy that the extent of Me-O bond cleavage with oxonium ion **14** is substantially greater than for the five-membered, oxonium ion[1a] **3**, and its behaviour, in this respect, resembles more closely that of the *O*-methyltetrahydropyranium ion[1d]. Steric factors are probably responsible for the absence of rearranged products which might result from solvent attack at C-3 of oxonium ion **14**. Examination of a molecular model of oxonium ion **14**, based on the conformation shown, revealed that the approach of nucleophiles to C-3 is hindered by the neighbouring, axial substituents.

EXPERIMENTAL

General methods. — Thin-layer chromatography (t.l.c.) was performed on silica gel with detection by vanillin–perchloric acid[17]. N.m.r. spectra were obtained with a Varian A-60 or Perkin–Elmer R-10 spectrometer for *ca.* 10% solutions in deuteriochloroform with tetramethylsilane as internal reference. Infrared spectra were recorded on Nujol mulls with a Perkin–Elmer 257 spectrometer, and mass spectra were measured on an A.E.I. MS9 spectrometer.

Light petroleum refers to the fraction having b.p. 40–60°.

*Methyl 4,6-*O*-benzylidene-2,3-di-*O*-methyl-β-*D*-galactopyranoside.* — Sodium hydride (2.5 g) was added in small portions to a stirred solution of methyl 4,6-*O*-benzylidene-β-D-galactopyranoside[18] (2.9 g) in *N*,*N*-dimethylformamide (100 ml), and, after 1 h, methyl iodide (6 ml) was added dropwise. Stirring was continued for 24 h, whereupon the excess of reagents was destroyed by the careful addition of dry methanol (70 ml), and the solvents were removed. The residue was extracted with chloroform (150 ml), and the extract was washed with 3% sodium thiosulphate solution (150 ml) and water (4 × 150 ml), and dried ($MgSO_4$). Removal of the solvent gave a residue which was decolorised (ethanol) and then recrystallised from chloroform–light petroleum to yield the product (2.4 g), m.p. 144–146°, $[\alpha]_D$ +15° (*c* 1, methanol); lit.[19], m.p. 148°, $[\alpha]_D$ +18° (chloroform).

*Methyl 6-*O*-methanesulphonyl-2,3-di-*O*-methyl-β-*D*-galactopyranoside* (**8**). — To a solution of diol **7** (1.1 g, obtained by graded, acidic hydrolysis of the previous compound essentially as described in the literature[10]) in dry pyridine (50 ml) was gradually added a cooled (0°) solution of methanesulphonyl chloride (0.42 ml) in pyridine (20 ml), and the solution was set aside overnight at room temperature. Processing in the usual manner, with chromatography on silica gel (100 g, elution with chloroform–methanol, 9:1), gave methanesulphonate **8** (0.64 g), m.p. 84–86° (from ethyl acetate–light petroleum), $[\alpha]_D$ $-11°$ (*c* 2, chloroform) (Found: C, 39.75; H, 6.8; S, 10.8. $C_{10}H_{20}O_8S$ calc: C, 40.0; H, 6.7; S, 10.7%). N.m.r. data: τ 5.60 (1-proton doublet, $J_{1,2}$ 6.5 Hz, H-1); 6.47, 6.49, 6.52 (3-proton singlets, methoxy groups); and 6.96 (3-proton singlet, Me.SO_2).

*The solvolysis of methyl 6-O-methanesulphonyl-2,3-di-O-methyl-β-*D*-galactopyranoside* (**8**). — The title sulphonate (0.57 g) in 50% aqueous methanol (120 ml) containing sodium acetate trihydrate (0.6 g) was heated under reflux for 95 h, after which time, t.l.c. (ethyl acetate) indicated the virtual disappearance of starting material and the formation of three products. The solvents were removed under diminished pressure, and toluene (2 × 15 ml) was distilled from the residue to remove the last traces of solvents. The residue was extracted with ethyl acetate (3 × 25 ml), and the combined extracts were concentrated to *ca.* 20 ml and chromatographed on silica gel (100 g) by elution with ethyl acetate and, for the last component, with methanol; the following compounds were eluted. (*i*) Methyl 3,6-anhydro-2-*O*-methyl-β-D-galactopyranoside (**9**) (128 mg, 35%), m.p. 46–47° (from ether–light petroleum), $[\alpha]_D$ $-85°$ (*c* 0.5, chloroform) (Found: C, 49.4; H, 7.45. $C_8H_{14}O_5$ calc: C, 50.5; H, 7.4%). N.m.r. data: τ 5.51 (1-proton singlet, H-1); 6.62, 6.64 (3-proton singlets, two methoxy groups). (*ii*) Methyl 2,3,6-tri-*O*-methyl-β-D-galactopyranoside (**12**) (81 mg, 18%), $[\alpha]_D$ $-22°$ (*c* 0.6, chloroform); lit.[10], $[\alpha]_D$ $-16°$ (*c* 0.7, chloroform); n.m.r. data: τ 6.47, 6.50, 6.55, and 6.63 (3-proton singlets, four methoxy groups). (*iii*) Methyl 2,3-di-*O*-methyl-β-D-galactopyranoside **7** (180 mg, 43%), $[\alpha]_D$ *ca.* 0° (*c* 2.5, methanol); n.m.r. data: τ 6.52, 6.53, and 6.59 (3-proton singlets, three methoxy groups).

Identification of anhydro-sugar **9**. — This was achieved by its conversion into methyl 3,6-anhydro-2,4-di-*O*-methyl-β-D-galactopyranoside (**10**) and the 4-*O*-trideuteriomethylated compound **11** as follows.

A solution of anhydro-sugar **9** (0.125 g) in *N,N*-dimethylformamide (10 ml) was treated with sodium hydride (50 mg) and methyl iodide (0.5 ml) by the standard procedure[20]. The crude product was chromatographed on silica gel (ethyl acetate as eluant) to give methyl 3,6-anhydro-2,4-di-*O*-methyl-β-D-galactopyranoside (**10**) (0.11 g), m.p. 72–74° (from water), $[\alpha]_D$ $-78°$ (*c* 0.8, methanol). The infrared spectrum of the product showed no absorption attributable to hydroxyl groups, and it could not be distinguished from a sample prepared according to the literature procedure[11]; this material also had m.p. 72–74° (from water) which was not depressed on admixture with the compound described above. The values m.p. 83–84°, $[\alpha]_D$ $-82°$ (*c* 0.5, methanol) are reported by Haworth, Jackson, and Smith[11] for compound **10**. The n.m.r. spectrum was consistent with the structure assigned and showed, *inter alia*, three methoxy resonances.

A similar procedure was used in trideuteriomethylation of anhydro-sugar **9** to give methyl 3,6-anhydro-2-*O*-methyl-4-*O*-trideuteriomethyl-β-D-galactopyranoside (**11**), m.p. 76–78°.

Characteristic fragmentations of anhydro-sugars **10** and **11**, based on Heyns and Scharmann's[12] interpretation of the mass spectra, are described in the text. The mass spectrum of anhydro-sugar **10** also contained peaks at *m/e* 141 ($M - Me\dot{O} - MeOH$, A_2^2), 109 ($M - Me\dot{O} - MeOH - MeOH$, A_3^1), 175 ($M - \dot{C}HO$, L_1), 143 ($M - \dot{C}HO - MeOH$, L_2), 131 ($M - MeO\dot{C}HCHO$, M_1), 99 ($M - MeO\dot{C}HCHO - MeOH$, M_2), and 73 ($\cdot Me\overset{+}{O} = CHCHO$, N_1). Ions belonging to the *L*, *M*, and *N* series are considered by Kochetkov and Chizhov[21] to be characteristic of methyl 3,6-anhydro-2,4-di-*O*-methylhexopyranosides, since they arise from rupture of the C-5–C-6 bond, followed by C-5–C-1 rearrangement and subsequent fragmentation.

Identification of methyl 2,3,6-tri-O-methyl-β-D-galactopyranoside (**12**) *and methyl 2,3-di-O-methyl-β-D-galactopyranoside* (**7**). — Compound **12** (41 mg) in pyridine (20 ml) was treated with methanesulphonyl chloride (0.1 ml) for 4 h, and the reaction mixture was then processed in the usual manner. Chromatography of the crude product on silica gel (30 g, elution with ethyl acetate) gave methyl 4-*O*-methanesulphonyl-2,3,6-tri-*O*-methyl-β-D-galactopyranoside **13** (45 mg), m.p. 141–142° (from acetone–light petroleum) (Found: C, 42.3; H, 6.8; S, 10.4. $C_{11}H_{22}O_8S$ calc: C, 42.0; H, 7.05; S, 10.2%). The t.l.c. properties and i.r. spectrum of this material were indistinguishable from those of the product resulting from methanesulphonylation of authentic[10] compound **12**; on admixture, the samples had m.p. 141–142°.

Methanesulphonylation of diol **7**, as already described, gave methyl 6-*O*-methanesulphonyl-2,3-di-*O*-methyl-β-D-galactopyranoside (**8**), m.p. and mixed m.p. 84–86°. The i.r. spectrum of the methanesulphonate was identical to that found previously.

ACKNOWLEDGMENTS

The authors thank Professor M. Stacey, C.B.E., F.R.S., for his interest, the University of San Marcos, Lima, for granting one of us (O.A.C.) a leave of absence, and Dr. E. F. Mooney for measuring a number of n.m.r. spectra.

REFERENCES

1 E. L. Allred and S. Winstein, *J. Amer. Chem. Soc.*, 89 (1967) (*a*) p. 3991, (*b*) p. 3998, (*c*) p. 4008, and (*d*) p. 4012.
2 S. Winstein, E. Allred, R. Heck, and R. Glick, *Tetrahedron*, 3 (1958) 1.
3 B. Capon, *Quart. Rev.* (London), 18 (1964) 45.
4 R. U. Lemieux and B. Fraser-Reid, *Can. J. Chem.*, 42 (1964) 539.
5 C. L. Stevens, R. P. Glinski, K. G. Taylor, P. Blumbergs, and F. Sirokman, *J. Amer. Chem. Soc.*, 88 (1966) 2073.
6 N. A. Hughes and P. R. H. Speakman, *J. Chem. Soc.* (C), (1967) 1182.
7 J. S. Brimacombe and O. A. Ching, *Carbohyd. Res.*, 5 (1967) 239; *J. Chem. Soc.* (C), (1968) 1642.
8 G. R. Gray, F. C. Hartman, and R. Barker, *J. Org. Chem.*, 30 (1965) 2020; J. S. Brimacombe and O. A. Ching, *Carbohyd. Res.*, 8 (1968) 376.
9 T. G. Bonner, E. J. Bourne, and S. McNally, *J. Chem. Soc.*, (1960) 2929.

10 N. R. WILLIAMS AND R. W. JEANLOZ, *J. Org. Chem.*, 29 (1964) 3434.
11 W. N. HAWORTH, J. JACKSON, AND F. SMITH, *J. Chem. Soc.*, (1940) 620.
12 K. HEYNS AND H. SCHARMANN, *Carbohyd. Res.*, 1 (1966) 371.
13 R. S. TIPSON, *Advan. Carbohyd. Chem.*, 8 (1953) 109.
14 A. K. MITRA, D. H. BALL, AND L. LONG, Jr., *J. Org. Chem.*, 27 (1962) 160; S. C. WILLIAMS AND J. K. N. JONES, *Can. J. Chem.*, 43 (1965) 3440.
15 J. S. BRIMACOMBE AND O. A. CHING, unpublished results.
16 E. L. ELIEL, N. L. ALLINGER, S. J. ANGYAL, AND G. A. MORRISON, *Conformational Analysis*, Interscience, New York, 1965, p. 413.
17 *Chromatography*, E. Merck A. G., Darmstadt, 2nd edn., p. 30.
18 E. G. GROS, *Carbohyd. Res.*, 2 (1966) 56.
19 J. W. OLDHAM AND D. J. BELL, *J. Amer. Chem. Soc.*, 60 (1938) 323.
20 J. S. BRIMACOMBE, B. D. JONES, M. STACEY, AND J. J. WILLARD, *Carbohyd. Res.*, 2 (1966) 167.
21 N. K. KOCHETKOV AND O. S. CHIZHOV, *Advan. Carbohyd. Chem.*, 21 (1966) 39.

Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

VINYLATION OF METHYL α-D-GLUCOPYRANOSIDE

J. T. MARVEL, J. W. BERRY, R. O. KUEHL, AND A. J. DEUTSCHMAN, JR.
Department of Agricultural Biochemistry, University of Arizona, Tucson, Arizona 85721 (U. S. A.)

(Received September 3rd, 1968)

ABSTRACT

Vinylation of methyl α-D-glucopyranoside (**1**) gives a mixture of products that usually contains all of the monovinyl ethers. Reaction conditions that favor increased conversion cause an increase in the proportion of divinyl ethers. Methyl 3-*O*-vinyl-α-D-glucopyranoside (**3**) was found to be isolable by an extraction procedure.

INTRODUCTION

The addition of an alcohol to acetylene in the presence of a base results in the formation of a vinyl ether. Industrial applications of this reaction have utilized various monohydric alcohols. The vinylation of a few alditols and carbohydrates has been studied in some detail, and it has been found that a mixture of rather closely related products generally results[1-6]. From the vinylation of methyl α-D-glucopyranoside (**1**), Kircher and Deutschman[7] isolated methyl 2-*O*-vinyl-α-D-glucopyranoside (**2**), and obtained evidence that the three other monovinyl ethers possible were also formed.

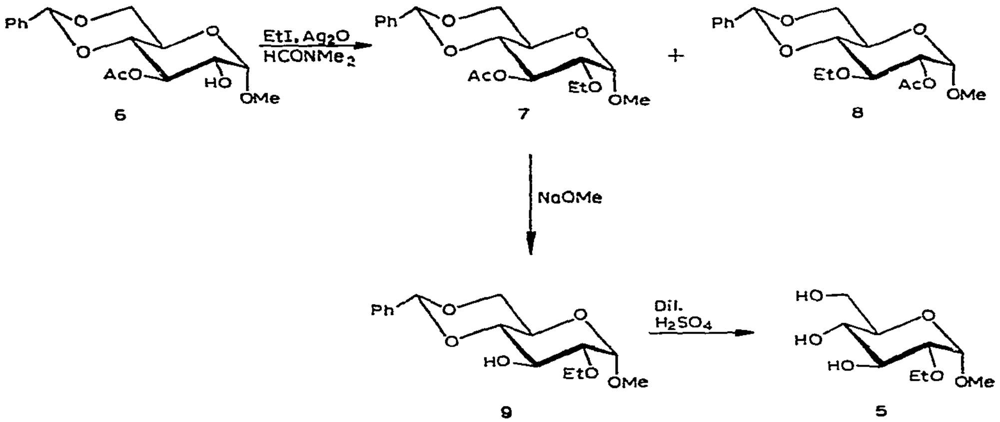
The purpose of the present work was to obtain more specific information about the effect of reaction conditions on the vinylation of **1**, and to characterize the mixture of vinylation products more completely.

DISCUSSION

Vinylations were performed by allowing acetylene to react with the glycoside **1** for a predetermined period of time, at a specified temperature and pressure, in a mixture of potassium hydroxide, *p*-dioxane, and water. The mixture was then treated with carbon dioxide, and evaporated to dryness. Successive extraction of the residue with solvents in the order of their increasing polarity (petroleum ether, benzene, ether) gave extracts containing products that increased in polarity and decreased in vinyl substitution, as judged by t.l.c. and g.l.c. behavior and i.r. spectra. However, a practical separation of all of the components in this way was not possible. Methyl 3-*O*-vinyl-α-D-glucopyranoside (**3**) was the only product that could be obtained pure by repeated extraction of the dried residue with petroleum ether. Methyl 2-*O*-vinyl-α-D-glucopyranoside (**2**) had previously been isolated from an aqueous solution of the carbonated reaction mixture by continuous extraction with ether[7].

A degree of substitution (D. S.) of unity was arbitrarily selected as the optimal extent of vinylation, because the monovinyl ethers appear to be more suitable for subsequent polymerization than more highly substituted products. More than thirty experiments were performed toward this goal, in accordance with a fractional-factorial, statistical design[8]. Empirically determined ranges for the variables were: time (1–9 h), temperature (115–175°), pressure (250–550 lb. in^{-2}, gauge), base concentration (20–104 mg per g of **1**), and volume percent of water in the solvent mixture (35–95%). In order to obtain a statistical yield-value for a given set of conditions, it was necessary to measure, by g.l.c., the mole fraction of each product and of **1** in the reaction mixture. An effort was made to prepare volatile derivatives of the methyl *O*-vinyl-α-D-glucopyranosides by trimethylsilylation, but considerable decomposition to **1** was observed. Therefore, the reaction mixture was hydrogenated, to give the corresponding methyl *O*-ethyl-α-D-glucopyranosides, per(trimethylsilyl) derivatives of which were readily prepared.

Methyl 3-*O*-ethyl-α-D-glucopyranoside (**4**), which has previously been synthesized by two routes[9], was obtained by hydrogenation of methyl 3-*O*-vinyl-α-D-glucopyranoside (**3**), the monovinyl ether isolated in this work. A synthesis of methyl 2-*O*-ethyl-α-D-glucopyranoside (**5**) has also been reported[7], but an alternative synthetic method was developed here (see Scheme 1). Ethylation of methyl 3-*O*-acetyl-4,6-*O*-benzylidene-α-D-glucopyranoside (**6**) gave a mixture of methyl 3-*O*-acetyl-4,6-*O*-benzylidene-2-*O*-ethyl-α-D-glucopyranoside (**7**) and methyl 2-*O*-acetyl-4,6-*O*-benzylidene-3-*O*-ethyl-α-D-glucopyranoside (**8**) (85 and 15%, respectively), from which the major product **7** was obtained by fractional recrystallization. Compound **7** was converted into methyl 4,6-*O*-benzylidene-2-*O*-ethyl-α-D-glucopyranoside (**9**) by saponification with sodium methoxide in methanol. The n.m.r. data for the intermediates are given in Table I. The spectrum of ethyl ether **9** further confirmed our previous assignment of hydroxyl resonances in other substituted methyl α-D-gluco-



Scheme 1.

pyranosides[9]. Conversion of **9** into methyl 2-*O*-ethyl-α-D-glucopyranoside (**5**) was accomplished by treatment with dilute sulfuric acid.

TABLE I

N.M.R. SPECTRA[a] OF SUBSTITUTED METHYL 4,6-*O*-BENZYLIDENE-α-D-GLUCOPYRANOSIDES IN METHYL SULFOXIDE-d_6

Compound	*Ph*	*PhCH*	*H-1*	*H-3*	*Other ring protons*	*OMe*	*2-OR*	*3-OR*
6[b]	3.33s	4.84s	5.62d (3.0)[c]	5.25m	6.08– 7.08	6.84s	5.09d (5.0)	8.06s
7	2.65s	4.44s	5.11d (3.2)	4.83m	5.86– 6.88	6.73s	9.02t (7.0)	8.08s
9	2.62s	4.46s	5.23d (3.3)	6.0– 7.0	5.86– 7.00	6.74s	9.02t (7.0)	4.84s (6.0)

[a]Chemical shifts are on the τ scale, relative to Me_4Si (in Me_2SO-d_6); spectra obtained at 60 MHz; d, doublet; m, multiplet; s, singlet; t, triplet. [b]Sample contained a trace of water. [c]Values in parentheses are coupling constants (in Hz).

Attempts were made to synthesize methyl 4-*O*-ethyl-α-D-glucopyranoside (**10**) and methyl 6-*O*-ethyl-α-D-glucopyranoside (**11**) from methyl 2,3-di-*O*-benzyl-6-*O*-trityl-α-D-glucopyranoside and methyl 2,3,4-tri-*O*-benzoyl-α-D-glucopyranoside, respectively. In both cases, insufficient amounts of product were obtained for satisfactory characterization, but the behavior of each on t.l.c. and g.l.c. was consistent with the zones and peaks identified by the technique to be described subsequently.

In order to substantiate the behavior of synthetic compounds on g.l.c., samples of polysaccharides containing (1→2), (1→3), (1→4), and (1→6) α-D-glucosidic linkages (namely, crown-gall polysaccharide, laminaran, amylose, and dextran, respectively) were vinylated (D.S. 1), the products hydrogenated, and the products methanolyzed[4]. The resulting mixtures of methyl *O*-ethyl-α-D-glucopyranosides were pertrimethylsilylated, and the ethers were subjected to g.l.c. The absence of known peaks on the chromatograms were taken to represent the position of the predominant repeating linkage in the respective polysaccharide structure, and the peaks on each chromatogram were assumed to show the positions of the free hydroxyl groups in the original polysaccharide. These data were correlated with those obtained with synthetic materials and with product mixtures from vinylation; the pertinent chromatograms are shown in Figure 1. The peak assignment for methyl 2,6-di-*O*-ethyl-α-D-glucopyranoside (**12**) was deduced from its relative position and from the fact that this peak appeared in the products of the ethylation and methanolysis of cellulose, amylose, and laminaran, in which O–2 and O–6 derivatives preponderate[4].

T.l.c. and g.l.c. showed that every reaction mixture contained methyl mono-*O*-vinyl-α-D-glucopyranosides, methyl di-*O*-vinyl-α-D-glucopyranosides, and unreacted **1**. In most cases, it was found that all of the possible monosubstituted products were present, whereas only one or two disubstituted products were present. The formation of tri- and tetra-substituted products was suppressed when at least 25% of water was present in the solvent mixture.

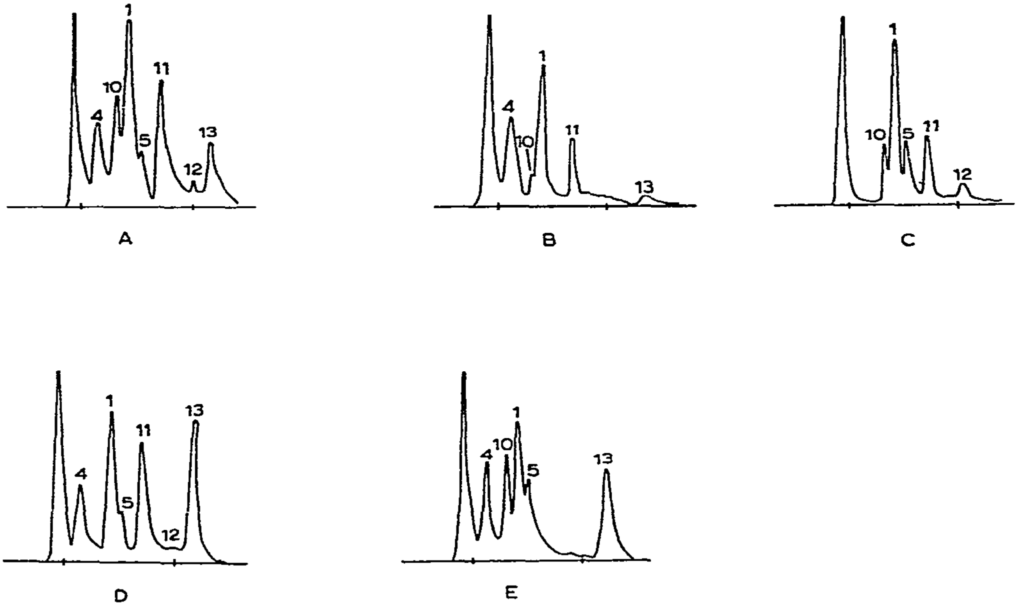


Fig. 1. Chromatograms of ethyl ethers of **1** derived from vinylation of: **1** (A), crown-gall polysaccharide (B), laminaran (C), amylose (D), and dextran (E).

Interpretation of the statistical data showed that interplay of the reaction variables resulted in alternative sets of reaction conditions that could be so selected as to provide a particular percentage of vinylation, and the compensation of any one of the variables by all of the others could be determined. An increase in the value of any of the variables time, temperature, pressure, and concentration of potassium hydroxide, or a decrease in the percentage of water, caused an increased percent conversion, but changes that favored increased percent conversion also favored an increase in the proportion of divinyl ethers. It was not found possible to select conditions that would favor the formation of a particular monosubstituted product over the others. The preponderant component in the disubstituted fraction was, in every instance, methyl 2,3-di-*O*-vinyl-α-D-glucopyranoside (**3**), and it was the only di-ether present in the product from about 75% of the experiments. In the rest of the experiments, minor proportions of methyl 2,6-di-*O*-vinyl-α-D-glucopyranoside and, occasionally, another component (thought to be methyl 3,6-di-*O*-vinyl-α-D-glucopyranoside) were present.

The order of elution observed for the pertrimethylsilylated ethyl ethers of **1** suggests that the relative retention times vary directly with the number of consecutive, oxygen-containing functional groups (ether or acetal) that are not trimethylsilylated. For the 3- and 4-ethyl ethers, some interference with the binding of the acetal oxygen atoms to the stationary phase apparently occurs; this would account for their lower retention-times as compared with those for the pertrimethylsilyl ethers of **1** and of the 2- and 6-ethyl ethers.

TABLE II

T.L.C. OF *O*-SUBSTITUTED METHYL α-D-GLUCOPYRANOSIDES

Compound	*Substituent*	R_F *values, benzene–butyl alcohol*		
		(49:1 v/v)	*(9:1 v/v)*	*(4:1 v/v)*
1			0.05	0.10
10	4-*O*-ethyl		0.19	0.24
4	3-*O*-ethyl		0.27	0.30
5	2-*O*-ethyl		0.29	0.31
11	6-*O*-ethyl		0.34	0.37
13	2,3-di-*O*-ethyl		0.42	
12	2,6-di-*O*-ethyl		0.48	
	4,6-*O*-benzylidene	0.08	0.45	
	4,6-*O*-benzylidene-3-*O*-ethyl	0.30	0.60	
9	4,6-*O*-benzylidene-2-*O*-ethyl		0.62	

EXPERIMENTAL

General. — Melting points are uncorrected. T.l.c. was performed on Silica Gel G (E. Merck, Germany), with benzene–ethyl alcohol or benzene–butyl alcohol. The spots were visibilized by spraying the dried chromatogram with a 10% (w/v) solution of phosphomolybdic acid, and heating for 5 min at 100°. G.l.c. was conducted in an apparatus constructed in this laboratory. An isothermal system, maintained by a Dynapac (Lab-Line Instruments, Inc.) proportional temperature-controller, was used with a Gow-Mac (Gow-Mac Instrument Company) tungsten-wire, detection cell. A 1-mV full-scale recorder provided with disc integration was used. The apparatus was constructed of Marinite (Johns–Manville Corp.), with a 2-in wall-thickness throughout. An internal fan assured even distribution of heat. The column (10 ft of stainless steel, 0.25-in diam.) was packed with 7% Carbowax 20M on Chromosorb W (60–80 mesh); helium was the carrier gas. 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 a Varian Associates A-60 spectrometer (60 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 the 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.

Trimethylsilylation of the hydrogenated vinylation-reaction mixtures was performed by the procedure of Sweeley *et al.*[10]; methyl β-D-xylofuranoside was employed as the internal standard.

Methyl 3-O-vinyl-α-D-glucopyranoside (3). — The equipment used, and the procedure for handling acetylene, have been described previously[3]. A mixture of 50 g (260 mmoles) of **1**, 2 g of potassium hydroxide, 50 ml of redistilled *p*-dioxane, and 50 ml of water was placed in a 300-ml autoclave and stirred. The autoclave was flushed with nitrogen, sealed, and heated to 150°. Acetylene, compressed to 400 lb. in^{-2} (gauge) was admitted, and this pressure was maintained for 12 h. The autoclave was cooled, and vented, and carbon dioxide was passed into the reaction mixture to saturation. The mixture was evaporated to dryness, and the residue was continuously extracted for 24 h with petroleum ether (b.p. 30–60°). The extract was evaporated to dryness, and, after one week, the residue had partially crystallized. Three recrystallizations from benzene and three from methanol gave 2.5 g of product, m.p. 125–125.5°, $[\alpha]_D^{25}$ +155.8° (*c* 1, ethanol), $\nu_{max}^{CHCl_3}$ 3600, 3400, 3100, 2910, 1640, 1450, 1360, 1315, 1160, 1080, 1020, 945, 895, and 840 cm^{-1}.

Anal. Calc. for $C_9H_{16}O_6$: C, 49.09; H, 7.27. Found: C, 48.95; H, 7.12.

Vinylation of methyl α-D-glucopyranoside (**1**) *for statistical treatment.* — The procedure was similar to that already described for compound 3. A mixture of 5 g of **1**, potassium hydroxide in the range of 0.1 to 0.7 g, water in the range of 35 to 95 ml, and *p*-dioxane in the range of 65 to 5 ml was placed in a 300-ml autoclave and stirred. The temperature, pressure of acetylene, and elapsed time of reaction were varied in the ranges of 115 to 175°, 250 to 550 lb. in^{-2} (gauge), and 1 to 9 h, respectively. After saturation with carbon dioxide, the mixture was evaporated to dryness and the residue was mixed with methanol. The suspension was filtered, and the filtrate was diluted to 200 ml with absolute methanol.

Hydrogenation of methyl O-vinyl-α-D-glucopyranosides. — A 100-ml portion of the methanol solution just described was treated with hydrogen in the presence of 5% palladium-on-carbon. When the reaction was complete, the catalyst was removed by filtration, and the filtrate was evaporated to dryness. The residue, a mixture of methyl *O*-ethyl-α-D-glucopyranosides, was analyzed by g.l.c. of the trimethylsilyl ethers[10].

Vinylation of polysaccharides. — A mixture of 2.5 g of the polysaccharide and 75 ml of redistilled *p*-dioxane was beaten in a Waring Blendor for 2 min. To this mixture was added a solution of 1.25 g of potassium hydroxide in 25 ml of water, and blending was continued for 3 min. The mixture was placed in a 300-ml autoclave which was then flushed with nitrogen and heated to 165° while the mixture was stirred. Acetylene gas was admitted, and its pressure maintained at 450 lb. in^{-2} (gauge) for 5 h. The autoclave was cooled and vented, and carbon dioxide was passed in until the base had been partially neutralized. The suspension was filtered, and the product was precipitated by pouring the filtrate into 400 ml of ethyl ether. The crude precipitate was filtered off, and treated with boiling methanol or *p*-dioxane, the suspension was filtered, and the product was precipitated with ethyl ether, and dried in a vacuum desiccator.

Vinylated polysaccharide so prepared had a D.S. in the range of 0.8 to 1.2, as measured by iodimetric analysis[3].

Hydrogenation of vinylated polysaccharide. — To a suspension of 800 mg of 5% palladium-on-carbon in 100 ml of methanol was added 200 mg of the vinylated polysaccharide. The mixture was hydrogenated for 70 h at 50° and 1,800 lb. in^{-2} (gauge). The catalyst was removed by filtration, and the filtrate was evaporated to dryness, yielding a pale-yellow, amorphous material.

Methanolysis of ethylated polysaccharide. — The product obtained by hydrogenation of the vinylated polysaccharide was mixed with 100 ml of methanol and 3 ml of conc. hydrochloric acid, and the mixture was boiled under reflux for 36 h and cooled. The mixture was stirred with Anion Resin CT-375 (Eli Lilly and Company) for 2 h at 25°, the suspension was filtered, and the filtrate was evaporated to dryness. The product, a mixture of methyl *O*-ethyl-α-D-glucopyranosides, was analyzed by g.l.c. of the trimethylsilyl ethers[10].

Methyl 3-O-acetyl-4,6-O-benzylidene-α-D-glucopyranoside (6). — The preparation of this compound has been described[11]; m.p. 175–176°, $\nu_{\max}^{\mathrm{CCl_4}}$ 3650, 2950, 2880, 1750, 1455, 1405, 1370, 1310, 1230, 1145, 1125, 1100, 1078, 1060, 1050, 1035 (sh), 992, 918, 895, and 695 cm^{-1}.

Methyl 3-O-acetyl-4,6-O-benzylidene-2-O-ethyl-α-D-glucopyranoside (7). — A solution of 3.2 g (10 mmoles) of 6 in 50 ml of *N,N*-dimethylformamide and 15.6 g (100 mmoles) of ethyl iodide was stirred with 11.6 g (50 mmoles) of silver oxide for 28 h at room temperature. The mixture was filtered, and the filtrate was evaporated to dryness. Based on comparison of areas in the n.m.r. spectrum at τ 4.83 (3-*O*-acetyl) and τ 5.2 (2-*O*-acetyl), the crude product was estimated to be a mixture of the desired product 7 (85%) and its isomer **8** (15%). Fractional recrystallization from petroleum ether (b.p. 60–65°) gave 7, yield 1.9 g (54%), m.p. 135°, $\nu_{\max}^{\mathrm{CCl_4}}$ 2970, 2900, 2875 (sh), 1750, 1460 (sh), 1450, 1440 (sh), 1365, 1320, 1305, 1270, 1225, 1195 (sh), 1180, 1090, 1055, 1035, 990, 915, 897, 875, and 695 cm^{-1}.

Anal. Calc. for $C_{18}H_{24}O_7$: C, 61.35; H, 6.86. Found: C, 61.27; H, 6.64.

Methyl 4,6-O-benzylidene-2-O-ethyl-α-D-glucopyranoside (9). — A solution of compound 7 (1.77 g, 5 mmoles) in 20 ml of 5% sodium methoxide in methanol was boiled for 2 h under reflux, evaporated to dryness, made neutral with M sulfuric acid, and extracted with chloroform. The extract was evaporated to dryness, and the resulting crude product was recrystallized from petroleum ether (b.p. 60–65°) to yield 0.93 g (60%) of crystalline product; m.p. 129–130°, $\nu_{\max}^{\mathrm{CHCl_3}}$ 3600, 2950, 1400, 1382, 1320, 1150 (sh), 1140 (sh), 1125 (sh), 1092, 1088 (sh), 1060, and 1000 cm^{-1}.

Anal. Calc. for $C_{16}H_{22}O_6$: C, 61.92; H, 7.15. Found: C, 61.82; H, 7.11.

Methyl 2-O-ethyl-α-D-glucopyranoside (5). — A solution of 780 mg (2.5 mmoles) of 9 in 15 ml of 50 mM methanolic sulfuric acid was stirred for 1 h at room temperature. After neutralization with Anion Resin CT-375, the suspension was filtered, and the filtrate was concentrated to 8 ml. Some starting material was recovered by filtration. The filtrate was concentrated to 5 ml, and crude product gradually separated; this was recrystallized from ethyl acetate to yield 22 mg (40%) of 5, m.p. 135–136°,

$\nu_{\max}^{\mathrm{KBr}}$ 3450, 2940, 1630, 1482 (sh), 1440, 1410, 1380, 1360, 1338, 1322, 1280, 1240, 1198, 1160, 1120, 1038, 925, 908, 890, 852, 835, 762, and 715 cm^{-1}.

Methyl 3-O-ethyl-α-D-glucopyranoside (**4**) *from methyl 3-O-vinyl-α-D-glucopyranoside* (**3**). — To a suspension of 200 mg of 5% palladium-on-carbon in 70 ml of methanol was added 1.25 g (5.6 mmoles) of **3**. The mixture was hydrogenated for 4 h at room temperature and 400 lb. in^{-2} (gauge). Recrystallization from methanol–ether gave **4**, yield 1.0 g (80%); m.p. 139–140°, $[\alpha]_D^{25}$ +152° (*c* 1, ethanol), $\nu_{\max}^{\mathrm{KBr}}$ 3450, 2900, 1630, 1460, 1400, 1365, 1335, 1320, 1280, 1240, 1225, 1205, 1190, 1155, 1130, 1115, 1085, 1060, 1045, 1000, 910, 842, 818, and 745 cm^{-1}.

Methyl 4-O-ethyl-α-D-glucopyranoside (**10**). — Ethylation of methyl 2,3-di-*O*-benzyl-6-*O*-trityl-α-D-glucopyranoside[12] was conducted as for the preparation of **7**. The expected product, methyl 2,3-di-*O*-benzyl-4-*O*-ethyl-6-*O*-trityl-α-D-glucopyranoside, was obtained only as a clear syrup, despite repeated purification by chromatography on alumina; $\nu_{\max}^{\mathrm{CCl_4}}$ 3080, 2950, 1950, 1740, 1600, 1495, 1450, 1375, 1360, 1260, 1195, 1160, 1100, 1045, 920, 902, and 698 cm^{-1}.

Subsequent reduction of this product by hydrogenolysis in the presence of palladium-on-carbon, or with sodium in liquid ammonia, gave a crude mixture. Separation of the more polar fraction of this mixture on silica gel, followed by g.l.c. of the trimethylsilyl ethers showed two peaks. The major peak corresponded to pertrimethylsilylated **10**, and the minor peak to methyl tetrakis-*O*-(trimethylsilyl)-α-D-glucopyranoside.

Attempted preparation of methyl 6-O-ethyl-α-D-glucopyranoside (**11**). — Detritylation of methyl 2,3,4-tri-*O*-benzoyl-6-*O*-trityl-α-D-glucopyranoside[13] by either hydrogen bromide in glacial acetic acid or hydrogen bromide in chloroform at 0° gave mixtures in which the products were only partially detritylated. Higher reaction-temperatures yielded detritylated mixtures which, when ethylated as described for **7**, yielded a mixture of all four methyl mono-*O*-ethyl-α-D-glucopyranosides, as shown by g.l.c. of the pertrimethylsilyl ethers.

Methyl 2,3-di-O-ethyl-α-D-glucopyranoside (**13**). — The preparation of this compound has been described previously[4].

ACKNOWLEDGMENT

This study was supported by the Agricultural Research Service, U. S. Department of Agriculture, contract No. 12-14-100-7165(71), administered by the Northern Utilization Research and Development Division, Peoria, Illinois.

REFERENCES

1 N. SHACHAT, H. SCHNEIDER, J. NEDWICK, G. MURDOCH, AND J. BOGNELL, *J. Org. Chem.*, 26 (1961) 3712.

2 N. SHACHAT AND N. BOGNELL, *J. Org. Chem.*, 27 (1962) 471.

3 J. W. BERRY, H. TUCKER, and A. J. DEUTSCHMAN, JR., *Ind. Eng. Chem. Process Des. Develop.*, 2 (1963) 318.

4 J. W. BERRY, A. J. DEUTSCHMAN, JR., AND J. P. EVANS, *J. Org. Chem.*, 29 (1964) 2619.

5 J. W. BERRY, H. TUCKER, A. J. DEUTSCHMAN, JR., AND J. P. EVANS, *Ind. Eng. Chem. Process Des. Develop.*, 5 (1966) 165.
6 J. W. BERRY, J. T. MARVEL, S. J. SEN, AND A. J. DEUTSCHMAN, JR., *Winter Meeting Am. Chem. Soc.*, Phoenix, Arizona, January 1966.
7 A. J. DEUTSCHMAN, JR., AND H. W. KIRCHER, *J. Amer. Chem. Soc.*, 83 (1961) 4070.
8 W. COCHRAN AND G. COX, *Experimental Designs*, Wiley, New York, 1957, p. 335.
9 J. T. MARVEL, S. SEN, J. W. BERRY, AND A. J. DEUTSCHMAN, JR., *Carbohyd. Res.*, 8 (1968) 148.
10 C. C. SWEELEY, R. BENTLEY, M. MAKITA, AND W. W. WELLS, *J. Amer. Chem. Soc.*, 85 (1963) 2497.
11 R. W. JEANLOZ AND D. A. JEANLOZ, *J. Amer. Chem. Soc.*, 79 (1957) 2579.
12 J. KENNER AND G. RICHARDS, *J. Chem. Soc.*, (1955) 1810.
13 B. HELFERICH AND J. BECKER, *Ann.*, 440 (1924) 1.

Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

KINETICS OF THE HYDROLYSIS OF 5-BROMO-4-CHLOROINDOL-3-YL β-D-GLUCOPYRANOSIDE BY ALMOND EMULSIN

JEROME P. HORWITZ*, CHITTUR V. EASWARAN**,
Detroit Institute of Cancer Research, Division of the Michigan Cancer Foundation; Department of Oncology, Wayne State University School of Medicine, Detroit, Michigan 48201 (U. S. A.)

AND LEON S. KOWALCZYK
Department of Chemical Engineering, The University of Detroit, Detroit, Michigan 48221 (U. S. A.)

(Received September 25th, 1968)

ABSTRACT

A kinetic study has been made of the hydrolysis of 5-bromo-4-chloroindol-3-yl β-D-glucopyranoside (**1**) by almond emulsin. The rate of hydrolysis depends on the nature of the buffer, and decreases in the following order of buffers: phosphate > acetate > citrate > "Tris" hydrochloride. The rate in phosphate buffer was found to pass through a sharp maximum at pH 6.23, and detailed studies were made at pH 6.10 to 6.75 for a range of substrate concentrations and temperatures. Up to a substrate concentration of 500 mM, the Michaelis–Menten law is obeyed accurately over the range of pH investigated. At higher concentrations of substrate, there is some falling off of the rate. The K_M for **1**, calculated from a Lineweaver–Burk plot, is 1.38 mM. The average energy of activation is 12.4 kcal/mol, which is within the range reported for the hydrolysis of other aryl β-D-glucopyranosides by this enzyme. The study demonstrates the potential utility of the indigogenic principle in the evaluation of the kinetic parameters of other hydrolytic enzymes.

INTRODUCTION

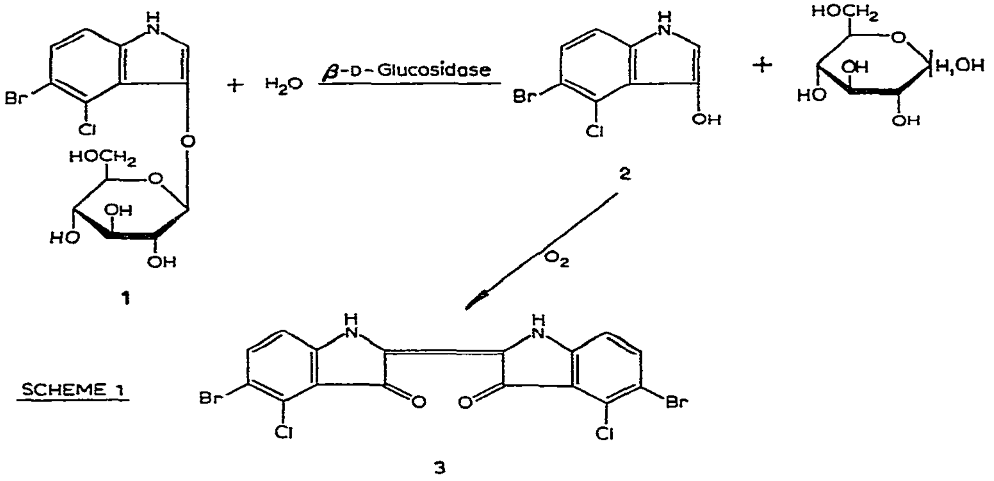
Recent reports from this laboratory have described the synthesis of a number of indol-3-yl β-D-glycopyranosides[1] that have found application as agents for the histochemical localization of corresponding glycosidases in mammalian tissue[2-5]. The chromogenic reaction-sequence underlying what has come to be known[6] as the "indigogenic principle" is initiated by enzymic release of an intermediate indoxyl (indol-3-yl) derivative (**2**). On air oxidation, the latter (*cf.*, Scheme 1) is rapidly and irreversibly transformed into an essentially insoluble (and highly colored) indigo derivative (**3**) that is deposited at the sites of activity.

---

*Inquiries concerning this paper should be addressed to this author at the Detroit Institute of Cancer Research, Division of the Michigan Cancer Foundation, 4811 John R. Street, Detroit, Michigan 48201, U. S. A.

**This paper is taken from a dissertation submitted by this author to the Graduate Division of The University of Detroit, in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

A kinetic study of the acid-catalyzed hydrolysis of several substituted indol-3-yl β-D-glucopyranosides[7] failed to disclose for the reaction any unusual mechanistic features that could be directly attributed to the particular aglycon. Instead, the investigation indicated the potential utility of other *O*-indoxyl derivatives for the kinetic characterization of acid- and base-catalyzed reactions.



SCHEME 1

Although almond emulsin is usually regarded as being a β-D-glucosidase, it shows differing glycosidase activity toward a range of aryl β-D-glycosides and disaccharides[8,9], but these activities have been clearly demonstrated to reside in a single enzyme[8]. We now describe extension of the indigogenic principle to evaluation of the kinetic parameters for the hydrolysis of 5-bromo-4-chloroindol-3-yl β-D-glucopyranoside (**1**) by almond emulsin.

EXPERIMENTAL

5-Bromo-4-chloroindol-3-yl β-D-glucopyranoside (**1**). — Was prepared as already described[1]; m.p. 240–243° (dec.), $[\alpha]_D^{23}$ $-89°$ (*c* 1.0, *N,N*-dimethylformamide). The purity of **1** was checked by t.l.c. on silica gel with 43:7 (v/v) butyl-alcohol–water. Compound **1** is available from Pierce Chemical Co., Rockford, Illinois.

Almond emulsin was purchased from Sigma Chemical Co., St. Louis, Missouri. A 1% solution of the enzyme was assayed with salicin according to the procedure of Joubert and Van der Walt[10]. A unit of β-D-glucosidase is defined as the amount of enzyme that liberates 200 μg of D-glucose during 10 min at 30°.

Rates were determined spectrophotometrically with a Cary Model 11 recording spectrophotometer and cells equipped with jackets and thermostated by a Haake Type F constant-temperature bath. The jackets and bath were joined in series to a Thermo-Cool heat-exchanger; the arrangement provided regulation of temperature to ±0.02° over the desired range (25–37°). Rates were followed by observing the formation of 5,5′-dibromo-4,4′-dichloroindigo (**3**) at[11] 660 nm.

Beer–Lambert plots were used for ascertaining the quantity of 3 formed in the oxidation step. These plots, in turn, afforded a measure of the intermediate indoxyl compound (2) generated in the hydrolysis step. The procedure of Cotson and Holt[11] was used in preparing the plot, which is based on the spectrophotometric measurement of the rate of appearance of 3. When the oxidation is effected in aqueous media, 3 is deposited as a colloidal suspension, the stability of which is not suitable for optical measurement. The dye sol can be stabilized by inclusion of 0.5% of polyvinyl alcohol, so that the dye sol does not change during several hours.

A linear relationship was observed in a plot of the concentration of 3 *versus* optical absorbance at 660 nm, over the desired range of concentration, in phosphate, citrate–phosphate, acetate, and "Tris" maleate buffers. The plot depicted in Fig. 1 is considered to be typical.

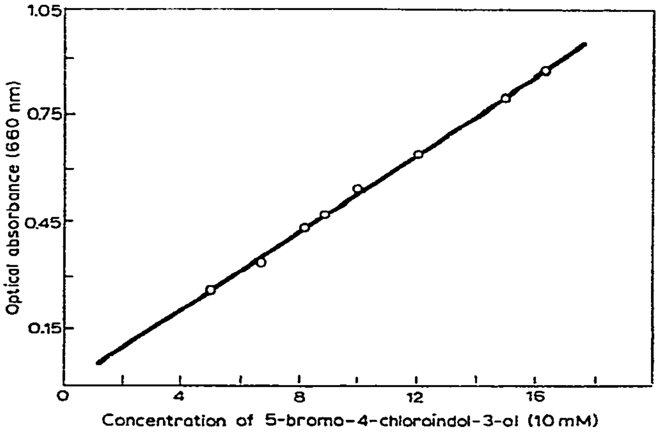


Fig. 1. Beer–Lambert plot for 5,5′-dibromo-4,4′-dichloroindigo in 200 mM phosphate buffer at pH 6.23.

The choice of a phosphate buffer system for rate studies was based on a comparison of the amount and rate of hydrolysis of 1 in the systems already described. The course of hydrolysis is given in Fig. 2 by plotting log $[S/(S-X)]$ *versus* t according to the first-order rate-expression

$$t = \frac{2.303}{k} \log \frac{[S]}{[S-X]},$$

where $[S]$ is the initial concentration of substrate, X is the amount of substrate hydrolyzed by the enzyme in t min, and k is the velocity constant observed. A comparison of the buffers tested showed that the amount and rate of hydrolysis decreases in the following order: phosphate > acetate > citrate–phosphate > "Tris" maleate. From Fig. 2, it is apparent that first-order kinetics are followed up to *ca.* 65% completion of the reaction. An explanation of the deviation from linearity is provided later.

The procedure in each experiment was similar, and the following is typical.

The reactants, consisting of 2 ml of oxygenated phosphate buffer* (200 mM, pH 6.75), 2 ml of the stock solution of substrate (490 mM), and 2 ml of enzyme (1.2 mg), were separately warmed to 30 ±0.02°, mixed, and immediately transferred to a 3.5-ml cuvet (10-mm pathway). The addition of enzyme, the transfer of the reaction mixture to the optical cells, and the introduction of the cells into the spectrophotometer required less than 30 sec. In all measurements, the reference cell contained a solution having the same concentration of polyvinyl alcohol and of salt, and having the same pH, as for the reaction mixture. The rate of appearance of the indigo derivative (**3**) was followed at 660 nm. From a knowledge of the concentration of **3** and the initial concentration of **1**, the fraction of unreacted substrate (**1**) could be calculated.

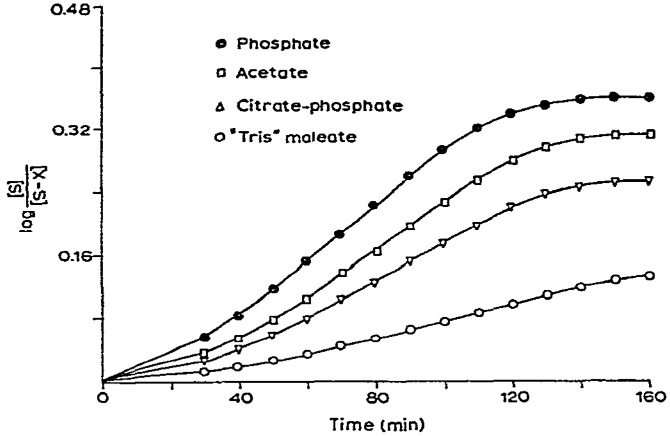


Fig. 2. Rate of hydrolysis of 5-bromo-4-chloroindol-3-yl β-D-glucopyranoside at pH 6.45 in several buffer systems (200 mM) at 30°; $[S]_0$ = 163.1 mM and $[E]_0$ = 33.2 nM.

RESULTS AND DISCUSSION

Aerobic oxidation of indoxyl derivatives to indigo dyes (*e.g.*, **2** to **3**) has been studied kinetically over the range of pH of 6 to 8.5 ("Tris" buffer)[11]. Above pH 8.5, the rate of oxidation is too high to be measured accurately by conventional techniques. At pH 6, data are obtained that indicate for the reaction an apparent, first-order rate that increases with time. However, measurements of $t_{0.5}$ at initial concentrations of indoxyl derivative of 125 to 500 mM gave the same value, namely, 48 ±5 min at 19.4°. At 30°, the reaction is too rapid to be followed by this method. By contrast, a $t_{0.5}$ value of 108 ±4 min was obtained for the enzymic conversion of **1** into **2** at 30° in phosphate buffer at pH 6.1 over a range of concentration of substrate of 110 to 400 mM. This result precludes the possibility that the (nonspecific) air oxidation is the slow step in the overall reaction. It is of interest that the aerobic oxidation of **2**

---

*Cotson and Holt[11] have shown that sufficient oxygen is present in the solution to maintain an adequate excess during an experiment.

was also found to be not rate-determining in the acid-catalyzed hydrolysis of this substrate[7]. Accordingly, the conversion of **2** into **3** has been ignored in our subsequent treatment of the kinetics of the hydrolysis of **1** by almond emulsin.

In systems for which Michaelis–Menten kinetics are applicable, the rate of the reaction increases with the concentration of the substrate up to a certain point, after which it remains constant[12]. However deviations of the type indicative of the phenomenon of substrate inhibition were observed in the hydrolysis of **1** at fixed concentrations of enzyme and various concentrations of the D-glucoside. Thus, a plot of the data (see Fig. 3), in the form of v *versus* $[S]$ at pH 6.75, reveals that the rate passes through a maximum as the concentration of substrate increases, and then decreases; this finding indicates that the deviation arises because of substrate inhibition[12].

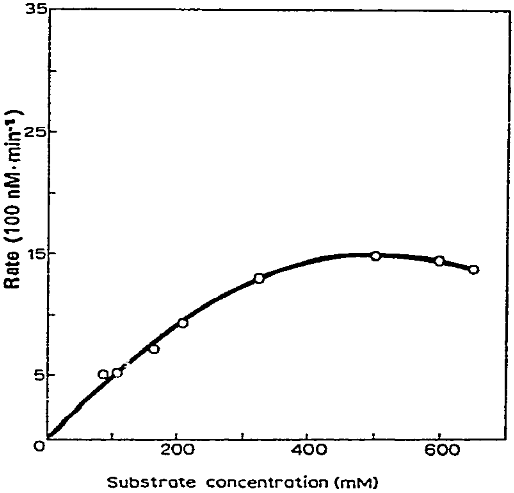


Fig. 3. Plot of rate *versus* substrate concentration for the hydrolysis of 5-bromo-4-chloroindol-3-yl β-D-glucopyranoside by almond emulsin in 200 mM phosphate buffer at pH 6.75. Assay conditions as for Fig. 2.

Inhibition by products. — An examination of the variation of initial rates with concentration indicated interference by products in the course of the hydrolysis of **1**. The phenomenon was investigated by analyzing the change of concentrations of reactant and products with time, by use of the graphical version of Van 't Hoff's differential method[13].

The results of applying this procedure to the present data are shown in Fig. 4, which is a plot of log rate *versus* log $[S]$. Each of the dotted curves represents a determination of the rate at a given initial concentration and, therefore, corresponds to the change in concentration with time. The complete curve indicates the change in initial rate with initial concentration, the initial rates having been derived from the dotted curves by extrapolation. At low concentration, the plot of log v against log $[S]$ has a slope of unity, showing that the rate varies linearly with the initial concentration. A significant feature of Fig. 4 lies in the discrepancy between the order with respect to time (*i.e.*, the slopes of the dotted curves) and that with respect to concentration (the slopes of the full curve). It may be noted that, in the vicinity of the maximum, where the order with respect to concentration is close to zero, the slopes of the dotted curves are very high, indicating a rapid decline in the rate as the reaction proceeds. That the products interfere with the course of reaction[13] is indicated by a higher order with respect to time than is observed with respect to concentration.

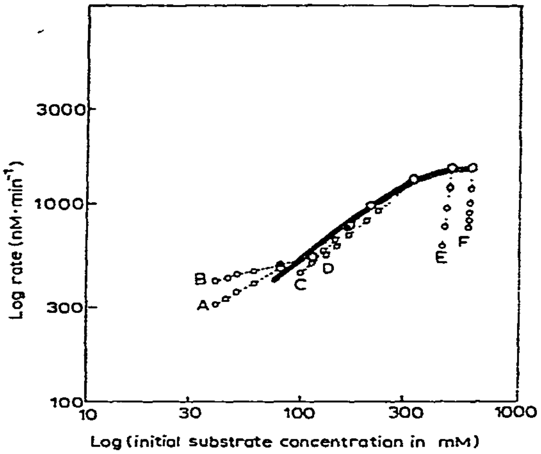


Fig. 4. Plot of log rate *versus* log [*S*]; the broken lines are for concentration changes in a single experiment, and the full curve represents the initial rates and concentrations. The apparent order is given by the slope. The broken curves relate to the following initial concentrations of 5-bromo-4-chloroindol-3-yl β-D-glucopyranoside: A = 81.6 mM; B = 111 mM; C = 208.1 mM; D = 322.1 mM; E = 499.6 mM; and F = 599.4 mM. Assay conditions as for Fig. 2, pH 6.75.

The effect of pH, over the range of 6.1 to 6.75, on the rate of hydrolysis of **1** is shown in Fig. 5. The rates are at a maximum between pH 6.2 and 6.3 for substrate

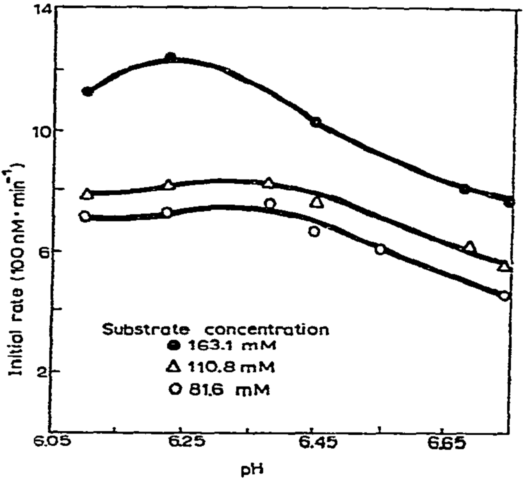


Fig. 5. The effect of pH (6.1 to 6.75, phosphate buffer) on the rate of hydrolysis of 5-bromo-4-chloroindol-3-yl β-D-glucopyranoside. Assay conditions as for Fig. 2, $[S]_0$ = 81.6 to 163.1 mM.

concentrations of 81.6 to 163.1 mM. However, the reaction at the optimal pH (6.23) was observed to fall considerably below the asymptotic approach to 100% hydrolysis that would be given by normal, first-order kinetics (see Fig. 6).

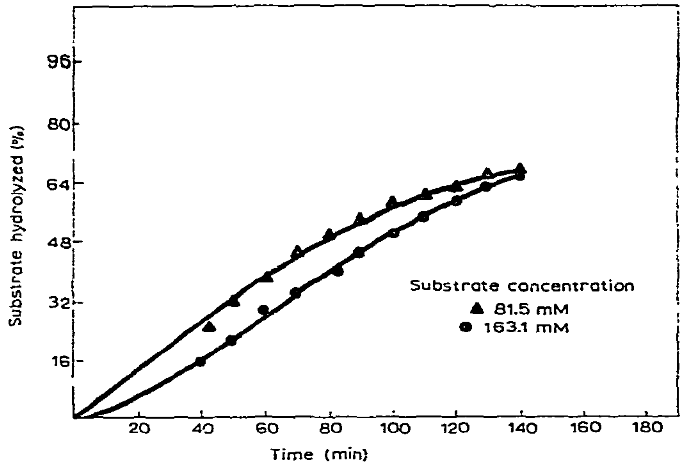


Fig. 6. Course of hydrolysis of 5-bromo-4-chloroindol-3-yl β-D-glucopyranoside by almond emulsin in 200 mM phosphate buffer, pH 6.23. Assay conditions as for Fig. 2.

A similar phenomenon has been observed in the hydrolysis of *p*-nitropheny β-D-glucopyranoside both by emulsin[8] and *Stachybotrys atra*, a fungal β-D-glucosidase[14]. It was suggested that the D-glucose liberated is a powerful, competitive inhibitor of the enzyme, because the phenol derivatives tested did not exert a comparable effect. The interference observed in the present system must, almost certainly, be due to D-glucose, as compounds related to the aglycon do not usually act as competitive inhibitors[15].

Inhibition by metal ions. — A slight, but nonetheless significant, inhibition of activity was observed with both Ag^+ and Hg^{2+}. By contrast, the effect of Zn^{2+} was almost negligible (*cf.*, Table I). For the same cations, Wallenfels and Fischer[16] observed inhibition, of a comparable magnitude, of the hydrolysis of *p*-nitrophenyl

TABLE I

INHIBITION BY METAL ION OF THE HYDROLYSIS OF 5-BROMO-4-CHLOROINDOL-3-YL β-D-GLUCOPYRANOSIDE BY ALMOND EMULSIN[a]

Metal ion	*Concentration,* mM	*Inhibition,* %
Ag^+	29	19.0
Hg^{2+}	13	17.0
Zn^{2+}	14	0.08

[a] The enzyme and the inhibitor were incubated for 10 min at 30°, and the reaction was initiated by addition of substrate (163.1 mM); phosphate buffer, pH 6.2.

β-D-galactopyranoside by calf-intestine β-D-galactosidase; the findings were attributed to a nonspecific binding that caused changes in the tertiary structure of the protein.

K_M *and the energy of activation.* — The initial velocity (v) of the hydrolysis of **1** was measured at several concentrations of substrate over the pH range of 6.1 to 6.75. From Fig. 3, it is apparent that application of the Michaelis–Menten law to the determination of K_M is inappropriate, because the rate falls off beyond a substrate concentration of 500 mM. Therefore, K_M was determined by use of a Lineweaver–Burk[17] plot, which yielded an essentially invariant value, 1.36 (±0.02) mM, over the pH range of 6.1 to 6.75. This constant compares favorably with the value (2.59 mM) reported[8] for the hydrolysis, by commercial, almond emulsin, of p-nitrophenyl β-D-glucopyranoside. In fact, K_M values having the same order of magnitude have been recorded[18,19] for the hydrolysis of o-nitrophenyl β-D-glucopyranoside with a more highly purified form of the same enzyme.

The effect of temperature on the rate of hydrolysis of **1** was examined over the temperature range of 25 to 40°. Plotted in the conventional, Arrhenius way, the results are linear in the neighborhood of the optimal pH (see Fig. 7). The departure of the curve from linearity occurs only at temperatures at which the enzyme is, apparently, inactivated. The average energy of activation is 12.4 kcal/mol, which is within the range of values reported for the hydrolysis of other aryl β-D-glucopyranosides by almond emulsin[10].

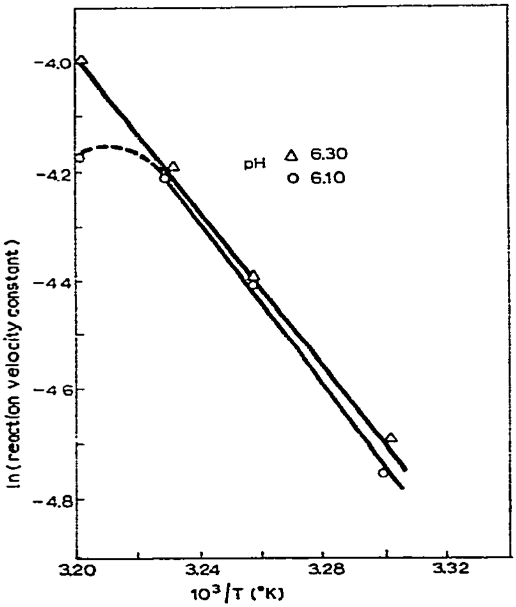


Fig. 7. Effect of temperature on the hydrolysis of 5-bromo-4-chloroindol-3-yl β-D-glucopyranoside by almond emulsin in 200 mM phosphate buffer, pH 6.1 and 6.3. Assay conditions as for Fig. 2.

It has been shown that almond emulsin displays a lack of specificity toward C-4 and C-5 of the sugar moiety[18,19]. The present study indicates that, as might be expected, the enzyme is also relatively insensitive to the nature of the aglycon. Of greater significance is the fact that the present work constitutes a clear demonstration of the potential application of the indigogenic principle to the study of other hydrolytic enzymes.

ACKNOWLEDGMENTS

This investigation was supported, in part, by U.S. Public Health Service Research Grant No. CA-02624 from the National Cancer Institute, and, in part, by an institutional grant from the United Foundation of Greater Detroit to the Detroit Institute of Cancer Research Division of the Michigan Cancer Foundation.

REFERENCES

1 J. P. Horwitz, J. Chua, R. J. Curby, A. J. Tomson, M. A. Da Rooge, B. E. Fisher, J. Mauricio, and I. Klundt, *J. Med. Chem.*, 7 (1964) 574.
2 B. Pearson, M. Andrews, and F. Grose, *Proc. Soc. Exp. Biol. Med.*, 108 (1961) 619.
3 B. Pearson, P. Wolf, and J. Vazquez, *Lab. Invest.*, 12 (1963) 1249.
4 B. Pearson, P. Wolf, and M. Andrews, *Lab. Invest.*, 12 (1963) 712.
5 P. L. Wolf, J. P. Horwitz, J. Vazquez, J. Chua, and M. A. Da Rooge, *Amer. J. Clin. Path.*, 44 (1965) 307.
6 S. J. Holt, in J. F. Danielli (Ed.), *General Cytochemical Methods*, Vol. 1, Academic Press, New York, 1958, p. 375.
7 J. P. Horwitz, C. Easwaran, and L. Kowalczyk, *J. Org. Chem.*, 33 (1968) 3174.
8 R. Haworth and P. G. Walker, *Biochem. J.*, 83 (1962) 331.
9 D. J. Manners and D. C. Taylor, *Carbohyd. Res.*, 7 (1968) 497.
10 F. J. Joubert and T. N. Van der Walt, *J. S. Afr. Chem. Inst.*, 17 (1964) 79.
11 S. Cotson and S. J. Holt, *Proc. Roy. Soc.* (London), B148 (1958) 506.
12 K. J. Laidler, *The Chemical Kinetics of Enzyme Action*, Oxford University Press, London, 1958, pp. 101, 58.
13 K. J. Laidler and J. P. Hoare *J. Amer. Chem. Soc.*, 71 (1949) 2699.
14 M. A. Jermyn, *Aust. J. Biol. Sci.*, 8 (1955) 563.
15 M. A. Jermyn, *Rev. Pure Appl. Chem.*, 11 (1961) 92.
16 K. Wallenfels and J. Fischer, *Z. Physiol. Chem.*, 321 (1960) 223.
17 H. Lineweaver and D. Burk, *J. Amer. Chem. Soc.*, 56 (1934) 658.
18 J. Conchie, A. L. Gelman, and G. A. Levvy, *Biochem. J.*, 103 (1967) 609.
19 J. Conchie, A. L. Gelman, and G. A. Levvy, *Biochem. J.*, 106 (1968) 135.

Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

DÉPLACEMENTS EN SÉRIE MÉTHYL FURANOSIDE. SYNTHÈSE DE DÉRIVÉS DU 2,3,5-TRIAMINO-2,3,5-TRIDÉSOXY-D-ARABINOSE ET -D-XYLOSE*

JEAN HILDESHEIM, JANINE CLÉOPHAX, ANNE-MARIE SÉPULCHRE ET STEPHAN D. GÉRO
Institut de Chimie des Substances Naturelles, C. N. R. S., 91 Gif-sur-Yvette (France)

(Reçu le 17 juillet 1968)

SOMMAIRE

Le méthyl 5-azido-5-désoxy-2,3-di-*O*-*p*-tolylsulfonyl-β-D-ribofuranoside, traité par l'azothydrate de sodium dans la *N*,*N*-diméthylformamide, fournit après 2,5 h à 145°, le diazide **3** caractérisé par un dérivé di-*N*-benzoylé cristallin. Le méthyl 5-benzamido-2,3-benzoylépimino-2,3,5-tridésoxy-β-D-lyxofuranoside (**7**) a été obtenu avec un rendement de 61 % à partir du diazide **3**, par traitement de ce dernier avec l'aluminohydrure de lithium, suivi d'une benzoylation. L'azidolyse de l'aziridine **7** a fourni deux composés dans le rapport 1,8:1 qui, après hydrogénation et *N*-benzoylation, ont donné respectivement les méthyl 2,3,5-tribenzamido-2,3,5-tridésoxy-β-D-arabinoside et -xyloside correspondants. L'absence de réactivité du groupement tosyloxy sur C-2, vis-à-vis de déplacements du type S_N2 est discutée.

ABSTRACT

Methyl 5-azido-5-deoxy-2,3-di-*O*-*p*-tolylsulfonyl-β-D-ribofuranoside was treated with sodium azide in *N*,*N*-dimethylformamide for 2.5 h at 145° to give the diazido compound **3**, characterised as crystalline di-*N*-benzoyl derivative. Methyl 5-benzamido-2,3-benzoylepimino-2,3,5-trideoxy-β-D-lyxofuranoside (**7**) was obtained in 61 % yield from diazide **3** by treatment with lithium aluminum hydride, followed by direct benzoylation. Azidolysis of **7** with sodium azide in *N*,*N*-dimethylformamide gave two compounds in a ratio 1.8:1, which were readily converted, after hydrogenation and *N*-benzoylation to the corresponding methyl 2,3,5-tribenzamido-2,3,5-trideoxy-β-D-arabinoside and -xyloside, respectively. The lack of reactivity of the tosyloxy group at C-2 towards S_N2-type displacements is discussed.

INTRODUCTION

La découverte dans la nature de composés aminés et polyaminés, possédant des propriétés antibiotiques et antitumorales[1-3], a éveillé l'intérêt de nombreux auteurs et

---

[*] 14e Communication sur les déplacements en série tétrahydrofurannique. Pour la 13e communication, voir J. Cléophax, J. Hildesheim, A. M. Sépulchre et S. D. Géro, *Bull. Soc. Chim.*, sous presse. Une communication préliminaire a été publiée : J. Cléophax, S. D. Géro et J. Hildesheim, *Chem. Commun.*, (1968) 94.

a stimulé la synthèse de monosaccharides possédant des fonctions azotées. Ces efforts ont, en particulier, porté sur l'élaboration de dérivés polyaminés et récemment[4], la synthèse d'un hexose de forme pyranoïde contenant quatre fonctions aminées a été réalisée.

Bien souvent, les problèmes soulevés pour la réalisation d'une telle polyfonctionnalité ont pu être résolus, soit à l'aide de réactions du type S_N2, soit par des réactions du type anchimérique sur des molécules substituées par des sulfonates convenablement situés. Il est néanmoins surprenant de constater le nombre restreint[5,6] de travaux ayant pour objet l'introduction de multiples fonctions azotées dans des dérivés tétrahydrofuranniques.

La partie glycosidique de la puromycine[7] est un «3-amino-3-désoxy-D-ribose». Cet antibiotique possède des propriétés biologiques du plus grand intérêt. En conséquence, l'élaboration de dérivés diaminés et triaminés du type furanoïde (précurseurs synthétiques des di- et triamino-nucléosides correspondants), exerce une certaine attraction aussi bien du point de vue chimique que du point de vue biologique.

Dans ce travail, nous décrivons l'introduction successive de trois fonctions azotées dans un pentofuranoside, le produit de départ étant le méthyl 2,3,5-tri-*O*-*p*-tolylsulfonyl-β-D-ribofuranoside.

RÉSULTATS ET DISCUSSION

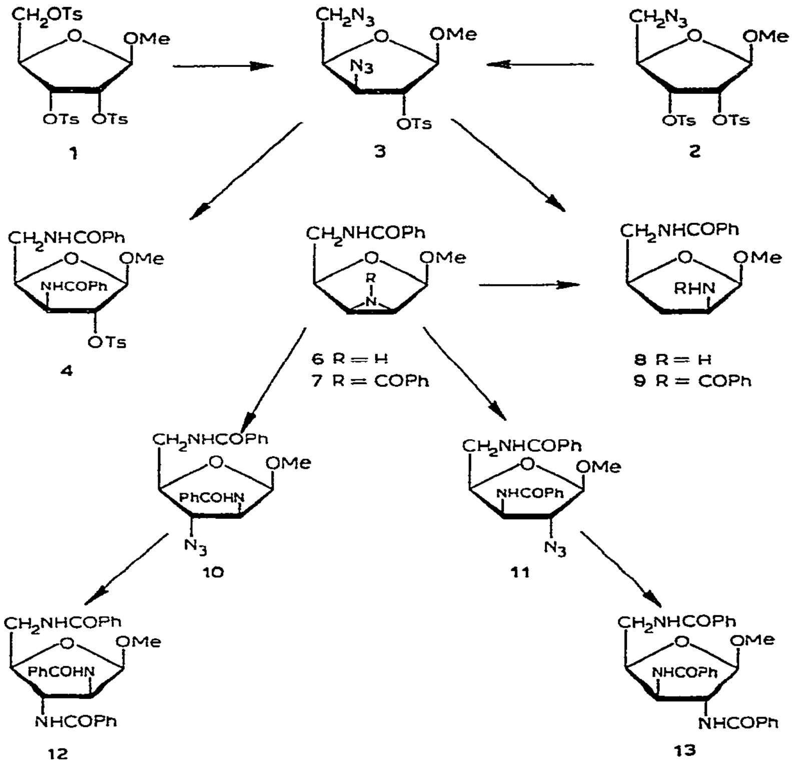
L'action de l'azothydrate de sodium dans la diméthylformamide pendant 30 min à 100° sur le méthyl 2,3,5-tri-*O*-*p*-tolylsulfonyl-β-D-ribofuranoside[8] (**1**) a mené, avec un rendement de 90%, au méthyl 5-azido-5-désoxy-2,3-di-*O*-*p*-tolylsulfonyl-β-D-ribofuranoside (**2**).

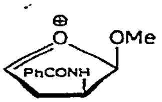
Une seconde fonction azide peut être introduite dans l'oside **2** par le déplacement direct de l'un des deux sulfonates secondaires. Ceci a été réalisé en augmentant, d'une part la température à 145° et, d'autre part, le temps de chauffage. Le méthyl 3,5-diazido-3,5-didésoxy-2-*O*-*p*-tolylsulfonyl-β-D-xylofuranoside (**3**) a été ainsi obtenu avec un rendement de 60%. En outre, le diazide **3** étant une huile, le composé a été caractérisé, après hydrogénation suivie d'une benzoylation, par son dérivé di-*N*-benzoylé cristallin **4**.

Il est à noter que le dérivé diazidomonotosyle **3** peut être obtenu directement à partir du tritosyle **1** par traitement direct de celui-ci avec de l'azothydrate de sodium.

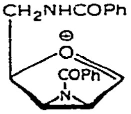
Le spectre de r.m.n. du diazide **3** montre le proton glycosidique à 4,87 p.p.m.; il apparaît sous forme d'un doublet de faible couplage, $J_{1,2}$ 2 Hz. Ceci est en accord avec une relation *trans* des protons sur C-1 et C-2. On remarque, d'autre part, que l'hydrogène de C-3 qui, dans le composé **2** apparaissait vers 4,75 p.p.m., a subi un déplacement diamagnétique. Il apparaît dans le diazide **3**, vers 4,4 p.p.m., comme un multiplet non résolu. Les données de la r.m.n. suggèrent donc fortement que le déplacement s'est produit avec inversion de configuration sur C-3.

Il ne nous a pas été possible en revanche d'introduire une troisième fonction azide par cette voie dans le diazide **3**, des conditions réactionnelles plus poussées menant invariablement à une décomposition extensive. Cette dernière observation, c'est-à-dire qu'un déplacement ne peut avoir lieu sur C-2 dans le composé **3**, même en présence d'un nucléophile puissant comme l'est l'ion azide, suggère que C-2 est un site non réactif. Ce fait peut être rationalisé en fonction de l'environnement électronique de C-2 qui a, dans sa proximité immédiate, d'une part, l'oxygène du méthoxyle anomérique, d'autre part, l'oxygène de l'hétérocycle. Au cours d'un processus de substitution du type S_N2, il en résulterait des interactions dipolaires qui s'opposeraient à la formation d'un état de transition sur C-2. Il est vraisemblable que le haut degré de sélectivité observé ici et dans d'autres cas similaires[9], lors de déplacements nucléophiles sur des composés possédant le système *cis*-ditosyloxy (par exemple **1** ou **2**), est dû à l'absence quasi totale de réactivité du groupement tosyloxy sur C-2.

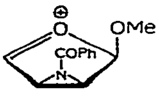




14
m/e 220

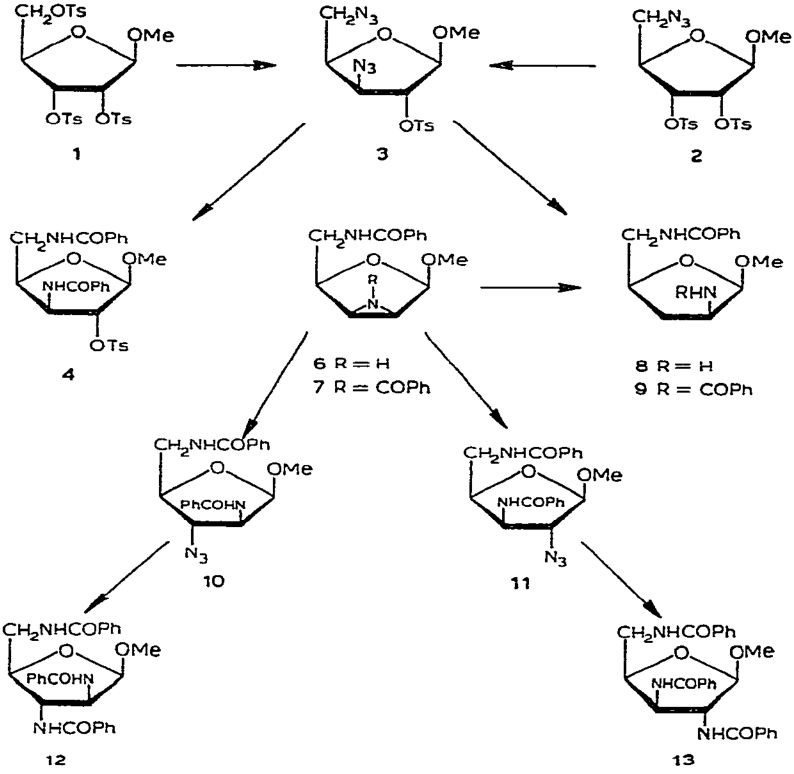


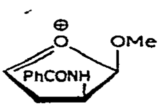
15
m/e 321



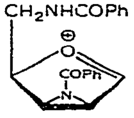
16
m/e 218

Afin de prouver de façon définitive la relation *trans* des groupements azide et tosyle dans le composé **3**, nous avons tenté de le convertir en aziridine corresmenant invariablement à une décomposition extensive. Cette dernière observation, c'est-à-dire qu'un déplacement ne peut avoir lieu sur C-2 dans le composé **3**, même en présence d'un nucléophile puissant comme l'est l'ion azide, suggère que C-2 est un site non réactif. Ce fait peut être rationalisé en fonction de l'environnement électronique de C-2 qui a, dans sa proximité immédiate, d'une part, l'oxygène du méthoxyle anomérique, d'autre part, l'oxygène de l'hétérocycle. Au cours d'un processus de substitution du type S_N2, il en résulterait des interactions dipolaires qui s'opposeraient à la formation d'un état de transition sur C-2. Il est vraisemblable que le haut degré de sélectivité observé ici et dans d'autres cas similaires[9], lors de déplacements nucléophiles sur des composés possédant le système *cis*-ditosyloxy (par exemple **1** ou **2**), est dû à l'absence quasi totale de réactivité du groupement tosyloxy sur C-2.

