

hexenals was *cis*-2-hexen-1-al, while the first was another isomer.

Octanal. Retention volume data corresponded. Canary yellow derivative had an infrared spectrum identical to that of octanal-dinitrophenylhydrazone. Paper chromatographic data agreed.

Octenal (?). Retention volume data checked. Melting point and paper chromatographic data agreed. However, infrared spectra compared poorly. Elemental analysis of the unknown derivative indicated an eight- or nine-carbon, unsaturated compound as the parent compound.

Furfural (?). Retention volume data agreed. Characteristic color of derivative checked. This was a trace component and not detected in all samples.

Neral (Citral b). The retention volume was the same as that of neral obtained from gas chromatographic fractionation of commercial citral. The infrared spectra and paper chromatographic data were essentially the same for the derivatives of both geranial and neral, but the geranial derivative melted at a much lower temperature.

Geranial (Citral a). Retention volume data, infrared spectra, melting point, and paper chromatographic data corresponded.

Carvone. Retention volume data agreed. Bright red color, infrared spectrum, and paper chromatographic data of dinitrophenylhydrazone checked. This peak formed as a shoulder on the peak for geranial. The combined materials were condensed and reacted with dinitrophenylhydrazine, and the mixed derivatives separated paper chromatographically on unimpregnated Whatman No. 3 paper, using heptane as the developing solvent.

Discussion

This procedure gives good identification of small amounts of aldehydes and ketones in gas chromatographic effluents. In some cases the melting points tend to be slightly lower than those reported in the literature, largely because of the small quantities involved, which make repeated recrystallizations impractical. However, good infrared spectra and paper chromatographic data can be obtained even when traces of impurities are present. The infrared spectra are sufficiently characteristic to permit even octanal and nonanal-dinitrophenylhydrazones to be distinguished.

Two carbonyl compounds encountered in this study—geranial and neral—reacted more slowly with dinitrophenylhydrazine than did the others and could not be directly precipitated by bubbling the effluent gas through the reagent solution. Their derivatives were formed by condensing the effluent from the suspected peaks and reacting it with dinitrophenylhydrazine sulfate according to the normal procedures. Some other carbonyl compounds will probably behave in this manner.

Hexenal and octenal have not previously been reported as constituents of orange juice. Their detection may have been the result of the large degree of concentration of all the components in the essence, or they may be artifacts.

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FOOD PLANT POLYSACCHARIDES

Structure of the Polysaccharide of the Japanese Water Plant, *Brasenia schreberi* J. F. Gmel.

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Composition of the Polysaccharide and Isolation of 2-O-(β -D-Glucopyranosyluronic Acid)-D-Mannose

DURING investigations on the relationship between structure and physical properties of polysaccharides attention had been drawn to the polysaccharide which forms the main component of the mucilaginous coating of the Japanese water plant, Junsai (*Brasenia schreberi* Gmel.). This plant, which belongs to the lotus family (*Nymphaeaceae*) (15), grows in swampy areas, possesses branching roots, and develops fine long stems under water with elliptical leaves that protrude through the surface. In summer, dark reddish violet flowers appear on the surface of the water. The transparent gelatinous mucilage which

covers the leaves and stems of the plant is commonly used as a food in Japan.

The polysaccharide was conveniently isolated from plant stems and leaves by treating them with hot water or with dilute sodium hydroxide at room temperature (16) followed by addition of ethanol to the extract to precipitate the required polysaccharide. Purification was effected by use of the fact that the polysaccharide forms an insoluble copper hydroxide complex when treated with Fehling solution (16). The polysaccharide, recovered from the copper complex as a white amorphous powder, constitutes 0.4 to 0.5% of the weight

