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Estimations of Uronic Acids as Quantitative Measures of Extracellular and Cell Wall Polysaccharide Polymers from Environmental Samples

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The extracellular polysaccharide polymers can bind microbes to surfaces and can cause physical modification of the microenvironment. Since uronic acids appear to be the components of these extracellular films that are most concentrated in a location outside the cell membrane, a quantitative assay for uronic acids was developed. Polymers containing uronic acids are resistant to quantitative hydrolysis, and the uronic acids, once released, form lactones irreproducibly and are difficult to separate from the neutral sugars. These problems were obviated by the methylation of the uronic acids and their subsequent reduction with sodium borodeuteride to the corresponding alcohol while they were in the polymer and could not form lactones. This caused the polymers to lose the ability to adhere to their substrates, so they could be quantitatively recovered. The hydrolysis of the dideuterated sugars was reproducible and could be performed under conditions that were mild enough that other cellular and extracellular polymers were not affected. The resulting neutral sugars were readily derivatized and then were separated and assayed by glass capillary gas-liquid chromatography. The dideuterated portion of each pentose, hexose, or heptose, identified by combined capillary gas-liquid chromatography and mass spectrometry, accurately provided the proportion of each uronic acid in each carbohydrate of the polymer. Examples of the applications of this methodology include the composition of extracellular polymers in marine bacteria, invertebrate feeding tubes and fecal structures, and the microfouling films formed on titanium and aluminum surfaces exposed to seawater.

Extracellular polymers are becoming increasingly important in microbial ecology. These polymers are important in conferring virulence on pathogenic bacteria by inhibiting phagocytosis and the action of serum antimicrobial factors. They protect organisms from predation and impede desiccation in soil microorganisms. Exopolysaccharides regulate the ionic traffic at the cell surface, particularly the traffic of magnesium ions. They concentrate nutrients such as amino acids, phosphates, and silicon and protect microbes from toxic heavy metals or antibacterial agents often used in antifouling treatments. In nitrogen-fixing organisms, these polymers act as carbon storage materials (15). Surfaces are particularly important in microbial ecology (29), and all known marine periphytic microbes attach irreversibly to surfaces containing acid polysaccharides (10–14, 17). Indirect effects of these polymers include the stabilization of sediments and soils by the presence of invertebrate feeding tubes and microbial extracellular polysaccharide polymers (19, 20, 36, 49). Recent evidence has indicated that microbially produced extracellular polymers may be particularly important in heat transfer resistance by the microfouling community that is formed when metal surfaces are exposed to rapidly flowing seawater (33, 34).

Since these extracellular polymers are of great importance, the means to quantitatively measure their formation and catabolism needed to be developed. For this type of analysis, a component found outside the cytoplasmic membrane but not found in intercellular polymers, such as the hydroxyproline in collagen or the desmosine in elastin, would be ideal. From an examination of compendia of extracellular and cellular polymer compositions (2, 18, 30, 35, 42, 43), it is clear that uronic acids are almost unique to the polymers found outside the cytoplasmic membrane of the cells. Uronic acids are also found in the polysaccharide polymers of higher plant cell walls, in gram-positive microbes grown with phosphate limitation (16), and in some gram-negative microbial lipopolysaccharides. Aminouronic acids found in the walls of micrococci (45) cannot be assayed by the methods described in this study. However, the electron micro-

graphic examination of environmental samples shows extensive extracellular polymers stained by ruthenium red (polymers containing uronic acid) surrounding the bacteria (13, 14).

Uronic acids are often estimated by their acid-catalyzed decarboxylation under controlled conditions (24). Although this method is very sensitive, it is not specific and does not differentiate between the various uronic acids. The carbazole reaction has been used to measure the uronic acids in hydrolysates (4). This is a relatively insensitive colorimetric reaction, and the extinction coefficients are different for different uronic acids and depend on other components in the polymers (38). This method, like the decarboxylation method, does not differentiate between the different uronic acids.