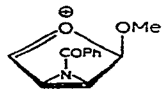




14
m/e 220



15
m/e 321



16
m/e 218

Afin de prouver de façon définitive la relation *trans* des groupements azide et tosyle dans le composé **3**, nous avons tenté de le convertir en aziridine corresvation du spectre de r.m.n. à 100 MHz. Le proton glycosidique de l'isomère *xylo* **11** se présente sour forme d'un doublet de faible couplage : $J_{1,2}$ 1 Hz, à 4,87 p.p.m., alors que le proton de C-2 apparaît comme un quadruplet vers 3,92 p.p.m. Par irradiation de ce dernier proton, on observe la disparition du couplage du proton glycosidique ainsi qu'une simplification du multiplet vers 4,82 p.p.m., dû à l'hydrogène de C-3. Le proton de C-4, qui apparait sous forme d'un multiplet complexe vers 3,1 p.p.m., ne modifie pas, après irradiation, l'apparence du proton vicinal à la fonction azide sur C-2. Un tel comportement est en faveur de la structure *xylo* **11**.

L'hydrogénation catalytique des deux isomères **10** et **11**, suivie d'une benzoylation *in situ*, fournit respectivement sous forme cristalline, le méthyl 2,3,5-tribenzamido-2,3,5-tridésoxy-β-D-arabinofuranoside (**12**) et le méthyl 2,3,5-tribenzamido-2,3,5-tridésoxy-β-D-xylofuranoside (**13**).

PARTIE EXPÉRIMENTALE

Les chromatographies sur colonne ont été effectuées sur gel de silice Davison (100–200 Mesh). Les séparations en couche mince ont été faites à l'aide de Kieselgel G (E. Merck, Darmstadt).

Les spectres de r.m.n. ont été, sauf mention contraire, mesurés dans le chloroforme, à la fréquence de 60 MHz avec un spectrophotomètre Varian A-60. Les déplacements chimiques sont mesurés à partir de la raie du tétraméthylsilane, prise comme zéro de référence et exprimés soit en Hz soit en δ.

Les spectres de masse ont été effectués dans le service de l'Institut sur un appareil MS 9 de la compagnie A. E. I.

Les points de fusion ont été déterminés sur un appareil Koffler et sont corrigés; les pouvoirs rotatoires ont été mesurés sur un appareil « Quick » de Roussel et Jouan.

Toutes les analyses ont été fournies par le service de micro-analyse du C. N. R. S.

*Méthyl 5-azido-5-désoxy-2,3-di-*O-p-*tolylsulfonyl-β-*D-*ribofuranoside* (**2**). — Le tritosylate **1** (3,098 g) est dissous dans la *N,N*-diméthylformamide anhydre (30 ml) est additionné d'azothydrate de sodium (520 mg, 62% d'excès). Le tout est porté à 100° pendant 30 min. La solution réactionnelle est alors refroidie et versée dans une ampoule contenant du chloroforme. La solution chloroformique est lavée 3 fois à l'eau, puis est séchée sur sulfate de sodium, évaporée à pression réduite et finalement sous vide poussé afin de chasser les dernières traces de *N,N*-diméthylformamide. Le résidu cristallise dans le méthanol (2,2 g; 90%). Une recristallisation dans le même solvant donne un p.f. 122–123°; $[\alpha]_D^{28}$ +80,5° (*c* 0,63, chloroforme).

Anal. Calc. pour $C_{20}H_{23}N_3O_8S_2$: C, 48,29; H, 4,62; N, 8,45; S, 12,87. Trouvé : C, 48,29; H, 4,66; N, 8,23; S, 13,09.

*Méthyl 3,5-diazido-3,5-didésoxy-2-*O-p-*tolylsulfonyl-β-*D-*xylofuranoside* (**3**). *Voie A* — Le monoazide **2** (10,13 g), dissous dans la *N,N*-diméthylformamide anhydre (100 ml) est additionné d'azothydrate de sodium (6,65 g, 5 éq.). Le tout est porté, sous agitation magnétique à 145° pendant 2,5 h. Après refroidissement, la solution réactionnelle est extraite comme pour le produit **2**. Le résidu brun montre alors deux taches sur couche mince (solvant : acétate d'éthyle–éther de pétrole 1:4). La tache migrant le moins (*R*$_F$ 0,50) est du produit de départ n'ayant pas réagi. L'autre tache (*R*$_F$ 0,55) est le diazide **3**. Le sirop est dissous dans le méthanol chaud (40 ml) qui, par refroidissement, laisse déposer 2,4 g de cristaux du produit de départ **2**. Les eaux mères sont concentrées et chromatographiées sur une colonne de gel de silice (300 g) préalablement équilibrée dans un mélange d'acétate d'éthyle–éther de pétrole (1:4). L'élution se fait à l'aide du même solvant. On recueille ainsi 3,2 g de diazide pur (55,8 %), $[\alpha]_D^{24}$ $-56°$ (*c* 0,53, chloroforme).

Voie B. — Le tritosylate **1** (55 g) dissous dans la *N*,*N*-diméthylformamide (500 ml) est additionné d'azothydrate de sodium (39,5 g; 3,47 éq.). Le tout est porté à 145° pendant 2,5 h. Après refroidissement, le mélange réactionnel est traité comme pour la voie A; 10,04 g de monoazide **2** sont récupérés par cristallisation. Le reste du produit fournit, après chromatographie, 14,56 du diazide **3** (rdt. 58,5 %, compte tenu du monoazide **2** formé).

*Méthyl 3,5-dibenzamido-3,5-didésoxy-2-*O*-*p*-tolylsulfonyl-β-*D*-xylofuranoside* (**4**). — Le diazide **3** (300 mg) dissous dans le méthanol (15 ml) est hydrogéné pendant 45 min en présence de platine d'Adams (100 mg). Après ce laps de temps, on filtre sur Célite, rince au méthanol et concentre le volume à environ 10 ml et ajoute 550 mg (50 % d'excès) d'anhydride benzoïque. Après 2 h d'agitation magnétique à température ordinaire, le précipité formé est filtré (200 mg), p.f. 170–177°, homogène en couche mince (*R*$_F$ 0,8); acétate d'éthyle–éther de pétrole (4:1). Une recristallisation dans le méthanol donne un p.f. 173,5–175°, $[\alpha]_D^{23}$ $-79,2$ (*c* 0,65, chloroforme).

Anal. Calc. pour $C_{27}H_{28}N_2O_7S$: C, 61,82; H, 5,38; N, 5,34; S, 6,10. Trouvé : C, 62,06; H, 5,44; N, 5,55; S, 6,39.

*Méthyl 5-benzamido-3-benzoylépimino-2,3,5-tridésoxy-β-*D*-lyxofuranoside* (**7**). — Une solution de diazide **3** (2,5 g) dissous dans le tétrahydrofuranne (25 ml) fraîchement distillé est versée goutte à goutte dans une suspension d'aluminohydrure de lithium (1,14 g) et de tétrahydrofuranne (10 ml) refroidie à 0°, sous agitation magnétique. Le bain est ainsi conservé à 0° durant 1,5 h, après quoi, le mélange réactionnel est porté à reflux pendant 2 h. L'excès d'hydrure est ensuite détruit par des petites portions d'éther humide. Le tout est filtré sur Célite et le précipité, abondamment lavé à l'aide de tétrahydrofuranne. Le filtrat est évaporé et le sirop résiduel, dissous dans le méthanol (25 ml). On ajoute 4,75 g d'anhydride benzoïque et la solution est gardée une nuit à température ordinaire. Le méthanol est ensuite évaporé à pression réduite et le résidu est agité avec du bicarbonate de sodium aqueux. Après 1 h de ce traitement, le tout est transvasé dans une ampoule contenant du chloroforme et la phase organique est lavée à l'eau jusqu'à neutralité. Après séchage sur sulfate de sodium et distillation du solvant, le résidu est cristallisé dans un mélange d'acétate d'éthyle–éther de pétrole, 1,322 g (53 %). La chromatographie des eaux mères augmente le rendement de 5 à 10 %, p.f. 126–128°; $[\alpha]_D^{23}$ $-92,5°$ (*c* 0,4, chloroforme).

Anal. Calc. pour $C_{20}H_{22}O_5N_2$: C, 68,17; H, 5,72; N, 7,95. Trouvé : C, 67,87; H, 5,73; N, 8,17.

*Méthyl 2,5-dibenzamido-2,3,5-tridésoxy-β-*D-thréo-*pentofuranoside* (**9**). — Le diazide **3** est dissous dans le méthanol (50 ml) et additionné d'hydrate d'hydrazine à 98% (3,5 ml). On ajoute alors avec précaution 4 à 5 grosses spatulées de nickel Raney vieilli. Il se produit un échauffement. Après 10 min la suspension est portée à reflux durant 1 h 20 min. Une filtration sur Célite donne une solution claire qui est ensuite concentrée à 30 ml. On ajoute alors 1,380 g (50% d'excès) d'anhydride benzoïque. Le mélange réactionnel est gardé 1 h à température ordinaire sous agitation magnétique, après quoi il est évaporé à sec. Le résidu est ensuite trituré pendant 1 h avec du bicarbonate de sodium aqueux et le tout est versé dans une ampoule contenant du chloroforme. Après lavage à l'eau, l'extrait chloroformique est séché sur sulfate de sodium et concentré en un sirop jaune pâle. Ce sirop est chromatographié sur une colonne de gel de silice préalablement équilibrée avec du chloroforme. Ce même solvant élue les impuretés. La substance est éluée avec le chloroforme–méthanol (98:2), 255 mg (34,4%), p.f. 161–163°, $[\alpha]_D^{22}$ $-23,5°$ (*c* 0,68, chloroforme).

Anal. Calc. pour $C_{20}H_{22}N_2O_4$: C, 67,78; H, 6,26; N, 7,91. Trouvé : C, 68,01; H, 6,26; N, 8,07.

*Méthyl 3-azido-2,5-dibenzamido-2,3,5-tridésoxy-β-*D-*arabinofuranoside* (**10**) *et méthyl 2-azido-3,5-dibenzamido-2,3,5-tridésoxy-β-*D-*xylofuranoside* (**11**). — L'aziridine **7** (310 mg) est dissoute dans la N,N^1-diméthylformamide (7,5 ml) et additionnée d'azothydrate de sodium (225 mg, 4 éq.). Le tout est porté à 140° pendant 45 min. Le mélange réactionnel est alors traité de la manière habituelle. Le résidu est dissous dans un peu de benzène et chromatographié sur une colonne de gel de silice (30 g) équilibrée dans ce même solvant. La polarité est alors augmentée rapidement jusqu'à 60% à l'aide du chloroforme. Le premier isomère **11** est élué avec ce système. On recueille 102 mg (29,3%) de cristaux homogènes en couche mince (R_F 0,65; acétate d'éthyle–éther de pétrole 4:1). Une recristallisation dans un mélange d'acétate d'éthyle–éther de pétrole donne un p.f. 193–195°; $[\alpha]_D^{27}$ $-130°$ (*c* 0,95; chloroforme).

Anal. Calc. pour $C_{20}H_{21}N_5O_4$: C, 60,75; H, 5,35; N, 17,71. Trouvé : C, 60,84; H, 5,35; N, 17,56.

Le second isomère **10** est obtenu en augmentant la polarité du solvant jusqu'à 80% avec le chloroforme. On obtient 191 mg (54,7%), d'un produit homogène sur plaque, (R_F 0,55; acétate d'éthyle–éther de pétrole 4:1), qui cristallise dans un mélange d'acétate d'éthyle–éther de pétrole, p.f. 158–160°; $[\alpha]_D^{26}$ $-4°$ (*c* 1,56, chloroforme).

Anal. Calc. pour $C_{20}H_{21}N_5O_4$: C, 60,75; H, 5,35; N, 17,71. Trouvé : C, 60,75; H, 5,41; N, 17,41.

*Méthyl 2,3,5-tribenzamido-2,3,5-tridésoxy-β-*D-*arabinofuranoside* **12**. — Le dérivé **10** (100 mg) est dissous dans le méthanol (10 ml) et hydrogéné pendant 30 min en présence d'oxyde de platine (40 mg). La suspension est filtrée sur Célite et le précipité est rincé à l'aide de méthanol. Le volume du filtrat est concentré à 10 ml et l'on ajoute 80 mg (1,3 éq.) d'anhydride benzoïque. La solution est gardée quelques heures à température ordinaire. Le précipité formé est alors filtré, rincé au méthanol et recristallisé dans un mélange de chloroforme–éther de pétrole (80 mg), p.f. 304–306°; $[\alpha]_D^{20}$ $-64°$ (*c* 0,17, chloroforme).

Anal. Calc. pour $C_{27}H_{27}N_3O_5$: C, 68,48; H, 5,75; N, 8,87. Trouvé : C, 68,34; H, 5,76; N, 8,65.

*Méthyl 2,3,5-tribenzamido-2,3,5-tridésoxy-β-*D*-xylofuranoside* (**13**). — Le processus d'obtention est essentiellement le même que pour le dérivé **12**; il est nécessaire néanmoins de doubler le volume de méthanol et au besoin de chauffer afin de solubiliser la substance, lors de l'hydrogénation; 89 mg sont obtenus à partir de 100 mg de **11**, p.f. 282–284°; $[\alpha]_D^{24}$ $-20,6°$ (*c* 2,9, chloroforme).

Anal. Calc. pour $C_{27}H_{27}N_3O_5$: C, 68, 48; H, 5,75; N, 8,87. Trouvé : C, 68,36; H, 6,03; N, 8,54.

REMERCIEMENTS

Ce travail a bénéficié d'une aide matérielle de la Délégation Générale à la Recherche Scientifique et Technique auprès du Premier Ministre, Comité de Biologie Moléculaire (Convention n° 66-00.308). Nous remercions M. le Professeur E. Lederer pour l'intérêt qu'il a porté à ce travail.

BIBLIOGRAPHIE

1 J. D. Dutcher, *Advan. Carbohyd. Chem.*, 18 (1963) 259.
2 G. F. Gause, *Chem. Ind.* (London), (1966) 1506.
3 Z. Vănek et J. Majer, *Antibiotics*, Springer-Verlag, Heidelberg, 1967.
4 Y. Ali et A. Richardson, *Chem. Commun.*, (1967) 554.
5 J. Cléophax, J. Hildesheim, R. E. Williams et S. D. Géro, *Bull. Soc. Chim. France*, (1968) 1415.
6 L. Goodman, *Advan. Carbohyd. Chem.*, 22 (1967) 109.
7 J. Fox, K. A. Watanabe et A. Bloch, *Progr. Nucleic Acid Res. Mol. Biol.*, 5 (1966) 251.
8 J. Cléophax, J. Hildesheim et S. D. Géro, *Bull. Soc. Chim. France*, (1967) 4111.
9 J. Cléophax, J. Hildesheim et S. D. Géro, résultats inédits.
10 R. D. Guthrie et D. Murphy, *J. Chem. Soc.*, (1963) 5288.
11 A. D. Barford et A. C. Richardson, *Carbohyd. Res.*, 4 (1967) 408.
12 D. H. Buss, L. Hough et A. C. Richardson, *J. Chem. Soc.*, (1965) 2736.
13 G. Casini and L. Goodman, *J. Amer. Chem. Soc.*, 86 (1964) 1427.

Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

A NEW CHEMICAL SYNTHESIS OF α,α-TREHALOSE

G. J. F. CHITTENDEN
Microbiological Chemistry Research Laboratory, Department of Organic Chemistry, University of Newcastle upon Tyne (Great Britain)

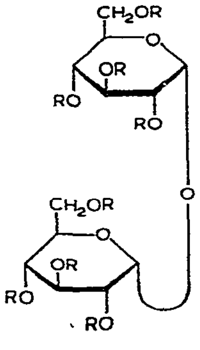
(Received June 7th, 1968; in revised form, July 17th, 1968)

ABSTRACT

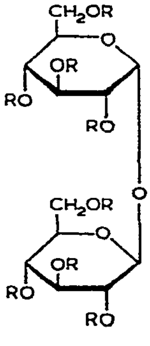
2,3,4,6-Tetra-*O*-benzyl-D-glucopyranose has been treated with 2 equivalents of the corresponding glycosyl chloride under modified Koenigs–Knorr reaction conditions. Hydrogenolysis of the protecting groups of the syrupy product, followed by chromatography on Dowex-1 (HO^-) resin gave α,α-trehalose (α-D-glucopyranosyl α-D-glucopyranoside) and α,β-trehalose (α-D-glucopyranosyl β-D-glucopyranoside) in yields of 18 and 2%, respectively.

INTRODUCTION AND DISCUSSION

α,α-Trehalose[1] (α-D-glucopyranosyl α-D-glucopyranoside) (**1**), one of Nature's "reserve" sugars and the only naturally occurring isomer of the series, has been synthesised, but only in very low yield, by the acid reversion of D-glucose[2,3] and (together with α,β-trehalose) by condensation[4] of Brigl's anhydride with 2,3,4,6-tetra-*O*-acetyl-D-glucose (containing 65% of the α-anomer). Haq and Whelan[5,6] obtained a small proportion of trehalose during the synthesis of nigerose (3-*O*-α-D-glucopyranosyl-D-glucose). Recently[7], both α,α- (**1**) and α,β-trehalose (**2**) were isolated as by-products during the synthesis of some glucosides of glycerol and ribitol.



1 R=H
3 R=Ac



2 R=H
4 R=Ac

The main difficulty encountered in the synthesis of disaccharides of this type lies in specifically obtaining the α-D-glycosidic linkage. It has been shown that α-linked glycosides can be prepared in moderate yield by using per-*O*-benzylated glycosyl chlorides under modified Koenigs–Knorr conditions[7]. Glycosyl chlorides are not normally used in such reactions as they are relatively unreactive[8]. However, it has been demonstrated that removal of the halogen from these compounds by silver ions is much faster, if, instead of depending on the heterogeneous reaction with silver carbonate, a mixture of this salt and a small amount of soluble silver perchlorate is used[9]. The yields of α-D-glycosides[7] indicate that, in the presence of silver perchlorate, the glycosyl chloride largely dissociates into an ion pair[9,10] before reacting with an aglycon. Thus, the configuration of the glycosidic linkage in the product does not depend entirely upon the configuration at the anomeric centre in the chloride.

Under these conditions[7,9], 2,3,4,6-tetra-*O*-benzyl-D-glucose was treated with 2 mol of 2,3,4,6-tetra-*O*-benzyl-D-glucosyl chloride, which appears to be preponderantly the α-anomer[7]. Elution of the crude product from alumina with benzene–ether (20:1) gave a clear syrup, which was hydrogenated over palladium to give material having the same mobility on paper chromatograms as α,α-trehalose. Elution with ether–ethanol (9:1) gave 2,3,4,6-tetra-*O*-benzyl-D-glucose, some of which probably resulted from the hydrolysis of unreacted chloride during the isolation procedure.

Elution of the disaccharide fraction from Dowex-1 (HO^-) resin[11] with water gave α,α-trehalose (**1**), as the dihydrate, and α,β-trehalose (**2**), in yields of 18 and 2%, respectively, which were characterised as the octa-acetates (**3**) and (**4**). No β,β-trehalose seemed to be formed in the reaction.

The above result illustrates further the usefulness of 2,3,4,6-tetra-*O*-benzyl-D-glucosyl chloride–silver carbonate–silver perchlorate in the synthesis of α-D-glucosides which are normally difficult to prepare.

EXPERIMENTAL

I.r. spectra were determined on Nujol mulls. Evaporations were carried out *in vacuo* at 40°. Column chromatography was carried out on alumina (B.D.H. grade) and on Dowex-1 (HO^-) resin (2% cross-linked, 200–400 mesh). T.l.c. was performed on silica gel G (Merck) with benzene–ether (5:3) and detection with 3% ethanolic sulphuric acid at 140°. Light petroleum was the fraction having b.p. 60–80°. Paper chromatography was carried out on Whatman No. 1 paper with the organic phase of butyl alcohol–ethanol–water–conc. ammonia (40:10:49:1)[12], and the descending technique. The periodate–Schiff[13,14] and silver nitrate–sodium hydroxide[15] sprays were used as location reagents.

*Reaction between 2,3,4,6-tetra-*O*-benzyl-*D*-glucopyranosyl chloride and 2,3,4,6-tetra-*O*-benzyl-*D*-glucopyranose.* — 2,3,4,6-Tetra-*O*-benzyl-D-glucopyranose[7] (3.0 g), silver carbonate[9] (10 g), and Drierite (20 g) were suspended in dry benzene (125 ml), and the mixture was stirred at room temperature in the dark for 18 h. Silver perchlorate (0.35 g), from which several portions (50 ml) of dry benzene had been evaporated, was then added, followed by a solution of 2,3,4,6-tetra-*O*-benzyl-D-glucopyranosyl chloride[7] (3.1 g, 1.0 mol) in dry benzene (100 ml) during 8 h with continuous stirring. All operations were conducted in the dark. Stirring was continued for 24 h, and a further portion (3.1 g, 1.0 mol) of the chloride in dry benzene (100 ml) was added over 4 h. On completion of this final addition, stirring was continued for a further 8 days. The mixture was filtered through "Celite", the inorganic material was washed well with benzene (100 ml), and the combined filtrate and washings washed with water (4 × 250 ml), dried (Na_2SO_4, K_2CO_3), and evaporated. A solution of the resulting syrup (8.09 g) was eluted from alumina (300 g) with benzene–ether (20:1, 1200 ml), and concentration of the eluate gave a clear syrup (1.54 g). Elution with ether—ethanol (9:1), followed by evaporation, gave a thick, semi-crystalline residue which was recrystallized from ether–light petroleum to give 2,3,4,6-tetra-*O*-benzyl-D-glucose (3.47 g), m.p. 148–150°, $[\alpha]_D^{21}$ +20° (*c* 1.7, chloroform); lit.[16], m.p. 148°, $[\alpha]_D^{20}$ +21.2° (chloroform).

The material eluted with benzene–ether (20:1) was hydrogenolysed (310-ml uptake in 27 h) in the presence of palladium (from 2.5 g of the oxide) in ethanol–*p*-dioxane (150 ml, 1:1). Removal of the catalyst and evaporation of the filtrate gave material (0.49 g) which had the same mobility on paper chromatograms as authentic α,α-trehalose (R_G 0.48). It was dissolved in water (2 ml) and applied to a column (40 × 3.5 cm) of Dowex-1 (HO^-) resin; elution was carried out with CO_2-free water, and the eluate was collected in 20-ml fractions. Fractions 12–17 gave, on evaporation, and crystallization of the residue from aqueous ethanol, α,α-trehalose (**1**) as the dihydrate (378 mg, 18%), m.p. 98–105°, $[\alpha]_D^{22}$ +175° (*c* 1.4, water); lit.[4,17], m.p. 97–98°, $[\alpha]_D^{20}$ +178°; *cf.* β,β-trehalose[18], m.p. 135–140°, $[\alpha]_D^{17}$ −40°. With acetic anhydride–anhydrous sodium acetate, in the usual manner, compound **1** gave the known octa-acetate (**3**), m.p. and mixed m.p. 98–100°, $[\alpha]_D^{22}$ +160° (*c* 1.1, chloroform); lit.[4], m.p. 101°, $[\alpha]_D^{20}$ +163°.

Fractions 20–26 contained α,β-trehalose (**2**, 42 mg, 2%) which, after recrystallization from aqueous ethanol, had m.p. 138–145°, $[\alpha]_D^{22}$ +93° (*c* 0.78, water); lit.[19], m.p. 145–150°, $[\alpha]_D$ +95°. Acetylation as above gave the octa-acetate (**4**), m.p. and mixed m.p. 140–142°, $[\alpha]_D^{22}$ +84.5° (*c* 0.68, chloroform); lit.[19], m.p. 140–141°, $[\alpha]_D$ +85°.

On t.l.c., compounds **3** and **4** had R_F values (0.155 and 0.205, respectively) that were identical with those of the authentic compounds.

ACKNOWLEDGMENTS

The author thanks Dr. P. W. Austin for gifts of 2,3,4,6-tetra-*O*-benzyl-D-glucose and authentic samples of α,α- and α,β-trehalose octa-acetates, and Dr. J. G. Buchanan for helpful discussion.

REFERENCES

1 See G. G. Birch, *Advan. Carbohyd. Chem.*, 18 (1963) 201, for complete review.
2 J. C. Sowden and A. Spriggs, *J. Amer. Chem. Soc.*, 78 (1956) 2503.

3 E. M. Montgomery and F. B. Weakly, *J. Assoc. Offic. Agr. Chemists*, 36 (1953) 1096.
4 R. U. Lemieux and H. F. Bauer, *Can. J. Chem.*, 32 (1954) 340.
5 S. Haq and W. J. Whelan, *Nature*, 178 (1956) 1222.
6 S. Haq and W. J. Whelan, *J. Chem. Soc.*, (1958) 1342.
7 P. W. Austin, F. E. Hardy, J. G. Buchanan, and J. Baddiley, *J. Chem. Soc.*, (1964) 2128; (1965) 1419.
8 L. J. Haynes and F. H. Newth, *Advan. Carbohyd. Chem.*, 10 (1955) 207.
9 M. L. Wolfrom, A. O. Pittet, and I. C. Gillam, *Proc. Natl. Acad. Sci. U. S.*, 47 (1961) 700.
10 C. G. Swain and E. E. Pegues, *J. Amer. Chem. Soc.*, 80 (1958) 812.
11 P. W. Austin, F. E. Hardy, J. G. Buchanan, and J. Baddiley, *J. Chem. Soc.*, (1963) 5350.
12 E. L. Hirst, L. Hough, and J. K. N. Jones, *J. Chem. Soc.*, (1949) 928.
13 J. Baddiley, J. G. Buchanan, R. E. Handschumacher, and J. F. Prescott, *J. Chem. Soc.*, (1956) 2818.
14 F. E. Hardy and J. G. Buchanan, *J. Chem. Soc.*, (1963) 5881.
15 W. E. Trevelyan, D. P. Procter, and J. S. Harrison, *Nature*, 166 (1950) 444.
16 O. T. Schmidt, J. Auer, and H. Schmadel, *Ber.*, 93 (1960) 556.
17 L. C. Stewart, N. K. Richtmyer, and C. S. Hudson, *J. Amer. Chem. Soc.*, 72 (1950) 2059.
18 B. Helferich and K. Weiss, *Ber.*, 89 (1956) 314.
19 W. N. Haworth and W. J. Hickinbottom, *J. Chem. Soc.*, (1931) 2847.

Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

SEPARATIONS OF CARBOHYDRATES BASED ON ION EXCLUSION AND GEL PERMEATION

S. A. BARKER, B. W. HATT, J. F. KENNEDY, AND P. J. SOMERS
Department of Chemistry, The University, Birmingham 15 (Great Britain)

(Received July 1st, 1968; in revised form, July 19th, 1968)

ABSTRACT

A method is described for the fractionation of oligosaccharides by molecular size on columns of cation-exchange resin with water as the sole eluant. The same system allowed separation of inorganic ions and acidic carbohydrates from neutral components. The elution of amino acids and peptides is described, and the application to separation problems in carbohydrate chemistry is discussed.

INTRODUCTION

The fractionation of molecules of different size on columns of porous particles may be regarded as a partition between the liquid inside the porous medium and the external solution. Larger molecules have a greater degree of exclusion and are thus eluted before those of smaller size. When the porous medium contains a high concentration of fixed ionic groups, charged molecules and electrolytes may be excluded from the matrix. Such ion exclusions are based on the Donnan distribution of an electrolyte between the liquid inside the porous medium and the external solution[1]. Ion-exchange resins, by virtue of their ionic groups and cross-linked matrix, should provide the basis for separation by both principles. This paper describes an investigation of the application of these principles to the separation of carbohydrates, carbohydrate derivatives, amino acids, peptides, and inorganic ions.

METHODS AND RESULTS

Column preparation. — Cation-exchange resins AG-50W x2, x4, and x8 (Bio-Rad Laboratories, 200–400 mesh, H^+) were converted into the appropriate cationic form by washing successively with distilled water (5 bed-volumes), aqueous lithium, calcium, or barium chloride (N, 10 bed-volumes), and distilled water (10 bed-volumes).

The regenerated resins were packed into vertical glass columns by the addition of the resin slurry to the water-filled column, the resin being allowed to settle under gravity. Analytical-scale columns (150 × 0.6 cm diameter) and preparative columns (210 × 3.8 cm diameter) were prepared, constant-flow conditions being achieved with the aid of peristaltic pumps (0.23 and 1.9 ml/min, respectively). The columns were maintained at 25° unless otherwise stated.

Analytical procedures. — Column eluates were monitored continuously with the aid of automated, spectrophotometric procedures. Hexose-containing components were determined[2] by the cysteine—sulphuric acid method[3]. Formaldehyde obtained from periodate oxidation of carbohydrates was determined by the pentane-2,4-dione procedure[4,5]. Amino acids were analysed by the ninhydrin method, using the standard Technicon procedure[6].

Technicon Auto-analyser modular equipment was employed for all automated analysis methods.

Investigation of the effect of cationic form and degree of crosslinking of the resin. — Analytical-scale columns of 2, 4, and 8 % cross-linked resin in the lithium, calcium, and barium forms were loaded with solutions of carbohydrate mixtures (100 μl) containing amylopectin, isomaltotriose or raffinose, and D-glucose (50–100 μg of each component). Because of the reduction in bed volume under pressure, particularly with the resins of low cross-linking, the columns were loaded under conditions of working pressure. The columns were eluted with de-aerated, distilled water, and the eluates monitored by the automated cysteine–sulphuric acid procedure.

In order to present the results in terms independent of column volume and dimensions, the results throughout this paper are given as a retention factor defined as the ratio of the elution volume of the component to the bed volume of the column under operating conditions. Table I and Fig. 1 show the results obtained.

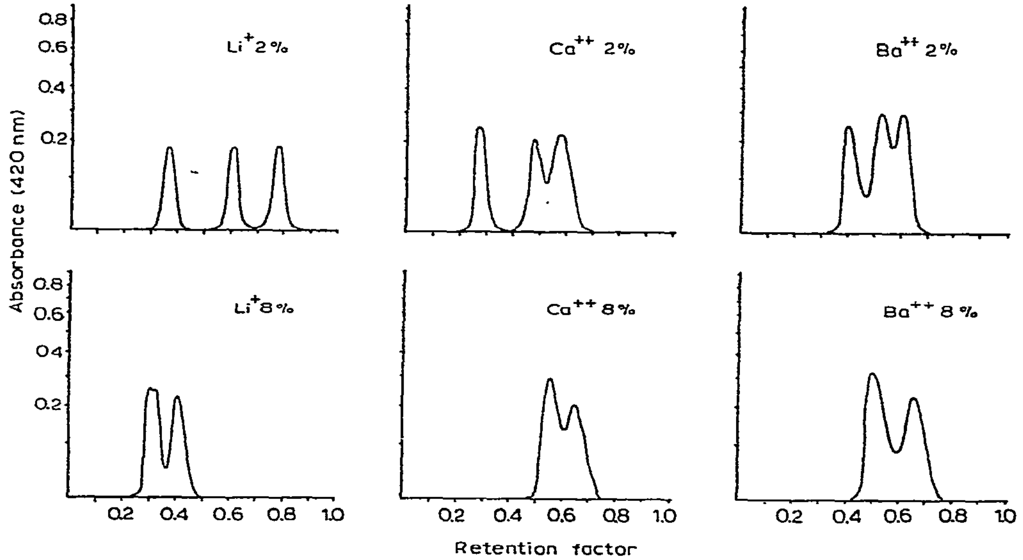


Fig. 1. Separation of amylopectin, trisaccharide, and D-glucose on resins of various cross-linking and ionic form. Analysis by the cysteine–sulphuric acid method.

Investigation of the effect of temperature. — A jacketed column of AG-50W x2 (Li^+) resin was loaded with mixtures of amylopectin and D-glucose, and eluted with

TABLE I

EFFECT OF CATION AND DEGREE OF CROSS-LINKING ON THE FRACTIONATION OF CARBOHYDRATES

Ion	*Cross-linking %*	*Retention factor* *Amylopectin*	*Isomaltotriose*	D-*Glucose*
	2	0.37	0.61	0.78
Li^+	4	0.29	0.41	0.49
	8	0.30	0.31	0.40
Ca^{2+}	2	0.27	0.47	0.59
	8	0.55	0.55*	0.64
Ba^{2+}	2	0.40	0.53	0.61
	8	0.49	0.49*	0.66

*Raffinose

water at various temperatures, as previously described. The results obtained are shown in Table II.

TABLE II

EFFECT OF TEMPERATURE ON THE SEPARATION OF AMYLOPECTIN AND D-GLUCOSE

Temperature (degrees)	*Retention factor* *Amylopectin*	D-*Glucose*
25	0.36	0.77
50	0.37	0.78
75	0.37	0.78

Fractionations of carbohydrates. — Various carbohydrates were independently loaded and eluted from an analytical column of AG-50W x2 (Li^+) resin. The column eluate was monitored by the cysteine–sulphuric acid and/or the periodate–pentane-2,4-dione procedures, as appropriate. The retention factors obtained are listed in Table III. The results obtained when mixtures of these compounds were fractionated showed no significant deviation from the elution profiles of individual components. A typical multi-component separation is shown in Fig. 2.

Quantitative recovery of monosaccharides from the column, when present in admixture, was confirmed by g.l.c. analysis of the trimethylsilyl ethers, using SE30 (10% on Celite) as stationary phase. The positions and integrated peak areas were identical with those obtained from the original sample.

The separations were not affected when samples of carbohydrates containing salts, *e.g.*, sodium phosphate or lithium sulphate, were fractionated. Sodium ions were exchanged for lithium ions during the elution, and the elution position of the exchanged lithium ions was determined by flame photometry.

TABLE III

FRACTIONATIONS OF CARBOHYDRATES

Compound	*Retention factor*	*Compound*	*Retention factor*
Amylopectin	0.37	Laminaritriose	0.61
Dextran 80	0.37	Laminaribiose	0.70
Li^+	0.37	Maltose	0.70
N-Acetylneuraminic acid	0.37	D-Glucose	0.78
D-Gluconic acid	0.37	D-Mannose	0.78
Glyceric acid	0.37	D-Galactose	0.78
Maltohexaose	0.55	L-Fucose	0.78
Maltohexaitol	0.55	Galactitol	0.78
Laminaripentaose	0.59	2-Acetamido-2-deoxy-D-glucose	0.78
Raffinose	0.61	2-Acetamido-2-deoxy-D-mannose	0.78

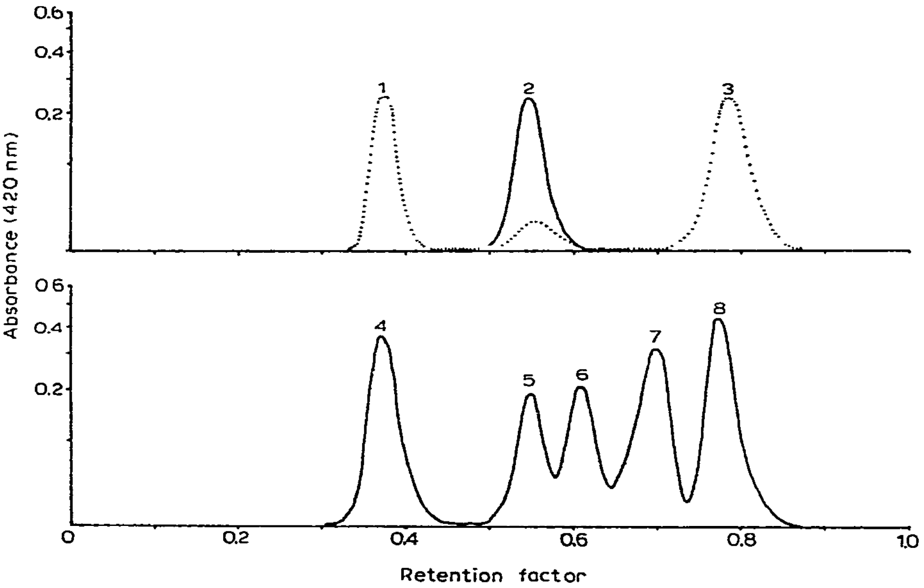


Fig. 2. Fractionation of various carbohydrates on a column of AG-50W x2 (Li^+) resin. Analysis by the cysteine–sulphuric acid (———) and periodate–formaldehyde (.........) methods. (1) D-Gluconic acid, (2) maltopentaitol, (3) 2-acetamido-2-deoxy-D-glucose, (4) amylopectin, (5) laminaripentaose, (6) laminaritriose, (7) maltose, and (8) D-glucose.

Fractionation of amino acids, peptides, and protein. — A series of amino acids, peptides, and protein was independently loaded and eluted from an analytical-scale column of AG-50W x2 (Li^+) resin. The column eluate was monitored by the ninhydrin procedure, and the retention factors observed are recorded in Table IV. The results obtained with mixtures did not significantly deviate from those obtained with single components, and a typical separation is shown in Fig. 3.

TABLE IV

SEPARATIONS OF AMINO ACIDS AND PEPTIDES

Compound	*Retention factor*	*Compound*	*Retention factor*
Glutamic acid	0.37	Phenylalanine	4.12
Bovine serum albumin	0.37	Glycyl-glycyl-glycine	6.04
Glutamine	1.13	Glycyl-glycyl-glycyl-glycyl-glycine	8.65
Serine	1.13	Lysine	∞
Proline	1.27		
Glycine	1.41		

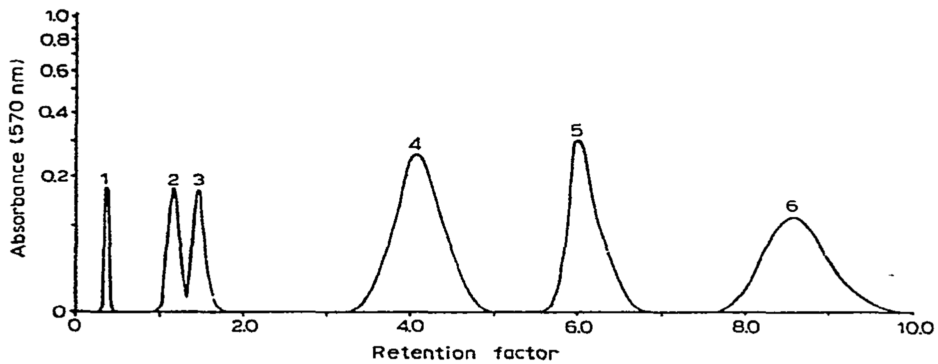


Fig. 3. Separation of amino acids and peptides on a column of AG-50W x2 (Li^+) resin. Analysis by the ninhydrin method. (1) Glutamic acid, (2) Glutamine, (3) Glycine, (4) Phenylalanine, (5) Glycyl-glycyl-glycine, and (6) Glycyl-glycyl-glycyl-glycyl-glycine.

Separations on a preparative scale. — Solutions (2–10 ml) containing mixtures of amylopectin, various oligosaccharides, and D-glucose (*ca.* 2 g of each component) were loaded and eluted from a preparative column of AG-50W x2 (Li^+) resin. A suitable proportion of the eluate was monitored by the automated cysteine–sulphuric acid method, and the residual eluate collected in fractions. The purity of components obtained was confirmed by chromatography, and the retention factors observed are shown in Table V.

TABLE V

SEPARATIONS OF OLIGOSACCHARIDES ON A PREPARATIVE SCALE

Compound	*Retention factor*	*Compound*	*Retention factor*
Amylopectin	0.38	Panose	0.61
Isomaltotetraose	0.55	Isomaltose	0.67
Laminaritriose	0.61	Sucrose	0.70
Melezitose	0.61	D-Glucose	0.78

DISCUSSION

A column packed with an ion-exchange resin will contain two physically distinguishable, liquid environments: the liquid inside the matrix of the cross-linked resin and that occupying the interstices between the particles of porous resin. The distribution of non-ionic solutes between these two phases will be dependent on their molecular dimensions. For large, solute molecules, which cannot enter the stationary phase, the distribution coefficient is zero, and the molecule will be eluted in a volume equivalent to the interstitial volume. Small, solute molecules, which may be distributed equally between the two phases and hence have a distribution coefficient of unity, are eluted in a volume equivalent to the total liquid volume of the column. The factors affecting the equilibrium distribution of small alditols, such as glycerol and D-glucitol, on various ion-exchange resins have been discussed by Mathisson and Samuelson[7].

The ionizable groups on the matrix influence the distribution coefficient of ionic solute molecules between the two phases, thus introducing a parameter which does not exist with such non-ionic matrices as cross-linked dextran. With a resin containing completely ionised matrix substituents, *e.g.*, sulphonic acid, cation-exchange resins, ionic solutes are virtually excluded from the matrix. A combination of these two principles should provide an important technique for the fractionation of carbohydrates by charge and molecular size.

Jones *et al.*[8] employed a cation-exchange resin for the fractionation of certain mono- and di-saccharides, and suggested that cross-linked (2% divinylbenzene) polystyrenesulphonic acid was the most satisfactory. This resin also permitted partial resolution of *O*-alkylated monosaccharides. Wheaton and Bauman[9] have developed ion exclusion, using cross-linked polystyrenesulphonic acid, for the fractionation of a series of organic acids, those with higher dissociation constants being eluted first with water.

Since a combination of ion exclusion and fractionation by molecular size was desired and the latter is the more affected by environmental changes in the resin, initial investigations were concerned with the fractionation of a polymer–, oligomer–, monomer series. The elution positions (with water) of amylopectin, isomaltotriose, and D-glucose showed predictable changes when both the degree of cross-linking and the cationic form of the resin were varied. To provide a basis for direct comparison of results, the elution volumes were expressed as a factor (retention factor) of the total bed-volume of the column under operating conditions. An increase in the percentage of divinylbenzene cross-linking from 2 to 8% resulted in a decreased exclusion limit of the resin matrix, so that, on the x8 resin, amylopectin and the trisaccharide were eluted together (Table I, Fig. 1). Furthermore, the resolution of polymer and monomer was diminished. Change in the cation from Li^+ to Ca^{2+} and Ba^{2+} also resulted in decreased separations between monomer, trimer, and polymer. The optimal resin conditions for fractionation of oligosaccharides by molecular size appeared to be 2% cross-linking with lithium counter-ion, and, in this form, Dowex-50 gives a highly swollen matrix in water[7]. The separation between amylopectin and D-glucose was unaffected by changes in operating temperature between 25 and 75° (Table II).

Examination of the elution profiles of various neutral carbohydrates (Fig. 2, Table III) illustrates the application of this type of column to the fractionation of oligosaccharides by molecular size. No separation was observed between oligosaccharides of similar chain length but differing linkages, although small differences in retention factors were observed. Similarly, alditols and *O*-glycosylalditols, obtained by reduction of the corresponding aldose or substituted aldose with sodium borohydride, were fractionated by size and eluted in the same position as the parent compound.

The utilisation of the ion-exclusion principle is revealed in the elution of acidic carbohydrates (Table III). Thus, *N*-acetylneuraminic acid, D-gluconic acid, and glyceric acid were excluded from the resin matrix and eluted in the same position (0.37) as species of large molecular weight. By this technique, D-gluconic acid was separated from D-glucono-1,4-lactone, the lactone being eluted as a small, neutral molecule. This provides a convenient method for separation of acidic carbohydrates from neutral entities of low molecular weight, with the advantage that water is the sole eluting agent. Salts containing cations other than lithium are essentially excluded from the matrix, the cation being exchanged for lithium. Neutral carbohydrates of low molecular weight may thus be separated from inorganic ions, and acid hydrolysates, after neutralisation with lithium hydroxide or carbonate, can be desalted. Similarly, inorganic ions and protein in enzymic digests of carbohydrates may be removed from the products of low molecular weight. No observable change in the elution pattern of oligosaccharides was found in the presence of inorganic ions, and recoveries of carbohydrates from the columns were quantitative as determined by gas–liquid chromatography. Similar elution profiles were obtained for fractionations of oligosaccharides on a preparative scale, the retention factors being in direct comparison with those obtained for the analytical experiments (Table V).

Examination of the elution profiles of various amino acids (Table IV, Fig. 3) indicated that acidic amino acids (*e.g.*, glutamic acid) were excluded from the resin matrix. All other amino acids investigated were retarded to varying extents, being eluted in volumes greater than the bed volume of the column. Phenylalanine was severely retarded, whilst lysine was not eluted with water, presumably due to exchange with lithium cations. Oligopeptides of the glycine series were separated, those of greater chain-length being the most strongly retarded.

Fractionation on AG-50W x2 (Li^+) resin thus combines two separation principles. The use of water as an eluant for fractionation by molecular dimensions or charge (ion exclusion) provides the basis for a mild method of separation and simplifies both recovery from, and analysis of, materials in the column eluates. The use of an analytical-grade resin, in which there is a closer control of mesh size ($>$ 95% within the 200–400 mesh range), enables higher flow-rates to be employed without loss of resolution, gives greater reproducibility, and precludes the need for special techniques for column packing.

ACKNOWLEDGMENTS

We thank Professor M. Stacey, C.B.E., F.R.S., for his interest in this work. Ranks Hovis McDougall (Research) Ltd. (B.W.H.) and the Medical Research Council (J.F.K.) are thanked for financial assistance.

REFERENCES

1 W. C. Bauman and J. Eichorn, *J. Amer. Chem. Soc.*, 69 (1947) 2830.
2 S. A. Barker, R. G. Jones, A. R. Law, and P. J. Somers, unpublished results.
3 Z. Dische, L. B. Shettles, and M. Osnos, *Arch. Biochem.*, 22 (1949) 169.
4 O. Samuelson and H. Stromberg, *Carbohyd. Res.*, 3 (1966) 89.
5 T. Nash, *Biochem. J.*, 55 (1953) 416.
6 *Techniques in Amino Acid Analysis*, Technicon Instruments Company Ltd., Chertsey, England, 1966, p. 127.
7 M. Mathisson and O. Samuelson, *Acta Chem. Scand.*, 12 (1958) 1386.
8 J. K. N. Jones, R. A. Wall, and A. O. Pittet, *Can. J. Chem.*, 38 (1960) 2285; J. K. N. Jones and R. A. Wall, *ibid.*, 38 (1960) 2290.
9 R. M. Wheaton and W. C. Bauman, *Ind. Eng. Chem.*, 45 (1953) 228.

Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

PROTON MAGNETIC RESONANCE SPECTRA OF ETHYL 3-(GLYCOSYLAMINO)CROTONATES

A. GÓMEZ SÁNCHEZ, M. TENA ALDAVE,

Departamento de Química Orgánica, Universidad de Sevilla, and Instituto de Química Orgánica General del Patronato "Juan de la Cierva", C. S. I. C., Seville (Spain)

AND U. SCHEIDEGGER

Research Laboratory, Varian A. G., Zürich (Switzerland)

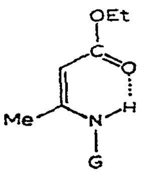
(Received July 29th, 1968; in revised form, August 23rd, 1968)

ABSTRACT

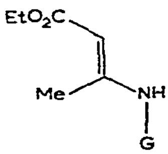
The p.m.r. spectra of ethyl 3-(glycosylamino)crotonates derived from D-glucose, D-galactose, D-mannose, and L-rhamnose, and those of their corresponding *O*-acetyl derivatives, have been examined. The results confirm that the amino and ethoxycarbonyl groups of the enamine portion of these substances are in *cis* disposition and intramolecularly bonded. No other isomeric or tautomeric forms could be detected under the conditions used. Pyranose ring sizes and anomeric configurations (β-L for the rhamnose derivative, and β-D for the remaining compounds) are assigned to the *O*-acetyl derivatives on the basis of the chemical shifts and coupling constants observed. The same pyranose structures and anomeric configurations are proposed for the parent compounds after considering the chemical shifts and coupling constants of the anomeric protons.

INTRODUCTION

Glycosylamines react with ethyl acetoacetate yielding[1] ethyl 3-(glycosylamino)crotonates (**1a**). The formulation of these substances with the amino and ethoxycarbonyl groups in *cis* disposition (compounds referred to as *cis* isomers in this paper) was based on spectroscopic (u.v. and i.r.) data which did not exclude the possibility of their existence in solution in an equilibrium with minor proportions of the *trans* isomer (**1b**), and/or the tautomeric imino ester (**1c**). It is known[2,3] that simple 3-(alkylamino)crotonic esters exist in the liquid state and in solution as a mixture of the geo-

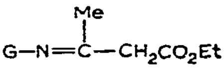


1a



G = glycosyl group

1b



$$\mathrm{G{-}N{=}\overset{\displaystyle Me}{\overset{|}{C}}{-}CH_2CO_2Et}$$

1c

metric isomers similar to structures **1a** and **1b** in which the *cis* form usually preponderates. These isomers can be easily distinguished by p.m.r. spectroscopy[2,3].

The assignment of the α-D anomeric configuration to compounds of the type **1a** was based on the values of their optical rotations, and was tentative, since only one isomer was isolated in each case.

In order to clarify the dubious points in the formulation of ethyl 3-(glycosylamino)crotonates and to have further confirmation of the complete structure, an investigation of the p.m.r. spectra of these substances and those of their *O*-acetyl derivatives has now been carried out.

EXPERIMENTAL

Compounds. — Ethyl 3-(glycosylamino)crotonates derived from D-glucose, D-galactose, D-mannose, and L-rhamnose, and their corresponding *O*-acetyl derivatives, were prepared as described previously[1]. Ethyl 3-(D-galactosylamino)crotonate had m.p. 179–180°, which was higher than that (m.p. 159–160°) previously given; the remaining properties agreed with those reported.

Anal. Calc. for $C_{12}H_{21}NO_7$: C, 49.5; H, 7.3; N, 4.8. Found: C, 49.8; H, 7.2; N, 4.5.

Acetylation of this compound gave a tetra-acetate having the same physical constants as previously described[1].

P.m.r. spectra. — Spectra were measured at 100 MHz and 26–28° with a Varian HA-100 spectrometer, using sweep-widths of 1000 and 500 Hz, and sweep rates of 2.0 and 1.0 Hz/sec. Solvents used were methyl sulfoxide-d_6 for the 3-(glycosylamino)-crotonic esters, and chloroform-d for their *O*-acetyl derivatives. The concentrations of solutions were 10–20%. Tetramethylsilane was used as the internal standard. Deuterations were effected by shaking the solutions with a few drops of deuterium oxide.

RESULTS AND DISCUSSION

O-Acetylated ethyl 3-(glycosylamino)crotonates

The p.m.r. data of the *O*-acetylated ethyl 3-(glycosylamino)crotonates, compounds **2**, **4**, **6**, and **8**, are presented in Table I.

The signals due to the enamine portion of these compounds were easily identified by comparison with the absorption typical of 3-(alkylamino)crotonic esters[2,3]. The *cis* forms (similar to structure **1a**) of these substances show a broad signal at δ 8.0–8.9 due to the intramolecularly bonded amino group, and a singlet at δ 1.8–1.9 produced by the :C-Me group. The corresponding *trans* forms (similar to structure **1b**) show the N-H signal at δ 5.50–6.60, and the :C-Me singlet at δ 2.2–2.4. The olefinic proton of a particular *cis* isomer comes into resonance at a higher field than the same proton of the corresponding *trans* form; however, the small difference (approximately 0.1 p.p.m.) between the chemical shifts of these protons precludes their use

TABLE I

CHEMICAL SHIFTS (δ, p.p.m.) AND COUPLING CONSTANTS (Hz) OF *O*-ACETYLATED ETHYL 3-(GLYCOSYLAMINO)CROTONATES AT 100 MHz IN CHLOROFORM-*d*

Substance	*N-H*	*:C-H*	*:C-Me and OAc*	*OEt*	*H-1*	*H-2*	*H-3*	*H-4*	*H-5*	*H-6*	*H-6'*
2	8.86d[a]	4.66	1.98; 2.02[b]	1.23t; 4.10q	4.75t	5.06t	5.32t	5.05q	3.73m	4.24q	4.08q
	$J_{NH,1}$ 9.0		2.07[c]	J 7.1	$J_{1,2}$ 9.1	$J_{2,3}$ 9.0	$J_{3,4}$ 9.0	$J_{4,5}$ 10.5			
4	8.91d	4.66	1.97; 1.98;	1.23t; 4.12	4.70t	5.29q	5.09q	5.40q	3.84–4.30		
	$J_{NH,1}$ 9.0		2.02; 2.04; 2.15	J 7.0	$J_{1,2}$ 8.7	$J_{2,3}$ 10.0	$J_{3,4}$ 3.0	$J_{4,5}$ 1.0			
6	9.15d	4.65	1.98; 1.99;	1.22t; 4.06	4.95q	5.47q	5.10q	5.20t	3.70m	4.0–4.35	
	$J_{NH,1}$ 10.0		2.05; 2.07; 2.31	J 7.0	$J_{1,2}$ 1.3	$J_{2,3}$ 3.2	$J_{3,4}$ 10.0	$J_{4,5}$ 10.0			
8	9.13	4.62	1.99[c]; 2.06;	1.22t; 4.05q	4.92	5.43q	4.98–5.15		3.57		1.26d[d]
	$J_{NH,1}$ 10.0		2.31	J 7.1	$J_{1,2}$ 1.6	$J_{2,3}$ 3.0					

[a] Signal multiplicities are indicated as following: d, doublet; t, triplet; q, quartet; m, multiplet; the absence of any indication implies that a singlet was observed. [b] Signal of intensity nine. [c] Signal of intensity six. [d] C-5 methyl group.

in the assignment of configurations in the cases where only one isomer is available. In the spectrum of ethyl 3-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosylamino)-crotonate (**2**) (see Table I and Fig. 1), the broad doublet ($J_{NH,1}$ 9.0 Hz), of intensity one, at δ 8.86 is ascribed to the chelated amino proton; the splitting is produced by the coupling with the anomeric proton (H-1). The olefinic proton appears as a singlet at δ 4.66, superimposed on the high-field branch of the triplet due to H-1 (see below). The methyl singlets, of total intensity fifteen, which appear at δ 1.98–2.07, are ascribed to the four acetoxyl groups of the sugar portion of the molecule, and to the :C-Me group of the *cis*-3-aminocrotonic ester. The triplet, of intensity three, at δ 1.23 and the quartet at δ 4.10, which overlaps the signal due to the two protons on C-6 of the sugar portion (see below), are assigned to the ethoxycarbonyl function. No signal was observed in the region δ 2.2–2.4 which could be ascribed to the :C-Me group of the *trans* form. It is then concluded that this compound exists in chloroform solution in the chelated *cis* form **2**.

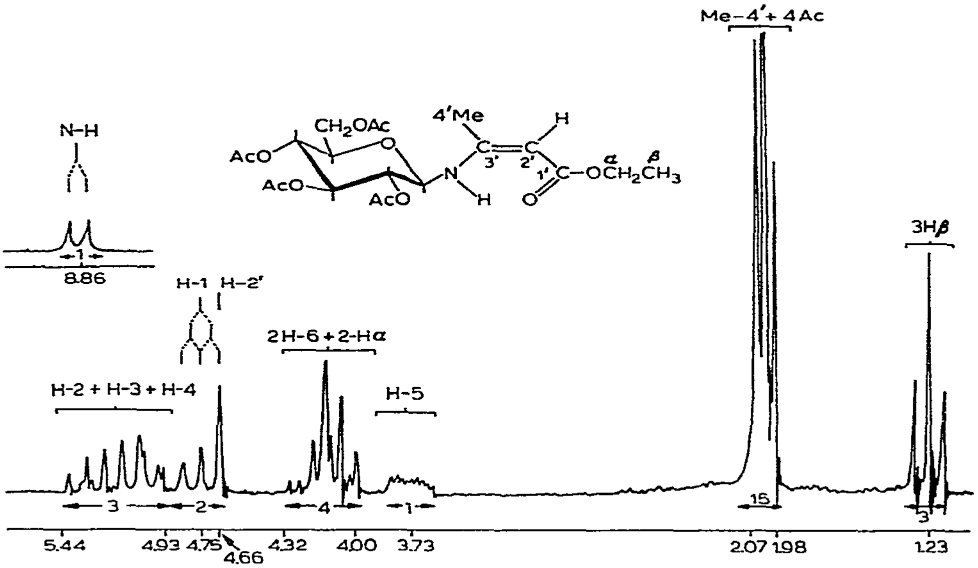
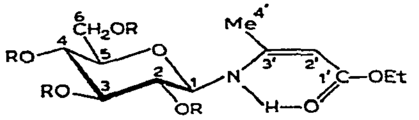
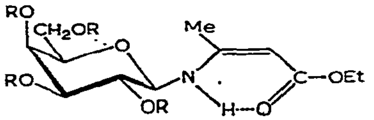


Fig. 1. The 100-MHz p.m.r. spectrum of ethyl 3-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosylamino)-crotonate (**2**) in chloroform-*d*.

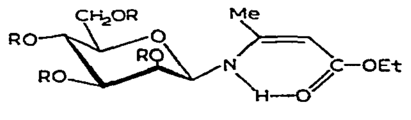
The pyranose structure of the sugar portion of compound **2** has been demonstrated previously by chemical means[1]. The interpretation of the spectrum of this part of the molecule is as follows. The triplet at δ 4.75 is due to the anomeric proton and was identified by a double-resonance experiment with irradiation of the N-H proton. Under these conditions, this triplet collapsed to a well-defined doublet whose spacing (9.1 Hz) was taken as $J_{1,2}$. The value of $J_{1,2}$ indicates that H-1 and H-2 are in *trans*-axial disposition and, therefore, that compound **2** has the β-D anomeric configuration. The multiplet at δ 3.73 is assigned to H-5 by analogy with the spectra of β-D-glucopyranose penta-acetate[4] and of penta-acetyl β-D-glucopyranosylamine[5]; the two quartets at approximately δ 4.08 and δ 4.24, which appear partly obscured by the methylene signal of the ethoxycarbonyl group, are due[4,5] to the two H-6 signals (total intensity of four for the resulting multiplet). The multiplets of total intensity three in the region δ 4.93–5.44, arise from H-2, H-3, and H-4. Of these multiplets, the quartet centered at δ 5.05 is assigned to H-4 because irradiation of H-5 caused the collapse of this quartet to a doublet whose spacing (9.0 Hz) was taken as $J_{3,4}$. Presumably, the triplet at δ 5.06, which appears superimposed on the H-4 quartet, is due to H-2 which is co-axial to H-4 on the same side of the pyranose ring[4]. The remaining triplet at lowest field (δ 5.32) is then ascribed to H-3. It can be seen that, with the exception of H-1 which appears at relatively high field, the remaining sugar protons of compound **2** show chemical shifts and coupling constants very similar to those of penta-acetyl β-D-glucopyranosylamine[5], thus providing a further confirmation of the β-D-glucopyranose structure.



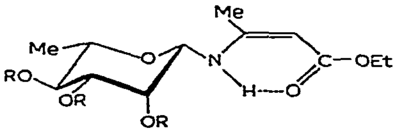
2 R = Ac
3 R = H



4 R = Ac
5 R = H



6 R = Ac
7 R = H



8 R = Ac
9 R = H

The *cis* structure and anomeric configuration of ethyl 3-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosylamino)crotonate (**4**) could also be easily deduced from its p.m.r. spectrum (Table I). The doublet ($J_{NH,1}$ 9.0 Hz) at δ 8.91 is due to the intramolecularly bonded amino proton. The singlet of intensity three at δ 2.15 is attributed to the axial C-4 acetoxyl group of the pyranose ring rather than to a *trans* :C-Me group. The group of singlets, of total intensity twelve, at δ 1.97–2.04 is considered to include the two equatorial acetoxyl groups of the sugar ring, the C-6 acetoxyl group, and the *cis* :C-Me group. The olefinic singlet, at δ 4.66, appears also in this compound very close to the triplet at δ 4.70 due to the anomeric proton. Irradiation of the aminic proton resulted in the collapse of this triplet to a doublet, the spacing of which ($J_{1,2}$ 8.7 Hz) indicated the β-D anomeric configuration of the compound. The remaining signals of this spectrum are assigned as follows. The quartet ($J_{3,4}$ 3.0 Hz; $J_{4,5}$ 1.0 Hz) at δ 5.40 is ascribed to the equatorial H-4, because of its position at lower field than the other protons of the pyranose ring and of the coupling constants involved. The quartet centered at δ 5.09 is clearly due to H-3 which is strongly coupled ($J_{2,3}$ 10.0 Hz) to axial H-2 and weakly ($J_{3,4}$) to equatorial H-4. The quartet ($J_{1,2}$; $J_{2,3}$ 10.0 Hz) with centre at δ 5.29, which collapsed upon irradiation of H-1, is assigned to H-2. The protons on C-5 and C-6 give rise to a complex ABC spin system in the region δ 3.84–4.30, overlapping the quartet of the ethoxycarbonyl group. The triplet at δ 1.23 is also due to the latter group. The chemical shifts and coupling constants of the sugar protons of this substance demonstrate the pyranose structure previously assigned[1].

In the spectrum of ethyl 3-(2,3,4,6-tetra-*O*-acetyl-β-D-mannopyranosylamino)-crotonate (**6**) (Table I), the presence of the doublet ($J_{NH,1}$ 10.0 Hz) at δ 9.15 due to the intramolecularly bonded N-H group was taken as indication of the *cis* disposition of the enamine portion. The singlet of intensity three at δ 2.31 is assigned to the axial C-2 acetoxyl group, and the remaining methyl singlets, of total intensity twelve, between δ 1.98 and 2.07, are considered to arise from the other acetoxyl groups of the sugar moiety and the :C-Me group in *cis* disposition. The singlet at δ 4.65 is assigned to the olefinic proton. The anomeric proton appears at δ 4.95 as a quartet ($J_{NH,1}$; $J_{1,2}$ 1.3 Hz) which collapsed into a doublet of spacing equal to $J_{1,2}$ upon irradiation of the N-H proton. The equatorial H-2 was identified as the quartet ($J_{1,2}$; $J_{2,3}$ 3.2 Hz) at δ 5.47, because of the position at low field of this signal and of the coupling constants involved. The quartet at δ 5.10, having a spacing equal to $J_{2,3}$ and a larger one ($J_{3,4}$ equal to 10.0 Hz), is assigned as H-3. This quartet overlaps the high-field branch of the triplet ($J_{4,5}$; $J_{3,4}$ 10.0 Hz) at δ 5.20, due to H-4. As in the D-glucose derivative **2**, protons on C-5 and C-6 give rise to an ABX spin system in the δ 3.60–4.35 region.

The chemical shifts and coupling constants of protons H-2 to H-6 in compound (**6**) are very similar to those observed[5] in penta-acetyl β-D-mannopyranosylamine; this confirms the pyranose structure previously given to the former compound on a chemical basis[1]. Assignment of anomeric configurations based on the $J_{1,2}$ values is not feasible for D-mannose derivatives. However, a tentative assignment can be made for compound **6** by comparing the chemical shifts of H-1 and H-5 with the chemical shifts of the same protons in the β-D-glucopyranose derivative **2**. For compound **6** H-1 appears at a field of 0.18 p.p.m. lower than for the D-glucose derivative **2**. This paramagnetic shift of the axial H-1 can be ascribed to the different shielding effect of the acetoxyl group on C-2 when in an axial instead of an equatorial position. Deshielding effects of the same order of magnitude have been observed in acetylated aldopyranoses[4,6] and in penta-acetyl glycopyranosylamines[5]. Compound **6** would then have H-1 in axial disposition and, therefore, the β-D anomeric configuration. Assuming that the rules enunciated by Lemieux and Stevens[4] for the estimation of chemical shifts in acetylated aldopyranoses also hold for the compounds under discussion, the chemical shift of the equatorial H-1 of the α-D anomer of compound **6** would be *ca.* 5.15. Furthermore, the chemical shifts of H-5 in compounds **2** and **6** are almost identical, thus suggesting that, in both cases, H-5 has the same neighbouring substituents on C-1 and C-3. It seems reasonable to assume, by analogy with the observations made on acetylated aldopyranoses[4], that, in the α-D anomer of compound **6**, the axial amino group would produce a deshielding effect on the opposing axial H-5.

The signals due to the enamine portion of the L-rhamnose derivative **8** (see Table I) appear at almost the same positions as in the D-mannose derivative **6**, thus indicating that compound **8** is also a *cis* isomer. Likewise, the chemical shifts and coupling constants of H-1 in both compounds are almost identical; therefore, compound **8** is formulated with a pyranose structure and, tentatively, with the β-L anomeric configuration. As in previous cases, the anomeric proton was identified by irradiation of N-H which caused the collapse of the H-1 quartet to a doublet having spacing equal to $J_{1,2}$ (1.6 Hz). A quartet ($J_{1,2}$; $J_{2,3}$ 3.0 Hz) is produced by H-2 at δ 5.43; irradiation of this proton removed $J_{1,2}$ and resulted in collapse of the H-1 quartet to a doublet having a spacing equal to $J_{NH,1}$ (10.0 Hz). For this compound, H-3 and H-4 produce a complex multiplet between δ 5.0 and 5.15. The assignment of the signals of the remaining protons are straightforward.

Ethyl 3-(glycosylamino)crotonates

The p.m.r. spectra of these substances were measured in methyl sulfoxide-d_6, in order to prevent effects due to hydrolysis and/or mutarotation[7]. Under these conditions, the signals produced by the protons of the enamine portion can be readily observed. Treatment of the samples with deuterium oxide eliminated the signals of the easily exchangeable hydroxyl protons but not the doublet produced by the intramolecularly bonded N-H group, which seems to undergo a much slower hydrogen exchange. The multiplet due to the anomeric proton then became apparent, and was identified by irradiation of N-H. As expected, the signals deriving from the other protons of the sugar moiety appear at higher field (δ 3.00–3.75). For example, in the spectrum of ethyl 3-(β-D-glucopyranosylamino)crotonate (**3**) (see Table II), the assignments of the doublet ($J_{NH,1}$ 8.4 Hz) at δ 8.77 to the intramolecularly bonded N-H, the singlet at δ 1.96 to the *cis* :C-Me group, and the quartet (J 7.0 Hz) at δ 3.98 and triplet at δ 1.15 to the ethoxycarbonyl group are straightforward. The integral values for this part of the spectrum, which are in agreement with the above interpretation, and the absence of any signal between δ 2.0 and 2.4 indicate the absence of the *trans* form. A multiplet, of intensity three, at δ 4.44, included the signals due to the olefinic proton, one hydroxyl proton, and H-1. Irradiation of N-H strongly affected the structure of this multiplet. Treatment of the sample with deuterium oxide changed the multiplet into a triplet ($J_{1,2} \simeq J_{NH,1} \simeq$ 8.5 Hz) centered at δ 4.44 and assigned to H-1, and a singlet at δ 4.49 belonging to the olefinic proton. The value of $J_{1,2}$ is the expected one for a *trans*-axial relationship of H-1 and H-2 on a pyranose ring, and indicates the β-D anomeric configuration of this compound.

The absorptions due to the enamine portion of the remaining ethyl 3-(glycosylamino)crotonates (Table II) are almost identical to the ones of the D-glucose derivative **3**. This indicates that all of these substances are *cis* isomers. The anomeric proton of the D-galactose derivative **5** (see Table II) appears at almost the same field, δ 4.41, as that for the D-glucose analogue; however, the value of $J_{1,2}$ (5.0 Hz) does

TABLE II

CHEMICAL SHIFTS (δ, p.p.m.) AND COUPLING CONSTANTS (Hz) OF ETHYL 3-(GLYCOSYLAMINO)CROTONATES AT 100 HMz IN METHYL SULFOXIDE-d_6

Substance	*N-H*	*:C-H*	*:C-Me*	*OEt*	*H-1*	*H-2*[a]	*H-4*	*H-5*	*OH*
3	8.77d[b] $J_{NH,1}$ 8.4	4.49	1.96	1.15t; 3.98q J 7.0	4.44t $J_{1,2}$ 9.0				4.49; 4.91d (J 4.0); 5.00d (J 4.0); 5.25d (J 5.5)
5	8.78d $J_{NH,1}$ 8.9	4.49	1.98	1.18t; 4.00q J 7.0	4.41q $J_{1,2}$ 5.0		3.71q J 4.5 J' 2.0		4.41; 4.54; 4.73d (J 5.0); 5.09 (J 5.0)
7	9.12d $J_{NH,1}$ 9.2	4.48	1.98	1.16t; 3.99q J 7.0	4.73q $J_{1,2}\sim$1.0	3.74[c]		3.12m	4.42t (J 5.8); 4.74; 4.98d (J 5.3)
9	9.10d $J_{NH,1}$ 9.6	4.47	1.95	1.17t; 3.98q J 7.0	4.70q $J_{1,2}\sim$1.0	3.74q $J_{2,3}$ 2.7		[d]	4.67; 4.99d (J 5.4)

[a] Signals due to H-3 and H-6 were not located for any of the compounds. [b] Signal multiplicities are indicated as follows: d, doublet; t, triplet; q, quartet; m, multiplet; the absence of any indication implies that a singlet was observed. [c] Broad. [d] The C-5 methyl signal appears at δ 1.17d ($J_{5,6}$ 5.0).

not allow any definite conclusion to be drawn with regard to the anomeric configuration. Therefore, formula **5** for this substance is tentative.

In the D-mannose and L-rhamnose derivatives, H-1 shows the splittings ($J_{NH,1}$ 9.2–9.6 Hz; $J_{1,2} \simeq 1.0$ Hz) anticipated for glycopyranoses having these configurations. However, these H-1 protons appear at a field 0.3 p.p.m. lower than for the β-D-glucopyranose derivative **3**. This paramagnetic shift is of the same order of magnitude as that produced on the axial H-1 of glycopyranoses by changing the hydroxyl group on C-2 from the equatorial to the axial position[6,8]; it can be ascribed to the same effect. On this assumption, H-1 in the compounds under discussion should be axial, and these substance should have the β-D and β-L anomeric configurations as indicated in formulae **7** and **9**, respectively.

The above results show that ethyl 3-(glycosylamino)crotonates have a larger tendency to adopt the *cis* configuration **1a** than do simple 3-(alkylamino)crotonic esters[3]. The difference is particularly noticeable in methyl sulfoxide solution in which ethyl 3-(methylamino)crotonate exists in the *trans* form to an extent of *ca.* 40%, whereas compounds **3**, **5**, **7**, and **9**, exist exclusively in the *cis* form **1a**. The greater stability of the *cis* forms of these compounds might be ascribed to the electron-withdrawing effect of the glycosyl group which most likely increases the electronegativity of the nitrogen atom and strengthens the hydrogen bond.

ACKNOWLEDGMENTS

The authors express their gratitude to Professor F. García González for his interest, and to Dr. J. Calderón, Instituto de Química Orgánica General, Madrid, for the microanalyses.

REFERENCES

1 A. GÓMEZ SÁNCHEZ AND J. VELASCO DEL PINO, *Carbohyd. Res.*, 1 (1965) 421.
2 G. O. DUDEK AND G. P. VOLPP, *J. Amer. Chem. Soc.*, 85 (1963) 2697.
3 A. GÓMEZ SÁNCHEZ, M. TENA ALDAVE, AND U. SCHEIDEGGER, *J. Chem. Soc.* (*C*), (1968) 2570.
4 R. U. LEMIEUX AND J. D. STEVENS, *Can. J. Chem.*, 43 (1965) 2059.
5 A. GÓMEZ SÁNCHEZ, M. TENA ALDAVE, A. CERT VERTULÁ, AND U. SCHEIDEGGER, unpublished results.
6 L. D. HALL, *Tetrahedron Lett.*, (1964) 1457.
7 B. CASU AND M. REGGIANI, *Tetrahedron Lett.*, (1964) 2839; B. CASU, M. REGGIANI, G. G. GALLO, AND A. VIGEVANI, *Tetrahedron*, 22 (1967) 3061.
8 R. U. LEMIEUX AND J. D. STEVENS, *Can. J. Chem.*, 44 (1966) 249.

Note

Preferential toluene-*p*-sulphonylation of methyl 6-*O*-trityl-α-D-mannofuranoside.
Part II*. Synthesis of 3,5,6-tri-*O*-methyl-D-mannose**

Until recently, only two methyl ethers of D-mannofuranose (namely, the 2,3,5-tri- and 2,3,5,6-tetra-*O*-methyl derivatives) were known[1,2], although derivatives of 5,6-di-*O*-methyl-D-mannose have been described. We have recently reported[4] on the synthesis of 3,5-di-*O*-methyl-D-mannose, and we now describe the synthesis of 3,5,6-tri-*O*-methyl-D-mannose.

Methyl 3,5-di-*O*-methyl-2-*O*-tosyl†-6-*O*-trityl-α-D-mannofuranoside[4] (**1**), on successive detritylation, methylation, detosylation, and hydrolysis, yielded crystalline 3,5,6-tri-*O*-methyl-D-mannose (**5**) having m.p. 73–74.5°, $[\alpha]_D^{26}$ $+12.8°$ (water). The constitution of the sugar follows from its mode of synthesis, and confirmation was obtained by the following observations: (*a*) demethylation[5] produced mannose, (*b*) the high M_G value in borate buffer showed that position 2 is unsubstituted, (*c*) oxidation with bromine water yielded a crystalline product having properties characteristic of a γ-lactone[6], and (*d*) the derived tri-*O*-methylhexitol consumed one mole of periodate per mole, and the oxidized sugar produced arabinose on demethylation.

EXPERIMENTAL

The general experimental methods have been described previously[7].

*Methyl 3,5-di-*O*-methyl-2-*O*-tosyl-α-*D*-mannofuranoside* (**2**). — A solution of methyl 3,5-di-*O*-methyl-2-*O*-tosyl-6-*O*-trityl-α-D-mannofuranoside[4] (**1**) in dry chloroform saturated with dry hydrogen chloride was kept for 30 min at 0°. Neutralization (silver carbonate), filtration, and evaporation gave a syrup that was purified by chromatography on a column of silica gel, with acetone–chloroform (1:10) as eluant, to give a homogeneous (t.l.c.) syrup (80%), $[\alpha]_D^{24}$ $+54.9°$ (*c* 6.2, chloroform).

Anal. Calc. for $C_{16}H_{24}O_8S$: C, 51.05; H, 6.43; S, 8.52; OCH_3, 24.73. Found: C, 51.16; H, 6.29; S, 8.58; OCH_3, 24.22.

*Methyl 3,5,6-tri-*O*-methyl-2-*O*-tosyl-α-*D*-mannofuranoside* (**3**). — Compound **2** (0.90 g) was treated with methyl iodide (50 ml) and silver oxide (5 g). Filtration and evaporation gave a homogeneous (t.l.c.) syrup (0.90 g, 95%), $[\alpha]_D^{27}$ $+62.2°$ (*c* 3.05, chloroform).

Anal. Calc. for $C_{17}H_{26}O_8S$: C, 52.32; H, 6.27; S, 8.22; OCH_3, 31.79. Found: C, 51.92; H, 6.35; S, 7.88; OCH_3, 31.50.

---

*For Part. I, see Ref. 4.

**Contribution No. 107 of the Food Research Institute, Canada Department of Agriculture.

†Tosyl = toluene-*p*-sulphonyl.

*Methyl 3,5,6-tri-*O*-methyl-α-*D*-mannofuranoside* (**4**). — A solution of compound **3** (0.866 g) in dry methanol (25 ml) was mixed with a solution of sodium methoxide (from 1.2 g of sodium) in dry methanol (25 ml). After the mixture had been refluxed for 16 h, it was cooled to room temperature and evaporated, water (15 ml) was added, and the suspension was extracted with chloroform (3 × 15 ml). Evaporation of the extract yielded a syrup (0.456 g, 87%), $[\alpha]_D^{29}$ +61.8° (*c* 5.5, water). Paper chromatography of the glycoside produced a single spot having R_F 0.84 (solvent *A*) and giving a blue stain with spray *B* (Found: OCH_3, 51.81. Tri-*O*-methylhexoside calc.: 52.5%).

