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Note

A micro method for the determination of the structure of substituted 2-acetamido-2-deoxy-D-galactitol and -D-glucitol residues by periodate oxidation*

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The compounds resulting from the periodate oxidation of glycoconjugates and oligosaccharides containing amino sugars (2-acetamido-2-deoxy-D-galactose and -D-glucose) are generally identified by paper[2] and ion-exchange chromatography[3] and electrophoresis[4], and more recently, by g.l.c.–mass spectrometry[1]. Because of their limits of detection, these techniques are, however, of restricted use in the study of the extremely small quantities of carbohydrate-containing substances that are obtained from cultured cells, or produced *in vitro* by biosynthetic reactions. In the study of the surface glycoproteins of the TA3-Ha ascites tumor-cell[5] and of the products resulting from glycosyltransferase reactions *in vitro*[6], a highly sensitive method determining the position of substitution of 2-acetamido-2-deoxy-D-galactitol and -D-glucitol residues was needed. This paper reports a method in which aminodeoxy-[^{3}H]alditols resulting from periodate oxidation, sodium borotritide reduction, and hydrolysis of nmole amounts of substituted 2-acetamido-2-deoxyalditols are separated by t.l.c. and detected by autoradiography.

The nature of the 2-amino-2-deoxyalditol resulting from periodate oxidation followed by sodium borohydride reduction and acid hydrolysis (Smith degradation) of a substituted 2-acetamido-2-deoxyalditol depends on the position of substitution, the number of substituents, and the nature of the 2-acetamido-2-deoxy sugar residues. Oligosaccharides having a terminal 2-acetamido-2-deoxyalditol residue are readily formed from *O*-glycoproteins (*O*-glycosyl linkage to serine and threonine, or mucin type) by reductive, alkaline treatment[7]. More-drastic hydrolytic conditions are

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needed in order to produce oligosaccharides having terminal 2-amino-2-deoxyalditol residues from *N*-glycoproteins (*N*-glycosylamine linkage to asparagine, or serum type)[3]. Reduced oligosaccharides may also be derived from both types of glycoprotein by enzymic cleavage of the glycopeptide or of the di-*N*-acetylchitobiosyl bond, followed by sodium borohydride reduction[4,8].

In order to develop a very sensitive method for determining the resulting 2-amino-2-deoxyalditol, thin-layer chromatography was combined with autoradiography. The sensitivity of this method is limited only by the specific radioactivity of the 2-amino-2-deoxyalditol residue. It was found that the standard 2-amino-2-deoxyalditols could conveniently be separated on thin-layer plates of Silica gel 60 by a continuous-flow technique (see Fig. 1). The migration distances are reported in

TABLE I

MIGRATION OF 2-AMINO-2-DEOXYALDITOLS ON THIN-LAYER PLATES OF SILICA GEL[a]

2-Amino-2-deoxy compounds of	$R_{2\text{-amino-1,3-propanediol}}$
L-Threitol (2)	0.72
L-Arabinitol (3)	0.66
D-Xylitol (4)	0.46
D-Galactitol (5)	0.33
D-Glucitol (6)	0.26

See legend to Fig. 1 for conditions.

Table I. The radioactive label was conveniently introduced by reduction with sodium borotritide during the Smith degradation. The method was tested with several, authentic, substituted 2-acetamido-2-deoxyalditols. Amounts of 5–30 nmoles of material could be handled with ease, and 30–50 nCi of tritium per nmole could generally be recovered in the tritium-labelled alditol. In the case where the method was tested with 2-acetamido-1,3,4,5,6-penta-*O*-acetyl-2-deoxy-D-glucitol, which is resistant to periodate oxidation, no significant radioactivity was found in the final compound. Autoradiography of the chromatogram of the 2-amino-2-deoxy[ω-^{3}H]-alditols produced from authentic samples showed that, in all instances, only one radioactive product, the one expected, had been formed (see Fig. 2).

EXPERIMENTAL

Materials. — The standards of 2-amino-2-deoxy-L-threitol, 2-amino-2-deoxy-D-xylitol, 2-amino-2-deoxy-D-galactitol, and 2-amino-2-deoxy-D-glucitol were prepared from the respective 2-acetamido-2-deoxyalditols by hydrolysis with 4M hydrochloric acid for 4 h at 100°. 2-Acetamido-2-deoxy-L-threitol and 2-acetamido-2-deoxy-D-xylitol were synthesized in this laboratory[1]. 2-Acetamido-2-deoxy-D-galactitol and -D-glucitol[10] were synthesized by reduction of 2-acetamido-2-deoxy-D-galactose and -D-glucose. 2-Amino-2-deoxy-L-arabinitol was prepared from 2-amino-2-deoxy-L-

