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Purification and Properties of a Phytotoxic Polysaccharide Produced by Corynebacterium sepedonicum[1]

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Summary. A polysaccharide possessing a capacity to wilt plant cuttings has been isolated and purified from cultures of *Corynebacterium sepedonicum.* The molecular weight, based on the average of molecular weights determined by 3 physical methods, is 21,450. The empirical formula of the polysaccharide is $C_{48}H_{96}O_{48}N$. It is antigenic and the borate complex migrates in an electric field. It has an intrinsic viscosity of 0.125 and an S_{20w} of 0.76. A hydrolysate of the compound yields glucose, mannose, 2 unidentified reducing compounds and 1 unidentified non-reducing compound. The nitrogen in the toxin can be accounted for in 6 amino acids. Although the toxin is primarily polysaccharide it might more aptly be termed a glycopeptide.

Some information regarding the physiological activity of this compound as well as a discussion of how it acts to bring about wilting is included.

Corynebacterium sepedonicum causes ring rot of potato. This disease is characterized by chlorosis, flagging of leaflets as well as stunting and wilting in some cases. The disease is found in all potato growing areas of the world and is a constant menace to the potato industry.

Hodgson et al. (3) showed that polysaccharides produced by a bacterial pathogen may be responsible for wilting symptomatology. Paquin, Lachance, and Coulombe (6) showed *C. sepedonicum* did not produce detectable quantities of pectic enzymes reported to be responsible for disease symptoms in other situations and suggested that a bacterial secretion might be responsible for the symptoms. Spencer and Gorin (9), following this hypothesis, reported the occurrence of physiologically active gums in cultures of *C. insidiosum* and *C. sepedonicum* and showed that crude polysaccharide preparations possessed the ability to wilt plant cuttings. There was circumstantial evidence of the presence of bacterial polysaccharides in naturally infected plants. The present report describes the purification and properties of a toxic polysaccharide from *C. sepedonicum.* Information regarding the physiological activity of this compound is also presented.

Methods

A pure culture of *C. sepedonicum* was obtained from a potato tuber showing typical ring rot symptoms. The culture was grown on the media described by Spencer and Gorin (9). Polysaccharide was produced by growing the bacterium in 0.5 liter of the liquid medium, incubated 1 week aerobically at 23° in 1 liter flasks.

The crude toxic polysaccharide was prepared by centrifugation of the medium at 20,000 × *g*; passage of the supernatant liquid through a 20.0 cm × 2.5 cm column of Dowex 1 200 to 400 mesh (formate form)

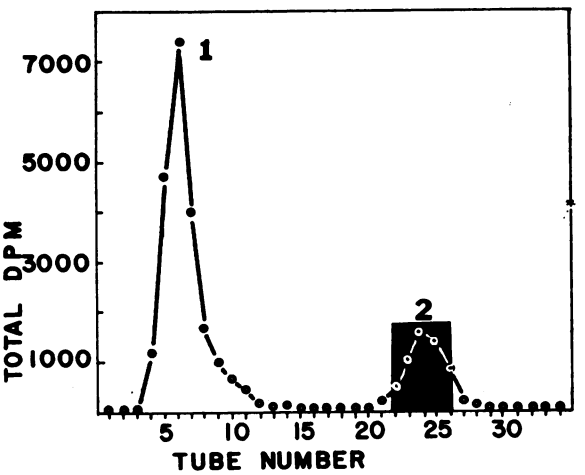

FIG. 1. The separation of labeled polysaccharides by Sephadex column chromatography. Ten mg of a crude preparation with a specific activity of 8000 dpm/mg was placed on a Sephadex G-200 column and eluted with distilled H_2O. Fractions (1.5 ml) were collected and 0.1 ml aliquots counted by liquid scintillation spectroscopy. The total dpm in each tube is plotted against the tube number. The shaded area represents the tubes that contained the toxin polysaccharide as manifested by the tomato cutting assay test.

