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Extracellular Polysaccharide Produced from Glucose by *Arthrobacter viscosus* NRRL B-1973: Chemical and Physical Characterization*

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Synopsis

The polysaccharide produced in good yields by *Arthrobacter viscosus* NRRL B-1973 when grown in liquid medium containing glucose has been isolated from the culture fluids, purified and characterized. The constituents of this water-soluble polysaccharide have been shown to be D-glucose, D-galactose, and D-mannuronic acid (as the potassium salt) in approximately equimolar proportions, and about 25% of *O*-acetyl groups. The rarity of known occurrences of D-mannuronic acid in polysaccharides other than alginates is documented. Properties which have been observed as significant for utilization include, for both native and deacetylated forms of the polysaccharide: high viscosity of dispersions in water and in solutions containing chlorides, stability of viscosity to shear and to pH change in the normal range, and excellent quality of unsupported films; and for the native polysaccharide: stability in storage and the favorable influence of alcohol–water solvent on rate of dispersion and viscosity attained.

INTRODUCTION

This study continues our series on microbial polysaccharides obtained freely dispersed in liquid cultures containing starch-derived sugars as an essential substrate and considered to have practical potentialities.[1–3] The physical and chemical constitution of these polysaccharides govern their specific properties,[2] and we have sought to establish fundamental correlations among properties, constitution, and structure.[4]

The heteropolysaccharide from *Arthrobacter viscosus* NRRL B-1973[5] differs distinctly in some aspects of composition from our previously reported bacterial polysaccharide from *Xanthomonas campestris* NRRL B-1459.[2] Preliminary indications[6] have been confirmed[7] that polysaccharide B-1973 is composed of D-glucose, D-galactose, and D-mannuronic acid (as the potassium salt) in the ratio of 1:1:1. Also present is 25% *O*-acetyl groups, but unlike polysaccharide B-1459, this polysaccharide does not contain pyruvic acid.[8]

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Very rarely has mannuronic acid, other than as a constituent of alginates, been identified in natural products. Heretofore, D-mannuronic acid has been reported only in an extracellular[9] and an endocellular[10] polysaccharide from pathogenic microorganisms. The occurrence of D-mannuronic acid in polysaccharide B-1973 is the first known in an extracellular polysaccharide from a nonpathogen. The ease of obtaining this polysaccharide, as well as the high percentage content of D-mannuronic acid, makes it valuable for fundamental studies related to this uronic acid. Already significant are the observations that aldobiuronic acid from this polysaccharide hydrolyzes with anomalous ease[6] and the establishment of the mechanism of biosynthesis of D-mannuronic acid in strain B-1973.[11]

Reported here are general chemical and physical characterizations of this polysaccharide. Detailed observations on structure[7] and on the effects of certain salts and of heating[12] are reported elsewhere.

EXPERIMENTAL

Production and Laboratory Purification of Native Polysacchoride B-1973

For purification and isolation as described here, culture fluids were used from a 12-liter experimental fermentation by strain B-1973 on a medium containing: commercial glucose 3% (as $C_6H_{12}O_6$), enzyme-hydrolyzed casein 0.25%, dipotassium hydrogen phosphate 0.4%, magnesium sulfate 0.07%, and manganous sulfate 0.005%. The initial pH of the medium was 7.0; fermentation was for 4 days with moderate aeration.[13] When fermentation was terminated, the culture fluids showed pH 5.6 and viscosity 12000 cpoise and had a noncohesive, pasty, and somewhat hydrophobic appearance.

Subsequent treatment of the culture fluid followed our general procedure for purifying and isolating polysaccharides suitable for compositional and structural analysis.[2,3]

After inactivation of the cells in 2000 ml. of culture fluid, the pH was adjusted to about 6, and more water, ethanol, and potassium chloride were added until the viscosity was reduced to about 100 cpoise at concentrations (based on water volume) of 35% ethanol (v/v) and 1% salt (w/v). Four successive passes of this diluted fluid through a Sharples continuous supercentrifuge were required to remove the bacterial cells. Increasing the ethanol concentration of the supercentrifugate to 70% (v/v) produced a voluminous, somewhat stringy, precipitate which settled partially upon standing. The precipitated polysaccharide gum was separated from supernatant liquor by decantation and supercentrifugation, and although it appeared rather hydrophobic in water, it hydrated and dispersed readily. To the aqueous dispersion of the gum (1.5%, estimated w/v) was added ethanol to give a concentration of about 50% (v/v) and also potassium chloride (as a filtered 30% aqueous solution) to give a concentration of 1% (w/v of water). Further increase in ethanol concentration to 70% repre-

cipitated the gum, which was recovered and the reprecipitation procedure was repeated once.