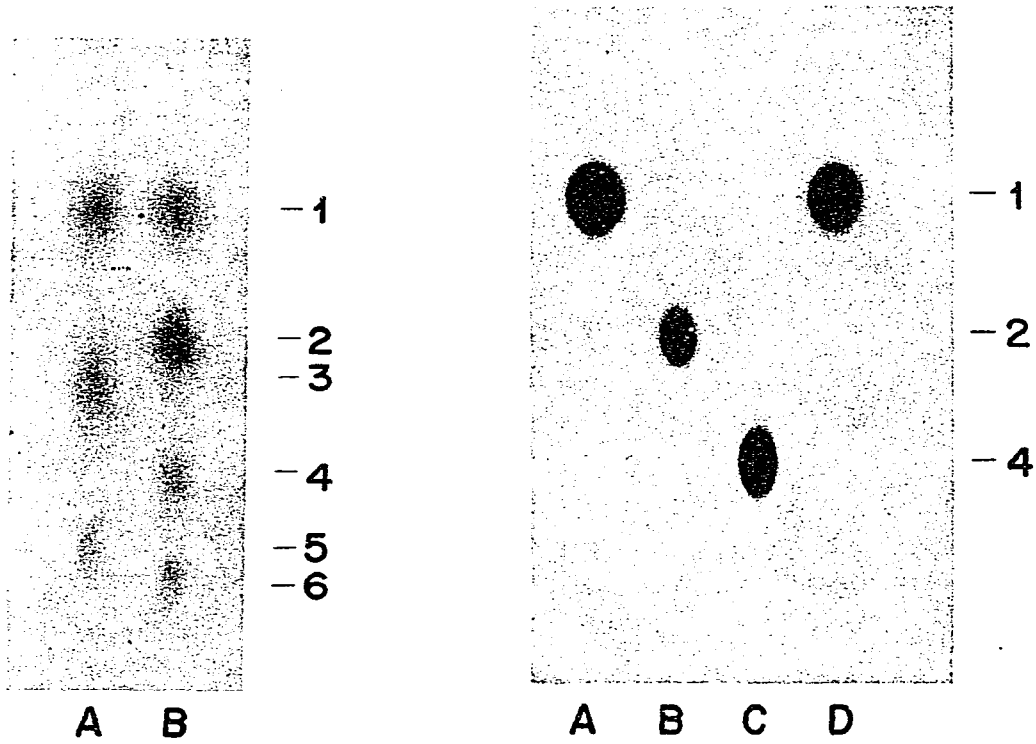

Fig. 1. Chromatograms on thin-layer plates precoated with Silica gel 60 and irrigated with 6:3:1:1 (v/v) 1-propanol–ethyl acetate–acetic acid–water under continuous flow[15] (jar height, 15 cm) for 16 h. Spots were detected with ninhydrin: A, 2-amino-1,3-propanediol (**1**), 2-amino-2-deoxy-L-arabinitol (**3**), and 2-amino-2-deoxy-D-galactitol (**5**); B, 2-amino-1,3-propanediol (**1**), 2-amino-2-deoxy-L-threitol (**2**), 2-amino-2-deoxy-D-xylitol (**4**), and 2-amino-2-deoxy-D-glucitol (**6**).

Fig. 2. Autoradiogram of aminodeoxy[^{3}H]alditols obtained by $NaIO_4$ oxidation, $NaBT_4$ reduction, hydrolysis, and separation as described in the Experimental section and in the legend to Fig. 1. The spots were detected by autoradiography as described by Randerath[15]. The compounds oxidized were: A, 2-acetamido-2-deoxy-D-glucitol; B, 2-acetamido-2-deoxy-3-*O*-β-D-galactopyranosyl-D-galactitol; C, 2-acetamido-2-deoxy-4-*O*-β-D-galactopyranosyl-D-glucitol; and D, 2-acetamido-2-deoxy-6-*O*-α-neuraminosyl-D-galactitol. The compounds obtained were: 2-amino-1,3-propanediol (**1**), 2-amino-2-deoxy-L-threitol (**2**), and 2-amino-2-deoxy-D-xylitol (**4**).

