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AN EXTRACELLULAR MICROBIAL POLYSACCHARIDE COMPOSED OF 2-ACETAMIDO-2-DEOXY-D-GLUCOSE AND 2-ACETAMIDO-2-DEOXY-D-GLUCURONIC ACID: RADIOCHEMICAL AND GAS-CHROMATOGRAPHIC ANALYSIS OF THE PRODUCTS OF METHANOLYSIS*

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ABSTRACT

When the polysaccharide from the black yeast NRRL Y-6272, composed of 2-acetamido-2-deoxy-D-glucose (**1**) and 2-acetamido-2-deoxy-D-glucuronic acid (**2**), is hydrolyzed, extensive humin formation occurs by decomposition of component residues, especially the hexosaminuronic acid. Methanolysis avoids this decomposition by forming stable methyl glycosides amenable to quantitation by both radiochromatographic techniques and gas chromatography. Unlike hydrolysis, which results in incomplete depolymerization, refluxing methanol–HCl (M, 16–24 h) completely depolymerizes polysaccharide Y-6272 to the methyl glycosides of its component sugars. Use of ^{14}C-methanol–HCl allows quantitation of **1** and **2** by counting the individual ^{14}C-methyl glycosides after separation by paper chromatography. As the methyl glycosides derived from the hexosaminuronic acid in polysaccharide Y-6272 consist of both a methyl ester and a lactone, for quantitation it was necessary to convert these two glycoside forms into a common derivative of known ^{14}C-methyl content by treatment with mild alkali. Methanolysis by using radioisotopes affords an extremely valuable method for detecting and quantitating amino sugars in polysaccharides; it is rapid and sensitive and it should be especially applicable for analyzing other polysaccharides and proteins that contain constituents labile to normal hydrolytic conditions.

INTRODUCTION

The extracellular polysaccharide produced by the black yeast strain NRRL Y-6272, composed[1] of 2-acetamido-2-deoxy-D-glucose (**1**) and 2-acetamido-2-deoxy-D-glucuronic acid (**2**), undergoes extensive humin formation and almost complete

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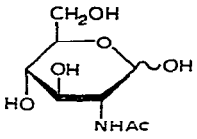
decomposition of **2** when subjected to hydrolysis by aqueous acid such as M HCl at 100°. As ease of degradation by aqueous acid is an established characteristic of the hexosaminuronic acids[2], an alternative method of depolymerization was necessary for further study of this novel polysaccharide. We have found that methanolysis is advantageous in that it depolymerizes the polysaccharide readily but does not decompose compound **2**, and the stable reaction products (methyl glycosides) are amenable to quantitation by both radioisotopic and gas-chromatographic methods. The method of choice, however, is methanolysis with ^{14}C-methanol and quantitation by measuring the radioactivity with a liquid scintillation-counter. Both **1** and **2** are readily quantitated by radiochemical counting of their individual ^{14}C-methyl glycosides after separation by paper chromatography. The need of standards for the tedious determination of the gas-chromatographic response-factor[3] for each component is avoided, as quantitation is dependent only on the number of ^{14}C-methyl groups incorporated per mole of sugar and the specific activity of the ^{14}C-methanol.

EXPERIMENTAL

Materials and reagents. — ^{14}C-Methanol (23 mCi/mmole) and ^{14}C-D-glucitol (7 mCi/mmole) were purchased from New England Nuclear Corp., and 2,5-diphenyloxazole (PPO scintillation grade), from Beckman Instruments, Inc. All other chemicals were reagent grade. Dry methanol, prepared by distillation from and storage over molecular sieves (4 Å, Davidson Chemical Co.), was added to the ^{14}C-methanol to give a final specific activity of 16 μCi/mmole. All lots of ^{14}C-methanol were found to contain a radioactive contaminant that could be removed by double distillation. This radioactive contaminant is characterized by (*a*) its consistent mobility [R_1 2.5, butanol–ethanol–water (4:1:5, upper phase)], (*b*) its partial solubilization from paper chromatograms by the scintillation fluid (PPO/toluene, 4 g/l), and (*c*) its volatility (it evaporates slowly from paper chromatograms). ^{14}C-Methanol–HCl was prepared by adding acetyl chloride (0.07 ml) to ^{14}C-methanol (0.93 ml) to give a solution that was 0.4 mCi/ml and M in acid[4]. ^{14}C-D-Glucitol, purified by preparative, paper chromatography, was added to the ^{14}C-methanol–HCl as an internal standard (0.8 μCi of ^{14}C-D-glucitol per ml of ^{14}C-methanol–HCl).