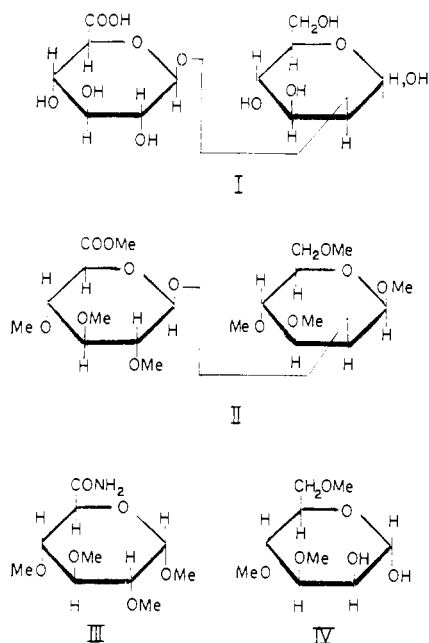
of the wet plant or about 40% of the dry weight of the plant. The polysaccharide, which is acidic in character (equivalent weight, 1040), after further purification via its acetate ($[\alpha]_D^{25} - 6^\circ$ in acetone and in chloroform), showed $[\alpha]_D^{25} + 9^\circ$ in water, in contrast to the product isolated previously (16) in 1940, which had $[\alpha]_D^{25} + 28^\circ$ in water. The substance, which revealed no evidence of heterogeneity by electrophoresis on glass paper (14), did not reduce boiling Fehling solution. It gave a negative test with iodine solution, but a strong positive reaction for uronic acid by the carbazole test (4).

A polysaccharide extracted from the water plant, *Brasenia schreberi*, with either hot water or dilute sodium hydroxide, is shown by hydrolysis to be an L-arabo-L-fuco-D-galacto-D-glucurono-D-manno-L-rhamno-D-xyloglycan. It consists of L-arabinose (5.9%), L-fucose (10.9%), D-galactose (34.1%), D-glucuronic acid (17.3%), D-mannose (13.4%), L-rhamnose (11.4%), and D-xylose (7.0%). Acid hydrolysis gives a mixture of the above sugars and an aldobiouronic acid, the structure of which has been established as 2-O-(β -D-glucopyranosyluronic acid)-D-mannose.

Acid hydrolysis of the acidic polysaccharide, followed by column and paper chromatographic analyses of the neutral components of the hydrolyzate, gave the following analysis (16): L-arabinose (5.9%), L-fucose (10.9%), D-galactose (34.1%), D-mannose (13.4%), L-rhamnose (11.4%), and D-xylose (7.0%) (Table I). D-Glucuronic acid (17.3%) was also shown to be present in the polysaccharide by titration and by the carbazole method. All of these components were identified by isolation of characteristic crystalline compounds (Table II).

The acid component present in the hydrolyzate consisted largely of an aldobiouronic acid (I) which showed the same electrophoretic mobility as the aldobiouronic acid, 2-O-(β -D-glucopyranosyluronic acid)-D-mannose, isolated from *Virgilia oroboides* gum (20).

Since the acid (I) showed $[\alpha]_D^{25} - 33^\circ$ in water and yielded, upon hydrolysis, D-glucuronic acid and D-mannose, it appeared to be a D-glucosyluronic acid-D-mannose with a beta linkage.



Treatment of I first with methyl sulfate and alkali and then with silver oxide and methyl iodide furnished the fully methylated crystalline derivative (II) previously obtained (20) by methylating the aldobiouronic acid, 2-O-(β -D-

Table I. Column Chromatographic Separation of the Neutral Components of the Water Plant Polysaccharide

Fractions	Tube No.	Yield, Mg.	Components
1	50-66	97.7	L-Rhamnose and traces of L-fucose
2	67-72	32.0	L-Rhamnose (84%) and L-fucose (16%) ^a
3	73-78	24.7	L-Fucose
4	79-85	25.0	L-Fucose (44%) and D-xylose (56%) ^a
5	86-89	23.5	D-Xylose
6	90-111	57.0	D-Xylose (trace), L-arabinose and D-mannose
7	112-125	26.0	L-Arabinose, D-mannose and D-galactose (trace) ^b
8	126-189	208.2	D-Galactose

Recovery = 492.1 mg. or 95%

^a Calculated from the specific optical rotation.

^b Components determined by paper chromatography.