A solution of the twice reprecipitated polysaccharide was dialyzed against deionized water; initially there was 0.25% polysaccharide concentration, pH 6.3, and about 350 cpoise viscosity. The presence of toluene maintained asepsis of the solution. After dialysis for 6 days, the retentate[14] was adjusted from pH 4.15 to 6.25, filtered through sintered glass to remove extraneous matter, and concentrated *in vacuo* to about 0.5% polysaccharide concentration for lyophilization.

The yield of purified polysaccharide, based on the initial weight of glucose in the medium, was 32%. Analysis showed 12.50% sulfated ash and 0.07% nitrogen.

Additional polysaccharide material (1.5% by weight of the main product) was obtained from the original supernatant culture liquors after concentrating them *in vacuo* to $^1/_{60}$ their initial volume and after removing most of the salt as crystalline solid. The polysaccharide material then was precipitated by 65% ethanol and purified by reprecipitation, removal of protein,[15] and dialysis.

Polysaccharide Materials

The product purified as described in the preceding section was used for all observations on composition and chemical characterization. When larger samples were required, as for measurement of viscosity and factors influencing it, two other preparations were used. These were: (A) a preparation from a 30-gal. fermentation partially purified on a pilot-plant scale[16] and (B) preparation A after further purification in the laboratory by dialysis, supercentrifugation, and lyophilization. Analysis of preparations A and B gave respective values as follows: sulfated ash, 20.60% and 12.20%; nitrogen, 0.75% and 0.22%. For 1% polysaccharide solutions in distilled water, pH values for A and B were 7.5 and 4.9, respectively.

Preparation B was used almost exclusively; the few cases when A was used are specified.

Analytical Methods

Procedures for humidifying, storing, sampling, and analyzing the polysaccharide products for moisture were the same as those reported previously.[3] All calculations for other analyses were made on a dry-weight basis.

Constituent sugars were identified by previously described methods,[3] except for paper chromatographic differentiation of mannuronic acid. For that, the developing solvent was pyridine, ethyl acetate, acetic acid, and water (5:5:1:3); the saturating solvent was pyridine, ethyl acetate, and water (11:40:6) (both were v/v.)[17] Mannuronic acid was measured by an adaptation of Gregory's method based on the use of borate to enhance the mannuronic acid–carbazole color reaction.[18] Sensitivity was improved

by several modifications.[19] *O*-Acetyl groups and neutral equivalent were measured as previously reported.[3] Methyl pentose was measured by the cysteine-sulfuric acid method.[20]

Optical rotation measurements were made with a Bates-type adjustable-sensitivity saccharimeter.

Viscosity measurements were made with Brookfield viscometers, types LVF and LVT, at 25 °C. and 30 rpm unless indicated otherwise. All values are based on equilibrium readings.

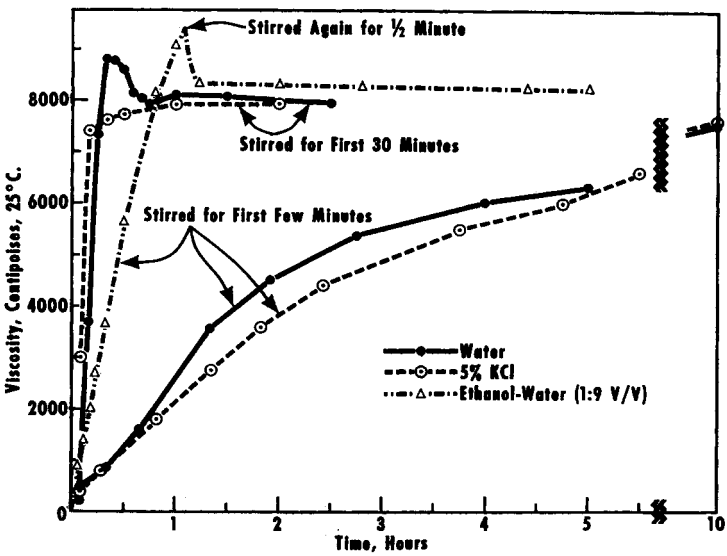


Fig. 1. Effect of solvent and stirring on rate of solvation and dispersion of polysaccharide B-1973, preparation A (1% concentration).