*3,5,6-Tri-*O*-methyl-*D*-mannose* (**5**). —Compound **4** (0.425 g) was hydrolyzed with 0.5N sulphuric acid (4 ml) for 3 h at 100°. Neutralization (barium carbonate), filtration, and evaporation of the hydrolysate gave a syrup that showed two minor, faster-moving impurities, besides the main component (t.l.c.). The impurities were removed by chromatography on a column of silica gel with chloroform–acetone (1:1). The syrup (0.380 g, 95%), on paper-chromatographic (solvent *A*) and electrophoretic examination showed a single component having R_F, R_G, and M_G values of 0.60, 0.77, and 0.52, respectively. Immediate crystallization was induced on storage of the syrup in a vacuum desiccator. Recrystallization from isopropyl ether, first at room temperature and then at 0°, gave compound **5**, m.p. 73–74.5°, $[\alpha]_D^{26}$ +10.6 (3 min)→12.8° (5 h, equil.) (*c* 1.5, water).

Anal. Calc. for $C_9H_{18}O_6$: C, 48.64; H, 8.15; OCH_3, 41.90. Found: C, 48.95; H, 8.42; OCH_3, 41.99.

*3,5,6-Tri-*O*-methyl-*D*-mannono-1,4-lactone* (**6**). — A mixture of compound **5** (0.07 g), water (2 ml), barium carbonate (0.15 g), and bromine (20 drops) was kept in the dark for 70 h. Bromine was removed by aeration, and the solution, after acidification with hydrochloric acid, was extracted continuously with chloroform for 24 h. The extract was dried (Na_2SO_4), filtered, and concentrated to a syrup which was distilled at 140–145° (bath)/0.01 mm to give the lactone. Crystallization from isopropyl ether containing a few drops of ethanol gave compound **6**, m.p. 132–133°, $[\alpha]_D^{26}$ +43.2 (initial)→ +37.5° (14 days, incomplete) (*c* 0.53, water), ν_{max}^{KBr} 1780 cm^{-1}.

Anal. Calc. for $C_9H_{16}O_6$: C, 49.09; H, 7.27. Found: C, 49.19; H, 7.20.

*Periodate oxidation of 3,5,6-tri-*O*-methyl-*D*-mannitol.* — 3,5,6-Tri-*O*-methyl-D-mannose (0.068 g) was reduced with sodium borohydride[8]. The resulting, syrupy hexitol (0.064 g) was oxidized in aqueous solution (50 ml) containing 0.1M sodium periodate (20 ml). The consumption of periodate, determined by the arsenite method[9], was 1.08 moles per mole of hexitol after oxidation for 2 and 24 h.

The solution remaining after periodate oxidation was extracted with chloroform (12 × 20 ml). The syrupy product was recovered, and a portion (0.005 g) on demethylation with boron trichloride[5] gave arabinose (identification by paper chromatography)

Food Research Institute,
Canada Department of Agriculture,
Ottawa (Canada)

I. R. SIDDIQUI

REFERENCES

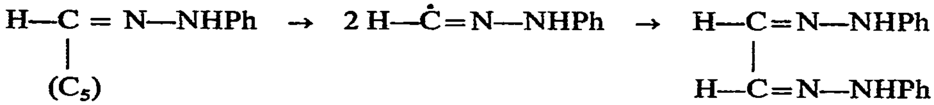
1 D. Heslop and F. Smith, *J. Chem. Soc.*, (1944) 574.
2 W. N. Haworth, E. L. Hirst, and J. I. Webb, *J. Chem. Soc.*, (1930) 651.
3 J. C. Irvine and B. M. Patterson, *J. Chem. Soc.*, 105 (1914) 898.
4 I. R. Siddiqui and V. L. N. Murty, *Carbohyd. Res.*, 8 (1968) 477.
5 S. Allen, T. G. Bonner, E. J. Bourne, and N. M. Saville, *Chem. Ind.* (London), (1958) 630.
6 S. A. Barker, E. J. Bourne, R. M. Pinkard, and D. H. Whiffen, *Chem. Ind.* (London), (1958) 658.
7 I. R. Siddiqui and B. Urbas, *Carbohyd. Res.*, 5 (1967) 210.
8 M. A. Abdel-Akher and F. Smith, *Nature*, 166 (1950) 1037.
9 P. P. Fleury and J. Lange, *J. Pharm. Chim.*, 17 (1933) 107.

Received July 8th, 1968)

Note

The reaction of D-glucose benzoylhydrazone with base

It was reported by Pinkus[1] that D-glucose reacts with benzoylhydrazine in aqueous alkali to yield glyoxal bis(benzoylhydrazone) (**1**) and pyruvaldehyde bis-(benzoylhydrazone). Diels and coworkers[2] obtained glyoxal bis(phenylhydrazone) in high yield when D-*arabino*-hexulose phenylosazone was refluxed in 1% alcoholic potassium hydroxide for 5 h. Simon and Moldenhauer[3] observed that hot 0.1M alcoholic potassium hydroxide converts D-*arabino*-hexulose phenylosazone into glyoxal bis(phenylhydrazone) in 85% yield, and into 2,4-dihydroxybutyric acid in about 45% yield. Wolfrom and coworkers[4] obtained glyoxal bis(phenylhydrazone) when either D-galactose phenylhydrazone or D-arabinose phenylhydrazone was heated with methanolic or ethanolic pyridine at 120–130°. These investigators suggested that homolytic fission occurs, followed by the union of two C_1 fragments to yield the osazone.



$$\begin{array}{l}\mathrm{H{-}C{=}N{-}NHPh}\\ \quad|\\ (C_5)\end{array} \rightarrow 2\,\mathrm{H{-}\dot{C}{=}N{-}NHPh} \rightarrow \begin{array}{l}\mathrm{H{-}C{=}N{-}NHPh}\\ \quad|\\ \mathrm{H{-}C{=}N{-}NHPh}\end{array}$$

We have observed that, when D-*arabino*-hexulose benzoylhydrazone (**2**) is refluxed in 1% potassium hydroxide in ethanol for 1 h, the solution is acidified with acetic acid, and sufficient water is added to dissolve the brown residue, glyoxal bis(benzoylhydrazone) (**1**) separates in low yield. In order to determine the source of the glyoxal carbon atoms, D-glucose-*1*-^{14}C benzoylhydrazone (**2a**) and D-glucose-*2*-^{14}C benzoylhydrazone (**2b**) were prepared, and subjected to the same alkaline decomposition. The results are described in Table I.

The results indicate that, in forming osazone **1**, a labeled entity derived from C-1 and C-2 of **2** competes with glycolaldehyde fragments derived from the non-labeled atoms C-3 to C-6 of **2** for reaction with the benzoylhydrazone groups. Dilution of the product from the labeled fragment with that from the nonlabeled fragments accounts for the specific activity observed. The fragmentation could result from a reversed-aldol mechanism[5].

Supporting this approach is our observation that the reaction of **2** with glyoxal in the presence of acetic acid produces **1** in good yield. Simon and coworkers[6] reported the conversion of 2-(phenylhydrazino)acetaldehyde phenylhydrazone ($\mathrm{PhNHNHCH_2{-}CH{=}N{-}NHPh}$) into the osazone in high yield in nonaqueous medium in the presence of acetic acid and the absence of phenylhydrazine. They also reported the ready conversion of glycolaldehyde into glyoxal bis(phenylhydrazone) in the presence of phenylhydrazine in methanolic acetic acid.

TABLE I

RADIOACTIVITY DATA

Ratio of activity of glyoxal bis(benzoylhydrazone) to that of D-*glucose benzoylhydrazone*	*Activity, dpm/mg*	
	Reactant	*Product* *Glyoxal bis(benzoylhydrazone)* (*Mol. wt.* 294)
	D-*glucose*-1-^{14}C *benzoylhydrazone* (*Mol. wt.* 298)	
0.333	61,8000	20,500
0.284	78,000	22,000
0.288	111,500	32,000
	D-*glucose*-2-^{14}C *benzoylhydrazone*	
0.319	64,000	20,400
0.308	62,400	19,200
0.269	101,500	27,300

EXPERIMENTAL

Materials and methods. — D-Glucose-*1*-^{14}*C* and -*2*-^{14}*C* were purchased from Nuclear Chicago. Radioactivity was measured with a Nuclear Chicago Liquid Scintillation Counter.

D-*Glucose benzoylhydrazone* (**2**, **2a**, *and* **2b**). — A solution of D-glucose (1.00 g) and benzoylhydrazine (1.00 g) in 95% ethanol (10 ml) was refluxed for 8 h, and then cooled to give **2**, as colorless crystals; yield 1.19 g (71%), m.p.[7] 185–187° (dec.), that could be recrystallized from 95% ethanol.

Reaction of **2**, **2a**, *and* **2b** *with base.* — A solution of D-glucose benzoylhydrazone (100 mg) in 1% ethanolic potassium hydroxide was refluxed. The mixture turned dark brown almost immediately. After 1 h, the solution was acidified with glacial acetic acid, and water was added to dissolve the brown residue. Yields of 1–4 mg of **1** were obtained in different experiments. Compound **1** was identified by its i.r. spectrum, m.p. and mixed m.p.

Samples (1–2 mg) for counting were solubilized with Nuclear Chicago NBS (10 drops), scintillation solution (5 ml)* (Nuclear Chicago Liquafluor diluted 25:1 with toluene) was added, and then acetic acid was added until the yellow color had been discharged or until the solution was acidic, and the milky sample was immediately counted. Compounds **1** and **2** were counted at the same time in the same way.

ACKNOWLEDGMENT

This work was supported, in part, by a City University Faculty Research Grant.

Department of Chemistry,
The City College of The
City University of New York,
New York, N.Y. 10031 (U.S.A.)

C. S. RUSSELL
ROBERT LYONS

---

*Samples were counted with 5 ml and 10 ml of scintillation solution to maximize the counts.

REFERENCES

1 G. PINKUS, *Chem. Ber.*, 31 (1898) 31.
2 O. DIELS, R. MEYER, AND O. ONNEN, *Ann.*, 525 (1936) 94.
3 H. SIMON AND W. MOLDENHAUER, *Chem. Ber.*, 100 (1967) 3121.
4 H. EL KHADEM, M. L. WOLFROM, Z. M. EL SHAFEI, AND S. H. EL ASHRY, *Carbohyd. Res.*, 4 (1967) 225.
5 W. L. EVANS, *Chem. Rev.*, 31 (1942) 537.
6 H. SIMON, G. HEUBACH, AND H. WACKER, *Chem. Ber.*, 100 (1967) 3106.
7 B. HOLMBERG, *Arkiv Kemi*, 7 (1954) 50.

(Received July 6th, 1968; in revised form, August 12th, 1968)

Note

Photochemical degradation of some 2,4-dinitroanilino derivatives of sugars*

Photochemical procedures for effecting preparative transformations in the carbohydrate field, introduced in earlier work from this laboratory[1], have been demonstrated in several different systems[2,3]. The present report describes the behavior of 1-deoxy-1-(2,4-dinitroanilino)-D-glucitol (4) and the sodium salt (2) of 2-deoxy-2-(2,4-dinitroanilino)-D-gluconic acid upon irradiation with ultraviolet light.

Akabori and co-workers[4] observed that *N*-(2,4-dinitrophenyl) derivatives of α-amino acids are photosensitive, whereas the ε-*N*-(2,4-dinitrophenyl) derivative of lysine is stable to light. Russell[5,6] showed that photolysis of *N*-(2,4-dinitrophenyl)-leucine gives 3-methylbutyraldehyde, carbon dioxide, and 4-nitro-2-nitrosoaniline; other *N*-(2,4-dinitrophenyl) derivatives of α-amino acids also undergo photolytic degradation, although 4-nitro-2-nitrosoaniline is not formed in all instances. Neadle and Pollitt[7] established that 6-nitrobenzimidazole 1-oxide may be a minor product accompanying 4-nitro-2-nitrosoaniline, and the proportions of these two products depend upon the pH of the solution being photolyzed.

Since *N*-(2,4-dinitrophenyl) derivatives of amino sugars are frequently used for chromatographic separation and for characterization of the parent amino sugars, it was of interest to study the behavior of such derivatives upon irradiation. The objectives of this investigation were to determine whether degradations of the type observed[5] with *N*-(2,4-dinitrophenyl)leucine take place in carbohydrate derivatives containing an α-amino acid system, and also to assess the stability to light of the 2,4-dinitroanilino group when it is substituted on an alditol chain.

1-Deoxy-1-(2,4-dinitroanilino)-D-glucitol[8-10] (4) was prepared in essentially quantitative yield by treatment of 1-amino-1-deoxy-D-glucitol oxalate[11] with 1-fluoro-2,4-dinitrobenzene in the presence of sodium hydrogen carbonate, and the product was further characterized as its pentaacetate (see Experimental section). Irradiation of an aqueous methanolic solution of **4** for 96 h, with a mercury-arc lamp enclosed in a quartz finger immersed in the solution[2], caused no decomposition of **4** that could be detected by chromatography. Addition of sodium hydrogen carbonate to the solution had no marked effect, although very slight decomposition was observed

---

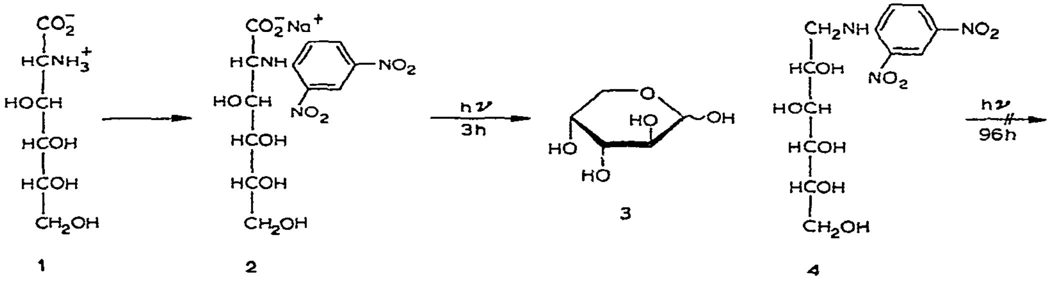
*Supported by the National Institutes of Health, Public Health Service, Department of Health, Education, and Welfare, Bethesda, Maryland, 20014; Grant No. GM-11976-04 (The Ohio State University Research Foundation Project 1820).

after irradiation for 72 h. It was concluded that compound **4** is not appreciably changed by u.v. irradiation unless a very extended period of exposure is used.

A 2-deoxy-2-(2,4-dinitroanilino)aldonic acid was prepared for photochemical study by treating 2-amino-2-deoxy-D-gluconic acid[12] (**1**) with 1-fluoro-2,4-dinitrobenzene and sodium hydrogen carbonate in aqueous ethanol, to give 2-deoxy-2-(2,4-dinitroanilino)-D-gluconic acid as its sodium salt (**2**). The crude reaction product contained **2** as the major component; by preparative t.l.c.[13], **2** was obtained free from minor side-products of greater chromatographic mobility. The salt **2**, obtained as a chromatographically homogeneous, yellow glass, showed i.r. spectral absorption for the ionized carboxylate group; absorptions for un-ionized CO_2H groups or $C=O$ groups of a lactone were absent.

Irradiation of **2** in water or aqueous sodium hydrogen carbonate, by the procedure used for the alditol derivative **4**, led to complete decomposition of **2** in 2.5 h, as revealed by t.l.c. and paper chromatography. The u.v. spectrum of the solution showed characteristic changes over this period of time (see Experimental section); irradiation for longer periods did not cause noticeable further changes in the u.v. spectrum of the solution or in chromatograms of the product mixture.

The product mixture contained a yellow component that could be extracted into ethyl acetate. Evaporation of the extract gave a yellow solid that contained no carbohydrate moiety, as shown by n.m.r. spectroscopy. The chromatographic and spectral properties of the yellow solid indicated that it was probably 4-nitro-2-nitrosoaniline[5], together with a second, minor product. The colorless, aqueous solution remaining after extraction with ethyl acetate was found to contain a single component, a carbohydrate indistinguishable from D-arabinose (**3**). Acetylation of this product gave tetra-*O*-acetyl-α,β-D-arabinopyranose, indistinguishable by n.m.r. spectrum from an authentic sample prepared by acetylating syrupy α,β-D-arabinose.



The results indicate that the amino sugar derivative **2** is readily degraded by u.v. light to the corresponding lower aldose **3**, whereas the alditol derivative **4** is stable. This difference in behavior may be a useful diagnostic tool in structural work. The ease with which compound **2** is degraded by u.v. light serves to emphasize the need for caution in spectrophotometric assays or chromatographic resolution of similar compounds, if interference from artifacts of photolytic degradation are to be avoided.

It may be possible to use the reaction 2→3 as a method for one-carbon descent of the chain with 2-amino-2-deoxyaldoses, by oxidation of an *N*-(2,4-dinitrophenyl) derivative to the aldonic acid, followed by photolysis of the latter. Identification of the resultant aldose would serve to establish the total structure of the amino sugar, except for the configuration at C-2. Such a method might be a useful complement to the one, useful for free 2-amino-2-deoxy sugars, that is based on degradation with ninhydrin[14].

EXPERIMENTAL

General. — Solutions were concentrated below 40° under diminished pressure. Melting points were determined with a Thomas–Hoover "Unimelt" apparatus and are uncorrected. 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-60A or HA-100 n.m.r. spectrometers. Chemical shifts are given on the τ-scale, for solutions in chloroform-*d* with tetramethylsilane as the internal standard. 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 performed by W. N. Rond. T.l.c. was conducted with Silica Gel G (E. Merck, Darmstadt, Germany), and colorless components were detected with sulfuric acid. Paper chromatography was performed on Whatman No. 1 paper, and colorless components were detected with alkaline silver nitrate and aniline hydrogen phthalate.

Irradiation procedure. — The unfiltered output of a mercury-arc lamp was used, by the procedure described by Horton and Jewell[2].

*1-Deoxy-1-(2,4-dinitroanilino)-*D*-glucitol* (4). — To a solution of 1-amino-1-deoxy-D-glucitol oxalate[11] (22.6 g, 100 mmoles) and sodium hydrogen carbonate (16.8 g, 200 mmoles) in a mixture of water (250 ml) and acetone (250 ml) was added 1-fluoro-2,4-dinitrobenzene (18.6 g, 100 mmoles) and the mixture was stirred for 18 h at 30°. The slightly turbid solution was filtered, and the filtrate was concentrated to 100 ml. The resulting suspension was kept for 1 h at 0°, and the product was filtered off, washed with two 50-ml portions of water at 0°, dried, and recrystallized from hot water (500 ml) to give 4, yield 33.5 g (97%), m.p. 164–165.5°; λ_{max} (40% methanol in water) 214.1 (ε 1.33×10^4), 256.9 (9.57×10^3), and 361.1 nm (1.82×10^4).

For this compound, prepared by somewhat different procedures, Weygand *et al.*[8] gave m.p. 166°; Foye and Feldmann[9] gave m.p. 167–169°; and den Otter[10] gave m.p. 151–152°.

*2,3,4,5,6-Penta-O-acetyl-1-deoxy-1-(2,4-dinitroanilino)-*D*-glucitol.* — A solution of 4 (1.00 g) in pyridine (10 ml) and acetic anhydride (5 ml) was kept for 18 h at 25° and then poured onto ice (60 g). The mixture was stirred for 3 h, during which time the gum that was precipitated initially solidified. The product was filtered off, washed with water, and dried; yield 1.60 g (99%). Recrystallization from boiling ethanol (50 ml) gave the product as large, lemon-colored prisms; yield 1.58 g (98%), m.p. 124–126°, $[\alpha]_D^{21}$ $+2.9 \pm 1.2°$ (*c* 1.7, chloroform); X-ray powder diffraction data: 11.55 w, 8.67 vw, 7.44 vs (2), 6.41 vw, 5.87 s (3,3), 5.03 w, 4.70 m, 4.50 m, 4.12 vs (1), 3.75 s (3,3), 3.61 vw, and 3.25 m.

Anal. Calc. for $C_{22}H_{27}N_3O_{14}$: C, 47.39; H, 4.89; N, 7.54. Found: C, 47.35; H, 5.13; N, 7.85.

*Irradiation of 1-deoxy-1-(2,4-dinitroanilino)-*D*-glucitol* (4) — *A. In aqueous methanol.* A solution of 4 (720 mg) in 80% aqueous methanol (150 ml) was irradiated[2] under nitrogen for 96 h at room temperature. Throughout the experiment, only starting material (R_F 0.85, papergram, 4:1:5 butyl alcohol–acetic acid–water) could be detected, before or after spraying the paper with aniline hydrogen phthalate.

B. In the presence of sodium hydrogen carbonate. To a solution of **4** (520 mg) in warm methanol (60 ml) were added water (40 ml) and a solution of sodium hydrogen carbonate (1.5 g) in water (50 ml), and the solution was irradiated for 72 h. At the end of this time, chromatographic examination showed that **4** remained essentially unchanged, although a trace of material having the chromatographic characteristics of D-glucose was present, together with a trace of a yellow component having R_F ~0.9.

*Sodium 2-deoxy-2-(2,4-dinitroanilino)-*D*-gluconate* (**2**). — To a solution of 2-amino-2-deoxy-D-gluconic acid[12] (**1**, 2.6 g) and sodium hydrogen carbonate (4.66 g) in water (60 ml) was added a solution of 1-fluoro-2,4-dinitrobenzene (4.96 g) in ethanol (60 ml), and the mixture was stirred for 3 h at room temperature. The solution was concentrated *in vacuo* to remove ethanol, and the aqueous solution was washed 3 times with ether to remove excess of 1-fluoro-2,4-dinitrobenzene. The aqueous solution was brought to pH 7 by adding M hydrochloric acid, and then evaporated below 35°, and the residue was dried in a vacuum desiccator. The residue was extracted with methanol (50 ml) and the extract was filtered from insoluble salts. The extract was evaporated, and the residue was dried, re-extracted with methanol, and the extract freed of insoluble salts by filtration. Evaporation of the solvent gave crude **2** as a yellow syrup in essentially quantitative yield. This product showed moderate absorption at 5.82 μm in its i.r. spectrum (film), and t.l.c. (3:2 chloroform–methanol) showed the presence of a yellow, major component having R_F ~0.2 and minor, yellow components having R_F ~0.6 and ~0.8. The major component was isolated from 320 mg of the crude syrup by preparative t.l.c.[13] on 4 plates coated with a 1-mm layer of adsorbent, to give chromatographically homogeneous, syrupy **2**, yield 219 mg (68%), $[\alpha]_D^{21}$ $-44 \pm 2°$ (*c* 0.8, water); R_F 0.86 (papergram, 4:1:5 butyl alcohol–acetic acid–water), 0.54 (papergram, 4:1 *tert*-butyl alcohol–4.25M aqueous ammonia); λ_{max} (in 4% aqueous $NaHCO_3$) 231.1 (ε 9.1 × 10^3), 267.1 (9.1 × 10^3), 365.1 nm (1.5 × 10^4); λ_{max}^{film} 2.95 (OH), 6.12 (CO_2^-), 6.38, 6.54, 6.67 sh (aryl C=C), 7.04, 7.45, 7.80 sh, 8.05 sh, 8.60, 8.72, 9.00, 9.55 (broad), 10.75, 12.1 (broad), and 13.38 μm. The product was dried *in vacuo* for 3 days at 100°.

Anal. Calc. for $C_{12}H_{14}N_3NaO_{10}$: C, 37.60; H, 3.68; N, 10.96; Na, 6.00, Found: C, 37.77; H, 4.29; N, 10.87; Na, 5.71.

The i.r. spectrum of the chromatographically purified **2** resembled that of the crude product, except that no absorption near 5.8 μm was observed. The elemental analysis indicated the presence of water of hydration even after prolonged drying.

*Photolysis of sodium 2-deoxy-2-(2,4-dinitroanilino)-*D*-gluconate* (**2**). — A solution of the salt **2** (192 mg) in 4% aqueous sodium hydrogen carbonate (150 ml) was photolyzed at room temperature, and the progress of the reaction was monitored by chromatography and by u.v. spectroscopy of aliquot samples. T.l.c. (3:2 chloroform–methanol) revealed that the starting material had been completely decomposed after 2.5 h. The u.v. spectrum (which, at the outset, showed maxima at 365 and 267 nm, in approximately 2:1 relative intensity) changed until, after 3 h, maxima were observed at 349 and 279 nm in approximately equal intensities (compare refs. 5 and 6). The absorption at 231 nm, observed for **2** at the outset, shifted to 215 nm after 3 h, with little change in intensity. Irradiation for a further 3 h gave no further change in the product, as revealed by chromatographic or u.v. spectral examination.

The photolysis was halted after 6 h, the suspension was filtered to remove a small amount of brown, insoluble material, and the yellow filtrate was extracted with three 30-ml portions of ethyl acetate. Evaporation of the combined extracts gave a yellow solid (~60 mg) that showed no signals above τ 4.0 in its n.m.r. spectrum; paper chromatography (4:1 *tert*-butyl alcohol–4.25M aqueous ammonia) revealed the presence of two yellow components having R_F 0.85 (minor) and 0.90 (major), and no additional components were revealed when the paper was sprayed with aniline hydrogen phthalate. Paper chromatography of the aqueous phase revealed no colored component; and, when the paper was sprayed with aniline hydrogen phthalate, a single component, having chromatographic characteristics identical with those of D-arabinose (**3**), was observed.

Evaporation of the aqueous phase, and acetylation of the residue with acetic anhydride–sodium acetate gave tetra-*O*-acetyl-α,β-D-arabinopyranose, which was chromatographically homogeneous (R_F 0.65, t.l.c., 9:1 benzene–methanol) and, by t.l.c., i.r. spectrum, and n.m.r. spectrum, identical with a sample of the tetraacetate prepared by acetylating a mutarotated sample of D-arabinose. The n.m.r. spectrum (100 MHz) showed that the α-D pyranose anomer (τ 4.32, doublet, $J_{1,2}$ 6.3 Hz, H-1; τ 7.86, 7.88, 7.93, 7.95, acetyls) was the major component; these data coincide with those determined for an authentic sample of α-D-arabinopyranose tetraacetate. The β-D-pyranose anomer (τ 7.84, 7.98, acetyls) was also present.

Longer periods of irradiation were required for decomposition of all of the starting material when larger samples of **2** were employed.

In a second experiment, a solution of **2** (315 mg) in salt-free water (150 ml) was photolyzed for 6 h at room temperature under nitrogen. The solution was extracted with ethyl acetate to remove yellow material (~79 mg) and the colorless, aqueous phase was concentrated to ~20 ml. The solution was passed through a column of Amberlite MB-3 mixed-bed resin (15 ml), and the column was washed with water (200 ml). Evaporation of the combined effluents gave chromatographically homogeneous D-arabinose (**3**); yield 65 mg (52%). Crystallization from aqueous methanol gave the β-D anomer of 3, m.p. 158–159°, $[\alpha]_D^{21}$ $-103°$ (equil., *c* 1.0, water) (lit.[15] m.p. 159–160°, $[\alpha]_D$ $-105.1°$ in water), identical with an authentic sample by mixed m.p. and X-ray powder diffraction pattern.

ACKNOWLEDGMENTS

The authors thank Dr. A. K. Chatterjee for assistance with some of the experiments, J. H. Lauterbach for recording the n.m.r. spectra, and Dr. G. N. Bollenback for a sample of 1-amino-1-deoxy-D-glucitol oxalate.

Department of Chemistry,
The Ohio State University,
Columbus, Ohio 43210 (U.S.A.)

A. E. EL ASHMAWY
D. HORTON
KERSTIN D. PHILIPS

REFERENCES

1 D. HORTON AND W. N. TURNER, *Chem. Ind.* (London), (1964) 76; *Carbohyd. Res.*, 1 (1966) 444.
2 D. HORTON AND J. S. JEWELL, *J. Org. Chem.*, 31 (1966) 509; R. FUNAKOSHI, D. HORTON, AND W. D. PARDOE, to be published.
3 R. H. BELL, D. HORTON, AND DIANA M. WILLIAMS, *Chem. Commun.*, (1968) 323; *idem*, to be published.
4 S. AKABORI, T. IKENAKA, Y. OKADA, AND K. KOHNO, *Proc. Japan Acad.*, 29 (1953) 509.
5 D. W. RUSSELL, *J. Chem. Soc.*, (1963) 894.
6 D. W. RUSSELL, *Biochem. J.*, 87 (1963) 1.
7 D. J. NEADLE AND R. J. POLLITT, *J. Chem. Soc. C*, (1967) 1764.
8 F. WEYGAND, R. LÖWENFELD, AND E. F. MÖLLER, *Ber.*, 84 (1951) 101.
9 W. O. FOYE AND E. G. FELDMANN, *J. Org. Chem.*, 22 (1957) 475.
10 H. P. DEN OTTER, *Rec. Trav. Chim.*, 56 (1937) 1196.
11 J. W. LONG AND G. N. BOLLENBACK, *Methods Carbohyd. Chem.*, 2 (1963) 79.
12 H. PRINGSHEIM AND G. RUSCHMANN, *Ber.*, 48 (1915) 680.
13 D. HORTON AND T. TSUCHIYA, *Carbohyd. Res.*, 5 (1967) 426.
14 S. GARDELL, F. HEIJKENSKJÖLD, AND A. ROCHNORLUND, *Acta Chem. Scand.*, 4 (1950) 970; P. J. STOFFYN AND R. W. JEANLOZ, *Arch. Biochem. Biophys.*, 52 (1954) 373; P. J. STOFFYN, *J. Org. Chem.*, 24 (1959) 1360.
15 O. RUFF, *Ber.*, 32 (1899) 550.

(Received July 20th, 1968)

Note

Mutarotation of D-glucose and D-mannose in aqueous solution*

D-Glucose (1) and D-mannose (2) exhibit pseudo-first-order kinetics during mutarotation, as monitored by the changes in optical rotation (o.r.) in a suitable solvent. Each of these hexoses is available as the α- and the β-pyranose in crystalline form. Both anomers of each sugar exhibit the same kinetics of mutarotation; hence, it would be expected that, at equilibrium in water, these two hexoses are present in only two major forms, namely, the α- and the β-pyranoses. Optical rotation studies, however, cannot exclude the possible presence of other tautomers (*e.g.*, the α- and β-furanoses) which, due to compensation, might not contribute to the optical rotatory data. Recently, the trimethylsilylation procedure[1] for preparing volatile derivatives of sugars has been applied[2,3] to the measurement, by g.l.c., of the individual tautomers of D-galactose in solution in pyridine. Bentley and Botlock[4] developed a trimethylsilylation procedure, and prepared pertrimethylsilyl derivatives of sugars without apparent change in the position of the anomeric equilibrium that exists in water.

Identification of the pertrimethylsilyl derivatives of tautomers separated by g.l.c. is a prerequisite to use of the procedure of Bentley and Botlock[4] for the study of the tautomerization of sugars. The pertrimethylsilyl derivatives of D-glucopyranose and D-mannopyranose tautomers obtained by g.l.c.[3] have now been characterized by elementary analysis, polarimetry, and p.m.r. and i.r. spectrometry. For both of these hexoses during mutarotation and at equilibrium in aqueous solution, the kinetic and thermodynamic data obtained by g.l.c.[4] have been compared with those calculated from o.r.** for the same sample, in order to determine the extent to which the trimethylsilylation procedure alters the position of the reaction, either during mutarotation or at equilibrium.

The gas–liquid chromatograms of the pertrimethylsilyl derivatives of mutarotated D-glucopyranose and D-mannopyranose showed two peaks (see Fig. 1). For characterization, each compound was isolated by preparative g.l.c. as previously described[3]. The data used to establish the identity of each peak, in descending order of elution from an (ethylcyano)silicone column are shown in Table I. The molecular rotations found are consistent with those reported for the peracetylated tautomers of D-glucopyranose and D-mannopyranose[6]. The 100-MHz p.m.r. spectra of the four

---

*Presented, in part, at the 155th meeting of the American Chemical Society at San Francisco, California, in April, 1968.

**The rate and equilibrium constants were calculated according to the procedure described by Isbell and Pigman[5].

compounds in chloroform-*d* showed three general types of protons. (*a*) The first signal of three sets occurred at τ 4.5–5.5, and is assigned to the anomeric proton.

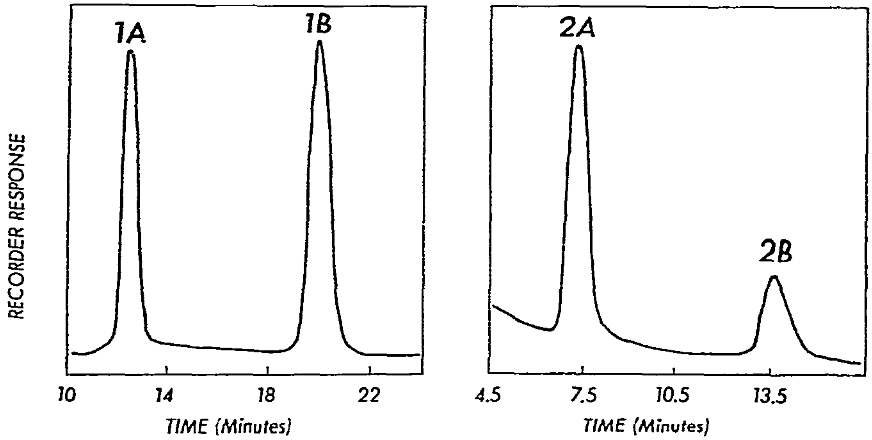


Fig. 1. Gas–liquid chromatogram of per-*O*-trimethylsilylated tautomers of D-glucose (1) and D-mannose (2) in aqueous solution at the equilibrium.

TABLE I

DATA CHARACTERIZING THE TRIMETHYLSILYL TETRA-*O*-(TRIMETHYLSILYL)-D-HEXOSIDES STUDIED

Pertrimethylsilyl ether of trimethylsilyl	$[\alpha]^{23}_{D}$, *degrees*	*[M]*	*100-MHz, p.m.r. data*		*Elementary analysis*		
			Chemical shift of H-1 signal, τ	$J_{1,2}$ *coupling (Hz)*	*C*	*H*	*Si*
					percent[a] *found*		
α-D-Glucopyranoside	+80.5	+43,500	4.6 (doublet)	3.0	46.71	9.61	25.53
β-D-Glucopyranoside	+ 4.6	+ 2,530	5.2 (doublet)	7.0	46.69	9.58	25.29
α-D-Mannopyranoside	+40.5	+21,870	5.1 (doublet)	1.2	46.06	9.70	25.08
β-D-Mannopyranoside	−20.1	−10,870	5.3 (singlet)	0.0	46.62	9.59	25.58

[a] Calc. for $C_6H_7O_6(SiMe_3)_5$; C, 46.66; H, 9.63; Si, 25.92%.

According to Lemieux *et al.*[7], the anomeric proton of an α-D-aldohexopyranose in the *CI* (D) conformation resonates at a lower chemical shift than for the β-D anomer in the same conformation, owing to interaction of this proton of the former with the *p*-orbitals of the ring-oxygen atom. For the four compounds studied, the signals given by the anomeric proton and their first-order coupling-constants ($J_{1,2}$) are in good agreement with the anomeric configurations assigned. (*b*) The second set of signals, at τ 5.5–7.0, is assigned to the remaining methine and methylene protons. (*c*) The third set, resonating at about τ 10, is assigned to protons of the *O*-trimethylsilyl groups. The integrated areas of these three sets of proton signals were in the ratios of 1:6:45, consistent with the view that the pairs of compounds isolated are the pertrimethylsilyl ethers of the anomers of trimethylsilyl D-glucopyranoside and D-mannopyranoside, respectively. Moreover, i.r. analysis failed to reveal any bands attributable to hydroxyl or carbonyl absorption. The results of elementary analysis of these four compounds were in agreement with the values calculated for $C_6H_7O_6(SiMe_3)_5$. The structural and configurational assignments for each compound were verified by trimethylsilylation immediately after dissolution of each pure, crystalline compound (**1** and **2**) in water; g.l.c. then revealed only one major peak for the compound having the configuration assigned.

TABLE II

RATE CONSTANTS[a] OF $\alpha \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \beta$ TRANSFORMATION DETERMINED BY G.L.C. AND O.R.

Sugar	*Rate constant*	*g.l.c. (× 10⁻²)*	*o.r. (× 10⁻²)*
D-Glucopyranose	$k_1^{25°}$	0.63	0.70
	$k_2^{25°}$	0.39	0.41
	$k_1^{35°}$	1.69	1.69
	$k_2^{35°}$	1.02	1.03
D-Mannopyranose	$k_1^{15°}$	0.48	0.44
	$k_2^{15°}$	1.04	1.02
	$k_1^{25°}$	1.31	1.25
	$k_2^{25°}$	2.78	2.84

[a] Calculated as $\log_{10}$ and expressed in $\min^{-1}$.

The information obtained from these data indicates that 1A and 1B (see Fig. 1) are trimethylsilyl tetra-*O*-(trimethylsilyl)-α- and -β-D-glucopyranoside, respectively, and 2A and 2B are trimethylsilyl tetra-*O*-(trimethylsilyl)-α- and -β-D-mannopyranoside, respectively.

The rate constants of the $\alpha \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \beta$ transformation, determined by both g.l.c. and o.r. during mutarotation and at equilibrium, are shown in Table II. The thermodynamic properties of the $\alpha \rightleftharpoons \beta$ transformation, calculated from the data given, are summarized in Tables III and IV. The value of ΔH obtained for D-glucopyranose is in agreement with that calculated from the optical rotatory data of Isbell and Pigman[8] (−59 cal/mol); however, these values are about 20% of those measured by calorimetry[9,10]. The reason for this discrepancy is not yet known. The thermodynamic data of activation, E_a and $\Delta S^{\ddagger}$ for D-glucopyranose are 5 to 10% lower than those reported by Schmid and Bauer[11] and Los and Simpson[12]. The value of $\Delta G^{\ddagger}$ for D-glucopyranose is about 10% lower than those reported[11]. It is interesting that, from the thermodynamic data, the observed entropy of activation for both of these hexoses is about 20–30 times the entropy difference between α- and β-D-hexopyranoses. Therefore, we suspect that the structure of the transition state is significantly different from that of either of the two pyranose anomers.

TABLE III

THERMODYNAMICS OF EQUILIBRIUM FOR $\alpha \rightleftharpoons \beta$ TRANSFORMATION[a]

	D-*Glucopyranose*[b]		D-*Mannopyranose*[c]	
	g.l.c.	*o.r.*	*g.l.c.*	*o.r.*
ΔG	−311	−313	+440	+480
ΔH	−50	−50	+254	+313
ΔS	+0.87	+0.84	−0.64	−0.57

[a]Expressed in cal. mol^{-1}, except for entropy values, which are given in cal. mol^{-1}.deg^{-1}.
[b]Calculated for 30°.
[c]Calculated for 20°.

TABLE IV

THERMODYNAMICS OF ACTIVATION FOR $\alpha \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \beta$ TRANSFORMATION[a]

Sugar		*g.l.c.*		*o.r.*	
		k_1	k_2	k_1	k_2
D-Glucopyranose[b]	$\Delta G^{\ddagger}$	+22.11	+22.78	+22.08	+22.78
	E_a	+18.01	+17.57	+16.09	+16.81
	$\Delta S^{\ddagger}$	−13.5	−17.1	−19.8	−19.6
D-Mannopyranose[c]	$\Delta G^{\ddagger}$	+21.89	+21.44	+21.92	+21.43
	Ea	+17.04	+16.85	+17.75	+17.43
	$\Delta S^{\ddagger}$	−16.49	−15.66	−14.22	−13.66

[a]Expressed in kcal. mol^{-1}, except for entropic values, which are in cal. mol^{-1}.deg^{-1}.
[b]Calculated at 30°.
[c]Calculated at 20°.

The kinetic and thermodynamic data obtained from g.l.c. are in excellent agreement ($r = 0.99$) with those calculated from optical rotation results. The results of the present study increase the potential usefulness of this g.l.c. technique as a method in the study of tautomerization of sugars, especially for those sugars that exhibit complex mutarotation.

ACKNOWLEDGMENTS

We thank R. M. Butts for the g.l.c. separations, and Gorton Wood for the p.m.r. determinations. This study was supported, in part, by a grant from the National Institutes of Health.

New York State Agricultural Experiment Station,
Cornell University,
Geneva, New York 14456 (U. S. A.)

C. Y. LEE
T. E. ACREE
R. S. SHALLENBERGER

REFERENCES

1 C. C. Sweeley, R. Bentley, M. Makita, and W. W. Wells, *J. Amer. Chem. Soc.*, 85 (1963) 2479.
2 R. S. Shallenberger and T. E. Acree, *Carbohyd. Res.*, 1 (1966) 495.
3 T. E. Acree, R. S. Shallenberger, and L. R. Mattick, *Carbohyd. Res.*, 6 (1968) 492.
4 R. Bentley and N. Botlock, *Anal. Biochem.*, 20 (1967) 312.
5 H. S. Isbell and W. W. Pigman, *J. Res. Nat. Bur. Stand.*, 18 (1937) 141.
6 F. J. Bates *et al.*, *Polarimetry, Saccharimetry and the Sugars, Nat. Bur. Stand. Circ. C 440*, 1942, pp. 729 and 746.
7 R. U. Lemieux, R. K. Kullnig, H. J. Bernstein, and W. G. Schneider, *J. Amer. Chem. Soc.*, 80 (1958) 6098.
8 H. S. Isbell and W. W. Pigman, Ref. 6, p. 449.
9 J. M. Sturtevant, *J. Phys. Chem.*, 45 (1941) 127.
10 M. A. Kabayama, D. Patterson, and L. Piche, *Can. J. Chem.*, 36 (1958) 557.
11 H. Schmid and G. Bauer, *Monatsh.*, 97 (1966) 866.
12 J. M. Los and L. B. Simpson, *Rec. Trav. Chim.*, 76 (1957) 267.

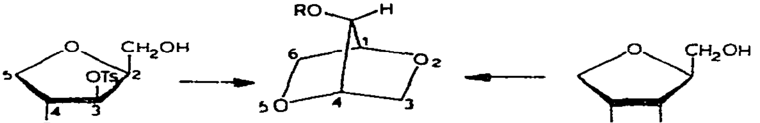
(Received September 7th, 1968)

Preliminary communication

The synthesis of (1S,4S)-7-toluene-*p*-sulphonyloxy-2,5-Dioxabicyclo[2.2.1]heptane[1]

As a part of a programme[1] directed towards the synthesis and reactions of bicyclic systems containing two heteroatoms, we now report on base-promoted cyclization reactions of sulphonates 1 and 2 leading to derivatives of dioxabicyclo[2.2.1]-heptane.

Treatment of the *trans*-disulphonate[2] 1 with boiling, methanolic sodium methoxide (2.2 mol) for 5 h gave a product (80%), m.p. 73–74°, $[\alpha]_D$ −55° (*c* 1, chloroform), which had a molecular weight of 270 (mass spectrometry) and an elemental analysis corresponding to the molecular formula, $C_{12}H_{14}O_5S$. The presence of the sulphonic ester group and the absence of a hydroxyl group were confirmed by infrared spectroscopy. These data suggested that the product was (1*S*,4*S*)-7-toluene-*p*-sulphonyloxy-2,5-dioxabicyclo[2.2.1]heptane (3). The formation of 3 can be rationalized in terms of an intramolecular, nucleophilic displacement. The hydroxyl group in compound 1 is *trans* to the C-4 toluene-*p*-sulphonyloxy group and thus suitably disposed, in the presence of methoxide ion, for rearward attack to give the dioxabicycloheptane 3.



1

3 R = Ts
4 R = H
5 R = Ac

2

Structure 3 is supported unequivocally by the n.m.r. spectrum which showed the following signals: a 3-proton singlet at 2.45 p.p.m. (methyl); a 4-proton multiplet centered at 7.60 p.p.m. (aromatic); 2-proton singlets at 3.86 and 3.95 p.p.m. (C-3 and C-6 methylene groups); 1-proton multiplets at 4.22 and 4.26 p.p.m. (H-1 and H-4); and a 1-proton quartet at 4.78 p.p.m. (H-7).

Compound 3 is totally asymmetric; the chiral centres at C-1 and C-4 have the *S* configuration, and C-7 has no chirality. The cyclization 1 → 3 involves the loss of the original asymmetry of C-3. From this type of geometry, it follows that the *cis*-di-*O*-sulphonyloxy system 2 should also give rise to the compound 3.

When the *cis*-disulphonate[3] 2 was heated with methanolic sodium hydroxide in methanol (2.2 mol.) for 5 h, the starting material 2 was recovered in high yield (80%). After heating had been continued for 18 h, the starting *cis*-disulphonate 2 was still the major component of the mixture, but compound 3 was also present and was subsequently isolated in 15% yield.

The relative rates at which compounds 1 and 2 undergo cyclization is remarkable. The ready formation of 3 from 1 may be attributed to the removal, during cyclization,of the severe gauche interaction between the C-2-CH_2OH and C-3-OTs groups in 1. In contrast, the *cis*-disulphonate 2 gives rise to 3 only under forcing conditions and in low yield (15%).

Having at hand the bridged bicyclic system 3, it was of interest to examine its behaviour towards substitution and solvolytic reactions. Only a limited amount of information is available on solvolytic reactions of heterocyclic analogues of bridged derivatives[4-6]. When sulphonate 3 was heated with an excess of either sodium azide or sodium benzoate at 150° in *N,N*-dimethylformamide for periods of 24–48 h, the starting product was recovered in high yield. Infrared spectroscopic examination of the mother liquors did not reveal any trace of azide or ester (OCOPh) bands. It is apparent that the lone electron pair of O-2 in 3 is not favourably directed towards the 7 position to provide anchimeric assistance in the displacement reactions. However, when 3 was treated with lithium aluminium hydride in tetrahydrofuran, O–S cleavage occurred, and the resulting alcohol 4 was characterized as its oily acetate 5, b.p. 55°/0.05 mm, $[\alpha]_D$ −35° (*c* 1, chloroform). Tosylation of alcohol 4 gave the starting sulphonate 3. Thus, it is apparent that no skeletal changes had occurred during the detosylation step.

ACKNOWLEDGMENTS

Professor E. Lederer is thanked for his constant encouragement. The work was aided by a grant from the Délégation Générale à la Recherche Scientifique et Technique auprès du Premier Ministre, no. 67-0192.

Institut de Chimie des Substances Naturelles,
C.N.R.S.,
91-Gif-sur-Yvette (France)

J. CLÉOPHAX
S. D. GÉRO
A. GAUDEMER
A. M. SÉPULCHRE

REFERENCES

1 Part XVII; part XVI "Déplacement et cyclisation dans la série furanosique", see J. Hildesheim, J. Cléophax, and S. D. Géro, *Compt. Rend.*, in press.
2 J. Cléophax, J. Hildesheim, and S. D. Géro, *Compt. Rend.*, 265 (1967) 257.
3 J. Cléophax, J. Hildesheim, and S. D. Géro, *Bull. Soc. Chim. France*, (1967) 4111.
4 J. C. Martin and P. D. Bartlett, *J. Amer. Chem. Soc.*, 79 (1957) 2533.
5 J. D. Hobson and W. D. Riddell, *Chem. Commun.*, (1968) 1180
6 A. K. Chatterjee, D. Horton, and J. S. Jewell, *Carbohyd. Res.*, 7 (1968) 212.

(Received November 25th, 1968)

Preliminary communication

Molecular-sieve chromatography with "Sepharose 4B"; the detection of artefacts of high molecular weight in acidic polysaccharides

Molecular-sieve chromatography (M-S.C.) with "Biogel P300" and "DEAE-Sephadex" has been used to estimate the molecular weights of some acidic polysaccharides and their degradation products[1-6] and to separate the components of polysaccharide mixtures[7]. Unfortunately, several polysaccharides were found[1,2,4] to have molecular weights higher than the exclusion limits of these cross-linked dextran and cross-linked polyacrylamide gels. However, spherical, porous silica beads, porous glass beads, and agarose gel beads (*e.g.*, "Sepharose"; Pharmacia, Uppsala) giving useful fractionation ranges for much higher molecular weights are now available. We report the chromatographic behaviour of nine acidic polysaccharides on "Sepharose 4B" (M_w fractionation range *ca.* 3–30 × 10^5).

Specimens of the gum polysaccharides from *Acacia arabica*[2], *A. campylacantha*[6], *A. laeta*[4], *A. nilotica*[8], *A. nubica*[9], *A. pycnantha*[10], *A. senegal*[11] [samples *BB(a)* and *QN1*], and *A. seyal*[5] were available; they had been prepared from aqueous solutions of the native gum by filtration, dialysis, and freeze-drying, and had been stored for different periods of up to 1 year in the essentially neutral, salt form, not the free-acid form.

Of these specimens, all except *A. pycnantha* gum gave distinct elution patterns involving 2 peaks, the first (corresponding to material of very high molecular weight) being at the void volume (V_o) of the column. The relative proportions of the two peaks, and the elution volume (V_e) of the component of lower molecular weight, were reproducible for each individual specimen. The gums from *A. seyal, A. nubica,* and *A. campylacantha* gave small peaks at V_o; *A. arabica, A. nilotica,* and *A. senegal* gave much larger peaks at V_o [the *QN1* sample of *A. senegal* gum had a higher, relative proportion of the peak at V_o than the *BB(a)* sample, which has[11] a lower intrinsic viscosity]. For *A. laeta* gum, the major peak in the elution pattern was at V_o; this species is known[12] to be unusual in forming mucilaginous gels if solutions are allowed to stand for several days. The single peak given by *A. pycnantha* gum, which has[13] an unusually low M_w for an *Acacia* exudate, was at virtually the same elution volume (V_i) as sucrose; "Sepharose 4B" is therefore an ineffective molecular sieve for this particular species.

These results appeared to give clear evidence that *Acacia* polysaccharides may be polydisperse[14]; recent studies[1-5] were interpreted on the assumption, based largely on the absence of marked discontinuities in experiments involving electrophoresis and ion-exchange chromatography, that the polysaccharides were polymolecular[14]. It had, however, been noted previously[11,12,15] that *Acacia* exudates that had been stored for periods of up to a year in the freeze-dried form tended either to be insoluble or to give much more-viscous solutions than had been given, for the same concentration, immediately after freeze-drying. The apparently polydisperse specimen of *A. senegal QN1* gum had indeed been isolated about 1 year previously; in contrast, a fresh preparation of this gum did not give a peak at V_0 when examined immediately after freeze-drying.

The possibility that the component of apparent high molecular weight at V_0 was an artefact, arising either from the purification or freeze-drying stages, or from some molecular aggregation process during storage in the freeze-dried form, was therefore investigated. Fresh solutions of each of the gums involved were therefore prepared direct from the natural exudates; the solutions were filtered and dialysed, but not freeze-dried. Although *A. pycnantha* gum gave an elution pattern identical with that observed previously, each of the other eight samples now gave only 1 peak which was, moreover, at the same value of V_e observed for the component of lower molecular weight in the polydisperse samples.

These values of V_e on "Sepharose 4B" clearly indicated the relative order of molecular size found independently in light-scattering measurements, *e.g.*, *A. nilotica*[13], M_w 2.27 × 10^6, V_e 76 ml; *A. arabica*[16], M_w 1.87 × 10^6, V_e 80 ml; *A. nubica*[16], M_w 0.87 × 10^6, V_e 90 ml; *A. seyal*[5], M_w 0.85 × 10^6, V_e 90 ml; *A. laeta*[13], M_w 0.72 × 10^6, V_e 95 ml; *A. senegal QN1*[17], M_w 0.60 × 10^6, V_e 100 ml; *A. campylacantha*[6], M_w 0.30 × 10^6, V_e 110 ml; *A. pycnantha*[13], M_w 0.06 × 10^6, V_e 120 ml.

In the ultracentrifuge, each of the fresh gum solutions gave symmetric boundaries. In contrast, solutions of the aged, freeze-dried specimens of *A. arabica, A. nilotica,* and *A. senegal* gums each gave asymmetric boundaries; for *A. laeta* gum, separation into two components was achieved.

When examined[18] on "Bio-Glas 500", a purified sample of *A. arabica* gum gave a shoulder at V_0 in addition to the major peak; *Citrus limonia* gum behaved similarly. On "Bio-Gel P300", several freeze-dried *Araucaria* exudates gave[19] a major peak in the useful fractionation range of that gel, but some samples showed a second peak at or near V_0.

Genera other than *Acacia* may also therefore be prone to molecular aggregation in purified or freeze-dried forms. Although it is already clear that molecular weight determinations and M-S.C. studies of acidic polysaccharides should always be made on freshly purified material, the phenomenon of apparent molecular aggregation demands further investigation.

EXPERIMENTAL

Molecular-sieve chromatography was carried out on columns (1.5 × 45 cm) of "Sepharose 4B" with M sodium chloride as eluant. To prevent "wall effects", the glass columns were pretreated with 1% dichlorodimethylsilane in benzene at 60° and then packed with the pre-swollen gel. Eluant was allowed to flow for 2 days to remove the bacteriostatic agent, sodium azide. The column was calibrated in terms of V_0 ("Blue dextran 2000", component of high molecular weight) and V_i (sucrose). Polysaccharide (10 mg) dissolved in 1.5M sodium chloride (1 ml) was applied to the column by layering beneath the M sodium chloride. Fractions, collected from a 2-ml syphon by an automatic collector, were screened by the phenol–sulphuric acid method. Elution volumes (V_e) were estimated to the nearest ml from the peak maxima.

Ultracentrifugation was carried out (through the courtesy of Professor R. Brown. F.R.S.) by Mr. R. Hart, using a Beckman Model E Ultracentrifuge at 20,410 r.p.m. Boundaries were detected by using Schlieren optics.

Chemistry Department,
The University,
Edinburgh 9 (Great Britain)

D. M. W. ANDERSON
I. C. M. DEA
A. C. MUNRO

REFERENCES

1 D. M. W. Anderson, Sir Edmund Hirst and J. F. Stoddart, *J. Chem. Soc. (C)*, (1966) 1959.
2 D. M. W. Anderson, Sir Edmund Hirst and J. F. Stoddart, *J. Chem. Soc. (C)*, (1967) 1476.
3 D. M. W. Anderson and I. C. M. Dea, *Carbohyd. Res.*, 6 (1968) 104.
4 D. M. W. Anderson, I. C. M. Dea, and R. N. Smith, *Carbohyd. Res.*, 7 (1968) 320.
5 D. M. W. Anderson, I. C. M. Dea and Sir Edmund Hirst, *Carbohyd. Res.*, 8 (1968) 460.
6 A. C. Munro, unpublished results.
7 S. A. Barker, S. M. Bick, J. S. Brimacombe and P. J. Somers, *Carbohyd. Res.*, 1 (1965) 393.
8 D. M. W. Anderson and R. A. Karamalla, *Carbohyd. Res.*, 2 (1966) 403.
9 D. M. W. Anderson and G. M. Cree, *Carbohyd. Res.*, 6 (1968) 385.
10 D. M. W. Anderson and G. M. Cree, *Carbohyd. Res.*, 6 (1968) 214.
11 D. M. W. Anderson, I. C. M. Dea, K. A. Karamalla and J. F. Smith, *Carbohyd. Res.*, 6 (1968) 97.
12 D. M. W. Anderson and R. N. Smith, *Carbohyd. Res.*, 4 (1967) 55.
13 D. M. W. Anderson and I. C. M. Dea, *Carbohyd. Res.*, accepted.
14 D. M. W. Anderson and J. F. Stoddart, *Carbohyd. Res.*, 2 (1966) 104.
15 D. M. W. Anderson and I. C. M. Dea, *Carbohyd. Res.*, 5 (1967) 461.
16 D. M. W. Anderson and S. Rahman, *Carbohyd. Res.*, 4 (1967) 298.
17 D. M. W. Anderson, Sir Edmund Hirst, S. Rahman and G. Stainsby, *Carbohyd. Res.*, 3 (1967) 308.
18 J. F. Stoddart and J. K. N. Jones, *Carbohyd. Res.*, 8 (1968) 29.
19 D. M. W. Anderson and A. C. Munro, *Carbohyd. Res.*, submitted.

(Received December 23rd, 1968)

Preliminary communication

Occurrence of glucofuranose residues in sialoglycolipids from *Echinodermata*

We have reported[1,2] the isolation, from gonads of the sea urchin *Strongylocentrotus intermedius,* of a group of closely related sphingoglycolipids containing sialic acid. These compounds are ceramide–oligoaldosides containing oligosaccharide chains of up to five sugar residues[2], of which glucose is the sole, neutral monosaccharide, as shown by paper chromatography of acid hydrolysates (2N sulphuric acid, 100°, 2 h) and g.l.c. of the trimethylsilylated[3] products of methanolysis (0.5N methanolic hydrogen chloride, 80°, 24 h).

The periodate-oxidation data for these sialoglycolipids indicate that, at least, a proportion of the glucose residues are in the furanose form. The mixture of glycolipids was oxidized with 0.01M sodium periodate for 24 h at 4°, followed by reduction with sodium borohydride and methanolysis. The product mixture was trimethylsilylated and subjected to g.l.c. which revealed the presence of equal amounts of glucose and xylose. Removal of the trimethylsilyl residues (80% methanol, 50°, 1 h) afforded methyl glucoside and methyl xyloside, identified by t.l.c. (silica gel, chloroform–methanol–water, 6:4:1, detection with orcinol–sulphuric acid). Acid hydrolysis (2N sulphuric acid, 80°, 2 h) of the mixture of methyl glycosides gave rise to glucose and xylose, identified by paper chromatography (butyl alcohol–pyridine–water, 6:4:3, detection with aniline hydrogen phthalate). The formation of xylose in this manner from the glycolipids can be attributed to the presence of glucofuranose residues having free hydroxyl groups at C-5 and C-6.

The presence of glucofuranose residues also explains the lability of the sialoglycolipids towards mild, acid hydrolysis[2]. Thus, the treatment of these glycolipids with 0.01N sulphuric acid for 1 h at 80° results not only in removal of sialic acid residues (as is usual for gangliosides of mammalia) but also in extensive cleavage of the oligosaccharide chain, leading to the formation of a ceramide–monoglucoside.

Similar results, indicative of the presence of glucofuranose residues, have been obtained with sialoglycolipids isolated from tissues of another representative of the *Echinodermata,* namely, the starfish *Patiria pectinifera.* Following the application in sequence of periodate oxidation, borohydride reduction, and methanolysis, xylose was detected, although the original sialoglycolipids contained only glucose, galactose, and arabinose as neutral monosaccharides.

The occurrence of glucofuranose residues in sialoglycolipids from *Echinodermata* is uncommon, since, hitherto, the glucose residues of glycolipids of animal, vegetable, and bacterial origin have been shown to have the pyranose structure.

Institute for Organic Chemistry,
Academy of Sciences,
Moscow (U.S.S.R.)

I. G. ZHUKOVA
G. P. SMIRNOVA

REFERENCES

1 N. K. Kochetkov, I. G. Zhukova, G. P. Smirnova and V. E. Vaskovsky, *Dokl. Akad. Nauk SSSR*, 177 (1967) 1472.
2 N. K. Kochetkov, I. G. Zhukova and G. P. Smirnova, *Dokl. Akad. Nauk SSSR*, 180 (1968) 996.
3 C. C. Sweeley, R. Bentley, M. Makita and W. W. Wells, *J. Amer. Chem. Soc.*, 85 (1963) 2497.

(Received January 11th, 1969)

Carbohydrate Research
Elsevier Publishing Company Amsterdam
Printed in Belgium

FRACTIONATION OF DEXTRAN AND HYALURONIC ACID ON POROUS SILICA BEADS*

S A BARKER, B W HATT, J B MARSTERS, AND P J SOMERS
Department of Chemistry, The University, Birmingham 15 (Great Britain)

(Received September 27th, 1968)

ABSTRACT

The evaluation of porous silica beads in the fractionation of polysaccharides in aqueous solution by molecular size is described Beads having pore sizes in two different ranges have been calibrated for dextran, and another pore-size range has been calibrated for hyaluronic acid The effect of sample concentration, flow rate, and temperature on the elution curves is examined, and the distribution of molecular weight for a dextran sample determined The advantages of porous silica beads over an organic molecular-sieve material, due to their rigidity and chemical inertness, are discussed

INTRODUCTION

Molecular-sieve chromatography provides a technique for the separation of solutes on a basis of molecular size, both on an analytical and a preparative scale The calibration of a chromatographic column of a molecular sieve with the appropriate standards may lead directly to a close estimation of the average molecular weight ($\overline{M}_w$ or $\overline{M}_n$) of a polymolecular sample and, in particular, to an evaluation of the distribution of molecular weight

In the past few years, much work has been published on the use of organic-based, molecular-sieve materials in the fractionation of biological compounds With their recent commercial availability, porous glass and silica beads having pore sizes in well-defined ranges appear to offer certain advantages over other sieve materials in the routine fractionation and analysis of biochemical polymers

The present study shows the evaluation and calibration of porous silica beads in the fractionation of dextran and hyaluronic acid in aqueous solution

MATERIALS AND METHODS

Porous silica beads (Porasil) were obtained from Waters Associates (Instruments) Ltd (Stockport, Cheshire, Great Britain) Porasil Types C (20–40 nm), D (40–80 nm), and E (80–150 nm) of quoted 75–100 mesh range were employed

Fractionated dextran samples, having a defined range of molecular weight, were obtained from Pharmacia (Uppsala, Sweden)

---

*Dedicated to Professor Stanley Peat, F R S

Hyaluronic acid, prepared from human umbilical cords, was obtained from Koch–Light Laboratories (Colnbrook, Bucks), Batch No 35944.

Preparation of columns — Before packing, an aqueous suspension of the Porasil beads was thoroughly deaerated under diminished pressure Jacketed, glass columns (140 5 × 0 8 cm, measured volume 70 6 cm^3; or 148.0 × 1 1 cm, measured volume 136.0 cm^3) were packed with the appropriate Porasil beads in water, the bed being allowed to settle under a flow rate of 20 $ml/h/cm^2$. The column was gently vibrated during packing until a stabilised bed was obtained

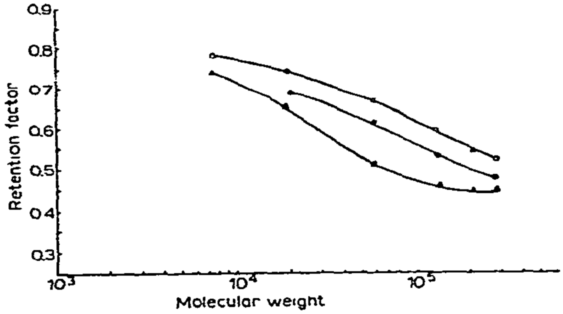
Analytical system — Column eluates were continuously monitored for total hexose content by the automated cysteine–sulphuric acid reaction[1] with Technicon modular AutoAnalyzer components

Hyaluronic acid in eluate fractions was determined by the carbazole–sulphuric acid reaction[2] for hexuronic acids

EXPERIMENTAL AND RESULTS

Calibration with dextran samples — A column of Porasil-D (140 5 × 0 8 cm) was eluted with deaerated water (25°) at a flow rate of 84 $ml/h/cm^2$, maintained with a peristaltic pump Aqueous solutions (0 2 ml) of Dextran-10→500 samples (nominal $\bar{M}_w$ 10→500 × 10^3, 3.0 mg/ml) and D-glucose (3 0 mg/ml, 0 2 ml) were applied to the column without interruption of the flow. Similar series of experiments were performed with a second batch of Porasil-D (148 0 × 1 1 cm) at flow rates of 6 7 and 84 $ml/h/cm^2$, and with a column of Porasil-C (140 8 × 0 8 cm) at a flow rate of 84 $ml/h/cm^2$

From the elution profile of each sample, the elution volume corresponding to elution of half the sample by weight was obtained. The retention factor (elution volume/total bed-volume) was plotted against the molecular weight corresponding to 50% by weight on the cumulative weight curve for the whole sample (obtained from Pharmacia) on a logarithmic scale (Fig. 1)



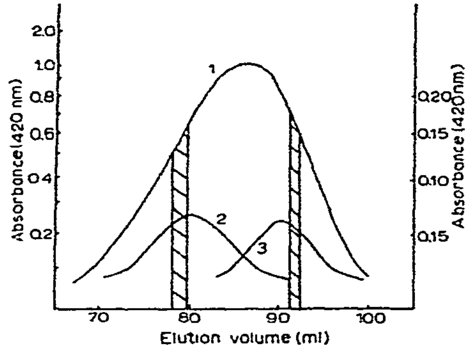


Fig 1 Calibration of dextrans on Porasil-D and Porasil-C —O—, Porasil-D, first batch, —●—, Porasil-D, second batch, —▲—, Porasil-C

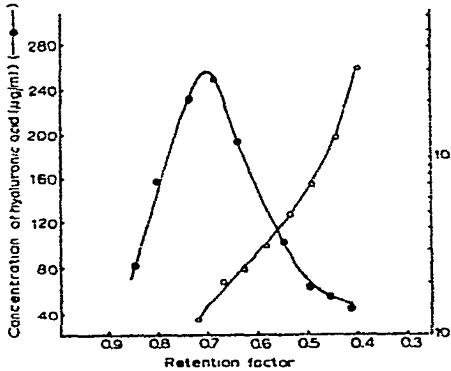
Fig 2 Fractionation of Dextran-80 on Porasil-D and refractionation of two portions of eluate 1, Original fractionation of Dextran-80 2, Fractionation of fraction 77 9–79 5 ml 3, Fractionation of fraction 90 9–92 0 ml

A solution of Dextran-80 (6.0 mg/ml; 0.2 ml) was applied to a column of Porasil-D (148.0 × 1.1 cm) and eluted at 6.7 ml/h/cm^2. The eluates corresponding to elution volumes of 77.9 to 79.5 ml and 90.9 to 92.0 ml were collected and, after concentration to 0.2 ml, separately reapplied to the column and eluted under identical conditions. The elution profiles obtained are shown in Fig. 2.

Calibration with hyaluronic acid. — Hyaluronic acid (13.9 mg) was dissolved in aqueous sodium chloride (0.2M, 4.0 ml), and ovine testicular hyaluronidase (1 mg, Sigma Chemical Company Ltd., Type II, 300 NF units/mg) was added. During incubation at 37°, aliquots (0.4 ml) were removed after 0, 2, 4, 6, 8, 12, 14, 17, and 25 min, and the enzymic activity was terminated by immersion in a boiling-water bath for 1 min. The samples obtained were remixed to give a solution containing a wide range of molecular weights.

Aliquots (4 × 0.9 ml) were repetitively fractionated with aqueous sodium chloride (0.2M) on a column (81.6 × 0.8 cm) of Porasil-E, and fractions (0.94 ml) were collected. Corresponding fractions from the four separations were combined, and the hexuronic acid content was determined by the carbazole–sulphuric acid[1] method.

The intrinsic viscosity of the fractions was obtained with a suspended-level viscometer (flow time for sodium chloride, 0.2M, 34.51 ± 0.03 sec; capacity 3.0 ml) at 20°. Sequential dilutions with aqueous sodium chloride (0.2M, 0.1, 0.4, 0.5, 1.0, 1.0, and 3.0 ml) were employed to enable extrapolation to zero concentration. The concentration of hyaluronic acid was redetermined by the carbazole–sulphuric acid[2] reaction. A plot of the retention factor against log $\overline{M}_n$ is shown in Fig. 3.



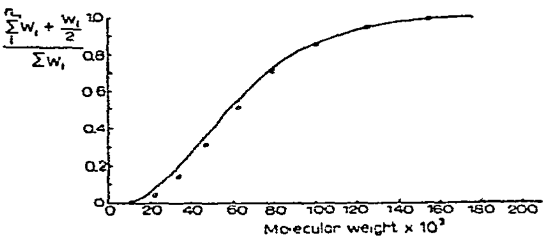


Fig. 3. Fractionation and calibration of hyaluronic acid on Porasil-E

Fig. 4. Molecular weight distribution of Dextran-80 using Porasil-D
———, Pharmacia, cumulative distribution of molecular weight ○, Cumulative distribution of molecular weight from fractionation on Porasil-D

Effect of flow rate, temperature, and solute concentration on fractionation. — Solutions of Dextran-80 (3.0 mg/ml, 0.8 ml) were applied to a column (140.5 × 0.8 cm) of Porasil-D and eluted at flow rates of 132, 84, and 60 ml/h/cm^2. A similar experiment was performed with solutions of Dextran-80 (0.53–2.86 mg/ml, 0.8 ml) at a flow rate of 132 and 60 ml/h/cm^2 with the same column.

Solutions of Dextran-80 (2 86 mg/ml, 0 2 ml) were applied to a column (148 0 × 1 1 cm) of Porasil-D, eluted at 6 7 ml/h/cm^2, with a jacket temperature of 25 and 75°.

The results obtained are shown in Table I, no effect of change in dextran concentration on peak elution-volume occurred in the range studied.

TABLE I

EFFECT OF FLOW RATE AND TEMPERATURE ON THE FRACTIONATION OF DEXTRAN-80 ON PORASIL-D

Flow rate (ml/h/cm^2)	*Column*[a]	*Temperature (degrees)*	*Retention factor of excluded peak*	*Retention factor of fractionated peak*	*"Broadness" (ml)*
132	A	25	0 483	0 674	13 2
84	A	25	0 479	0 674	12 1
60	A	25	0 481	0 667	11 2
6 7	B	25	0 415	0 628	14 4
6 7	B	75	0 471	0 598	13 2

[a]Column A first batch of Porasil-D, 140 5 × 0 8 cm, column B second batch of Porasil-D' 148 0 × 1 1 cm

DISCUSSION

Determination of the distribution of molecular weight for a chemically homogeneous species by a molecular-sieve technique requires calibration of the column with that polymer. This calibration involves individual determinations of molecular weight on samples of the polymer having a narrow distribution of molecular weight These fractions may be prepared by an independent method, and their elution volumes from the column measured Alternatively, they may be prepared by fractionation on the column of a sample having a broad distribution of molecular weight, and the molecular-weight determinations may be performed on the eluate fractions The former method was used to calibrate Porasil-C and Porasil-D for dextran, and the latter was used in the calibration of Porasil-E for hyaluronic acid

The Pharmacia-fractionated samples of dextran had been obtained from *Leuconostoc mesenteroides* B512 and had 5–10% of non-(1→6)-linkages The heterogeneity ($\bar{M}_w/\bar{M}_n$) of the samples varied from 1 3 to 2 0 Berger and Schultz[3] have calculated the theoretical elution curves for linear polymers and have shown that, for the molecular weight values of the peaks, $\bar{M}_n < \bar{M}_{gpc}{}^* \leqslant \bar{M}_w$ To avoid the assumption that $\bar{M}_{gpc} = \bar{M}_w$, the molecular weight of a species eluted at a volume corresponding to elution of half the sample by weight was equated with the molecular weight at 50% on the Pharmacia plot of cumulative weight for that sample In fact, for many of the samples, the peak values and the half-elution volumes were the same.

Many authors have reported that plots of the logarithm of molecular weight against elution volume (or functions of it) give an S-shaped curve, the central portions

---

*$\bar{M}_{gpc}$, molecular weight determined by gel-permeation chromatography

of which are essentially linear. This has been given a theoretical basis by Anderson and Stoddart[4] in terms of the Stokes radius of the molecules, Fig. 1 shows these plots made for the fractionation of dextran on Porasil-C and Porasil-D. The difference in the two calibrations of Porasil-D is due either to variations in packing or to variations in the two batches used

Comparison of the elution curves of Dextran-80 when the sample concentration was increased up to 3.0 mg/ml showed negligible change in peak shape. Thus, the concentration value above which "viscous fingering" may occur had not been reached. The effect of flow rate and temperature on elution volume and peak shape were investigated, and the results are shown in Table I "Broadness", defined as width at half-peak height, was taken as a convenient parameter. The peak elution volumes of excluded and fractionated material showed little variation with a flow rate up to 132 ml/h/cm^2. However, the "broadness" decreased significantly with decreasing flow-rate, suggesting that low flow-rates should be used for the accurate determination of molecular weight distributions, but that higher flow-rates may be used to obtain $\overline{M}_w$ or $\overline{M}_n$ of a sample, according to the type of assay used. The effect of temperature on the elution volumes of both the excluded and fractionated material is significant At 75°, the two peaks move 40% closer together, and the "broadness" of the fractionated peak is reduced from 14.4 to 13.2 ml.

Analysis of the elution peak of Dextran-80 fractionated at 6 7 ml/h/cm^2 (25°) was calculated as a cumulative percentage by weight against molecular weight, and Fig. 4 shows this curve and the calibration curve for the sample supplied by Pharmacia.

The validity of the analysis of the whole elution curve was checked by re-running cuts from either side of the peak The peaks of the re-run fractions moved slightly towards the peak side of the original distribution, as shown in Fig 2 The statistical distribution of species between the mobile and stationary phases results in cuts, receiving more material from the peak side than from the low side and, together with longitudinal diffusion, will result in a broadening of the re-run fraction and a slight shift of its position towards the original peak The "broadness" of the re-run fractions increased from 1 to 4 ml, but their elution curves are still narrow compared with the original distribution

During elution, the broadening of the zone of a single solute species, due to its statistical distribution, leads to a method of determining the height equivalent of a theoretical plate (HETP) for the column In ideal conditions, the elution curve approximates to a Gaussian curve, non-ideal conditions due to too high a flow rate and non-ideal packing cause a further broadening of the zone and a skewing of the elution curve Glueckauf[5] derived a formula relating the number of theoretical plates (N) to the width (β) at 1/e of the maximum peak height for an ideal, Gaussian elution curve of a single solute species, as follows

$$N = 8[(V_e)^2/\beta],$$

where V_e is the peak elution volume The HETP is given by the ratio of the column length (l) and the number of theoretical plates (N); HETP $= l/N$

This treatment was applied to the elution curve of D-glucose on a column of Porasil-D. D-Glucose, which may be regarded as the monomer unit of dextran, gave for the column a HETP of 0.65 cm and a reduced plate height (HETP/av. diameter of particle) of 3.9. These values are of the same order as those determined by de Vries and co-workers[6] for benzene in tetrahydrofuran on Porasil-C.

The calibration of Porasil-E for hyaluronic acid required a sample having a broad distribution of molecular weight. This was prepared artificially by degrading hyaluronic acid of high molecular weight to different extents and combining the resulting samples. The limiting or intrinsic viscosity of the eluant fractions was measured. The Mark–Houwink[7] modification of Staudinger's original equation[8] relates the intrinsic viscosity (η_i) of a solute species to a function of its molecular weight (M):

$$\eta_i = KM^a,$$

where K and a are constants for the species. Laurent[9] has shown that for hyaluronic acid this equation becomes

$$\eta_i = 0.36 \times M^{0.78}$$

Thus, the viscosity-average molecular weight ($\bar{M}_\eta$) for each fraction was calculated, and Fig. 3 shows the plot of log $\bar{M}_\eta$ against retention factor as well as the original elution curve.

It is clear that molecular-sieve columns of porous silica beads may be used in the rapid determination of the average molecular weights of samples of polysaccharides and in the calculation of the molecular weight distribution of such species. The assay used with the dextran determinations led directly to $\bar{M}_w$, but if an end-group assay were used the $\bar{M}_n$ of a sample would be given.

The porous silica "gel" has definite advantages over organic gels due to its inorganic nature. Once such a column has been calibrated, it may be used indefinitely as a routine analytical and preparative tool in the fractionation of polymers according to molecular size. There is no possibility of sample contamination due to bacteriological or chemical degradation of the bed, and the column may be cleaned *in situ* with, for example, hot nitric acid. The rigidity of the beads results in a constant, total bed-volume, even when the column is operated at high back-pressures, due either to high flow-rates or fine-mesh particles.

ACKNOWLEDGMENTS

The authors thank Professor M. Stacey, C.B.E., F.R.S., for his interest in this work. They also gratefully acknowledge financial support from the Science Research Council, Ranks Hovis McDougall (Research) Ltd. (to B. W. H.), and the Birmingham (Dudley Road) Group of Hospitals (to J. B. M.).

REFERENCES

1 Z. DISCHE, L. B. SHETTLES, AND M. OSNOS, *Arch. Biochem.*, 22 (1949) 169.
2 T. BITTER AND H. M. MUIR, *Anal. Biochem.*, 4 (1962) 330.