Table II. Composition of the Acidic Polysaccharide from *Brasenia Schreberi* (Japanese Water Plant; Junsai)

Component	Per Cent	Identified as
L-Arabinose	5.7	Diphenylhydrazone, m.p. 204° C.
L-Fucose	12.2	Free sugar, m.p. 145° C., $[\alpha]_D^{25} - 74.5^\circ$ (H ₂ O); diphenylhydrazone, m.p. 202° C.
D-Galactose	34.0	Free sugar, m.p. 165° C., $[\alpha]_D^{25} + 80^\circ$ (H ₂ O), <i>N</i> - <i>p</i> -nitrophenylglycosylamine, m.p. 219° C.
D-Mannose	13.2	Phenylhydrazone, m.p. 195° C.
L-Rhamnose	12.8	Free sugar, m.p. 98° C. (hydrate), $[\alpha]_D^{25} + 8^\circ$ (H ₂ O); <i>p</i> -nitrophenylhydrazone, m.p. 191° C.
D-Xylose	6.6	Free sugar, m.p. 145° C., $[\alpha]_D^{25} + 18.8^\circ$ (H ₂ O), di- <i>O</i> -benzylidene dimethylacetal, m.p. 211° C.
D-Glucuronic acid	17.3 ^a	<i>N</i> - <i>p</i> -nitrophenylglycosylamine, m.p. 130° C.

^a Determined separately by the carbazole method (4).

glucosyluronic acid)-D-mannose, isolated from *Virgilia oroboides* gum. Hydrolysis of the latter gave rise to 2,3,4-tri-*O*-methyl-D-glucuronic acid, identified as the crystalline amide, methylglycoside (III) (18), and 3,4,6-tri-*O*-methyl-D-mannose (IV) which crystallized directly. There seems little doubt therefore that aldobiouronic acid produced from the water plant polysaccharide by acid hydrolysis has the structure (I) and that it must be designated as 2-O-(β -D-glucosyluronic acid)-D-mannose, a compound previously obtained from damson (8) and cherry (12) gum.

Experimental

All evaporations were carried out under reduced pressure at 40° to 50° C. Paper chromatography was performed by the descending method on filter paper (Whatman No. 1) using the following solvent systems: (A) 1-butanol: ethanol:water (4:1:5) (17); (B) ethyl

acetate:pyridine:water (2:1:2) (17); (C) 1-butanol:acetic acid:water (2:1:1) (10); (D) ethyl acetate:acetic acid: formic acid:water (18:3:1:4) (13). Components were detected with aniline oxalate (9) or *p*-anisidine trichloroacetate (10). Chromatographic mobilities are quoted relative to that of L-rhamnose (R_{Rk}).

Isolation of the Water Plant Polysaccharide. Commercial young leaves of *Brasenia schreberi* J. F. Gmel. (Junsai), collected and bottled in the Hyogo Prefecture, Japan, were used in these studies.

METHOD A. The fresh leaves (396 grams, water content of 98%) were washed thoroughly with water and immersed in 0.2*N* sodium hydroxide (700 ml.). After 5 hours, when most mucilage on the leaves had dissolved, the solution was filtered through cloth and the residue washed with water. The filtrate was centrifuged to remove plant debris not removed during pre-

vious filtration, neutralized with acetic acid, and the solution (1200 ml.) poured with stirring into ethanol (4000 ml.). The flocculent precipitate was recovered (centrifuge) and redissolved in water (500 ml.). After adding 5*N* hydrochloric acid to a pH of 2, the solution was dialyzed against distilled water for 3 days. The solution (700 ml.) was centrifuged and poured with stirring into ethanol (2500 ml.). The polysaccharide thus obtained was centrifuged, washed successively with ethanol (thrice), diethyl ether (twice), and petroleum ether (once) and dried in vacuo; yield, 1.95 grams or 0.49% of the wet leaves.

METHOD B. The wet leaves (200 grams) were heated for 3 hours at 80° C. with water (1000 ml.). The hot extract was filtered through cloth, centrifuged, and treated with Fehling solution (600 ml.). The precipitated copper complex was recovered (centrifuge) and washed twice with Fehling solution. The complex was shaken with 90% aqueous ethanol containing 4% hydrochloric acid until no more color remained in the precipitate. The polysaccharide so obtained was reprecipitated from aqueous solution with ethanol and dried as above; yield, 0.85 gram or 0.43% of the wet weight of leaves.