Dispersions for viscosity measurements and other physical tests usually were prepared by mixing the finely divided polysaccharide solid with one tenth to one half the final amount of water and allowing the polysaccharide to hydrate either 2 hr. or overnight. The remainder of the water then was added gradually to the mechanically stirred mixture, and the dispersion was allowed to stand until constant viscosity had been reached; usually 30 min. sufficed. For measurement of rate of solvation (Fig. 1), all the water was added at once either to the polysaccharide alone or to the polysaccharide already mixed with salt or ethanol. Vigorous mechanical stirring then was applied. Stirring the polysaccharide in water or ethanol–water for 2 min., or in 5% potassium chloride for 5 min., resulted in homogeneous mixtures that did not separate upon standing.

The effects of salt addition, pH, and temperature on the polysaccharide viscosity were measured as described previously.[3]

Preparation of Deacetylated Polysaccharide

Deacetylation would be most suitably carried out as an additional step during purification and isolation of the polysaccharide from culture fluids. In experimental deacetylation, we obtained equivalent results by using either crude, native polysaccharide gum after the first precipitation from the cell-free culture fluid or dry, solid, native polysaccharide preparation A.

An approximately 0.25% solution of native polysaccharide was deacetylated by treatment at 25°C. for 2 hr. with 0.034*N* potassium hydroxide in the presence of 1% potassium chloride in oxygen-free solutions under an atmosphere of nitrogen. Viscosity of the reaction solution decreased from the initial 470 cpoise to a steady value, 35 cpoise. The solution was neutralized by hydrochloric acid and supercentrifuged to remove some extraneous suspended matter; addition of methanol to give a concentration of about 35% then precipitated the deacetylated polysaccharide. The fibrous precipitate was collected by centrifugation, redissolved in water, and reprecipitated from a 0.1% potassium chloride solution by addition of methanol to 50% (v/v). The purified, deacetylated polysaccharide was collected by centrifugation, dehydrated by slurrying in methanol, and freed of methanol by desiccation *in vacuo* at room temperature. Recovery was about 66% of the initial weight of preparation A and 85% of the calculated value. Analysis of the deacetylated polysaccharide showed 37.5% uronic acid, 15.85% sulfated ash, and 0.10% nitrogen.

RESULTS AND DISCUSSION

Composition and Chemical Constitution

Polysaccharide B-1973, being composed of approximately equal proportions of D-glucose, D-galactose, and D-mannuronic acid, might be expected to be quite simple structurally. No other component sugars were found in this main polysaccharide product, but the byproduct polysaccharide isolated in low yields from the supernatant culture liquors did contain fucose (17%).

The presence of 25% *O*-acetyl groups, which is 62.5% of the theoretical for complete substitution, does not inhibit dissolution of polysaccharide B-1973 in water, but diminishes the rate and extent of hydration, and increases the compatibility with alcohols. Thus the viscosity of the polysaccharide increases over 10% as ethanol content of the aqueous solvent is increased up to 33%; such dispersions are homogeneous and stable. The influence of 10% ethanol on rate of solvation and dispersion is shown in Figure 1. Heat also increases the extent of hydration.

Removal of *O*-acetyl substituents by dilute alkali in the presence of an electrolyte progresses relatively slowly at room temperature and changes the solubility characteristics and intermolecular relationships of polysaccharide B-1973. Thus, the deacetylated polysaccharide is precipitated from aqueous solution (containing 1% potassium chloride) by about one-

half the concentration of alcohol required by the native polysaccharide. Molecules of the deacetylated polysaccharide appear to align readily and to form firm intermolecular bonds. This is evidenced by the tough, fibrous precipitates; by the behavior with certain salts;[21] and by the formation of clear, strong, flexible films when aqueous dispersions are cast and dried.[12] The dry, deacetylated product disperses completely in water, but if the amount of water is restricted initially, a tough, cohesive, stringy gum results.