3 H L Berger and A R Shultz, *J Polymer Sci*, A3 (1965) 3643
4 D M W Anderson and J F. Stoddart, *Anal Chim. Acta*, 34 (1966) 401
5 E Glueckauf, *Ion Exchange and its Applications*, Soc Chem Ind, London, 1955, p 34
6 A J de Vries, Madeleine Le Page, R. Beau, and C L Guillemin, *Anal Chem*, 39 (1967) 935
7 R Houwink, *J Prakt Chem*, 157 (1940) 15
8 H. Staudinger and W Hueur, *Ber*, 63 (1930) 222; H Staudinger and R Nodzu, *ibid*, 63 (1930) 721.
9 T C Laurent, M Ryan, and A Pietruszkiewicz, *Biochim Biophys Acta*, 42 (1960) 476

Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

METABOLISM OF THE RESERVE POLYSACCHARIDE OF *Streptococcus mitis*

PROPERTIES OF MALTODEXTRIN PHOSPHORYLASE*

Gwen J. Walker, Margaret C. Lukas, and Alexandra Lavrova
Institute of Dental Research, 2 Chalmers Street, Sydney (Australia 2010)

(Received September 17th, 1968)

ABSTRACT

A maltodextrin phosphorylase found in cell extracts of several strains of *Streptococcus mitis* has been purified and freed from several other enzymes concerned with glycogen synthesis and degradation. Maltodextrins were the best primers for the synthetic reaction; glycogen was a poor primer for this phosphorylase. Further indications that the enzyme was not a glycogen phosphorylase were (*i*) its lack of binding with glycogen, (*ii*) the high Km value for glycogen which was 130-fold higher than that of muscle phosphorylase, (*iii*) the low rate of phosphorolysis of glycogen, and (*iv*) the low limit of conversion of glycogen into D-glucopyranosyl phosphate. A second phosphorylase was found with particulate glycogen in the pellet obtained on centrifuging sonicated cells. This was considered to be the enzyme involved in the degradation of glycogen.

INTRODUCTION

Many of the enzymes found in micro-organisms that store glycogen are similar to those used by plants and animals for the synthesis and degradation of starch or glycogen. The enzymes concerned have analogous functions and may be distinguished from each other mainly by differences in their specificity. For instance, muscle phosphorylase and potato P-enzyme degrade glycogen and amylopectin, respectively, in reactions that are chemically identical, yet the plant enzyme has little[1] or no action[2] on glycogen.

There have been few studies on bacterial phosphorylases. Cell extracts of *Streptococcus bovis*[3] and *Arthrobacter sp.*[4] were shown to contain phosphorylases that degraded soluble starch, and phosphorylase activity in an extract of *Agrobacterium tumefaciens*[5] was measured by the incorporation of D-glucose-^{14}C from D-glucopyranosyl phosphate into glycogen. A detailed study was made of the maltodextrin phosphorylase[6] of *E. coli*, and evidence was produced of a glycogen phosphorylase[7] in *E. coli*.

---

*Dedicated to Professor Stanley Peat, F.R.S.

Streptococcus mitis can store intracellular, iodophilic polysaccharide when the cells are provided with excess of D-glucose[8,9], and catabolism of this polysaccharide, which provides energy in a utilizable form[10], would be expected to occur by means of a phosphorolytic mechanism accompanied by the hydrolysis of α-(1$\rightarrow$6)-D-glucosidic, branch linkages Two enzymes of *S mitis* that can hydrolyse the branch linkages of glycogen and its degradation products have been isolated[11,12]. In the present work, the properties of a purified phosphorylase isolated from cell extracts of *S mitis* are described. In view of its greater reactivity towards maltodextrins than toward glycogen, the enzyme is termed a maltodextrin phosphorylase The possibility is discussed that this enzyme may provide an amylose-like precursor as substrate for branching enzyme, which could then synthesize a primer suitable for glycogen synthetase Such a synthetic activity for phosphorylase would recall the concept of pseudo-amylose, introduced by Peat and co-workers[13] to describe the course of synthesis of amylopectin by P-enzyme and Q-enzyme Frydman and Cardini[14] have recently published evidence that phosphorylase may be involved in the first stages of starch synthesis through the formation of an amylose-like precursor which Q-enzyme could act on to form a branched polysaccharide. An intracellular polysaccharide of this type was isolated from tuber slices, and was found to be an excellent primer for starch synthetase

MATERIALS AND METHODS

Carbohydrates — Waxy-maize starch, amylose, and maltodextrins were prepared as described by Walker[9] Soluble starch, oyster glycogen, and dextrine were purchased from B D H Ltd , U K , Calbiochem , Los Angeles, and T Kerfoot, Lancashire; respectively D-Glucopyranosyl phosphate was isolated from the products of the action of potato juice on starch, and contained no primer for phosphorylase *S. mitis* glycogen was isolated from strain S3 grown in Gibbons and Kapsimalis[8] (GK) medium (20 l) containing 1% of D-glucose The inoculated medium was incubated anaerobically (N_2+CO_2, 95 5) for 18 h at 39° The cells were collected in a Sharples Super-Centrifuge, washed twice with 0.067M phosphate buffer (pH 7.1), resuspended in water, and freeze-dried (yield 6 5 g, 32 5% glycogen) Crude glycogen was extracted by the method of Palmstierna[15], but the precipitations with acetic acid were omitted, and glycogen was purified by four precipitations with 60% ethanol The purity of the final product was found to be 95% by the cysteine–H_2SO_4 assay[16] and also by reducing-sugar estimation[17] after hydrolysis in 1 5N H_2SO_4 for 2 h at 100°. Rabbit-liver glycogen was a gift from Professor D J Manners

Enzymes. — Rabbit-muscle phosphorylase[18], potato phosphorylase[19], and pullulanase[20] from *Aerobacter aerogenes* were prepared by literature methods.

Cell extracts — The dialysed cell-extracts were prepared as described by Walker[12]. The extent of carbohydrate storage that occurs in *S mitis* depends on the strain used, and on the amount of D-glucose added to the broth Four of the strains studied by Walker[9] were able to store polysaccharide when D-glucose was present in excess, and two strains were never found to contain reserve polysaccharide. In the present work, the three representative strains chosen for study were S3 and 439, which store the most and least polysaccharide, respectively, and strain RB1633 which is unable to store polysaccharide. Unless stated otherwise, the strains were grown in the presence of 0.5% of D-glucose.

Phosphorylase activity was assayed in digests (0.8 ml) that contained 0.125M D-glucopyranosyl phosphate (0.2 ml), 1% soluble starch or alternative primer (0.2 ml), 0.05M citrate buffer (pH 6.7, 0.16 ml), 10mM copper sulphate (0.04 ml), and cell extract or purified phosphorylase (0.20 ml). The digests, and controls without added primer, were incubated at 30°, and portions (0.2 ml) were withdrawn at 0, 30, and 60 min into 4% trichloroacetic acid (0.3 ml). Water (1 ml) was added, the protein was removed by centrifuging, and inorganic phosphate in the supernatant solution (1.0 ml) was determined[21]. The results were expressed as μg of P released in the activity digest. Copper sulphate completely inhibited the phosphatase and phosphoglucomutase of cell extracts and had no effect on phosphorylase activity. When purified phosphorylase was assayed, both copper ions and the trichloroacetic acid precipitation were omitted. There was a linear relationship between enzyme concentration and release of inorganic phosphate.

Isolation of purified phosphorylase from cell extracts. — All operations were carried out at 1–2°. *Step 1.* A cell extract (10 ml) was dialysed overnight against 0.05M citrate buffer (pH 6.7), and then saturated ammonium sulphate (pH 7) was added to give 45% saturation. The precipitate, which contained pullulanase, was removed by centrifuging for 10 min at 10,000 *g*, and the supernatant solution was treated with ammonium sulphate to give 60% saturation. The precipitate, which contained most of the phosphorylase activity and none of the phosphatase or phosphoglucomutase activity of the original extract, was dissolved in 0.05M citrate buffer (1.5 ml).

Step 2. A Pharmacia column (83 × 1.5 cm) was packed with Bio-Gel P-150 (10 g), and washed with 3 vol. of 0.05M citrate buffer (pH 6.7). The sample was applied, and the column was eluted with the same buffer at a flow rate of 10 ml/h. The fractions (2 ml) were tested for phosphorylase and α-(1→6)-D-glucosidase activity. Most of the phosphorylase was found in two tubes (11 and 12), α-(1→6)-D-glucosidase, which was retarded on the column, appeared in tubes 15–25. Step 2 was omitted in the case of strain RB1633, which does not contain α-(1→6)-D-glucosidase.

Step 3. The fractions containing phosphorylase were dialysed for 1 h against a buffer solution containing 0.01M sodium-β-glycerophosphate, 0.001M EDTA, and 0.1M NaCl, and then applied to a DEAE-Sephadex (A-50) column (1 × 25 cm) that had been washed with the same buffer. The column was eluted with increasing concentrations of NaCl (see Fig. 1) at a flow rate of 10 ml/h. The mixing chamber contained 250 ml of buffer, and the reservoir contained 0.6M NaCl dissolved in the same 0.01M glycerophosphate–0.001M EDTA buffer at pH 6.7 (500 ml). The fractions (6 ml) were collected into tubes containing 0.05M citrate buffer (pH 6.7, 0.5 ml). In this way, phosphorylase was deprived of the stabilising effect of citrate for the shortest possible time. It was not practicable to elute DEAE-Sephadex columns with citrate buffer, because the maximum flow-rate possible was only 10 ml/day. Phosphorylases from strains S3, 439, and RB1633 were eluted with 0.35, 0.38, and 0.28M NaCl, respectively, while the corresponding transglucosylases were eluted with 0.30, 0.41, and 0.40M NaCl. Thus, it was necessary to rechromatograph the fractions of S3 and 439 that contained peak phosphorylase activity in order to remove traces of transglucosylase. Branching enzyme in all cases was eluted between 0.20 and 0.28M NaCl, and only the fractions of phosphorylase from strain RB1633 which were completely free from branching enzyme were pooled for use. Finally, the appropriate fractions were dialyzed overnight against 0.05M citrate buffer (pH 6.7).

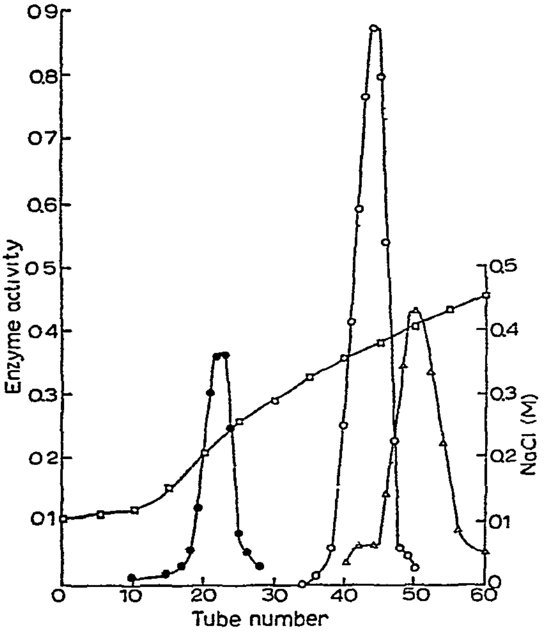


Fig. 1. Purification of phosphorylase from *S. mitis* strain 439. Step 3. Chromatography on a DEAE-Sephadex column with NaCl gradient (□). Separation of phosphorylase (○) from branching enzyme (●) and transglucosylase (△). Branching enzyme[39] was determined in transglucosylase activity digests with D-glucose omitted.

The increase in specific activity of the enzyme obtained after Steps 1, 2, and 3 was 8, 21, and 108, respectively. Losses of up to one-third of the original activity occurred in Step 1. There were no further losses in Steps 2 or 3, but approximately half of the fractions containing phosphorylase activity were discarded after Step 3 owing to the presence of traces of transglucosylase in the case of strains S3 and 439 and of branching enzyme in strain RB1633.

RESULTS

Phosphorylase activity of cell extracts. — Phosphorylase activity in dialysed cell-extracts of several strains of *S. mitis* was determined in activity digests containing oyster glycogen as primer. Glycogen primer was chosen at this stage because the phosphorylase was thought to be concerned in the breakdown of reserve glycogen. The bacteria were cultivated in trypticase soy broth, a medium in which growth occurs when D-glucose is omitted, thus affording an opportunity to test for induction of phosphorylase in the presence of D-glucose No effect of added D-glucose was observed (Table I) When the bacteria were divided into two groups according to their ability to store intracellular polysaccharide, it was found that phosphorylase activities in cell extracts of the two groups were widely different The group containing little or no polysaccharide (LP) comprised all the strains having low phosphorylase activity, whereas the high polysaccharide (HP) group had specific activities for phosphorylase which were on average 6 times higher than those of the LP group. The strains of *S mitis* in the HP group contained high phosphorylase activity even when grown under conditions where no storage of polysaccharide was possible

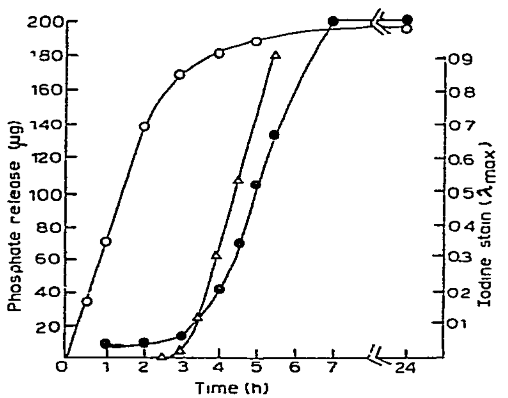
TABLE I

PHOSPHORYLASE ACTIVITY IN CELL EXTRACTS OF SEVERAL STRAINS OF *S mitis* WITH GLYCOGEN PRIMER

Group	*Strain*	*Score for polysaccharide content of cells grown in excess* D-*glucose*	*Score for presence of pullulanase in cell extracts*	D-*Glucose in broth* (%)	*Phosphorylase activity* µg of *P/h*	*Activity per mg of N*
LP	RB1633	—	—	0 5	13	16 6
LP	RB1633	—	+++	0	13	75 2
LP	FW251	—	+++	0 5	1 9	8 2
LP	FW251	—	+++	0	15	133
HP	439	+	+	0 5	175	277
HP	439	+	++	0	90	299
HP	FW225	++	—	0	61	493
HP	FW213	+++	—	0	89	403
HP	S3	++++	—	0 5	256	433
HP	S3	++++	—	0	87	390

Effect of pullulanase on determination of phosphorylase activity. — Cell extracts of *S mitis* strains RB1633, FW251, and 439 contain pullulanase[12], an enzyme that hydrolyses the α-(1→6)-D-glucosidic branch-linkages of glycogen[22]. The linear maltodextrins released in this reaction were found to have higher priming ability than glycogen for phosphorylase in cell extract (see later) Therefore, the phosphorylase activities shown in Table I for several members of the LP group could clearly be too high

The first evidence of the effect of pullulanase on the determination of phosphorylase activity was the release of phosphate, with concomitant synthesis of iodine-staining polysaccharide, that occurred in control digests containing cell extract and D-glucopyranosyl phosphate. In strain RB1633, this effect occurred after a lag period (Fig. 2), during which the action of pullulanase on branched polysaccharide in the cell extract was producing a suitable primer for phosphorylase Thus, although strain RB1633 is in the LP group, the cells must contain a minimal amount of branched polysaccharide The weight of total carbohydrate in the cell extract was determined to be less than 1% of the weight of glycogen primer added to activity digests. There was more carbohydrate in cell extracts of strain 439 (HP group), and the lag period was less than 30 min The effect was not observed with cell extracts of strains FW251, 439, or RB1633 grown in the absence of D-glucose Synthesis by phosphorylase occurred in control digests only when the cell extract contained pullulanase and branched polysaccharide



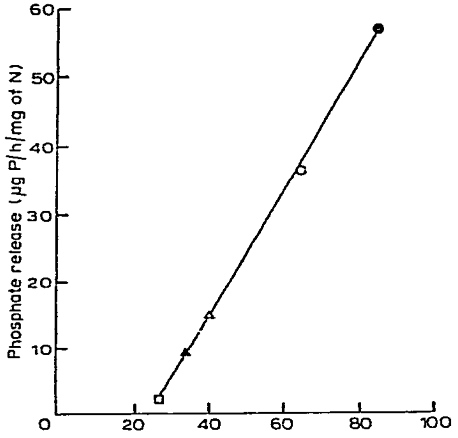


Fig 2 Phosphorylase activity of a cell extract of *S mitis* strain RB1633 grown in GK medium containing 0 5% of D-glucose Phosphate release in activity digest (○) with glycogen primer, and in control digest (●) without added primer Portions (0 1 ml) of the control digest (△) were stained with iodine [0 1 ml of a solution that contained iodine (2 g/l) and KI (20 g/l)] and diluted to 1 ml

Fig 3 Synthesis by phosphorylase in control digests without added primer. Extracts of *S mitis* strain 439, made from cells harvested after 12 h (●), 16 h (○), 20 h (△), 24 h (▲), and 36 h (□) growth in GK medium, were incubated with D-glucopyranosyl phosphate Branched polysaccharide in the cell extracts was determined by the cysteine–H_2SO_4 method[16]

The dependence of polysaccharide synthesis in control digests on the presence of branched polysaccharide is shown in Fig 3 When dialysed cell-extracts of strain 439 containing equal pullulanase and phosphorylase activities were incubated with D-glucopyranosyl phosphate, the extent of synthesis was proportional to the amount of branched polysaccharide in the cell extract.

Since the presence of pullulanase had such a marked effect on polysaccharide synthesis in control digests when small amounts of branched polysaccharide were present, a similar effect was expected in activity digests containing glycogen primer This was tested by pre-incubating glycogen (8 mg) with pullulanase (0 2 ml of FW251 cell extract) at pH 6 5. After 2 h at 30°, the solution was boiled, and its efficiency as a primer was compared with that of normal glycogen in phosphorylase activity digests with S3 cell extract The pullulanase-treated glycogen was the better primer by a factor of 2 Longer incubation with pullulanase raised this factor to 6

These observations led to two conclusions Firstly, the true phosphorylase activity of the LP group was likely to be only half of that recorded in Table I. Secondly, less highly branched polysaccharides were better primers than glycogen for *S mitis* phosphorylase.

Relative priming efficiency of polysaccharides. — Several polysaccharides were tested in activity digests that contained 2 mg of primer (Table II) Maltotetraose was the most efficient of the primers tested, and was given a score of 100 in each case. During the purification of phosphorylase from strain 439, the relative activity towards branched polysaccharides fell, so that glycogen became a very poor primer for phosphorylase. The removal of pullulanase activity from the phosphorylase preparation could account for this; there was no evidence that a second phosphorylase having a special affinity for glycogen had been separated during the purification procedures The results in Table II indicated a similarity between *S mitis* phosphorylase and potato phosphorylase, the bacterial enzyme was clearly different from the glycogen phosphorylase of muscle

TABLE II

COMPARISON OF PRIMER SPECIFICITIES OF VARIOUS PHOSPHORYLASES

Primer	*Cell extract Strain 439*	*Purified* S mitis *phosphorylase*			*Potato phosphorylase*	*Muscle phosphorylase*
		439	*S3*	*RB1633*		
Maltotetraose	100	100	100	100	100	100
Soluble starch	37	28	32	34	43	500
Amylopectin	23	12	15	21	39	160
Glycogen	13	3	5	6	21	950

Chen and Segel[23] found that maltose induced a 10-fold increase in phosphorylase activity of an *E coli* K-12 cell extract towards dextrin, but only a 2-fold increase when glycogen was used as substrate These results suggested the presence of two phosphorylases having different primer specificities The phosphorylase activities of *S mitis* cell extracts (strains S3 and FW213) with maltohexaose as primer were increased by only 50% when maltose or maltotriose replaced glucose in the growth medium An increase of the same order was noted in digests containing glycogen primer

Adsorption on polysaccharides — The adsorption of *S. mitis* phosphorylase on retrograded amylose was tested in a similar manner to that described for potato phosphorylase[24]. A cell extract of strain RB1633 (3 ml) was treated successively with 0.03, 0 135, and 0 10 g of amylose, the ethanol concentration being kept at 25% (v/v) Adsorption did not occur, and all of the phosphorylase activity was recovered ~~from the supernatant solution~~

De la Haba[25] showed that muscle phosphorylase was strongly bound to potato-starch granules, and use was made of this property in the isolation of pure phosphorylase from a crude, muscle extract. When portions (1 5 ml) of a cell extract of strain RB1633 were shaken for 1 h at 0° with 15 mg of potato, maize, and waxy-maize starch granules, respectively, no phosphorylase activity was adsorbed on the granules

Madsen and Cori[26] measured the binding of muscle phosphorylase with corn glycogen, and the binding of muscle phosphorylase to rat-liver glycogen has also been demonstrated[27-29] Barber *et al* [30] found that the distribution of phosphorylase in rat-liver homogenates after zonal centrifugation in sucrose density-gradients was similar to that of glycogen A cell extract of *S mitis* strain S3 grown on 1% of D-glucose, which contained 15 6 mg of reserve polysaccharide per ml, was centrifuged at 105,000 *g* for 1 h at 2°. Although this procedure sedimented over 90% of the polysaccharide, all of the phosphorylase activity remained in the supernatant solution A portion of the solution (1 5 ml) was then stirred overnight at 2° with a solution (5%, 1.5 ml) of oyster glycogen The mixture was centrifuged at 105,000 *g* for 1 h, but again enzyme was not brought down with the glycogen

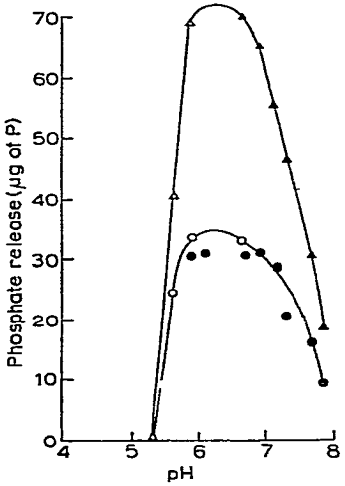
In a second experiment, an extract of strain S3, grown on 0 1% of D-glucose, and containing 0 38 mg of polysaccharide per ml, was centrifuged at 105,000 *g* for 1 h, and a portion (3 ml) of the supernatant solution, which retained all of the phosphorylase activity, was stirred overnight at 2° with a solution (2%, 1 ml) of *S mitis* glycogen The glycogen was then sedimented at 105,000 *g*, and once more all of the phosphorylase was found in the supernatant solution

Selinger and Schramm[31] have emphasized that the amount of insoluble complex formed when muscle phosphorylase interacts with soluble glycogen is dependent on the phosphorylase/glycogen ratio in the system. A cell extract of strain S3 was therefore passed through a column packed with Sephadex G-200, and the carbohydrate content was thereby reduced to 6 μg/ml Solutions (0 3 ml) of oyster glycogen were added to portions of this enzyme solution (2 25 ml) to give final concentrations of 0.02, 0.10, 0 50, 1.0, and 2 5% The mixtures were stirred overnight at 2°, and, after centrifugation, the phosphorylase activities in the residues and supernatant solutions were determined Phosphorylase was fully recovered in the supernatant solution in each case.

These results, which show that *S mitis* phosphorylase does not bind to amylose, starch granules, or glycogen, apply only to the enzyme in cell extracts A phosphorylase was also found in association with the sediment obtained on centrifuging disrupted cells Cell suspensions of strain S3 grown on 1% of D-glucose (2 l) were sonicated, and then centrifuged at 27,000 *g*. The sediment, which gave a strong glycogen-type stain with iodine, was washed with 0 05M citrate buffer (30 ml) and resuspended in the same buffer (4 ml), and a portion (0 25 ml) was tested for phosphorylase in the usual activity digest containing glycogen primer. The release of inorganic phosphate was 267 μg of P/h. This value remained the same when glycogen primer was omitted, since the weight of glycogen added with the suspension (4 mg) was an excess.

It was not possible to solubilize phosphorylase by washing the sediment with 0 05M citrate buffer or with *S mitis* glycogen solution (2%) at 30°, and this strong binding of the phosphorylase to 'particulate glycogen' suggested that the enzyme was different from the phosphorylase in the cell extract. A further indication that two separate phosphorylases were present in *S. mitis* was provided by the effect of adding maltohexaose (2 mg) to an activity digest that already contained glycogen primer (10 mg). Maltohexaose caused a 10-fold increase in release of inorganic phosphate by purified phosphorylase from a cell extract of strain S3, but the maltodextrin had no effect on the activity of the phosphorylase that was with 'particulate glycogen'. In strain S3, phosphorylase activity associated with the sediment obtained on centrifuging sonicated cells accounted for 30% of the combined activity of the sediment plus cell extract, when calculated with glycogen as primer Disrupted cells of FW251 (LP group) had little phosphorylase activity in the sediment.

Effects of various conditions on phosphorylase activity — All of the experiments that follow were carried out with purified phosphorylase isolated from cell extracts Enzyme from strain RB1633 was incubated with D-glucopyranosyl phosphate and maltotetraose at various pH values. The pH optimum was 6.0–7 0 (Fig 4) The effect of temperature on the reaction was studied between 16° and 50° The optimum temperature was 40–50° (Fig 5), but 30° was chosen for experiments with phosphorylase



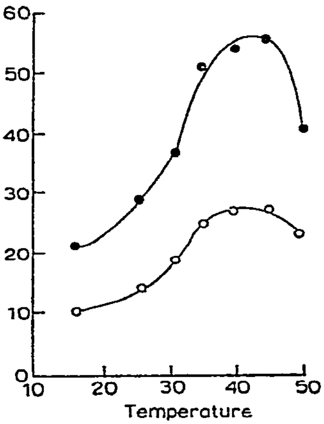


Fig 4 Effect of pH on phosphorylase activity The digests were incubated at 40° for 30 min (○) and 60 min (△) The open symbols refer to citrate buffer, and the closed symbols to Tris–citrate buffer.

Fig 5 Effect of temperature on phosphorylase activity The digests were incubated at pH 6 5 for 15 min (○) and 30 min (●)

The enzyme was completely stable at 2°, provided it had been dialysed against 0 05M citrate. After freezing and thawing, 70% of the original activity was retained. Phosphorylase in glycerophosphate–EDTA buffer lost 66% of its activity in 1 month at 2°, and the activity could not be restored by incubating with cysteine, glutathione, or mercaptoethanol. Freezing the enzyme in glycerophosphate–EDTA led to complete loss of phosphorylase activity. The concentration of citrate added to all incubation mixtures to obtain full phosphorylase activity was 7 mM. Phosphorylase activity remained constant when the citrate concentration was further raised to 20 mM.

Addition of pyridoxal phosphate (5×10^{-4}M) or adenosine 5′-phosphate (1×10^{-3}M) to activity digests containing soluble starch as primer did not increase phosphorylase activity. The enzyme was not inhibited by α-Schardinger dextrin.

Kinetic properties. — The priming ability of several maltodextrins was tested in phosphorylase activity digests containing various weights of acceptor. The Km values for the acceptors (Table III) indicated that the priming efficiency of maltodextrins increased with chain length. Maltose did not prime the reaction, but phosphate released in a maltotriose digest after incubation for 24 h was similar to that in a maltotetraose digest after 1 h.

TABLE III

Km VALUES FOR VARIOUS PHOSPHORYLASES

Substrate	S. mitis *RB1633* (mM)	S. mitis *439* (mM)	*Potato* (mM)	*Muscle* (mM)
G 1-P[a]	1.1	—	2.6[b]	1.7[e]
Maltoheptaose	—	1.4	—	—
Maltohexaose	1.1	1.8	0.66[b]	—
Maltopentaose	2.0	2.3	—	—
Maltotetraose	—	5.4	—	—
	g/l	*g/l*	*g/l*	*g/l*
Glycogen	—	27.8	1.38[c]	0.20[f]
Soluble starch	—	15.4	0.97[b]	—
Amylopectin	—	14.3	0.13[d]	—
Dextrine	2.3	—	—	—

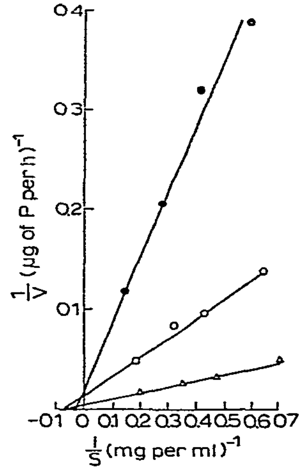
[a]D-Glucopyranosyl phosphate. [b]Data from ref. 33. [c]Data from ref. 35. [d]Data from ref. 32. [e]Data from ref. 36. [f]Data from ref. 34.

The Km value for maltohexaose (1.1×10^{-3}M) was the same order as that (0.66×10^{-3}M) reported[33] for potato phosphorylase, and that (0.5×10^{-3}M) for the maltodextrin phosphorylase[6] of *E. coli*.

Lineweaver–Burk plots for branched polysaccharides and for maltodextrins are shown in Fig. 6 and Fig. 7, respectively. The Km values for the polysaccharides were 100-fold greater than those shown in Table III for muscle and potato phosphorylases. These results provide additional evidence that *S. mitis* phosphorylase has low affinity for branched polysaccharides.

Inhibition by nucleoside diphosphate sugars. — The glycogen phosphorylase of *Agrobacterium tumefaciens* is competitively inhibited[36] by uridine 5-(D-glucopyranosyl dihydrogen pyrophosphate) (UDPG), the Ki being 0.67×10^{-3}M. Chen and Segel[37] similarly found that the glycogen phosphorylase of *E. coli* was inhibited by both UDPG and adenosine 5-(D-glucopyranosyl dihydrogen pyrophosphate) (ADPG). The

Ki values were $2\ 0 \times 10^{-3}$M and $0\ 7 \times 10^{-3}$M, respectively. *S mitis* phosphorylase was also competitively inhibited by UDPG (Ki = $1\ 5 \times 10^{-3}$M, strain RB1633), and by ADPG (Ki = $2\ 8 \times 10^{-3}$M, strain S3) Thus, the presence of nucleoside diphosphate sugar in actively growing cells supplied with excess of D-glucose would tend to prevent the slow degradation of branched polysaccharide that could otherwise occur.



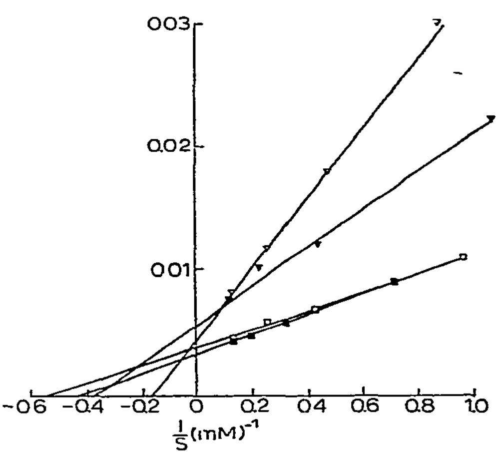


Fig 6 Lineweaver–Burk plots for *S mitis* glycogen (●), waxy-maize starch (○), and soluble starch (△) Purified phosphorylase (0 133 ml) from strain 439 was incubated with D-glucopyranosyl phosphate and various weights of primer in the usual activity digest (0 8ml)

Fig 7 Lineweaver–Burk plots for maltotetraose (▽), maltopentaose (▼), maltohexaose (■), and maltoheptaose (□) Purified phosphorylase (0 133 ml) from strain 439 was incubated with D-glucopyranosyl phosphate and various weights of maltodextrin primer in the usual activity digest (0 8 ml)

Degradation of branched polysaccharides — Phosphorolysis digests (2 5 ml) contained polysaccharide (10 mg), phosphate buffer (pH 6 5) (0 4 mmole), and enzyme (0 4 ml) Adenosine 5′-phosphate (1 mM) was also added to muscle phosphorylase digests D-Glucopyranosyl phosphate was determined after removal of excess phosphate as magnesium ammonium phosphate *S mitis* phosphorylase degraded waxy-maize starch to the same extent (44%) as muscle phosphorylase (Fig 8), but *S mitis* glycogen proved to be more resistant to phosphorolysis by the bacterial enzyme than by muscle phosphorylase. The reaction became very slow after the conversion into D-glucopyranosyl phosphate reached 15%. Addition of more enzyme at intervals slowly increased the extent of degradation, but a true limit was difficult to obtain Potato phosphorylase also degraded glycogen very slowly

The limit dextrin prepared by the action of *S mitis* phosphorylase on amylopectin was incubated overnight with *Aerobacter aerogenes* pullulanase, and the products of the reaction were examined by paper chromatography The paper was developed for 50 h in ethyl acetate–pyridine–water (10 4 3, by vol), and then dipped in $AgNO_3$–NaOH solution[38] The α-(1→6)-D-glucosidic branch linkage had been hydrolysed to give mainly maltotetraose, with smaller amounts of higher maltodextrins, and traces of maltose and maltotriose. The action of *S. mitis* phosphorylase on amylopectin therefore terminated when the A chains were 4 units long.

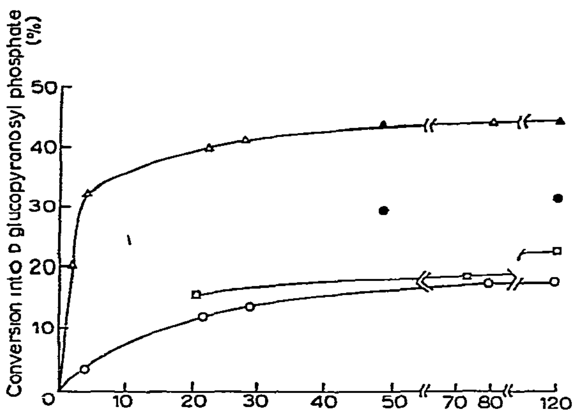


Fig. 8. Phosphorolysis of *S. mitis* glycogen (○, ●), rabbit-liver glycogen (□), and waxy-maize starch (△, ▲). The open symbols refer to purified phosphorylase from *S. mitis* strain RB1633, and the closed symbols to rabbit muscle phosphorylase. After 25 h and 80 h, more enzyme (0.25 ml) was added to each digest.

Rate of phosphorolysis. — A linear production of D-glucopyranosyl phosphate over 30 min was obtained in the digests described above, when the enzyme concentration was reduced to one-quarter. The conversion into D-glucopyranosyl phosphate of soluble starch, waxy-maize starch, *S. mitis* glycogen, rabbit-liver glycogen, and oyster glycogen were 3.09, 2.56, 1.23, 0.92, and 0.80%, respectively, in 30 min. Although these results indicate the relative rate of action on amylopectin and *S. mitis* glycogen to be 2:1, this initial rate was not long maintained for glycogen, whereas the reaction with amylopectin rapidly reached a true limit (Fig. 8).

DISCUSSION

The maltodextrin phosphorylase of *S. mitis* described in this communication has few features in common with the well-studied mammalian muscle phosphorylase. The bacterial enzyme was not activated by AMP, and glycogen was a poor substrate both in the direction of synthesis and of degradation. The enzyme resembled potato phosphorylase with respect to the high affinity for maltodextrins, but the Km values for glycogen and amylopectin were 20- and 100-fold higher, respectively, for *S. mitis* phosphorylase than for the plant enzyme. The properties of *S. mitis* maltodextrin phosphorylase were similar to those of the maltodextrin phosphorylase of *E. coli*. Both enzymes had a high affinity for maltodextrins and a low affinity for glycogen.

Chen and Segel[37] found both maltodextrin phosphorylase and glycogen phosphorylase in extracts of early log-phase cells of *E. coli*. Only maltodextrin phosphorylase was found in cell extracts of *S. mitis*. The bacteria were harvested at a stage when the cells contained up to one-third of their dry weight of glycogen, and preliminary results indicated that strains in the HP group contained a glycogen phosphorylase found exclusively in the sediment obtained on centrifuging disrupted cells

Schwartz and Hofnung[6] suggested that maltodextrin phosphorylase, which is an inducible enzyme in *E coli*, was concerned in the intermediary metabolism of maltose The function of the enzyme was to catalyse the phosphorolysis of the maltodextrins synthesized by amylomaltase. Two other functions for *S. mitis* maltodextrin phosphorylase may be envisaged. First, the enzyme could rapidly degrade glycogen when acting simultaneously with pullulanase Such a mechanism for degradation might occur in strains that were devoid of glycogen phosphorylase.

The second possible function of maltodextrin phosphorylase could be the synthesis of long-chain maltodextrins from molecules of primer provided by *S mitis* transglucosylase Such an amylose-type chain containing 20 or more D-glucosyl residues is a substrate for *S mitis* branching enzyme[39], and the branched polysaccharide thus formed would be a suitable primer for the glycogen synthetase[40] of *S mitis*. A synthetic activity of this kind was proposed for potato phosphorylase[13] before the discovery of the granule-bound and soluble starch synthetases[41-43], which led to a complete change in the conception of starch biosynthesis Thereafter, the pathway of starch synthesis was based on nucleoside diphosphate glucose, and the role of phosphorylase was held to be purely degradative

Nevertheless, there remained the problem of providing a suitable primer for starch synthetase, and, at present, the concept of synthesis by phosphorylase is being reconsidered In their search for a soluble polysaccharide intermediate in starch synthesis, Frydman and Cardini[14] found a polysaccharide that was probably synthesised from D-glucopyranosyl phosphate by potato phosphorylase Tsai and Nelson[44] found that one of the two phosphorylases isolated from the endosperm of *Zea mays* appeared only at the stage of rapid biosynthesis of starch, and was not found when the endosperm was being mobilised during seed germination Present indications are that the isolation from bacteria and plants of two or more phosphorylases having different functions will become a common occurrence

In recent reports of polysaccharide synthesis by potato[45,46] and muscle[47,48] phosphorylase in absence of primer, evidence of *de novo* synthesis has not been conclusive, because the incubation mixtures contained traces of primer introduced either with D-glucopyranosyl phosphate[49] or with the enzyme[50]. Kamogawa *et al* [50] observed no release of inorganic phosphate when charcoal-treated D-glucopyranosyl phosphate was incubated with glucoamylase-treated potato phosphorylase. Addition of α-amylase to this system resulted, nevertheless, in a rapid release of phosphate The effect of pullulanase on the activity of *S mitis* maltodextrin phosphorylase in control digests without added primer was somewhat similar The synthesis of polysaccharide that occurred when a cell extract of strain RB1633 was incubated with D-glucopyranosyl phosphate is shown in Fig 2 When changes were made in the medium of growth of the bacteria, which resulted in (*a*) absence of pullulanase or (*b*) absence of branched polysaccharide in the cell extracts, no synthesis occurred. This was therefore an example of a system in which "*de novo*" synthesis occurred not directly because of primer in D-glucopyranosyl phosphate or in phosphorylase, but because of the presence of a hydrolytic enzyme that converted a few micrograms of ineffective primer into a larger number of molecules of efficient primer

ACKNOWLEDGMENTS

This investigation was supported by grants from the National Health and Medical Research Council of Australia, and the Dental Board of New South Wales

REFERENCES

1 A M Liddle, D J Manners, and A Wright, *Biochem J*, 80 (1961) 304
2 W J Whelan and G J Walker, unpublished data
3 R N Ushijima and R H McBee, *Proc Montana Acad Sci*, 17 (1957) 33
4 L P T M Zevenhuizen, *Mededel Landbouwhogeschool, Wageningen*, Nederland, 66-10 (1966)
5 N B Madsen, *Biochim Biophys Acta*, 50 (1961) 194
6 M Schwartz and M Hofnung, *European J Biochem*, 2 (1967) 132
7 G Chen and I H Segel, *Bacteriol Proc Amer Soc Microb*, P89 (1966) 85
8 R. J. Gibbons and B Kapsimalis, *Arch. Oral Biol*, 8 (1963) 319
9 G J Walker, *Biochem J*, 101 (1966) 861
10 R J Gibbons, *J Bacteriol*, 87 (1964) 1512
11 G J Walker and J E Builder, *Biochem J*, 105 (1967) 937
12 G J Walker, *Biochem J*, 108 (1968) 33
13 E J Bourne and S Peat, *J Chem Soc*, (1945) 877
14 R B Frydman and C E Cardini, *Plant Physiol*, 42 (1967) 628
15 H Palmstierna, *Acta Chem Scand*, 10 (1956) 567
16 Z. Dische, L B Shettles, and M Osnos, *Arch Biochem*, 22 (1949) 169.
17 N Nelson, *J Biol Chem*, 153 (1944) 375
18 G T Cori, B Illingworth, and P. J Keller, *Methods Enzymol*, 1 (1955) 200
19 W J Whelan, *Methods Enzymol*, 1 (1955) 192
20 H Bender and K. Wallenfels, *Biochem Z*, 334 (1961) 79
21 R J L Allen, *Biochem J*, 34 (1940) 858
22 M Abdullah, B J Catley, E Y C Lee, J Robyt, K Wallenfels, and W J Whelan, *Cereal Chem*, 43 (1966) 111
23 G S Chen and I H Segel, *Arch Biochem Biophys*, 127 (1968) 164
24 H Baum and G A Gilbert, *Nature*, 171 (1953) 983
25 G De La Haba, *Biochim Biophys Acta*, 59 (1962) 672
26 N B Madsen and C F Cori, *J Biol Chem*, 233 (1958) 1251
27 E W. Sutherland and W D Wosilait, *J Biol Chem*, 218 (1956) 459
28 J R Tata, *Biochem J*, 90 (1964) 284
29 H -G Sie, A Hablanian, and W H Fishman, *Nature*, 201 (1964) 393
30 A A Barber, S A Orrell, and E Bueding, *J Biol Chem*, 242 (1967) 4040
31 Z Selinger and M Schramm, *Biochem Biophys Res Commun*, 12 (1963) 208
32 Y -P Lee, *Biochim Biophys Acta*, 43 (1960) 18
33 C. Weibull and A Tiselius, *Arkiv Kemi, Mineral Geol*, A19, No 19 (1945)
34 E Helmreich, S Karpatkin, and C F. Cori, *Control of Glycogen Metabolism, Ciba Found Symp*, 1964, p 211
35 D S Feingold, E F. Neufeld, and W Z Hassid, *Modern Methods of Plant Analysis*, 7 (1964) 474
36 N B Madsen, *Biochem Biophys Res Commun*, 6 (1961) 310
37 G. S Chen and I H Segel, *Arch Biochem Biophys*, 127 (1968) 175
38 W E Trevelyan, D. P Procter, and J S Harrison, *Nature*, 166 (1950) 444
39 G. J. Walker, to be published
40 J E Builder and G J Walker, to be published

41 L F LELOIR, M A R DE FEKETE, AND C E CARDINI, *J Biol Chem*, 236 (1961) 636
42 R B FRYDMAN AND C E CARDINI, *Arch Biochem Biophys*, 116 (1966) 9
43 R B FRYDMAN AND C E CARDINI, *J Biol Chem*, 242 (1967) 312
44 C Y TSAI AND O E NELSON, *Plant Physiol*, 43 (1968) 103
45 D E. GREEN AND P K STUMPF, *J Biol Chem*, 142 (1942) 355
46 J WATKINS, D. E NORMANSELL, AND G A GILBERT, *Nature*, 207 (1965) 857
47 B ILLINGWORTH, D H. BROWN, AND C F. CORI, *Proc Natl Acad Sci U S*, 47 (1961) 469
48 D H BROWN, B ILLINGWORTH, AND C F. CORI, *Proc. Natl Acad Sci U S*, 47 (1961) 479
49 M ABDULLAH, E H FISCHER, M Y QURESHI, K N SLESSOR, AND W J WHELAN, *Biochem J*, 97 (1965) 9P
50 A KAMOGAWA, T FUKUI, AND Z NIKUNI, *J Biochem.* (Tokyo), 63 (1968) 361

Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

SULPHATES OF MONOSACCHARIDES AND DERIVATIVES

PART VII[1]. SYNTHESIS OF SOME DISULPHATES AND A NEW SYNTHESIS OF D-GALACTOSE 4-SULPHATE*

M J HARRIS AND J R TURVEY
Department of Chemistry, University College of North Wales, Bangor, Caernarvonshire (Great Britain)

(Received September 26th, 1968)

ABSTRACT

Sulphation of a sugar derivative with the pyridine–sulphur trioxide reagent in pyridine solution has been shown to be an equilibrium reaction. For polysulphation to occur, it is necessary to use enough reagent to oversaturate the pyridine and to form a reagent-rich, second phase With these conditions, barium salts of a number of sugar and glycoside disulphates have been prepared by definitive routes. β-D-Galactose 4-sulphate and two D-galactoside 4-sulphates have been synthesized by an improved method.

INTRODUCTION

Although definitive syntheses of many monosaccharide monosulphates have been reported in the literature[1,2], only a few monosaccharide disulphates have been synthesized by strictly definitive routes The recognition that certain polysaccharides contain monosaccharide disulphates as constituent units[3] led us to attempt the synthesis of some monosaccharide and glycoside disulphates, as model compounds for a study of the chemical and physical properties of this class of compound. This paper describes the preparation of a range of these compounds and some important features of the reactions leading to their syntheses For comparison purposes, a stock of D-galactose 4-sulphate was also required, and a new route for its synthesis is included.

DISCUSSION

When a sugar derivative containing more than one free hydroxyl group is treated in pyridine solution with the reagent, pyridine–sulphur trioxide[4], a mixture of starting compound and mono- and poly-sulphates results In the past, in order to obtain polysulphation, it has been the practice[2] to use an excess of the sulphating reagent and prolonged reaction times at temperatures of 60–80°. From experience with the syntheses described here, it became apparent that the sulphation process is

---

*Dedicated to Professor Stanley Peat, F.R.S.

an equilibrium reaction, and that equilibrium is attained in less than 0 5 h at 60°, longer periods of reaction serve no useful purpose. Even with the axial C-4 hydroxyl group on a galactopyranose derivative, the sulphation of which has been reported to be difficult[5], it was possible to obtain complete sulphation in 0 5 h at 60° under suitable conditions

For disulphation to occur, it seems essential to have a high concentration of the sulphating reagent The reagent, however, is not very soluble in pyridine and, in a homogeneous, saturated solution, sulphation is rarely complete For instance, when 4,6-*O*-ethylidene-D-glucose was treated with the reagent (2 mol) in homogeneous pyridine solution, the preponderant product was the monosulphate An increase in the amount of reagent to 5 mol resulted in the separation of two phases, the lower phase containing essentially pure disulphate mixed with excess of the reagent. It is evident that only in this lower, reagent-rich phase can disulphation go to completion That the sulphation is an equilibrium reaction was demonstrated by treating methyl β-D-galactopyranoside 2,3-di-(barium sulphate) with the reagent in pyridine for 2 5 h at 60° The products of this reaction included monosulphates and trisulphates, in addition to unchanged disulphate

In order to follow these reactions, it became necessary to monitor the reactions at regular intervals For this purpose, paper electrophoresis has been used extensively but suffers from the apparent disadvantage that glycoside sulphates cannot readily be detected by conventional spray reagents However, with an acetic acid–pyridine buffer for paper electrophoresis, it was found that the migrated zones of sugar sulphates were readily detected by viewing in ultraviolet light, after drying the paper. Presumably, the sugar sulphates are present on the paper as the pyridinium salts, and this results in absorption of ultraviolet light in the region of the sulphates Where it was important to detect also non-sulphated glycosides, thin-layer electrophoresis on silica gel proved extremely useful, since the plate could then be developed with any of the highly acidic, spray reagents

The choice of blocking groups used in the syntheses was governed by the usual considerations Acetals were generally preferred, since they are quite stable under the conditions of sulphation but are readily removed selectively by acid hydrolysis Furthermore, with the disulphates containing cyclic acetals as blocking groups, it was found that these groups were more acid-labile than usual For example, benzylidene groups were completely hydrolysed in aqueous solution of pH 3 5 at room temperature in a few hours Thus, it proved impracticable to store many of the intermediate *O*-benzylidene disulphates, since they underwent an autocatalytic decomposition even in the solid state, the catalyst being benzoic acid produced by air oxidation of liberated benzaldehyde Isopropylidene groups were readily removed from disulphates by treatment in aqueous solution at pH 3 and 60° Benzyl ethers were also used as blocking groups but, as noted earlier[5], were resistant to hydrogenation when attached to a sulphated sugar, and were thus difficult to remove, except when present at the anomeric centre Thus, benzyl glycoside sulphates were converted into the free sugar sulphates by one treatment with hydrogen over palladium, whereas methyl 2,3-di-*O*-benzyl-α-D-glucopyranoside 4,6-di-(barium sulphate) required several, consecutive treatments with hydrogen, and even then removal of the benzyl ether groups was incomplete

There is a possibility that sulphate migration might have occurred during the hydrolytic removal of blocking groups, but this is unlikely for two reasons Firstly, as will be reported elsewhere, a study of the physical properties and periodate-oxidation patterns of the synthetic compounds is in full agreement with the assigned structures Secondly, sugar sulphates, when dissolved in dilute, aqueous acid, do not appear to undergo sulphate migration When D-glucose 3-, D-glucose 6-, D-galactose 4-, and D-galactose 6-(sodium sulphate) were separately heated at 100° in 0 33N sulphuric acid, the only detectable reaction was slow hydrolysis, no products of sulphate migration could be detected by paper chromatography These conditions are more acidic than any used in the removal of blocking groups

Table I lists the sugar and glycoside sulphates synthesized, together with the essential intermediates The majority of the sulphates were isolated as crystalline barium salts, and only a few of the decomposition points proved to be reproducible; for the most part, the decomposition point varied with the rate of heating (see Table I) Aqueous ethanol was generally useful for crystallisation of the disulphates and also for some barium salts of monosaccharide monosulphates previously synthesized in these laboratories

The barium salts of all the sulphates listed in Table I were soluble in water, but this solubility did not always extend to the intermediate sulphates having blocking groups still intact Methyl 4,6-*O*-benzylidene-α-D-glucopyranoside 2,3-di-(barium sulphate), 2,3-di-*O*-benzyl-D-glucose 4,6-di-(barium sulphate), and methyl 2,3-di-*O*-benzyl-α-D-glucopyranoside 4,6-di-(barium sulphate) were only sparingly soluble in water, but the corresponding sodium salts were all soluble

The synthesis of crystalline β-D-galactose 4-sulphate was based on a selective benzoylation procedure[6] The procedure worked well with the 2,3,6-tribenzoates of both methyl α- and benzyl β-D-galactopyranoside The yield of 4-sulphates obtained by these routes was very much better than that obtained by the previous route[5].

A detailed study of the physical properties of these sulphates and of their reactions with periodate will be given in a later communication

EXPERIMENTAL

General methods. — The methods for paper chromatography and paper electrophoresis in pyridine–acetic acid buffer have been described[7]. For thin-layer electrophoresis, plates (30 × 20 cm) were coated with a layer (0 5 mm) of Silica-gel G slurried in 0 1M acetic acid–pyridine buffer (pH 6). When the surface moisture had evaporated, leaving the layer still damp, spots of the unknown compounds were applied. the ends of the plates were connected by filter-paper wicks to electrode vessels, and a voltage gradient of *ca* 10 volts/cm applied for 3 h. The plates were then dried at 110° and sprayed with the reagent, prepared by dissolving *p*-anisidine

TABLE I

DATA FOR CERTAIN SUGAR SULPHATES

Compound synthesized	*Intermediate used*	*Dec temp (degrees)*[b]	$[\alpha]_D^{20}$ *in water (degrees)*
α-D-Glucose 2,3-di-(barium sulphate)	4,6-*O*-Benzylidene D glucose	194	+ 40.0 → +37.5
Methyl α-D-glucopyranoside 2,3-di-(barium sulphate)	Methyl 4,6-*O*-benzylidene-α-D-glucopyranoside	146	+ 61.5
α-D-Galactose 2,3 di (barium sulphate)	4,6-*O*-Benzylidene-D galactose	152	+ 66.0 → +50.0
Methyl α-D-galactopyranoside 2,3-di-(barium sulphate)	Methyl 4,6-*O*-benzylidene-α-D-galactopyranoside	132	+ 79
Methyl β-D-galactopyranoside 2,3 di-(barium sulphate)	Methyl 4,6-*O*-benzylidene β-D-galactopyranoside	146 (r)	+ 2.5
D-Galactose 2,6 di (barium sulphate)[a]	Benzyl 3,4-*O*-isopropylidene-β-D galactopyranoside	155 (r)	+ 40.0
Benzyl β-D galactopyranoside 2,6 di (barium sulphate)	Benzyl 3,4-*O*-isopropylidene-β-D galactopyranoside	195	− 15.5
Methyl α-D-galactopyranoside 2,6 di-(barium sulphate)[a]	Methyl 3,4-*O*-isopropylidene-α-D galactopyranoside	158	+ 79.0
D-Glucose 4,6-di (barium sulphate)	2,3-di-*O*-Benzyl D-glucose	148 (r)	+ 35.5
Methyl α-D-glucopyranoside 4,6-di-(barium sulphate)	Methyl 2,3-di-*O*-benzyl-α-D-glucopyranoside	200	+ 84
β-D-Galactose 4-(barium sulphate)	Benzyl 2,3,6 tri *O*-benzoyl β-D-galactopyranoside	189 (r)	+ 35.0 → +49.5
Benzyl β-D-galactopyranoside 4 (barium sulphate)[a]	Benzyl 2,3,6 tri-*O*-benzoyl β-D galactopyranoside	218	− 20.5
Methyl α-D-galactopyranoside 4-(barium sulphate)	Methyl 2,3,6 tri *O*-benzoyl-α D-galactopyranoside	214	+111.0

[a] Obtained only as amorphous salt [b] Not reproducible unless indicated by symbol (r)

hydrochloride (1 g) and conc sulphuric acid (5 ml) in butyl alcohol (100 ml) On warming at 110° for 10 min, all carbohydrates developed as brown zones

T l.c of sulphates was performed on Silica-gel G (1 part) and Kieselguhr G (1 part), activated for 1 h at 100°, and employed the solvent system (*a*) ethyl acetate–acetic acid–water (6 4 2) containing 2% (w/v) of cetylpyridinium chloride In this system, monosulphates have greater mobilities than disulphates, but the presence of blocking groups results in even greater mobilities It is thus possible to separate a glycoside sulphate from the parent sugar sulphate For non-sulphated sugar derivatives and for certain sulphated derivatives, activated Silica-gel G was used with the solvents (*b*) benzene–ethyl acetate (7 3) and (*c*) benzene–methanol–chloroform (3 3 7) After being dried, the sugar zones were developed with the *p*-anisidine–conc sulphuric acid spray-reagent

The general methods used in the sulphation reaction, in isolating the products after sulphation, and in their purification by cellulose-column chromatography have all been described[7] Barium salts were converted into sodium salts by percolation of the sugar sulphate through a column of ZeoKarb 225 (Na$^+$), followed by evaporation to dryness

*Sulphation of 4,6-O-ethylidene-*D*-glucose* — To 4,6-*O*-ethylidene-D-glucose[8] (160 mg) in pyridine (5 ml) was added pyridine–sulphur trioxide reagent (1 mol), and the mixture was heated at 60°. Similar mixtures were prepared with 2 and 5 mol of reagent After 1 h, water (5 ml) was added, and the products were examined by thin-layer electrophoresis Visual estimation of the products showed that, with 1 mol of reagent, approximately equal proportions of non-sulphated and monosulphated compound were formed With 2 mol of reagent, a second layer was just formed in the reaction mixture, and the products contained a small proportion of disulphate With 5 mol of reagent, two layers formed in the reaction mixture, and disulphation was complete

Reversibility of the sulphation reaction. — To pyridine–sulphur trioxide (0.08 g) in pyridine (2 5 ml) was added methyl β-D-galactopyranoside 2,3-di-(barium sulphate) (0 12 g), and the mixture was stirred for 2 5 h at 60° The products were isolated, and examined by paper electrophoresis Monosulphates and trisulphates were detected, in addition to the disulphate Incorporation of water (23 mg) in a similar reaction mixture did not alter the yield of each product

*α-*D*-Glucose 2,3-di-(barium sulphate)* — 4,6-*O*-Benzylidene-D-glucose[9] (20 g), m p 186–188°, $[\alpha]_D^{20}$ −3 0° (methanol), was treated with pyridine–sulphur trioxide reagent (40 g) in dry pyridine (600 ml) for 4 h at 65° The reaction mixture was monitored by paper electrophoresis, which showed that reaction had ceased after 0.5 h, and that monosulphate was the major product. Pyridine–sulphur trioxide (55 g) was then added, and, after 1 5 h at 65°, disulphation was virtually complete The product was recovered in the usual manner but could not be stored, since it spontaneously lost benzaldehyde, even in the solid state The disulphate (35 g) was suspended in water, and the pH adjusted to 3 5 with sulphuric acid At intervals, the pH was readjusted to 3 5 with dilute barium hydroxide until, after 6 h at room temperature, hydrolysis was complete After the pH had been adjusted to 7, the solution was filtered and evaporated at 35° to dryness (yield, 12 g) The product (6 g) was purified by cellulose-column chromatography to give D-glucose 2,3-di-(barium sulphate), which crystallised spontaneously from the column eluent The product was recrystallised from aqueous ethanol (yield, 2 8 g). A portion (100 mg) was converted into the sodium salt for analysis (Found S, 16 5. $C_6H_{10}Na_2O_{12}S_2$ calc S, 16 7%)*

Methyl α-D-glucopyranoside 2,3-di-(barium sulphate). — Methyl 4,6-*O*-benzylidene-α-D-glucopyranoside[10] (12 g) was treated with pyridine–sulphur trioxide (41 5 g) in pyridine (375 ml) After 2 h at 65°, examination showed that reaction was complete. Crystalline methyl 4,6-*O*-benzylidene-α-D-glucopyranoside 2,3-di-(barium sulphate) (12 6 g), $[\alpha]_D^{20}$ +25 5° (*c* 0 5, water) was isolated This product (7 g) was treated as above to remove the benzylidene group, and the product purified on a cellulose column Methyl α-D-glucopyranoside 2,3-di-(barium sulphate) (2 7 g) crystallised from concentrated, aqueous solution (Found Ba, 27 6 $C_7H_{12}BaO_{12}S_2$ calc Ba, 28 0%)

α-D-Galactose 2,3-di-(barium sulphate) — 4,6-*O*-Benzylidene-D-galactose[11] (12 g), m p 187–189°, $[\alpha]_D^{20}$ +113° (methanol), was treated with pyridine–sulphur trioxide (32 g) in pyridine (170 ml) for 2 h at 65° Paper electrophoresis indicated that disulphation was incomplete, and more reagent (17 g) was added. After 1 h at 65°, the upper layer of the reaction mixture was discarded, and the lower layer used for recovering the sulphated product T l c [solvent (*a*)] revealed partial hydrolysis of the benzylidene groups, and hydrolysis was completed by dissolving the product in aqueous solution at pH 3 5 The product was purified on a cellulose column and then crystallised from aqueous ethanol at 4° to yield α-D-galactose 2,3-di-(barium sulphate) (3.9 g), which was converted into the sodium salt (Found S, 16 5 $C_6H_{10}Na_2O_{12}S_2$ calc S, 16.7%) The barium salt was indistinguishable chromatographically from that synthesised previously[1].

Methyl α-D-galactopyranoside 2,3-di-(barium sulphate) — Methyl 4,6-*O*-benzylidene-α-D-galactopyranoside[12] (2 0 g) was treated with pyridine–sulphur trioxide (5 g) in pyridine (175 ml) for 2 5 h at 65°, and then further reagent (4 g) was added to effect complete disulphation After 1 h, the sulphated product was isolated, and recrystallised from ethanol–water The methyl 4,6-*O*-benzylidene-α-D-galactopyranoside 2,3-di-(barium sulphate) (4 1 g) had $[\alpha]_D^{20}$ +92 5° (*c* 0 4, water) A portion (1 8 g) was warmed to 60° in aqueous solution at pH 3 5 for 5 h; constant attention was paid to the pH. Paper electrophoresis indicated that debenzylidenation was then complete Evaporation of the solvent, after removal of barium sulphate, and slow crystallisation of the residue from aqueous ethanol gave methyl α-D-galacto-

---

*In this and subsequent cases where only a Ba or S analysis is given, the compounds were too hygroscopic for determination of C and H, good values for C were obtained, but the absorption of water between weighing and introduction of the sample into the automatic analyser invalidated the values for H pyranoside 2,3-di-(barium sulphate) (0.75 g) (For the sodium salt, found. S, 16.1. $C_7H_{12}Na_2O_{12}S_2$ calc S, 16.1%)

Methyl β-D-galactopyranoside 2,3-di-(barium sulphate) — Methyl 4,6-*O*-benzylidene-β-D-galactopyranoside[13] (4.5 g) was sulphated with pyridine–sulphur trioxide (18 g) in pyridine (175 ml) at 65° until electrophoretic examination showed that only disulphate was present. The product furnished crystalline methyl 4,6-*O*-benzylidene-β-D-galactopyranoside 2,3-di-(barium sulphate) (8.5 g), $[\alpha]_D^{20}$ +29.2° (*c* 1.5, water) This compound (6.8 g) was hydrolysed as described for the α-D-anomer and gave crystalline methyl β-D-galactopyranoside 2,3-di-(barium sulphate) (6.0 g) (For the sodium salt, found S, 16.1 $C_7H_{12}O_{12}S_2Na_2$ calc. S, 16.1%)

Benzyl β-D-galactopyranoside 2,6-di-(barium sulphate) and D-galactose 2,6-di-(barium sulphate) — Benzyl 3,4-*O*-isopropylidene-β-D-galactopyranoside[14] [15] (10 g), m.p 118°, $[\alpha]_D^{20}$ −1.6° (*c* 1.5, chloroform), was treated with pyridine–sulphur trioxide (35 g) in pyridine (300 ml) at 65°, and the reaction was monitored by paper electrophoresis After 0.5 h, disulphation was complete, and the product was recovered as a syrup in the usual manner Attempts to crystallise this syrup failed, due to constant gel formation in the aqueous ethanol used as solvent. T l c and paper electrophoresis showed that the product was homogeneous The product was dissolved in water (250 ml), the pH was adjusted to 3.1 with dilute sulphuric acid, and the solution was warmed for 5 h at 60° Examination of the product by paper electrophoresis and n m r spectroscopy showed that the isopropylidene group had been removed by this treatment The product was recovered, as described above, and crystallised slowly from warm, aqueous ethanol to give benzyl β-D-galactopyranoside 2,6-di-(barium sulphate) (12 g) as large, white crystals (Found Ba, 24.6. $C_{13}H_{16}BaO_{12}S_2$ calc Ba, 24.4%)

A solution of benzyl β-D-galactopyranoside 2,6-di-(barium sulphate) (6 g) in water (100 ml) was hydrogenated at 4 atmos over 5% palladium-on-carbon[16] At intervals, the pH was adjusted to 8 by the addition of barium hydroxide. After 6 h, the catalyst was removed, and the filtrate was concentrated Purification on a cellulose column gave amorphous D-galactose 2,6-di-(barium sulphate) (3.7 g) [For the sodium salt, found S, 17.0 $C_6H_{10}Na_2O_{12}S_2$ calc S, 16.7%]

Methyl α-D-galactopyranoside 2,6-di-(barium sulphate) — Syrupy methyl 3,4-*O*-isopropylidene-α-D-galactopyranoside[14] [16.3 g, homogeneous by t l c in solvents (*b*) and (*c*), and characterised as the crystalline di-*O*-*p*-tolylsulphonyl derivative[17], m p 150°] was sulphated with pyridine–sulphur trioxide (48 g) in pyridine (300 ml) for 3 h at 65° During the isolation of the product, acidity developed in the solution, with consequent hydrolysis of the isopropylidene group and slight desulphation The product was purified on a cellulose column to give amorphous methyl α-D-galactopyranoside 2,6-di-(barium sulphate) (7 g) (For the sodium salt, found: S, 16.0. $C_7H_{12}Na_2O_{12}S_2$ calc S, 16.1%)

D-*Glucose 4,6-di-(barium sulphate)*. — 2,3-Di-*O*-benzyl-D-glucose[18] (3.0 g) m p 120°, $[\alpha]_D^{20}$ +48° (ethanol), was treated with pyridine–sulphur trioxide (8.0 g) in pyridine (170 ml) for 45 min at 65° A further portion (5 g) of pyridine–sulphur trioxide was then added, and, after 30 min, disulphation was almost complete, as indicated by electrophoretic examination The product, 2,3-di-*O*-benzyl-D-glucose 4,6-di-(barium sulphate) (1.6 g), crystallised from aqueous solution but slowly decomposed on storage A portion (1 g) was suspended in 50% aqueous ethanol and hydrogenated at 5 atmos over 5% palladium-on-charcoal (6 g) for 19 h, the pH being adjusted at intervals Electrophoretic examination of the mixture suggested that the major product was a mono-*O*-benzyl disulphate The hydrogenation was repeated with fresh catalyst (6 g) to give D-glucose 4,6-di-(barium sulphate) (183 mg) which crystallised slowly from aqueous ethanol (Found Ba, 28 9 $C_6H_{10}BaO_{12}S_2$ calc Ba, 29.1%)

Methyl α-D-glucopyranoside 4,6-di-(barium sulphate) — Methyl 2,3-di-*O*-benzyl-α-D-glucopyranoside[19] (14 g) was treated with pyridine–sulphur trioxide (31 g) in pyridine (300 ml) for 2 5 h at 65° From the mixture, crystalline methyl 2,3-di-*O*-benzyl-α-D-glucopyranoside 4,6-di-(barium sulphate) (17.5 g), $[\alpha]_D^{20}$ +64 5° (*c* 0 6, water), was recovered (Found Ba, 20 8 $C_{21}H_{24}BaO_{12}S_2$ calc Ba, 20 5%) This product was dissolved in aqueous ethanol and hydrogenated at 4 atmos over 5% palladium-on-carbon, a total of 5 treatments was necessary The mixture was separated on a cellulose column, and the methyl α-D-glucopyranoside 4,6-di-(barium sulphate) (1 5 g) crystallised from aqueous ethanol (For the sodium salt, found S, 16 0 $C_7H_{12}Na_2O_{12}S_2$ calc S, 16 1% For Ba salt, found C, 17 3; H, 2 45 $C_7H_{12}BaO_{12}S_2$ calc.: C, 17 2; H, 2 45%)

Benzyl β-D-galactopyranoside 4-(barium sulphate) and β-D-galactose 4-(barium sulphate) — Benzyl β-D-galactopyranoside (18 g) was selectively benzoylated[6] at −40° to give benzyl 2,3,6-tri-*O*-benzoyl-β-D-galactopyranoside (12 g), m p 163–165° (from aqueous ethanol), $[\alpha]_D^{20}$ +22 5° (chloroform) (Found C, 69 9; H, 5.19 $C_{34}H_{30}O_9$ calc · C, 70.1; H, 5 19%)

The tribenzoate (10 g) was sulphated with pyridine–sulphur trioxide (21 g) in pyridine (300 ml) for 0 5 h at 65° T l c then indicated complete sulphation Crystalline benzyl 2,3,6-tri-*O*-benzoyl-β-D-galactopyranoside 4-(barium sulphate) (10 g), $[\alpha]_D^{20}$ +19 5° (*c* 1.72, pyridine), was obtained from the reaction This product (8 5 g) was debenzoylated (barium methoxide) to give amorphous benzyl β-D-galactoside 4-(barium sulphate) A portion (7 8 g) of this was catalytically hydrogenated for 3 h at 4 atmos to give β-D-galactose 4-(barium sulphate) (1.1 g), after crystallisation from aqueous, ethanolic solution (For the sodium salt, found S, 11.3 $C_6H_{11}O_9SNa$ calc S, 11 3%) The sodium salt was indistinguishable chromatographically and electrophoretically from authentic[5] D-galactose 4-(sodium sulphate)

Methyl α-D-galactopyranoside 4-(barium sulphate) — Methyl 2,3,6-tri-*O*-benzoyl-α-D-galactopyranoside[6] (17 g) was treated with pyridine–sulphur trioxide (42 g) in pyridine (350 ml) for 6 h at 65°. During the usual isolation procedure, some methyl 2,3,6-tri-*O*-benzoyl-α-D-galactopyranoside 4-(barium sulphate) precipitated from the aqueous mixture This was extracted from the barium sulphate impurity with hot ethanol, and the combined extract and filtrate was concentrated The product, $[\alpha]_D^{20}$ +93° (*c* 1 3, chloroform), precipitated from aqueous solution and was catalytically debenzoylated (barium methoxide) Purification on a cellulose column gave methyl α-D-galactopyranoside 4-(barium sulphate) (3 4 g) which crystallised slowly from aqueous ethanol [Found C, 24 6, H, 3 9. $(C_7H_{13}O_9S)_2Ba$ calc C, 24 6; H, 3 84%]

Treatment of sugar sulphates with dilute acid — Sodium salts (10 mg each) of D-glucose 3-, D-glucose 6-, D-galactose 4-, and D-galactose 6-sulphate were separately heated in 0 33N sulphuric acid (3 ml) at 100°. After intervals of 0.5, 1, and 3 h, aliquot portions (*ca* 1 ml) were neutralised ($BaCO_3$), centrifuged, and evaporated Examination by paper chromatography (ethyl acetate–acetic acid–water, 6 3 2, which resolves isomeric monosulphates) showed that only starting material and the free sugar were present in all of the samples

ACKNOWLEDGMENTS

We thank Professor Stanley Peat, F R S , for his interest and advice, and one of us (M J H) thanks the Science Research Council for financial support

REFERENCES

1 Part VI S Peat, D M Bowker, and J R Turvey, *Carbohyd Res* , 7 (1968) 225
2 J R Turvey, *Advan Carbohyd Chem* , 20 (1965) 183
3 D A Rees, *J Chem Soc* , (1963) 1821
4 P Baumgarten, *Ber* , 59 (1926) 1166
5 J R Turvey and T P Williams, *J Chem Soc* , (1962) 2119
6 J M Williams and A C Richardson, *Tetrahedron*, 23 (1967) 1369
7 S Peat, J R Turvey, M J Clancy, and T P Williams, *J Chem Soc* , (1960) 4761
8 R C Hockett, D V Collins, and A Scattergood, *J Amer Chem Soc* , 73 (1951) 599
9 H B Wood, H W Diehl, and H G Fletcher, Jr , *J Amer Chem Soc* 79 (1957) 1986.
10 N K Richtmyer, *Methods Carbohyd Chem* , 1 (1962) 107, B Capon, W G Overend, and M Sobell, *Tetrahedron*, 16 (1961) 106
11 E G Gros and V Deulofeu, *J Org Chem* , 29 (1964) 3647
12 E Sorkin and T Reichstein, *Helv Chim Acta*, 28 (1945) 1
13 J W H Oldham and D J Bell, *J Amer Chem Soc* , 60 (1938) 323
14 M L Wolfrom, F Shafizadeh, R K Armstrong, and T M Shen Han, *J Amer Chem Soc* , 81 (1959) 3716
15 A Levy, H M Flowers, and N Sharon, *Carbohyd Res* , 4 (1967) 305
16 R Mozingo, *Org Syn* , 26 (1946) 78
17 B Iselin and T Reichstein, *Helv Chim Acta*, 29 (1946) 508
18 F Micheel and A Klemer, *Ber* , 91 (1958) 663
19 D J Bell and J Lorber, *J Chem Soc* , (1940) 453

Carbohydrate Research
Elsevier Publishing Company Amsterdam
Printed in Belgium

QUANTITATIVE ANALYSIS OF GENTIOBIOSE AND ISOMALTOSE IN ADMIXTURE, AND ITS APPLICATION TO THE CHARACTERIZATION OF DEXTRINS*

M L WOLFROM AND G SCHWAB
Department of Chemistry, The Ohio State University, Columbus, Ohio 43210 (U S A)

(Received September 26th, 1968)

ABSTRACT

Gentiobiose in admixture with isomaltose was selectively hydrolyzed by emulsin, and the resultant D-glucose was separated from unchanged isomaltose by thin-layer chromatography on borohydride-reduced microcellulose The separated sugars were located, eluted, and assayed photometrically by the anthrone method.

Representative, acid-modified dextrins were partially acid-hydrolyzed under conditions wherein reversion was negligible, and disaccharide formation was maximal Inorganic salt was removed by extraction of the neutralized, evaporated hydrolyzate with *N*,*N*-dimethylformamide under anhydrous conditions, and the extract was analyzed by the above method without solvent removal The method gives comparable results for the characterization of dextrination and is more rapid than those previously utilized

INTRODUCTION

In connection with experiments on dextrin formation, a method for the quantitative analysis of mixtures of gentiobiose (6-*O*-β-D-glucopyranosyl-D-glucose) and isomaltose (6-*O*-α-D-glucopyranosyl-D-glucose) was needed. The extremely small differences in the R_F values of these closely related disaccharides, in all the well-established developer systems, render their separation by chromatographic methods very difficult Their separation was first achieved by Ough[1], who used twenty-day, continuous, descending, paper chromatography

We have applied herein the selective cleavage of gentiobiose by emulsin, so that the resultant mixture of isomaltose and D-glucose reduces the problem to the simple separation of isomaltose from D-glucose Suitable conditions for the action of emulsin on gentiobiose are stated[2] to be a substrate concentration of 5 g/100 ml in acetate buffer (pH 5.0) at 40° The buffer could not be employed, however, since its relatively high ionic strength interfered with the subsequent separation of the sugars by thin-layer chromatography Removal of the ions with ion-exchange resins

---

*Dedicated to Professor Stanley Peat, F R S

was not advisable either, since the analysis was devised for microgram amounts of the sugars. It was found, however, that the cleavage reaction could be effected in neutral solutions if the reaction time was prolonged to 48 h, and if the emulsin concentration was sufficiently high Isomaltose was not attacked by emulsin under these conditions.

The separation of the resultant isomaltose and D-glucose was made by thin-layer chromatography on glass plates coated with sodium borohydride-reduced, microcrystalline cellulose[3] For the subsequent, quantitative determination, the anthrone reaction was used, essentially following the method described by Vomhof, Truitt, and Tucker[4].

The analytical method was then applied to the characterization of two typical commercial dextrins These are generally manufactured by the combined action of heat and mild acidity on solid starches, a process which produces chain degradation and glycosyl transfer with the formation of new linkages, wherein those formed on the C-6 primary alcohol groups predominate[5 6]. The new linkages are not produced stereospecifically, and both new α-D-(1→6) and β-D-(1→6) linkages are produced

This laboratory has been concerned with the investigation, by fragmentation procedures, of the linkages formed, whereas other workers[7 8] have utilized periodate and methylation techniques The fragmentation method embodies partial, acid hydrolysis under conditions of such low sugar concentration and acidity that the amount of new linkages formed by the hydrolytic procedure is negligible[9 10] The hydrolytic conditions were selected so that the maximum yield of disaccharides was obtained When these conditions were applied to the dextrin samples, it was necessary to remove quantitatively the inorganic salt (sodium chloride) formed on neutralization of the hydrolysis mixture This posed a problem Malpress and Morrison[11] have reported that this could be effected by extraction of the dry salt mixture with dry pyridine at 100° We have found it more convenient to use *N*,*N*-dimethylformamide under anhydrous conditions and at room temperature. An aliquot of the extracting solution could then be applied directly to the thin-layer plates without solvent removal This procedure was shown to give quantitative separation of the sugar from the salt An established analytical method was then used to determine the amount of total gentiobiose and isomaltose present and, from this, the amount of each by difference after removal of the gentiobiose by emulsin and determination of the residual isomaltose

EXPERIMENTAL

Analysis of mixtures of gentiobiose and isomaltose materials — The isomaltose was an amorphous solid prepared[12] from its crystalline β-octaacetate The emulsin, β-D-glucosidase (970 International Units per mg), was obtained from the Worthington Biochemical Corporation, Freehold, New Jersey The anthrone reagent[13] was prepared by dissolving 1 g of anthrone in 50 ml of redistilled ethyl acetate and was stored in a brown-glass bottle[14]

Emulsin digestion. — To neutral, aqueous solutions containing 20–100 mg each of isomaltose and gentiobiose, each at a gentiobiose concentration of 1 mg/ml, emulsin was added until the enzyme concentration was 0.75 mg/ml (930 International Units of β-D-glucosidase per mg of substrate gentiobiose). The solutions were shaken vigorously in stoppered tubes, two drops of toluene were added, and the tubes were placed in a thermostat at 37±0.5°. After 48 h, the tubes were heated in a boiling water-bath until a flocculent precipitate of denaturated enzyme appeared. The solutions were then centrifuged for 10 min, and the supernatant was ready for t.l.c.

Thin-layer chromatography (t.l.c.). — Glass plates ($20 \times 20 \times 0.4$ cm) were coated[3] with 0.5-mm layers of sodium borohydride-reduced, microcrystalline cellulose. The emulsin-digested sugar solutions were applied to the plates in 1.5–2.0 μl quantities with a syringe microburette. The plates were dried between applications with a hair drier, until the desired amount of solution was spotted on the plates. Comparison spots were applied at the two edges and in the middle of the plates, using the largest amount in the series to be analyzed. The plates were developed twice with ethyl acetate–pyridine–water[15] (2:1:2, v/v, upper phase), stopping the development when the solvent front had travelled 15 cm from the origin. The plates were then dried and carefully masked, leaving the locator strips open at the sides and in the middle. These strips were then sprayed with aniline hydrogen phthalate[16,17] and heated with a hair drier until the spots for D-glucose and isomaltose appeared.