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and then a 2.0 cm × 2.5 cm column of Dowex 50 200 to 400 mesh (H^+). The effluent was flash evaporated to 20 to 30 ml and the polysaccharides precipitated by the addition of 3 volumes of 95 % ethanol. The precipitate was centrifuged and stored in a vacuum desiccator over P_2O_5. Further purification was carried out by placing 10 to 100 mg of crude polysaccharide preparation on a 1.4 × 25 cm column of Sephadex G-200 and eluting with H_2O. Approximately one-third of the polysaccharide preparation, by weight, was recovered as toxic polysaccharide. A typical elution curve of the separation of ^{14}C labeled polysaccharides is shown in figure 1. All studies in this report were done with the polysaccharide illustrated as peak No. 2 in figure 1. Peak No. 1 was a polysaccharide, but did not possess biological activity. Polysaccharides were labeled by growing *C. sepedonicum* in 25 ml of the regular medium in the presence of 25 μc of mannose ^{14}C-UL with a specific activity of 4.9 mc/mmole (Nuclear Chicago Corp.).

Radioactivity on all samples was determined with a model 6804 Nuclear Chicago liquid scintillation spectrometer. The channels ratio method was used to correct for quenching. The sample, up to 0.1 ml aqueous solution, was placed in a vial along with 3.0 ml of absolute methanol. Added to this was 12.0 ml of scintillation solution containing 4.0 g 2,5-diphenyloxazole and 100 mg of *p-bis-2*(5-phenyloxazolyl)-benzene per liter of toluene.

The biological activity of the polysaccharide was assayed by the time required for a 7.5 cm young tomato cutting to begin wilting at the margin of the primary leaf. There was a linear relationship of the time required to wilt and the toxin concentration (fig 2).

The polysaccharide was acid hydrolysed by refluxing (0.2 %) in 0.5 N H_2SO_4 for varying periods of time after which the acid was neutralized with an excess of $BaCO_3$. The precipitate was removed by centrifugation and the supernatant liquid taken to dryness.

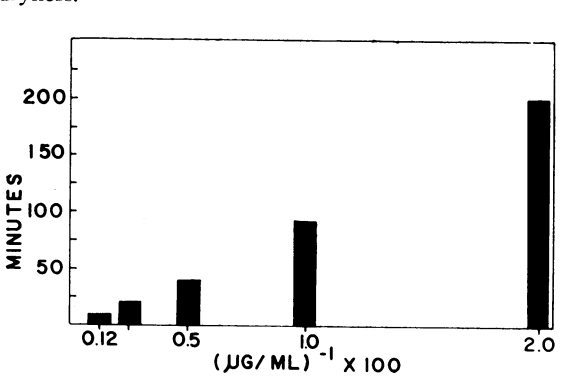

FIG. 2. Time, in minutes, of 7.5 cm tomato leaves prior to wilting as a function of purified polysaccharide concentration. Polysaccharide concentration is expressed as the reciprocal of μg/ml × 100. The values on the graph (left to right) are equal to 0.8, 0.4, 0.2, 0.1 and 0.05 % solutions, respectively.

Separation of the sugar residues was done on Whatman No. 1 paper using ethyl acetate: pyridine: H_2O 8:2:1. The sugars were detected with the reagent of Trevelyan et al. (10). After elution from chromatograms, reducing sugars were determined by the method of Nelson (4). Amino acids were determined qualitatively and quantitatively with a Technicon Amino Acid Analyzer.

Nuclear magnetic resonance studies were done on a Varian NMR A60. The samples were prepared by dissolving 100 mg of polysaccharide in D_2O, removing the D_2O by flash evaporation and repeating the process 2 to 3 times. Finally 1.0 ml of D_2O was added to the polysaccharide for analysis. An important resonance line of D_2O remained about 4.75 ppm down field from tetramethylsilane.

The antiserum used in this study was prepared by Mr. P. V. Rai of this department. It was obtained from the serum of rabbits injected intermuscularly twice at weekly intervals with 10 mg of crude toxin with adjuvant. A titer of 1:4 was obtained in this antigen-antibody system.

Carbon, hydrogen and oxygen were determined by Schwarzkopf Microanalytical Laboratory, Woodside, New York. Nitrogen was determined by the microkjeldahl technique. Number average molecular weight was done by Dr. George Bogart, Hewlett Packard Corporation, using the 500 series membrane osmometer.