Polysaccharide[1] Y-6272 was converted into its free carboxyl form by passing a dilute solution over a column of AG 50W-X4 resin (20–50 mesh, H^+ form, Bio-Rad Laboratories) before lyophilization to dryness. Methyl 2-acetamido-2-deoxy-α and β-D-glucopyranoside (**3** and **4**) were prepared by treating **1** with dry methanol and dry AG 50W-X4 (20–50 mesh, H^+ form) resin according the method of Zilliken *et al.*[5] Compound **3** was separated from **4** on a 62 × 1 cm column of AG 1-X4 (200–400 mesh, Bio-Rad Laboratories) resin in the OH^- form according to the method of Matsushima *et al.*[6], which is much simpler and faster than chromatography on charcoal.[5] Fractions from the column containing only **3** or **4** were combined and crystallized from ethanol. The ratio of crystalline **3** to **4** was 73:27. Compound **3** had m.p. 192.5–193.5°, $[\alpha]_D^{20}$ + 131.5° (*c* 0.2, water) [lit.[7] m.p. 185–187°, $[\alpha]_D^{20}$ + 131°; lit.[8]

m.p. 190–193; $[\alpha]_D^{20}+128.2°$]. Compound **4** had m.p. 197–202°, $[\alpha]_D^{20}-41.0°$ (*c* 0.39, water) [lit.[8] m.p. 196–199°, and $[\alpha]_D^{20}-43°$, lit.[5] m.p. 204-205°, $[\alpha]_D-44.3°$]. The foregoing assignments of configuration were confirmed by n.m.r. spectroscopy.



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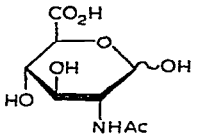

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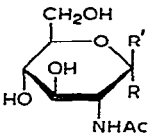

3 R = OMe, R' = H
4 R = H, R' = OMe

The methyl glycosides of 2-acetamido-2-deoxy-D-galactose (**5**), 2-acetamido-2-deoxy-D-mannose (**6**), and *N*-acetylneuraminic acid (**7**) were prepared by methanolysis of the reducing sugars. These glycosidic mixtures were used in paper chromatography without resolution and isolation of the pure anomers. Resolution on paper chromatograms, however, showed a profile of migration behavior characteristic of each mixture, as shown in Table I.

Methanolysis.–The overall scheme used in applying methanolysis to analysis of polysaccharide Y-6272 by radiochemical and gas-chromatographic methods is outlined in Fig. 1.

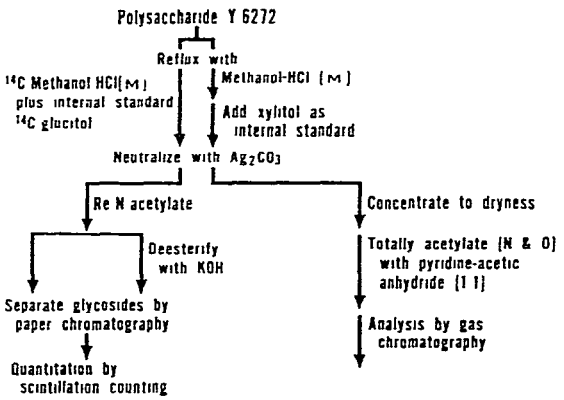

Fig. 1. Methanolysis of polysaccharide Y-6272: general reaction scheme.

Polysaccharide samples contained in tubes (15 × 125 mm) equipped to take a Teflon-lined screw cap were dried *in vacuo* over P_2O_5, first for 16 h at room temperature and then for 4 h at 78°. To the dried samples were added ^{14}C-methanol–HCl (M) to give a 1% solution and a small Teflon-coated magnetic stirrer, and the tubes were capped. The mixture was stirred and samples were heated for at least 4 h at reflux (75–77°). Solubilization of polysaccharide Y-6272 was complete after 20 h of treatment. These solutions, which remained clear and developed no more than a