Consequently, a method that allowed quantitative recovery of each uronic acid was sought. The known microbial exopolysaccharides contain D-glucuronic acid, D-galacturonic acid, D-mannuronic acid, and L-gulonic acid (15). Possibly, other components, such as those isolated from marine sediments by Mopper and Larsson (31), could be measured in such an analysis. If each component were isolated in the analysis, it would be possible to use isotopes to monitor rates of formation and catabolism. Polymers containing uronic acids resist acid hydrolysis because the carboxylic acid moiety stabilizes the glycosidic linkage (25). This stabilization causes very low yields of galacturonic acid when polygalacturonic acid polymers are acid hydrolyzed. Once hydrolyzed, the uronic acids become extensively lactonized. The extent of the lactonization in our experiments and as reported previously (5) is irreproducible. Since quantitative analysis requires the separation of the uronic acids from the neutral aldoses (6), anion-exchange columns are usually used. Uronic acids react with and thus are not quantitatively recoverable from the weak anion-exchange columns necessary to separate the acids from the neutral sugars (32). The lactones will not sorb to strong anion-exchange columns in the acetate form and can cause degradation of the aldoses and uronic acids in the hydroxide form (6). The conversion of the lactones to the free acids or to salts by using weak alkali causes the formation of colored degradation products unless very carefully titrated. If the anomeric carbons of the aldoses and uronic acids are reduced, the resulting alditols and aldonic acids can be separated on anion-exchange columns (23).

The problems associated with irreproducible lactonization could be eliminated if it were possible to reduce the uronic acids to the corresponding alcohols while they were still in the polymers. Consequently, a method for derivatization of the component uronic acids in the extracellular polymers, as employed by Smith (41), was adapted for the analysis of environmental samples. The method involves the reduction of the uronic acids to primary alcohols while they are still a part of the polysaccharide polymers. Uronic acids as monomers are very difficult to quantitatively reduce to the corresponding neutral carbohydrate, even with repeated treatments with $NaBH_4$ (39). However, in the polymer, the uronic acids can be quantitatively reduced with one treatment with NaB^2H_4, provided the methyl ester is formed first (41).

After much frustration with methods that were designed to reproducibly hydrolyze, separate, and assay uronic acids, a method in which the uronic acids are reduced to dideuterated neutral sugars while still in the polymer and then are assayed quantitatively was adapted.

MATERIALS AND METHODS

Materials. Glass-distilled solvents (Burdick and Jackson, Muskegon, Mich.), freshly distilled chloroform, and derivatizing reagents (Pierce Chemical Co., Rockford, Ill.; Aldrich Chemical Co., Milwaukee, Wis.; and PCR Research Chemicals, Inc., Gainesville, Fla.) were used. Standard sugars and uronic acid-containing gums were purchased from Sigma Chemical Co., St. Louis, Mo. Sodium borodeuteride (98 atom% of deuterium) was acquired from Merck and Co., Inc./Isotopes, St. Louis, Mo.

Bacterial cultures. *Pseudomonas marina* (ATCC 27129) and *Pseudomonas atlantica* strain T_6C (the gift of W. A. Corpe, Columbia University) were maintained on Difco marine agar (3). Bacteria that were forming mucoid colonies were isolated from marine sediments near the Florida State University marine laboratory on sterilized glass slides that had been left in the sediment for 3 days (11). These organisms were maintained on agar containing 1% (wt/vol) glucose and 0.5% (wt/vol) peptone in sea salts (Instant Ocean, Aquarium Systems Inc., East Lake, Ohio) with a salinity of 25 μg/liter or were frozen in medium containing 15% glycerol at −70°C. The extracellular polymers for analysis were recovered by the modified Bligh and Dyer chloroform-methanol-water extraction (47) after precipitation.

Collection of invertebrates. Maldanid polychaete worms of *Clymenella* spp. were collected by the sieving of subtidal mud flat sediments. The worm tubes were washed extensively, frozen, and lyophilized before the analysis for uronic acids.

Fresh fecal mounds formed by the enteropneust *Ptychodera bahamensis* were identified on the sediment surface and carefully collected by divers with SCUBA equipment as previously described (44). Control sediments were collected at least 25 cm from the fecal mounds (5 to 12 cm in diameter). The cores were frozen and returned to the laboratory, the lipids were extracted, the residue was lyophilized, and the uronic acids were determined. The site of these measurements in the Florida Panhandle was 29° 54.0′ N, 84° 37.8′ W.

Microfouling samples. Samples of titanium (commercial grade) and 5052 aluminum pipes were exposed to

seawater that was flowing at 1.85 m/s at the Naval Coastal Systems Center, Panama City, Fla. The pipe samples were removed from the system, drained, frozen, and returned to the laboratory; the lipids were extracted; and the pipes were allowed to dry (33). The extracellular polymer was recovered by the exposure of the insides of the pipes to a suspension of 25% (wt/vol) acid-washed, 00-mesh, silicate rock chips in distilled water containing 10% chloroform in a specially constructed shaker that subjected the inside surfaces to the abrading action of the chips. The resulting material was then lyophilized.