Ultracentrifugation analyses were done on a Spinco model E analytical ultracentrifuge using 1.0 ml of a 1 % aqueous solution of polysaccharide for the sedimentation constant determination. The short column method of Van Holde and Baldwin (12) for equilibrium sedimentation was employed for molecular weight determination using 0.2 ml of a 0.02 % solution. Sedimentation calculations were based on those of Schachman (8). The partial specific volume of the toxic polysaccharide was assumed to be 0.62.

A Cannon-Ubbelohde semi-micro viscometer, model 75, (Cannon Instrument Co.) was used to determine the specific viscosity of the polysaccharide at concentrations of 5.0, 3.3 and 2.5 mg/ml at 25°. The intercept of the plot of specific viscosity/concentration as a function of concentration was the intrinsic viscosity.

Results

Elemental analysis of the polysaccharide yielded the empirical formula of $C_{48}H_{96}O_{48}N$. The infrared spectrum of the polysaccharide was consistent with those generally observed for polysaccharides; absorption bands at 3300 cm^{-1} and 1100 cm^{-1} for OH groups and a sharp band at 2900 cm^{-1} for $-CH-$. No assignment, however, has been given to a band at 1615 cm^{-1}.

Acid hydrolysis was done by refluxing for 8 hours on 2 mg of polysaccharide containing 132,000 dpm. The solution was neutralized and passed through a

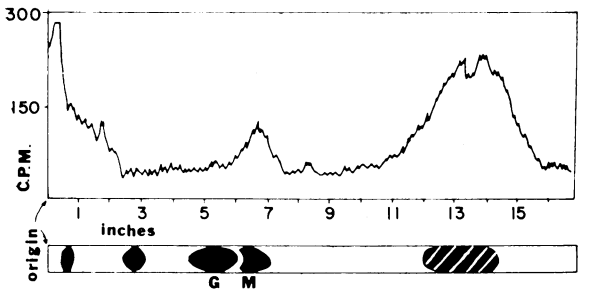

FIG. 3. A radiochromatoscan of the neutral-anion fraction of the polysaccharide after acid hydrolysis. The instrument scanned at 1 cm/min with a colliminator width of 5.0 mm and a time constant of 30. The dark areas on the chromatogram represent reducing compounds. G, and M, indicated the location of glucose and mannose, the other reducing compounds are unidentified, and the non-reducing compound is represented by the slashed area on the chromatogram.

small column of Dowex 50 (H^+). The eluate contained the neutral and anion fraction, and the cation fraction was eluted with 2.0 ml of 6 N HCl. After chromatography, elution from the chromatograms, and analysis, the ratio of glucose to mannose was 1.5:1.0. Two other reducing compounds and 1 non-reducing compound were also present but the quantities were not determined. A radiochromatoscan and reproduction of the chromatogram of the neutral-anion fraction obtained from hydrolysis of the labeled polysaccharide are illustrated in figure 3. The cation fraction yielded threonine, serine, glycine, alanine, lysine, and methionine in a ratio of 1:1:1:1:1:2.

Partial acid hydrolysis of the polysaccharide resulted in the drastic reduction of biological activity illustrated in figure 4. Virtually all of the activity was lost after 3 minutes of refluxing in 0.5 N H_2SO_4.

Ten mg of a labeled crude toxin preparation with a specific activity of 9230 dpm/mg was applied to the Sephadex G-200 column and elution was begun. The tubes containing radioactivity were pooled and taken to dryness by flash evaporation. The tomato cutting assay was used for a 0.2 % solution of polysaccharide from each peak. No wilting was observed in the tomato leaf placed in a solution prepared from peak 1 whereas the leaf wilted within 30 minutes in the solution prepared from peak 2. Thus, the polysaccharide isolated from peak 2 was considered as the toxin. After 2 hours in the solutions the leaves were removed and placed on Kodak-no-screen X-ray film for 2 weeks at −10°. An illustration of a leaf and a positive print of the x-ray film of the same leaf is shown in figure 5. A small amount of exposed film corresponding to the veins and veinlets was throughout the leaf for the solution from peak 1. However, for the toxin treated leaf, radioactivity accumulated at the junction of the leaflets and the petiole, (see arrows in fig 5). This leaf was extracted with 2.0 ml of distilled H_2O, centrifuged, and the supernatant liquid taken to dryness. The extract was made up to 0.2 ml and assayed for its capacity to wilt a tomato leaf. After 250 minutes the primary leaflet began to wilt. The remaining solution was tested by the Ouchterlony (5) double diffusion gel method against the antiserum to the crude toxin. An antigen-antibody precipitation band was observed between the 2 wells. No reaction was observed with an extract from a control plant treated with H_2O.