TABLE I

COMPONENTS IN METHANOLYZATES OF AMINO SUGARS AND POLYSACCHARIDE Y-6272: PAPER-CHROMATOGRAPHIC PROFILES OF R_1[a] VALUES

Parent material	*Component of methanolyzates*				*Detecting reagent*	
	Number	*Relative amount (%)*	*Identity*	R_1	*NaOCl spray*[13] *for amino sugars*	NH_2OH–Fe^{3+} *spray*[14] *for esters*
1[b]			**1**	1.0	+	–
	1	10	ND	1.2	+	–
	2	25	**4**	1.5	+	–
	3	65	**3**	1.8	+	–
6[c]			**6**	1.1	+	–
	1	25	ND	1.4	+	–
	2	25	ND	1.7	+	–
	3	50	ND	2.0	+	–
5[c]			**5**	0.9	+	–
	1	20	ND	1.3	+	–
	2	40	ND	1.6	+	–
	3	20	ND	1.9	+	–
	4	20	ND	2.1	+	–
7[c]			**7**	0.1 (streak)	+	–
	1	35	ND	1.4	+	+
	2	65	ND	2.1	+	+
Polysaccharide Y-6272[b]			PS Y-6272	0.0	+	–
	1	6	ND	1.2	+	–
	2	17	**4**	1.5	+	–
	3	43	**3**	1.8	+	–
	4	ND[d]	ND	2.3	+	+
	5	ND[d]	ND	2.8	+	+
	4+5 de-esterified	33	Me glycoside of **2**	0.4	+	–

[a]Abbreviations used. R_1 = mobility relative to 2-acetamido-2-deoxy-D-glucose (**1**); ND = not determined; PS Y-6272 = polysaccharide Y-6727. [b]For this substance, the relative amounts of anomeric glycosides listed in column 3 were determined by ^{14}C-methanolysis. [c]For this substance, the relative amounts of anomeric glycosides listed in column 3 were estimated visually after use of the NaOCl spray[13]. [d]Undefined mixture of ester and lactone forms prevented quantitative determination.

slight yellow color, were in dramatic contrast to hydrolyzates (M HCl, 4–16 h, 100°), which were dark in color and contained precipitated humin. After methanolysis, the acid was neutralized by solid silver carbonate. As extensive de-*N*-acetylation[9] occurs during methanolysis, the methanolysis products were re-*N*-acetylated[10] by addition of acetic anhydride (0.2 ml/1.0 ml of methanolyzate) to the same tubes containing the neutralized products over $AgCl$–Ag_2CO_3. The tubes were recapped and the reaction mixtures were stirred with a Vortex mixer before keeping the samples for 16–20 h at room temperature.

The reaction mixtures were then centrifuged and the supernatant of each was decanted. Twice the precipitate was washed with methanol and sedimented by centrifugation, and the supernatant was decanted. Combined supernatants were then concentrated by evaporation at room temperature in a stream of nitrogen to near dryness; methanol was then added and similarly evaporated off (thrice). The residues were kept overnight *in vacuo* over P_2O_5 and solid KOH. This method of Clamp *et al.*[10] for re-*N*-acetylation was found to be equivalent to the acetic anhydride–$NaHCO_3$–Dowex-50 (H^+) method[11] and much simpler.

Deesterification of methanolysis products[12]. — After re-*N*-acetylation and concentration to dryness, the methanolysis products (5 mg) were deesterified with 25 mM KOH (2 ml) under nitrogen for 4.5 h at room temperature before neutralizing with 0.5M HCl. This solution was concentrated to dryness *in vacuo* at 30° and dissolved in a known amount of water before spotting on paper chromatograms.

Paper chromatography. — Samples of re-*N*-acetylated methanolysis products were dissolved in methanol, spotted on chromatograms (Whatman No. 1 paper) and developed in butanol–ethanol–water [4:1:5 (v/v), upper phase]. Amino sugar glycosides, both free amino and *N*-acetylated forms, were located on paper chromatograms with the NaOCl–KI–amylose sprays[13]. Free amino sugars were detected with a ninhydrin dip[11]. Glycosides containing methyl-esterified uronic acids were detected by spraying with hydroxylamine and ferric chloride[14]. Alkaline silver nitrate[11], used as a dip to detect amino sugars, gave weak spots for most amino sugar glycosides. The R_1 values are listed in Table I.

Radiochromatography. — Radiochromatograms, after development on Whatman paper (No. 1, 2 × 46 cm) in butanol–ethanol–water (4:1:5, upper phase), were cut into 1-cm segments, each of which was placed in a scintillation vial and counted in 10 ml of scintillation fluid (PPO/toluene, 4 g/l) in the liquid-scintillation counter (Beckman LS-250).