arabinose[11] by *O*-peracetylation with acetic anhydride–pyridine, reduction and concomitant *O*-deacetylation with sodium borohydride ($NaBH_4$) in 0.1M sodium hydroxide, and *N*-deacetylation with 4M hydrochloric acid. 2-Amino-1,3-propanediol was purchased from I.C.N. Pharmaceuticals, Life Science Group, Cleveland, Ohio 44128. 2-Acetamido-2-deoxy-3-*O*-β-D-galactopyranosyl-D-galactitol and 2-acetamido-2-deoxy-4-*O*-β-D-galactopyranosyl-D-glucitol were prepared by reduction with sodium borohydride, at pH 7.8, of 2-acetamido-2-deoxy-3-*O*-β-D-galactopyranosyl-D-galactose[12] and 2-acetamido-2-deoxy-4-*O*-β-D-galactopyranosyl-D-glucose[13], respectively. 2-Acetamido-2-deoxy-6-*O*-α-D-neuraminosyl-D-galactitol was obtained by treatment of ovine submaxillary mucin with M $NaBH_4$–0.1M NaOH for 3 days at 37°;

the reduced disaccharide was isolated by passing the reduction mixture through a column of Dowex 50 X-2 (H^+) ion-exchange resin. 2-Acetamido-1,3,4,5,6-penta-*O*-acetyl-2-deoxy-D-glucitol[11] was prepared by acetylation of 2-acetamido-2-deoxy-D-glucitol with acetic-anhydride–pyridine for 30 min at 80°. Sodium borotritide ($NaBT_4$, 334 Ci/mol) was purchased from New England Nuclear, Boston, MA 02118, and thin-layer plates precoated with Silica gel 60, from Merck (Darmstadt, Germany). All other chemical compounds were of analytical grade, and obtained commercially.

Procedure. — A 10-fold molar excess of sodium periodate ($NaIO_4$) was added to the sample (5–30 nmoles) in 0.1M sodium acetate buffer (pH 4.5, 100 µl). After 24 h at 0–6° in the dark, the oxidation was terminated by the addition of 1,2-ethanediol (5 µl), and the sample was lyophilized. The dry residue was dissolved in 0.1M Tris, pH 9.0 (50 µl), and $NaBT_4$ (1.0–1.4 µmoles, 334 Ci/mol) in 0.1M Tris, pH 9.0 (50 µl) was added. After 1 h at room temperature, an excess of $NaBH_4$ (27 µmol) in 0.1M Tris (pH 9.0, 50 µl) was added, and the sample was kept for another hour. The excess of $NaBH_4$ was decomposed by the addition of 4M acetic acid (20 µl). The radioactive water was removed by drying the sample in a vacuum desiccator over P_2O_5, followed by two additions of water (100 µl), and drying. The cations were removed by dissolving the residue in 10mM formic acid (0.5 ml), and passing the solution through a column (0.6 × 2 cm) of Dowex 50 X-16 (H^+) cation-exchange resin (200–400 mesh). The column was washed with 10mM formic acid (2.5 ml), and the eluate and wash were combined and lyophilized.

The following step was performed only when sialic acid was present in the original sample. The dry sample was dissolved in 0.05M H_2SO_4 (500 µl), and the solution was heated for 1 h at 80°. The hydrolyzate was passed through a column (0.6 × 2 cm) of Dowex 1 X-8 (AcO^-) anion-exchange resin (200–400 mesh), which was washed with water (2.5 ml). The eluate and wash were combined and lyophilized.

The sample was hydrolyzed by heating with 4M HCl (600 µl) for 4 h at 100°. The acid was removed by additions of toluene–ethanol, and evaporation under a stream of nitrogen. The 2-amino-2-deoxyalditol derivative was isolated by passing the sample, in 10mM formic acid (1 ml), through a column (0.6 × 2 cm) of Dowex 50 X-16 (H^+) cation-exchange resin (200–400 mesh). The column was washed with 10mM formic acid (3 ml), and the wash was discarded. The 2-amino-2-deoxyalditol was eluted with 1.5M NH_4OH (2.5 ml), and the eluate was lyophilized.

The dry sample was dissolved in 7:3 (v/v) ethanol–water (100 µl) and aliquots containing 25–30 nCi of ^3H were deposited on a thin-layer plate of Silica gel 60. Samples (3 µg in 1 µl) of each of the following standard compounds were added to the unknown sample as carrier for the radioactive compounds, and were also deposited in reference lanes: 2-amino-1,3-propanediol and 2-amino-2-deoxy-L-threitol, -L-arabinitol, -D-xylitol, -D-galactitol, and -D-glucitol. The chromatogram was developed by the continuous-flow technique[14] (jar height, 15 cm) with 6:3:1:1 (v/v) 1-propanol–ethyl acetate–acetic acid–water for 16 h.

The autoradiography (fluorography) was performed by the Randerath method[15], with an exposure time of 10 days at −75°. After autoradiography, the

standards on the chromatogram were detected with ninhydrin. The radioactive products were identified by comparing the migration distance and shape of the spot on the autoradiogram with those on the chromatogram.

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