Analysis of chromatographically separated isomaltose and D-glucose. — After zone location, all of the microcellulose was scraped off the plate except for the regions of the D-glucose and isomaltose zones. These regions were sectioned into rectangular areas of about equal size, each containing all of the material corresponding to a sugar spot. These areas plus one blank area were scraped off with a razor blade and transferred quantitatively into dry, 100 × 14 mm, culture tubes. The material was broken up with a thin, glass rod. To each tube 2 ml of water was added, and the tubes were stoppered and left for 1 h at room temperature with occasional, gentle shaking. The samples were then filtered with suction through a 15-ml fritted-glass filter, of medium porosity, into dry, 15-ml, centrifuge tubes having ground-glass joints. The centrifuge tubes were then placed into an iced-water bath, and 0.5 ml of anthrone reagent and 4 ml of concentrated sulfuric acid, in that order, were slowly added to each tube. The tubes were closed with glass stoppers and shaken vigorously. They were then kept at 80° for 30 min. After being cooled to room temperature in a water bath, the samples were transferred to photometric glass cells (1 cm), and their absorbances were measured in a Beckman DU spectrophotometer at 620 nm against the absorbance of the blank.

It was established that Beer's law was obeyed from 0–100 μg of isomaltose and from 0–100 μg of gentiobiose cleaved by emulsin. The calibration data are shown in Table I and these plotted to give a straight line to the origin. Analytical data on known mixtures of the two disaccharides are given in Table II.

Characterization of dextrins by fragmentation, and determination of gentiobiose and isomaltose. — *(a) Removal of sodium chloride from aqueous, sugar mixtures.*

TABLE I

ANALYTICAL CALIBRATION FOR GENTIOBIOSE AND ISOMALTOSE AFTER TREATMENT OF EACH WITH EMULSIN

Sugar, µg	*Absorbance*		*Average of average deviation*
	Average[a]	*Average deviation*	
Gentiobiose			
20	0.164	0.009	
40	0.346	0.009	
60	0.508	0.014	
80	0.678	0.006	
100	0.825	0.008	0.009 (≙ 1.1 µg)
Isomaltose			
20	0.166	0.005	
40	0.347	0.010	
60	0.514	0.010	
80	0.652	0.011	
100	0.814	0.009	0.009 (≙ 1.1 µg)

[a] Average of five readings

D-Glucose (50 mg), gentiobiose (50 mg), and sodium chloride (105 mg) were dissolved in 25 ml of water. This solution was taken to dryness under diminished pressure in a 50-ml round-bottomed flask containing a small amount of clean, sea sand. A small magnetic stirrer and 10 ml of *N*,*N*-dimethylformamide, previously dried for 1 week over Molecular Sieve-Type 4A (Fischer Scientific Co., Fair Lawn, N. J.), were then added, and the mixture was heated, with stirring, for 30 min at 100° under reflux (calcium chloride guard-tube). The flask was rotated repeatedly so that the solvent could reach all of the material adhering to the walls of the flask. The mixture was then centrifuged in a dry, stoppered tube, and the solution was used directly for t.l.c. and determination of the sugars with anthrone, as described above. This procedure was repeated with a like mixture but with the substitution of isomaltose for gentiobiose. The recovery of all sugars was quantitative, within the limits of the analysis (±1 µg). This procedure was found not to be applicable to phosphate-buffered solutions.

(*b*) *Analysis of dextrins.* It was established by analysis of the unseparated gentiobiose and isomaltose, by the above procedure but with the omission of the emulsin treatment, that the maximum, combined yield of these two disaccharides was obtained in 2 h of hydrolysis of the dextrin (dark canary) under the hydrolytic conditions described below.

Two samples of typical, acid-modified, commercial dextrins (Table III) were dried for 24 h at 98° under diminished pressure over Drierite (calcium sulfate as soluble anhydrite). The water contents found were in the range 5.7–7.2%. The dried samples (100 mg) were dissolved in 25 ml of 0.15N hydrochloric acid (*c* 0.16), and the solution was heated for 2 h at 98° under reflux. The solution was then neutralized with sodium hydrogen carbonate and divided into two equal parts. To one part, emulsin was added to a concentration of 0.75 mg/ml, the solution was covered with

TABLE II

ANALYSES OF MIXTURES OF GENTIOBIOSE AND ISOMALTOSE AFTER EMULSIN TREATMENT

Sugar in mixture, µg		*Absorbance*					
Gentiobiose	*Isomaltose*	*Gentiobiose*			*Isomaltose*		
		Average[a]	*Average deviation*	*Average of average deviation*	*Average*[a]	*Average deviation*	*Average of average deviation*
20	20	0.167	0.007		0.170	0.006	
40	40	0.358	0.010		0.327	0.017	
60	60	0.513	0.014		0.516	0.009	
80	80	0.666	0.011		0.660	0.009	
100	100	0.816	0.011	0.011 (≈ 1.3 µg)	0.820	0.006	0.009 (≈ 1.1 µg)
100	20	0.825	0.010 (≈ 1.2 µg)		0.169	0.006 (≈ 0.7 µg)	
20	100	0.165	0.008 (≈ 1.0 µg)		0.820	0.009 (≈ 1.1 µg)	

[a] Average of five readings

a thin layer of toluene, and the vessel was stoppered and placed in a thermostat for 48 h at 37° The other half of the neutralized hydrolyzate was evaporated to dryness under diminished pressure and extracted with 10 ml of dry *N*,*N*-dimethylformamide, as described above Spots of 125 μl of the solution were then applied to the thin-layer plates and chromatographed as described above, except that the development was repeated three times with intermediate drying After location and elution of the zone containing gentiobiose and isomaltose, their combined amount was determined by the anthrone reaction, as described previously The part treated with emulsin was analyzed in the same way, except that only the isomaltose was determined, the D-glucose was developed off the plate The analytical data are recorded in Table III

TABLE III

FRAGMENTATION ANALYSES OF DEXTRINS

Dextrin type (acid-modified)[a]	*Gentibiose + isomaltose*			*Isomaltose*			*Gentiobiose, by difference,*
	Average[b] *absorbance*	*μg equiv*	%	*Average*[b] *absorbance*	*μg equiv*	%	%
Dark canary	0 532	64 8	6 5	0 336	40 9	4 1	2 4
White	0 416	50 7	5 1	0 290	35 4	3 5	1 6

[a] Kindly supplied by the Clinton Corn Processing Co , Clinton, Iowa, a division of Standard Brands, Inc , produced from corn starch [b] Average of 9–12 readings

DISCUSSION

Solutions of isomaltose and of gentiobiose alone were subjected to the same conditions as the mixtures and were used for calibration curves Correspondence of the absorbance values found for the mixtures with those of the calibration values (Table I) showed the cleavage of gentiobiose in the mixtures, as well as the separation of the resultant isomaltose and D-glucose mixture, to be quantitative (Table II). The separation was good, even if one of the sugars was present in a five-fold excess over the other. The upper limit for successful separation of the two sugars was not determined, but 100 μg of isomaltose could be separated quantitatively from 100 μg of gentiobiose The accuracy of these analyses was ± 1 μg (1 0–1 2 μg)

There are no β-D-(1$\rightarrow$6) linkages present in natural starches, so that gentiobiose, containing a β-D-(1$\rightarrow$6) intersugar linkage, provides a measure of the amount of such linkages formed in the dextrination process At the same time, there will be about an equal amount of α-D-(1$\rightarrow$6) linkages formed, and these will appear in the new isomaltose units so produced A certain number of α-D-(1$\rightarrow$6) linkages are present in starches at their branch-points Now the acid fragmentation method is necessarily not quantitative, but, under rigorously controlled conditions, the results can be comparable It has been established[6], by isolative methods, that these disaccharide units are present in dextrins so that non-isolative, thin-layer chromatography then becomes a valid procedure for such known constituents The procedure herein described is rapid and is probably more nearly quantitative than the extrusive, silicate column, acetate chromatography used previously in this laboratory

The data of Table III indicate a larger formation (reversion) of new linkages (2.4 × 2 = 4 8%) in the dark canary dextrin than in the white dextrin (1.6 × 2 = 3 2%), formed under milder conditions If these values (twice the gentiobiose found) are subtracted from the comparative total amount of isomaltose, the differences show a 1 7 and 1 9% initial value for isomaltose, which should be present in the original corn starch used This compares with the value 0 8% (calculated from the value 1% obtained on waxy-maize amylopectin[18] and here corrected for the average amylose content of dent corn starch (1 00 − 0 24 = 0 76) The lower value of 0 8 was obtained on the undoubtedly less-quantitative, and more-laborious, acetate column, isolative method[18].

ACKNOWLEDGMENT

The support of The Corn Industries Research Foundation is acknowledged.

REFERENCES

1 L D Ough, *Anal Chem*, 34 (1962) 660

2 E M Crook and B A Stone, *Biochem J*, 65 (1957) 1

3 M L Wolfrom, R M de Lederkremer, and G Schwab, *J Chromatog*, 22 (1966) 474

4 D W Vomhof, J Truitt, and T C Tucker, *J Chromatog*, 21 (1966) 335

5 A Thompson, K Anno, M L Wolfrom, and M Inatome, *J Amer Chem Soc*, 76 (1954) 1309.

6 A Thompson and M L Wolfrom, *J Amer Chem Soc*, 80 (1958) 6618

7 J D Geerdes, B A Lewis, and F Smith, *J Amer Chem Soc*, 79 (1957) 4209

8 G M Christensen and F Smith, *J Amer Chem Soc*, 79 (1957) 4492

9 M L Wolfrom, E N Lassettre, and A N O'Neill, *J Amer Chem. Soc*, 73 (1951) 595

10 A Thompson, M L Wolfrom, and E J Quinn, *J Amer. Chem Soc*, 75 (1953) 3003

11 F H Malpress and A B Morrison, *Nature*, 164 (1949) 963

12 M L Wolfrom, L W Georges, and I L Miller, *J Amer Chem Soc*, 71 (1949) 125

13 R Dreywood, *Ind Eng Chem*, *Anal Ed*, 18 (1946) 499

14 F A Loewus, *Anal Chem*, 24 (1952) 219

15 M A Jermyn and F A Isherwood, *Biochem J*, 44 (1949) 402

16 S M Partridge, *Nature*, 164 (1949) 443

17 C M Wilson, *Anal Chem*, 31 (1959) 1199

18 M L Wolfrom, J T Tyree, T T Galkowski, and A N O'Neill, *J Amer. Chem Soc*, 73 (1951) 4927

Carbohydrate Research
Elsevier Publishing Company Amsterdam
Printed in Belgium

THE ACTIVE CARBOHYDRATE METABOLITES OF THE BROWN SEAWEED, *Fucus vesiculosus*[1]*

E. J. BOURNE, PAMELA BRUSH, AND ELIZABETH PERCIVAL

Chemistry Department, Royal Holloway College (University of London), Englefield Green, Surrey (Great Britain)

(Received October 2nd, 1968)

ABSTRACT

Biosynthetic studies with $Na_2{}^{14}CO_3$ on *Fucus vesiculosus* by Bidwell[2] have shown that D-mannitol is the main respiratory substrate, but that a proportion of the radioactivity is incorporated into fucoidin, alginic acid, alkali-soluble alcohol-soluble material, and into the insoluble residue remaining after the removal of the other materials by acid and alkali extraction. The quantity of seaweed used in the biosynthetic studies prevented a complete investigation of the various materials. Large-scale extraction, under the conditions used in the above studies, has now revealed that the "fucoidin" is indeed a mixture of fucoidin and laminarin, and that the "alginic acid" is contaminated with a sulphated glucuronoxylofucan which has been found in this genus for the first time The alkali-soluble, alcohol-soluble material is a partially degraded portion of this glucuronoxylofucan Further extraction of the insoluble residue gives additional, crude glucuronoxylofucan, and an acid-insoluble and an acid-soluble glucan are separated by extraction with 6N alkali after mild treatment with chlorite Tentative evidence is advanced for the presence of (1→3)- and (1→4)-linked D-glucose units in these glucans and in the final residue

INTRODUCTION

Fucus vesiculosus, a brown seaweed, has previously been shown to metabolise the following carbohydrates: D-mannitol[3], laminarin[4], fucoidin[5], alginic acid[6], and cellulose[7] Recent, biosynthetic studies by Bidwell[2] with $Na_2{}^{14}CO_3$ have demonstrated that, although D-mannitol is the main respiratory metabolite in this weed, radioactivity is also incorporated into the polysaccharides The "fucoidin" was extracted from the weed by acid at pH 2, and the "alginic acid" with dilute sodium carbonate, and each was precipitated from these solutions with 75% ethanol. The mannitol remained in the acidic, alcoholic supernatant, and the alkaline, alcoholic supernatant also contained radioactive material, as did the insoluble residue Bidwell found that 90% of the radioactivity was incorporated after 4 5 h incubation, and, thereafter, unlabelled sodium carbonate was supplied for a total of 74 h, with alternating 12 h periods of light and dark After 24 h, the radioactivity in the insoluble residue decreased in the light and increased in the dark, indicating that it also

---

*Dedicated to Professor Stanley Peat, F R S

contained an "active metabolite" which was being broken down and resynthesised from newly supplied material Although the major proportion (*ca* 82% after 74 h) of the radioactivity was incorporated into mannitol, this decreased slowly after the first 12 h, and, of the remainder of the activity (18%) after 74 h, "fucoidin" had approximately 17%, "alginic acid" 25 5%, the alkali-soluble alcohol-soluble material 6 5%, and the insoluble residue 51%

The quantity of weed necessarily used in these experiments precluded the characterisation (apart from the mannitol) of the different materials, and the identification of the "fucoidin" and "alginic acid" was based on the results of earlier extractions and on their solubility characteristics The importance of these studies made it desirable to confirm the characterisation, to determine the proportions of these polysaccharides, and to investigate the nature of the material in the ethanolic, alkaline, supernatant liquid and in the residual, insoluble material. With Professor Bidwell's approval, therefore, large-scale extraction of newly harvested fronds of *F. vesiculosus* was carried out under the exact conditions described by him[2], and recent methods of analysis and characterisation were applied to each of the fractions

DISCUSSION

The acid-soluble, alcohol-insoluble material amounted to *ca.* 22% of the dry weight of the weed It had a half-ester sulphate content of 27% and gave fucose and glucose, in the molar proportions of about 2 7·1 5, on hydrolysis Earlier work[4] had revealed that *Fucus* species synthesise up to 7% of laminarin which is extractable with dilute acid, and it is reasonable to conclude on this evidence that the glucose in the hydrolysate was derived from laminarin, the only soluble glucan found in brown seaweeds Allowing for the fact that *ca* 24% of this extract therefore consists of an unsulphated laminarin, the sulphate content of the remainder is *ca* 40% and is comparable with that (38%) previously recorded for the fucoidin isolated from *F vesiculosus*[5] This extract therefore contains *ca.* 77% of fucoidin and 24% of laminarin, and the distribution of the activity between these two polysaccharides must await further radioactive studies.

The alkali-soluble, alcohol-soluble material contained carbohydrate corresponding to only 0 47% of the dry weight of the weed. If it is assumed that the majority of the radioactivity in this fraction is in the carbohydrate, it must be relatively high, since the fraction contains 6 5% of the total "polysaccharide" activity This material was divided into dialysable and nondialysable substances The former was a mixture of fucose, 4,5-unsaturated uronic acid, traces of xylose, and amino acids. The last compounds were presumably derived from protein, possibly combined with polysaccharide The 4,5-unsaturated acids were detected, after periodate oxidation, with thiobarbituric acid[8].

Since fucose and xylose are soluble in acid, if present initially, they would have been removed from the seaweed in the earlier extraction with acid The present sugars can, therefore, only have been derived by alkaline degradation of polymeric material.

The nondialysable fraction contained polysaccharide of low molecular weight (not precipitated with 3 vol of ethanol) consisting of fucose, xylose, and glucuronic acid This material also contained a proportion of 4,5-unsaturated acids The Norwegian workers[9] had shown previously that ascophyllan (a glucuronoxylofucan, combined with protein, extracted from *Ascophyllum nodosum*) was degraded by N sodium hydroxide at 80° by the mechanism of β-alkoxycarbonyl elimination from the glucuronic acid residues and their conversion into 4,5-unsaturated derivatives of hexuronic acids These workers made no mention of the release of free sugars in this reaction, but it must be remembered that their conditions were considerably more drastic than the present extraction with 3% sodium carbonate at 50° The effect of treating a glucuronoxylofucan (previously characterised[10]) with sodium carbonate was therefore examined. Free fucose and 4,5-unsaturated acids were produced, confirming the origin of these substances in the alkali-soluble, alcohol-soluble fraction which therefore consists of a partially degraded glucuronoxylofucan combined with a certain amount of protein The absence of any mannuronic or guluronic acids in this fraction is not surprising, since it is known that alginic acid is not degraded to alcohol-soluble fragments under these mild conditions.

Of the "alginic acid" fraction, which represents *ca.* 29% of the dry weight of the weed, *ca* 10% consisted of a glucuronoxylofucan This was separated by conversion into the soluble calcium salt, the alginic acid being precipitated in the presence of Ca^{2+} That the insoluble portion was indeed calcium alginate was confirmed by (*a*) hydrolysis, and the identification of mannuronic acid and guluronic acid and their lactones by paper chromatography, and (*b*) conversion of these acids into their methyl ester methyl glycosides, reduction, and hydrolysis to mannose and gulose Paper chomatography and ionophoresis confirmed the identity of these two sugars

Trace quantities of fucose and xylose were also detected in the hydrolysate, indicating that a small proportion of the calcium-soluble material had been co-precipitated with the alginic acid That this was indeed very small was confirmed by g l c of the corresponding alcohols

The calcium-soluble portion of this fraction had a half-ester sulphate content of *ca* 11%, and contained 11 4% of protein, and fucose, xylose, and glucuronic acid in molar proportions of *ca* 3 5 1 1. It is most probably the parent polysaccharide from which the alcohol-soluble material had been derived by alkali degradation. In view of the relatively high radioactivity of the latter, it is very probable that a considerable proportion of the radioactivity attributed to alginic acid is in fact incorporated in the glucuronoxylofucan It should be pointed out that this is the first time that this type of polysaccharide has been found in species of *Fucus*

The insoluble residue (*ca* 27% of the dry weight of the weed) consisted of dark-brown, intractable material containing, among other things, polymeric phenolic substances and inorganic material. However, hydrolysis gave glucose with small proportions of fucose, xylose, and acidic materials Since ammonium oxalate–oxalic acid (pH 2 8) extracts a glucuronoxylofucan from similar residual material from *Ascophyllum nodosum*[10], the present, insoluble residue was subjected to this treatment.

A crude glucuronoxylofucan (*ca* 5% of the dry weight of weed) was separated from this extract. Attempted purification of this material was unsuccessful. The insoluble residue (*ca* 21% of the dry weight of the weed) remaining after this extraction was given a mild treatment with chlorite, which dissolved *ca.* 3% of protein and *ca* 0 5% of a glucuronoxylofucan (both based on the dry weight of the weed). Extraction of the residual solid with 6N alkali left *ca* 1% of a white, fibrous material, and from the alkaline solution, an acid-soluble glucan (*ca* 0.2%) and an acid-insoluble glucan (*ca* 0.2%) were separated Both glucans gave glucose on acid hydrolysis and were readily hydrolysed to glucose by a specific β-(1→3)-D-glucanase, and the acid-insoluble material was also hydrolysed by cellulase Insufficient acid-soluble glucan remained for incubation with cellulase Parallel enzymic hydrolyses were carried out with laminarin and lichenin The acid-insoluble material gave no change of colour when tested with zinc chloride and iodine The insoluble, fibrous material gave glucose on acidic and enzymic hydrolyses (cellulase), and a trace of glucose with β-(1→3)-D-glucanase It was soluble in Schweizer's reagent, and gave the characteristic blue colour for cellulose with zinc chloride and iodine It may be concluded from these results that the alkali-soluble materials contain a mixture of β-(1→3)- and (1→4)-linked D-glucose units, and that the insoluble residue consists mainly of cellulose, with a small proportion of a (1→3)-linked glucan.

Percival and Ross[7] demonstrated the presence of a small proportion of cellulose in this species of weed, but these studies clearly indicate the presence of other polysaccharides, in addition to cellulose, in the "insoluble residue" It is unlikely that the cellulose is the active metabolite, but it is not possible from these results to say which of the remaining materials constitutes a respiratory substrate

When considering the proportions of the different polysaccharides in this sample of *F vesiculosus*, it must be borne in mind that these quantities may vary somewhat, depending on the season of collection and the particular environment of the seaweed

This work forms a necessary basis for any further biosynthetic work on *F vesiculosus* It is intended, however, that further studies using ^{14}C will be carried out in collaboration with Professor Bidwell

EXPERIMENTAL

All solutions were evaporated under diminished pressure below 50°. Paper chromatography was carried out by the descending method on Whatman No 1 paper The following solvent systems (v/v) were used (*1*) butyl alcohol–ethanol–water (40 11.19), (*2*) ethyl acetate–acetic acid–formic acid–water (18·3 1 4); (*3*) pyridine–ethyl acetate–acetic acid–water (5 5 1 2), as descending eluant equilibrated with pyridine–ethyl acetate–water (11.40 60), (*4*) ethyl acetate–pyridine–water (10 4.3); (*5*) butanone–acetic acid–water (9 1·1) saturated with boric acid Papers were sprayed with a saturated, aqueous solution of aniline oxalate (*a*), ninhydrin (*b*), or dipped in silver nitrate[11] (*c*). Gas–liquid chromatography of the trimethylsilylated (TMS) sugars and alditols was carried out as described before[10] Unless otherwise stated, hydrolyses were carried out with 90% formic acid in sealed tubes in an atmosphere of carbon dioxide for 6 h at 100°, followed by hydrolysis of formic esters by dilution (5 vol) and further heating for 2 h at 100°. All reductions were carried out with potassium borohydride in aqueous solution The carbohydrate content of the polysaccharides was determined by the phenol–sulphuric acid method[12] Sulphate was determined[13] after digestion of the polysaccharide (10 mg) with conc nitric acid (1 ml; "Analar") and a few mg of sodium chloride in a sealed tube for 18 h at 100°. This was followed by evaporation to dryness, the addition of conc hydrochloric acid (1 ml, "Analar"), evaporation to dryness, and heating the dry tube in an oven at 110° for 2 h The contents were then dissolved in water (1 ml), and appropriate aliquots were removed for the determination.

Extraction of the seaweed — Wet, freshly harvested fronds of *Fucus vesiculosus* (200 g; dry weight, 64.4 g), collected on the west coast of Scotland during January 1968, were frozen in liquid nitrogen and ground to a powder The frozen powder was then subjected to exhaustive extractions[2] with dilute acid and dilute sodium carbonate From the combined, acidic extracts, 75% ethanol precipitated polysaccharide material (14 g) (Found carbohydrate content[12] 55, SO_4^{2-}, 27%) which, on hydrolysis, gave mainly fucose with a lesser amount of glucose and a trace of xylose (paper chromatography, and g l c of the TMS derivatives). The glucose was also confirmed with glucose oxidase[14]. The molar proportions of fucose to glucose were found to be *ca* 2 7 1.5 by reduction to the corresponding alditols and g l c examination of the TMS derivatives[10]

Similarly, 75% ethanol gave from the combined, alkaline extracts a cream precipitate (*A*, 18 4 g).

Soluble "other compounds" — The alcoholic, supernatant from the alkaline extract was concentrated to remove the alcohol, and the derived, aqueous, alkaline solution (carbohydrate content, 0 47% of dry weight of weed as fucose), termed by Bidwell "other compounds", was dialysed in a closed system against distilled water The first two changes of water were concentrated (to 5 ml, hereinafter called solution *B*) An aliquot (0 2 ml) of solution *B* gave a positive test for uronic acid[15], and paper chromatography of solution showed mainly fucose, with trace amounts of xylose This was confirmed by g l c of the TMS derivatives of the sugars and their derived alditols.

The solution from the dialysis sac was made alkaline with ammonia, and freeze-dried to a dark-green solid A portion gave a positive test for uronic acid[15], and, after hydrolysis of a second portion, fucose, xylose, and amino acids were detected (paper chromatography, solvents *1*, *2*, and *3*) No spots having the chromatographic mobilities of glucuronic, mannuronic, guluronic, or galacturonic acids could be detected, although a spot having R_{Fuc} 0 56 and 0 63 (solvents *1* and *2*, respectively) was present [cf. 3-*O*-(β-D-glucopyranosyluronic acid)-L-fucose[10]]

A portion of solution *B* and of the above, dark-green solid (360 μg of carbohydrate) were separately treated with 0 025N sodium periodate in 0.125N sulphuric acid (0 5 ml) After 20 min at room temperature, 2% sodium arsenite in 0 5N hydrochloric acid (1 0 ml) was added with shaking, and the mixture was set aside for 2 min Thiobarbituric acid (0 3%, pH 2 0, 4 ml) was added with stirring, and the mixture was heated for 10 min at 100°, cooled, and filtered The absorption of the resulting pink solutions was measured at 510 nm to 590 nm, and gave maximum absorption at 548–554 nm. The glucuronoxylofucan (1 g) extracted from *Ascophyllum nodosum*[10] was treated with 3% aqueous sodium carbonate under the conditions used for the extraction of *F. vesiculosus* The derived solution contained free fucose and a trace of xylose, and, when subjected to the above test with thiobarbituric acid after periodate oxidation, gave a maximum absorption at 548 nm

Alkali-soluble, alcohol-insoluble fraction. — A portion of precipitate *A* (20 mg) was hydrolysed, and chromatographic examination of the hydrolysate gave spots having the mobilities of fucose, xylose, and glucuronic, mannuronic, and guluronic acids (solvents *1*, *2*, and *3*). A second portion of *A* (12 5 g) was dissolved in water to give a concentration of about 1%, and, to this, 2% aqueous calcium chloride was added slowly, with stirring, until precipitation was complete The mixture was kept in the ice-chest overnight, and the gelatinous precipitate was then removed on the centrifuge It was washed with water and freeze-dried to a cream solid (calcium alginate, 6 5 g The supernatant liquid was concentrated to *ca.* 500 ml and dialysed against distilled water until free from Cl^- It was then concentrated and freeze-dried to a white solid (1 2 g) (Found· SO_4^{2-}, 11; N_2, 1 78%) Chromatographic examination of a hydrolysate revealed the presence of fucose (major), xylose, and acid spots (solvents *1*, *2*, and *3*) A portion of the hydrolysate was converted into the TMS derivative, and the presence of xylose and fucose was confirmed by g l c A second portion of the hydrolysate in water (0 5 ml) was reduced with sodium borohydride overnight. The mixture was treated with Amberlite IR-120 (H^+) resin, and the borate was removed by repeated evaporations with methanol. The derived alditols and acid were converted into the TMS derivatives Examination on g l c gave peaks having the retention times of the TMS derivatives of fucitol, xylitol, and gulonic acid Reference to standard graphs obtained by plotting peak heights against weights[10] gave the proportions by weight of fucose xylose.glucuronic acid as 3 75·1.1:1 3

The calcium-insoluble material (50 mg) was hydrolysed Mannuronic acid and guluronic acid, and their respective lactones, were the major constituents of the hydrolysate, with trace quantities of fucose and xylose (paper chromatography, solvents *2* and *3*) A second portion of the insoluble material (100 mg) was heated for 6 h under reflux with 4% methanolic hydrogen chloride The derived solution was neutralised with silver carbonate, and the filtrate and methanolic washings of the silver salts were combined and evaporated to dryness The resulting solid was dissolved in water (1 ml), and the esterified acid residues were reduced as above. Hydrolysis of the product gave mannose and gulose (solvent *5*). A second paper, run in solvent *1* and sprayed with glucose oxidase[14], gave a single, pink spot having the mobility of mannose; this spot appeared about 3 h after the paper had been sprayed, as did a control spot of mannose. No evidence for the presence of glucose could be obtained. Ionophoresis in borate buffer gave two spots, M_G 0.59 and 0.46 (ref. 16), identical, respectively, with mannose and gulose run as controls.

Insoluble residue. — Paper chromatography of a formic acid hydrolysate of the dried, insoluble residue (50 mg) (total dry weight, 17 g) gave glucose (major), fucose, xylose, and a slower spot R_{Fuc} 0.56 and 0.64 (solvents *1* and *2*, respectively). A portion (0.3 g) was added to ice-cold sulphuric acid (80% w/w; 0.3 ml), and the mixture was kept for 18 h at 20°. It was then diluted to give an acid concentration of 2N, heated in a sealed tube for 5 h at 100°, neutralised with barium carbonate, and filtered. The filtrate was concentrated, and examined by paper chromatography. Glucose and trace quantities of fucose and xylose were detected. The glucose was confirmed with glucose oxidase[14].

Extraction of the insoluble residue with ammonium oxalate–oxalic acid. — The wet, insoluble residue (15 g, dry weight) was extracted with a solution of ammonium oxalate and oxalic acid (each 0.25% by weight; pH 2.8) for 20 h at 80°. The residual, insoluble material *C* (dry weight, 12 g) was removed by centrifugation, and the solution was dialysed until free from oxalate. It was then concentrated and freeze-dried to a dark solid (3 g). This gave a positive test for uronic acid[15], and hydrolysis of a portion (50 mg) gave fucose, xylose, and glucuronic acid. Attempts to purify the rest of this material were unsuccessful.

Mild treatment of insoluble residue C *with chlorite, followed by alkali extraction.* — The insoluble residue *C* (6 g, dry weight) in dilute acetic acid (250 ml, containing 1 ml of glacial acetic acid) was treated with sodium chlorite (3 g) hourly, for a total of 6 h. The chlorite solution was dialysed until free from Cl^- (10 days). Concentration of the solution from the dialysis sac gave a cream glass *D* (1 g, carbohydrate content, *ca.* 12%). The insoluble, off-white, gelatinous residue, separated from the chlorite solution by centrifugation, was washed with water (3×50 ml). It was then stirred with 6N potassium hydroxide (125 ml) for 2 days at room temperature. A residue was again removed by centrifugation, washed until neutral, and freeze-dried to a white, fibrous solid *E* (305 mg, carbohydrate content, 100%). The supernatant liquid was acidified (pH 5) with glacial acetic acid. The derived, white precipitate was freed from adsorbed alkali by dialysis, and the resulting slurry was freeze-dried to a white solid *F* (50 mg, carbohydrate content, 100%). The acidic, supernatant solution was dialysed, concentrated, and freeze-dried to an off-white solid *G* (40 mg, carbohydrate content, 96%).

A portion of each of the solids *D*, *E*, *F*, and *G* was hydrolysed, and the hydrolysates were examined by paper chromatography. That from *D* gave spots corresponding to a variety of amino acids (spray *b*) and small proportions of fucose, xylose, and acidic oligosaccharides (sprays *a* and *c*). The major spot from all the other materials had the mobility of glucose (confirmed with glucose oxidase), but trace amounts of fucose, xylose, and acid spots were detected in the hydrolysates of *G* and *F*.

Enzymic hydrolyses of glucans E, F, *and* G. — (*a*) *β-(1→3)-*D*-Glucanase.* Glucan *E* (14.4 mg), *F* (9 mg), *G* (5 mg), laminarin (9.4 mg), and lichenin (23.3 mg), each in citrate buffer at pH 4.5 (1 ml), were separately incubated with a specific β-(1→3)-D-glucanase (9 mg in each) (kindly given by Dr E T. Reese) at 38° After 4 h, portions (0 5 ml) were removed, diluted (2 ml), and heated to boiling for 5 min. The cooled solution was treated with Bio-deminrolit mixed-bed resin (carbonate form) to remove inorganic ions. The residual, incubation mixtures were treated similarly after incubation for 18 h All of the solutions, after concentration to dryness, were examined by paper chromatography. All (except those from *E* which gave only faint spots) gave strong spots for glucose (confirmed with glucose oxidase[14]), and all except *E* (including those from laminarin and lichenin) gave a single, oligosaccharide spot, R_G 0.55 (*cf.* laminaribiose and cellobiose, R_G 0 72 and 0 59, respectively) (solvent *2*) All of the spots were considerably stronger in the 18-h incubates

(*b*) *Cellulase.* Glucans *E* (8 mg), *F* (6 mg), and lichenin (6 6 mg) in citrate buffer at pH 4.5 (1 ml) were each incubated with cellulase (4 mg) (Sigma) for 4 and 18 h at 47° as above Paper chromatography revealed only glucose in all of the incubates Again, the spots from the 18-h incubation were more intense than those from the 4-h incubation No oligosaccharides were revealed Trace quantities of galactose were detected in the cellulase

ACKNOWLEDGMENT

The authors are grateful to the Wellcome Trust for the gift of the gas chromatograph

REFERENCES

1 A brief account of this work has been presented at the VIth International Seaweed Symposium at Santiago, Spain, September, 1968
2 R G S Bidwell, *Can J Botany*, 45 (1967) 1557
3 See Elizabeth Percival and R H McDowell, *Chemistry and Enzymology of Marine Algal Polysaccharides*, Academic Press, London, 1967
4 W A P Black and E T Dewar, *J Marine Biol Assoc U K*, 28 (1949) 673
5 E G V Percival and A G Ross, *J Chem Soc*, (1950) 717
6 F G Fischer and H Dorfel, *Z Physiol Chem*, 301 (1955) 224, 302 (1955) 186
7 E G V Percival and A G Ross, *J Chem Soc*, (1949) 3041
8 A Weissbach and J Hurwitz, *J Biol Chem*, 234 (1959) 705
9 B. Larsen, A Haug, and T. J Painter, *Acta Chem Scand*, 20 (1966) 219
10 E Percival, *Carbohyd Res*, 7 (1968) 272
11 D J Bell, in *Modern Methods of Plant Analysis*, K Paech and M V Tracey (Eds), Vol 2, Springer, Berlin, 1955, p 9
12 M Dubois, K A Gilles, J K Hamilton, P A Rebers, and F Smith, *Anal Chem*, 28 (1956) 350
13 A S Jones and D S Letham, *Chem Ind* (London), (1954) 662
14 M R Salton, *Nature*, 186 (1960) 966
15 B Tollens, *Ber*, 41 (1908) 1788
16 D W Drummond, E L Hirst, and E Percival, *Chem Ind* (London), (1958) 1088

Carbohydrate Research
Elsevier Publishing Company Amsterdam
Printed in Belgium

ASPECTS OF THE P.M.R. SPECTRA OF BRANCHED-CHAIN METHYL GLYCOPYRANOSIDES*

R. D. King and W. G. Overend

Department of Chemistry, Birkbeck College (University of London), Malet Street, London, W.C.1 (Great Britain)

(Received November 25th, 1968)

ABSTRACT

The chemical shifts and proton coupling constants of various methyl *O*-isopropylideneglycopyranosides have been measured. It has been shown that the variation in the chemical shift of the *endo*-methyl group of the isopropylidene residue provides a convenient method for the determination of the configuration of branched-chain glycopyranosides.

INTRODUCTION

A series of investigations reported from this laboratory has been concerned with the preparation[1-6] of branched-chain sugars and with the assignment of configuration at the branch point[7]. Configurational studies have involved an examination of intramolecular hydrogen-bonding by infrared spectral measurements and of the chromatographic mobilities of the branched-chain glycosides in solvent systems incorporating benzeneboronic acid. Also, we have studied the p.m.r. spectra of a range of glycosides of this type to evaluate the applicability of such measurements in assignments of configuration at the site of branching. The present report describes the results obtained.

DISCUSSION

The spectra obtained were consistent with the structures assigned to the glycosides on chemical evidence, but it was not possible to assign complete structures on the basis of the n.m.r. spectral measurements alone. However, one trend in the spectra of a series of *O*-isopropylidenated branched-chain glycosides was particularly noticeable. This concerns the chemical shifts of the methyl groups of the isopropylidene residue and can be used as a means of determining the configuration at the branch point. In Table I, the chemical shifts are listed for the *endo*- and *exo*-methyl groups of the isopropylidene group for a series of glycosides (see Table I, compounds **1–17**) having the general formulae *A*, *B*, and *C*. The *endo*-methyl group can be expected to be deshielded by the pyranoid ring and so is assigned to the downfield signal[8,9]. It can be seen from the values given in Table I that the chemical shift of

---

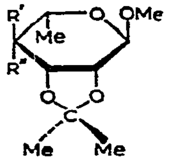
*Dedicated to Professor Stanley Peat, F.R.S.

the *exo*-methyl group (high-field signal) is fairly constant for all of the glycopyranosides examined. The *endo*-methyl signal, however, has a fairly constant value (τ *ca.* 8.53) when the branching substituent is *cis* to the isopropylidene ring (compounds **13**–**17**) and a lower value (τ *ca.* 8.42) when the substituent is *trans* to the isopropylidene ring (compounds **6**–**12**) As the variation within each group of branched-chain glycosides is fairly large, a comparison of substances epimeric at the branch-point is to be recommended It is notable that, for the compounds studied, the nature of the branching group has little effect on the signals

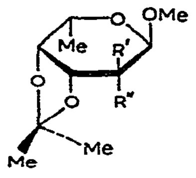
TABLE I

CHEMICAL SHIFTS OF ISOPROPYLIDENE SIGNALS

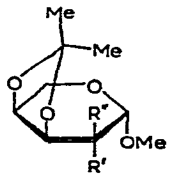
Compound No	*Reference for preparation*	*Chemical shift (τ) of methyl groups of isopropylidene residue*	
		endo	exo
1 (*A*, $R' = OH$, $R'' = H$)	10	8.48	8.65
2 (*A*, $R' = H$, $R'' = OH$)	11	8.42	8.62
3 (*B*, $R' = OH$, $R'' = H$)	12	8.48	8.63
4 (*C*, $R' = OH$, $R'' = H$)	13	8.46	8.64
5 (*C*, $R' = H$, $R'' = OH$)	11	8.44	8.63
6 (*A*, $R' = CH_2NO_2$, $R'' = OH$)	6	8.44	8.62
7 (*A*, $R' = Me$, $R'' = OH$)	—	8.43	8.66
8 (*B*, $R' = CH_2NO_2$, $R'' = OH$)	6	8.42	8.63
9 (*C*, $R' = CH_2NO_2$, $R'' = OH$)	6	8.40	8.61
10 (*C*, $R' = Me$, $R'' = OH$)	1	8.40	8.62
11 (*A*, $R' = CH_2NHAc$, $R'' = OH$)	6	8.42	8.65
12 (*C*, $R' = CH_2NH_2$, $R'' = OH$)	6,14	8.45	8.66
13 (*A*, $R' = OH$, $R'' = CH_2NO_2$)	6	8.51	8.65
14 (*A*, $R' = OH$, $R'' = Me$)	—	8.50	8.65
15 (*B*, $R' = OH$, $R'' = CH_2NO_2$)	6	8.54	8.64
16 (*C*, $R' = OH$, $R'' = CH_2NO_2$)	6	8.52	8.62
17 (*B*, $R' = OH$, $R'' = Me$)	—	8.56	8.68



(A)



(B)



(C)

From Table I (compounds **1**–**5**), it can be seen that an adjacent hydroxyl group which is *cis* to the isopropylidene ring has only a small, additional, deshielding effect on the *endo*-methyl group.

Since the chemical shift of the *endo*-methyl group of the acetal ring seems to be dependent on the configuration at the branch-point in the glycoside, it is postulated that when the configuration is that in which the substituent is *cis* to the adjacent isopropylidene group, then, if there is a 1,3-diaxial interaction between the substituent and the isopropylidene ring, the preferred conformation will be a skew form rather than the more favoured of the chair forms. In the skew form, the *endo*-methyl group will be less deshielded than would be the case if the pyranoid ring had a chair conformation. Examination of the coupling constants (see Table II) supports a skew conformation for these substances.

TABLE II

COUPLING CONSTANTS[a] OF PYRANOID RING PROTONS OF BRANCHED-CHAIN GLYCOPYRANOSIDES

Compound No	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$
6	~1	6.0	—	—
7	~1	6.0	—	—
13[c]	5.2	7.1	—	—
15[d]	—	—	7.4	2.0
17	—	—	7.2	[b]
3	3.5	6.4	6.0	1.0

[a] In Hz [b] Unresolved signal [c] Pyridine solution [d] Benzene solution

Using the Karplus equation with the modified parameters of Coxon and Hall[15], an estimate of the dihedral angle between protons attached to adjacent carbon atoms can be made (see Table III). The dihedral angle between the bridge-head protons of the conformationally stable compounds **3**, **6**, and **7** is the same, there being a negligible dependence on the nature of the substituent. However, in the compounds for which a skew conformation is postulated, this dihedral angle is reduced by 5–7°, which means that further flattening of the ring occurs for compounds **13**, **15**, and **17**.

TABLE III

DIHEDRAL ANGLES[a] CALCULATED FROM *J* VALUES LISTED IN TABLE II

Compound No	H_1H_2	H_2H_3	H_3H_4	H_4H_5
6	73	38	—	—
7	73	38	—	—
13	44	31	—	—
15	—	—	28	65
17	—	—	30	—
3	54	35	38	73

[a] In degrees

Three new branched-chain methyl glycopyranosides (**7**, **14**, and **17**) are reported. They were prepared by standard procedures previously developed. They provide a test of the principle now reported, because the configurations as determined by consideration of the chemical shifts of the *endo*-methyl groups were identical with those indicated by examination of the chromatographic mobilities of the deacetonated glycosides Thus, deacetonation of compound **7** gave methyl 6-deoxy-4-*C*-methyl-α-L-talopyranoside, the paper-chromatographic mobility of which in butyl alcohol–ethanol–water (4 1 5) was increased when 1% of benzeneboronic acid was added to the solvent system This indicated the presence of a *cis,cis* triol system in the compound which consequently must have the L-*talo* configuration. The deacetonated products from compounds **14** and **17** showed no increase in paper-chromatographic mobility when examined in the same way, addition of benzeneboronic acid to the solvent had no effect This indicated that compound **14** had the L-*manno* configuration and compound **17** belonged to the L-*galacto* series.

EXPERIMENTAL

General methods — N m r spectra were measured at 60 MHz with a Varian A-60 n m r spectrometer with tetramethylsilane as internal reference Unless stated otherwise, deuteriochloroform was used as solvent Spectra were measured at ambient temperature on solutions containing 50 mg of compound in ~0 5 ml of solvent Spectral analyses are first-order, and the *J* values recorded refer to the observed splittings

Paper-chromatographic separations were effected on Whatman No. 1 paper The chromatograms were developed by the descending method to give a front which had moved about 40 cm from the start line The solvent system was butyl alcohol–ethanol–water (4 1 5, *v/v/v*) alone (*A*) and containing 1% of benzeneboronic acid (*B*)

Melting points and boiling points are uncorrected Optical rotations were measured at room temperature Solutions were evaporated below 50° under diminished pressure

Methyl 1',4-anhydro-6-deoxy-4-C-hydroxymethyl-2,3-O-isopropylidene-α-L-mannopyranoside — Diazomethane (1 g) in ether (100 ml) was added to a solution of methyl 6-deoxy-2,3-*O*-isopropylidene-α-L-*lyxo*-hexopyranosid-4-ulose[11] (1 3 g) in methanol (25 ml) at room temperature After storage for 12 h at 4° and subsequently for 24 h at room temperature, the solution was evaporated The residue distilled as a clear syrup (1 g, 85%), b.p. 72°/0 1 mm, $[\alpha]_D$ −89° (*c* 1 2, dichloromethane)

Anal Calc for $C_{11}H_{18}O_5$ C, 57 4, H, 7 9 Found C, 57 2, H, 8 0.

Methyl 6-deoxy-2,3-O-isopropylidene-4-C-methyl-α-L-mannopyranoside (**14**) — The 1'4,-anhydride (0 5 g) described above was heated under reflux for 1 5 h with an excess of lithium aluminium hydride in ether Excess of the hydride was destroyed by addition of water, and the ethereal layer was separated by decantation and dried (Na_2SO_4) Filtration and subsequent evaporation of the solution afforded a clear syrup (0 4 g, 80%), b p 78°/0 1 mm, $[\alpha]_D$ −70° (chloroform)

Anal Calc for $C_{11}H_{20}O_5$ C, 56.9; H, 8 7. Found C, 56 8; H, 8 9.

Methyl 6-deoxy-2,3-O-isopropylidene-4-C-methyl-α-L-talopyranoside (**7**) — Methyl 6-deoxy-2,3-*O*-isopropylidene-α-L-*lyxo*-hexopyranosid-4-ulose[11] (3 g) in ether

(50 ml) was added to an ethereal solution of methylmagnesium iodide [prepared from methyl iodide (7 74 g) and magnesium (1 44 g) in dry ether (50 ml)] After heating under reflux for 1 5 h, the product was isolated in standard fashion as a clear syrup (1 49 g, 45%), b p 60°/0 06 mm, $[\alpha]_D$ −23° (*c* 1 2, chloroform).

Anal Calc for $C_{11}H_{20}O_5$· C, 56 9, H, 8 7. Found C, 56 9, H, 8 8.

Methyl 6-deoxy-3,4-O-isopropylidene-2-C-methyl-α-L-galactopyranoside (**17**) — Methyl 6-deoxy-3,4-*O*-isopropylidene-α-L-*lyxo*-hexopyranosidulose[11] (1 g) in dry ether (50 ml) was added dropwise with stirring to a cold solution of a Grignard reagent prepared from methyl iodide (1 g), magnesium (0 4 g), and ether (30 ml) The reaction was conducted, and the product isolated, as described above Compound **17** was obtained as a colourless syrup (0 77 g, 72%), b p 60°/0 2 mm, $[\alpha]_D$ −70° (chloroform)

Anal Calc for $C_{11}H_{20}O_5$ C, 56 9, H, 8 7 Found C, 56 7; H, 8.7

Deacetonation of compounds **7**, **14**, *and* **17** — The *O*-isopropylidene compound (~3 mmoles) was dissolved in methanol (30 ml) containing conc hydrochloric acid (0 3 ml), and the solution was warmed for 1 h at 50° The cooled solution was neutralised with silver carbonate and filtered, and the filtrate was evaporated The products had the following constants

Compound	*Yield* (%)	$[\alpha]_D$	*Chromatographic mobility* (R_F)	
			Solvent A	*Solvent B*
Methyl 6-deoxy-4-*C*-methyl-α-L-talopyranoside	89	−94° (EtOH)	0 73	0 92
Methyl 6-deoxy-4-*C*-methyl-α-L-mannopyranoside	30	—	0 70	0 72
Methyl 6-deoxy-2-*C*-methyl-α-L-galactopyranoside[a,b]	81	−151° (MeOH)	0 82	0 84

[a]Found C, 49 6, H, 8 2 Calc for $C_8H_{16}O_5$ C, 49 99, H, 8 4 [b]m p 155° (from ethyl acetate)

ACKNOWLEDGMENT

The n m r. measurements were carried out at the School of Pharmacy (University of London), and Professor W B Whalley is thanked for providing facilities for these determinations

REFERENCES

1 J S Burton, W G Overend, and N R Williams, *J Chem Soc*, (1965) 3433
2 W G Overend and N R Williams, *J Chem Soc*, (1965) 3446
3 A A Feast, W G Overend, and N R Williams, *J Chem Soc*, (1965) 7378
4 R D King, W G Overend, J Wells, and N R Williams, *Chem Commun*, (1967) 726
5 R F Butterworth, W G Overend, and N R Williams, *Tetrahedron Lett*, (1968) 3239
6 S W Gunner, S McNally, R D King, W G Overend, and N R Williams, unpublished results S W Gunner, Ph D Thesis (University of London), 1964, R D King, Ph D Thesis (University of London), 1968

7 R. J Ferrier, W G. Overend, G A Rafferty, H M Wall, and N R. Williams, *Proc. Chem Soc*, (1963) 133

8 N. Baggett, K W Buck, A B Foster, R J Jefferis, B H Rees, and J M. Webber, *J Chem Soc.*, (1965) 3382.

9 J G. Buchanan and A R Edgar, *Chem Commun*, (1967) 29.

10 P A Levene and J Compton, *J Amer Chem Soc*, 57 (1935) 2306.

11 P. M Collins and W G Overend, *J. Chem. Soc*, (1965) 1912

12 E. E. Percival and E G V Percival, *J. Chem. Soc.*, (1950) 690

13 M A Oldham and J Honeyman, *J Chem Soc*, (1946) 986

14 R J. Ferrier, W G Overend, G A Rafferty, H M Wall, and N R Williams, *J Chem Soc (C)*, (1968) 1091.

15 B Coxon and L D Hall, *Tetrahedron*, 20 (1964) 1685

Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

SOME STRUCTURAL FEATURES OF THE MUCILAGE FROM THE BARK OF *Ulmus fulva* (SLIPPERY ELM MUCILAGE)*

R. J. Beveridge, J. F. Stoddart, W. A. Szarek, and J. K. N. Jones
Department of Chemistry, Queen's University, Kingston, Ontario (Canada)

(Received September 30th, 1968)

ABSTRACT

Slippery elm mucilage contains residues of L-rhamnose, D-galactose, 3-*O*-methyl-D-galactose, and D-galacturonic acid. The methylated polysaccharide yields 3-*O*- and 4-*O*-methyl-L-rhamnose, 2,3,4,6-tetra- and 2,3,6-tri-*O*-methyl-D-galactose, and 2,3,4-tri- and 2,3-di-*O*-methyl-D-galacturonic acid, in addition to trace amounts of 2,3,4-tri- and 3,4-di-*O*-methyl-L-rhamnose and 2,4,6-tri-*O*-methyl-D-galactose. Borohydride reduction of the periodate-oxidised polysaccharide yields a polyalcohol, which, on partial hydrolysis with acid, affords *O*-(3-*O*-methyl-D-galactopyranosyl)-(1→4)-*O*-(3-*O*-methyl-D-galactopyranosyl)-(1→4)-*O*-(3-*O*-methyl-D-galactopyranosyl)-(1→4)-L-rhamnose Mild, acid hydrolysis yields a Smith-degraded polysaccharide Methylation analyses are reported for the polyalcohol and for the Smith-degraded polysaccharide It is concluded that the polysaccharide contains chains of 3-*O*-methyl-D-galactose residues attached to the C-4 positions of certain L-rhamnose residues, and that 3-*O*-methyl-D-galactose residues occur in some cases as non-reducing end-groups D-Galactose is attached as single residues or as 4-*O*-substituted residues to the C-3 positions of some L-rhamnose residues This evidence indicates that the polysaccharide is more highly branched than was at one time supposed

INTRODUCTION

Earlier investigations[1-7] on the structural features of a water-soluble polysaccharide obtained by autohydrolysis of slippery elm mucilage have shown it to contain D-galacturonic acid[1], L-rhamnose[1], D-galactose[1], and 3-*O*-methyl-D-galactose[6], in the approximate molar proportions[6] of 2·2 1·1, respectively. Graded, acid hydrolysis afforded an aldobiouronic acid containing D-galacturonic acid and L-rhamnose[1], which was later shown[2] to be 2-*O*-(α-D-galactopyranosyluronic acid)-L-rhamnose In addition, examination[4] of the methylated polysaccharide, obtained after methylation with methyl iodide and thallium hydroxide and thallium ethoxide[8], showed that it contained 3,4-di-(2 parts) and 4-*O*-methyl-L-rhamnose (2 parts), 2,3,4,6-tetra-(2 parts), 2,3,6-(1 part) and 2,4,6-tri-*O*-methyl-D-galactose (1 part), and

---

*Dedicated to Professor Stanley Peat, F R S

2,3,4-tri-(a trace) and 2,3-di-*O*-methyl-D-galacturonic acid (4 parts) Later, it was reported[5] that rhamnose was also present in the *O*-methyl derivative of the polysaccharide Prior to the characterisation[6] of 3-*O*-methyl-D-galactose, a possible structural fragment (**1**) was proposed[3] for the polysaccharide In addition to reporting a re-investigation of the methylated polysaccharide, this paper presents evidence that chains of 3-*O*-methyl-D-galactose residues are attached to L-rhamnose residues in the polysaccharide

RESULTS AND DISCUSSION

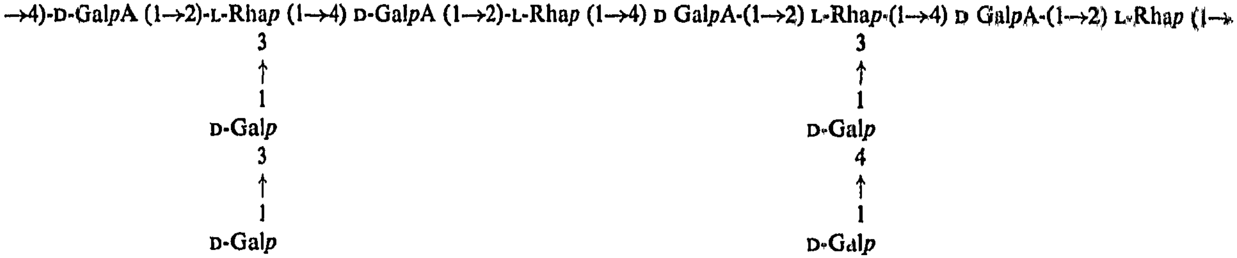
After extraction of the elm bark with methanol, acetone, and 80% aqueous methanol, the crude product was obtained by extraction with hot water Purification was effected through precipitation as a Cetavlon complex and then by two precipitations from ethanol Most of the proteinaceous material was removed by filtration after heating the crude product in 0.1M phosphate buffer (pH 6 9) on a steam-bath for 24 h to denature the protein This procedure is somewhat milder than that described previously[2] to obtain the polysaccharide The present sample was shown to contain D-galacturonic acid (36%), and L-rhamnose, D-galactose, and 3-*O*-methyl-D-galactose, in the approximate molar proportions of 1 00 2 70 2 08* Since the sample migrated as a single peak on free-boundary electrophoresis, it was judged suitable for structural investigations

It is worthy of note that sucrose was isolated and characterized from the organic-solvent extractions of the bark; Schirmer[9] reported that glucose and fructose are components of the acid hydrolysate of the bark.

A re-examination of the methylated polysaccharide indicated the presence of 3-*O*-(+/2) and 4-*O*-methyl-L-rhamnose (+ +), 2,3,4,6-tetra-(+ + +) and 2,3,6-tri-*O*-methyl-D-galactose (+ + +), and 2,3,4-tri-(+/2) and 2,3-di-*O*-methyl-D-galacturonic acid (+ +), in addition to trace amounts of 2,3,4-tri- and 3,4-di-*O*-methyl-L-rhamnose and 2,4,6-tri-*O*-methyl-D-galactose Comparison of the g l c patterns given by the methyl glycosides of the *O*-methyl sugars from this methylated polysaccharide with that from a sample[4] of an *O*-methyl derivative of the mucilage obtained previously showed that they were almost identical. The earlier sample[4] contained *slightly* more 3,4-di- and *slightly* less 4-*O*-methyl-L-rhamnose, in addition to small amounts of rhamnose (*cf* ref. 5) As well as showing that 3,4-di-*O*-methyl-L-rhamnose is present in much smaller proportion than was reported originally[4], these results indicate only trace amounts of 2,4,6-tri-*O*-methyl-D-galactose, which may be separated from the 2,3,6-tri-*O*-methyl isomer by double development in solvent (*b*) (see Experimental) This means that D-galactose and 3-*O*-methyl-D-galactose residues are either terminal, non-reducing groups and/or 4-*O*-substituted (assuming pyranose forms are

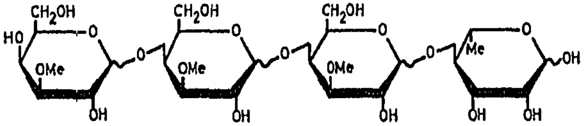
---

*The configurational assignments of these sugars were established in the earlier investigations[1–6]. The discrepancy between these molar proportions and those reported earlier[6] has not yet been explained

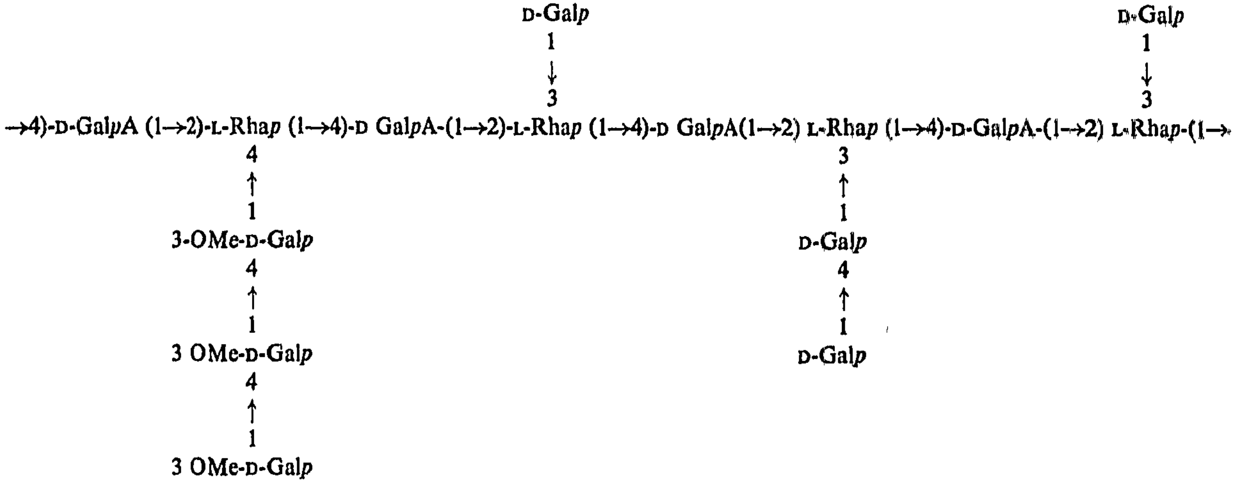


$$\begin{array}{lclcl}
\rightarrow 4)\text{-D-Gal}p\text{A }(1\rightarrow 2)\text{-} & \text{L-Rha}p & (1\rightarrow 4)\ \text{D-Gal}p\text{A }(1\rightarrow 2)\text{-L-Rha}p\ (1\rightarrow 4)\ \text{D Gal}p\text{A-}(1\rightarrow 2)\ & \text{L-Rha}p & \text{-}(1\rightarrow 4)\ \text{D Gal}p\text{A-}(1\rightarrow 2)\ \text{L-Rha}p\ (1\rightarrow \\
 & 3 & & 3 & \\
 & \uparrow & & \uparrow & \\
 & 1 & & 1 & \\
 & \text{D-Gal}p & & \text{D-Gal}p & \\
 & 3 & & 4 & \\
 & \uparrow & & \uparrow & \\
 & 1 & & 1 & \\
 & \text{D-Gal}p & & \text{D-Gal}p &
\end{array}$$

1



2



$$\begin{array}{lclclclcl}
 & & & \text{D-Gal}p & & & & \text{D-Gal}p & \\
 & & & 1 & & & & 1 & \\
 & & & \downarrow & & & & \downarrow & \\
 & & & 3 & & & & 3 & \\
\rightarrow 4)\text{-D-Gal}p\text{A }(1\rightarrow 2)\text{-} & \text{L-Rha}p & (1\rightarrow 4)\text{-D Gal}p\text{A-}(1\rightarrow 2)\text{-} & \text{L-Rha}p & (1\rightarrow 4)\text{-D Gal}p\text{A}(1\rightarrow 2)\ & \text{L-Rha}p & (1\rightarrow 4)\text{-D-Gal}p\text{A-}(1\rightarrow 2)\ & \text{L-Rha}p & \text{-}(1\rightarrow \\
 & 4 & & & & 3 & & & \\
 & \uparrow & & & & \uparrow & & & \\
 & 1 & & & & 1 & & & \\
 & \text{3-OMe-D-Gal}p & & & & \text{D-Gal}p & & & \\
 & 4 & & & & 4 & & & \\
 & \uparrow & & & & \uparrow & & & \\
 & 1 & & & & 1 & & & \\
 & \text{3 OMe-D-Gal}p & & & & \text{D-Gal}p & & & \\
 & 4 & & & & & & & \\
 & \uparrow & & & & & & & \\
 & 1 & & & & & & & \\
 & \text{3 OMe-D-Gal}p & & & & & & &
\end{array}$$

3

present in the polysaccharide) Very occasionally, some D-galactose residues are 3-*O*-substituted. If there are alternating chains of 2-*O*-substituted L-rhamnopyranose and 4-*O*-substituted D-galactopyranosyluronic acid residues in the polysaccharide, as shown in structural fragment **1**, D-galactose and 3-*O*-methyl-D-galactose residues, or chains containing these residues, are attached to the C-3, and occasionally to the C-4, positions of L-rhamnopyranose residues

An idea of the distribution of D-galactose and 3-*O*-methyl-D-galactose residues in side chains attached to the C-3 and C-4 positions of L-rhamnose residues was obtained by isolation of an oligosaccharide, containing 3-*O*-methyl-D-galactose and rhamnose, from the partial, acid hydrolysate of the polyalcohol afforded on borohydride reduction of the periodate-oxidized polysaccharide A partial n m r spectrum of the oligosaccharide in deuterium oxide is shown in Fig 1 The doublet at τ 8 53 is assigned to the methyl group of the rhamnose residue, and the signals at τ 6 37 and 6.40 to the methoxyl groups of the 3-*O*-methylgalactose residues Integration of the spectrum suggested that the oligosaccharide is a trisaccharide or a tetrasaccharide containing two 3-*O*-methyl-D-galactose residues to one L-rhamnose residue, or three 3-*O*-methyl-D-galactose residues to one L-rhamnose residue, respectively An unequivocal decision between these two possibilities was not possible on account of the appearance of an HOD peak at τ 5 2 The observation that the molar proportion of 3-*O*-methyl-D-galactose to L-rhamnose is 2 7:1* in the oligosaccharide, and the fact that it is homogeneous on paper chromatograms in four solvent systems, lends support to the proposal it is a tetrasaccharide The fact that borohydride reduction, followed by acid hydrolysis, gives rhamnitol proves that the reducing end-group is a rhamnose residue Methylation analysis shows that the *O*-methyl derivative contains 2,3,4,6-tetra- and 2,3,6-tri-*O*-methyl-D-galactose and 2,3-di-*O*-methyl-L-rhamnose, in the approximate molar proportions of 1 2 1 Assuming that all of the residues are in

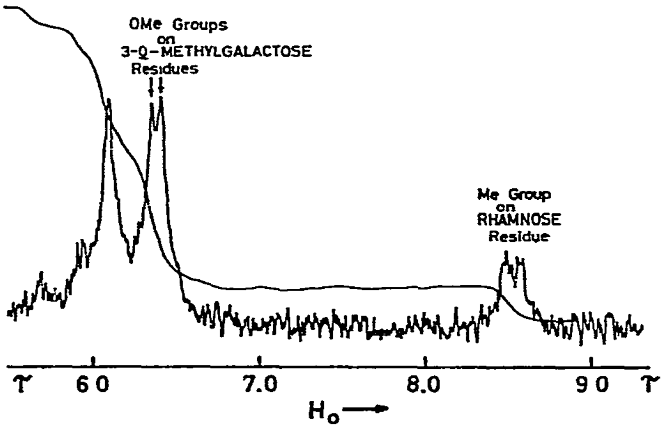


Fig. 1 Partial n m r spectrum of tetrasaccharide 2 in deuterium oxide

---

Note added in proof Subsequent work has yielded a ratio of 2 34 1, a value which suggests that the fragment may be a trisaccharide the pyranose form, the experimental evidence is consistent with the formulation of the oligosaccharide as *O*-(3-*O*-methyl-D-galactopyranosyl)-(1→4)-*O*-(3-*O*-methyl-D-galactopyranosyl)-(1→4)-*O*-(3-*O*-methyl-D-galactopyranosyl)-(1→4)-L-rhamnose (2) Although the configurations of the glycosidic linkages are unknown, the low, positive value (+13°) for the specific rotation would suggest that at least some of them are β-D The isolation of this component from the partial, acid hydrolysate of the polyalcohol indicates that the 6-deoxyhexopyranosyl bond is more labile than hexopyranosyl bonds to acid-catalysed hydrolysis Also, it may be concluded, in conjunction with the methylation analysis on the methylated polysaccharide, that chains of 3-*O*-methyl-D-galactose residues are attached to the C-4 positions of some L-rhamnose residues.

In addition to small two, three, and four carbon fragments, the polyalcohol was shown to contain D-galacturonic acid, 3-*O*-methyl-D-galactose, and L-rhamnose, but only traces of D-galactose Thus, nearly all of the D-galactose residues are periodate vulnerable and therefore either occur as non-reducing end-groups or as 4-*O*-substituted residues A few are periodate-resistant and so are almost certainly 3-*O*-substituted. These conclusions are consistent with the methylation evidence for the polysaccharide An examination of the methylated polyalcohol* showed the presence of 3,4-di- (trace) and 3-*O*-methyl-L-rhamnose (+), 2,3,4,6-tetra- (+), and 2,3,6- (+ + +) and 2,4,6-tri-*O*-methyl-D-galactose (trace), and 2,3-di-*O*-methyl-D-galacturonic acid (trace) The presence of the *O*-methyl derivative of D-galacturonic acid indicates that the periodate oxidation of 4-*O*-substituted D-galacturonic acid residues in the polysaccharide is complete and is to be compared with the incomplete oxidation of 4-*O*-substituted D-glucuronic acid residues in gum arabic[10], and 4-*O*-substituted D-mannuronic and L-guluronic acid residues in alginic acid[11] The identification of some 2,3,4,6-tetra-*O*-methyl-D-galactose is proof that some periodate-resistant 3-*O*-methyl-D-galactose residues are non-reducing end-groups in the polysaccharide

Mild hydrolysis of the polyalcohol[12] with N sulphuric acid at 23 5° yielded a small proportion of non-dialysable material, which contained D-galacturonic acid, 3-*O*-methyl-D-galactose, and L-rhamnose. Methylation analysis showed the presence of 2,3-di-*O*-methyl-L-rhamnose (+) in addition to those *O*-methyl sugars already reported in the methylated polyalcohol, except that the proportion of 4-*O*-methyl-L-rhamnose was greatly diminished, and 2,4,6-tri-*O*-methyl-D-galactose was not present These observations are further evidence that chains of 3-*O*-methyl-D-galactose residues are attached to the C-4 position of certain L-rhamnose residues, whilst some D-galactose or 4-*O*-substituted D-galactose residues are attached to the C-3 positions of other L-rhamnose residues. Trace amounts of 3,4-di-*O*-methyl-L-rhamnose in the methylated polysaccharide indicate that a very few L-rhamnose residues do not carry single residues or side-chains at the C-3 and C-4 positions The revised structural features of this polysaccharide are presented as a fragment in array 3 Although the overall, molecular architecture of the polysaccharide remains to be elucidated, it is evident that it is more highly branched than was at one time supposed

---

*During methylation of the polyalcohol, care was taken to ensure that the reaction mixture was always basic.

EXPERIMENTAL

Melting points were determined on a Fisher–Johns apparatus and are uncorrected. Optical rotations were measured with a Perkin–Elmer Model 141 polarimeter at 23 ±1°. Infrared (i.r.) absorption spectra (KBr discs) were recorded on a Beckman model IR 5A spectrophotometer. Nuclear magnetic resonance (n.m.r.) spectra were measured at 60 MHz in deuterium oxide with sodium 4,4-dimethyl-4-silapentane-1-sulphonate (τ 10.00) as external standard. Paper chromatography was carried out on Whatman Nos. 1 and 3MM papers with the following solvent systems (v/v): (*a*) ethyl acetate–acetic acid–formic acid–water (18:3:1:4), (*b*) butanone–water–ammonia (*d* 0.88) (200:17:1), (*c*) butyl alcohol–ethanol–water (4:1:5, upper layer), (*d*) butyl alcohol–ethanol–water (3:1:1), (*e*) ethyl acetate–pyridine–water (10:4:3), (*f*) butyl alcohol–pyridine–water–benzene (5:3:3:1, upper layer). R_{Gal} and R_{Rha} values of sugars refer to distances moved relative to those of galactose and rhamnose, respectively. 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 (g.l.c.) (F and M chromatograph, type 402) of mixtures of methyl glycosides of *O*-methyl sugars was carried out at helium flow-rates of *ca.* 100 ml/min on columns of (*i*) 15% by weight of butane-1,4-diol succinate polyester on 60–80 mesh acid-washed Chromosorb W (110 × 0.4 cm) at 175°, and (*ii*) 15% by weight of ethylene glycol adipate polyester 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. Uronic acid percentages were estimated by the carbazole procedure[13], and molar ratios of neutral sugars were determined after their conversion[14] into their acetylated alditols, which were examined by g.l.c. on column (*i*) at 260°. Peak areas were measured by triangulation, and standard curves were obtained for known molar ratios of galactose to rhamnose, and 3-*O*-methylgalactose to rhamnose, after conversion into their acetylated alditols. Unless otherwise stated, polysaccharides were methylated successively with methyl sulphate and sodium hydroxide, and with methyl iodide and silver oxide, and methanolyses were performed under reflux for 6 h with 5% methanolic hydrogen chloride. Authentic specimens of 2,3,4-tri-, 2,3- and 3,4-di-, and 3- and 4-*O*-methyl-L-rhamnose, and 2,3,4,6-tetra- and 2,3,6- and 2,4,6-tri-*O*-methyl-D-galactose were available for comparison purposes on g.l.c. after conversion into their methyl glycosides. An authentic sample of the methyl ester of methyl 2,3,4-tri-*O*-methyl-α-D-galactopyranosiduronic acid was obtained on methylation of the methyl ester of methyl α-D-galactopyranosiduronic acid[15] with methyl sulphate and powdered sodium hydroxide in tetrahydrofuran[16]. An authentic mixture of methyl glycosides of the methyl ester of 2,3-di-*O*-methyl-D-galacturonic acid was obtained after methanolysis of methylated pectic acid[17] obtained from orange pectin.

Extraction of polysaccharide from elm bark. — Chips (1500 g) of elm bark [obtained from Botanical and Allied Products, S. B. Penwick and Company, 100 Church Street, New York (lot No. WHB-299)] were treated sequentially with methanol (2 × 6 l), acetone (6 l), and 80% aqueous methanol (2 × 6 l). The combined extracts were filtered and concentrated to a syrup (189 g, 12 6%) Crystallisation from acetone–water gave sucrose, m p. and mixed m p 185°, $[\alpha]_D$ +63 5° (*c* 2 2, water) Acid hydrolysis yielded glucose and fructose The chips were extracted five times by adding boiling water (20-l portions) The combined, aqueous extracts were filtered through linen, and a 10% solution of hexadecyltrimethylammonium bromide was added to the filtrate until precipitation of the complex was complete The precipitate was collected by filtration through linen After being washed with water, the complex was broken down in 10% sodium chloride solution (8 l) The precipitate, obtained on addition of ethanol (2 vol), was dissolved in water (8 l) and reprecipitated with ethanol (2 vol) The precipitate was washed twice with ethanol and twice with ether, and dried at 40° to give a mucilage (258 g, 17 9%)

The mucilage (30 g) was suspended in 0 1M phosphate buffer (pH 6 9) (1 2 l) and heated on a steam-bath for 24 h with two agitations in a Waring Blender after 8 h and after 12 h The protein-rich material[2], which precipitated during this time, was removed by centrifugation, washed twice with water, and dried *in vacuo* to give the product (5 2 g, 15%) The supernatant was dialysed against running tap-water for 48 h, concentrated to a volume of 2 l, and poured into ethanol (3 vol) The precipitate was washed twice with ethanol and twice with ether, and dried *in vacuo* to give the polysaccharide (22 5 g, 75%), $[\alpha]_D$ +60° (*c* 1 5, water) (Found N, 0 12, galacturonic acid anhydride, 36%; molar ratios of rhamnose–galactose–3-*O*-methylgalactose, 1 00 2 70 2 08) After dialysis of a 1% solution against 0 05M sodium tetraborate for 48 h, free-boundary electrophoresis on a Tiselius apparatus (Perkin–Elmer Model 38-A) at 150 volts and 20 mamps indicated the presence of one component A small "concentration peak" remained at the "starting line" and flattened out during the runs

Methylation of the polysaccharide — The polysaccharide (1 1 g) was methylated to give a product (510 mg), $[\alpha]_D$ −31 5° (*c* 1 7, chloroform) (Found OMe, 40 4%) Methanolysis of a sample (20 mg) of this product, followed by g l c of the mixture of methyl glycosides, gave the results shown in Table I Hydrolysis of the methyl glycosides with 0 5N sulphuric acid for 4 h on a steam-bath, followed by paper-chromatographic examination in solvents (*b*) and (*c*), confirmed the presence of the neutral *O*-methyl sugars identified by g l c

Methanolysis of a sample of a methylated polysaccharide prepared several years ago[4], followed by g l c of the mixture of methyl glycosides, gave the results shown in Table II Hydrolysis of the methyl glycosides as described above, followed by paper-chromatographic examination in solvents (*b*) and (*c*), indicated the presence of rhamnose in addition to the neutral *O*-methyl sugars identified by g l c

Preparation of the Smith-degraded polysaccharide — Solutions of the polysaccharide (10 28 g) in water (500 ml) and sodium metaperiodate (42 17 g) in water (500 ml) were mixed After 196 h, ethylene glycol (50 g) was added, and the solution was dialysed against running tap-water for 48 h Sodium borohydride (6 5 g) was added to the solution with stirring over a period of 12 h After stirring had been continued for a further 12 h, the excess of sodium borohydride was destroyed by

TABLE I

EXAMINATION OF METHANOLYSIS AND HYDROLYSIS PRODUCTS FROM METHYLATED POLYSACCHARIDE

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-rhamnose	0 49	0.51	—	Trace
3,4-Di-*O*-methyl-L-rhamnose	0.96	0.98	—	Trace
3-*O*-Methyl-L-rhamnose	3 32	3 24	0 61	+/2
4-*O*-Methyl-L-rhamnose	(3 72)[a]	3 64	0 65	++
2,3,4,6-Tetra-*O*-methyl D-galactose	1 75	1 73	0 88	+++
2,3,6-Tri-*O*-methyl-D-galactose	2 81 (3.72) (4 20)	2 99 (3 91) (4.35)	0 78[b]	+++
2,4,6-Tri *O*-methyl-D-galactose	(3 72) (4 20)	(3.91) (4 35)	0.78[b]	Trace
2,3,4-Tri *O*-methyl-D-galacturonic acid[c]	6 53	6 72	—	+/2
2,3-Di-*O*-methyl-D-galacturonic acid[c]	4 72, 17.0, 20.0	4.76, 17 3, 19 9	—	++

[a] Figures in parentheses indicate *T* values of components not completely resolved [b] Separated and identified by double development on paper in solvent (*b*). [c] As methyl ester methyl glycosides

TABLE II

EXAMINATION OF METHANOLYSIS AND HYDROLYSIS PRODUCTS FROM METHYLATED POLYSACCHARIDE[a]

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-rhamnose	0.46	0.48	—	Trace
3,4-Di-*O*-methyl-L-rhamnose	1.00	0.91	—	Trace
3-*O*-Methyl-L-rhamnose	3.24	3.15	0.61	+/2
4-*O*-Methyl-L-rhamnose	(3.73)[b]	3.55	0.67	++
L-Rhamnose	12.4	—	0.38	Trace
2,3,4,6-Tetra-*O*-methyl-D-galactose	1.72	1.70	0.90	+++
2,3,6-Tri-*O*-methyl-D-galactose	2.89 (3.73) (4.14)	2.87 (3.70) (4.17)	0.74[c]	+++
2,4,6-Tri-*O* methyl-D-galactose	(3.73) (4.14)	(3.70) (4.17)	0.74[c]	Trace
2,3,4-Tri-*O*-methyl-D-galacturonic acid[d]	6.49	6.75	—	+/2
2,3-Di-*O*-methyl-D-galacturonic acid[d]	6.72, 16.9, 19.0	4.62, 17.4, 18.65	—	++

[a] Sample used in earlier investigation (see ref 4). [b] Figures in parentheses indicate *T* values of components not completely resolved [c] Separated and identified by double development on paper in solvent (*b*) [d] As methyl ester methyl glycosides

addition of acetic acid, and the solution was dialysed against running tap-water for 48 h, concentrated to a small volume, and freeze-dried to give the polyalcohol (5.61 g), $[\alpha]_{\mathrm{D}}$ -28° (*c* 1.7, water) (Found: galacturonic acid anhydride, 7.7%, rhamnose:3-*O*-methylgalactose, 1:8.1).

The polyalcohol (2.40 g) was hydrolysed[12] with N sulphuric acid (100 ml) for 30 h at 23.5°, and the solution was neutralised with barium carbonate, filtered, passed through Rexyn-101 (H^+), concentrated to a small volume (*ca.* 50 ml), and dialysed against distilled water (2×4 l) for 24 h. The dialysate was concentrated to a syrup (1.62 g), which was not studied further, and the non-dialysable portion was freeze-dried to give the Smith-degraded polysaccharide (274 mg), $[\alpha]_{\mathrm{D}}$ -3.5° (*c* 1.8, water) (Found: galacturonic acid anhydride, 14%, rhamnose:3-*O*-methylgalactose, 0.60:1.00).