Analysis by gas chromatography. — Methanolysis [methanol–HCl (M), 2.0 ml] was performed on preweighed dry samples (2 mg) in Teflon-lined screw-cap tubes by heating for 24 h at 80° before cooling to room temperature and centrifuging. A known amount of xylitol was added on an internal standard, and the solution was neutralized with solid Ag_2CO_3 and centrifuged before removing an aliquot that was concentrated *in vacuo* at 40° and then stored *in vacuo* over P_2O_5 and KOH pellets. The dry products were acetylated[15] by adding 0.2 ml of a 1:1 mixture of dry pyridine and acetic anhydride. Acetylation times of 1, 2, 24, and 120 h gave equivalent results.

Samples (1–2 μl) were analyzed in a F and M Model 810 gas chromatograph (Hewlett Packard) on a 6-ft column of Apiezon-L. The temperature, initially 150°, was allowed to rise 1°/min to 180°. The detector response K for **1** was determined by the formula:

$$K = \frac{\text{Weight of xylitol}}{\text{Weight of } \mathbf{1}} \cdot \frac{\text{Area of } \mathbf{1}}{\text{Area of xylitol}}.$$

Peak areas were determined by use of the disc integrator. The detector response and

quantitation of **2** was not possible by gas chromatography because of the lack of an authentic standard sample.

RESULTS

Radiochemical analysis of the products of methanolysis

Compound **1**. — Treatment with ^{14}C-methanol–HCl converts **1** into ^{14}C-labeled anomeric glycosides, which were readily quantitated by scintillation counting (Fig. 2). Deacetylation of amino groups during methanolysis resulted in formation of multiple peaks. Re-*N*-acetylation resulted in two main unified peaks which consisted of **3** and **4** in the approximate ratio of 75:25.

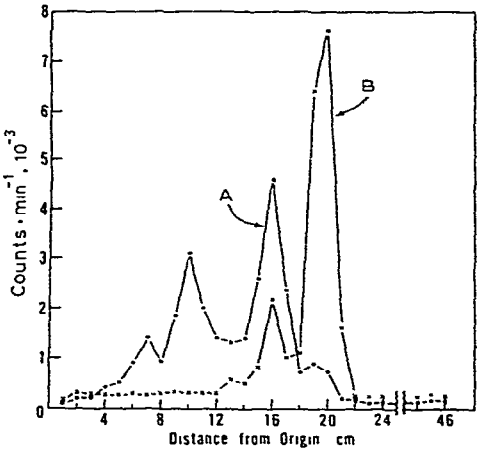

Fig. 2. Methanolysis of 2-acetamido-2-deoxy-D-glucose: A (●—●), before re-*N*-acetylation; B (×—×), after re-N-acetylation with $AgCO_3$–AgCl and acetic anhydride. Methanolysis conditions: methanol–HCl (M), 20 h.

Polysaccharide Y-6272. — The major products expected from ^{14}C-methanolysis and subsequent re-*N*-acetylation of the repeating unit[1] of polysaccharide Y-6272 are singly labeled anomeric glycosides of **1** and doubly labeled anomeric glycosides of the methyl ester of **2**. Paper-chromatographic separation of the re-*N*-acetylated products of methanolysis from polysaccharide Y-6272 showed no significant amount of oligosaccharide products, partial resolution of **3** and **4**, and separation of these from the faster-moving glycosides of **2** (Fig. 3A). The suspected presence of lactone in peaks 4 and 5 (Fig. 3) was confirmed by deesterification of the components of these peaks by treating the total methanolysis reaction-mixture with dilute potassium hydroxide solution. Paper chromatography of the resultant mixture (Fig. 3B) showed total absence of the former peaks 4 and 5 (Fig. 3A) and presence of a single new slow-moving peak composed of anomeric methyl glycosides of **2** (K^+ form). Quantitation of ^{14}C in this peak established the content of **2** in polysaccharide Y-6272. ^{14}C-D-

Glucitol, added initially to the methanolysis reaction mixture, served as an internal standard for determining the percent of recovery at various stages of sample manipulation.