METHOD C. To secure enough polysaccharide for the studies reported herein, dried leaves (26 grams, moisture content of 2.8%) were suspended in water (2000 ml.) until the mucilage had swollen and heated at pH 7.0 and 80° C. for 5 hours. The extract was filtered through cloth and centrifuged. The polysaccharide was recovered from the extract by pouring into ethanol (four volumes), redissolved in water, dialyzed as in method A and recovered by alcoholic precipitation as before; yield, 7.5 grams.

The mucilaginous residue remaining on the cloth was further extracted with 0.2*N* NaOH at room temperature. Isolation of the product via copper complex formation as in method B gave 3.5 grams of polysaccharide, making a total of 11 grams or 42% of the original weight of dried leaves.

Purification of Polysaccharide by Acetylation. Since the polysaccharide preparations obtained above as in methods A, B, and C did not give clear solutions in water, the polysaccharide (2.5 grams) was dispersed in formamide (50 ml.) by shaking at room temperature and was treated with pyridine (80 ml.) followed by acetic anhydride (160 ml.). After shaking for 50 hours at room temperature, the reaction mixture was poured with stirring into ice water (3000 ml.). The white, flocculent precipitate was collected by filtration, washed with water, and dried. The acetate was dissolved in acetone (25 ml.) and the solution poured with

stirring into petroleum ether (b.p. 40° to 60° C.). The polysaccharide acetate was centrifuged, washed with petroleum ether, and dried in vacuo; yield, 1.2 grams, $[\alpha]_D^{25} - 6^\circ$ in chloroform (concentration, 4.2 grams per 100 ml. of solution), -6° in acetone (concentration, 2.7 grams per 100 ml. of solution). (Found: COCH₃, 43.2%.)

The polysaccharide acetate (281 mg.) was dissolved in acetone (15 ml.) and deacetylated by heating for 3 hours under reflux with 20% aqueous sodium hydroxide (10 ml.). The solution was cooled, neutralized with acetic acid, and poured with stirring into ethanol. The precipitated polysaccharide was collected (centrifuge) and washed successively with ethanol and diethyl ether, and dried in vacuo; yield, 110 mg., $[\alpha]_D^{25} + 9^\circ$ in water (concentration, 1.7). The polysaccharide did not reduce boiling Fehling solution nor did it give a color with iodine solution, but it gave a strong positive carbazole test for uronic acid. Electrophoresis on glass-filter paper (74) indicated that the polysaccharide was homogeneous. Analysis. Found: uronic acid, 17.5% by the carbazole method (4).

A solution of polysaccharide (46.5 mg.) in water (10 ml.) was passed through a column (10 × 1 cm.) of Amberlite IR 120 (H form). The eluate was adjusted to 50 ml. and 20-ml. aliquots containing 18.6 mg. of polysaccharide were titrated with 0.01*N* NaOH. Found: equivalent weight, 1040, corresponding to 17.5% of glucuronic acid.

Hydrolysis of Polysaccharide and Identification of Components. A. The polysaccharide (100 mg.) was dissolved in 1*N* sulfuric acid (5 ml.) and heated (sealed tube) for 8 hours at 100° C. Neutralization (BaCO₃), filtration, and passage through Amberlite IR 120 (H form) exchange resin gave an acidic eluate which after concentration was found by paper chromatography (using solvent A) to contain rhamnose (*R_{Rh}*, 1.0), fucose (*R_{Rh}*, 0.82), xylose (*R_{Rh}*, 0.67), galactose (*R_{Rh}*, 0.39), and arabinose and/or mannose (*R_{Rh}*, 0.59). Using solvent D and paper electrophoresis it was found that both arabinose and mannose were present. Using solvent C, glucuronolactone (*R_{Rh}*, 0.95), glucuronic acid (*R_{Rh}*, 0.58), and an aldobiouronic acid, 2-*O*-(β-D-glucosyluronic acid)-D-mannose (*R_{Rh}*, 0.40), were detected in addition to the above neutral sugars.