Analysis of the uronic acid polymers. Lyophilized polymers, sediment samples, or the material from the pipes was suspended in 10 volumes of "magic" methanol (methanol-chloroform-concentrated HCl; 10:1:1, vol/vol) and was stirred for 24 h at 25°C. The suspension was neutralized with 1 N $NaHCO_3$ and dialyzed overnight against distilled water. The contents of the dialysis sack were frozen with dry ice-acetone and lyophilized. The residue was reduced in aqueous 2.44 mM NaB^2H_4 (pH 7.0) and stirred for 24 h at 4°C. If the samples contain metallic chlorides, such as deep-sea sediments, colored complexes will result, and extra borodeuteride should be added until the effervescence stops. These metallic chlorides catalyze the decomposition of borodeuteride (37). Once the effervescence stops, the solution is cooled and the uronic acid esters reduced. During the reaction, the pH increases as metaborate ions are formed by reaction with water. After 24 h, the solution is warmed to room temperature and residual borodeuteride destroyed by the addition of 10% acetic acid until effervescence ceases. The solution was dialyzed overnight and lyophilized. The polymer was hydrolyzed for 2 h at reflux temperature in 2 N HCl. The monosaccharide alditals were reduced to alditols in 130 mM $NaBH_4$ in 0.054 M NaOH. The reaction was performed at 25°C for 1 h. The solution was acidified with 10% (vol/vol) acetic acid, and the sample was added to a 2-g column of Dowex 50 (H^+ form) in a champagne column (Supelco, Inc., Bellefonte, Pa.). Quantitative elution of the neutral carbohydrates was achieved with five 1-ml washes of water. The injection standard, 100 μl of 20 mM 1,9-nonanediol in methanol, was added, and the remaining borate was removed by the addition of methanol and three evaporations with a stream of nitrogen to remove the methyl borate azeotroph (54.6°C bp). An internal standard of 100 μl of a 20 mM solution of inositol was added. The sugars were then peracetylated in acetic anhydride-pyridine (1:1, vol/vol) and heated for 1 h at 55 to 60°C. The reaction was terminated by the addition of two volumes of chloroform–20% (wt/vol) tartaric acid and mixing with a Vortex mixer for 4 min. The tartaric acid was removed, and a second volume was added and after mixing was removed. The chloroform was evaporated in a stream of nitrogen, and the carbohydrates were dissolved in tetrahydrofuran.

Preparation of pentose and heptose standards. D-Ribonic acid-1,4-lactone and D-glucoheptonic acid-1,4-lactone (Sigma Chemical Co., St. Louis, Mo.) were hydrolyzed in magic methanol for 72 h at 60°C, which partially hydrolyzed the lactones and formed the methyl esters. The esters were reduced with sodium borodeuteride and peracetylated. These were compared with peracetylated D-ribitol and with the alcohol formed after reduction of the glucoheptose for differences in fragmentation patterns.

GLC. Gas-liquid chromatography (GLC) was performed with a Varian model 3700 gas chromatograph with flame ionization detectors, a model 8000 autosampler, and a CDS-111 data system. Samples (1 μl) were injected onto a 25-m glass capillary column of 0.25-mm inside diameter coated with Silar 10C, a polysiloxane with phenyl and cyanoalkyl functional groups (Applied Science Laboratories, State College, Pa.), with splitless injection and a 0.5-min venting time. The temperature was programmed to rise from 45 to 90°C at 5°/min and from 90 to 190°C at 2°/min, with an isothermal period to the end of the 180-min analysis. The helium carrier gas was operated at 12 lb/in^2 with a flow rate of 1.35 ml/min. The injection port was held at 225°C, and the detector was held at 250°C. The data were fed into a programmable Hewlett-Packard 3502 laboratory data system for analysis. A chromatographic tracing of the separation of authentic standards is illustrated in Fig. 1. The Trenzahl (Tz) separation number (22) measured between galactose and glucose was 6.7, with $Tz/m = 0.27$ (m = 25 m). The calculated number of theoretical plates with glucose was n = 3,066, or n/m = 132. The response factors averaged 0.86 for pentoses and 0.79 for hexoses, relative to 1,9-nonanediol (7).

Gas chromatography-mass spectrometry. Gas chromatography-mass spectrometry (GC/MS) was performed with the Hewlett-Packard 5995A GC/MS with the column described above operated from 45 to 190°C at 2°C/min and with other conditions the same as in the GLC analysis. The mass spectrometer was autotuned with decafluorotriphenylphosphine at a scan speed of 380 amu/s (four samples per 0.1 amu) with a delay time of 0.5 s between scans. The electron multiplier voltage was 1,800 V, and the sensitivity was 15 out of 10,000. Operation in the peakfinder mode provided a tracing of the total ion current at an m/e of 115; all fragmentation patterns greater than 100 linear counts at this m/e were saved. The accelerating voltage for the electron impact was 70 μV.