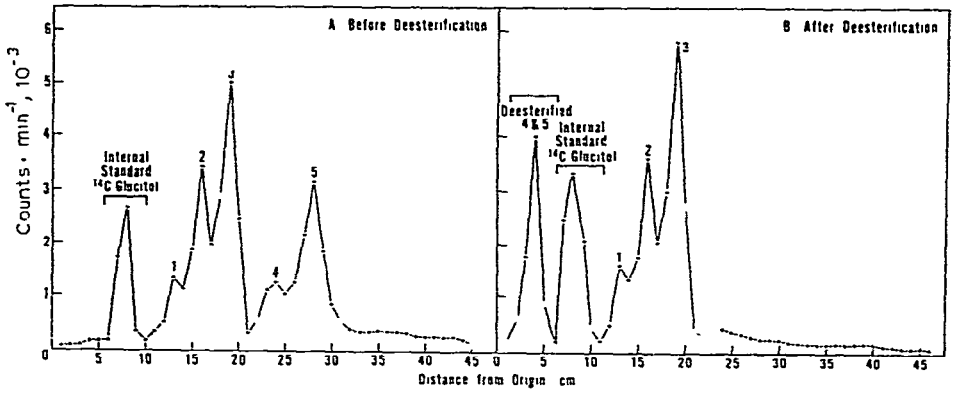

Fig 3. Radiochromatography of the products of methanolysis of polysaccharide Y-6272 after re-*N*-acetylation. Methanolysis conditions: methanol–HCl (M), 20 h. A, before deesterification; B, after deesterification with KOH (25mM). ^{14}C-Glucitol was added initially as an internal standard. Peaks 1, 2, and 3 are assigned to the ^{14}C-methyl glycosides 3+4 [(*3*+*4*)-*Me*-^{14}*C*] of 2-acetamido-2-deoxy-D-glucose (**1**). Peaks 4 and 5 are assigned to the ^{14}C-methyl glycosides of 2-acetamido-2-deoxy-D-glucuronic acid (**2**) (in A, as ^{14}C-methyl ester; in B, as potassium salt)

Effect of reaction time in methanol–HCl, and stability of glycosides. — A time study of incorporation of ^{14}C into the products when polysaccharide Y-6272 was refluxed in ^{14}C-methanol–HCl (M) revealed that maximum depolymerization was reached between 9 and 20 h. Continued treatment, even up to 5 days, did not affect the yield of glycosides of either **1** or **2**.

The findings of Clamp and associates[3,10,16,17] indicate that release of carbohydrates from glycoproteins and oligosaccharides was complete within 3 h in methanol–HCl (M) at 85°. No loss of the glycosides occurred with 24 h of treatment.

Identification of the parent constituents in polysaccharide Y-6272

Compound **1**. — Compounds **3** and **4** in the ratio of 74:26 were isolated from methanolyzates of polysaccharide Y-6272 by resin chromatography, as described for the resolution of **3** and **4** (see Experimental). Correspondence was thus established between peak 2 material (Fig. 3A) and **4**, and peak 3 and **3**. The other fast-moving peaks (4 and 5, Fig. 3A) remained on the resin under all elution conditions tried.

The identity of the hexosamine moiety of polysaccharide Y-6272 was confirmed further by correspondence of the paper-chromatographic migration pattern of the glycosides with that of authentic **3** or **4** and lack of correspondence with patterns of the methyl glycosides of **5**, **6**, and **7** (see Table I).

Compound **2**. — Various evidence suggests that the fast-moving peaks (4 and 5 in Fig. 3A) in polysaccharide Y-6272 methanolyzates are primarily methyl glycosides

of the hexosaminuronic acid methyl ester. First, these peaks were shown to react positively with a spray (NH_2OH–$FeCl_3$) used for detecting esters[14]. Second, treatment of the glycosidic mixture from polysaccharide Y-6272 with KOH completely converted these fast-moving peaks into one slow-migrating, acidic peak (Fig. 3B). Third, treatment of re-*N*-acetylated methanolysis (unlabeled methanol) products of polysaccharide Y-6272 with NaB^3H_4 (to reduce the methyl ester) converted the fast-moving peaks into ^3H-labeled peaks that cochromatographed (Fig. 4) with the methyl

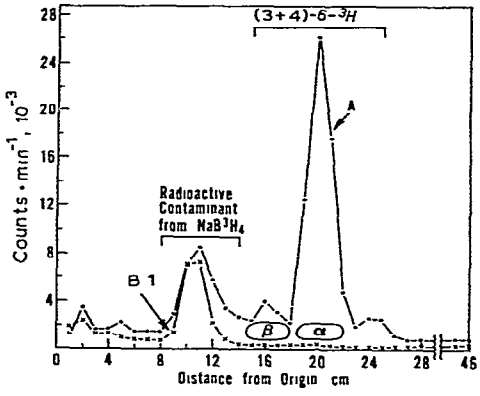

Fig. 4. Radiochromatography of the methanolysis products after re-N-acetylation and NaB^3H_4 reduction. A (●—●), polysaccharide Y-6272; B (×—×), **1** (control). Methanolysis conditions: unlabeled methanol–HCl (M), 20 h.