B. A solution of polysaccharide (1.5 grams) in 1*N* sulfuric acid (60 ml.) was heated for 8 hours at 100° C. The solution was neutralized (BaCO₃), passed through a column (2 × 20 cm.) of Amberlite IR 120 (H form) cation exchange resin and evaporated to dryness; yield of sirup, 1.25 grams. The sirupy hydrolyzate was dissolved

in water (15 ml.) and the solution passed through a column (2 × 20 cm.) of Duolite A₄ (OH form) anion exchange resin, the column being washed with water until no more reducing sugar could be detected (Molisch test) in the eluate. Evaporation of the eluate gave a sirupy mixture of neutral sugars; yield, 920 mg.

The Duolite A₄ anion resin column was then eluted with 10% formic acid (70 ml.) and the eluate evaporated. Since the sirupy product was found to contain incompletely hydrolyzed material, the product was rehydrolyzed for 3 hours at 100° C. with 1*N* sulfuric acid. The solution was neutralized (BaCO₃) and the acidic components (203 mg.) and neutral sugars (94 mg.) were isolated.

Isolation and Characterization of Neutral Sugars. A portion (520 mg. out of a total of 1014 mg.) was dissolved in a minimum volume of methanol: water (1:1) and the solution put on the cellulose-hydrocellulose column (6), after which separation was effected with solvent A. Fractions (7 ml.) of the eluate were collected every 30 minutes in the usual way. Results of column chromatography are recorded (Table I).

Examination of Fraction 1 and Identification of L-Rhamnose. This fraction crystallized spontaneously, and recrystallization from aqueous ethanol gave L-rhamnose monohydrate m.p. and mixed m.p. 91° to 93° C. $[\alpha]_D^{25} + 8^\circ$, equilibrium value in water (concentration, 2). Traces of L-fucose were detected in mother liquor by paper chromatography. Treatment with *p*-nitrophenylhydrazine in the usual way gave the corresponding L-rhamnose *p*-nitrophenylhydrazone, m.p. and mixed m.p. 191° C. (Table II).

Examination of Fraction 3 and Identification of L-Fucose. This fraction crystallized spontaneously and after recrystallization from aqueous ethanol afforded L-fucose, m.p. and mixed m.p. 145° C., $[\alpha]_D^{25} - 74.5^\circ$ equilibrium value in water (concentration, 1.3 grams per 100 ml. of solution). Treatment with diphenylhydrazine in ethanol in the usual manner gave L-fucose diphenylhydrazone, m.p. and mixed m.p. 202° C., after recrystallization from aqueous ethanol (Table II).

Examination of Fraction 5 and Identification of D-Xylose. This fraction, which crystallized spontaneously, was recrystallized from methanol giving D-xylose, m.p. and mixed m.p. 145° C., $[\alpha]_D^{25} + 19^\circ$ equilibrium value in water (concentration, 1.2 grams per 100 ml. of solution). Treatment with methanolic hydrogen chloride containing benzaldehyde gave di-*O*-benzylidene-D-xylose dimethylacetal (2), m.p. and mixed m.p. 211° C. (Table II).

Examination of Fraction 6 and Identification of L-Arabinose and D-Mannose. Since this fraction was

shown to consist of two components corresponding to L-arabinose and D-mannose by paper electrophoresis (using a borate buffer) and to contain also a small proportion of D-xylose (revealed by paper chromatography using solvent A), a portion (17 mg.) of the sirupy material was applied to a paper chromatogram to remove xylose, the faster moving component, using solvent A. The zone of the slower moving component consisting of a mixture of L-arabinose and D-mannose was extracted with water and the solution evaporated to a sirup. The latter containing the two components, L-arabinose and D-mannose, was separated by paper (Whatman No. 1) electrophoresis (600 volts, 3.5 hours) using 0.1M borate buffer (3). The separate components were freed from borate by treatment (3) with 1% methanolic hydrogen chloride followed by treatment with ion exchange resins. Evaporation of solutions in each case gave L-arabinose [4.5 mg. $[\alpha]_D^{25} + 80^\circ$ in water (concentration, 0.25 gram per 100 ml. of solution)] and D-mannose (3.7 mg. $[\alpha]_D^{25} + 12^\circ$ in water (concentration, 0.75 gram per 100 ml. of solution)). In view of the relatively small amounts available, the former was transformed into L-arabinose diphenylhydrazone, m.p. and mixed m.p. 204°C ., after recrystallization from ethanol and the latter into D-mannose phenylhydrazone, m.p. and mixed m.p. 195°C ., without recrystallization. Polarimetric observation on the two sugars after separation revealed their series configuration (Table II).