Lipid extraction and GLC of the fatty acids. The modified Bligh and Dyer single-phase chloroform-methanol-water extraction adapted for sediments (47) and for pipe sections (33) yielded the lipids. The lipids were hydrolyzed in mild acid, purified by thin-layer chromatography, analyzed by GLC, and identified by GC/MS (8, 46). Extractable lipid phosphate, a measure of membrane biomass, was measured (47).

RESULTS

Detection of uronic acids in polymers. If the uronic acids were quantitatively reduced to neutral sugars while still in the polymers, then the polymer could be readily hydrolyzed into aldoses that can be efficiently separated and analyzed by capillary GLC (Fig. 1). If NaB^2H_4 was employed in the reduction, then glass capillary GLC separation coupled with mass spectrometry of the isolated neutral carbohydrates of a second portion of the hydrolyzed polymer could be used after hydrolysis and derivatization to detect enrichment of the fragments with deuterium. The enrichment of fragments of each sugar that was a uronic acid in the polymer could be

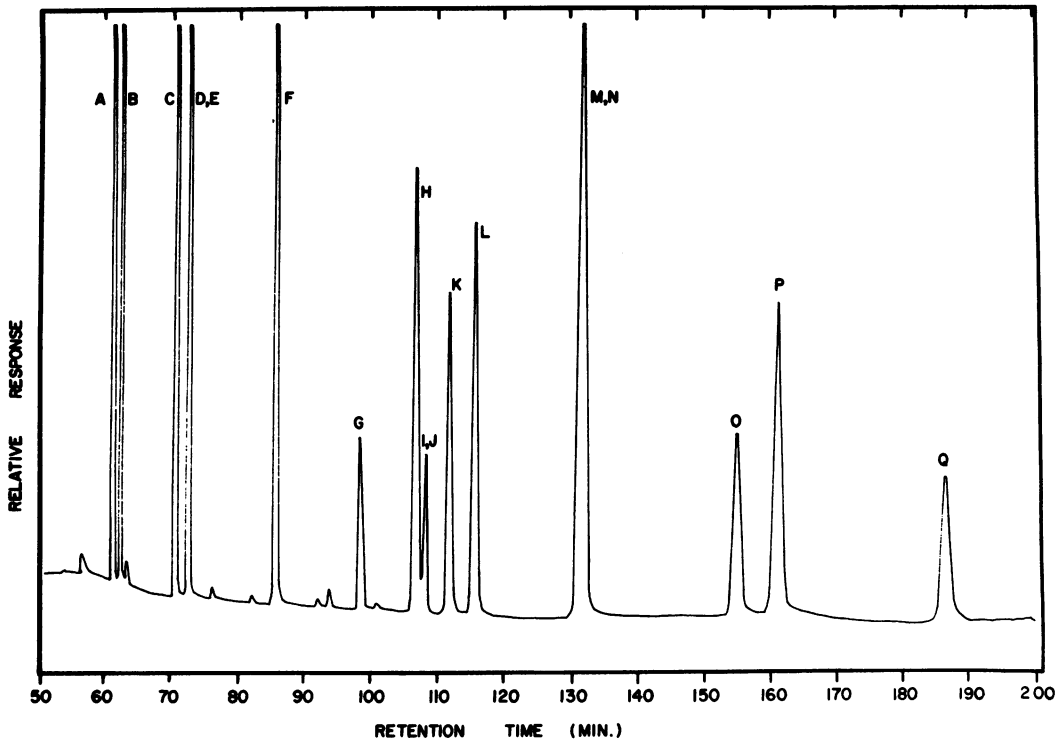

FIG. 1. Glass capillary gas chromatographic separation of peracetylated alditols on a 25-m, Silar 10C-coated column with sensitivity of 8×10^{-11} A/mV with a splitless injection. There was a 1-min venting at 45°C with an increase to 70°C at 5°/min, an increase to 190°C at 2°/min, and an isothermal run to the end of the program. The helium carrier gas flow was 1.35 ml/min at 12 lb/in^2. A, rhamnose; B, fucose; C, ribose; D, arabinose; E, lyxose; F, xylose; G, allose; H, mannose; I, altrose; J, talose; K, 3-*O*-methyl glucose; L, galactose; M, glucose; N, gulose; O, inositol; P, idose; and Q, glucohepulose.

used to calculate the proportion of each sugar that was a uronic acid in the polymer.