A tendency to lactonization or internal esterification in the native polysaccharide is suggested by the high neutral equivalent value from titration of decationized polysaccharide (664) as compared with the weight of polysaccharide per uronic acid unit (618) calculated from the measured uronic acid content (28.5%). This tendency is suggested also by the observation that products which have undergone dialysis and pH adjustment to about 6.0–6.5 before lyophilization show pH near 5 when redissolved (1% concentration).

Consistent readings for specific optical rotation of dilute solutions of native polysaccharide B-1973 in water, 35% ethanol, or 1% potassium chloride were obtained only after allowing tube and contents to stand undisturbed for about 3 days. Expressed as $[\alpha]_D^{25}$, these values were $-50°$ to $-55°$ ($c = 0.25$; 1 dm). These values are believed to be due in part to optical effects of solution structure since dispersions in water after 15 min. autoclaving showed $[\alpha]_D^{25}$ $-10°$. The $[\alpha]_D^{25}$ value for the deacetylated polysaccharide in water was $-24°$ and reliably indicates the presence of β-linkages in the polysaccharide.

Factor Influencing Viscosity

Storage of the polysaccharide powder (preparation A, moisture content about 14%) for 2 yr. at 20–25°C. had no detectable influence upon viscosity of dispersions.

The effects of the solvent and the manner of solvation of the polysaccharide powder upon viscosity are shown in Figure 1. Essentially the same viscosity is reached by fast solvation as by slow, and solvation in 5% potassium chloride is only slightly slower than in water alone. The presence of ethanol facilitates solvation and dispersion in water (Fig. 1) Some soft, gelatinized particles still remaining at the end of 1 hr. were dispersed by brief stirring. Other concentrations of ethanol or even other alcohols might serve as well.

The relation between concentration and viscosity for three forms of polysaccharide B-1973 is shown in Figure 2 and compared with two reference polysacolloids.[22] Restricted hydration of native polysaccharide B-1973 is believed responsible for the break in the curve above about 0.5% concentration. Brief autoclaving at 2% concentration and serial dilution to the lower concentrations appears to improve hydration. The deacetylated polysaccharide shows excellent viscosity characteristics.

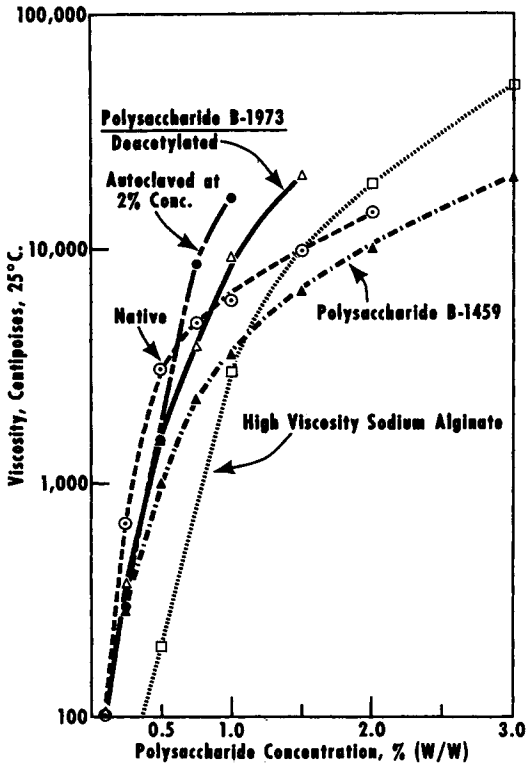


Fig. 2. Viscosity vs. concentration relationships of native, deacetylated, and autoclaved forms of polysaccharide B-1973 compared with those of representative polysacolloids. (The native and autoclaved forms were preparation B.)