Examination of Fraction 8 and Identification of D-Galactose. This fraction which also crystallized spontaneously was recrystallized from aqueous methanol giving D-galactose, m.p. and mixed m.p. 165°C ., $[\alpha]_D^{25} + 80^\circ$ equilibrium value in water (concentration, 1.3 grams per 100 ml. of solution). Treatment with *p*-nitroaniline in methanol containing hydrochloric acid (0.07%) gave rise to *N-p*-nitrophenyl- β -D-galactosylamine, m.p. and mixed m.p. 219°C ., after recrystallization from methanol (27) (Table II).

Quantitative Determination of Component Sugars of Polysaccharide. The polysaccharide (45 mg.) was hydrolyzed by heating (sealed tube) for 10 hours at 100°C . with 2*N* sulfuric acid. The solution was neutralized (BaCO_3) and separated into neutral (31.1 mg., 75.2%) and acidic components (10.2 mg., 24.8%) by means of anion and cation exchange resins as described above.

The mixture of neutral sugars was resolved into its components by paper (Whatman No. 1) chromatography using solvent A for 55 hours. Components L-fucose, D-galactose, L-rhamnose, and D-xylose were extracted with water from appropriate sections of the chromatograms and determined by the phenol-

sulfuric acid method (5); hexoses being determined at 490 $m\mu$ and the other sugars at 480 $m\mu$. The fifth component consisting of a mixture of L-arabinose and D-mannose was separated into its two components by paper electrophoresis using a borate buffer (3) and determined by the phenol-sulfuric acid method (5). Results for the polysaccharide before and after purification by copper complex formation are given in Table III.

Calculation of Per Cent Composition of All Polysaccharide Components. Titration of the polysaccharide in the acid form (equivalent weight 1039) and determination of uronic acid by the carbazole method (4) showed that the polysaccharide contained 17.3% glucuronic acid. Since the acid fraction of the polysaccharide hydrolyzate, amounting to 24.8%, consisted only of glucuronic acid and mannose (ascertained by further acid hydrolysis, followed by paper chromatography), the mannose content of the acid fraction is 7.5%—i.e., $24.8 - 17.3$. Consequently, the over-all mannose content of polysaccharide is 13.4% ($5.9 + 7.5$). The percentage composition of the polysaccharide (purified via copper complex) is therefore as follows: L-arabinose (5.9%), L-fucose (10.9%), D-galactose (34.1%), D-mannose (13.4%), L-rhamnose (11.4%), D-xylose (7.0%), and D-glucuronic acid (17.3%).

Examination of the Acid Fraction of the Polysaccharide Hydrolyzate. The acid fraction eluted from the anion resin was separated by paper (Whatman No. 3) chromatography using solvent C. In this manner, four fractions were isolated: fraction 1, 9 mg., R_{Rf} 0.95; fraction 2, 14.7 mg., R_{Rf} 0.58; fraction 3, 85.5 mg., R_{Rf} 0.40 $[\alpha]_D^{25} - 33^\circ$ in water (concentration, 1.4 grams per 100 ml. of solution); and fraction 4, 27.5 mg. R_{Rf} 0.13, $[\alpha]_D^{25} - 17^\circ$ in water (concentration, 2.7 grams per 100 ml. of solution).

Identification of D-Glucuronic Acid. Since fractions 1 and 2 correspond to D-glucurono-(6 \rightarrow 3)-lactone and D-glucuronic acid, respectively, they were combined and heated at 80°C . for 1 hour in the presence of acetic acid to effect complete lactonization. After removal of acetic acid in vacuo, the residue was treated with *p*-nitroaniline in methanolic hydrogen chloride (7) for 15 minutes. This provided the *p*-nitroaniline derivative of D-glucuronic acid, m.p. and mixed m.p. 130°C .