The electron impact fragmentation of the peracetylated hexose alditols into primary fragments by cleavage of carbon-carbon bonds yielded abundant ions at M−73 and at M−73−n72, where n = 1, 2, and 3 (Table 1). The uronic acids in the polymers that were reduced with the borodeuteride yielded fragments enriched in M+2−73 and M+2−73−n72 ions (Ta-

TABLE 1. Abundance of fragments from peracetylated alditols and dideuterated alditols reduced with NaB^2H_4

m/e[a]	Amt[b] in:					
	Galactitol	Dideuterated galactitol	Ribitol	Dideuterated ribitol	Glucoheptatol	Dideuterated glucoheptatol
73	14.1	13.7	12.3	13.6	22.8	29.4
75	0.6	3.1	<0.01	2.5	<0.01	4.7
145	48.4	47.8	63.3	82.1	38.6	53.1
147	0.9	26.1	1.1	1.2	<0.01	<0.01
217	35.5	36.3	17.0	35.2	12.4	16.1
219	0.7	30.9	<0.01	0.7	<0.01	<0.01
289	27.1	25.6				
291	0.8	20.5				
361	5.9	3.7				
363	0.3	3.0				

[a] Mass spectrum of eluent of a Silar 10C-coated glass capillary column scanned at 380 amu/s with a 0.5-s delay and a 50-to-500-amu scan, with the electron multiplier at 1,800 V.

[b] Abundance in percent compared with fragment at *m/e* = 115.

TABLE 2. Analysis of uronic acid in gum arabic recovered from sand and after double reduction with NaB^2H_4

Component	Amt (μmol/mg of gum) for following treatment of 50 mg of gum arabic:		
	A[a]	B[b]	C[c]
Rhamnose	0.33 (0.04)[d]	0.77 (0.03)[e]	0.30 (0.08)
Arabinose	0.07 (0.06)	0.09 (0.06)	0.05 (0.03)
Galactose	2.46 (0.14)	2.67 (0.16)	1.40 (0.23)[e]
Galacturonic acid	0.075 (0.05)	0.03 (0.03)	0.065 (0.084)
Glucose	0.034 (0.067)	0.047 (0.02)	0.098 (0.06)
Glucuronic acid	0.45 (0.15)	0.45 (0.04)	0.44 (0.14)

[a] None, $n = 4$.

[b] Mixed with 20 g of acid-washed, ignited sand; $n = 3$.

[c] Methylated and reduced twice with NaB^2H_4; $n = 5$.

[d] Numbers within parentheses are standard deviations.

[e] There was a significantly greater difference between the means than within the means by analysis of variance, $p < 0.01$.

ble 1). These fragmentation patterns, which were for the peracetylated alditols of authentic standards of galactose, glucose and mannose, were similar to those observed for the uronic acids of these sugars in various polymers (Tables 2 and 3). Similar values have been reported in the literature (27, 40).

To examine the fragmentation patterns of pentoses, authentic D-ribose was reduced, peracetylated, and compared with the gamma lactone of D-ribonic acid which had been hydrolyzed, methylated, reduced with NaB^2H_4, peracetylated, and analyzed. Unfortunately, the clean primary fragmentation patterns of the hexatols were not detected in the ribitol (Table 1). Possibly the ions with m/e's of 73 and 75 could be used. We examined the properties of hepturonic acids by the reduction of D-glucoheptonic acid with NaB^2H_4 and $NaBH_4$ (Table 1). Heptoses, exemplified by glucohepulose, did not give the primary fragmentation patterns of the hexatols. Apparently, the formation of 2,6-lactones disrupts the fragmentation, yielding only the pair with *m/e*'s of 73 and 75 as a possible marker.

Analysis of gum arabic. We used the ratio of the peak heights at *m/e*'s of 217/(217 + 219) to determine the proportions of each hexuronic acid in unknown polymers. The absolute amount of each uronic acid in a polymer was calculated from this ratio, which was determined with the mass spectrometer. The total yield of each neutral sugar (which also contained its dideuterated neutral sugar) was determined from GLC analyses like that illustrated in Fig. 1. The calculations included the response factors for each sugar, which had been determined previously for the flame ionization detector (7). The analyses for the uronic acids and neutral carbohydrates from authentic gum arabic agree with published data (Table 2) (1).

Completeness of the reduction. The completeness of reduction was examined by reduction of gum arabic, remethylation, and repetition of the reduction before hydrolysis and analysis. Two reductions of the methylated uronic acids in gum arabic did not significantly change the yield of glucuronic or galacturonic acid (Table 2, columns A and C).

Reproducibility. Polygalacturonic acid was treated to yield dideuterated galactose. The dideuterated galactose from the polymer was mixed in various proportions with authentic galactose, and the mixtures were reduced to the alditols, peracetylated, and analyzed by GC/MS. The percentages of the dideuterated galactose were plotted against the ratios of the peak heights at *m/e*'s of 217/(217 + 219), which yielded a straight line (correlation coefficient r = 0.99) (Fig. 2).