glycosides of **1** (Fig. 2). Similar treatment of methanolysis products from **1** with NaB^3H_4 failed to incorporate any radioactivity into its methyl glycosides. Hydrolysis of the NaB^3H_4-reduced glycoside mixture from polysaccharide Y-6272 produced radioactive 2-amino-2-deoxy-glucose but not 2-amino-2-deoxy-galactose or 2-amino-2-deoxy-mannose, as shown by cochromatographing with authentic hexosamines.

Gas-chromatographic analysis of polysaccharide Y-6272. — Methanolyzates of many carbohydrates have been quantitated by gas chromatography after trimethylsilylation[3,10,16,17]. Limitations in this method arising from incomplete silylation led us to abandon it. More-satisfactory results were obtained after total acetylation (both N- and O-)[15] of methanolyzates of polysaccharide Y-6272, as shown in Fig. 5. The chromatogram for the polysaccharide derivatives consisted of one major and two minor peaks for **1** and two additional, more-volatile peaks (Fig. 5B), arbitrarily attributed to derivatives of **2**. After the laborious calculation of the response factor for **3** and **4**, this component (**1**) of the polysaccharide was quantitated (Table II); however, lack of an authentic sample of the other component, the methyl glycoside of **2**, prevented its quantitation. As shown here, standardization of each individual sugar constituent is not necessary in radiochromatographic analysis of the products of methanolysis, as results were uniform regardless of the parent sugar being analyzed.

Quantitation of components of polysaccharide Y-6272. — The percentage com-

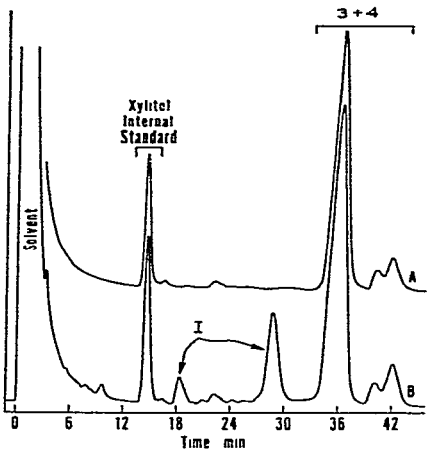

Fig. 5. Gas-chromatographic separation of the totally acetylated methanolysis products of A, *1*; B, polysaccharide Y-6272; I, methyl glycoside methyl ester of *2*.

position of polysaccharide Y-6272 as determined by various methods is summarized in Table II. There is excellent agreement in the values for percent of **1** when determined by methanolysis either by radiochromatography (61.8%) or by gas chromatography (62.9%) and the values (61.0%) previously determined by NaB^3H_4 reduction of nitrous acid-treated hydrolyzates[1,18,19].

TABLE II

COMPOSITION OF THE EXTRACELLULAR POLYSACCHARIDE FROM BLACK YEAST NRRL Y-6272

Method of determination	*Residues of* **1** (%, *by wt.*)	*Residues*[a] *of* **2** (%, *by wt.*)
I. Methanolysis (M HCl)		
A. Radiochromatography		
1. ^{14}C-methanolysis	61.8	20–40[b]
2. ^{14}C-methanolysis and deesterification by KOH	61.8	33 0[c]
B. Gas chromatography of totally acetylated methyl glycosides	62.9	—
II. NaB^3H_4 microreduction after:		
A. Hydrolysis (M HCl)	49.0	—
B. Hydrolysis (M HCl) and nitrous acid treatment[1]	61.0	—
III. Calculated from neutral equivalent determined by titration[1]	—	34.9
IV. Calculated values of repeat unit [$\mathbf{1}_2 \rightarrow \mathbf{2}_1$], as the K salt (percent potassium, 5.8)	61.4	32.8

[a]Calculated as free acid. [b,c]Calculated from ^{14}C-methyl glycosides of the ^{14}C-methyl ester–lactone mixture and the K-salt, respectively.