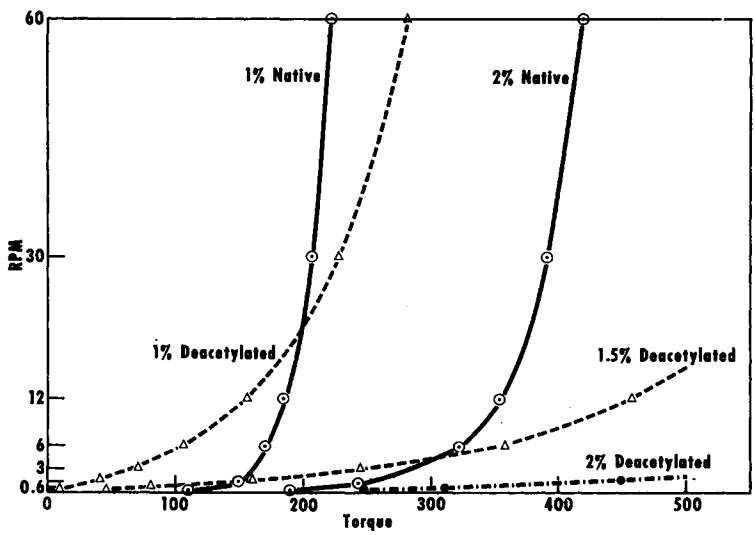


Fig. 3. Torque vs. rpm curves for native (preparation B) and deacetylated polysaccharides B-1973, 25°C.

Rate of shear versus torque measurements show that dispersions of the native polysaccharide have plastic rheological characteristics;[23] after the yield value is exceeded, resistance to increasing rate of shear levels off rapidly (Fig. 3). Dispersions of the deacetylated polysaccharide show

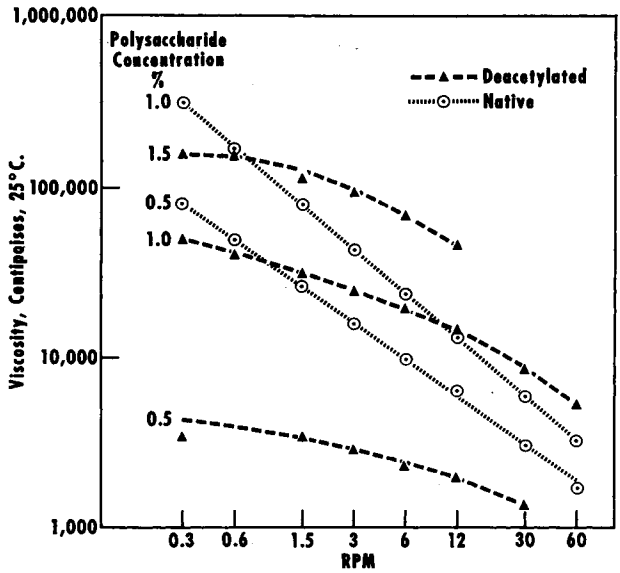


Fig. 4. Viscosity vs. rpm relationships for native (preparation B) and deacetylated polysacchardies B-1973.