TABLE 3. Uronic acid content of extracellular polysaccharide polymers

Component	Amt (nmol/g [dry weight]) in:					Amt (nmol/cm²) in:	
	P. marina	*Pseudomonas* sp.	Maldanid worm tubes	*Ptychodera* fecal mounds	Control sand	Film from titanium	Film from aluminum
Rhamnose		1.95 (0.03)	35.0 (6.0)	58.0 (1.0)	48.0 (0.9)	0.66	0.114
Fucose			270 (4.0)	39.0 (1.0)	51.0 (3.0)	0.35	0.03
Ribose	6.6 (3.6)[a]			10.0 (1.0)	32.0 (2.0)	0.07	
Arabinose	1.80 (0.01)	3.57 (2.27)	8.0 (2.0)	24.0 (2.0)	39.0 (3.0)	0.59	0.065
Xylose	21.0 (10.8)	7.78 (1.62)	65.0 (10.0)	57.0 (3.0)	100 (6.0)	0.57	0.082
Mannose	6.25 (0.6)	1480 (208)	49.0 (1.0)	59.0 (3.0)	57.0 (6.0)	1.48	0.161
Mannuronic acid	0.05 (0.00)	4.6 (0.65)	3.0 (0.1)	2.0 (0.1)	1.3 (0.04)	0.31	
Galactose	10.8 (6.7)	64.2 (8.42)	116.0 (12.0)	110 (14)	159.0 (12.0)	1.76	0.166
Galacturonic acid	3.53 (2.3)	168.0 (16.2)	13.0 (1.0)	16.0 (6.0)	4.0 (0.9)	0.50	0.197
Glucose	58.2 (27.6)	121.2 (11.7)	387.0 (75.0)	272.0 (9.0)	671.0 (43.0)	10.4	0.66
Glucuronic acid		27.8 (4.5)	27.0 (5.0)	18.0 (5.0)	13.0 (1.0)	3.46	0.19

[a] Numbers within parentheses are standard deviations.

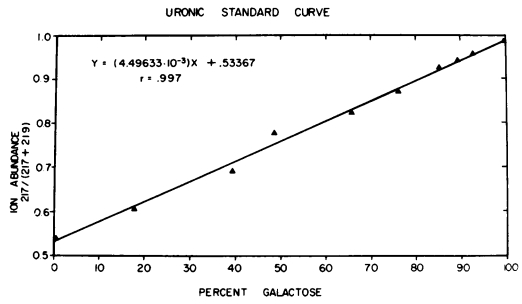

FIG. 2. Relationship between the percentages of the peracetylated alditols of dideuterated galactoses and galactose and the ratios of fragments with *m/e*'s of 217/(217 + 219), determined by GC/MS.

Sensitivity. The limit of detectability of the enrichment in *m/e*'s of 217/(217 + 219) under the conditions used allowed the detection of 237 pmol of dideuterated galactose. Presumably this sensitivity could be increased by modifications of the inlet system of the mass spectrometer.

Hydrolysis of the polymer. After reduction of the uronic acid residues of the polymer to dideuterated carbohydrates, the polymer was completely hydrolyzed in 2 N HCl at 100°C for 2 h. Continued hydrolysis caused decreased yields of neutral carbohydrates, particularly of galactose (Fig. 3). The yield of galacturonic acid from polygalacturonic acid that was refluxed with 6 N HCl for 36 h was 13%.

Recovery from sediment. Gum arabic dissolved in distilled water was added to 20 g of acid-washed, ignited sand, stirred for 1 h, and lyophilized. The sand was then analyzed, and recoveries of glucose, glucuronic acid, and galactose that were linear with additions of 10 to 100 mg of polymer were observed (Fig. 4). This was also true for the other three components of gum arabic (rhamnose, arabinose, and galacturonic acid). When more than 100 mg of gum arabic was added to the sand, there was a loss of linearity in the recovery of galactose. The direct analysis of the polymer and the analysis of an equivalent amount of polymer added to sand showed statistically insignificant differences in the amounts of galactose, galacturonic acid, glucose, and glucuronic acid (Table 2, columns A and B).

Analysis of environmental extracellular polysaccharides. Table 3 shows the quantitative analysis of a number of extracellular polysaccharide polymers. Extracellular polymer was isolated from chloroform-methanol precipitates of the growth media of two marine pseudomonads. *P. marina* produced an extracellular polymer rich in galacturonic acid, and a pseudomonad isolated from the sediment near the Florida State University marine laboratory produced an extracellular polymer with a trace of mannuronic acid, a large amount of galacturonic acid, and a lesser amount of glucuronic acid.