The percent of **2** was determined precisely by radiochromatography of its ^{14}C-methyl glycosides after deesterification, and it agrees well with both the value calculated from titration data and that calculated for a hypothetical repeating unit (Table II). Precise results could not be obtained by direct methanolysis because the methyl glycoside of **2** existed in two forms, lactone and methyl ester.

DISCUSSION

Methanolysis appears to be as efficient as hydrolysis for cleaving polysaccharides[20–25] and causes less decomposition of constituent sugars[26,27] than does aqueous acid, yet its use as an analytical technique has been limited by the methods used to analyze the resulting mixture of methyl glycosides. Methyl glycosides have been analyzed as the polyacetyl derivatives[28,29] by gas chromatography, but thermally catalyzed changes[30] occur that limit their use. Methyl glycosides of sugars can also be analyzed by gas chromatography of their *O*-trimethylsilyl derivatives, yet great care must be taken to eliminate both water and inorganic salts to ensure complete derivatization and quantitative results. Liu *et al.*[12] have shown that the Group B and C meningococcal polysaccharides, which contain sialic acid, are cleaved readily by methanolysis and that neuraminic acid can be quantitated as its methyl glycoside (potassium salt) in the amino acid analyzer[12]. In the analysis of glycosidic mixtures by either gas chromatography or with an amino acid analyzer, each sugar has the potential of giving a different molar response and, therefore, for quantitation an authentic sample must be available for determining its molecular-response factor. Quantitation of unknown sugars by these methods is thus excluded.

Radioisotopic methanolysis is not only free of such disadvantages but also has several other distinct advantages. First, unlike hydrolysis, methanolysis gives complete depolymerization of polysaccharide Y-6272 in one step. With a polysaccharide such as this one, which contains amino sugars, the problem of incomplete hydrolysis arises from the extremely slow release of the *N*-deacetylated hexosamine residues. Treatment of such a partial hydrolyzate with nitrous acid permits complete depolymerization by conversion of both free and bound 2-amino-2-deoxy-D-glucosamine to 2,5-anhydro-D-mannose, a reducing sugar that can be quantitated by the NaB^3H_4 microreduction method[18]. Methanolysis and analysis by radiochromatography or gas chromatography, however, was found to be not only as accurate but also much simpler than the nitrous acid technique for quantitating **1** in polysaccharide Y-6272. Comparative results are shown in Table II. Second, methanolysis prevents the gross decomposition of **2** that otherwise occurs during hydrolysis; methanolysis blocks the reducing end-group, which apparently is needed for formation of humin. Unlike hydrolyzates, which are always dark and contain precipitated humin, methanolyzates of polysaccharide Y-6272 remained clear even after 72 h at reflux [methanol HCl (M)]. The amount of **2** in polysaccharide Y-6272 was readily determined for the first time by radiochemical counting of the deesterified ^{14}C-methyl glycosides of **2** after separation by paper chromatography. A third advantage of methanolysis with use of radioiso-

topes is that the direct quantitation of both **2** and **1** can be made on the same sample. Fourth, in contrast to analysis by gas chromatography or with an amino acid analyzer, radioisotopic methanolysis allows rapid quantitation without requirement for authentic samples. The unavailability of authentic **2** led to development of the radioisotopic methanolysis procedures reported here.

The new technique of methanolysis plus radiochromatography will be valuable in study of other polysaccharides that contain not only **2**[31–33] but other hexosaminuronic acids[2,34–37], which are being discovered with increasing frequency. Polysaccharide Y-6272 is unique among these hexosaminuronic acid-containing polysaccharides in that it is the only one obtained extracellularly, and, with one exception, from an apparent nonpathogen.

The paper-chromatographic solvent system, butanol–ethanol–water (4:1:5, upper phase) separates **3** and **4**, and thus permits quantitative determination of these anomers. The ratio of **3** to **4**, formed from either authentic **1** or polysaccharide Y-6272 and with either HCl or AG-50(H^+) ion-exchange resin as catalyst, is approximately 75:25. The equilibrium ratio of methyl glycoside anomers of **2** formed during methanolysis of polysaccharide Y-6272 appears to be 85α to 15β, as the ratio of *3,6*-^{3}H to *4,6*-^{3}H, formed by NaB^3H_4 reduction of the methyl ester-lactone form of **2**, was found to be 85:15. Thus the ratio of the α to β anomer of the methyl glycosides of **2** is slightly higher than the corresponding anomers of **3** and **4** (75:25).

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