Identification of 2-O-(β -D-Glucopyranosyluronic Acid)-D-Mannose. Fraction 3, a colorless, glassy solid, showed the same chromatographic mobility using solvent C as aldobiouronic acid isolated from *Virgilia oroboides* polysaccharide (20). When hydrolyzed by heating (sealed tube) in water with a cation exchange resin (IR 120, H form) for 30 hours on a boiling water bath, it gave four components cor-

Table III. Composition^a of Neutral Fraction of Polysaccharide Hydrolyzate

Component Sugars	Direct Hydrolysis	Hydrolysis after Purification	
		via Copper Complex, %	Polysaccharide, ^b %
L-Arabinose	7.0	7.9	5.9
L-Fucose	17.8	14.6	10.9
D-Galactose	40.8	45.3	34.1
D-Mannose	7.0	7.9	5.9
L-Rhamnose	18.9	15.2	11.4
D-Xylose	8.1	9.3	7.0

^a Determined by paper chromatography (5).

^b Since ion exchange separation showed that the polysaccharide hydrolyzate consisted of 75.2% neutral fraction and 24.8% acid fraction, values in this column were obtained by multiplying those in column 2 by 75.2/100.

responding chromatographically (solvent C) to D-glucurono-(6 \rightarrow 3)-lactone, D-glucuronic acid, D-mannose, and unchanged aldobiouronic acid.

Methylation of the Aldobiouronic Acid. Fraction 3 (65 mg.) was dissolved in water (1 ml.) and methylated with methyl sulfate (5 ml.) and 30% (w./w.) sodium hydroxide (15 ml.) in the usual way for 3 hours in an ice bath and for 3 hours at room temperature in a nitrogen atmosphere. The reaction mixture was then heated for 20 minutes at 40°C . After cooling, the reaction mixture was neutralized with dilute sulfuric acid and made slightly alkaline with ammonium hydroxide. The solution was treated with methanol to a composition of 60% by volume after which salts were filtered off and washed with methanol. Solvent was removed and the residue retreated with methyl sulfate and 30% sodium hydroxide as before at room temperature for 5 hours and at 55°C . for 2 hours. The reaction mixture was cooled, acidified with dilute sulfuric acid, and extracted thrice with chloroform (100 ml.). The combined extracts were dried (MgSO_4) and evaporated to give the crude methylated aldobiouronic acid (43 mg.). Further treatment of this product with silver oxide (1 gram) and methyl iodide (8 ml.) under reflux for 8 hours gave the fully methylated product which was freed from solvent and purified by extraction with diethyl ether. The product (36 mg.) so obtained crystallized spontaneously m.p. 142°C . and $[\alpha]_D^{25} - 19^\circ$ in chloroform (concentration, 1 gram per 100 ml. of solution) after recrystallization from diethyl ether. This product gave no depression of the melting point when mixed with authentic methyl 2-O-[methyl(2,3,4-tri-O-methyl-D-glucosyluronate)]3,4,6-tri-O-methyl-D-mannoside

(78). Analysis. Calculated for $C_{20}H_{36}O_{12}$: C, 51.27; H, 7.76. Found: C, 51.86; H, 7.77.

Hydrolysis of Methylated Aldobiouronic Acid and Identification of 2,3,4-Tri-*O*-methyl-*D*-glucuronic Acid and 3,4,6-Tri-*O*-methyl-*D*-mannose.