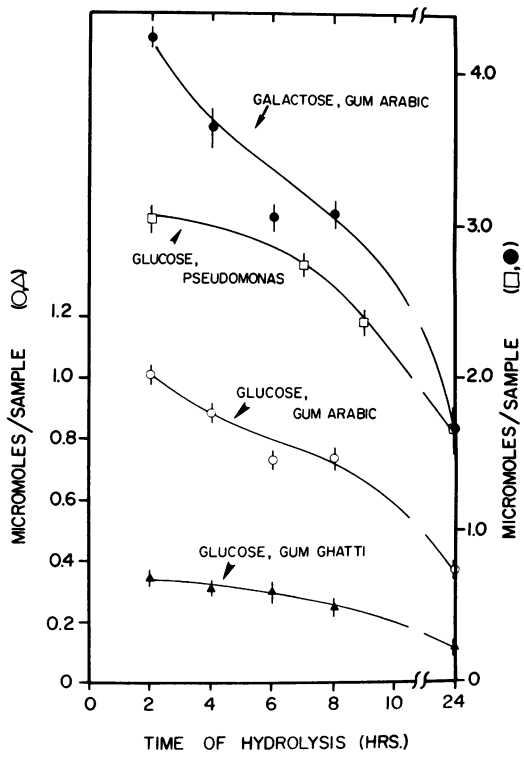

FIG. 3. Yield of the hydrolysis in 2 N HCl at 100°C of polymers in which the uronic acid was reduced before hydrolysis. Symbols: ▲, glucose recovered from 4 mg of gum ghatti, of which 88% was dideuterated; ○, glucose recovered from 4 mg of gum arabic, of which 92% was dideuterated; □, glucose recovered from 5 mg of exopolymer derived from a marine pseudomonad isolated locally, of which 28% was dideuterated; ●, galactose in 4 mg of gum arabic, of which 3% was dideuterated. Each determination was done in triplicate, and the means and standard deviations are indicated.

The maldanid polychaete worms of the species *Clymenella* construct tubes in soft sediments that can be recovered. These tubes contained a polymer with neutral carbohydrate hexoses enriched in fragments with an *m/e* of 219, of which mannuronic, galacturonic, and glucuronic acids were identified. The enteropneust *P. bahamensis* releases clearly definable large fecal pellets that were enriched in galacturonic acid.

Studies of heat transfer resistance across metal pipes exposed to rapidly flowing seawater have shown that the extracellular polymer films secreted by the microfouling community are of primary importance (32, 33). The polymer re-

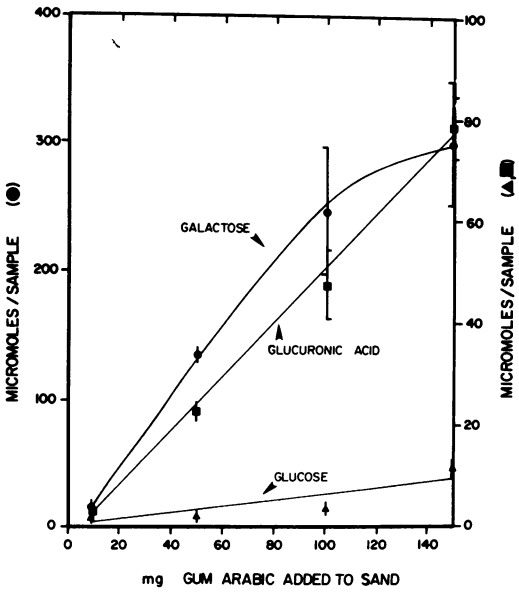

FIG. 4. Recovery of glucose (▲), glucuronic acid before reduction (■), and galactose (●) from gum arabic added in increasing amounts to acid-washed, ignited sand. The sand and dissolved gum were mixed for 1 h and then lyophilized before analysis. Each analysis was done in triplicate, and the means and standard deviations are indicated.

covered from the film formed on aluminum was greatly enriched in galacturonic acid compared with the polymer recovered from the film formed on titanium. In each of these extractions, the lipids were removed before the uronic acid analyses.