Methylation of the Smith-degraded polysaccharide. — The Smith-degraded polysaccharide (100 mg) was methylated to give a product (76 mg), $[\alpha]_{\mathrm{D}}$ -22° (*c* 2.1, chloroform) (Found: OMe, 36.0%). Methanolysis, followed by g.l.c. of the mixture of methyl glycosides, indicated the presence of 3,4- (trace) [(*i*) *T* 0.91, (*ii*) 0.90] and 2,3-di-(+) [(*i*) *T* 1.44, (*ii*) 1.45], and 3-(+) [(*i*) *T* 3.24, (*ii*) 3.20] and 4-*O*-methyl-L-rhamnose (trace) [(*i*) *T* (3.80), (*ii*) 3.62], and 2,3,4,6-tetra-(+) [(*i*) *T* 1.72, (*ii*) 1.75] and 2,3,6-tri-*O*-methyl-D-galactose (+++) [(*i*) *T* 2.88, (3.80), 4.21, (*ii*) 2.92, 3.90, 4.32], and 2,3-di-*O*-methyl-D-galacturonic acid methyl ester (trace) [(*i*) *T* 4.81, 17.6, 20.0, (*ii*) 4.72, 17.5, 20.1]. Hydrolysis of the methyl glycosides with 0.5N sulphuric acid for 4 h at 100°, followed by paper-chromatographic examination of the free sugars in solvents (*b*) and (*e*), confirmed these results.

Methylation of the polyalcohol. — The polyalcohol (760 mg) was methylated to give a product (230 mg), $[\alpha]_{\mathrm{D}}$ -48° (*c* 2.5, chloroform) (small i.r. absorption in the range 3400–3600 cm^{-1}). Methanolysis, followed by g.l.c. [column (*ii*)] of the mixture of methyl glycosides, indicated the presence of 3,4- (trace) (*T* 0.91) and 3-(+) (*T* 3.23) and 4-*O*-methyl-L-rhamnose (++) (*T* 3.62), and 2,3,4,6-tetra-(+) (*T* 1.77) and 2,3,6-tri-*O*-methyl-D-galactose (+++) (*T* 2.94, 3.88, 4.35), and 2,3-di-*O*-methyl-D-galacturonic acid methyl ester (trace) (*T* 4.62, 16.9, 19.9). Hydrolysis of the methyl glycosides with 0.5N sulphuric acid for 4 h at 100°, followed by paper-chromatographic examination of the free sugars in solvents (*b*) and (*c*), indicated the presence of 2,4,6-tri-*O*-methyl-D-galactose, in addition to those neutral *O*-methyl sugars identified by g.l.c.

Partial, acid hydrolysis of the polyalcohol. — The polyalcohol (2.5 g) was hydrolysed with 0.5N sulphuric acid (100 ml) for 1 h on a steam-bath. The cooled solution was neutralized with barium carbonate, filtered, treated with Rexyn-101 (H^+), and concentrated to a syrup (1.44 g). This syrupy mixture was dissolved in water (50 ml) and passed through a column of Duolite A-4 (OH^-) to remove the acidic components. The eluent was concentrated to a syrup (895 mg), which on paper-chromatographic examination in solvent (*f*) showed the presence of three components having R_{Gal} values of 1.63, 1.27, and 1.03 (corresponding R_{Rha} values were 0.86, 0.67, and 0.55, respectively) in addition to 3-*O*-methylgalactose and rhamnose. Since the component having R_{Gal} 1.27 was present in largest amount, it was purified by separation on Whatman No 3MM paper in solvents (*f*) and (*a*) successively to give an oligosaccharide (7 mg), $[\alpha]_D$ $+11° \rightarrow +13°$ (equil.) (*c* 0 7, water), which was homogeneous on paper chromatograms in four solvents [R_{Rha} 0 25, in solvent (*c*), 0 27 in solvent (*d*), 0 86 in solvent (*e*), and 0 59 in solvent (*f*)] N m r data in deuterium oxide τ 8 54 (doublet, CH_3 group on rhamnose residue); 6 37, 6 44, OCH_3 groups on 3-*O*-methylgalactose residues) On hydrolysis with N sulphuric acid for 7 h at 100°, the oligosaccharide yielded 3-*O*-methylgalactose and rhamnose in the molar proportions of 2 7 1. Borohydride reduction of the oligosaccharide, followed by acid hydrolysis and g l c of the derived acetates, showed the presence of rhamnitol and 3-*O*-methylgalactose A portion (6 mg) of the oligosaccharide was methylated twice with methyl iodide and silver oxide in *N*,*N*-dimethylformamide[18] G l c of the methanolysis products revealed peaks having retention times corresponding to those of the methyl glycosides of 2,3,4,6-tetra- (+) [(*i*) *T* 1.72, (*ii*) 1 77] and 2,3,6-tri-*O*-methyl-D-galactose (+ +) [(*i*) *T* 3 00, 4.04, 4 35, (*ii*) 3 00, 4 10, 4.45], and 2,3-di-(+) [(*i*) *T* 1 45, 1 72, (*ii*) 1.43, 1 80] and 3-*O*-methyl-L-rhamnose (trace) [(*i*) *T* 3 30, (*ii*) 3 37] Hydrolysis of the methyl glycosides, followed by paper chromatography in solvent (*b*), confirmed the presence of 2,3,4,6-tetra-(R_G 0 84) and 2,3,6-tri-*O*-methyl-D-galactose (R_G 0 58), and 2,3-di-*O*-methyl-L-rhamnose (R_G 0 68)

ACKNOWLEDGMENT

The authors thank the National Research Council of Canada for the award of a scholarship (to R J B) and for a grant which made this work possible

REFERENCES

1 E ANDERSON, *J Biol Chem*, 104 (1934) 163
2 R E GILL, E L HIRST, AND J K N JONES, *J Chem Soc*, (1939) 1469.
3 E L HIRST, *J Chem Soc*, (1942) 70
4 R E GILL, E L HIRST, AND J K N JONES, *J Chem Soc*, (1946) 1025
5 E L HIRST, *J Chem Soc*, (1949) 522
6 E L HIRST, L HOUGH, AND J K N JONES, *Nature*, 165 (1950) 34, *J Chem Soc*, (1951) 323
7 F SMITH AND R MONTGOMERY, *The Chemistry of Plant Gums and Mucilages*, Reinhold, New York, 1959, p 370
8 C M FEAR AND R C MENZIES, *J Chem Soc*, (1926) 937
9 W SCHIRMER, *Arch Pharm*, 250 (1912) 230, *Chem Zentr*, 2 (1912) 195
10 D M W ANDERSON, SIR EDMUND HIRST, AND J F STODDART, *J Chem Soc* (*C*), (1966) 1959
11 D W DRUMMOND, E L HIRST, AND E PERCIVAL, *J Chem Soc*, (1962) 1208, D A REES AND J W B SAMUEL, *J Chem Soc* (*C*), (1967) 2295
12 I J GOLDSTEIN, G W HAY, B A LEWIS, AND F SMITH, *Methods Carbohyd Chem*, 5 (1965) 361
13 Z DISCHE, *J Biol Chem*, 167 (1947) 189, 183 (1950) 480
14 D G LANCE AND J K N JONES, *Can J Chem*, 45 (1967) 1995
15 J K N JONES AND M STACEY, *J Chem Soc*, (1947) 1340
16 E L FALCONER AND G A ADAMS, *Can J Chem*, 34 (1956) 338, D G LANCE AND J K N. JONES, *ibid*, 45 (1967) 1533, J F STODDART AND W A SZAREK, *ibid*, 46 (1968) 3061
17 S LUCKETT AND F SMITH, *J Chem Soc*, (1940) 1506
18 R KUHN, H TRISCHMANN, AND I LOW, *Angew Chem*, 67 (1955) 32

Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

STUDIES ON CARBOHYDRATE-METABOLISING ENZYMES

PART XXI*. THE α-GLUCOSIDASE AND D-ENZYME ACTIVITY OF EXTRACTS OF CARROTS AND TOMATOES

D J MANNERS AND KAREN L ROWE**
Department of Brewing and Biochemistry, Heriot-Watt University, Edinburgh 1 (Great Britain)

(Received October 3rd, 1968)

ABSTRACT

Extracts of carrot roots and tomato fruits show a range of carbohydrase activities, including substantial maltase, nigerase, and isomaltase activity In addition, the extracts unexpectedly showed disproportionation activity with maltotriose and higher maltosaccharides. This latter activity (D-enzyme) can be separated from the α-glucosidases by continuous electrophoresis The purified D-enzymes show a similar catalytic activity to the corresponding potato enzyme, but differ in optimum pH and other properties.

INTRODUCTION

During a survey of the carbohydrase activities of various plant tissues, Hutson and Manners[2] noted that extracts of carrot roots (*Daucus carota*) and tomato fruits (*Solanum lycopersicum*) showed unexpectedly high α-glucosidase activity The latter has now been investigated further, and, during purification, evidence for the presence of a maltosaccharide disproportionating-enzyme similar to potato D-enzyme was obtained Since D-enzyme is one of several starch-metabolising enzymes first isolated by Professor Peat and his co-workers[3], we now report details of the carrot and tomato enzymes as an appropriate contribution to this volume.

METHODS AND MATERIALS

General methods. — The following solvent systems were used for paper chromatograms (*A*) propyl alcohol–ethyl acetate–water[4] (14 2 7), (*B*) isopropyl alcohol–acetic acid–water[5] (54·8 18); (*C*) methyl ethyl ketone–acetic acid–water[6] (9 1 1) A Beckman Spinco Model CP continuous electrophoresis cell was used at a constant voltage of 700 volts and 40 mamps for the fractionation of protein material. Protein and glucose were analysed by using a colorimetric method[7] and a glucose oxidase–Tris reagent[8], respectively.

Preparation of enzyme extracts — All operations were carried out at 2° Minced carrot tissue, from 336 g of roots, was homogenised in 1 litre of 0 4M sodium

---

[*] Dedicated to Professor Stanley Peat, F R S For Part XX, see Ref 1

[**] Present address: Department of Pathology, University of British Columbia, Vancouver 8, Canada

citrate buffer (pH 6.5) for 5 min by using an Ultra-Turrax TP 18 homogeniser. The homogenate was further extracted in the citrate buffer for 2 h and centrifuged at 4,000 *g*, and the solution was dialysed against running tap-water for 18 h. Protein was precipitated by the addition of solid ammonium sulphate (to 0.70 saturation) and collected by centrifugation, and an aqueous solution was dialysed against running tap-water for 24 h. The dialysate was made 0.01M with respect to sodium citrate, and freeze-dried to yield 2.1 g of a tan powder having a protein content of 1.3%.

Fresh tomato fruits (388 g) were homogenised in 0.02M McIlvaine buffer (pH 7.0) for 5 min and then extracted for 1 h, and the homogenate was centrifuged at 4,000 *g*. Protein was precipitated from the centrifugate (*ca.* 560 ml), as described for the carrot extract, and freeze-drying of the final solution gave 1.2 g of a tan powder having a protein content of 3.0%.

Preparation of nigerose. — Nigerose was prepared from an enzymic hydrolysate of nigeran[5]. The polysaccharide was extracted from mycelia of *Aspergillus japonicus* (QM 332), and nigeranase was extracted from the mycelia of *Penicillium melanii* (QM 1931) as described by Reese and Mandels[5]. The crude enzyme preparation showed substantial amylase and α-glucosidase activity which were eliminated by gel filtration on Sephadex A-50 (DEAE). The purified nigeranase preparation (150 mg, freeze-dried powder) was incorporated into a digest (total volume, 15 ml) containing 1.0 g of nigeran which was incubated for 12 h at 50°. The enzyme was inactivated by heating (10 min, 100°), denatured protein was removed by centrifugation, and the solution was concentrated to *ca.* 5 ml. Preparative, paper chromatography of the concentrate (solvent *B*) yielded 190 mg of chromatographically pure nigerose.

Assay of α-glucosidase activity. — Digests containing disaccharide (1 mg) and enzyme preparation in 0.01M McIlvaine buffer (pH 4.5) in a total volume of 2 ml were incubated for 1 h at 37°. The enzyme was inactivated by heating, and the glucose content was determined by using the glucose oxidase–Tris reagent[8]. In digests containing between 0.03 and 0.48 mg of protein, there was a linear relationship between the extent of hydrolysis and enzyme concentration, with both the carrot and tomato preparations.

Assay of D-enzyme. — D-Enzyme activity may be followed by measurement of the iodine-staining power of higher maltosaccharides formed by disproportionation from maltohexaose[3]. The method was modified, and digests containing maltopentaose (1 mg) in 0.5 ml of 0.01M McIlvaine buffer (pH 5.5) and enzyme preparation (2 mg in 0.5 ml of water) were incubated at 37°. Aliquot portions (0.2 ml) were withdrawn at intervals and added to standard iodine reagent (3 ml), and the absorbance at 500 or 510 nm was measured. Provided the period of incubation did not exceed 2.5 h, these conditions could be used for the assay of D-enzyme.

RESULTS

Preliminary examination of extracts. — By paper-chromatographic analysis, the carrot and tomato extracts were shown to have strong hydrolytic activity towards the following substrates maltose, nigerose, isomaltose, sucrose, lactose, higher maltosaccharides (DP>9), and amylopectin β-dextrin. There was appreciable activity towards panose, isomaltotetraose, and laminarin, but no activity against cellobiose, salicin, phenyl α-D-glucopyranoside, melezitose, α-limit dextrin, pullulan, nigeran, dextran, levan, or alfalfa galactomannan Control digests showed that neither enzyme extract underwent autolysis Incubation of either extract with maltotriose or maltotetraose yielded glucose and maltosaccharides of both higher and lower DP than the original substrate This type of transferase activity seemed to resemble that of potato D-enzyme[3] and was different from the more-usual trans-α-glucosylation in which maltose serves as a substrate[9].

Studies of the α-glucosidase activity. — In view of the marked activity towards maltose, isomaltose, and nigerose, attempts were made to determine whether this was due to a group-specific α-glucosidase or to a mixture of specific disaccharidases

The effect of pH on the three activities was examined (see Fig. 1) With both extracts, maltase and nigerase activity was optimal at pH 4 5–4 8, whereas isomaltase activity was highest at pH 4 8–5 1 In general, the pH activity curves were rather similar, although the isomaltase curve could be distinguished from the other two activities

Samples of the carrot preparation (1 0 g, containing 13 mg of protein) and the tomato preparation (0 6 g, containing 18 mg of protein) were dissolved in water (10 ml) and dialysed at 2° against 0 01M citrate buffer (pH 5 3) for 3 h Each dialysed solution was then subjected to continuous electrophoresis for 30 h at 2°, using 0 01M citrate buffer (pH 5 3) The protein content of the fractions was measured spectrophotometrically at 280 nm Fractions were assayed for amylase, maltase, and D-enzyme activity, using amylopectin β-dextrin as a substrate for the detection of amylases The results (Fig 2) showed an anodic migration of the D-enzyme, while the maltase and amylase activities migrated slightly towards the cathode Appropriate fractions were combined as follows *carrot extract*, fractions 7-18 from racks A-E were used as α-glucosidase, fraction 19-31 as D-enzyme, *tomato extract*, fractions 7-18 from racks A, D, and E, and 7-20 from racks B and C as α-glucosidase, fractions 19-31 from racks A, D, and E, and 21-31 from racks B and C were used as D-enzyme The pH of each combined solution was adjusted to 6.0 with 0 01M sodium citrate buffer, and the resulting solutions were freeze-dried, and the protein contents determined by the Lowry method[7] (see Table I) The total yields of protein recovered from the electrophoresis were 85% of the carrot extract, and 67% of the tomato extract

Qualitative examination of the α-glucosidase fractions showed strong disaccharidase activity, but no action on panose or on maltotriose The latter shows the absence of D-enzyme The two fractions still contained amylase activity, but as this enzyme has no action on maltose, nigerose, or isomaltose, its presence would not interfere with the assays for α-glucosidase activity

The ratios of the three disaccharidase activities were measured in digests of pH 4 5 containing substrate (5 mg) and α-glucosidase fraction (5 mg) in a total volume of 4 ml The extent of hydrolysis, after incubation for 1 h at 37°, is given in Table I, and indicates maltase nigerase isomaltase activities of 100 91 81 for the carrot preparation, and 100 92 65 for the tomato fraction

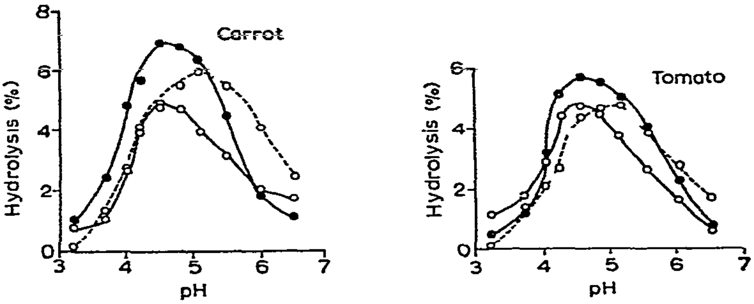


Fig 1 Effect of pH on the α-glucosidase activity of the carrot and tomato preparations acting on maltose (●———●), nigerose (○———○), and isomaltose (○– – –○)

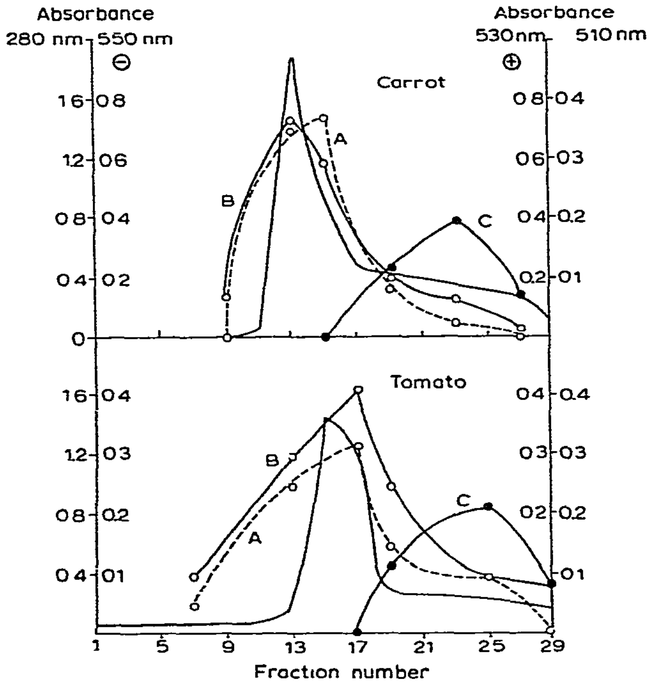


Fig 2 Continuous electrophoresis of carrot and tomato extracts The distribution of protein (280 nm) is shown by the continuous line Curve A (○– – –○) represents the decrease in iodine staining-power (550 nm) of amylopectin β-dextrin due to α-amylase activity Curve B (○———○) represents maltase activity assayed by glucose oxidase (530 nm) Curve C (●———●) represents D-enzyme activity as measured by iodine staining (510 nm) with maltopentaose as substrate The results show the activities of the fractions from Rack C

The effect of temperature on the stability of the enzymes was examined by pre-incubating the enzyme preparation for 1 h at various temperatures, cooling to 20°,

TABLE I

RELATIVE α-GLUCOSIDASE ACTIVITY OF ELECTROPHORETIC FRACTIONS FROM CARROT AND TOMATO EXTRACTS

	Carrot		*Tomato*	
	α-Glucosidase	*D-Enzyme*	*α-Glucosidase*	*D-Enzyme*
Weight, g	0.215	0.320	0.220	0.290
Protein content, %	3.2	1.3	3.1	1.8
Maltase activity[a]	10.8	—	8.4	—
Nigerase activity[a]	9.9	—	7.8	—
Isomaltase activity[a]	8.8	—	5.5	—

[a] Results are expressed as percentage hydrolysis under the conditions described in the text.

and incorporating into standard digests. The results (Table II) showed that there was no significant, selective inactivation of any of the three activities, although the isomaltase activity was slightly more thermostable than that towards maltose and nigerose.

TABLE II

EFFECT OF TEMPERATURE ON THE STABILITY OF THE α-GLUCOSIDASES

Temperature	*Activity* (%)			
	20	45	55	65
Carrot preparation				
Maltase	100	94	84	75
Nigerase	100	93	84	77
Isomaltase	100	100	92	84
Tomato preparation				
Maltase	100	93	85	76
Nigerase	100	93	87	77
Isomaltase	100	100	93	80

Since Tris has been widely used as an inhibitor of α-glucosidases[10], its effect on the three disaccharidase activities was examined. However, the results (Table III) showed that no selective inhibition of any one activity occurred. Tris buffer (pH 7.6) inhibited both enzyme preparations by *ca.* 50% with each substrate, whereas the pH 8.5 buffer caused *ca.* 70% inhibition.

Since the disaccharidase activities could not be differentiated by heat inactivation or inhibitor methods, an attempted separation of the three activities by fractionation with ammonium sulphate was carried out. Fresh samples of carrots and tomatoes were extracted as described previously, and protein was precipitated by the addition of ammonium sulphate to 15, 30, and 75% saturation. The six protein fractions were then assayed against the disaccharides. The results (Table IV) show a significant variation in the ratios of the relative maltase to nigerase and isomaltase activities,

TABLE III

EFFECT OF TRIS BUFFER ON α-GLUCOSIDASE ACTIVITIES[a]

Digest	*Control, pH 4.5*	*Tris, pH 7.6*	*Tris, pH 8.5*
Carrot preparation			
Maltase	12.6	6.3	3.8
Nigerase	11.2	5.6	3.4
Isomaltase	10.5	5.3	3.2
Tomato preparation			
Maltase	10.6	5.1	2.2
Nigerase	9.6	4.8	2.9
Isomaltase	5.5	3.8	2.2

[a]Results are expressed as percentage hydrolysis under standard conditions

suggesting that more than one α-glucosidase was present in the original tomato and carrot extracts

TABLE IV

ACTION OF AMMONIUM SULPHATE FRACTIONS ON THE THREE DISACCHARIDES

Carrot preparation	*Fraction I*	*Fraction II*	*Fraction III*
Ammonium sulphate saturation, %	0–15	16–30	31–75
Weight, g	0.49	0.91	1.15
Nitrogen content, %	13.1	11.5	14.5
Maltase activity[a]	1.8	3.7	9.7
Nigerase activity[a]	0.9	2.4	7.5
Isomaltase activity[a]	0.9	2.6	8.3
Tomato preparation			
Ammonium sulphate saturation, %	0–15	16–30	31–75
Weight, g	0.34	0.85	1.21
Nitrogen content, %	14.1	11.0	16.2
Maltase activity[a]	1.0	3.7	9.9
Nigerase activity[a]	0.9	2.5	7.6
Isomaltase activity[a]	0.9	1.8	6.8

[a]Results are expressed as percentage hydrolysis under standard conditions

Studies of the D-enzyme activity — Qualitative analysis of the carrot and tomato D-enzyme preparations showed the absence of isomaltase, nigerase, panase, invertase, and lactase activity, and the presence of traces of maltase activity. On incubation with maltotriose, maltotetraose, or maltopentaose, rapid disproportionation took place with the production of glucose and higher maltosaccharides. Initially, maltotriose gave a mixture of glucose and maltopentaose, whereas maltotetraose gave a mixture of glucose and maltoheptaose. After 4 h, a homologous series of oligosaccharides had been produced from both substrates. However, maltosaccharides were not formed from glucose and maltose under similar conditions When maltotriose was incubated with a mixture of D-enzyme and a low concentration of β-amylase (25 units[11] per mg of maltotriose), it was converted into a mixture of maltose and glucose β-Amylase alone had no action on maltotriose (*cf.* Whelan, Bailey, and Roberts[12]). This provides evidence for the synthesis of oligosaccharides containing only α-(1→4)-linked D-glucose residues.

The effect of pH on enzyme activity was examined in standard digests prepared in McIlvaine buffer having pH 4 0–6 5 The increases in iodine staining-power after 2 h are depicted in Fig 3(*a*), and show maximal activity at pH 5 2–5 5 with both the carrot and tomato preparations. 0 001M Tris buffer (pH 7 6 or 8 5) had no effect on the activity of either enzyme preparation.

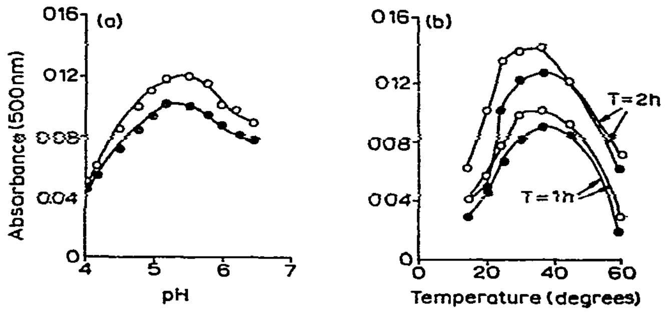


Fig 3 Effect of pH (*a*) and temperature (*b*) on the D-enzyme activity of the carrot (○———○) and tomato (●———●) preparations

The effect of temperature on enzyme activity was investigated by using digests at pH 5.3 which were incubated at various temperatures for 1 and 2 h The results [Fig 3(*b*)] show maximal activity for both enzyme preparations at about 37°

The action of potato D-enzyme on maltotriose leads to the production of an equilibrium mixture of glucose and higher, iodine-staining maltosaccharides[3]. This equilibrium is disturbed by the addition of D-glucose which reverses the reaction Similar experiments were therefore carried out on the carrot and tomato preparations Maltohexaose (2 mg) and enzyme preparation (2 mg) were incubated at pH 5 3 in a total volume of 2 ml, and samples (0 1 ml) were removed at intervals for iodine staining The results (Table V) showed that maximal iodine staining-power was reached after 6 h This result was not due to inactivation of the enzyme, since, on the addition of maltohexaose (2 mg) after 20 h, a further small increase in iodine staining-power occurred The digests were then divided; D-glucose (2 mg in 1 ml of water) was added to one half of each digest, and water (1 ml) to the other half The iodine staining-power of the digests containing D-glucose fell rapidly, whereas that of the control digests remained constant over the next 20 h (Table VI). The latter result also provides good evidence for the absence of amylases from the D-enzyme preparation

TABLE V

EFFECT OF ENZYME PREPARATIONS ON MALTOHEXAOSE AS MEASURED BY IODINE STAINING[a]

Time (h)	*0*	*0 5*	*1 0*	*2 0*	*4 0*	*6 0*	*10 0*	*20 0*	*40 0*
Carrot preparation	0 02	0 08	0 13	0 25	0 36	0 38	0 39	0 39	0 41[b]
Tomato preparation	0 01	0 06	0 10	0 15	0 28	0 32	0 32	0 32	0 34[b]

[a]Results are expressed as absorbance readings at 500 nm [b]After addition of further maltohexaose

TABLE VI

EFFECT OF D-GLUCOSE ON THE EQUILIBRATED MALTOHEXAOSE SYSTEM AS MEASURED BY IODINE STAINING[a]

Time (h)	*1*	*5*	*10*	*15*	*20*
Carrot preparation[b]	0 37	0 34	0 16	0 07	0 06
Tomato preparation[c]	0 30	0 25	0 08	0 06	0 03

[a]Results are expressed as absorbance readings at 500 nm [b]Control digest had an absorbance of 0 37 after 1 and 20 h [c]Control digest had an absorbance of 0 30 after 1 and 20 h

To confirm that D-glucose could serve as acceptor substrate, digests at pH 5 3 containing maltotriose (2 mg) and enzyme preparation (2 mg) in a total volume of 1 ml were incubated for 10 h D-Glucose-^{14}C (1–1 5 μCi) was added to each digest, and samples were withdrawn at intervals and applied to Whatman 3 MM paper, together with appropriate, reference maltosaccharides After development in solvent *A*, the presence of radioactive sugars on each paper strip was detected by using a Panax RCMS-2 scanner, a GSA-2 geiger/scintillation amplifier, and a Record Graphic recorder The ratios of the areas of radioactive maltosaccharides are given in Table VII and show that glucose was incorporated into the higher maltosaccharides produced by D-enzyme action A similar experiment with maltose as substrate did not produce any radioactive maltosaccharides.

TABLE VII

INCORPORATION OF D-GLUCOSE-^{14}C INTO MALTOSACCHARIDE[a]

Time (h)	*Maltotriose*		*Maltotetraose*		*Maltopentaose*	
	C	*T*	*C*	*T*	*C*	*T*
1	0 20	0 28	—	—	0 00	0 05
5	0 44	0 75	—	—	0 08	0 46
24	0 57	0 48	0 17	0 14	0 21	0 18

[a]The carrot and tomato preparations are denoted as *C* and *T*, respectively The results are expressed as areas corresponding to the amount of radioactive maltosaccharide, and are relative to glucose = 1 00

The action of potato D-enzyme on a mixture of D-glucose-^{14}C and a suitable donor substrate results in the formation of maltosaccharides in which the radioactivity is confined solely to the reducing D-glucose residue[13] The behaviour of the carrot and tomato enzymes was similar The maltosaccharides-^{14}C were eluted from the paper strips and combined to yield a carrot and a tomato maltosaccharide fraction After reduction with potassium borohydride, the solutions were neutralised with acetic acid and hydrolysed with 2N sulphuric acid (3 h at 100°) The neutralised hydrolysates were examined by paper chromatography in solvent *C* and shown to contain only D-glucose and D-glucitol. Scanning of the paper strips with the Panax apparatus showed that all the radioactivity was in the D-glucitol

The ability of other sugars to serve as acceptors was examined in digests at pH 5 3 containing maltopentaose (2 mg) and enzyme preparation (2 mg) in a total volume of 2 ml After the iodine staining-power had become constant (11 h), portions (0 2 ml) of each digest were added to solutions of acceptors (2 mg in 0 3 ml of water) and incubated for 5 h The efficiency of maltose and methyl α-D-glucopyranoside as acceptor substrates, relative to D-glucose, was 33 and 60% with the carrot enzyme, and 42 and 83% with the tomato enzyme

DISCUSSION

The present investigation confirms and extends the previous study[2] on plant α-glucosidases The relatively high nigerase activity of the carrot and tomato preparations is notable, and from the fractionation study with ammonium sulphate, it would appear that these tissues, like those of alfalfa, contain a mixture of α-glucosidases whose catalytic properties are very similar to each other. In contrast to alfalfa, the mixture of carrot and tomato α-glucosidases could not be separated by continuous electrophoresis The *in vivo* function of the α-glucosidases is not yet known, particularly since oligosaccharides or glucans containing a high proportion of α-(1→3)- or α-(1→6)-D-glucosidic linkages are not produced by these plants

The ability of the enzyme extracts to disproportionate maltosaccharides was unexpected. D-Enzyme was first isolated from potato juice[3] and is also present in the broad bean[14], but had not hitherto been described in other plant tissues However, certain sweet-corn extracts also show D-enzyme activity (unpublished work), and its distribution in the plant kingdom may be wider than was originally believed The carrot and tomato enzymes are similar to potato D-enzyme in their ability to transfer reversibly two or more D-glucose residues from maltotriose or a higher maltosaccharide to an acceptor which may be D-glucose, maltose, maltotriose or higher maltosaccharide, or to methyl α-D-glucopyranoside At equilibrium, the products stain with iodine and therefore contain more than about twenty D-glucose residues Since the product from maltotriose is completely degraded by β-amylase, it contains only α-(1→4)-linked D-glucose residues The transfer of D-glucose residues to the OH group at C-4 of D-glucose is also a characteristic of the potato enzyme.

The inability of the carrot and tomato enzymes to produce maltosaccharides from maltose, and to incorporate D-glucose into maltosaccharides in the presence of maltose, is a clear distinction between D-enzyme action and the relatively common trans-α-glucosylation catalysed by a maltase or α-glucosidase[9].

The carrot and tomato D-enzyme differ from the potato enzyme in some respects, the optimum pH and temperature of the latter being about 6 7 and 45°, respectively, and in the relative activities with maltose and methyl α-D-glucopyranoside acceptors (11 and 37% of that with D-glucose)[3]. The *in vivo* role of D-enzyme is not clear In the potato and broad bean, which are tissues actively concerned with the synthesis and degradation of starch, it has been suggested[15] that D-enzyme plays some part in the formation of the primers which are required for the synthesis of linear chains of α-(1→4)-linked D-glucose residues Since the starch content of the carrot root and tomato fruit is generally low (a figure of 0.02% has been quoted for the latter)[16], it seems unlikely that the relatively active D-enzyme is concerned with *in vivo* metabolism

ACKNOWLEDGMENTS

The authors are indebted to the Science Research Council and the Royal Society for equipment grants, and to the University of Edinburgh for a postgraduate scholarship (to K L R)

REFERENCES

1 D J Manners and K L Rowe, *Carbohyd Res* , 9 (1969) 107
2 D H Hutson and D J Manners, *Biochem J* , 94 (1965) 783
3 S Peat, W J Whelan, and W R Rees, *J Chem Soc* , (1956) 44
4 J D. Commerford, G T VanDuzee, and B L Scallett, *Cereal Chem* , 40 (1963) 482
5 E T Reese and M Mandels, *Can J Microbiol* , 10 (1964) 103
6 W R Rees and T Reynolds, *Nature*, 181 (1958) 767
7 O H Lowry, N. J Rosebrough, A L Farr, and R J Randall, *J Biol Chem* , 193 (1951) 265
8 J W White and M H Subers, *Anal Biochem* , 2 (1961) 380
9 D J Manners, *Bull Soc Chim Biol* , 42 (1960) 1789
10 J Larner and R E Gillespie, *J Biol Chem* , 223 (1956) 709, A Dahlqvist, *Biochem J* , 80 (1961) 547
11 P. N Hobson, W J Whelan, and S Peat, *J Chem Soc* , (1950) 3566
12 W J Whelan, J M Bailey, and P J P Roberts, *J Chem Soc* , (1953) 1293
13 G J Walker and W J Whelan, *Biochem J* , 67 (1957) 548
14 W R Rees, Ph D Thesis, University of Cambridge, 1953
15 W J. Whelan, in *Encyclopedia of Plant Physiology*, W Ruhland (Ed), Vol 6, Springer-Verlag, Berlin, 1958, p 154
16 L F J Brimble, *Intermediate Botany*, Macmillan, London, 1957, p 67

Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

CHARACTERISATION OF POLYSACCHARIDE STRUCTURES BY GLYCOSIDE STABILISATION WITH TOLUENE-*p*-SULPHONATES

MODEL EXPERIMENTS WITH DEXTRAN*

D A Rees, N G Richardson, N J Wight, and Sir Edmund Hirst
Chemistry Department, University of Edinburgh, West Mains Road, Edinburgh 9 (Great Britain)

(Received October 11th, 1968)

ABSTRACT

Toluene-*p*-sulphonylation (tosylation) in the primary position is proposed as a method for stabilising polysaccharide linkages to acid conditions The degree of stabilisation depends on the aglycon, as it does for uronic acid derivatives The method is convenient and can give semi-quantitative information but has the drawback that *O*-methyl-*O*-tosyl oligosaccharides are difficult to purify and must be identified indirectly Side reactions prevent the use of formic acid for glycoside hydrolysis when toluene-*p*-sulphonic esters are present, but aqueous chloroacetic acid or methanolysis is satisfactory

Successive triphenylmethylation (tritylation), methylation, detritylation, tosylation, and methanolysis of dextran gave mainly derivatives of isomaltose The structural implications of this and other evidence are discussed Tritylation of dextran occurs most rapidly at C-6 but is not as specific as in monosaccharide reactions

INTRODUCTION AND DISCUSSION

The characterisation of the branching pattern of amylopectin by Professor Stanley Peat and his colleagues[1] is one of the important structure proofs of carbohydrate chemistry By the elegant use of specific enzymes, the molecule was shown to correspond to the "random branched", rather than the "laminated" or "herring bone", model Most problems of this complexity still cannot be tackled by nonenzymic methods, and it is a challenge to the polysaccharide chemist to devise approaches through simple reactions which are equally effective It is especially desirable to have more methods by which particular linkages in polysaccharides can be broken selectively, and in this paper we describe an attempt to use the properties of toluene-*p*-sulphonates for the purpose.

These esters are themselves stable to acid and, when present at C-2, are known to stabilise glycoside linkages to acid hydrolysis[2]. Presumably, the carbonium ion at C-1, which would be a hydrolysis intermediate, is destabilised by the inductive effect. By analogy with pyranuronosides[3], toluene-*p*-sulphonates at C-6 should be sufficiently electron withdrawing to stabilise the glycoside to a useful extent. The measurements made by Hine and Ramsey[4] correspond to an inductive substituent

---

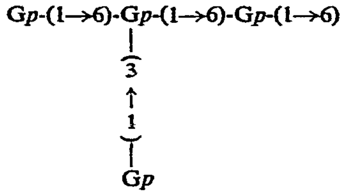
*Dedicated to Professor Stanley Peat, F R S

constant[5] (σ_I) for $-CH_2 \cdot OSO_2 \cdot C_6H_4 \cdot CH_3$ of about 0 37, compared[5] with 0 39 for $-CO_2H$ and 0 07 for CH_2OH This method would have obvious applications to such polysaccharides as dextran and pectic arabinan, in which unsubstituted, primary hydroxyl groups occur only at the end residues; if these could be selectively tosylated, the stabilisation effect should allow the terminal linkages to be characterised For both polysaccharides[6 7], this would give useful new information about fine structure The principle is the same as in the catalytic oxidation method[8], in which end groups of this type are converted into uronic acid residues Although the oxidation method is established as a useful tool in polysaccharide structure determination, it has some limitations, including the need for extended oxidation periods and, frequently, failure to oxidise more than a small proportion of end groups The use of toluene-*p*-sulphonic esters might be more convenient and give quantitative information When the present investigation was almost complete, Weigel and co-workers reported a successful application[9] of the oxidation method to dextran Therefore, we do not suggest that the sulphonic ester method has significant advantages for dextran, although it might be preferred for polysaccharides which are more difficult to oxidise

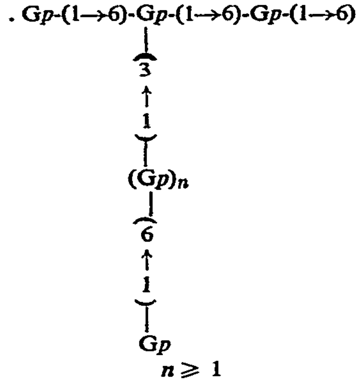
In preliminary experiments, the hydrolysis of methyl 6-*O*-toluene-*p*-sulphonyl-α-D-glucopyranoside was compared with that of methyl α-D-glucopyranoside by thin-layer chromatography The estimated half-lives from spot intensities indicated stabilisation by a factor of between 3 and 5 This effect is too small to be useful in structure analysis, but it is greater than for the corresponding uronoside[10]. It seemed possible that greater effects would be observed with other aglycons, as in the uronic acid series[11], and we therefore proceeded to experiment with dextran

The polysaccharide was prepared from a culture of *Leuconostoc mesenteroides* P m r. spectroscopy indicated *ca* 20% branching, the interpretation of the spectrum being based on the assignments by Pasika and Cragg[12]. This estimate was in approximate agreement with periodate oxidation and methylation analysis The major products of methylation analysis were identified by paper and gas–liquid chromatography as derivatives of 2,3,4,6-tetra-*O*-methylglucose, 2,3,4-tri-*O*-methylglucose, and 2,4-di-*O*-methylglucose The structure is therefore of a well-known dextran type[13], in which (1→6)-linked chains of D-glucopyranose residues are branched through position 3 Small quantities of 2,4,6-tri-*O*-methylglucose were also detected, but the amounts were small enough to be negligible in the present context The next question, to be answered through the experiments described below, is whether the side chains are single units of the type **1** usually thought most likely[6] and which have been established for two dextrans[9] Examples of other possibilities are structures **2** and **3**.

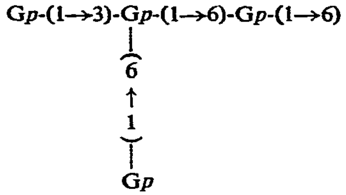
With the object of obtaining the most selective substitution possible, the tosyl groups were introduced after tritylation, methylation, and detritylation The first step was possible under homogeneous conditions in pyridine–methyl sulphoxide (1 1) The reaction was monitored by t l c , but tritylation of dextran was not completely specific (Fig 1) In contrast, the reaction of methyl α-D-glucopyranoside under similar conditions showed high specificity Part of the reason is no doubt that dextran



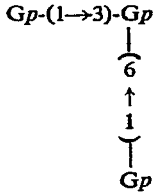
(1)



(2)



(3)



(4)

has five times as many secondary hydroxyl groups in relation to primary groups as the simple glycoside, but it would also seem likely that there is a genuine difference in the reactivity of some of the secondary hydroxyl groups Conditions were chosen for the preparative experiment that would ensure complete primary substitution of dextran, and the product was found to be over-tritylated to the extent of *ca* 20% Methylation, followed by methanolysis and g l c , gave no tetra-*O*-methylglucosides, proving the location of the majority of trityl groups The methyl ether was detritylated with hydrogen chloride in methanol and finally treated with toluene-*p*-sulphonyl chloride in pyridine

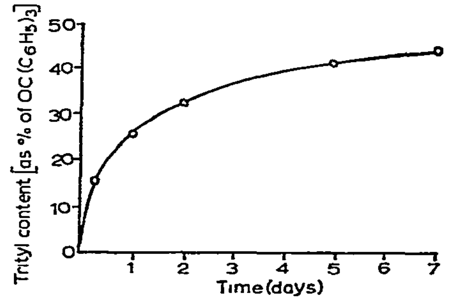


Fig 1 Tritylation of dextran at 23 5°, as shown by the analysis of samples withdrawn from the reaction mixture Complete and specific substitution of primary hydroxyl groups would correspond to a trityl content of 24 6%

Aqueous chloroacetic acid was used for hydrolysis because the *O*-methyl-*O*-tosyldextran was insoluble in dilute mineral acid, and there was evidence for reductive detosylation in aqueous formic acid (see Experimental) Measurement of the average degree-of-polymerisation[14], for samples withdrawn from the hydrolysate, showed that the reaction levelled off at *ca.* 80% hydrolysis as had been hoped Analysis by t l c of the cleavage products was greatly assisted by a specific spray for toluene-*p*-sulphonic esters[15], which showed the accumulation of a major and several minor tosylated carbohydrates during solvolysis with chloroacetic acid or methanolic hydrogen chloride, but not with formic acid

The methanolysate was treated with sodium amalgam to remove sulphonic esters, and then methylated and analysed by g l c. Tetra-*O*-methylglucosides, hepta-*O*-methylisomaltosides (derivatives of 6-*O*-α-D-glucopyranosyl-D-glucose), and hepta-*O*-methylnigerosides (derivatives of 3-*O*-α-D-glucopyranosyl-D-glucose) were detected in the approximate molar ratios 100 53 4, pointing to the surprising conclusion that the structure corresponds more closely to **2** or **3** rather than to **1*** The possibility that the isomaltose derivatives arose from nontosylated parts of the structure was excluded by an experiment which showed that the proportion of hepta-*O*-methyl disaccharide glycosides fell to one eighth when the treatment with amalgam was omitted As a check on the procedure, we may compare the experimental and calculated yields of tosylated disaccharide derivatives Analysis of the *O*-methyl-*O*-trityl polysaccharide showed 1 trityl group for every 4 10 sugar residues, therefore, if each trityl ether gave rise to one mole of disaccharide, the methanolysis mixture would contain monosaccharide and disaccharide derivatives in the molar ratio (4.10–2 00) 1, or 100 48 The experimental figure (see above) of 100 (53+4) includes some disaccharide from incomplete hydrolysis of the nontosylated regions This was estimated by g l c without detosylation (see Experimental) and subtracted from the total to give a corrected ratio, 100 44 This agrees well with the expected ratio of 100 48, and therefore excludes the presence of substantial amounts of undetectable fragments such as derivatives of the trisaccharide **4** Of the 53+4 disaccharide molecules in the methanolysate (see above), it is easily calculated, on the basis of 20% branching, that no more than 16 can have arisen by nonspecific substitution and/or incomplete methanolysis of nonstabilised linkages Therefore, 6-*O*-tosylisomaltosides must be present in at least ninefold excess over 6-*O*-tosylnigerosides, and indeed, it is more probable that there are none of these nigerose derivatives present These conclusions were confirmed by isolation of the mixture of tosylated fragments by using cellulose-column chromatography followed by t l c Isomaltose derivatives, but not nigerose derivatives, were detected by methylation, reductive detosylation, and remethylation

A further distinction between the possible structures is that only **2** should give derivatives of 2,4-di-*O*-methylglucose under cleavage conditions which retain the terminal linkage This sugar was isolated from the cellulose column during the purification of tosylated fragments (see above) In quantitative terms, the relative yields of 2,4-di- and 2,3,4-tri-*O*-methylglucose derivatives from structure **2** should be 1 2 To avoid calibration, as would otherwise be advisable with compounds which contain different numbers of hydroxyl groups, the ratio was determined by g l c

---

**Note added in proof* (*10th February 1969*) Another dextran structure of this type has been reported since the manuscript was submitted (B LINDBERG AND S SVENSSON, *Acta Chem Scand*, 22 (1968) 1907).

relatively to *O*-methyl-*O*-trityldextran, for which the ratio should be 1:4; structure **2** should therefore yield twice as much 2,4-di-*O*-methylglucoside as this polysaccharide. The experimental result was within 5% of this prediction, and we therefore conclude that our sample of dextran has a structure which approximates to **2**.

We have made many unsuccessful attempts to isolate the *O*-tosyl disaccharide derivatives for further characterisation, by column chromatography on silica gel, alumina, Sephadex LH-20, ion-exchange resins (using them as molecular sieves), and cellulose; and also by thick-paper chromatography. These compounds have a pronounced tendency to streak in all systems, and their purification is also made difficult by contamination with products from nonspecific substitution. It is probably a general drawback of the method that indirect structure proofs such as we have used in this paper will be the only ones practicable for tosylated disaccharide fragments. Although there is no ambiguity, it would be more satisfying to isolate the products in the pure state.

EXPERIMENTAL

Comparative stabilities of methyl glycosides. — Methyl α-D-glucopyranoside and methyl 6-*O*-toluene-*p*-sulphonyl-α-D-glucopyranoside, each at 1% concentration, were heated at 100° in N sulphuric acid and in N hydrochloric acid. Small samples were withdrawn from time to time and neutralised with silver carbonate before examination by t.l.c.; microscope slides coated with silica gel were used with double development in methanol–benzene (1:4). By visual comparison of the spot intensities with anisaldehyde–sulphuric acid spray[16], the half lives were estimated as 0.7 and 1.5 h for the glucoside in hydrochloric and sulphuric acids, respectively, and 3.5 and 4.5 h for the toluene-*p*-sulphonate.

Preparation and characterisation of dextran. — The polysaccharide was prepared from a culture of *Leuconostoc mesenteroides* NCIB 2706 from the Torry Research Station, Aberdeen. The medium contained sucrose (10%), yeast extract (1%), potassium hydrogen phosphate (0.5%), and peptone (0.25%), and was adjusted to pH 7.0 with sodium hydroxide. After incubation for 4 days at 24°, the polysaccharide was isolated by slow addition of ethanol (1.5 volumes) with stirring. The gummy product was further purified by dissolving in water, boiling to coagulate proteins, and centrifugation. The supernatant solution was filtered, and the polysaccharide was reprecipitated, dissolved, and isolated by freeze-drying. *Anal.* Found: N, 0.7.

The p.m.r. spectrum was recorded in deuterium oxide solution with a Perkin–Elmer R10 spectrometer; the results (see Discussion) were calculated from the integrated signals. Dr. D. H. Hutson (personal communication) has estimated by periodate-oxidation methods[13] that this dextran contains 18% of (1→3) linkages and 3% of (1→4) linkages.

Methylation analysis. — Dextran (0.5 g) was dissolved in *N*-methyl-2-pyrrolidone (20 ml), and the solution was cooled in ice. Methyl iodide (1.5 ml) in *N*-methyl-2-pyrrolidone (20 ml) was added, followed by barium hydroxide octahydrate (3.6 g). The mixture was shaken in ice for 2 h and then at room temperature overnight.

Methyl iodide (5 ml) and barium hydroxide octahydrate (12 g) were added, and shaking was continued for a further 24 h at room temperature. The mixture was diluted with water (200 ml) and extracted with chloroform (3 times) The extracts were washed with water (when necessary, emulsions were broken by addition of EDTA), dried over sodium sulphate, and concentrated to a yellow syrup which was diluted with a small volume of benzene and poured into a large volume of light petroleum (b p 40–60°). The white, fibrous product was purified by reprecipitation in the same way (0 50 g, 79%) *Anal.* Calc for tri-*O*-methylhexose polymer OCH_3, 45.6. Found OCH_3, 38 5.

The incompletely methylated material (0 50 g) was dissolved in *N*,*N*-dimethylformamide (10 ml), and methyl iodide (1 5 ml) was added with silver oxide (2 7 g) The mixture was shaken for 16 h at room temperature and diluted with *N*,*N*-dimethylformamide The filtrate was shaken with aqueous sodium cyanide and then extracted with chloroform, and the product (0 45 g, 71% overall) was isolated as above *Anal.* Found OCH_3, 40.0 The methylation with silver oxide was repeated at the boiling point of methyl iodide, under reflux, yield, 0 36 g from 0 50 g, 51% overall *Anal.* Found OCH_3, 43 6 The product was separated by fractional precipitation from chloroform–light petroleum (b p 40–60°) into four subfractions of approximately equal weights (*Anal* Found OCH_3, 45 3, 44 0, 42 9, 38 5) Methanolysis (3% methanolic hydrogen chloride for 12 h at 100°), followed by neutralisation with silver carbonate and g l c (Pye Argon Chromatograph with ^{90}Sr detector) on neopentyl glycol adipate (3% on Gas Chrom P), showed, at 175°, 2,4-di-*O*-methylglucosides (retention times relative to 2,3,4,6-tetra-*O*-methyl-β-D-glucoside were standard, 6 18, 8 54, dextran, 6 10, 8 54) but not 3,4-di-*O*-methylglucosides (standard, 7.54) or 2,3-di-*O*-methylglucosides (standard, 12 01) The identifications of 2,3,4,6-tetra-*O*-methyl-glucosides (standard and dextran, 1 00, 1 46), 2,3,4-tri-*O*-methylglucosides (standard, 2 32, 3 32, dextran, 2 33, 3 29), and 2,4,6-tri-*O*-methyl-glucosides (traces only; standard, 2 88, 4 48, dextran, 2 85, 4 38) were made with the same system but at 160°.

The presence of 2,3,4,6-tetra-*O*-methyl-, 2,3,4-tri-*O*-methyl, and 2,4-di-*O*-methyl derivatives was confirmed by hydrolysis, followed by chromatography in butyl alcohol–ethanol–water (4 1 5) and ammonia (sp gr 0 880)–water–ethyl methyl ketone (1 17 200) solvents Separation of a hydrolysate on filter sheets with the latter solvent gave 2,4-di-*O*-methylglucose (0 168 g after exhaustive drying at 40° *in vacuo*) and a mixture of tri- and tetra-*O*-methylglucoses (1.02 g). This corresponds to 15 1% branching

O-Triphenylmethyldextran. — Dextran was insoluble in pyridine and did not enter solution during prolonged heating with pyridine containing an excess of chlorotriphenylmethane It was soluble in methyl sulphoxide–pyridine mixtures, and experiments were therefore performed to assess the suitability of this solvent for alkylation The methyl sulphoxide was purified by distillation from barium oxide (diminished pressure), and the pyridine was dried over Type 4a molecular sieve Methyl α-D-glucopyranoside (7 mg) and chlorotriphenylmethane (100 mg, 10 mol) were dissolved in the 1 1 solvent mixture (1 ml), and the solution was heated at 100° The reaction was monitored by t l c with methanol–benzene (3.17) The involatile solvents did not affect the separations but interfered strongly with the anisaldehyde–sulphuric acid[16] spray For this reason, the plates were strongly heated before, as well as after, spraying Trityl ethers gave yellow spots during the first few seconds and, on further heating, the characteristic colours of carbohydrate derivatives. The results showed that tritylation was complete in 1 h but that traces of fast-moving compounds, presumably ditrityl ethers, were also formed. At 23 5°, the reaction was complete in 24 h, but over-tritylation could not be detected until 70 h

Trial experiments with dextran were carried out as follows Dextran (0 5 g) was dissolved by heating in methyl sulphoxide (8 3 ml), and the solution was diluted with pyridine (8 3 ml) Chlorotriphenylmethane (1.73 g) was added, and the solution was kept in a thermostat. At intervals, samples of the viscous solution were withdrawn, diluted with methanol, and then heated for a few minutes to destroy the residual chloride The solution was poured in a thin stream into a large volume of methanol, and the white, fibrous product was collected by centrifugation This was washed repeatedly with methanol until the washings no longer gave a yellow coloration with concentrated sulphuric acid, and then dried at 60° *in vacuo* Small samples (0 1 mg) were weighed into colorimeter tubes with the Cahn electrobalance for analysis by the method of Duffield and Nussbaum[17]. The calibration curve was constructed with triphenylcarbinol Typical results are shown in Fig 1 From the curves, it appeared that overtritylation was a significant complication, even at 0° For the preparative experiment, dextran (9 0 g) was dissolved in methyl sulphoxide (150 ml) and diluted with pyridine (150 ml) Chlorotriphenylmethane (31 g) was added, and the solution was kept at 23 5° for the period which had been judged from the small-scale experiments to be the minimum for complete tritylation of primary hydroxyl groups (27 h) The product (9 2 g, 79% on the basis of 20% branching) was isolated in the same way as for the small scale *Anal* Calc for 20% branching $OC(C_6H_5)_3$, 24 6 Found $OC(C_6H_5)_3$, 29 2

O-*Methyl*-O-*triphenylmethyldextran* — The product from the previous stage (5 0 g) was dissolved in *N*,*N*-dimethylformamide (100 ml) Methyl iodide (60 ml) and barium hydroxide octahydrate (150 g) were added at 0°, and the mixture was shaken for 2 h at this temperature, and then for 16 h at room temperature The product was isolated in the same way as after the similar methylation of dextran (above) A part (3 7 g of 4 4 g) was dissolved in *N*,*N*-dimethylformamide (100 ml) Methyl iodide (10 ml) and silver oxide (20 g) were added, and the mixture was heated under reflux (40°) with magnetic stirring A second addition of methyl iodide and silver oxide was made after 16 h, and heating and stirring were continued for a similar period The product (3 6 g, 73% from *O*-trityldextran) was isolated as described for the similar methylation of dextran. *Anal* Calc. for complete methylation of previous product $OC(C_6H_5)_3$, 24 8, OCH_3, 32 4 Found $OC(C_6H_5)_3$, 24 0, OCH_3, 32 3 There was no hydroxyl absorption in the infrared spectrum. A small sample was heated in a sealed tube at 100° with 3% methanolic hydrogen chloride;

after neutralisation with silver carbonate, filtration, and evaporation, g.l.c. (Pye Argon Chromatograph) showed no peaks corresponding to the methyl glycosides of 2,3,4,6-tetra-*O*-methylglucose.

O-Methyl-O-toluene-p-sulphonyldextran. — Heterogeneous detritylation of the product from the previous stage was found to be ineffective. *O*-Methyl-*O*-triphenylmethyldextran (3.0 g) was therefore dissolved in chloroform (20 ml), and methanolic hydrogen chloride was added. This had been prepared by mixing acetyl chloride (0.18 ml) and methanol (80 ml). After 16 h at room temperature, the solution was neutralised with silver carbonate, filtered, and evaporated. The resulting syrup was dissolved in chloroform, light petroleum (b.p. 40–60°) was added to incipient precipitation, and then the whole solution was poured into a large volume of light petroleum. The grey-white precipitate was removed on a filter and purified by reprecipitation; yield, 1.5 g, 65%. *Anal.* Calc. for previous product with trityl groups removed: $OC(C_6H_5)_3$, 0; OCH_3, 42.4. Found: $OC(C_6H_5)_3$, 0.4; OCH_3, 39.1.

The detritylated material (1.4 g) was dissolved in pyridine (20 ml) containing toluene-*p*-sulphonyl chloride (1.35 g) and left at 37° overnight. The reaction mixture was diluted with water (500 ml) and extracted with chloroform (4 × 200 ml). The combined extracts were washed with water (4 × 500 ml), dried over sodium sulphate, filtered, and evaporated to a syrup. The product was isolated by precipitation with light petroleum (b.p. 40–60°) and showed a slight hydroxyl absorption in the infrared. The toluene-*p*-sulphonylation was therefore repeated to give a pale-yellow powder (1.30 g, 78%). *Anal.* Calc. for complete toluene-*p*-sulphonylation of the previous product: S, 3.3; OCH_3, 35.4. Found: S, 2.9; OCH_3, 33.8. There was no hydroxyl band in the infrared spectrum.

Solvolysis of O-Methyl-O-toluene-p-sulphonylglycosides. — Owing to the solubility properties of these substances, systems that were mainly aqueous could not be used. The hydrolysis of methyl 2,3,4-tri-*O*-methyl-6-*O*-toluene-*p*-sulphonyl-α-D-glucopyranoside in aqueous formic acid (45% v/v) was followed by the methods described above, except that the neutralisation step was omitted, and methanol–benzene (1:10) was used for t.l.c. The main carbohydrate product had the mobility of a tri-*O*-methylglucose and gave no reaction with the spray for toluene-*p*-sulphonic esters[15]; this spray showed several components which did not react with the reagent for carbohydrates[16]. A very similar pattern was observed when *O*-methyl-*O*-tosyldextran was heated in formic acid. No such complications were apparent when either the glycoside or the dextran derivative was heated with 3% methanolic hydrogen chloride or with 50% aqueous chloroacetic acid, and the main products appeared to be carbohydrate toluene-*p*-sulphonates.

To follow the chloroacetic acid hydrolysis quantitatively, samples of *O*-methyldextran and *O*-methyl-*O*-tosyldextran (20 mg) were separately dissolved in aqueous chloroacetic acid (50% w/v, 2.0 ml) and heated in a thermostat bath. *O*-Methyldextran dissolved immediately, but the sulphonic ester was at first insoluble. Samples (0.010 ml) were removed from each tube at intervals and diluted with water (1.00 ml). A weighed amount (0.100 g) of sodium borohydride was added slowly, and the tube was left for 24 h or longer at room temperature, with occasional shaking After cautious acidification (20N sulphuric acid, 2 0 ml), the usual phenol–sulphuric acid determination[18] was carried out. The interpretation is the same as for the method of Peat, Whelan, and Roberts[14], which differs only in technical details The curves for the two polysaccharides diverged in a manner which clearly showed the presence of 15–20% of stable linkages in the toluene-*p*-sulphonate The optimal conditions were judged to be 72 h at 84 5°.

Identification of tosylated fragments — Samples (25 mg) of *O*-methyl-*O*-tosyldextran were heated in sealed tubes with 3% methanolic hydrogen chloride (2 5 ml) at 100° for 0 5, 1.5, 4.5, 13 5, and 40.5 h, respectively. The material dissolved instantly After neutralisation with silver carbonate, filtration, and evaporation to dryness, each residue was dissolved in *N*,*N*-dimethylformamide (2 0 ml) Methyl iodide (0 5 ml) and silver oxide (0 2 g) were added, and each tube was sealed and shaken in the dark for 48 h at room temperature before being processed in the usual way (see the similar methylation of dextran above) The procedure was repeated twice The products were dried *in vacuo* over phosphoric anhydride and then dissolved in dry carbon tetrachloride for infrared spectroscopy. No trace of hydroxyl absorption was observed for any sample, showing that remethylation had been complete Reference compounds for the analysis of these products by g l c. were prepared by methylation of isomaltose and nigerose in essentially the same way The products were shown to be separable from each other and from octa-*O*-methylsucrose on an ECNSS(M) column (10% on Gas Chrom P) at 210°, using the Pye 104 System The retention times (T_S) were expressed relative to octa-*O*-methylsucrose, and are in the range to be expected from the literature[19] octa-*O*-methylsucrose, T_S 1 00, methyl hepta-*O*-methylisomaltosides, T_S 1.44, 2 02; methyl hepta-*O*-methylnigerosides, T_S 1 82, 2 35 Analysis of the polysaccharide methanolysates in this way showed isomaltosides as the only detectable disaccharide products [T_S, 1 48, 2 00, 2 60 (minor and unidentified)]

To each polysaccharide methanolysate was added octa-*O*-methylsucrose as an internal standard, the solvent was evaporated, and the residue was dissolved in aqueous isopropyl alcohol (80%). Excess of sodium amalgam (2%) was added to the solution over 24 h at 60°, followed by addition of solid carbon dioxide, filtration, and evaporation The product was extracted from the salty residue with acetone which was then filtered and evaporated to dryness The absorption in the ultraviolet at 223 nm (toluene-*p*-sulphonates) was virtually undiminished, and the treatment with amalgam was therefore repeated, but with a very large excess which was added over 48 h to the solution in aqueous ethanol (80%) at 65°. Each product now showed substantially less absorption at 223 nm It was remethylated (see above), and the increase in methylated disaccharides was measured, using the internal standard, from the peak areas Both isomaltosides and nigerosides were identified on the basis of retention times [T_S, 1 48, 1 98, 2 34 (except in product after 0 5 h), 2 60 (minor)] The assignment of the peaks to disaccharide derivatives, rather than to any other detosylation products, was supported by the gradual build up of peak areas with methanolysis time. The maximum increase in the proportion of isomaltose plus nigerose derivatives, relative to octa-*O*-methylsucrose (8.2 fold), was observed after the 40.5 h period.

Further quantitative information about the methanolysate after 40.5 h was obtained by g.l.c. with the same apparatus and stationary phase as above, but with temperature programming from 140–210° (increasing by 6°/min to 210°, followed by 5 min at this temperature). It was then possible to measure the peak areas of tetra-*O*-methylglucosides, hepta-*O*-methylisomaltosides, and hepta-*O*-methylnigerosides by triangulation (2.16, 2.10, and 0.17 cm^2, respectively). The results are interpreted (see Discussion) on the assumption that the flame-ionisation detector gives a response on a weight basis for compounds of such related structures, and are probably no more accurate than ±10%.

In a separate experiment, *O*-methyl-*O*-tosyldextran (0.32 g) was dissolved in aqueous chloroacetic acid (45% w/v, 32 ml) and heated in a thermostat bath for 72 h at 84.5°. The aqueous acid was removed by careful evaporation under diminished pressure, and the residue (0.45 g) was dried *in vacuo*. The u.v. spectrum showed a band at 223 nm identical with that given by methyl 6-*O*-toluene-*p*-sulphonyl-α-D-glucopyranoside. The i.r. spectrum also showed bands attributable to toluene-*p*-sulphonate (1160 and 1380 cm^{-1}), together with strong carbonyl absorption, which was presumably due to chloroacetic esters or chloroacetic acid and which would account for the high weight of the hydrolysate. Aqueous ammonium carbonate (1% w/v, 10 ml) was added, and the solution was evaporated to dryness after standing for 2 h at room temperature. This treatment was repeated, and then water was distilled from the residue until the smell of ammonia was no longer detectable. The i.r. spectrum showed that most of the absorption at 1740 had shifted to 1600 cm^{-1}, and therefore that the chloroacetic acid had been converted into the ammonium salt. The small, residual band presumably indicated some chloroacetic ester, and the product was therefore treated briefly (30 min) with sodium methoxide in methanol (0.5% w/v), followed by neutralisation with solid carbon dioxide. Extraction with ethanol (6 × 10 ml), followed by evaporation of the extracts, gave a clear syrup (0.30 g) and left a salty residue which gave a negative reaction[18] for carbohydrate. The syrup was dissolved in ethanol (5 ml) and loaded onto a cellulose column (70 × 3.5 cm) which was eluted with a mixture of equal volumes of benzene–ethanol–water–light petroleum (b.p. 60–80°) (169:47:15:130) and benzene–ethanol–light petroleum (b.p. 60–80°) (169:47:130). Fractions (10 ml) were collected automatically, screened by spotting onto paper and spraying with *p*-anisidine hydrochloride solution followed by heating at 120°, and then combined into three larger fractions as follows.

Fraction 1 (0.105 g) showed the i.r. and u.v. absorptions of toluene-*p*-sulphonates, and also reacted with the spray for toluene-*p*-sulphonates[15]. T.l.c. (silica gel, benzene–ethanol, 2:1) showed two components; sequential use of iodine vapour, toluene-*p*-sulphonate spray[15], and anisaldehyde–sulphuric acid spray[16] suggested that the faster of these was a carbohydrate toluene-*p*-sulphonate and the slower was noncarbohydrate.

Fraction 2 (0 061 g) was shown by paper chromatography, t l c., and methanolysis and g l c (for methods, see above) to consist of 2,3,4-tri-*O*-methylglucose

Fraction 3 (0 017 g) was shown by the methods used for the preceding Fraction to consist of 2,4-di-*O*-methylglucose

Fraction 1 was separated by preparative chromatography on silica gel plates (ethyl methyl ketone) The faster component (60 mg) was located with iodine vapour and eluted with acetone Re-examination of this purified material by t l c showed at least four carbohydrate toluene-*p*-sulphonates, of which two were major components and, in retrospect, are probably anomeric forms of the same derivative The mixture was methylated, detosylated, remethylated, and examined by g l c., using the procedures described above Isomaltosides were detected [T_S 1.48, 2 00, 2 60 (*minor*)], but no nigerosides

Relative yields of methyl ethers from O-*methyl*-O-*trityldextran and* O-*methyl*-O-*tosyldextran* — Each polysaccharide (10 mg) in methanolic hydrogen chloride (3% w/v, 1 ml) was heated on a boiling water-bath for 41 h After neutralisation with silver carbonate, filtration, and evaporation, the products were analysed by g l c on a polyethylene glycol adipate (10% on Gas Chrom P) column at 175°, using the Pye Argon Chromatograph with ^{90}Sr detector When each mixture was loaded to give similar peak heights for 2,4-di-*O*-methylglucosides, the peak heights for the faster 2,3,4-tri-*O*-methylglucoside derived from *O*-methyl-*O*-trityldextran and *O*-methyl-*O*-tosyldextran were in the ratio 21 10 (The second 2,3,4-tri-*O*-methylglucoside peak was off the chart)

ACKNOWLEDGMENTS

We thank Professor D J Manners and Dr J. R Turvey for samples of nigerose and isomaltose, Dr D H Hutson for help with the preparation of dextran, and the Institute of Paper Chemistry for a grant under the Pioneering Research Program

REFERENCES

1 S Peat, W J Whelan, and G J Thomas, *J Chem Soc*, (1956) 3025
2 J K N Jones and W H Nicholson, *J Chem Soc*, (1955) 3050
3 B Capon and B C Ghosh, *Chem Commun*, (1965) 586
4 J Hine and O B Ramsay, *J Amer Chem Soc*, 84 (1962) 973
5 M Charton, *J Org Chem*, 29 (1964) 1222
6 W B Neely, *Advan Carbohyd Chem*, 15 (1960) 341
7 D A Rees and N G Richardson, *Biochemistry*, 5 (1966) 3099
8 G O Aspinall, I M Cairncross, and A Nicholson, *Proc Chem Soc*, (1959) 270
9 D Abbott, E J Bourne, and H Weigel, *J Chem Soc* (*C*), (1966) 827, and earlier papers
10 T E Timell, *Chem Ind* (London), (1964) 503
11 L K Semke, N S Thompson, and D G Williams, *J Org Chem*, 29 (1964) 1041
12 W M Pasika and L H Cragg, *Can J Chem*, 41 (1963) 293
13 A Jeanes, W C Haynes, C A Wilham, J C Rankin, E H Melvin, M J Austin, J E Cluskey, B E Fisher, H M Tsuchiya, and C E Rist, *J. Amer Chem Soc*, 76 (1954) 5041
14 S Peat, W J Whelan, and J G Roberts, *J Chem Soc*, (1956) 2258

15 M. Jackson and L D. Hayward, *J Chromatog* , 5 (1961) 166
16 E Stahl and U Kaltenbach, *J Chromatog* , 5 (1961) 451.
17 A. M. Duffield and A. L Nussbaum, *Anal. Biochem* , 7 (1964) 254
18 M Dubois, K A Gilles, J K Hamilton, P A Rebers, and F Smith, *Anal Chem* , 28 (1956) 350
19 C. T Bishop, *Advan Carbohyd Chem* , 19 (1964) 95, especially p 108.

Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

BUTYLIDENE ACETALS OF D-MANNITOL AND THEIR N.M.R. SPECTRA*

T G BONNER, E J BOURNE, D G GILLIES, AND D LEWIS

Chemistry Department, Royal Holloway College (University of London), Englefield Green, Surrey (Great Britain)

(Received September 16th, 1968)

ABSTRACT

The condensation of butyraldehyde and D-mannitol has been studied, and the 1,3 2,5 4,6-tri-, 1,3 4,6- and 1,3 5,6-di-, and 1,3- and 3,4-monoacetals have been characterised Their n m r spectra have been determined, and assignments made that confirm the ring sizes

INTRODUCTION AND DISCUSSION

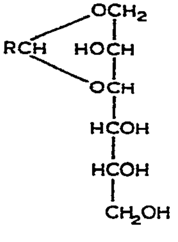
Holst[1] reported that condensation of butyraldehyde with D-mannitol in the presence of concentrated sulphuric acid yields a syrupy triacetal (79%) This product was not chemically characterised. In our hands, his method yielded diacetals (three g l c. peaks, 3% isolated) and triacetals (three g l c peaks, 60% isolated), but the only pure compound obtained was a trace (0 7%) of the crystalline 1,3 2,5 4,6-triacetal With phosphoryl chloride as catalyst, removal of the water of condensation by azeotropic distillation gave the mixed diacetals, but no 1,3 2,5 4,6-triacetal could be isolated However, use of aqueous hydrobromic acid as catalyst gave better yields (13–17%) of the crystalline 1,3 2,5 4,6-triacetal; when less aldehyde was used, the crystalline 1,3 5,6-diacetal (2%) was also obtained The main (*i e.*, syrupy) triacetal fraction obtained by Holst's method or by using phosphoryl chloride always yielded, on partial hydrolysis with acid, a mixture of 3,4- and 1,3-monoacetals, from which the crystalline 3,4-isomer (2–7%) could be obtained Partial hydrolysis of the 1,3 2,5 4,6-triacetal with acid yielded the crystalline 1,3 4,6-diacetal (24%) and crystalline 1,3-monoacetal (22%)

The structure of the 1,3-acetal **1** was proved as follows It consumed 1 94 mol of periodate and gave 1 05 mol. of formaldehyde and 0 975 mol of formic acid The major fission product of oxidation was identical (paper chromatography) with 2,4-*O*-butylidene-D-erythrose[2], and acetylation yielded 2,4-*O*-butylidene-D-erythrose acetate[2] (16%). The acetal **1** did not migrate when subjected to ionophoresis in molybdate[3] buffer (pH 5.0); when hydrolysed, it gave mannitol and butyraldehyde. It gave a crystalline tetrabenzoate and a crystalline benzeneboronate [but not a bis(benzeneboronate)]

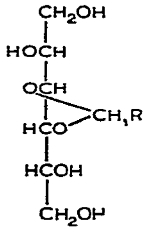
---

*Dedicated to Professor Stanley Peat, F. R S

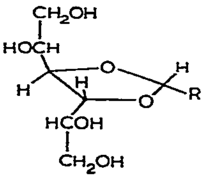
The 3,4-acetal **2** did not migrate during ionophoresis in molybdate buffer, consumed 2 06 mol of periodate, and liberated 1 96 mol of formaldehyde, but no acidity developed. These facts prove the structure. Acid hydrolysis yielded mannitol and butyraldehyde The acetal was characterised through its bis(benzeneboronate) and tetrabenzoate



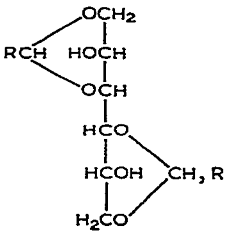
1



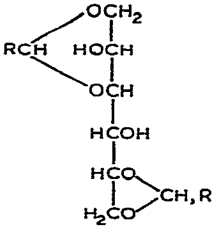
2



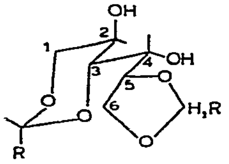
2a



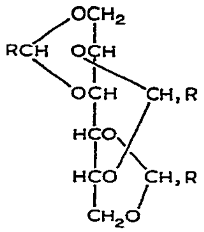
3



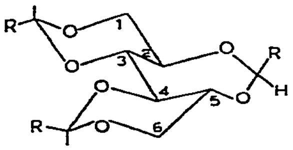
4



4a



5



5a

$R = CH_3CH_2CH_2—$ $2 = 2a$, $4 = 4a$, $5 = 5a$

The 1,3 4,6-diacetal **3** did not consume periodate Evidence for the presence of two hydroxyl groups was the isolation of a diacetate, a dibenzoate, and a syrupy dimethyl ether The ether on hydrolysis and then acetylation yielded 2,5-di-*O*-methyl-D-mannitol tetra-acetate[4]

The 1,3 5,6-diacetal **4** also did not consume periodate It yielded a dimethyl ether, a dibenzoate, and a monotoluene-*p*-sulphonate. The ether on acid hydrolysis gave the syrupy 2,4-di-*O*-methyl-D-mannitol, characterised as its crystalline tetra-acetate, which was synthesised by a definitive route from 1,6-di-*O*-benzoyl-2,4-*O*-methylene-D-mannitol[5] by methylation, debenzoylation, hydrolysis, and acetylation

The ring sizes of the various acetals were confirmed by n m r spectroscopy The acetals, in deuterochloroform (except where stated), gave a triplet signal for the proton on the acetal carbon Some spectra displayed more than one triplet feature The position of the acetal proton was identified by double resonance On irradiation of the methylene region of the propyl group, and observation of the spectrum at lower field, only the acetal triplet was perturbed The chemical shift of the acetal proton was related to the ring size by using an analysis based on that previously used for benzylidene[6] and furfurylidene[7] acetals

Acid-catalysed condensation of DL-propane-1,2-diol and butyraldehyde gave a product showing two triplets of comparable intensity at τ 5 10 and 4 99. These signals are assigned to the DL-pairs of five-membered ring acetals, the feature at high field to the acetal proton *trans* to the methyl group, that at low field to the *cis* proton 2,3-*O*-Butylidene-D-glucitol[8] in methyl sulphoxide-d_6 had a signal (τ 4 92) supporting the value assigned above to the dioxolane ring, assuming that there is no significant shift of the peak on change of the solvent 3,4-*O*-Butylidene-D-mannitol (**2**) in deuterium oxide or methyl sulphoxide-d_6 had several different resonances in the range τ 5.0–5 5 which obscured the acetal proton, and therefore its tetrabenzoate was used This gave a signal at τ 4 75, integration of which indicated 0 95 proton

The 1,3 5,6-diacetal **4** had signals at τ 5 05 and 5 41, which integration showed were each due to 1 1 protons The low-field signal is assigned to the dioxolane, but it is not possible to say whether the proton is *trans* or *cis* to C-4 The high-field signal must therefore arise from the dioxane ring, and this value is supported by the position of the peak (τ 5.48, 2 2 protons) found for the 1,3 4,6-diacetal **3** The tetrabenzoate of the 1,3-acetal **1** gave a signal at τ 5 45 In this spectrum, the high-field part of the triplet was obscured by other signals, and could not be integrated accurately The spectrum of the 1,3-acetal in deuterium oxide displayed several peaks in the region τ 5 1–5 4.