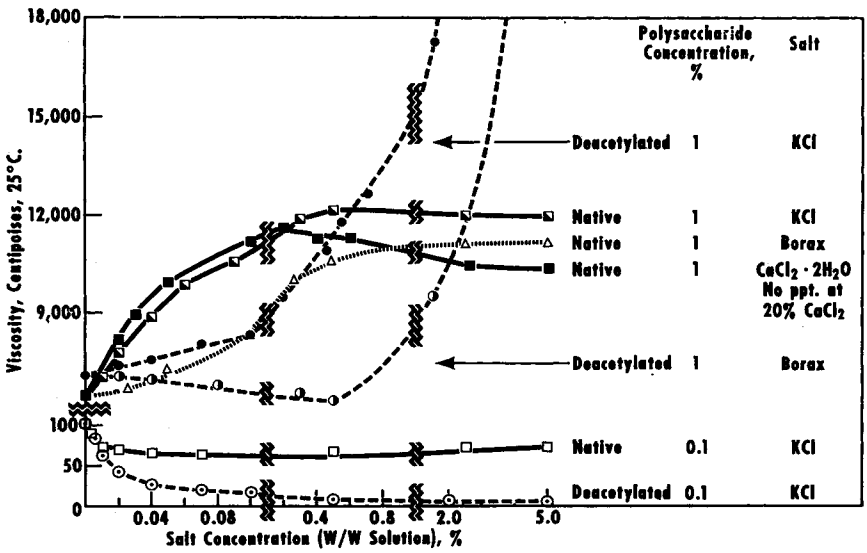
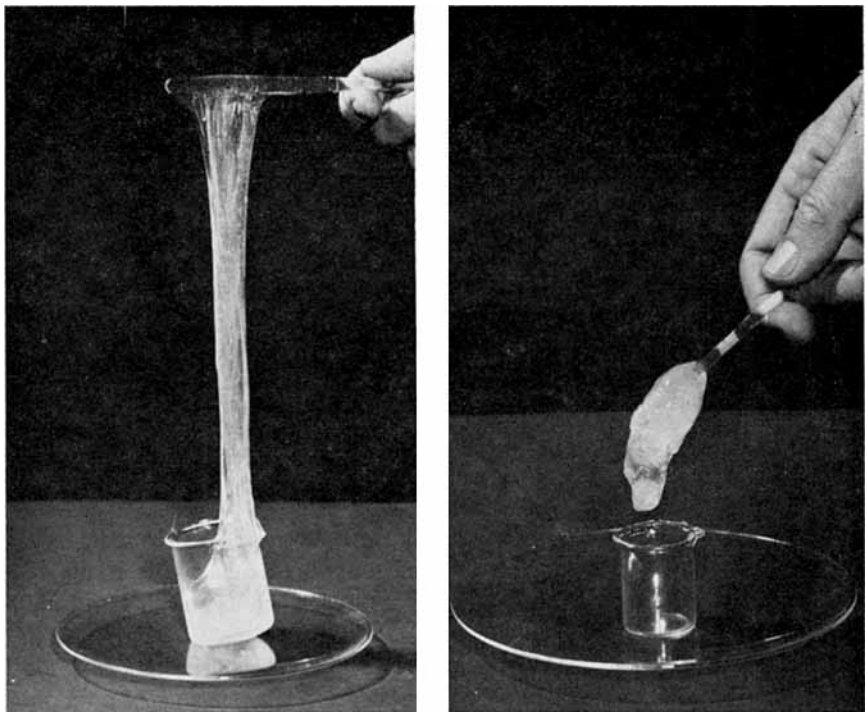


Fig. 5. Viscosity of native (preparation B) and deacetylated polysaccharides B-1973 in presence of salts.



(a) (b)

Fig. 6. Aqueous dispersions of deacetylated polysaccharide B-1973 (1% concentration) containing (a) 2% borax and (b) 3% potassium chloride.

pseudoplastic properties[23] and offer greater resistance to increasing shear than does the native polysaccharide. These differences are confirmed by viscosity versus rpm relationships (Fig. 4) and correlate with other indications that dispersions of the deacetylated polysaccharide are characterized by strong intermolecular cohesion in contrast to deformable entanglements among the native molecules.

Effects of Salts and pH. The effect of salts on the viscosity of aqueous dispersions of native polysaccharide B-1973 is influenced by polysaccharide concentration and purity and also by the specific salt. As shown in Figure 5 for our essentially salt-free preparation B, potassium (0.4%) and calcium (0.1%) chlorides increased the viscosity (1% polysaccharide) about 80%; increase in concentration of these salts (to saturation and to 20%, respectively) caused little further change. The effect of borax resembles that of potassium chloride and gives no indication of complex formation or crosslinking. For 0.1% native polysaccharide (preparation B), 0.04% potassium chloride produced a 35% decrease in viscosity, but additional salt caused a gradual increase. The crude native polysaccharide (preparation A), which contained considerable inorganic salt impurity, showed very little change in viscosity upon experimental addition of salt.

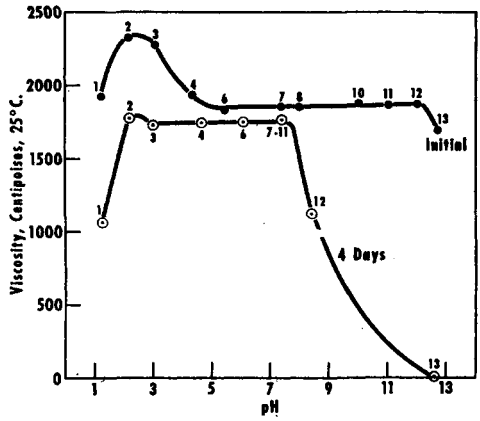


Fig. 7. Effect of pH and time on viscosity of native polysaccharide B-1973, preparation B (0.5% concentration, 25°C.). Numbers 1–13 designate individual test solutions.