DISCUSSION

Uronic acid assay. Uronic acids appear to be the most universal and specific indicators of extracellular polymers that are used by the invertebrates in feeding nets, fecal pelletization, or feeding tube structures in the benthic environment and of the polymers that protect and regulate the ionic traffic at the surface of the bacteria. These polymers are important in the stabilization of marine sediments (36, 49). The measurement of the uronic acid content of the extracellular polymers by the formation of the ester and the subsequent reduction of the carboxylic acid moiety to an alcohol eliminated the problems of resistance to hydrolysis and of quantitative recovery in separation from the neutral carbohydrates. The reduction of the uronic acid methyl esters while they were in the polymers was completed in one treatment (Table 2). If the polymers are compared for the proportions of dideuterated alditols before and after methylation, the methyl esters in the original polymer can be determined, as the reduction of the methyl esters of the uronic acids in polymers was quantitative. The recovery of the acidic polysaccharide polymer gum arabic from sand was quantitative (Fig. 4). The detection of the dideuterated neutral sugar component of each carbohydrate by mass spectrometry was reproducible (Fig. 2), and the detection was sensitive enough for analyses of marine sediments. The hydrolyses of the dideuterated polymers yielded monosaccharides under conditions that were mild enough that proteins and glycoproteins were not hydrolyzed. The use of the cation exchange column in the reduction of the reducing sugars to alditols before peracetylation separated any amines from the neutral carbohydrates. Microbial extracellular polymers containing aminouronic acids have been isolated from *Achromobacter georgiopolitanum* (41). These polymers are particularly resistant to hydrolysis. However, they can be deaminated by treatment with *n*-butyl nitrite in 1,2 dimethoxyethane at −25°C, as has been done for heparin (21), before methylation and reduction of the uronic acids. The resulting polymer is as easily hydrolyzable as those illustrated in Fig. 4.

Mopper and Larsson (31) have recovered numerous organic acids from marine sediments. The application of the extraordinary separative prowess of glass capillary GLC coupled with the sensitivity of mass spectrometry can be of great importance in the analysis of organic acids. The separation of the 3-pentose and 6-heptose isomers, as well as a heptose and several deoxypentoses, is illustrated in Fig. 1. Unfortunately, the derivatization procedure renders identical products for the aldoses of glucose and gulose, as well as for the aldoses of altrose and talose.

Use of the uronic acid analysis. Bacteria that produce uronic acid-containing extracellular polymers can be readily selected from soil enrichments in media containing sulfate (28). The production of these polymers by bacteria can be important in medicine. Pseudomonas recovered from patients with cystic fibrosis and other pneumonias produce extracellular polysaccharide polymers of acetylated D-mannuronic and L-guluronic acids (9, 26). Marine bacteria cultured under the proper conditions produce sufficient extracellular polysaccharide polymer to form mucoid colonies. Many of these are gram-negative, motile, pseudomonad-like organisms that may be important in the stabilization of marine sediments (36, 49). Sediments may also be stabilized by tube-building invertebrates (36, 49). The isolated tubes of the maldanid bamboo worms contain uronic acids (Table 3). The conditions that induce maximal polymer secretion (at least for the microbes) are generally not those for optimal growth (48), and the factors that

regulate the formation and catabolism of these polymers in sediments must be included in any basic explanation of sediment stability.

The fecal mounds created by the deposit-feeding enteropneusts form clearly defined areas that persist for days in the sediments. The mounds are enriched in galacturonic acid (Table 3). These polymers may be responsible for the total organic carbon, which is equal to that of the surrounding sediment in the face of half the inducible biomass, measured as the extractable lipid phosphate or as the total extractable lipid measured as palmitic acid. The fecal mounds are relatively depauperate in bacteria and microeucaryotes as measured by the fatty acid composition (8). Thistle (44) found that the densities of harpacticoid copepods in the fecal mounds were decreased sevenfold within hours of their formation. Within 24 h, these mounds contained the same density of harpacticoid copepods as did the surrounding sediments. There was a disproportionate abundance of two species in the initial colonization. Clearly, the extracellular polymers influence the recolonization and the sediment stability.

A particularly important role for extracellular polysaccharide polymers is in the increase of the heat transfer resistance that is produced by the microfouling community on metal surfaces exposed to rapidly flowing seawater. Extracellular polymer, estimated as the ratio of total organic carbon to cellular biomass measures (e.g., lipid phosphate and lipid palmitate), accumulates when the metal surfaces are brush cleaned as an antifouling countermeasure (33, 34). These polymer-rich films have a greater heat flow resistance for an equivalent amount of total organic carbon than do other films of equal cellular biomass. The data in Table 3 illustrate the enrichment in galacturonic acid in films formed on aluminum. Films formed on aluminum appear to have a greater heat flow resistance than films of greater biomass formed on titanium (34). The quantitative measurement of extracellular polysaccharide polymer films will be important in the definition of the microfouling films, their heat flow resistance, and their potentialities for facilitating corrosion.

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