The spectrum of the 1,3 2,5 4,6-triacetal **5** contained a 2-proton triplet at τ 5 50, which is assigned to the dioxane rings There was also a 1-proton triplet at τ 5 25, which must therefore be due to the dioxepane ring However, 2-propyl-1,3-dioxepane (neat liquid) gave a signal (0 94 proton) at τ 5 43 The discrepancy between these τ values could be relieved by suggesting that increased steric hindrance in the triacetal could result in a shift to low field The signal at τ 5 25 cannot, in a hexitol derivative, arise from a third dioxane ring, and it is at a higher field than for any dioxolane

The results thus show that the acetal protons of dioxolane rings studied appear at, or below, τ 5.10, and those of dioxane rings at or above τ 5.41, with dioxepane rings being roughly intermediate The two tetrabenzoates reported also obey this scheme

The only triacetals (from aldehydes, as distinct from ketones) of mannitol with proved structure are methylene[9] and ethylidene[4], both having a 1,3 2,5 4,6-arrangement and synthesised in good yield (94 and 72%, respectively) On this evidence, Barker and Bourne postulated[10] that such an arrangement is the preferred triacetal pattern for mannitol, and Mills[11], using somewhat different arguments, arrived at the same conclusion In the present work, although the 1,3 2,5 4,6-triacetal was isolated, the dominant triacetal fraction appears, from the evidence of partial hydrolysis, to be mixed 1,2·3,4 5,6-triacetals, an analogous situation was noted with butylidene acetals of D-glucitol[12] Thus, it seems that, under our conditions, the rules governing the preferential formation of acetal rings when D-mannitol reacts with an aldehyde are followed less closely as the size of the aldehyde increases However, these rules were developed on the basis of products isolated after long reaction times and must relate in most cases to thermodynamically controlled products It is now known[13] that the kinetically controlled acetals formed in the early stages of acetal synthesis may differ structurally from the ultimate products, and, perhaps, in the present work, the final stages of the syntheses were not reached It is of interest, in this connection, that the only diacetal isolated from the syntheses was 1,3 5,6-di-*O*-butylidene-D-mannitol (4) which carried one six-membered *β*-ring (favoured by the rules) and one five-membered *α*-ring (less favoured by the rules, but probably more favoured during kinetic control[13]) Models show that this diacetal would be unlikely to lead, by reaction at positions 2 and 4, to a triacetal, since the resulting *trans*-fused bicyclic system would carry the five-membered (5,6) ring as a large, axial substituent.

EXPERIMENTAL

Quantitative, periodate oxidations[14], formaldehyde[15], and formic acid[16] determinations used standard procedures Light petroleum refers to the fraction having b p. 60–80°. Gas–liquid chromatography involved a Pye Argon Chromatograph having a *β*-ionisation detector. Columns[17] *A* and *B* contained butane-1,4-diol succinate polyester at 175° and polyphenyl ether [*m*-bis(*m*-phenoxyphenoxy)benzene] at 175°, respectively. Hydroxy compounds were converted into their trimethylsilyl[18] ethers before injection. The n m r spectra were usually recorded on a Varian HA 60-IL spectrometer, with tetramethylsilane as an internal reference (except when using deuterium oxide)

Method *B* of Astle, Zaslowsky, and Lafyatis[19] was used to prepare (*a*) DL-propane-1,2-diol butylidene acetal, b p (after redistillation) 139–141° (lit [20], b p 141–144°), and (*b*) 2-propyl-1,3-dioxepane (29%), b.p (after redistillation) 174–176° (lit [21], b.p 169–171°)

Condensation of butyraldehyde and D-*mannitol* — (*a*) *Holst's method*[1] In our hands, the redistilled product (103 g) from this method had b p 162–166°/0 7 mm, $[\alpha]_D^{25}$ −2 5° (*c* 10 0, ethanol) The product partially solidified due to the presence of mixed diacetals Holst reported a product having b p. 160–161°/3 mm, $[\alpha]_D^{25}$ +3 6° Crystallisation of our product from light petroleum gave the mixed diacetals (3%, m p 94–97°, 3 peaks on column *B*), which did not afford a pure isomer by crystallisation, chromatography on alumina, or isolation of a derivative Chromatography of the triacetal product (3 peaks on column *A*) on alumina with light petroleum gave 1,3 2,5·4,6-tri-*O*-butylidene-D-mannitol (0 7% overall), m p 81–82 5° (from aqueous ethanol), $[\alpha]_D^{22}$ −32 0° (*c* 1 8, chloroform) (Found C, 63 0, H, 9 2 $C_{18}H_{32}O_6$ calc C, 62 8; H, 9.4%)

(*b*) *Azeotropic removal of the water of acetalisation* D-Mannitol (91 g), butyraldehyde (140 ml), benzene (310 ml), and phosphoryl chloride (6 drops) were refluxed together for 4 h (total of water collected, 14–17 5 ml) The product (55–85 g) had b p. 161–180°/0 7 mm , it yielded mixed diacetals (*ca* 1 g, m p. 92–94 5°) Chromatography on alumina gave no 1,3 2,5 4,6-triacetal (*c*) *Hydrobromic acid* Hydrobromic acid (80 ml, 48% w/w) was added to D-mannitol (160 g) and butyraldehyde (460 ml) with cooling and manual stirring After storage for *ca* 17 h, light petroleum was added, and the organic layer was washed with water The product (266–290 g) had b p. 160–176°/0 8 mm Crystallisation (five times) from aqueous ethanol yielded the 1,3 2,5·4,6-triacetal (38–50 g), m p. 80–82° With D-mannitol (91 g), butyraldehyde (132 ml) and hydrobromic acid (10 ml) gave, after vacuum distillation, and crystallisation from light petroleum, a mixed diacetal fraction (6.3 g, m p 94–97°) The main crystallisation liquors yielded material which, after four recrystallisations from light petroleum, gave needles of 1,3 5,6-di-*O*-butylidene-D-mannitol (3 5 g), m.p 99–101°, $[\alpha]_D^{21}$ −17.8° (*c* 1 7, chloroform) (Found· C, 58.2; H, 8 8 $C_{14}H_{26}O_6$ calc C, 57 9; H, 9.0%) The diacetal has a characteristic absorption at 975 cm^{-1} (KBr disc)

Periodate oxidation of the 1,3 5,6-*diacetal.* — The diacetal in a water–methanol solution (9 1, v/v) consumed no periodate (1 8 mol initially present) after 21 5 h

Derivatives of 1,3 5,6-*di-O-butylidene-*D-*mannitol.* — (*a*) The diacetal (1 0 g), *N,N*-dimethylformamide (3 ml), methyl iodide (2 5 ml), and silver oxide (1 5 g) were shaken for 40 h After working up in the usual manner, chromatography on alumina (25g) with light petroleum–benzene (1·1) yielded the 2,4-dimethyl ether (0.63 g) m p 24–26° (unchanged on crystallisation from aqueous ethanol), $[\alpha]_D^{24}$ −6 7° (*c* 1 1, chloroform) (Found 60 3, H 9 5; OMe, 19.3. $C_{16}H_{30}O_6$ calc C, 60 35, H, 9 5, OMe, 19 5%)

(*b*) The diacetal (0 5 g) gave the 2,4-dibenzoate (0 49 g), m p 90–92° (from 8 parts of light petroleum), $[\alpha]_D^{23}$ −58.0° (*c* 1 7, chloroform). (Found C, 67 5; H, 6 9; Bz, 42 5 $C_{28}H_{34}O_8$ calc C, 67 5, H, 6 9, Bz, 42 2%) Deacylation regenerated the parent diacetal, m p 98–100°

(*c*) The diacetal (0 2 g) in pyridine (1 2 ml) with toluene-*p*-sulphonyl chloride (0 3 g, 2 3 mol) for 20 h gave a monotoluene-*p*-sulphonate (0.06 g, 20%), m p 96–98° (from 30 parts of light petroleum) (Found C, 56 55; H, 7 2, S, 7 4 $C_{21}H_{32}O_8S$ calc C, 56.7; H, 7.3, S, 7 2%). The product decomposed on storage

Partial acid hydrolyses of the triacetals. — (*a*) *Syrupy triacetal fraction from Holst's method.* The triacetal (68 5 g) was treated with acetic acid–water (212 ml 89 ml) for 50 min at 88–90° The solution was evaporated at 45°. The residue was freed from water by co-distillation with ethanol, and then chloroform The light petroleum-insoluble portion (23 g) was purified on alumina (400 g), using ethanol–water (94 6, v/v) passing to industrial methylated spirit The first monoacetal fractions (3 3 g), m p. 132–133°, were a 1·1 mixture of the 1,3- and 3,4-isomers, which could not be resolved by g l.c The later monoacetal fractions gave pure 3,4-*O*-butylidene-D-mannitol (3 5g, 7%), m p 113–114° (from 5 parts of ethanol), $[\alpha]_D^{23}$ +28 3° (*c* 1 8, water) (Found C, 50 9, H, 8 3 $C_{10}H_{20}O_6$ calc C, 50 8, H, 8 5%) The acetal did not migrate on ionophoresis in molybdate[3] buffer (pH 5 0)

(*b*) *Syrupy triacetal obtained by azeotropic removal of the water of acetalisation* This material gave 2% of pure 3,4-monoacetal when treated as above

(*c*) *1,3 2,5 4,6-Triacetal* The triacetal (20 g) in ethanol (850 ml) and concentrated hydrochloric acid (85 ml) was kept for 3 days at 22–23°. After neutralisation with concentrated, aqueous sodium hydroxide, the mixture was filtered, the filtrate was evaporated, and the dried residue, in benzene, was purified on alumina (350 g). Elution with chloroform → ethanol → industrial methylated spirit–water (94:6, v/v) gave, successively, unchanged triacetal (5.2 g), crude 1,3:4,6-diacetal (4.0 g), m.p. up to about 83°, and 1,3-*O*-butylidene-D-mannitol (2.2 g) (Found: C, 50.9; H, 8.5%), m.p. 111–113°, which on admixture with an equal weight of the 3,4-isomer had m.p. 133–134°. The monoacetal, plates from ethyl acetate, had $[\alpha]_D^{23}$ −25.5° (*c* 1.85, water) and was non-migrating in molybdate buffer (pH 5.0). The diacetal was purified through its diacetate or dibenzoate (see below). Deacylation of the diacetate yielded the parent 1,3:4,6-di-*O*-butylidene-D-mannitol, m.p. 97–99° (from 60 parts of light petroleum), $[\alpha]_D^{21}$ +9.7° (*c* 1.7, chloroform) (Found: C, 57.9; H, 9.1%). The diacetal was obtained as needles or as a mixture of flakes and spheres. These dimorphs (both of the same m.p.) had different infrared spectra (KBr discs); one showed seven medium or weak intensity absorption bands between 990 and 870 cm^{-1}, and the other medium intensity absorption bands at 925, 895, and 850 cm^{-1}. Identical infrared spectra were obtained for solutions in carbon tetrachloride. The m.p. showed depression on admixture with the 1,3:5,6-diacetal, m.p. 99–101°.

*Periodate oxidation of 1,3:4,6-di-O-butylidene-*D*-mannitol.* — The diacetal consumed no periodate (2.3 mol. initially present) after 24 h.

*Derivatives of 1,3:4,6-di-O-butylidene-*D*-mannitol.* — (*a*) The diacetal gave the 2,5-diacetate (78%), m.p. 139–140° (from 45 parts of light petroleum), $[\alpha]_D^{23}$ −52.3° (*c* 1.2, chloroform) (Found: C, 57.8; H, 8.0; Ac, 22.8. $C_{18}H_{30}O_8$ calc.: C, 57.7; H, 8.1; Ac, 23.0%).

(*b*) The diacetal gave the 2,5-dibenzoate (70%), m.p. 154–156° (from 35 parts of methanol), $[\alpha]_D^{22}$ −4.8° (*c* 1.7, chloroform) (Found: C, 67.3; H, 7.0%).

(*c*) The diacetal (0.65 g) was methylated, and the product was purified, as described for the methylation of the 1,3:5,6-diacetal, to yield the syrupy 2,5-dimethyl ether (0.62 g), $[\alpha]_D^{24}$ −44.0° (*c* 2.7, chloroform) (Found: C, 60.6; H, 9.4; OMe, 19.5%).

*Hydrolysis of 1,3:4,6-di-O-butylidene-2,5-di-O-methyl-*D*-mannitol.* — The diether (0.07 g) was boiled for 25 min with 1.5 ml of a solution prepared from ethanol (8 ml) and N hydrochloric acid (2 ml). After evaporation, the residue was acetylated to yield 1,3,4,6-tetra-*O*-acetyl-2,5-di-*O*-methyl-D-mannitol (0.06 g), m.p. 107–108°. The melting point showed no depression on admixture with the authentic[4] acetate.

*Hydrolysis of 1,3:5,6-di-O-butylidene-2,4-di-O-methyl-*D*-mannitol.* — The diether (0.1 g) was hydrolysed, and the hydrolysate was acetylated, as described for the 2,5-di-*O*-methylacetal, to yield 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-D-mannitol (0.04 g), m.p. 88–89° (from light petroleum containing a trace of ethanol) (Found: C, 50.7; H, 7.15; OMe, 16.1. $C_{16}H_{26}O_{10}$ calc.: C, 50.8; H, 6.9; OMe, 16.4%).

*Periodate oxidation of 1,3-O-butylidene-*D*-mannitol.* — The acetal consumed 1.92, 1.97, 1.97, and 1.94 mol. of periodate (5.05 mol. initially present) after 0.75, 2.25, 7.5, and 24 h, respectively, and gave 1.05 mol. of formaldehyde and 0.975 mol. of formic acid.

The acetal (0.3 g) was oxidised as described[2] for 4,6-*O*-butylidene-D-glucose. The fission product (single spot, R_F 0.945) co-chromatographed with 2,4-*O*-butylidene-D-erythrose in butyl alcohol–ethanol–water (40:11:19, v/v). A portion (0.05 g) was acetylated and yielded the acetate (16%), m.p. and mixed m.p. 154–155°, the infrared spectrum (KBr disc) was identical to that of the authentic acetate.

*Derivatives of 1,3-*O*-butylidene-D-mannitol.* — (*a*) The acetal gave the 2,4,5,6-tetrabenzoate (40%), m.p. 125–127° (from methanol), $[\alpha]_D^{25}$ +26.2° (*c* 1.7, chloroform) (Found C, 69.9, H, 5.7, Bz, 64.4. $C_{38}H_{36}O_{10}$ calc. C, 69.9, H, 5.6, Bz, 64.4%). Deacylation regenerated the parent acetal.

(*b*) The acetal (0.15 g) was treated with benzeneboronic anhydride (0.066 g, 0.33 mol.) in methanol, to afford a monobenzeneboronate (0.18 g), m.p. 116–118° (from 4 parts of carbon tetrachloride), $[\alpha]_D^{22}$ −10.85° (*c* 1.7, chloroform) (Found C, 59.7; H, 7.4; B, 3.2. $C_{16}H_{23}BO_6$ calc. C, 59.65, H, 7.2; B, 3.4%).

*Periodate oxidation of 3,4-*O*-butylidene-D-mannitol.* The acetal consumed 1.97, 2.03, and 2.06 mol. of periodate (4.4 mol. initially present) after 0.6, 4.5, and 18 h, respectively, and gave 1.96 mol. of formaldehyde. No acidity developed during the oxidation.

*Derivatives of 3,4-*O*-butylidene-D-mannitol.* — (*a*) The acetal (0.4 g) was treated as described[22] for the preparation of D-arabinose bis(benzeneboronate), and gave the 1,2:5,6-bis(benzeneboronate) (0.58 g), m.p. 69–72° (from 5 parts of light petroleum), $[\alpha]_D^{27}$ +45.0° (*c* 1.7, chloroform) (Found C, 64.7, H, 6.3; B, 5.2. $C_{22}H_{26}B_2O_6$ calc. C, 64.7, H, 6.4, B, 5.3%).

(*b*) The acetal (0.15 g) gave the 1,2,5,6-tetrabenzoate (0.18 g), m.p. 88–90.5° (from methanol), $[\alpha]_D^{19}$ −8.75° (*c* 1.5, chloroform) (Found C, 69.9, H, 5.5, Bz, 64.1%). Deacylation regenerated the acetal, m.p. 114–115°.

Acid hydrolysis of the 1,3- and 3,4-monoacetals. — The acetal (0.16–0.2 g) in 0.2N acid was treated for 30 min as described[2] for the hydrolysis of 4,6-*O*-butylidene-D-glucitol. The 1,3- and 3,4-acetals gave 63 and 73% of D-mannitol hexa-acetate and 79 and 74% of butyraldehyde bisdimedone, respectively, the appropriate mixed melting-points showed no depression.

*1,6-Di-*O*-benzoyl-2,4-di-*O*-methyl-3,5-*O*-methylene-D-mannitol.* — 1,6-Di-*O*-benzoyl-2,4-*O*-methylene-D-mannitol[5] (10 g), *N,N*-dimethylformamide (25 ml), methyl iodide (15 ml), and silver oxide (10 g) were shaken for 41 h. After being processed in the usual manner, the product was purified on alumina (180 g) by using light petroleum–benzene (2:1) → benzene–chloroform (2:1). The first solid material eluted, m.p. 76–77°, was rejected as it could not be purified. The remaining material afforded the dimethyl ether (4.1 g), m.p. 78–80° (from 4 parts of ethanol), $[\alpha]_D^{23}$ +2.0° (*c* 1.7, chloroform) (Found C, 63.9, H, 6.1; OMe, 14.1; Bz, 48.3. $C_{23}H_{26}O_8$ calc. C, 64.2, H, 6.1, OMe, 14.4; Bz, 48.8%).

*1,3,5,6-Tetra-*O*-acetyl-2,4-di-*O*-methyl-D-mannitol.* — 1,6-Di-*O*-benzoyl-2,4-di-*O*-methyl-3,5-*O*-methylene-D-mannitol in chloroform was treated with methanolic sodium methylate in the usual way, and yielded crude, syrupy dimethylmethylene-D-mannitol. A portion (0.13 g) of this syrup was refluxed for 4.5 h with 0.6N hydrochloric acid (2 ml) in the presence of phloroglucinol[23] (0 05 g, 0 68 mol) The resulting 2,4-di-*O*-methyl-D-mannitol was contaminated with an impurity. Acetylation, and crystallisation from light petroleum–ethanol, afforded the tetra-acetate (*ca* 10% based on the crude dimethylmannitol), m p 86 5–87.5°, which showed no depression on admixture with the previously prepared tetra-*O*-acetyl-2,4-dimethyl ether Purification of the di-*O*-methyl-D-mannitol on cellulose (CF 11 grade), using butyl alcohol–light petroleum (b.p 100–120°)–water (15 15 1, v/v), followed by acetylation, also gave the pure tetra-acetate (2%)

ACKNOWLEDGMENTS

The authors thank Dr. E Percival of this Department for the use of a Pye Argon gas–liquid chromatograph, donated by the Wellcome Trust, Mrs. M. Hunt for technical assistance, and Imperial Chemical Industries Limited and S.C R for financial support

REFERENCES

1 W H. Holst, *U S Pat* 2,355,533 [*Chem Abstr*, 39 (1945) 191], *U S Pat* 2,387,662 [*Chem Abstr*, 40 (1946) 499]
2 T. G Bonner, E J Bourne, and D Lewis, *J Chem Soc*, (1965) 7453
3 E. J. Bourne, D H. Hutson, and H Weigel, *J Chem Soc*, (1960) 4252, (1961) 35, H Weigel, *Advan Carbohyd Chem*, 18 (1963) 61
4 E J Bourne, G T Bruce, and L F Wiggins *J Chem Soc*, (1951) 2708
5 W. N Haworth and L F Wiggins, *J Chem Soc*, (1944) 58, H G Fletcher, Jr, and H W Diehl, *J Amer Chem Soc*, 74 (1952) 3799
6 N Baggett, K W. Buck, A B Foster, M H Randall, and J M Webber, *J Chem Soc*, (1965) 3394, N Baggett, K W Buck, A B Foster, and J M Webber, *ibid*, (1965) 3401
7 T G Bonner, E J Bourne, S E Harwood, and D Lewis, *J Chem Soc (C)*, (1966) 2229.
8 T G Bonner, E J Bourne, P J V Cleare, and D Lewis, *J Chem Soc. (B)*, (1968) 822
9 A T Ness, R M. Hann, and C S Hudson, *J. Amer Chem Soc*, 65 (1943) 2215
10 S A Barker and E J Bourne, *Advan Carbohyd Chem*, 7 (1952) 137
11 J A Mills, *Advan Carbohyd Chem*, 10 (1955) 44
12 T G Bonner, E J Bourne, S E Harwood, and D Lewis, *J Chem Soc*, (1965) 121
13 T. G Bonner, E. J Bourne, P J. V. Cleare, and D. Lewis, *Chem Ind.* (London), (1966) 1268.
14 G O Aspinall and R J Ferrier, *Chem Ind* (London), (1957) 1216
15 J Mitchell, I M Kolthoff, E S Proskauer, and A Weissberger, *Organic Analysis*, Vol 1, Interscience, New York, 1953, p 288; W E A Mitchell and E. Percival, *J Chem Soc*, (1954) 1423
16 E L Hirst and J K N Jones, *J Chem Soc*, (1949) 1659
17 G O Aspinall, *J Chem Soc*, (1963) 1676
18 C C Sweeley, R Bentley, M Makita, and W W Wells, *J Amer Chem Soc*, 85 (1963) 2497.
19 M J Astle, J A Zaslowsky, and P G Lafyatis, *Ind Eng Chem*, 46 (1954) 787
20 P Mastagli, Mlle. P Lambert, and D Baladie, *Compt Rend*, 255 (1962) 2978
21 E D Bergmann and A Kaluszyner, *Rec Trav Chim*, 78 (1959) 337
22 T G Bonner, E J Bourne, and D Lewis, *Carbohyd. Res*, 2 (1966) 421
23 M L Wolfrom, B W Lew, and R M Goepp, Jr, *J Amer Chem. Soc*, 68 (1946) 1443.

Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

AMINOSACCHARIDES

PART IV* THE METHYL ETHERS OF 2-DEOXY-2-(2,4-DINITROANILINO)-D-GLUCOSE (Dnp-D-GLUCOSAMINE)**

P F LLOYD, B EVANS, AND R J FIELDER
Department of Chemistry, University College of North Wales, Bangor, Caernarvonshire (Great Britain)

(Received October 1st, 1968)

ABSTRACT

The methyl ethers of ethyl 2-deoxy-2-(2,4-dinitroanilino)-α-D-glucopyranoside (**2**) and the corresponding free sugar (**1**) have been synthesised They are crystalline and are readily separated and identified chromatographically Their structures were established by definitive synthesis, interconversion, and periodate-oxidation studies The 4- (**10**) and 6- (**11**) methyl ethers were synthesised *via* ethyl 3,4-di-*O*-acetyl-2-deoxy-2-(2,4-dinitroanilino)-6-*O*-trityl-α-D-glucopyranoside (**3**) and its de-*O*-tritylated derivative (**7**) When **7** was methylated, the acetyl group at C-4 migrated to C-6 (76%) The C-3 acetyl group in the 3,6-di-*O*-acetyl-4-*O*-methyl derivative **6** underwent methanolysis and acid hydrolysis abnormally slowly The 3,6-di- and the 4,6-dimethyl ethers, **12** and **13**, were synthesised by graded methylation of the 4- and 6-monoethers, **10** and **11** The 3-methyl ether **17** was synthesised *via* ethyl 4,6-*O*-benzylidene-2-deoxy-2-(2,4-dinitroanilino)-α-D-glucopyranoside (**15**), and the 3,4-dimethyl ether **20** was obtained by hydrolysis of the product of methylation of ethyl 2-deoxy-2-(2,4-dinitroanilino)-6-*O*-trityl-α-D-glucopyranoside (**18**) The 3,4,6-trimethyl ether **14** was obtained by methylation of glycoside **2**

INTRODUCTION

We have reported[1] recently a method of linkage analysis of mucopolysaccharides based upon the formation of *N*-2,4-dinitrophenyl (Dnp) derivatives of the de-*N*-acylated polysaccharides Application of this method to heparin has led to the isolation of, *inter alia*, a Dnp-disaccharide and a monosulphated Dnp-tetrasaccharide Our subsequent investigations on the structure of these oligosaccharides included methylation studies, and authentic specimens of the methyl ethers of 2-deoxy-2-(2,4-dinitroanilino)-D-glucose (Dnp-D-glucosamine) weie required as reference compounds This paper describes the synthesis of these ethers and their ethyl glycosides

RESULTS AND DISCUSSION

The synthesis of the three monomethyl, three dimethyl, and one trimethyl ethers of 2-amino-2-deoxy-D-glucopyranose have all been reported[2-7], and although

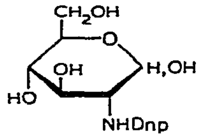
---

*Part III P F Lloyd and B Evans, *J Chem Soc* (C), (1968) 2753
**Dedicated to Professor Stanley Peat, F R S

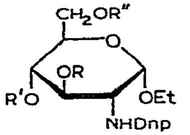
they have been characterised as the *N*-benzyloxycarbonyl, *N*-(2-hydroxynaphthylidene), or *N*-acetyl derivatives, only the acetyl group has been commonly used to protect the amino group during the synthesis.

One possible approach to the methyl ethers of Dnp-D-glucosamine consists of successive de-*N*-acetylation (by hydrolysis) and *N*-2,4-dinitrophenylation (using 1-fluoro-2,4-dinitrobenzene) of the *N*-acetyl derivatives prepared by existing methods However, a consideration of the experimental difficulties involved in the syntheses of *N*-acetyl derivatives, which include the formation of non-crystalline intermediates and the need for preparative, chromatographic analyses of non-coloured substances, together with the expectation, in some cases, of poor yields of intermediates, persuaded us that the use of the dinitrophenyl group as an *N*-blocking group, in parallel or alternative syntheses, would be preferable The Dnp group resists cleavage by acid and mild alkali[8,9], and since Dnp derivatives of amino sugars are crystalline and readily separable by chromatography, on account of their distinctive yellow colour, they can be widely used for synthesis[10–12]. The possibility that the migration of *O*-acetyl groups, in partially acylated derivatives, might occur was borne in mind from the outset, it being considered that such rearrangements could well facilitate the preparation of the complete range of ethers

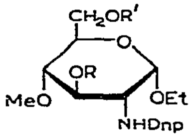
Treatment of 2-deoxy-2-(2,4-dinitroanilino)-D-glucose (**1**) (obtained now in much higher yield by an improved method) with ethanolic hydrogen chloride yielded ethyl 2-deoxy-2-(2,4-dinitroanilino)-α-D-glucopyranoside (**2**) (prepared earlier[11] by an indirect method) Treatment of **2** in successive stages with chlorotriphenylmethane and acetic anhydride furnished the trityl ether **3** which was separated from the main side-reaction product, ethyl 3,4,6-tri-*O*-acetyl-2-deoxy-2-(2,4-dinitroanilino)-α-D-glucopyranoside (**4**), by column chromatography on silica gel The triacetate **4** also appeared as a minor product after detritylation (acetic acid–hydrobromic acid) of **3** to give the 3,4-di-*O*-acetyl derivative **7**



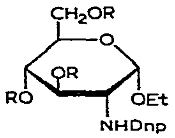
1



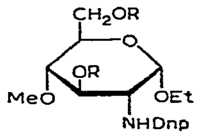
2 R=R=R″=H
3 R=R′=Ac, R″=Tr
4 R=R′=R″=Ac



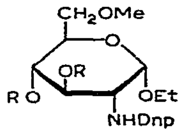
5 R=Ac, R′=H
6 R=R′=Ac



7 R=Ac, R=H
8 R=Ac, R=Me



9 R=Ac, R′=Me
10 R=R′=H



11 R=R=H 13R=H, R′=Me
12 R=Me, R=H 14R=R′=Me

Methylation of compound **7** with methyl iodide and silver oxide gave a chromatographically homogeneous product which did not show a sharp melting point

Deacetylation (methanolic ammonia), followed by chromatography on silica gel, gave ethyl 2-deoxy-2-(2,4-dinotroanilino)-6-*O*-methyl-α-D-glucopyranoside (**11**) in 16% yield, ethyl 2-deoxy-2-(2,4-dinitroanilino)-4-*O*-methyl-α-D-glucopyranoside (**10**) in 30% yield, and ethyl 3-*O*-acetyl-2-deoxy-2-(2,4-dinitroanilino)-4-*O*-methyl-α-D-glucopyranoside (**5**) in 46% yield, together with a small proportion (2% yield) of unchanged diacetate, which presumably was a mixture of **6** and **8**.

The structures of compounds **11**, **10**, and **5** were established by elemental and methoxyl analysis, and by the following experiments The monoether **11** was oxidised by sodium metaperiodate, and, since the interaction of Dnp-glucosamine derivatives with periodate is not anomalous[10], the methoxyl group must be located at C-6. Compound **10** was not attacked by periodate, indicating that it was etherified at C-3 or C-4. However, when the *N*-2,4-dinitrophenyl group was removed by hydrolysis with a basic ion-exchange resin[10], the resulting free amine was susceptible to oxidation by periodate, indicating the presence of a hydroxyl group adjacent to the amino group[13], and thus locating the methoxyl group in **10** at C-4

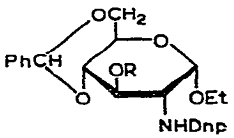
A reliable, qualitative test of the susceptibility of a Dnp compound to periodate consisted of chromatography before and after treatment with the oxidant For colourless, free amines, treatment with periodate was followed by replacement of the Dnp group Restoration of the characteristic yellow spot on the chromatogram indicated that oxidation had not occurred.

The chromatographic mobility of **5** suggested that it was a disubstituted derivative of ethyl 2-deoxy-2-(2,4-dinitroanilino)-α-D-glucopyranoside, and analysis revealed that it contained one methoxyl and one acetyl group Compound **5** was not oxidised by periodate, nor was the de-*N*-dinitrophenylated product, one substituent was therefore at position 3 The location of the methoxyl group was demonstrated when prolonged treatment (6 days) of **5** with methanolic ammonia removed the remaining acetyl group with formation of the 4-methyl ether **10**, **5** is therefore the 3-*O*-acetyl derivative of **10**

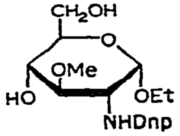
Exhaustive methylation of compounds **2**, **10**, and **11** gave a single product, ethyl 2-deoxy-2-(2,4-dinitroanilino)-3,4,6-tri-*O*-methyl-α-D-glucopyranoside (**14**) Partial methylation of **10** and **11** furnished two of the three isomeric dimethyl ethers of **2**, and, at the same time, confirmed the structures allocated to the monoethers Methylation of **11**, carried out with acetone as diluent, gave the 4,6-dimethyl derivative **13** (55%) and the 3,6-isomer **12** (21%) together with a small amount of the trimethyl ether **14** On the other hand, the 4-methyl ether **10** gave, under similar conditions, only one dimethyl ether, *viz*, the 4,6-derivative **13** (58%) The structure of **13** was deduced from the facts that it was formed from both compounds **10** and **11**, and was oxidised by periodate only after removal of the *N*-2,4-dinitrophenyl group The other dimethyl ether (21%) obtained from **11** was therefore the 3,6-derivative **12** Neither compound **12** nor its de-*N*-dinitrophenylation product was susceptible to periodate oxidation

Methylation of the mono-acetate **5** gave ethyl 3-*O*-acetyl-2-deoxy-2-(2,4-dinitroanilino)-4,6-di-*O*-methyl-α-D-glucopyranoside (**9**) which, like **5**, contained an acetyl group which could only be removed by prolonged methanolysis or acid hydrolysis Thus, acetate **9** was converted into **13** by methanolysis for six days

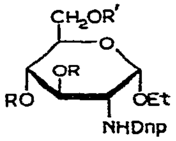
The definitive synthesis of ethyl 2-deoxy-2-(2,4-dinitroanilino)-3-*O*-methyl-α-D-glucopyranoside (**17**) was achieved as follows The ethyl glycoside **2** reacted rapidly with benzaldehyde to form the 4,6-*O*-benzylidene derivative **15** in good yield. Methylation then afforded the 3-*O*-methyl derivative **16**, which was converted into the required 3-*O*-methyl compound **17** by graded hydrolysis with 60% acetic acid Both **17** and its de-*N*-dinitrophenylation product resisted oxidation by periodate



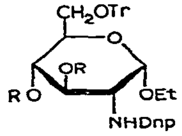
15 R = H
16 R = Me



17



18 R = H, R′ = Tr
19 R = Me, R = Tr
20 R = Me, R = H



21 R = Me, R′ = H
22 R = H, R′ = Me

Ethyl 2-deoxy-2-(2,4-dinitroanilino)-3,4-di-*O*-methyl-α-D-glucopyranoside (**20**) unexpectedly absent from the products of methylation of glycoside **10**, was synthesised by the following route The glycoside **2** was converted into the 6-*O*-trityl derivative **18**, and thence by methylation into the 3,4-di-*O*-methyl-6-*O*-trityl sugar **19** This route parallels that chosen by Jeanloz[5] for 2-acetamido-2-deoxy-3,4-di-*O*-methyl-D-glucose, and, in like manner, we isolated appreciable proportions of the 3- and 4-monomethyl ethers, formed, despite prolonged methylation, as a result, no doubt, of steric hindrance by the bulky substituent at C-6 The three trityl derivatives **19**, **21**, and **22** were not crystalline but were isolated as yellow glasses that were analytically pure De-*O*-tritylation of **19** with hydrobromic acid gave the 3,4-dimethyl ether **20** in high yield, and the isomeric 3- and 4-methyl ethers, **17** and **10**, were obtained in a similar manner from ethers **21** and **22**

The properties of the methyl ethers of **2** are summarised in Table I All melt sharply and are readily separated and identified by column and thin-layer chromatography Acid hydrolysis of these compounds gave the methyl ethers of the free sugar, 2-deoxy-2-(2,4-dinitroanilino)-D-glucose (**1**), each was obtained in about 70% yield These reducing compounds, although crystalline, did not display such sharp melting points as the glycosides but were readily characterised by their specific rotations and R_F values on t l c (Table II)

The schemes of synthesis described above are also routes to the corresponding ethers of 2-amino-2-deoxy-D-glucose (with free amino group or as an appropriate salt) and other *N*-substituted derivatives Conversion from the Dnp derivatives could be effected by removal of the Dnp group by hydrolysis[10] with an ion-exchange resin, followed by hydrolysis of the glycosidic linkage and introduction of the new acyl group, in an appropriate order

Certain features of the above syntheses are noteworthy Although there is ample precedent[14] for the migration of acetyl groups from other positions in a glucopyranose

TABLE I

PROPERTIES OF THE METHYL ETHERS OF ETHYL 2-DEOXY-2-(2,4-DINITROANILINO)-α-D-GLUCOPYRANOSIDE (2)

Compound	*M p.* *(degrees)*	$[\alpha]_D^{19}$ [a] *(degrees)*	R_F [b]
Parent compound 2	193–194	−18 3[c]	0 11
3-Methyl ether	146–147	−34 2	0 26
4-Methyl ether	176–177	+10 4	0 30
6-Methyl ether	165–166	−30 0	0 36
3,4-Dimethyl ether	99–100	+ 8 9	0 63
3,6-Dimethyl ether	124–125	−35 6	0 68
4,6-Dimethyl ether	142–143	+ 4 6	0 79
3,4,6-Trimethyl ether	129–130	+ 6 1	0 92

[a] In chloroform, *c* 0 2–0 4 [b] T l c on Kieselgel G in chloroform–ethanol (19 1 v/v) [c] *c* 0 05

TABLE II

PROPERTIES OF THE METHYL ETHERS OF 2-DEOXY-2-(2,4-DINITROANILINO)-D-GLUCOSE (1)

Compound	*M p* *(degrees)*	$[\alpha]_D^{19}$ [a]	R_F [b]		*Analysis*			
					C	*H*	*N*	*OMe*
Parent compound 1	194–196	+ 6 7	0 01					
3-Methyl ether	204–208	−13 9	0 03	Found	43 6	4 5	11 5	—
				Calc	43 5	4 7	11 7	8 6
4-Methyl ether	177–178	+49 0	0 05	Found	43 7	4 6	11 8	8 3
6-Methyl ether	188–195	+ 6 6	0 08	Found	43 1	4 6	11 6	8 2
3,4-Dimethyl ether	175–180	+26 8	0 16	Found	44 6	4 9	11 3	—
				Calc	45 0	5 1	11 2	16 9
3,6-Dimethyl ether	201–205	−18 2	0 22	Found	44 6	5 0	11 0	16 2
4,6-Dimethyl ether	200–210	+37	0 26	Found	44 8	5 0	11 4	16 0
3,4,6-Trimethyl ether	166–168	0 0	0 55	Found	46 0	5 2	10 6	22 8
				Calc	46 5	5 5	10 9	24 0

[a] In acetone, *c* 0 2–0 4 [b] T l c on Kieselgel G in chloroform–ethanol (19 1 v/v)

ring to C-6, the extent of rearrangement during methylation of acetate **7** (yield of **10**+**5** = 76%) was remarkably high despite the very mild, alkaline conditions that obtained This may reflect, in part at least, the ease with which the mixture of products was separated and crystallised in this case Preliminary studies suggest that when, in a parallel scheme of synthesis, acetyl groups are replaced by benzoyl groups, migration is almost completely inhibited.

Also of interest is the unusually high resistance, to both acid hydrolysis and alkali-catalysed methanolysis, of the C-3 acetyl group in the 3-*O*-acetyl-4-*O*-methyl-glycosamide **5** This, together with the observation that no methylation at C-3 occurs under mild conditions, may be taken to indicate a poor accessibility of this position in the ring to both electrophilic and nucleophilic reagents Steric hindrance by the Dnp group at C-2 may be involved here, but, since other derivatives having acetyl groups at C-3 and C-4 (*e g* , the 3,4,6-tri-*O*-acetyl and the 3,4-di-*O*-acetyl-6-*O*-methyl derivatives of **2**) are smoothly deacylated, the location of a methoxyl group at C-4 would also seem to be a necessary condition for this anomaly. Alternatively, compound **5** itself may be stabilised by chelation, but this is considered to be unlikely Considerations such as these may have consequences and applications in the acid hydrolysis of mucopolysaccharides

Although the highly polarised Dnp group causes anomalous optical rotational effects[15], such n m r data as are available for 2-deoxy-2-(2,4-dinitroanilino)-D-glucose derivatives[16] suggest that the predicted *C1* (D) conformation is adopted Further studies on the nature and conformation of glycoside **5** and related compounds are envisaged

EXPERIMENTAL

All evaporations were carried out under diminished pressure unless otherwise stated Optical rotations were measured with a Perkin–Elmer 141 polarimeter Thin-layer chromatography (t l c) and column chromatography were performed with Kieselgel G (Merck 7731) and chloroform–nitromethane, 9 1 v/v (*A*), or chloroform–ethanol, 19 1 v/v (*B*)

*2-Deoxy-2-(2,4-dinitroanilino)-*D*-glucose* (**1**) — 2-Amino-2-deoxy-D-glucose hydrochloride (45 0 g) and sodium carbonate (22 0 g) in water (225 ml) were mixed with a solution of 1-fluoro-2,4-dinitrobenzene (42 7 g) in methanol (900 ml) and shaken for 2 days at room temperature. On removal of the methanol by distillation, the product **1** crystallised It was washed with water, dried over P_2O_5, and used for the next stage without recrystallisation, yield, 56 3 g (78%), m p 192–194°, $[\alpha]_D^{21}$ +42 2° (*c* 1 0, ethanol); lit [10], m p 194–196°, $[\alpha]_D^{13}$ +41 2°

*Ethyl 2-deoxy-2-(2,4-dinitroanilino)-α-*D*-glucopyranoside* (**2**). — A solution of compound **1** (30 0 g) in dry ethanol (1.1 litre) containing 2 1% of hydrogen chloride was heated under reflux for 6 h The solution was neutralised (silver carbonate or barium carbonate), filtered, and evaporated to a small volume The glycoside **2**, which crystallised, was recrystallised, as yellow needles, from ethanol, yield, 25 6 g (80%), m.p 193–194°; $[\alpha]_D^{21}$ +23 1° (*c* 0 3, in acetone), lit [11], m p 193–195°, $[\alpha]_D^{19}$ +25 9°

*Ethyl 3,4-di-O-acetyl-2-deoxy-2-(2,4-dinitroanilino)-6-O-trityl-α-*D*-glucopyranoside* (**3**) — Compound **2** (25 0 g) was dissolved in dry pyridine, and recrystallised chlorotriphenylmethane (20 3 g) was added. The mixture was shaken until complete dissolution was achieved and then heated under reflux for 6 h Dry pyridine (350 ml) was added, the solution cooled to 0°, and acetic anhydride (350 ml) added After a further 2 days at room temperature, the reaction mixture was poured into ice–water (2 5 l), and the yellow precipitate was collected by filtration, washed with water, and dried over P_2O_5 Purification by column chromatography with solvent *A*, and recrystallisation from ethanol, gave product **3** (33 8 g, 62%), m p 181–182°, $[\alpha]_D^{21}$ +39.4° (*c* 0 35, chloroform) (Found C, 63 7; H, 5 3, N, 5 9 $C_{37}H_{37}N_3O_{11}$ calc. C, 63.5, H, 5 3, N, 6 0%)

Also isolated from the column was ethyl 3,4,6-tri-*O*-acetyl-2-deoxy-2-(2,4-dinitroanilino)-α-D-glucopyranoside[11] (**4**) (2 5 g), m p and mixed m p 220–221°, $[\alpha]_D^{21}$ +15 8° (*c* 0 3, chloroform)

*Ethyl 3,4-di-O-acetyl-2-deoxy-2-(2,4-dinitroanilino)-α-*D*-glucopyranoside* (**7**) — To a solution of compound **3** (10.0 g) in glacial acetic acid (220 ml) at 10° was added hydrobromic acid in glacial acetic acid (50% w/v, 10 ml), and the mixture was shaken for 1 min The precipitate was immediately removed by filtration and washed with a little cold acetic acid The filtrate and washings were poured into cold water (2 l), and the precipitate of crude product was extracted with chloroform The extract was washed (water), dried ($MgSO_4$), and evaporated, and the residue was recrystallised from ethanol Further purification was effected by column chromatography on silica gel (solvent *A*) and recrystallisation from ethanol. Compound **7**, thus obtained, had m p 241°, $[\alpha]_D^{22}$ −15 0° (*c* 0 6, chloroform) (Found C, 47 7; H, 5 3, N, 9.2. $C_{18}H_{23}N_3O_{11}$ calc C, 47.3; H, 5 1; N, 9 2%) The chief impurity, also isolated from the column, was compound **4** (0 44 g), m p 221° Yield of **7** was 3 5 g (45%)

*Methylation of ethyl 3,4-di-O-acetyl-2-deoxy-2-(2,4-dinitroanilino)-α-*D*-glucopyranoside* (**7**) — Compound **7** (5 0 g), dissolved in methyl iodide (100 ml), was magnetically stirred with silver oxide (freshly prepared, 5 0 g), and heated under reflux for 2 days Methylation was then complete (t l c) The silver residues were removed by filtration and washed (dry chloroform), and the combined filtrate and washings were evaporated to dryness The residue was dissolved in the minimal volume of chloroform, and the product crystallised by addition of pentane The yellow, crystalline solid obtained (3 8 g, 74%) had m.p 188–191°, $[\alpha]_D^{21}$ +23 8° (*c* 0 2, chloroform)

The methylation product (7 0 g) was dissolved in anhydrous methanol (700 ml) that had been saturated with ammonia at 0° and allowed to stand for 24 h The solvent was removed by distillation, and the yellow residue was analysed on a silica gel column with solvent *B* Three main products, obtained after evaporation of combined fractions from the column, were recrystallised from ethanol–light petroleum, to give the following compounds (*i*) Ethyl 2-deoxy-2-(2,4-dinitroanilino)-6-*O*-methyl-α-D-glucopyranoside (**11**) (1.6 g, 16%), m p. 165–166°, $[\alpha]_D^{21}$ −30 0° (*c* 0 2, chloroform) (Found· C, 47 2; H, 5 6; N, 10 9; OMe, 7 7 $C_{15}H_{21}N_3O_9$ calc C, 46 5; H, 5.5, N, 10.8; OMe 8 0%).

(*ii*) Ethyl 2-deoxy-2-(2,4-dinitroanilino)-4-*O*-methyl-α-D-glucopyranoside (**10**) (2 4 g, 30%), m p 176–177°, $[\alpha]_D^{21}$ +10 4° (*c* 0 4, chloroform) (Found C, 47 1, H, 5 3; N, 11 0, OMe, 7.2 $C_{15}H_{21}N_3O_9$ calc C, 46 5; H, 5 4, N, 10 8, OMe, 8 0%)

(*iii*) Ethyl 3-*O*-acetyl-2-deoxy-2-(2,4-dinitroanilino)-4-*O*-methyl-α-D-glucopyranoside (**5**) (3.0 g, 46%), m p 226–227°, $[\alpha]_D^{21}$ +36.4° (*c* 0 3, chloroform) (Found. C, 48 1; H, 5 3; N, 9 9; OMe, 6 8. $C_{17}H_{23}N_3O_{10}$ calc C, 47 6; H, 5.4; N, 9 8, OMe, 7.2%) Additionally, another small fraction (*iv*) was isolated, m p 191–193°, $[\alpha]_D^{21}$ +27 6° (*c* 0 3, chloroform), which, as judged by its high chromatographic mobility (R_F 0 91), was a mixture of unchanged diacetates **6** and **8** Elution from the column was in the order (*iv*), (*iii*), (*i*), (*ii*).

*Ethyl 2-deoxy-2-(2,4-dinitroanilino)-3,4,6-tri-O-methyl-α-*D*-glucopyranoside* (**14**) — Compound **2** (0 10 g) was methylated with methyl iodide (50 ml) and silver oxide (0 50 g), as described above, for 24 h T l c then indicated that the reaction was complete The trimethyl ether **14** (0 089 g, 80%), isolated by the method described above and recrystallised from chloroform–pentane, had m p. 129–130°, $[\alpha]_D^{21}$ +6 1° (*c* 0 4, chloroform) (Found C, 48 8, H, 6 2; N, 9 9, OMe, 21.9. $C_{17}H_{25}N_3O_9$ calc C, 49.1; H, 6 0, N, 9.8; OMe, 22 4%)

This compound was also isolated, in similar yield, after methylation of compounds **10** and **11** in a similar manner.

*Ethyl 3-O-acetyl-2-deoxy-2-(2,4-dinitroanilino)-4,6-di-O-methyl-α-*D*-glucopyranoside* (**9**). — Compound **9** was formed in 81% yield when the 3-*O*-acetyl-4-*O*-monomethylglycoside **5** was methylated in the manner described above, **9** had m p 201–202°, $[\alpha]_D^{21}$ +27 1° (*c* 0 2, chloroform) (Found· C, 48 5; H, 5 6; N, 9 6; OMe, 15 1 $C_{18}H_{25}N_3O_{10}$ calc C, 48 5, H, 5 6; N, 9.5; OMe, 14 0%)

Partial methylation of compounds **11** *and* **10** — Compound **11** (0 20 g) was dissolved in anhydrous acetone (50 ml), and methyl iodide (1 0 ml) and silver oxide (0 10 g) were added After heating under reflux and stirring for 24 h, the products of methylation were isolated as already described, dissolved in solvent *A*, and separated on a silica gel column by elution with solvent *A* In addition to small quantities of starting material **11** and the trimethyl ether **14**, the following compounds, recrystallised from chloroform–pentane, were obtained (*i*) Ethyl 2-deoxy-2-(2,4-dinitroanilino)-4,6-di-*O*-methyl-α-D-glucopyranoside (**13**) (0 114 g, 55%), m p 142–143°, $[\alpha]_D^{21}$ +4 6° (*c* 0 4, chloroform) (Found C, 47.7, H, 5 7; N, 10 5, OMe, 15 2 $C_{16}H_{23}N_3O_9$ calc C, 47 9, H, 5 7; N, 10 5, OMe, 15 5%)

(*ii*) Ethyl 2-deoxy-2-(2,4-dinitroanilino)-3,6-di-*O*-methyl-α-D-glucopyranoside (**12**) (0 043 g, 21%), m p 124–125°, $[\alpha]_D^{21}$ −35 6° (*c* 0 4, chloroform) (Found· C, 48 0, H, 5 8; N, 10 6; OMe, 15 3 $C_{16}H_{23}N_3O_9$ calc C, 47 9, H, 5 7, N, 10 5; OMe, 15 5%)

When compound **10** was subjected to graded methylation in a similar manner, small proportions of **10** and **14** were again isolated by chromatography, but the only dimethyl ether formed was the 4,6-derivative **13** (0 120 g, 58%), m.p. 142–143°

Deacetylation of compounds **5** *and* **9**. — The deacetylation of **5** in methanolic ammonia at room temperature was monitored by t l c. (solvent *A*) Thus, the slow conversion of **5** into **10** was observed, the reaction was complete after 6 days Evaporation of the reaction mixture afforded compound **10**, m p and mixed m p 176–177° In a similar manner, **9** was converted into **13** over a period of 6 days.

*Ethyl 4,6-O-benzylidene-2-deoxy-2-(2,4-dinitroanilino)-α-*D*-glucopyranoside* (**15**) — A mixture of finely ground compound **2** (4 75 g), anhydrous zinc chloride (5 g), and redistilled benzaldehyde (12 ml) was shaken overnight The yellow, crystalline solid formed was washed in turn with water, ethanol, water, ethanol, and light petroleum, and recrystallised from acetone to give compound **15**, m p 266–267°, $[\alpha]_D^{19}$ −56 4° (*c* 0 2, chloroform) (Found C, 54 3, H, 4 9; N, 8 9 $C_{21}H_{23}N_3O_9$ calc C, 54 7; H, 5 0; N, 9 1%)

*Ethyl 4,6-O-benzylidene-2-deoxy-2-(2,4-dinitroanilino)-3-O-methyl-α-*D*-gluco-*

pyranoside (**16**). — Compound **15** (0 50 g) was dissolved in methyl iodide (50 ml), silver oxide (5 g) was added, and the methylation was carried out (48 h) and the product isolated in the usual manner Recrystallisation from chloroform–pentane gave compound **16** (0 45 g, 85%), m p 193–195°, $[\alpha]_D^{19}$ −85° (*c* 0 2, chloroform) (Found C, 55 0, H, 5 3, N, 8 7 $C_{22}H_{25}N_3O_9$ calc C, 55 1, H, 5 3; N, 8 8%)

*Ethyl 2-deoxy-2-(2,4-dinitroanilino)-3-O-methyl-α-*D*-glucopyranoside* (**17**) — Compound **16** (0 30 g) was suspended in 60% acetic acid (20 ml) and heated on a water bath for 1 h at 100° The solution was evaporated, and the residual syrup was taken up in the minimal volume of chloroform, and light petroleum (b p 60–80°) was added Final purification by preparative t.l c (solvent *B*) gave compound **17** (0 21 g, 74%), m p. 146–147°, $[\alpha]_D^{19}$ −34 2° (*c* 0 2, chloroform) (Found C, 45 9, H, 5 4; N, 10 3 $C_{15}H_{21}N_3O_9$ calc C, 46 5, H, 5 5, N, 10 8)

*Ethyl 2-deoxy-2-(2,4-dinitroanilino)-6-O-trityl-α-*D*-glucopyranoside* (**18**) — The glycoside **2** (10 0 g) and freshly recrystallised chlorotriphenylmethane (6 0 g) in dry pyridine (150 ml) were heated under gentle reflux for 8 h The mixture was poured into ice–water and extracted with chloroform The extract was washed with potassium bisulphite solution and with water, and dried over magnesium sulphate The product was separated from small amounts of **2** and di-trityl derivatives by column chromatography with solvent *B* The trityl compound **18** (10 8 g, 65%), which was crystallised only with difficulty, was obtained as a light-yellow solid that did not have a sharp m p. (softening at 73°), $[\alpha]_D^{19}$ −28 2° (*c* 0 5, chloroform) (Found C, 64 7; H, 5 4, N, 6 4 $C_{33}H_{33}N_3O_9$ calc C, 64.4, H, 5 4; N, 6.8%).

Methylation of compound **18** — Compound **18** (9 0 g), silver oxide (10 g), and methyl iodide (100 ml) were heated under reflux with stirring for 48 h The products of methylation were isolated in the usual manner and separated by column chromatography with solvent *A* The following were obtained as light-yellow glasses (*i*) ethyl 2-deoxy-2-(2,4-dinitroanilino)-3,4-di-*O*-methyl-6-*O*-trityl-α-D-glucopyranoside (**19**) (2 6 g, 25%), softening at 97°, $[\alpha]_D^{19}$ +5 0° (*c* 0 1, chloroform) (Found C, 65 3, H, 5 8, N, 6 5 $C_{35}H_{37}N_3O_9$ calc C, 65 0, H, 5 8, N, 6 3%) (*ii*) ethyl 2-deoxy-2-(2,4-dinitroanilino)-4-*O*-methyl-6-*O*-trityl-α-D-glucopyranoside (**22**) (1.7 g, 19%), softening at 93°, $[\alpha]_D^{19}$ *ca* 0° (*c* 0 2, chloroform) (Found C, 64 2; H, 5 6; N, 6 3 $C_{34}H_{35}N_3O_9$ calc C, 64 8, H, 5 6, N, 6 7%) (*iii*) ethyl 2-deoxy-2-(2,4-dinitroanilino)-3-*O*-methyl-6-*O*-trityl-α-D-glucopyranoside (**21**) (0 20 g, 22%), softening at 88°, $[\alpha]_D^{19}$ −26 5° (*c* 0 2, chloroform) (Found C, 64 2, H, 5 6; N, 6 4. $C_{34}H_{35}N_3O_9$ calc C, 64 8; H, 5 6; N, 6 7%)

*Ethyl 2-deoxy-2-(2,4-dinitroanilino)-3,4-di-O-methyl-α-*D*-glucopyranoside* (**20**) — Compound **19** (1 00 g) was dissolved in glacial acetic acid (22 ml), and 50% hydrobromic acid in acetic acid (w/v, 1.0 ml) was added The mixture was shaken for 1 min, cooled, and filtered The filtrate was poured into water (200 ml), and the products were removed by extraction with chloroform The extracts were washed (water), dried ($MgSO_4$), and evaporated, and the residue was purified by column chromatography (solvent *A*), and recrystallisation from ethanol The resulting

3,4-dimethyl ether **20** (0 52 g, 84%) had m p. 99–100°, $[\alpha]_D^{19}$ +8 9° (*c* 0 4, in chloroform) (Found C, 48.1; H, 6.0, N, 9.8. $C_{16}H_{23}N_3O_9$ calc · C, 47.9, H, 5.7, N, 10 4%).

Detritylation of compounds **22** *and* **21.** — Detritylation, as described above, of compound **22** gave the 4-*O*-methyl derivative **10** (60%), m p and mixed m p 176–177° Similarly, compound **21** gave the 3-*O*-methyl derivative **17** (41%), m p 146–147°, $[\alpha]_D^{19}$ −32 8° (*c* 0 1, chloroform), which was identical with the product formed by the alternative method described above.

Hydrolysis of the methyl ethers of ethyl 2-deoxy-2-(2,4-dinitroanilino)-α-D-glucopyranoside — The methyl ether (0.10 g) was heated in 3N hydrochloric acid (100 ml) for 6 h at 100°. The solution was neutralised with silver carbonate, filtered, and evaporated to dryness The residue was dissolved in acetone and subjected to preparative t l c (solvent *B*) The reducing sugar was extracted from the excised band with acetone, and recrystallised from ethanol In this manner, compounds, **10, 11, 17, 20, 12, 13,** and **14** were converted into the corresponding reducing sugars in 60–70% yield. The properties and analyses for these products are given in Table II.

Periodate oxidation — (*a*) *Test for the presence of hydroxyl groups on contiguous carbon atoms.* The compound (5 mg) was dissolved in acetone–water (2 1 v/v, 5 ml), and sodium metaperiodate (20 mg) was added. After being shaken for 6 h, the mixture was filtered, concentrated, and examined by t.l c. The absence of the yellow spot corresponding to the starting material indicated that periodate oxidation had occurred

(*b*) *Test for the presence of hydroxyl and 2,4-dinitroanilino groups on contiguous carbon atoms* — The compound (10 mg) in acetone–water (2 1 v/v, 10 ml) was shaken with Amberlite IRA-401 (OH^-) (5 ml) until the solution became colourless (10–15 min) The resin was removed, sodium metaperiodate (40 mg) was added, and the solution was shaken for 6 h After filtration, 1-fluoro-2,4-dinitrobenzene (0 1 ml), sodium carbonate (20 mg), and ethanol (2 ml) were added. The mixture was shaken overnight, filtered, concentrated, and examined by t.l.c.; the absence of the spot corresponding to the starting material indicated that oxidation had occurred

ACKNOWLEDGMENTS

The authors thank Professor S Peat, F R S., for his continued interest and encouragement. The award of studentships by the Science Research Council (to B E and R J. F) and an Imperial Chemical Industries Fellowship tenable in the University of Wales (to B. E) are gratefully acknowledged, as is valuable technical assistance by Mrs. Olive Owen

REFERENCES

1 P F. Lloyd and B Evans, *Carbohyd Res* , 8 (1968) 372
2 W. O Cutler, W. N Haworth, and S. Peat, *J. Chem Soc* , (1937) 1974
3 W. N Haworth, W H G. Lake, and S Peat, *J Chem Soc* , (1939) 271
4 A. Neuberger, *J Chem Soc* , (1941) 50
5 R. W Jeanloz, *J Amer. Chem. Soc* , 74 (1952) 4597, 76 (1954) 555, 558
6 R W. Jeanloz and C. Gansser, *J. Amer. Chem. Soc* , 79 (1957) 2583

7 R. W Jeanloz, *Advan. Carbohyd Chem* , 13 (1958) 689
8 P. F Lloyd and M Stacey, *Chem Ind* (London), (1955) 917.
9 P F Lloyd and B Evans, *J. Chem Soc* , (1968) 2753
10 P F Lloyd and M Stacey, *Tetrahedron*, 9 (1960) 116
11 P F Lloyd and G P Roberts, *J Chem Soc* , (1963) 2962
12 P F Lloyd and G P Roberts, *J Chem Soc* , (1965) 6910
13 B H Nicolet and L A Shinn, *J Amer. Chem Soc.*, 61 (1939) 1615
14 B Helferich and W Klein, *Ann* , 450 (1926) 219; K Josephson, *ibid* , 472 (1929) 217 W N Haworth, E. L Hirst, and E G Teece, *J. Chem Soc* , (1930) 405, (1931) 2858
15 Y Wang and H -I Tai, *Acta Chim Sinica*, 24 (1958) 368
16 D Horton, *J Org Chem* , 29 (1964) 1776, D Horton, J B Hughes, J S Jewell, K D. Philips, and W N Turner, *ibid* , 32 (1967) 1073

Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

THE ACTION PATTERN OF D-ENZYME, A TRANSMALTODEXTRINYLASE FROM POTATO*

G JONES AND W. J WHELAN

Department of Chemistry, University College of North Wales, Bangor (Great Britain) and The Lister Institute of Preventive Medicine, London, S W. 1 (Great Britain)

(Received October 14th, 1968)

ABSTRACT

Potato D-enzyme is a transglycosylase that disproportionates maltotriose and higher maltodextrins by the transfer of maltodextrinyl radicals between maltodextrins, with the rupture and re-formation of α-(1→4)-D-glucosidic bonds. Only maltodextrins or similarly structured polymers are group-donating substrates D-Glucose and a variety of other sugars can act as acceptors. Earlier workers had noted that D-enzyme appears to operate on maltodextrins under constraint Maltose is not a donor substrate Only one bond in maltotriose, that at the reducing end, is acted on In the higher dextrins, there are two "forbidden" linkages The nonreducing-end bond, and that penultimate to the reducing end, cannot be split.

The present paper tests and fully supports the hypothesis of forbidden linkages and their locations in the donating molecule The tests have involved quantitative examination of the way in which (*a*) maltodextrins and D-glucose-^{14}C interact in the presence of D-enzyme, and (*b*) maltotriose-^{14}C is disproportionated into D-glucose and other maltodextrins It is noted that maltosyl groups are transferred more rapidly than any larger group, and that the rate of transfer of the maltosyl group to D-glucose is equal in malto-triose, -pentaose, and -hexaose (A maltosyl group cannot be transferred from the tetraose)

INTRODUCTION

D-Enzyme is a transglycosylase discovered in the potato by Peat, Whelan, and Rees[1]. It disproportionates maltodextrins by splitting off a maltosyl or larger group from a donor molecule and transferring the group to an acceptor molecule with the

---

*(By W J Whelan, at present at the Department of Biochemistry, University of Miami, Florida, U S A) This tribute to Professor Stanley Peat, F R S , comes from only one of the authors since Dr Glyn Jones died in 1960 He and I were formerly undergraduate and graduate students of Professor Peat (G J. 1951–57, W.J W 1942–48) and I an academic colleague during 1947–55 We both derived the utmost benefit from the inspiration and kindly affection shown to us by this dedicated and distinguished scientist. The present paper, which depended on his earlier work, and which was carried out with his help and encouragement, in seconding Dr Jones from Bangor to my laboratories in London, is dedicated to Professor Peat with respect and gratitude for his leadership to us and to science

re-formation of an α-D-(1$\rightarrow$4) bond The smallest donor molecule is maltotriose, and the smallest acceptor is D-glucose The action of the enzyme is readily revealed by paper chromatography of a maltodextrin digestion mixture, when the original substance is seen to have been converted into D-glucose and a mixture of maltodextrins The chromatogram reveals an intriguing feature, namely the virtual absence of maltose This sugar is not a donor substrate and has relatively weak acceptor activity[2] which, as discussed later, might now be traceable to the known presence of maltotriose in commercial preparations of maltose[3] To explain this aspect of D-enzyme specificity, Peat *et al* [1] proposed that two of the (1$\rightarrow$4)-linkages in a maltodextrin could not be acted on by the enzyme These are the nonreducing-end bond and the linkage penultimate to the reducing end (In maltotriose these are one and the same bond.) This paper is an attempt to test this hypothesis, if not to explain why the enzyme behaves in this way

EXPERIMENTAL

Substrates — Maltodextrins were prepared as by Peat, Whelan, and Kroll[4], and maltotriose was labelled[5] with ^{14}C in the reducing-end D-glucose unit, using D-glucose-^{14}C prepared by hydrolysis of ^{14}C-labelled starch (Radiochemical Centre, Amersham). The D-glucose had a specific activity of 42,600 counts/100 sec/mg and the maltotriose, 15,100 counts/100 sec/mg

Potato D-enzyme, free from amylase, phosphorylase, and Q- and R-enzymes, was prepared as by Peat, Turvey, and Jones[6]

Methods. — (*a*) *Fractionation and location of* ^{14}*C-labelled sugars* The mixtures of ^{14}C-labelled D-glucose and maltodextrins under examination (see below) were fractionated by paper chromatography on Whatman 3MM paper in propyl alcohol–ethyl acetate–water (6 1 3, by vol) The individual substances were located by radioautography in a 3–7 day exposure of the chromatogram to Ilford Industrial F X-ray film

(*b*) *Determination of radioactive sugars* The zones containing the sugars were cut from the paper chromatogram and eluted with water, as in descending chromatography[7] The water eluate dripped directly onto a flat, aluminium disc (1-inch diameter) The disc had previously been treated with 5 drops of 0.1% Teepol XL solution (Shell Chemicals Ltd) The detergent was spread over the disc, and the solution was evaporated to dryness under an infrared lamp About 1 ml of eluate was collected from each paper strip onto the coated disc, and the solution was again evaporated Control experiments had shown that when strips were eluted in this way[7] more than 98% of the sugar was contained in the first 4 drops It was also shown that there was a linear response between weight of sugar and counting rate in the range 0–1 3 mg of sugar per disc, and that the counting rate was unaffected by the added presence of citrate buffer (0 125 ml of a 2mM solution, pH 6 5), such as might be present when enzyme digests were fractionated (see below)

The discs containing the ^{14}C-labelled sugars were transferred for counting to a thin-end-window scaler (Ekco Electronics Ltd., Model N530C). For count rates greater than 300/100 sec, the time to collect 3000 counts was measured in duplicate. For count rates below this figure, counts were collected for two periods each of 300 sec. All rates are reported as counts/100 sec, corrected for background and coincidence.

Digestions with D-enzyme. — (*a*) *With* D-*glucose*-^{14}C *and unlabelled maltodextrins*. The final concentrations of solutes in a 200-μl digest were D-glucose-^{14}C (364 mM), maltodextrin (17 mM), and D-enzyme[6] in 50 mM citrate buffer (pH 7.0), diluted 3:8. Incubation was at 30°. At intervals, 30-μl portions of the digest were transferred to the starting line of a paper chromatogram where a 15-μl spot of 50 mM mercuric chloride had already been placed. Control experiments showed that admixture with the mercuric chloride immediately inhibited enzyme action. The chromatogram was irrigated, and the separated sugars were located as above.

(*b*) *With maltotriose*-^{14}C. The digestion mixture (250 μl), incubated at 30°, contained 41.2 mM maltotriose and D-enzyme/citrate as above, diluted 1:2. The progress of reaction was followed as in (*a*).

RESULTS AND DISCUSSION

Action of D-enzyme on maltodextrins and D-*glucose*-^{14}C. — In a series of experiments, D-enzyme was incubated with D-glucose-^{14}C and the individual maltodextrins in the series maltotriose–maltohexaose. At intervals, the radioactive products were separated chromatographically and their radioactivity determined. A large excess of D-glucose was employed (21:1, molar basis). A control experiment with maltohexaose and D-glucose had shown, by paper chromatography, that this excess of D-glucose was such as effectively to render it the only acceptor, so that no polymers larger than the initial maltodextrin substrate could be formed. This conclusion was borne out by the results of the experiment, listed in Table I and Fig. 1.

Walker and Whelan[5] showed that, in this type of system, D-enzyme splits a maltodextrin and transfers the nonreducing-end portion to the D-glucose. The D-glucose-^{14}C is always in the reducing-end position, never elsewhere. The amounts of radioactivity measured therefore represent relative molar amounts of product. The hypothesis of linkages that are not split by D-enzyme[1] (see above, termed "forbidden linkages") requires that in the present experimental system only the following reactions are possible (G = D-glucose, * = ^{14}C-labelled, other symbols as in Table I).

$$\begin{aligned} &M3+G^* \rightleftharpoons M3^*+G \\ &M4+G^* \rightleftharpoons M4^*+G \\ &M5+G^* \rightleftharpoons M5^*+G \\ & \rightleftharpoons M3^*+M3 \end{aligned} \qquad \begin{aligned} &M6+G^* \rightleftharpoons M6^*+G \\ & \rightleftharpoons M4^*+M3 \\ & \rightleftharpoons M3^*+M4 \end{aligned}$$

Table I shows these predictions to have been entirely fulfilled. The only exceptions to this generalization are small amounts of maltose and maltotetraose being formed from maltotriose, and of maltose from maltotetraose, after a prolonged period of incubation (24 h). Malto-pentaose and -hexaose gave only the expected

TABLE I

PRODUCTS FORMED DURING THE INCUBATION OF D-ENZYME WITH D-GLUCOSE-^{14}C AND MALTODEXTRINS

D-*Glucose*-^{14}C *incubated with*	^{14}C-*Labelled product (counts/100 sec)*	*Time (h)*				
		0.5	*1*	*1.5*	*2*	*24*
M3	M2[a]		0		0	45
	M3		445		672	1405
	M4+[b]		0		0	28
M4	M2+M3		0		0	46
	M4		226		296	772
	M5+		0		0	0
M5	M2	0	0	0	0	
	M3	186	323	475	649	
	M4	0	0	0	0	
	M5	74	224	343	648	
	M6+	0	0	0	0	
M6	M2	0	0	0	0	
	M3	180	351	515	687	
	M4	76	217	430	441	
	M5	0	0	0	0	
	M6	53	84	130	168	
	M7+	0	0	0	0	

[a] M2 = maltose, etc [b] "M4+" means that the area of the chromatogram extending from the expected position of maltotetraose to the origin was eluted onto a single counting planchette

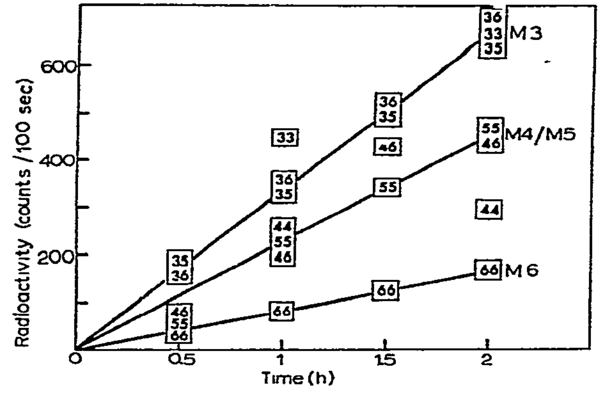


Fig 1 The formation of radioactive maltodextrins in the reaction between maltodextrin and D-glucose-^{14}C, catalysed by D-enzyme M3 denotes the formation of maltotriose, etc. The rectangles have been drawn over the experimental values (see Table I), and the symbolism 35, etc , means the formation, in this case, of maltotriose from maltopentaose

products during 2 h of incubation. These results, in a qualitative sense, simply confirm what had already been noted by Peat *et al.*[1] The quantitative measure of the products, however, now permits a more-detailed insight into the unique specificity of D-enzyme. In Fig 1, are plotted the rates of formation of malto-triose, -tetraose, -pentaose, and -hexaose from the four substrates, according to the various pathways indicated by the foregoing equations. The interesting finding is that the rate of formation of maltotriose, by the transfer of a maltosyl residue to D-glucose, occurs at the same rate regardless of whether the donor substrate is malto-triose, -pentaose, or -hexaose (Fig. 1) The transfer of a malto-triosyl or -tetraosyl residue seems to occur at equal rates, which are lower than for the maltosyl transfer Transfer of a maltopentaosyl residue occurs at the lowest rate For the two substrates where more than one type of transfer is possible, malto-pentaose and -hexaose, the rate of transfer decreases with the size of the group transferred, *i e*, for maltopentaose, a maltosyl residue is transferred more rapidly than maltotetraosyl. For maltohexaose, the rate decreases in the order maltosyl, maltotriosyl, and maltopentaosyl. An experiment in which amylopectin was incubated with D-glucose and D-enzyme, and the digest chromatographed, showed the same thing; *i e.*, maltotriose as the most-abundant product We conclude therefore that, at least for the case where D-glucose is an acceptor, D-enzyme prefers to transfer the smallest possible segment There may even be an upper limit to the length of group that can be transferred

Action of D-enzyme on maltotriose-^{14}C — D-Enzyme was incubated with maltotriose labelled with ^{14}C in the reducing unit At intervals, the products were separated and assayed Fig 2 shows the progress of the reaction. More details of the results are given in Table II, where it will be seen that virtually all of the original radioactivity could be accounted for each time the system was assayed The experiment permits the hypothesis of "forbidden linkages" to be tested in terms of the relative rates of appearance and, in some cases, decay of the products. The following equations show how all products from D-glucose to maltoheptaose should be formed from maltotriose In these equations, $G = \alpha$-D-glucose, G_R = reducing-end unit, $-$ = transferrable (1→4)-bond, $\sim$ = forbidden linkage The donor molecule is that on the left

$$G\sim G-G_R + G\sim G-G_R \rightleftharpoons G\sim G-G\sim G-G_R + G_R \tag{1}$$

$$G\sim G-G\sim G-G_R + G\sim G-G_R \rightleftharpoons G\sim G-G-G-G\sim G-G_R + G_R \tag{2}$$

$$G\sim G-G-G-G\sim G-G_R + G\sim G-G_R \rightleftharpoons G\sim G-G-G\sim G-G_R + G\sim G\sim G-G_R \tag{3}$$

Maltotriose is always shown as one of the reactants since, being the sole initial substrate, it will be in excess Other reactions between maltotriose and the pentaose and heptaose could occur but would not give the tetraose or hexaose Equations (1)–(3) predict that the sequence in which other maltodextrins should be formed from maltotriose is malto-pentaose, -heptaose, and -tetraose/-hexaose Fig 2 shows that this is exactly what happens Maltopentaose appears first, closely followed by the heptaose, and both then decay as the later products, the tetraose and hexaose, appear. Table II records the amounts of each product at 24 h, when it may be supposed that equilibrium had been reached. These values show that maltohexaose decays from the level it had reached at 3 h, while maltotetraose increases in amount between 3 and

24 h Maltotriose, the initial substrate, remains the major polymer at equilibrium It does not follow that this would be so for other substrates

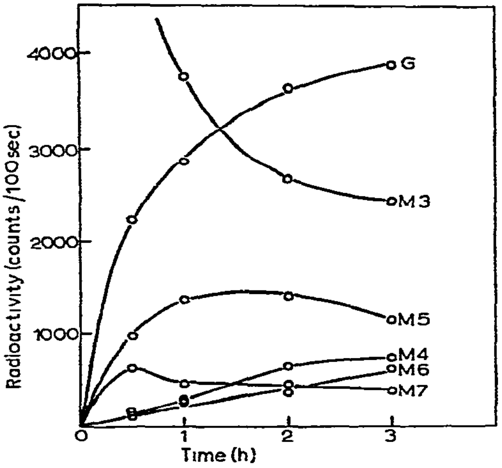


Fig 2 The reaction of D-enzyme with maltotriose-^{14}C (M3) to form D-glucose (G) and other maltodextrins (M4, etc) The complete results of the experiment, including the amounts of product at equilibrium, are given in Table II

TABLE II

ACTION OF D-ENZYME ON MALTOTRIOSE-^{14}C

Sugar	*Radioactivity [counts/100 sec at designated time (h)]*					*Moles of product as % of original maltotriose*[b]	
	0 5	*1*	*2*	*3*	*24*	*A*	*B*
D-Glucose	2210	2840	3640	3880	4490	46	42
Maltose	0	0	0	0	0	0	2 1
Maltotriose	5840	3730	2660	2420	1945	20	20
Maltotetraose	160	227	660	748	1006	10	11
Maltopentaose	980	1300	1340	1150	928	9 5	7 5
Maltohexaose	126	224	345	615	463	4 8	3 5
Maltoheptaose	628	472	456	378	602	6 2	
Higher maltodextrins	95	154	282	405			
Total maltodextrins, excluding maltotriose	1989	2377	3083	3296	2999		
Recovery of ^{14}C[a]	103	92	96	98	97		

[a]The initial radioactivity of the maltotriose was 9766 counts/100 sec [b]*A* = present work, *B* = Peat *et al*[4]

Having measured the relative rates of action of D-enzyme on bonds in different positions (Fig. 1), we see also that, apart from forbidden linkages dictating the route from maltotriose to the tetraose and hexaose, there would be another impedance to the formation of these two compounds We have seen that the favoured transfer is of the maltosyl residue. When maltopentaose is formed (equation 1), the reactions open to it are (2) and a preferred, but unproductive, reaction in which a maltosyl group is transferred, *i e*, in the terminology of Table I, $M5+M3 \rightleftarrows M3+M5$ Similarly, for maltoheptaose, the preferred reaction, if maltoheptaose is a donor, should be $M7+M3 \rightleftharpoons 2M5$ Even if maltopentaose reacts with D-glucose, the second most-abundant substrate, it can only re-form pentaose or give rise to triose. Therefore, even progress to the heptaose is held up, except that, if the roles of donor and acceptor are reversed in equation (2), there will now be a "preferred" reaction which leads to the same two products, maltoheptaose and D-glucose

Table II compares the products at equilibrium with yields of the same substances obtained by Peat *et al*[4] in a preparative-scale reaction between maltotriose and D-enzyme It will be seen that there is a good correlation between the two sets of data, obtained in quite different ways The only major difference is the 2 1% molecular proportion of maltose noted by Peat *et al*[4], where we find none. Almost certainly, this could be ascribed to amylase in the less-pure D-enzyme preparation used earlier

The role of maltose requires further consideration We have shown here conclusively that maltose is not formed by D-enzyme Since any reaction in which D-enzyme participates may be presumed to be reversible, it follows that, if maltose cannot be formed, it should not be taken up. Yet we have earlier reported that maltose can function as an acceptor in a reaction in which amylopectin was the donor[2] Relative to D-glucose (100), its acceptor activity of 11 was poor We now know that the commercial brand of maltose we used in the earlier experiments contains about 5% of maltotriose[3] Walker[8] and Palmer, Ryman, and Whelan[9] have noted that the rates of action of other transglycosylases on maltose are enhanced when maltotriose is added. It may be that the apparent acceptor activity of maltose with D-enzyme[2] is traceable to a maltotriose impurity

Conclusions — The foregoing experiments have proved correct the original hypothesis of Peat, Whelan, and Rees[1] to explain D-enzyme specificity as regards transferable and forbidden linkages in maltodextrins While the hypothesis is proven, we still await an explanation of why D-enzyme acts in this way As regards the forbidden nonreducing-end bond, an explanation can be advanced If this bond could be split, then, in a reaction with D-glucose, neither the D-glucosyl group transferred nor the accepting D-glucose would be polymeric, and contain α-(1→4)-D-glucosidic bonds. The parallel here is pullulanase, an enzyme specific for the hydrolysis of α-(1→6)-D-glucosidic bonds[10] The smallest substrate for this enzyme is the tetrasaccharide 6^2-α-maltosylmaltose The enzyme will not split α-(1→6)-D-glucosidic bonds when only a D-glucose unit lies on one side or other of the bond. Like pullulanase, D-enzyme may be specific for the transfer of maltodextrinyl residues, pullulanase to water, D-enzyme to another carbohydrate The paradox with D-enzyme is that the bond penultimate to the reducing end is also forbidden Except for the case of maltotriose, if this bond were to be split, the transferred fragment would always be polymeric. It may be that the inability to act on this bond arises out of the fact that, in maltotriose, the forbidden nonreducing-end bond is also the forbidden "penultimate" bond.