Although potassium and calcium chlorides, even at high concentration, do not insolubilize and precipitate the native polysaccharide from aqueous dispersion, it will be shown elsewhere that salts such as sulfates and phosphates act differently.[21]

At 0.1% concentration, the deacetylated polysaccharide showed more pronounced and extended decrease in viscosity in the presence of potassium chloride than did the native polysaccharide (Fig. 5). For 1% dispersions of deacetylated polysaccharide, both viscosity and general character are changed sharply when apparently critical concentrations of potassium chloride (about 0.1%) or borax (about 0.4%) are exceeded (Fig. 5). The formation of cohesive, tough yet mobile, stringy masses (Fig. 6) indicates partial dehydration and insolubilization of the molecules, and development of strong intermolecular binding. Further increase in potassium chloride concentration results in precipitation.

Viscosity of the native polysaccharide is essentially constant in the range of pH 4–11 although, with time, saponification of acetyl groups and lactone structure causes decrease in pH of solutions which initially had values $\geqq 8$ (Fig. 7). At pH >11, decrease in viscosity accompanies deacetylation.

Effect of Temperature. Measurement of viscosity of 0.1% and 1.0% dispersions of preparation A at temperatures up to 98°C. showed decreasing values which at 98°C. were lower than those at 25°C. by 60 and 13%, respectively. However, upon spontaneous cooling back to 25°C., viscosity was regained so that permanent losses were less than 10%. In the presence of 5% potassium chloride, viscosity of 1% polysaccharide dispersion remained constant throughout the temperature range.

We are pleased to acknowledge our indebtedness to Mr. M. C. Cadmus for experimental culture fluids, to Mr. S. P. Rogovin for pilot-plant-grade polysaccharide, and to Mrs. Clara McGrew for microanalyses.

Mention of trade names should not be construed as a recommendation or endorsement by the Department over those not mentioned.

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Résumé

Le polysaccharide, qui est produit avec un rendement élevé parl' *Arthrobacter viscosus* NRRL B-1973 lorsqu'on le laisse croître au sein d'un milieu liquide contenant du glucose, a été isolé du liquide de culture purifié et caractérisé. On a montré que ce polysaccharide soluble dans l'eau était constitué de D-glucose, D-galactose et d'acide D-mannuronique (sous forme de sel potassique) en proportion à peu près équimoléculaire et de 25% de groupements *O*-acétyles. On ne signale que de rares exemples de la présence d'acide D-mannuronique dans les polysaccharides autres que les alginates. On a observé certaines propriétés communes aux formes naturelles et désacylées du polysaccharide et présentant un intérêt pratique: une viscosité élevée des dispersions dans l'eau et dans

les solutions contenant des chlorures, une stabilité de la viscosité au cisaillement et aux variations de pH, du moins dans une domaine normal, et une qualité excellente des films sans support. En ce qui concerne le polysaccharide naturel en particulier, on observe une stabilité élevée lors de l'emmagasinage et une influence favorable du solvant mixte eau-alcool sur la vitesse de dispersion et sur la viscosité.

Zusammenfassung

Das durch *Arthrobacter viscosus* NRRL B-1973 beim Wachstum in flüssige Glucose enthaltendem Medium erzeugte Polysaccharid wurde aus der Kulturflüssigkeit isoliert gereinigt und charakterisiert. Es wird gezeigt, dass sich dieses in Wasser lösliche Polysaccharid aus D-Glucose, D-Galactose und D-Mannuronsäure (als Kaliumsalz) in annähernd äquimolarem Verhältnis und aus etwa 25% *O*-Acetylgruppen zusammensetzt. Das seltene Vorkommen von D-Mannuronsäure in Polysacchariden ausser Alginaten wird belegt. Die beobachteten, für die Verwendung wichtigen Eigenschaften, sowohl von nativen als auch deacetylierten Polysaccharidarten sind: hohe Viskosität von Dispersionen in Wasser und in chloridhältigen Lösungen, Stabilität der Viskosität in Bezug auf Scherungs- und pH-Wertänderungen im normalen Bereich, ausgezeichnete Qualität der trägerfreien Filme, und für das native Polysaccharid: Stabilität bei Lagerung und günstiger Einfluss von Alkohol-Wasser als Lösungsmittel auf Dispersionsgeschwindigkeit und Viskosität.

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