Fully methylated aldobiouronic acid was heated (sealed tube) with 2% methanolic hydrogen chloride (2 ml.) for 9 hours at 110° C. Neutralization, followed by filtration and evaporation, gave a sirupy product which was treated with methanolic ammonia in the usual way. Removal of solvent gave a crystalline residue, m.p. 223° C. after recrystallization from an ethanol-ether mixture. This appeared to be the amide of the unhydrolyzed, methylated aldobiouronic acid, since the melting point is higher than either that of the α - or β -anomers of methyl 2,3,4-tri-*O*-methyl-*D*-glucosiduronamide (19). The product (3 mg.) was therefore hydrolyzed by heating (sealed tube) for 18 hours at 100° C. with 2*N* sulfuric acid (0.7 ml.). After neutralization ($BaCO_3$), the filtered solution was passed first through a cation (Amberlite IR 120, H form) resin and then through an anion (Duolite A₄, OH form) resin. The neutral eluate gave a sirup upon evaporation and after purification by extraction with ether, crystalline 3,4,6-tri-*O*-methyl-*D*-mannose (1.5 mg.) was obtained [$R_{tetra-O-methyl-D-glucose}$ 0.86 (solvent A)], m.p. and mixed m.p. 104° C., $[\alpha]_D^{25} + 37^\circ$ in methanol (concentration, 0.4 gram per 100 ml. of

solution). The literature (7) gives m.p. 102° C., $[\alpha]_D^{25} + 36$ (methanol). Elution of the anion resin with 0.1*N* sodium hydroxide followed by passage of the eluate through the cation resin gave a solution containing 2,3,4-tri-*O*-methyl-*D*-glucuronic acid. Removal of solvent followed by treatment first with methanolic hydrogen chloride and then with methanolic ammonia (78) yielded crystalline methyl 2,3,4-tri-*O*-methyl-*D*-glucosiduronamide, m.p. and mixed m.p. 183° C.

Examination of Fraction 4. Hydrolysis of this fraction in water with the cation exchange resin for 20 hours at 100° C. (as described for fraction 3) gave components, the chromatographic mobilities (solvent C) of which corresponded to *D*-glucurono-(6 \rightarrow 3)-lactone, *D*-glucuronic acid, *D*-mannose, 2-*O*-(β -*D*-glucopyranosyluronic acid)-*D*-mannose, and the original component. It appears therefore that the fraction is a trisaccharide composed of one mole of *D*-glucuronic acid and two moles of *D*-mannose. The mode of attachment between the two *D*-mannose units remains to be determined.

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FEED ADDITIVES

Nitrofurazone Determination in Poultry Feed by Phenylhydrazine or Alkali Reaction

NITROFURAZONE, 5-nitro-2-furaldehyde semicarbazone (NFZ), is added to poultry feeds in small concentrations as a prophylactic against coccidiosis. For its determination the colorimetric method of Buzard, Ells, and Paul (3), which involves conversion to 5-nitro-2-furaldehyde phenylhydrazone, is commonly employed. The main difficulty is in the purification of feed extracts. Other feed compounds also give color reactions with the phenylhydrazine reagent, resulting in more or less extinction at the 440- $m\mu$ wave length, which is characteristic of the 5-nitro-2-furaldehyde phenylhydrazone. Nitrofurazone is insoluble in heptane- CCl_4 and hexane. Therefore the authors propose extraction using these solvents. Beckman (7) has described an improved

method which allows at the same time separation between NFZ and furazolidone. His procedure for determining NFZ by chromatography with an Al_2O_3 column was modified by Brüggemann and Bronsch (2); these authors determined NFZ either by phenylhydrazine or preferably by reaction with alkali. Our experiments are concerned with critical comparison of both methods applied to commercial feed mixtures and to laboratory preparations as well. They show that column chromatography and reaction of nitrofurazone with alkali are superior to the phenylhydrazine method in selectivity and accuracy.

Methods

Method 1. Modification of the

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Buzard, Ells, and Paul Method for Nitrofurazone Determination (3).

A. Weigh 5 grams of feed and fill into a coarse fritted-disk funnel. Apply suction and extract successively with the following solvents previously warmed to 50° to 60° C.:

1. Heptane, 30 ml.
2. Heptane- CCl_4 (1:1), 15 ml.
3. CCl_4 , 25 ml.
4. Heptane- CCl_4 (1:1), 15 ml.
5. Hexane, 15 ml.

Dry by use of full vacuum and transfer the sample to a 100-ml. Erlenmeyer flask. Add exactly 50 ml. of dimethylformamide (DMF). Shake for 30 minutes in a water bath heated to 70° C. protected from light and filter through a paper filter (Schleicher & Schüll No. 588). Cool and add 25 ml. of water to 25 ml. of the DMF extract. Return to