If maltotriose cannot be aligned with D-enzyme such that the enzyme acts on the nonreducing-end bond, then no other larger substrate can align itself to have its penultimate bond split, regardless of the size of the molecule and the size of the potentially transferable maltodextrinyl group This restriction does not impose any constraint on the ability of D-enzyme to transfer any given maltodextrinyl group. The only group it will not transfer is D-glucosyl Thus, if we look collectively at maltotriose–maltohexaose, we find examples (Table I) where maltosyl, malto-triosyl, -tetraosyl, and -pentaosyl can be transferred So the constraint, which prevents D-enzyme from transferring a D-glucosyl residue from maltotriose, persists in larger molecules so as to prevent a maltosyl residue being transferred from maltotetraose, maltotriosyl from maltopentaose, maltotetraosyl from maltohexaose, etc. This rather rigid specificity towards donor substrates is not nearly so marked with acceptor substrates. A wide variety of sugars[2], including L-sorbose[11], can act to receive transferred maltodextrinyl radicals

ACKNOWLEDGMENTS

We thank the Department of Scientific and Industrial Research for the award of a studentship (to G J), and the Agricultural Research Council and Imperial Chemical Industries Ltd. for research grants

REFERENCES

1 S Peat, W J Whelan, and W R Rees, *J Chem Soc*, (1956) 44
2 S Peat, W. J. Whelan, and G Jones, *J Chem Soc*, (1957) 2490.
3 E Y C Lee, Ph D Thesis, University of London, 1966
4 S Peat, W J Whelan, and G W F Kroll, *J Chem Soc*, (1956) 53
5 G. J Walker and W J Whelan, *Biochem J*, 67 (1957) 548
6 S Peat, J R Turvey, and G Jones, *J Chem Soc*, (1959) 1540
7 J. S D Bacon, *Biochem J*, 63 (1956) 200
8 G J Walker, *Biochem J*, 101 (1966) 861
9 T. N Palmer, B E Ryman, and W. J Whelan, *FEBS Letters*, 1 (1968) 1.
10 M Abdullah, B J Catley, E Y C Lee, J Robyt, K Wallenfels, and W J Whelan, *Cereal Chem*, 43 (1966) 111.
11 M Abdullah and W J Whelan, *Arch Biochem Biophys*, 112 (1965) 592

Carbohydrate Research
Elsevier Publishing Company Amsterdam
Printed in Belgium

QUANTITATIVE DETERMINATION OF MIXTURES OF ALKYL ETHERS OF D-GLUCOSE*

PART I ANALYTICAL METHOD

SUSAN HAWORTH, J G ROBERTS, AND B F. SAGAR
Shirley Institute, Didsbury, Manchester 20 (Great Britain)

(Received November 4th, 1968)

ABSTRACT

Analysis of complex mixtures of methyl ethers of D-glucose by gas–liquid chromatography of their trimethylsilyl derivatives is described. The ratio of the specific retention volumes for α- and β-anomers varies with the total number of methyl substituents and their position on the D-glucose molecule The ratio of the retention volume of any trimethylsilyl derivative relative to its methyl analogue is independent of the number of trimethylsilyl or methyl groups elsewhere in the molecule

Molar responses of the trimethylsilylated methyl ethers of D-glucose are almost independent of the number of methyl substituents Quantitative analysis of mixtures of all D-glucose methyl ethers substituted at C-2, C-3, and/or C-6 is possible with two columns

INTRODUCTION

Methyl ethers of glucose can be separated by column[1,2] and paper chromatography[3-5]. A combination of these techniques has been used by Croon[6] to determine the relative reactivities of the hydroxyl groups in cellulose towards a variety of methylating agents Gas–liquid chromatography has been used to separate methyl *O*-methyl-D-glucosides[7-9] The monomethyl ethers are, however, not sufficiently volatile to be separated in this way, and, even in the case of the dimethyl ethers, column temperatures of the order of 200° are required[10]. Increased volatility can be achieved by making the fully substituted *O*-acetyl[11] or *O*-trimethylsilyl derivatives[12-14]. Quantitative determinations of fully trimethylsilylated sugars have been made [15-18], but little information as to the molar responses of these derivatives has been reported[17,19]

In the work described here, the relative molar responses of a series of methyl ethers have been determined, and quantitative analyses of complex mixtures achieved.

RESULTS AND DISCUSSION

D-Glucose and a series of methyl ethers of D-glucose were examined by gas–liquid chromatography as their fully trimethylsilylated derivatives As the methyl

---

* Dedicated to Professor Stanley Peat, F R S

ethers were available in one anomeric configuration only, the second anomer was obtained in a mutarotated mixture by heating the ether in pyridine solution; comparison of the chromatograms of the pure anomer and the mutarotated mixture enabled the direct assignment of peaks to their correct anomers to be made in all cases except that of 2,6-di-*O*-methyl-D-glucose. This sugar has only been obtained as an anomeric mixture, and the two peaks found have been assigned by comparison with the other di-*O*-methyl-D-glucoses examined. Specific retention volumes (Vg^{θ}) for 22 D-glucose derivatives are shown in Table I.

TABLE I

SPECIFIC RETENTION VOLUMES OF *O*-TRIMETHYLSILYL DERIVATIVES OF *O*-METHYL-D-GLUCOSES AT 125°

	Specific retention volume Vg^{θ}							
Column[a]	*1*		*2*		*3*		*4*	
Anomer *Trimethylsilyl derivative of*	α	β	α	β	α	β	α	β
G[b]	11480	25000	41330	92930	10770	23540	23700	46300
G_{2}	9030	13720	27300	44850	5980	9350	14670	22500
G_{3}	6590	11680	21420	37160	4850	9590	11300	19000
G_{6}	13740	18470	38180	35100	7890	9780	18700	25700
G_{23}	5840	6740	13970	17060	2660	3480	7190	8920
G_{26}	10180	10900	21460	26130	4370	4370	10180	12300
G_{36}	7750	9090	17880	21590	3430	4030	8500	10500
G_{46}	8500	12410	17980	27830	3950	4910	—	—
G_{236}	6570	5700	10220	10180	1840	1610	4830	—
G_{2346}	4480	4080	4710	4710	1010	920	—	—
G_{12346}	5920	3880	3470	2640	1960	1150	—	—

[a] Columns 1, poly(decane-1,10-diol succinate) on glass beads; 2, *n*-hexatriacontane on glass beads, 3, poly(ethylene glycol succinate) on kieselguhr, 4, silicone gum rubber SE 30 on glass beads.
[b] G = D-glucose, G_{2} = 2-*O*-methyl-D-glucose, etc.

The ratio of Vg^{θ} (β-D-anomer)/Vg^{θ} (α-D-anomer) is markedly dependent on the number of methyl groups, ranging from 2.2 for trimethylsilyl 2,3,4,6-tetra-*O*-trimethylsilyl-D-glucoside down to 0.6 for methyl 2,3,4,6-tetra-*O*-methyl-D-glucoside, but is less affected by the position of the methyl groups or by the chemical nature of the stationary phase.

The mean, multiplicative factor associated with the replacement of a methyl group by a trimethylsilyl group at either position 2, 3, 4, or 6 varies from 2.57 to 0.87, depending on the configuration at C-1 and on the nature of the stationary phase, but it is relatively independent of substitution at any of the remaining positions (Table II). The effect of replacing a methyl group by the considerably bulkier trimethylsilyl group is generally greatest for the C-3 position and smallest at C-6 or C-1. These results suggest that retention data might be reliably predicted from a small selection of measured retention volumes for other analogous series of mixed sugar derivatives

TABLE II

EFFECT ON V_g^0 OF REPLACING –OMe BY –OSiMe$_3$ IN MIXED *O*-METHYL/*O*-TRIMETHYLSILYL DERIVATIVES OF D-GLUCOSE[a]

	Ratio $V_g^0(-OSiMe_3)/V_g^0(-OMe)$							
	Column 1		*Column 2*		*Column 3*		*Column 4*	
	α	β	α	β	α	β	α	β
Replacement at 6(OH)								
G/G_6	0.84	1.35	1.08	1.69	1.37	2.41	1.27	1.80
G_2/G_{26}	0.89	1.26	1.27	1.72	1.37	2.13	1.44	1.83
G_3/G_{36}	0.85	1.28	1.20	1.72	1.41	2.38	1.33	1.81
G_{23}/G_{236}	0.89	1.18	1.37	1.68	1.45	2.16	1.49	—
Mean	0.87	1.27	1.23	1.70	1.40	2.27	1.38	1.81
Replacement at 4(OH)								
G_6/G_{46}	1.62	1.49	2.12	1.98	2.00	1.99	—	—
G_{236}/G_{2346}	1.47	1.40	2.17	2.16	1.82	1.75	—	—
Mean	1.54	1.44	2.15	2.07	1.91	1.87	—	—
Replacement at 3(OH)								
G/G_3	1.74	2.14	1.93	2.50	2.22	2.45	2.10	2.44
G_2/G_{23}	1.55	2.03	1.95	2.63	2.25	2.68	2.04	2.52
G_6/G_{36}	1.77	2.03	2.13	2.55	2.30	2.43	2.20	2.45
G_{26}/G_{236}	1.54	1.91	2.10	2.57	2.38	2.71	2.11	—
Mean	1.64	2.03	2.03	2.56	2.29	2.57	2.11	2.47
Replacement at 2(OH)								
G/G_2	1.27	1.82	1.51	2.07	1.80	2.52	1.62	2.06
G_3/G_{23}	1.13	1.73	1.53	2.18	1.82	2.75	1.57	2.13
G_6/G_{26}	1.35	1.69	1.77	2.11	1.81	2.24	1.84	2.09
G_{36}/G_{236}	1.18	1.59	1.75	2.12	1.86	2.50	1.76	—
Mean	1.23	1.71	1.64	2.12	1.82	2.50	1.70	2.09
Replacement at 1(OH)								
G_{2346}/G_{12346}	0.76	1.05	1.36	1.79	0.52	0.80	—	—

[a] Abbreviations as in Table I

As sampling was carried out with capillary pipettes which do not give completely reproducible delivery volumes, an internal standard was used to enable molar responses to be measured. Ethyl myristate was found to be suitable since it has a V_g^0 value (15,280 on Column 1) clear of the sugars being examined Furthermore, the response of the ^{90}Sr detector to this compound was reasonably linear over the range 0 to 2×10^{-7} moles (Fig 1) Similarly, trimethylsilyl 2,3,4,6-tetra-*O*-trimethylsilyl α-D-glucoside gave a linear response over the range 0 to 1×10^{-7} mole, its molar response relative to that of ethyl myristate was 2.7. With this internal standard, the relative molar responses of the fully trimethylsilylated *O*-methyl-D-glucoses were then determined (Table III). No differences were found between values obtained for a single anomer and those obtained from mutarotated mixtures. All of the D-glucose derivatives examined gave the same value within the limits of experimental error. This makes the routine use of an internal standard unnecessary as the peak area of each individual component in a given mixture relative to the total peak area is a measure of the relative molar amount of that component in the mixture.

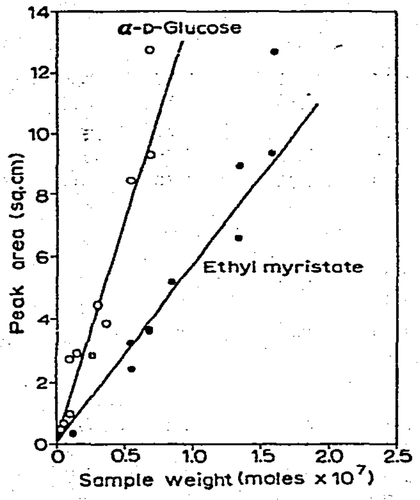


Fig. 1. Molar responses of ethyl myristate and trimethylsilyl 2,3,4,6-tetra-*O*-trimethylsilyl-α-D-glucoside.

TABLE III

RELATIVE MOLAR RESPONSES OF *O*-TRIMETHYLSILYL DERIVATIVES OF PARTIALLY METHYLATED D-GLUCOSES

Sugar	*Relative molar response (Ethyl myristate = 1)*
D-Glucose	2.66
2-*O*-Methyl-D-glucose	2.71
3-*O*-Methyl-D-glucose	2.76
6-*O*-Methyl-D-glucose	2.64
2,3-Di-*O*-methyl-D-glucose	2.78
2,6-Di-*O*-methyl-D-glucose	2.67
3,6-Di-*O*-methyl-D-glucose	2.81
4,6-Di-*O*-methyl-D-glucose	2.61
2,3,6-Tri-*O*-methyl-D-glucose	2.77
2,3,4,6-Tetra-*O*-methyl-D-glucose	2.75
Mean value	2.72 ±0.06

Mixtures of authentic *O*-methyl-D-glucoses were trimethylsilylated, and analysed on the basis of equal molar responses without using an internal standard. Recoveries varied between 90 and 107% with a mean of 100.0 ±4.8 (Table IV).

TABLE IV

RECOVERY OF *O*-METHYL-D-GLUCOSES FROM PREPARED MIXTURES[a]

Mixture	*Component*	*Relative amounts*		*Recovery*
		Made up by weight	*Found by g.l.c.*	(%)
1	α-G$_{3}$	51.3	50.3	98.1
	α-G$_{6}$	28.7	28.6	99.7
	$\alpha\beta$-G$_{26}$	20.0	21.1	105.5
2	β-G	36.5	37.3	102.2
	β-G$_{2}$	45.3	43.5	96.1
	β-G$_{23}$	18.2	19.2	105.4
3	$\alpha\beta$-G	44.5	46.4	104.4
	$\alpha\beta$-G$_{2}$	37.7	35.5	94.3
	$\alpha\beta$-G$_{23}$	17.8	18.0	101.1
4	α-G$_{36}$	26.3	23.8	90.5
	α-G$_{46}$	46.1	49.3	106.9
	α-G$_{236}$	27.6	26.9	97.8
5	α-G$_{3}$	34.2	34.7	101.5
	β-G$_{23}$	15.7	14.4	91.7
	α-G$_{36}$	28.0	28.0	100.0
	α-G$_{236}$	22.1	23.0	104.1
			Mean	100.0 ±4.8

[a]Abbreviations as in Table I.

Acid hydrolysis[6] of a commercial *O*-methylcellulose gave a mixture of *O*-methyl-D-glucoses which, on trimethylsilylation and examination by g.l.c., showed no more than eleven resolved peaks out of the possible sixteen (Figs. 2 and 3). Superposition of components varied with the column used; a complete analysis of peak areas of individual components was achieved when the sample was examined on three columns. Four commercial *O*-methylcellulose samples, differing in molecular weight but not in methoxyl content, were analysed (Table V). Preferential reaction of the hydroxyl groups at C-2 and C-6 compared with reaction at the C-3 hydroxyl group is apparent; the bulk of the methylation at the C-3 hydroxyl group occurs in fully methylated D-glucose residues. This is consistent with the presence of an intra-chain hydrogen bond from the C-3 hydroxyl group to the C-5′ ring oxygen atom of the adjacent D-glucose residue, as postulated on the basis of infrared studies[20]. The methoxyl content as calculated from the observed distributions is, in every case, in reasonable agreement with that measured by the Zeisel procedure. This method of analysis has

TABLE V

DISTRIBUTION OF METHOXYL GROUPS IN COMMERCIAL O-METHYLCELLULOSES

Sample	*Methoxyl*[c] *D. S*	*Moles % of*								*Degree of substitution*			
		G^d	G_2	G_3	G_6	G_{23}	G_{26}	G_{36}	G_{236}	C_2	C_3	C_6	*Overall*
Celacol[a] M450	1.71	5.8	15.9	0.6	10.8	9.1	29.3	5.0	23.0	0.77	0.38	0.69	1.84
Celacol[a] M5000	1.68	8.6	15.3	3.5	11.9	9.8	25.9	5.4	21.4	0.72	0.40	0.64	1.76
Celacol[a] MM10	1.92	3.8	14.7	3.3	7.8	11.8	26.4	5.1	27.5	0.80	0.48	0.67	1.95
B.D H O-methyl cellulose[b]	1.76	5.5	13.9	3.5	11.6	8.2	30.5	5.7	21.0	0.74	0.39	0.69	1.82

[a] Products of British Celanese Ltd. [b] British Drug Houses Ltd [c] By Zeisel procedure [d] G = D-glucose, G_2 = 2-O methyl-D-glucose, etc

been extended to a wide range of partially methylated cotton cellulose samples, and, in all cases, good agreement between calculated methoxyl content and that obtained by a Zeisel procedure was found (Fig 4) A detailed account of its application to a series of partly methylated cotton celluloses will be given in a later paper.

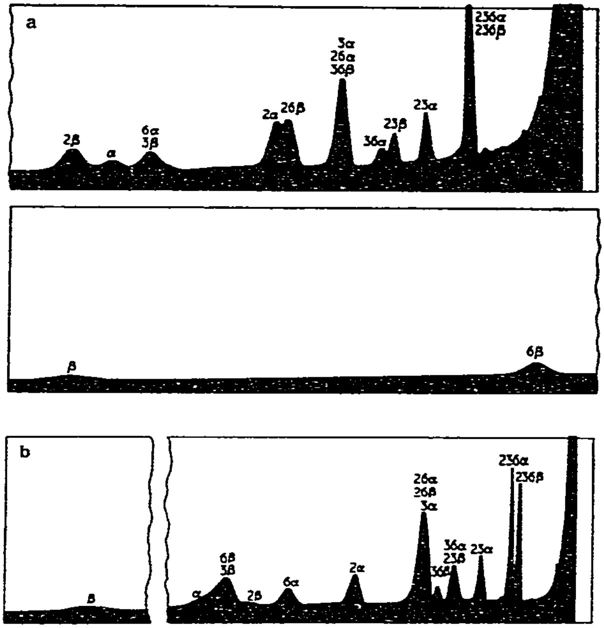


Fig 2 G l c separation of mixtures of *O*-trimethylsilyl derivatives of D-glucoses on (*a*) *n*-hexatriacontane (Column *2*) and (*b*) poly(ethylene glycol succinate) (Column *3*)

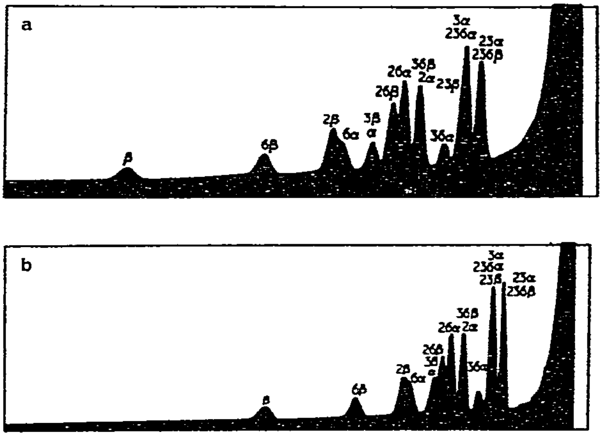


Fig 3 G l c separation of trimethylsilylated *O*-methyl-D-glucoses on a poly(1,10-decanediol succinate) stationary phase (Column *1*). The effect of a change in temperature from 123° (*a*) to 125° (*b*).

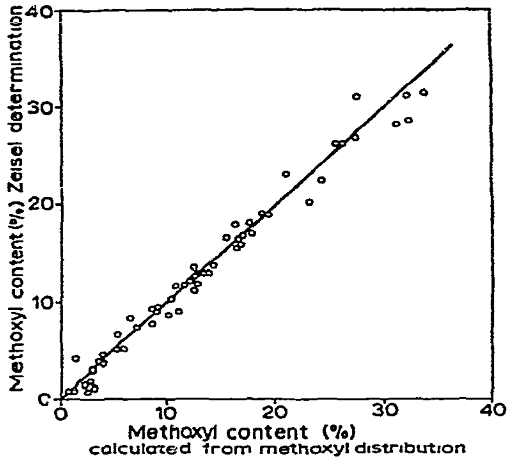


Fig 4 Comparison of methoxyl contents found by Zeisel determination with those calculated from methoxyl distribution measurements

EXPERIMENTAL

Methyl sugars — Thin-layer chromatography was performed on Silica Gel G (Merck) with methyl ethyl ketone saturated with water containing 1% of ammonium hydroxide Components were detected by spraying with an alcoholic solution of *p*-anisidine hydrochloride and heating, or by spraying with conc sulphuric acid and heating.

2-*O*-Methyl-β-D-glucose was obtained from the former British Rayon Research Association It was chromatographically homogeneous and had m p 158–159°, $[\alpha]_D^{22\ 5}$ +12 0 → +67 2° (*c* 1.0, water; lit [21], m p 157–159°, $[\alpha]_D$ +12 0 → +66 0°. 3-*O*-Methyl-α-D-glucose[22] had m p. 172–173°, $[\alpha]_D^{22\ 5}$ +110 → +60 5° (*c* 1 0, water), lit [22], m.p 164–167°, $[\alpha]_D$ +104 5 → +55 5° 6-*O*-Methyl-α-D-glucose[23], prepared from 6-*O*-acetyl-1,2 3,5-di-*O*-methylene-α-D-glucofuranose[23], had m p. 138–139 5°, $[\alpha]_D^{21\ 5}$ + 104 → +55° (*c* 1.0, water); lit.[21], m.p 143–145°, $[\alpha]_D$ +110 → +59°, 55°. 2,3-Di-*O*-methyl-β-D-glucose, prepared from 4,6-*O*-benzylidene-α-D-glucose[24], had m p 106–107°, $[\alpha]_D^{20\ 5}$ +7 → 48.7° (*c* 1.0, acetone); lit.[21], m p 110°, 121°, $[\alpha]_D$ +6 5 → 50 9° 2,6-Di-*O*-methyl-D-glucose was isolated from the hydrolysate of a partially methylated cellulose as a chromatographically homogeneous syrup (Found C, 43.6; H, 7 8; $C_8H_{16}O_6$ calc C, 46 1; H, 7 7%) 3,6-Di-*O*-methyl-α-D-glucose[25] had m p 116–117°; lit [21], 113–116°. 4,6-Di-*O*-methyl-α-D-glucose was obtained from the former British Rayon Research Association[26] The sample, after recrystallisation from ethanol, had m p 162–164°, previously reported[26], 163–163 5°. 2,3,6-Tri-*O*-methyl-α-D-glucose, prepared by acid hydrolysis of fully methylated cellulose, had m.p 123–125°, $[\alpha]_D^{22}$ +104 → +68°, lit.[21], m.p. 121–123°, $[\alpha]_D$ +70° (equil). 2,3,4,6-Tetra-*O*-methyl-α-D-glucose, prepared by acid hydrolysis of methyl 2,3,4,6-tetra-*O*-methyl-α-D-glucoside, had m p. 91–93° Methyl 2,3,4,6-tetra-*O*-methyl-α-D-glucoside was prepared from methyl α-D-glucopyranoside by complete methylation, it was chromatographically homogeneous but otherwise uncharacterized.

Cellulose hydrolysates — The procedure for hydrolysis of *O*-methylcellulose samples was similar to that described by Croon and Lindberg[27]. Methyl cellulose (3 g) was dissolved in 72% (v/v) sulphuric acid (16 7 ml) at room temperature; this usually took 30–60 min The solution was diluted with water to 150 ml and heated for 4 h at 100° The sulphuric acid was neutralized with barium carbonate, and the barium salts were removed by filtration and well washed. The combined filtrate and washings were concentrated under diminished pressure, and a solution of the syrupy residue in hot ethanol was filtered and concentrated to a syrup This procedure was scaled down to a sample size of 100 mg for the later experiments

Trimethylsilylation — Carbohydrate samples (10 mg), dried over phosphorus pentoxide *in vacuo*, were trimethylsilylated in pyridine solution by the procedure of Sweeley *et al.*[12]

Gas-liquid chromatography. — A modified Pye Argon Chromatograph was used. An aluminium, column heater-bar, constructed to take three columns, was wound with Nichrome tape (10 ohms/yard); the density of the windings was progressively increased towards the ends of the bar to give a constant temperature throughout its length Gas flows from the three columns were collected in a stainless steel adapter and fed to a single Lovelock ^{90}Sr detector situated in the aluminium bar and heated to the column temperature The columns were Pyrex tubes of 4 mm, i d and 1 2 m in length Columns *1*, *2*, and *4* were packed with well-washed, glass micro-beads (105–125 μm diameter), pretreated with 20% sodium hydroxide (30 min), conc hydrochloric acid (3 × 30 min), a mixture of hydrofluoric acid 4%, nitric acid 30%, and Teepol 2% (2 × 30 min), and finally with hydrofluoric acid 40% (7 min), the beads were washed with water between each treatment Column *1* was coated with poly-(decane-1,10-diol succinate), m p 69 5° (from chloroform–acetone, 1 1), 0 213% by weight, total weight of stationary phase, 0 0496 g Column *2* was coated with *n*-hexatriacontane (B Newton Maine Ltd), 0 204% by weight, total weight, 0 0441 g Column *4* was coated with silicone gum rubber SE30 (F & M Scientific Corporation), 0 226% by weight, total weight of stationary phase, 0 024 g. Column *3* was packed with 60–100 mesh Kieselguhr (M. and B 'Embacel' acid-washed) coated with poly(ethylene glycol succinate), m p. 103–104° (from chloroform–acetone, 1 1); 8 7% by weight, total weight of stationary phase, 0 329 g Before use, the columns were purged with nitrogen for two weeks at 160° (Column *3* was used at 175° for a number of weeks for another problem prior to being used for the present purpose; some loss of stationary phase occurred during this period at high temperature) Column temperatures were 125 ±0 1° Individual flow-rates, measured by a soap-bubble flowmeter after stopping the flow through the other two columns, were in the range 20–35 ml/min The samples, 0 025–0 1 μl, were introduced onto a plug of silica-glass wool on top of the column packing by means of capillary pipettes Specific retention volumes, V_g^θ, were corrected for column dead-volume and calculated at the column temperature

TABLE VI

COMPARISON OF METHODS OF COMPUTATION[a] IN METHOXYL DISTRIBUTION ANALYSES ON A SERIES OF REPEATEDLY METHYLATED COTTON-FIBRE SAMPLES

Component	*Number of methylations*	*Columns* 1 *and* 3		*Columns* 1 *and* 4		*Columns* 3 *and* 4		*Means*		*Column* 3 *alone*
		(i)	(a)	(ii)	(b)	(iii)	(c)	(i) (ii) (iii)	(a) (b) (c)	
G	2	69.2	72.8	71.4	72.8	70.6	74.4	70.4	73.3	74.4
	3	64.5	64.3	66.0	67.4	65.8	67.0	65.4	66.2	63.9
	4	61.8	63.1	63.9	64.6	62.9	64.2	62.9	64.0	62.8
	5	58.9	59.4	60.3	59.9	60.2	60.1	59.8	59.8	59.5
	8	57.0	56.4	58.6	60.5	56.7	56.9	57.4	57.9	52.8
	10	54.7	56.0	57.8	58.5	56.7	53.6	56.4	57.7	56.0
G_2	2	9.3	8.4	8.0	8.5	8.4	8.4	8.6	8.4	8.4
	3	8.5	7.7	7.9	7.6	7.5	7.7	8.0	7.7	7.7
	4	6.9	6.9	7.2	7.2	7.0	7.0	7.0	7.0	6.9
	5	7.3	6.6	7.4	7.1	7.0	6.8	7.2	6.8	6.6
	8	7.2	6.6	6.5	6.2	6.2	6.4	6.6	6.4	6.6
	10	6.6	6.4	6.4	6.4	5.6	6.4	6.2	6.4	6.4
G_3	2	—	—	—	—	—	—	—	—	—
	3	—	—	—	—	—	—	—	—	—
	4	—	—	—	—	—	—	—	—	—
	5	—	—	—	—	—	—	—	—	—
	8	0.4	0.4	—	—	0.8	0.4	0.4	0.3	0.4
	10	0.6	0.6	—	—	0.4	0.6	0.3	0.4	0.6
G_6	2	6.8	6.2	7.0	6.1	6.8	6.4	6.9	6.2	6.4
	3	7.6	7.0	7.3	6.8	7.0	7.1	7.3	7.0	7.1
	4	6.8	7.2	7.0	7.0	8.0	7.4	7.3	7.2	7.4
	5	7.7	7.6	7.6	7.5	8.3	7.7	7.9	7.6	7.7
	8	8.9	8.5	9.0	8.7	8.3	8.3	8.7	8.5	8.3
	10	8.6	8.6	8.4	8.5	8.1	8.8	8.4	8.6	8.8

G_{23}	2	3.3	2.6	3.0	3.0	3.2	2.8	3.2	2.8	2.6
	3	3.1	2.5	2.3	3.0	2.9	2.8	2.8	2.8	2.5
	4	2.6	2.4	2.0	2.3	2.6	2.3	2.4	2.3	2.4
	5	2.5	2.3	2.0	2.7	2.6	2.5	2.4	2.5	2.3
	8	2.1	2.1	1.8	2.3	2.6	2.2	2.2	2.2	2.1
	10	1.9	2.4	1.6	2.4	2.5	2.4	2.0	2.4	2.4
G_{26}	2	5.6	5.6	4.6	4.6	5.1	5.2	5.1	5.1	5.6
	3	6.6	7.0	5.7	5.7	6.2	6.6	6.2	6.4	7.0
	4	7.0	7.0	6.4	6.4	6.4	6.7	6.6	6.7	7.1
	5	6.8	7.5	6.8	6.8	6.7	6.9	6.8	7.1	7.4
	8	8.1	8.1	7.0	7.0	7.3	7.7	7.5	7.6	8.1
	10	7.2	9.0	7.1	7.1	7.1	7.7	7.1	7.6	8.0
G_{36}	2	1.1	0.6	0.9	1.0	0.6	0.3	0.9	0.6	0.3
	3	1.0	0.9	0.8	1.3	0.4	0.4	0.7	0.9	0.4
	4	1.3	0.9	1.1	1.4	0.5	0.4	1.0	0.9	0.4
	5	1.6	1.1	1.3	1.7	0.5	0.5	1.1	1.1	0.5
	8	1.2	1.0	1.2	1.4	0.7	0.5	1.0	1.0	0.5
	10	1.2	1.0	1.0	1.5	0.5	0.6	0.9	1.0	0.6
G_{236}	2	6.5	6.0	5.6	5.4	5.7	5.7	5.9	5.7	6.0
	3	10.2	9.6	9.7	10.0	9.8	9.8	9.9	9.8	9.6
	4	13.5	13.3	12.7	12.6	12.9	12.9	13.0	12.9	13.3
	5	15.7	15.3	14.6	14.8	15.1	15.1	15.1	15.1	15.3
	8	18.0	18.1	17.1	17.3	17.7	17.7	17.6	17.7	18.1
	10	17.6	18.1	15.9	16.2	17.1	17.1	16.9	17.1	18.1

---

[a] (*i*), (*ii*), and (*iii*) were computed from all available peaks as described in text, (*a*), (*b*), and (*c*) were computed from completely separated components only, assuming an α,β ratio of 1 1.

Relatively small variations in column temperature were found to produce surprisingly large differences in separation. The separation of α-D-glucose, 3-*O*-methyl-β-D-glucose and 2,6-di-*O*-methyl-β-D-glucose is markedly affected by a change in column temperature from 123 to 125° (Fig. 3) In this case, a small difference in separation of overlapping peaks is enhanced geometrically.

Analysis of gas–liquid chromatograms. — Peak areas were measured by planimeter, and the area attributable to individual components was computed in one of three ways, observing the following order of preferences in the calculation· (*1*) the fully separated peaks, (*2*) the peaks computed from combined peaks on one column and fully separated peak or peaks on a second column, and (*3*) peaks computed from a combined peak and fully separated peaks of the corresponding anomers, assuming a 1 1 anomeric ratio No computed value was used for further estimation of individual components of compound peaks The three methods were (*a*) by use of measurements from three columns, (*b*) by using results from pairs of columns (*1* and *3*, *1* and *4*, or *3* and *4*), and (*c*) by using one column only (column *3*) and assuming an anomeric ratio for all components of 1 1, which is a reasonable first approximation based on measured amounts of each anomer over a large number of determinations Table VI shows that reasonably good agreement was obtained between results computed by the abbreviated methods (*b*), using 3 different pairs of columns, and (*c*)

ACKNOWLEDGMENT

This work was financed in part by the United States Department of Agriculture under P.L. 480.

REFERENCES

1 W. J. Whelan and K Morgan, *Chem Ind* (London), (1954) 78
2 B Lindberg and B Wickberg, *Acta Chem Scand*, 8 (1954) 569
3 L Rebenfeld and E Pacsu, *Textile Res J*, 24 (1954) 941
4 R F Schwenker, T Kinoshita, K Beurling, and E Pacsu, *J Polymer Sci*, 51 (1961) 185.
5 W M. Doane, N L Smith, C R Russell, and C E Rist, *Stärke*, 17 (1965) 225
6 I Croon, *Svensk Papperstid*, 63 (1960) 247
7 G O Aspinall, *J Chem Soc*, (1963) 1676
8 W B Neely, J Nott, and C B. Roberts, *Anal Chem*, 34 (1962) 1423.
9 C. T. Bishop and F P. Cooper, *Can J Chem*, 38 (1960) 388
10 E Klein and C J Barter, *Textile Res J*, 31 (1961) 486
11 S W. Gunner, J K N. Jones, and M B Perry, *Chem Ind* (London), (1961) 255
12 C C Sweeley, R Bentley, M Makita, and W W Wells, *J Amer Chem Soc*, 85 (1963) 2497
13 R J Ferrier, *Chem Ind.* (London), (1961) 831
14 E J Hedgley and W. G Overend, *Chem Ind* (London), (1960) 378
15 H G Walker, *Abstracts Papers Amer. Chem Soc Meeting*, 148 (1964) 16D
16 H H Sephton, *J Org Chem*, 29 (1964) 3415
17 J M Richey, H G Richey, and R Schraer, *Anal Biochem*, 9 (1964) 272
18 R J Alexander and J T Garbutt, *Anal Chem*, 37 (1965) 303
19 V D Arendt and E Pacsu, *Textile Res J*, 35 (1965) 554
20 C. Y. Liang and R H Marchessault, *J Polymer Sci*, 37 (1959) 385
21 E J. Bourne and S Peat, *Advan. Carbohyd Chem*, 5 (1950) 145

22 W. L. Glen, G. S. Myers, and G. A. Grant, *J. Chem. Soc.*, (1951) 2568.
23 L. Hough, J. K. N. Jones, and M. S. Magson, *J. Chem. Soc.*, (1952) 1525.
24 J. C. Irvine and J. P. Scott, *J. Chem. Soc.*, (1913) 575.
25 D. J. Bell, *J. Chem. Soc.*, (1936) 1553.
26 J. Kenner and G. N. Richards, *J. Chem. Soc.*, (1956) 2916.
27 I. Croon and B. Lindberg, *Svensk Papperstid.*, 50 (1957) 843.

Carbohydrate Research
Elsevier Publishing Company Amsterdam
Printed in Belgium

SOME STRUCTURAL STUDIES OF BREA GUM (AN EXUDATE FROM *Cercidium australe* JONHST)*

A S CEREZO**, M STACEY, AND J M WEBBER
Department of Chemistry, University of Birmingham, P O Box 363, Birmingham 15 (Great Britain)

(Received December 11th, 1968)

ABSTRACT

The gum exudate (brea gum) from the leguminous tree *Cercidium australe* contains residues of L-arabinose, D-xylose, D-glucuronic acid, and 4-*O*-methyl-D-glucuronic acid, in the approximate molar ratios of 1 7 6 3 1 9 0 9 Autohydrolysis of the gum afforded (chromatographic identification) xylobiose (and homologues), 2-*O*- and 4-*O*-(α-D-glucopyranosyluronic acid)-D-xylose, and 2-*O*-(4-*O*-methylglucopyranosyluronic acid)-D-xylose The principal, neutral components of the methylated gum were 3-*O*- and 2,3,4-tri-*O*-methyl-D-xylose, by chromatographic methods, 2,3-di-*O*-methyl-D-xylose and -L-arabinose, and a tri-*O*-methylarabinose were also detected After reduction, the acidic products from the methylated gum gave, *inter alia*, 3,4-di-*O*-methyl-D-glucose and 2,3,4-tri-*O*-methyl-D-glucose (chromatographic identification)

The major structural features of brea gum appear to be a β-(1→4)-linked D-xylan backbone [possibly containing some (1→2)-linkages] that is heavily 2-substituted by short branch-chains containing residues of D-xylose (and L-arabinose) and D-glucuronic acid, in which both types of residue may be terminal Approximately one-third of the uronic acid is present as the 4-methyl ether, and, in the purified polysaccharide, a similar proportion of the acid residues is lactonised Supporting evidence for these features was obtained by periodate-oxidation studies of the carboxyl-reduced polysaccharide

In possessing this type of structure, which is not common amongst plant gums, brea gum appears to be closely related to sapote gum (from *Sapota achras*)

INTRODUCTION

Cercidium australe Jonhst is a leguminous tree that grows in dry areas of the central, western, and north-western regions of the Argentine Republic, where it is known as 'brea' The exudate (brea gum) of this tree forms slowly in wounds made in the bark of the trunk or main branches, the production of gum is high in dry, hot weather, but diminishes during the rainy season Brea gum is commonly used in Argentina as a substitute for gum arabic

---

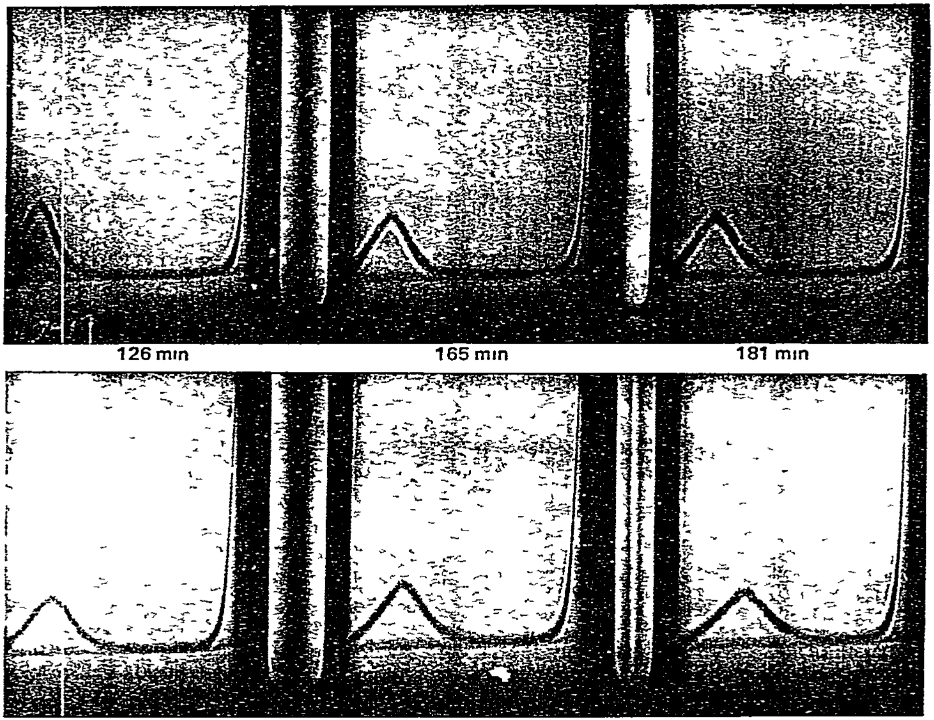
*Dedicated to Professor Stanley Peat, F R S

**Present address Departamento de Química Organica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina

Preliminary studies had indicated that brea gum is an acidic polysaccharide consisting mainly of residues of pentose (a xylose and an arabinose) and hexuronic acid This distribution of sugars is not common in plant gums[1] and indicated that brea gum might resemble the sapote gum[2] obtained from such trees as *Sapota achras*, which grow in Peru We now describe some structural studies of brea gum, which permit a preliminary comparison of the gums

RESULTS AND DISCUSSION

The natural gum was obtained as brown nodules, and the acidic polysaccharide was isolated as a fibrous material by addition of an acidified (pH *ca* 2), aqueous solution of the gum to ethanol Repeated application of this procedure gave a product ($[\alpha]_D$ −6 4° in water) that was examined for homogeneity by an attempted fractionation involving precipitation from aqueous solution by stepwise addition of ethanol Approximately 90% of the polysaccharide was precipitated at an ethanol concentration of 70–75%, and this behaviour has been held[3] to be indicative of essential homogeneity in a polysaccharide Since the product also sedimented as a single peak on ultracentrifugation (Fig 1) and migrated as a single band during zone electrophoresis



126 min 165 min 181 min

213 min 240 min 298 min

Fig 1 Ultracentrifugation of purified brea gum The sedimentation pattern was obtained in a Spinco Model E ultracentrifuge with a 1% solution of the polysaccharide in 0 1M sodium chloride, at a rotor speed of 42,040 r p m, and photographs were taken 126, 165, 181, 213, 240, and 298 min after obtaining full speed The sedimentation coefficient ($S_{20,w}$), calculated in the usual way, was $1\ 7 \times 10^{-13}$

on films of cellulose acetate with three different buffer systems, it was thought to be suitable for structural investigation

The polysaccharide contains *ca.* 24% of uronic acid residues, as determined by the decarboxylation[4] and hydroxamic acid[5] methods, and the latter procedure also showed the presence of an additional 10 4% of lactonised residues of uronic acid The values for the equivalent weight of the polysaccharide (*viz* , 810 ±10 by direct titration, and 540 ±10 by back titration) confirmed that approximately one third of the acid residues were in the lactone form Previous reports of lactonised residues of uronic acid in polysaccharides have involved alginic acid[5 6] and *dried* samples of acidic polysaccharides[5] The infrared spectrum of the purified polysaccharide shows a strong absorption (ν_{max} *ca* 1760–1765 cm^{-1}) that is attributed to lactone groups[7] rather than inter-chain linkages (ester carbonyl group, *ca* 1735–1750 cm^{-1}). The natural gum showed only a very weak absorption at 1725–1800 cm^{-1}, and it therefore seems probable that lactonisation occurs during the purification process A study[8] of the reduction of the lactonised residues with sodium borohydride has shown that there are two types of acid residue in brea gum, one type is readily lactonised, whereas the other is resistant to lactonisation It has been suggested[8] that a contributing factor to this behaviour may be the differing abilities of the two types of acid residue to assume a conformation that permits lactone formation.

The presence of uronic acid residues in the gum confers resistance to acid hydrolysis, and this method was unsuitable for use in the quantitative analysis of the constituent sugars, for example, prolonged treatment (24 h at 100°) with 2N sulphuric acid gave xylose, arabinose, and a mixture of acidic oligosaccharides Attention was therefore turned to the carboxyl-reduced polysaccharide which was prepared by reduction of the propionylated gum with diborane, the propionate was preferred to the acetate for this purpose[9], because of the greater solubility of the former ester in 2-methoxyethyl ether The reduced product had an equivalent weight of *ca* 11,800 by direct titration, but back titration gave a value of *ca* 1200, indicating an appreciable content of residual lactone, free acids are known[10] to be reduced more readily by diborane than are the corresponding lactones The reduction process was therefore completed by sequential treatment with methanolic hydrogen chloride and lithium aluminium hydride Acid hydrolysis of the resulting mixture of glycosides gave arabinose, xylose, glucose, and 4-*O*-methylglucose, in the approximate molar proportions of 1 7 6 3 1 9 0 9, as determined by the triphenyltetrazolium salt method[11] on the sugars eluted from paper chromatograms These values indicate a pentose–uronic acid ratio of *ca* 8 3 (glucuronic acid–4-*O*-methylglucuronic acid, *ca* 2.1), which is in satisfactory agreement with the values obtained (Table I) for the equivalent weights and uronic acid contents

Autohydrolysis (70 h at 100°) of the brea-gum polysaccharide gave a mixture of neutral sugars that was separated from the acidic products by chromatography on De-Acidite FF (CO_3^{2-}) resin Crystalline samples of L-arabinose and D-xylose were isolated from this mixture by chromatography on a cellulose column, and further characterised as crystalline derivatives Other products isolated were xylobiose

(4-*O*-β-D-xylopyranosyl-D-xylose) (chromatographic identification) and a mixture of xylose-containing products that are tentatively identified, from their chromatographic behaviour, as xylobiose and the homologous β-D-(1→4)-linked tri-, tetra-, and pentasaccharides This series of saccharides is obtained on hydrolysis of xylans[12], and it seems probable that brea gum contains a β-(1→4)-linked D-xylan backbone A trace of a galactose was detected in the autohydrolysate, but it is not known whether this component is of structural significance, or whether it arises from an impurity resembling the non-acidic contaminant[2] of sapote gum

TABLE I

ANALYTICAL DATA FOR PURIFIED BREA GUM

	Found	*Calc* [a]
Equivalent weight		
Direct titration	810	790
Back titration	540	527
‘ Uronic anhydride”, %	24 0[b,c]	22 0
Lactonised “uronic anhydride”, %	10 4[c]	10 3
Methoxyl, %	1 6	2 0

[a]For a unit containing pentose and glucuronic acid residues in the ratio of 8 3, one-third of the acid residues being lactonised and one-third as the 4-methyl ether [b]By decarboxylation [c]By the hydroxamic acid method[5]

Fractionation (cellulose column) of the acidic products of autohydrolysis gave D-glucuronic acid (characterised as 1,2-*O*-isopropylidene-α-D-glucofuranurono-6,3-lactone) but did not completely resolve the other components Products identified* by chromatographic and electrophoretic analyses included D-glucurono-6,3-lactone, 4-*O*-methyl-D-glucuronic acid, 2-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-D-xylose (**1**), and 2-*O*-(α-D-glucopyranosyluronic acid)-D-xylose (**2**) Additional evidence for the presence of disaccharide **2**, and also of 4-*O*-(α-D-glucopyranosyluronic acid)-D-xylose (**3**), was obtained when sequential treatment of a mixture ($[\alpha]_D^{20}$ +45 1°) of **2** and **3** with methanolic hydrogen chloride, lithium aluminium hydride, methyl iodide–silver oxide, and acid gave components that were chromatographically and electrophoretically indistinguishable from 3,4- and 2,3-di-*O*-methyl-D-xylose (together with tetra-*O*-methyl-D-glucose) In agreement with these assignments of structure for the aldobiouronic acids **2** and **3**, the latter gave a strongly positive reaction with triphenyltetrazolium bromide[13], whereas no reaction was obtained with the 2-substituted disaccharide **2**

Hydrolysis of methylated brea-gum (OMe, 39 6%) gave a mixture of neutral sugars that was separated from the acidic products on De-Acidite FF (CO_3^{2-}) resin Fractionation (cellulose column) of the neutral sugars gave 2,3,4-tri-*O*-methyl-D-

---

*These studies do not permit an unambiguous assignment of D or L configuration to these products, but the D configuration has been inferred as a result of the isolation of D-xylose and D-glucuronic acid xylose, 3-*O*-methyl-D-xylose, and a very small proportion of D-xylose*, each of which was characterised by preparation of a crystalline derivative. An intermediate fraction contained a 2,3-di-*O*-methylxylose and a 2,3-di-*O*-methylarabinose, which were identified by paper chromatography and by demethylation

An attempted fractionation of the acidic products from the degraded, methylated gum was incomplete, but, following reduction of individual fractions and chromatography on a cellulose column, chromatographic, electrophoretic, and demethylation studies resulted in the detection** of 2,3,4-tri-*O*-methyl-D-glucose, 2,3,4-tri-*O*-methyl-D-xylose, 3,4-di-*O*-methyl-D-glucose, 3-*O*-methyl-D-xylose, and traces of a tri-*O*-methylarabinose The 3,4-di-*O*-methyl-D-glucose was further characterised as its aniline derivative

From the foregoing results, it is possible to deduce some of the structural features of brea gum The isolation of 3-*O*-methyl-D-xylose as a principal product from the methylated gum indicates that a major proportion of the D-xylose residues are (*a*) (1→4)-linked and 2-substituted, or (*b*) (1→2)-linked and 4-substituted Of these alternatives, (*a*) seems more likely, since it is consistent with the autohydrolytic studies that showed that the gum probably has a β-(1→4)-linked D-xylan backbone, however, the presence of some (1→2)-linked residues cannot be discounted (*cf* later discussion) The presence of terminal, and a small proportion of unbranched, residues of D-xylose is indicated by the isolation of 2,3,4-tri-*O*- and 2,3-di-*O*-methyl derivatives of D-xylose from the methylated gum, and the detection of the corresponding derivatives of arabinose (as minor components) suggests that some of these D-xylose residues are replaced by L-arabinose residues A formal representation of these structural features is shown in Fig 2, this accords with the observation that the principal neutral products from the methylated gum are 3-*O*- and 2,3,4-tri-*O*-methyl-D-xylose For convenience, the unbranched pentose residues are represented in a side chain rather than in the backbone Treatment of brea gum with 0 1N sulphuric acid caused release of very little pentose, and it is concluded that the L-arabinose residues, as well as the D-xylose residues, are in the pyranoid form. Sapote gum[14] contains L-arabinopyranose residues, but in most plant gums, the L-arabinose residues occur in the furanoid form

The isolation of 3-*O*-methyl-D-xylose from the reduced, acidic products obtained from the methylated gum is consistent with the presence of uronic acid residues that are (1→2)-linked to the xylan backbone This feature also accords with the detection of 2-*O*-(α-D-glucopyranosyluronic acid)-D-xylose (**2**) and 2-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-D-xylose (**1**) amongst the acidic products of autohydrolysis The presence of units **4** and **5** in the side chains is indicated by the detection of 2,3,4-tri-*O*-methyl-D-xylose (and tri-*O*-methylarabinose) and 3,4-di-*O*-methyl-D-glucose amongst the reduced, acidic products obtained from the methylated

---

*It is not known whether the D-xylose is of structural significance, since it may result from incomplete methylation

**See footnote on p 508

gum. 2,3,4-Tri-*O*-methyl-D-glucose accompanied these products, indicating that some of the side chains must be terminated by, or simply consist of, a uronic acid residue The detection of 4-*O*-(α-D-glucopyranosyluronic acid)-D-xylose (**3**) in the autohydrolysate indicates that a small proportion of the D-xylose residues are not (1→4)-linked, and since 3-*O*-methyl-D-xylose was the only monomethylated sugar to be detected, a (1→2)-linkage is indicated Disaccharides **1**, **2**, and **3** have been variously isolated from previous hydrolytic studies of glucuronoxylans (*e g* , refs. 2 and 15)

D-Xyl*p*(or L-Ara*p*)-(1→2)-D-G*p*A-(1→ **4**

D-Xyl*p*(or L-Ara*p*)-(1→2)-4-*O*-Me-D-G*p*A-(1→ **5**

The unit of eleven residues shown in Fig 2 is not proposed as a repeating unit for the structure of brea gum, but represents an average unit for the structural features indicated by the foregoing studies Evidence to support this unit was provided by periodate-oxidation studies of the carboxyl-reduced polysaccharide, in which the consumption (after 124 h) of 8–9 moles of oxidant per unit was accompanied by the release of 2 9–3 0 moles of formic acid This is in satisfactory agreement with the expected consumption of 6 moles of periodate (with release of 3 moles of formic acid) by the terminal pentose residues, and a further 3 moles of periodate by the remainder of the unit, in which one of the uronic acid residues is lactonised and unreduced, and therefore resistant to oxidation

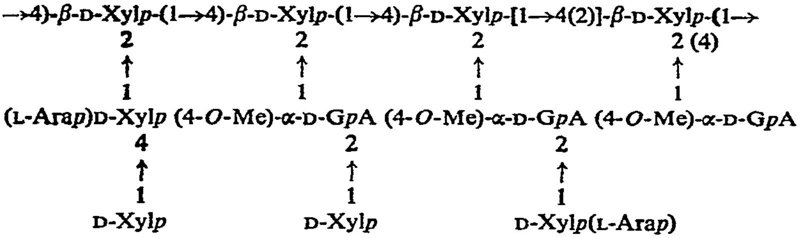


Fig 2 Schematic representation of the structural features that are proposed for brea gum

Acid hydrolysis of the polyalcohol recovered after periodate oxidation and reduction of brea gum gave (chromatographic detection) the products that would be expected for the average unit (Fig 2), *viz* , xylose, xylobiose, glycerol, and erythritol Glucose and 4-*O*-methylglucose were also detected and probably originate from lactonised residues of D-glucuronic acid (or its 4-methyl ether) that had not been reduced by diborane Such residues would survive the treatment with periodate and would then be reduced during the treatment with sodium borohydride Hydrolysis of the reduced oxopolysaccharide with sulphurous acid[16] at room temperature released glucose and 4-*O*-methylglucose, whereas similar treatment at 95° gave xylose as the main component; this behaviour is consistent with the presence of a backbone of periodate-resistant D-xylose residues

Brea gum thus appears to consist of a xylan backbone that is highly substituted by short branches containing uronic acid, D-xylose, and L-arabinose residues Although structural investigations of both gums are incomplete, these indicate a close resemblance in the structures of brea and sapote gums[2,14]; this type of structure has only rarely been found amongst plant gums[17]. A related polysaccharide has been found[17] in the exudate of the seed boxes of *Watsonia versveldii*

EXPERIMENTAL

General methods — Paper chromatography was performed on Whatman No. 1 paper with (*A*) butyl alcohol–ethanol–water (4 1 5, upper layer), (*B*) butyl alcohol–acetic acid–water (4 1 5, upper layer); (*C*) isopentyl alcohol–pyridine–water (1 1.1); (*D*) ethyl acetate–acetic acid-formic acid-water (18 3 1·4); or (*E*) butyl alcohol–pyridine–water (10 3 3) Whatman No. 17 paper was used for preparative, paper chromatography Paper electrophoresis was performed on Whatman No 3 paper with the following buffers (*F*) borate[18] (pH 10), (*G*) acetate (pH 5); (*H*) germanate[19] (pH 10 7), or (*I*) 0 5N sodium carbonate Detection was effected with aniline hydrogen phthalate or alkaline silver nitrate[20] R_{Xyl}, R_{TG}, and M_G refer to rates of movement relative to those of D-xylose, 2,3,4,6-tetra-*O*-methyl-D-glucose, and D-glucose, respectively Demethylations of methylated sugars were performed by the boron trichloride method[21] Relative intensities of infrared absorption peaks and of spots on paper chromatograms, estimated visually, are given as s, strong, m, moderate; w, weak; and vw, very weak

Determination of equivalent weight — Two procedures were employed (*a*) *Direct titration* The sample was titrated against 0 04N sodium hydroxide with phenolphthalein as indicator (*b*) *Back titration*[22]. A solution of the sample (*ca.* 0 5 g) in oxygen-free 0 04N sodium hydroxide (50 ml) was kept at room temperature in a nitrogen atmosphere At intervals, aliquot portions were titrated against 0 01N hydrochloric acid (phenolphthalein). After 24 h, there were only small changes in the titration values, and graphical extrapolation gave the value for calculation of the molecular weight

Purification of the gum — The natural gum (moisture, 13%) was obtained as brown nodules (contaminated with traces of bark). After being dried at 80°/12 mm for 8 h over phosphoric oxide, it had $[\alpha]_D^{16}$ $-4.8°$ (*c* 1.0, water) (Found. ash, 5 2%). The i r spectrum (Nujol mull) showed ν_{max} at, *inter alia*, 3400s, 2900w, and 1725–1800vw^{-1}.

The natural gum (50 g) was stirred with water (300 ml), the brown residue (2 8 g) was removed by filtration, and the cooled (5°) solution (pH *ca* 6–7) was adjusted to pH 2–2 5 by addition of dilute hydrochloric acid The solution was immediately poured into ethanol (1 litre), and the precipitated polysaccharide was collected by decantation, washed by suspension in ethanol (200 ml), and collected by filtration; the product had $[\alpha]_D^{20}$ -6 1° (*c* 1 0, water) The precipitation process was then repeated twice from acidic, aqueous solution and finally once from neutral, aqueous solution. The product, dried by solvent exchange (ethanol and ether) and by storage *in vacuo* at room temperature, was a white powder (37.8 g), $[\alpha]_D^{20}$ $-6.4°$ (c 0.6, water), ν_{max} (Nujol mull) 3400s, 2900w, *ca* 1765s, and *ca* 1655 cm^{-1} [Found (Table I): uronic acid (by decarboxylation[4]), 24; OMe, 1.6; ash, 0.2%; equivalent weight, 810 ±10 (direct titration), 540 ±10 (back titration)]. On electrophoretic analysis, using the enclosed-strip technique with a cellulose acetate support and detection by alkaline permanganate[23], the purified gum gave a single band in borate (pH 9.1), acetate (pH 5.5), and citrate (pH 5.1) buffers.

Ethanol precipitation. — Portionwise addition of ethanol to an aqueous solution of the purified gum (1 g in 20 ml) resulted in no precipitation up to a concentration of 2.5 vol. of ethanol. At this stage, the cloudy solution was clarified by filtration through a Celite pad, and the ethanol concentration was increased to 3 vol. The precipitated polysaccharide (0.92 g) had $[\alpha]_D^{17}$ $-6.3°$ (c 1.6, water); increase in the alcohol concentration caused no further precipitation.

Autohydrolysis of the gum. — An aqueous solution (300 ml) of the purified gum (20 g) was heated on a boiling-water bath for 70 h. The hydrolysis was followed by measurement of α_D for aliquot portions (5 ml) withdrawn as follows:

Time (h)	11	21	35	50	58	70
α_D (degrees)	+0.53	+0.74	+1.00	+1.10	+1.19	+1.19

During this hydrolysis, the solution acquired a deep, red-brown colour and deposited a dark-brown resin. After 70 h, the solution was filtered and poured into ethanol (1 litre), and the precipitated material was filtered off to yield *fraction 1* (1.7 g), $[\alpha]_D^{17}$ $+8.2°$ (c 0.8, water), equivalent weight (direct titration), 765. After ethanol had been removed by distillation, the supernatant solution from fraction 1 was treated with barium carbonate, filtered, and poured into methanol (1 litre). The resulting, white solid was washed with methanol–water to give *fraction 2* (8.6 g). Concentration of the supernatant solution from fraction 2 gave syrupy *fraction 3* (8.2 g) which was fractionated (solvent *A*) on a cellulose column (42 × 4 cm) to give the following fractions:

Fraction 4. Chromatographically homogeneous D-xylose (1.5 g), which, on recrystallisation from ethanol, had m.p. and mixed m.p. 145–146°, $[\alpha]_D^{15}$ $+18°$ (c 0.9, water). The product was further characterised as the di-*O*-benzylidene dimethyl acetal[24], m.p. and mixed m.p. 210–211°.

Fraction 5. A syrup (3.2 g) containing arabinose and xylose (paper chromatography, solvents *A* and *C*).

Fraction 6. Chromatographically homogeneous L-arabinose (0.71 g), which, on recrystallisation from ethanol, had m.p. and mixed m.p. 159–160°, $[\alpha]_D^{15}$ $+104.6°$ (c 1.1, water). The L-arabinose was further characterised as the *p*-nitroaniline derivative[25], m.p. and mixed m.p. 201–202°.

Fraction 7 (elution completed with water). A syrup (2.5 g) containing arabinose, xylose, galactose (traces), and components of lower mobility (R_G<0.5) on paper chromatograms (solvents *A* and *B*). This material, combined with the neutral sugars recovered from fractions *1* and *2* (see below), was subjected to preparative, paper chromatography (solvent *A*), to give the following components:

(*i*) A hexose (traces) which was indistinguishable from D-galactose by chromatography in solvents *A*, *B*, and *C*, and by electrophoresis in buffer *F*.

(*ii*) A component (*ca.* 18 mg) [R_{Xyl} 0.44 (solvent *A*)] which was indistinguishable from xylobiose on paper chromatograms (solvents *A*, *B*, and *E*) and electrophoretograms (buffers *F* and *H*). Acid hydrolysis gave only xylose.

(*iii*) Components having R_{Xyl} 0.44, 0.18, 0.056, and 0.02, and giving a straight-line plot[12b,26] for $\log(1/R_{Xyl}-1)$ against *D.P.* (2 to 5). On acid hydrolysis, these components gave xylose, and are identified[12b,26] as a homologous series of saccharides.

Fractions 1 and 2. The combined fractions (9.3 g) were treated with Amberlite IR-120 (H^+) resin, and an aqueous solution (300 ml) of the resulting syrup (6 g) was stirred for 24 h with DeAcidite FF (CO_3^{2-}, 100 ml of wet resin). The resin was transferred to a column, and elution of the neutral sugars was completed with water, total recovery, 0.15 g. Paper chromatography showed the same components as fraction 7, and the products were combined.

Acidic components. These were eluted from the DeAcidite FF resin with N aqueous ammonium carbonate. After repeated evaporation of water (5×200 ml) from the eluate, residual cations were removed with Amberlite IR-120 (H^+). A portion (3.5 g) of the recovered, acidic sugars (5.5 g) was fractionated (solvent *D*) on a cellulose column (45×4 cm) to give fractions (*a*) to (*e*).

Fraction (a) (*ca.* 1 litre). Paper chromatography (solvents *A–D*) and electrophoresis (buffers *F* and *I*) showed components having the same properties as D-glucurono-6,3-lactone, 4-*O*-methyl-D-glucuronic acid, D-xylose, and L-arabinose.

Fraction (b) (150 ml). This contained two components that were indistinguishable, by chromatography (solvents *B* and *D*) and electrophoresis (buffers *G* and *H*), from 2-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-D-xylose (**1**) (major component, no reaction with alkaline 2,3,5-triphenyltetrazolium bromide[13]) and D-glucuronic acid. Acid hydrolysis gave xylose, 4-*O*-methylglucuronic acid, and glucuronic acid (trace).

Fraction (c) (400 ml). A syrup (*ca.* 20 mg) containing D-glucuronic acid [identified by conversion into 1,2-*O*-isopropylidene-α-D-glucofuranurono-6,3-lactone*[27], m.p. and mixed m.p. 119–120°] and traces of disaccharide **1**.

Fraction (d) (1380 ml). A syrup (*ca.* 40 mg) that contained two components giving xylose and glucuronic acid on acid hydrolysis. The major component (R_{Xyl} 0.35, no reaction with alkaline 2,3,5-triphenyltetrazolium bromide[13]) was chromatographically (solvent *D*) and electrophoretically (buffers *G* and *H*) indistinguishable from 2-*O*-(α-D-glucopyranosyluronic acid)-D-xylose (**2**). The minor component (R_{Xyl} 0.32, *cf.* fraction (*e*) gave a strongly positive reaction with the triphenyltetrazolium salt.

Fraction (e) (780 ml). A syrup (97 mg), $[\alpha]_D^{20}$ +45.1° (*c* 0.4, water), R_{Xyl} 0.35

---

*Dextrorotary in methanol, lit.[27], $[\alpha]_D$ +70° in methanol.

and 0 32 (solvent *D*), M_G 0 71 and 0 62 By treatment with 4% methanolic hydrogen chloride, the mixture (80 mg) was converted into the methyl ester methyl glycosides which were then reduced with lithium aluminium hydride in tetrahydrofuran during 18 h at room temperature Acid hydrolysis of the resulting glycosides gave only glucose and xylose. Four treatments of the glycoside mixture with silver oxide–methyl iodide, followed by treatment with N sulphuric acid for 6 h at 95° and preperative, paper chromatography of the products (solvent *A*), gave tetra-*O*-methylglucose and a mixture containing components that were electrophoretically indistinguishable from 3,4- and 2,3-di-*O*-methyl-D-xylose (buffer *F*) having M_G 0 28, R_{TG} 0 80, and M_G 0 00, R_{TG} 0 80, respectively.

Fraction (f) (elution completed with water) A mixture (3 1 g, 89%) of acidic oligosaccharides (R_{Xyl} < 0 2), giving xylose and acidic components on acidic hydrolysis

Reduction of brea gum. — A suspension of the finely powdered, purified gum (5 g) in formamide (60 ml) was treated with pyridine (45 ml) and propionic anhydride (30 ml) After storage for 2 days at room temperature, the resulting solution was poured into ethanol (500 ml), the small amount of precipitated material was filtered off, and the solution was concentrated to *ca* 150 ml and poured into ice-cold 1% hydrochloric acid (1 litre) The precipitated material was filtered off, washed with ice-cold water, and dried *in vacuo* at 60° The resulting product (8 g) was re-treated with pyridine (100 ml) and propionic anhydride (12 ml) for 3 days to yield the propionylated acid (6 7 g) having ν_{max} 1750s (C=O) and 3500 cm^{-1} (OH)

To a solution of the esterified acid (2 25 g) in 2-methoxyethyl ether (diglyme) (60 ml) was added sodium borohydride (1.48 g) During 5 h, a solution of boron trifluoride etherate (6 g) in diglyme (16 ml) was added slowly, with stirring, to the resulting suspension. After storage overnight, followed by neutralisation with 0 1N sodium hydroxide, the solution was concentrated *in vacuo* The residue was suspended in 0.1N sodium hydroxide (60 ml) and heated for 1 h at 60–70°, and the resulting, clear solution was dialysed against running tap-water for 2 days and treated with Amberlite IR-120 (H$^+$) The recovered product (0 94 g) had ν_{max} 1720vw cm^{-1}; equivalent weight, *ca* 11,800 (direct titration), *ca* 1200 (back titration)

The reduced gum (200 mg) was boiled with 4% methanolic hydrogen chloride (50 ml) for 6 h, and the recovered glycosides were treated with lithium aluminium hydride (400 mg) in tetrahydrofuran (100 ml) overnight at room temperature. The resulting product showed no absorption at ν_{max} 1500–2000 cm^{-1}, and acid hydrolysis (N H_2SO_4, 3 5 h, 60–70°) gave (chromatographic analysis in solvent *A*), arabinose, xylose, glucose, and 4-*O*-methylglucose [proportions, 1 7 6 3 1 9 0 9 (by the triphenyltetrazolium salt method[11])].

Periodate oxidation of the reduced gum — Acetylation of the purified gum, followed by reduction of the acetate (by the procedure described for the propionate), gave a reduced gum having an equivalent weight of 8750 (direct titration)

(*a*) A solution of this gum (57.6 mg) in 0 0415M sodium metaperiodate (50 ml) was stored in the dark at 3°. The consumption of oxidant[28] and release of formic acid (titration with 0.01N NaOH) were as follows (expressed for a unit having molecular weight 1552)

Time (h)	1.25	24	46	124
Periodate (mol)	2.9	7.7	7.6	8.7
Formic acid (mol)	1.5	2.6	2.9	3.0

(*b*) A solution of the reduced gum (0.493 g) in 0.0415M periodate (500 ml) was kept at 3°. After 3 and 5 days, *ca* 8.5 mol of oxidant had been consumed, with release of 2.9 mol of formic acid (per unit of mol wt. 1552) After 5 days, ethylene glycol (5 ml) was added, and the solution was dialysed against running tap-water for 3 days and then freeze-dried, yield, 0.473 g.

A portion (51.5 mg) of this polyaldehyde was added, with stirring, to a solution of sodium borohydride (13 mg) in water (1.5 ml) at room temperature. The resulting solution was kept at room temperature overnight, dialysed against running tap-water for 2 days, and freeze-dried, yield, 28.7 mg. Acid hydrolysis (0.12N H_2SO_4, 4 h, 95°) gave components that were indistinguishable on paper chromatograms (solvents *A* and *C*) and electrophoretograms (buffer *F*) from xylose (s), arabinose (w), glucose (w), 4-*O*-methylglucose (w), xylobiose (w), glycerol (s), erythritol (vw), and erythronolactone (vw).

Acid hydrolysis of the reduced oxopolysaccharide with sulphurous acid[16] (room temperature overnight) gave glucose, 4-*O*-methylglucose, and traces of arabinose, at 95°, xylose was the main component, together with minor proportions of the foregoing sugars and xylobiose

Methylation of brea gum — An aqueous solution (200 ml) of the purified gum (20 g) was treated at 0° with methyl sulphate (90 ml) and 30% (w/v) sodium hydroxide (175 ml), added dropwise, with stirring, during 4 h. After stirring had been continued for 1 h at 0°, the solution was dialysed for 48 h against running tap-water and freeze-dried. The resulting product (24 g) (in aqueous acetone) was subjected to three further methylations, as follows: (*i*) final stirring (1.5 h) at 15–20° (yield, 22 g), (*ii*) final stirring at 20–25° (yield, 18 g), and (*iii*) addition of reagents at 35°, final stirring at 50°; yield, 17.5 g; $[\alpha]_D^{20}$ −22.3° (*c* 0.7, chloroform) (Found OMe, 32.3%) The final product (16 g) was converted into the silver salt by treatment with Amberlite IR-120 (H^+), followed by silver carbonate, yield, 16 g.

A suspension of the silver salt (16 g) in dry methanol (100 ml) and methyl iodide (100 ml) was stirred for 30 min, and the resulting solution was boiled for 6 h, stirred overnight at room temperature, and filtered After three repetitions of this procedure (heating for 9, 12, and 15 h), the methylated gum (8 g) had $[\alpha]_D^{20}$ −39.0° (*c* 1.3, chloroform), ν_{max} (liquid film) 1750s (C=O) and *ca* 3450vw cm^{-1} (OH) (Found OMe, 39.6%; unchanged by the final methylation)

Hydrolysis of the methylated gum. — A solution of the methylated gum (4 g) in 90% formic acid (40 ml) was heated for 1 h at 90–95° After evaporation of formic acid from the resulting, clear solution, the residue was heated with N sulphuric acid

(120 ml) for 7 h at 95°. The cooled solution was neutralised [$Ba(OH)_2$ and $BaCO_3$] and treated with Amberlite IR-120 (H^+) The resulting product was treated with DeAcidite FF (CO_3^{2-}; 70 ml), and the neutral sugars were recovered by elution with water; yield, 2 5 g Elution with N ammonium carbonate gave the methylated, acidic sugars, yield of free acids, 1 g.

Neutral sugars These products (2 1 g) were placed on a cellulose column (53 × 4 5 cm) which was then eluted with solvent *A* to give. *Fraction* A A chromatographically pure syrup (0 543 g), $[\alpha]_D$ +14° (*c* 0.1, water), R_{TG} 0.95, M_G 0 0, that was identified as 2,3,4-tri-*O*-methyl-D-xylose by conversion into 2,3,4-tri-*O*-methyl-D-xylonolactone[29], m p and mixed m p 52–54°.

Fraction B A syrup (1 39 g) that was refractionated as described below

Fraction C. A chromatographically pure syrup (0.513 g), R_{TG} 0 51, M_G 0 71, $[\alpha]_D^{16}$ +14° (*c* 0 7, water), that was identified as 3-*O*-methyl-D-xylose by conversion into the aniline derivative[30], m.p and mixed m.p 137°

Fraction D A chromatographically pure syrup (0 03 g) that was identified as D-xylose by conversion into the di-*O*-benzylidene dimethyl acetal[24], m p and mixed m p 210–211°.

Refractionation of Fraction B. Cellulose-column chromatography of this material gave components that were identical with fractions *A* and *C*, together with an intermediate fraction (0 3 g) that gave xylose (s) and arabinose (w) on demethylation In solvent *A*, the components of this intermediate fraction were chromatographically indistinguishable from 2,3-di-*O*-methyl-D-xylose (s) (R_{TG} 0 80) and 2,3-di-*O*-methyl-L-arabinose (w) (R_{TG} 0 74)

Acidic sugars The acidic sugars (1 g) were placed on a cellulose column (50 × 4 cm) which was eluted with solvent *D* to give *Fraction I* A syrup (0.18 g), R_F 0 75, which was treated with 4% methanolic hydrogen chloride, followed by reduction with lithium aluminium hydride Acid hydrolysis of the reduced product (N H_2SO_4, 55 h, 95°) gave 3-*O*-methylxylose (R_{TG} 0 51, M_G 0 71), 3,4-di-*O*-methylglucose (R_{TG} 0 63, M_G 0 30), and a small proportion of a product having R_{TG} 0.95, M_G 0 00 (probably 2,3,4-tri-*O*-methylxylose) (solvent *A*, buffer *F*)

Fractions II to V These fractions (total weight, 0 479 g), which respectively contained compounds having R_F 0 76, 0 76 and 0 57, 0 43, and 0 35, were individually treated as for fraction I, and all gave mixtures in which 3-*O*-methylxylose and 3,4-di-*O*-methylglucose were the main components

A portion (0 25 g) of the combined, reduced products from fractions I–V was fractionated on cellulose, as described for the neutral sugars, to give *Fraction* a. A syrup (0 1 g), R_{TG} 0 94 (s) (2,3,4-tri-*O*-methyl-xylose and -arabinose) and R_{TG} 0 87 (s) (2,3,4-tri-*O*-methylglucose) Demethylation gave xylose (s), arabinose (w), and glucose (s)

Fraction b A syrup (0 035 g), R_{TG} 0 63, $[\alpha]_D^{20}$ +87 2° (*c* 0 6, water), which was identified as 3,4-di-*O*-methyl-D-glucose by conversion into the aniline derivative[31], m p and mixed m p. 171–173°.

Fraction b′ A mixture (40 mg) of *b* and *c*

Fraction c A syrup (70 mg), $[\alpha]_D^{20}$ +14.2° (*c* 0 5, water), which was identical with the 3-*O*-methyl-D-xylose from fraction C

ACKNOWLEDGMENTS

We thank Drs B H. Howard, Elizabeth Percival, and T E Timell, and Professor A Wacek for gifts of sugars

One of us (A.S.C.) is indebted to the Consejo Nacional de Investigaciones Cientificas y Técnicas of the República Argentina for an external fellowship

REFERENCES

1 F Smith and R Montgomery, *The Chemistry of Plant Gums and Mucilages*, Reinhold, New York, 1959
2 R D Lambert, E E Dickey, and N S Thompson, *Carbohyd Res*, 6 (1968) 43
3 R L Whistler and J N BeMiller, *J Amer Chem Soc*, 78 (1956) 163; C T Bishop, *Can J Chem*, 35 (1957) 1010
4 S A Barker, A. B Foster, I R Siddiqui, and M Stacey, *Talanta*, 1 (1958) 216
5 M A G Kaye and P W Kent, *J. Chem Soc*, (1953) 79.
6 D. W Drummond, E L Hirst, and E Percival, *J Chem Soc*, (1962) 1208.
7 S A Barker, E J. Bourne, R M Pinkard, and D H Whiffen, *Chem Ind* (London), (1958) 658; R S Tipson, H S Isbell, and J E Stewart, *J Res Natl Bur Std*, 62 (1959) 259
8 A S Cerezo, *Anales Asoc Quim Argentina*, 55 (1967) 169
9 F Smith and A M Stephen, *Tetrahedron Lett*, (1960) 17
10 H C Brown, *Hydroboration*, Benjamin, New York, 1962
11 K Wallenfels, *Naturwissenschaften*, 37 (1950) 491
12 (*a*) J K Hamilton and N S Thompson, *J Amer Chem Soc*, 79 (1957) 6464, (*b*) D H Shaw and A. M Stephen, *Carbohyd Res*, 1 (1965) 400
13 G Avigad, R Zelikson, and S Hestrin, *Biochem J*, 80 (1961) 57
14 E V White, *J Amer. Chem Soc*, 75 (1953) 257.
15 R L Whistler and L Hough, *J Amer Chem Soc*, 75 (1953) 4918
16 J D Moyer and H S Isbell, *Anal Chem*, 29 (1957) 1862; C M Ewald and A S Perlin, *Can J Chem*, 37 (1959) 1254
17 D H Shaw and A M Stephen, *Carbohyd Res*, 1 (1966) 414
18 A B Foster, *J Chem Soc*, (1953) 982
19 B Lindberg and B Swan, *Acta Chem Scand*, 14 (1960) 1043
20 W E Trevelyan, D P Procter, and J S Harrison, *Nature*, 166 (1950) 444
21 T G Bonner, E J Bourne, and S McNally, *J Chem Soc*, (1960) 2929
22 R L Whistler and M S Feather, *Methods Carbohyd Chem*, 1 (1962) 467
23 D R Buggs, E F Garner, and F Smith, *Nature*, 178 (1956) 154
24 L J Breddy and J K N Jones, *J Chem Soc*, (1945) 738
25 F Weygand, W Perkow, and P Kuhner, *Ber*, 84 (1951) 594
26 E C Bate-Smith and R G Westall, *Biochim Biophys Acta*, 4 (1950) 427
27 L N Owen, S Peat, and W J G Jones, *J Chem Soc*, (1941) 339
28 E L Jackson, *Org Reactions*, 2 (1944) 341
29 S P James and F Smith, *J Chem Soc*, (1945) 739, 746
30 R A Laidlaw and E G V Percival, *J Chem Soc*, (1950) 528
31 D J Bell and G D Greville, *J Chem Soc*, (1950) 1912

ERRATA

p 72, line 11 below formulae, for epoxide read epoxide **4a**

p. 74, line 7 below formula, for V_0 read V^0

p. 74, line 13 up, for 10 00 read 1 00

p. 76, line 5, for *pentribose* read *pentose*