Molecular Weight Determinations. Column chromatography with Sephadex has been used effectively for molecular weight estimations of polysaccharides (2). Using Sephadex G-200, the equation for molecular weight estimation is $Ve = 3.20 \text{ to } 0.58 \log \frac{V_t}{\bar{V}_t}$ molecular weight. Where Ve equals the volume at which the sample is eluted from the column minus the void volume of the column and V_t equals the total volume of the column which was 35.5 ml. The void volume was determined by the use of blue dextran and was 10.5 ml whereas the sample volume was 36 ml. Thus Ve equaled 0.718. The molecular weight from this equation is 18,850.

The short column method of equilibrium centrifugation yielded a weight average molecular weight of 21,900. The number average molecular weight was 23,600 as determined by membrane osmometry. The average molecular weight from these 3 values is 21,450.

Other Physical Properties. The polysaccharide (0.5 mg) with a specific activity of 7200 dpm/mg

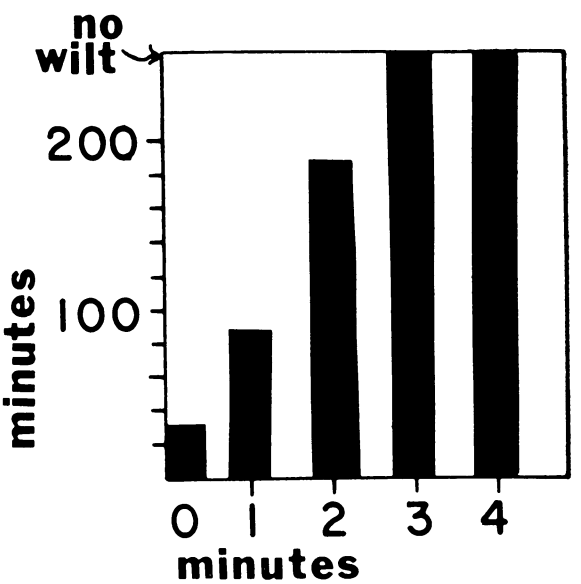

FIG. 4. Minutes of exposure of a 0.2 % solution of polysaccharide to refluxing in 0.5 N H_2SO_4 (X axis) plotted against minutes required to cause wilt in the tomato assay test (Y axis). After each exposure time, an aliquot of the acid solution was removed and neutralized with an excess of $BaCO_3$. After removal of the precipitate the solution was taken to dryness and redissolved in an amount of water resulting in a 0.2 % solution of the partially hydrolyzed polysaccharide.

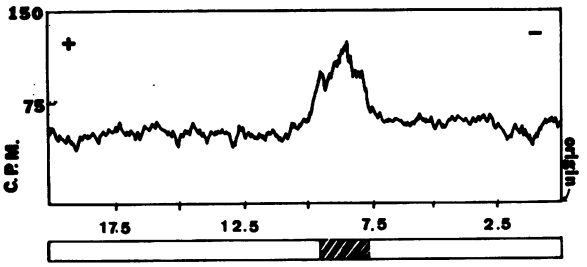

FIG. 6. Electrophoretic mobility of the purified polysaccharide in 0.1 M borate buffer pH 8.7. After electrophoresis, a portion of the strip exposed x-ray film for 2 to 3 weeks after which 1 spot at the location of the slashed area on the paper was noticed likewise this was the only area possessing weak reducing property as represented by the shaded area on the paper. Biological activity was detected between the 7.5 and 10.0 cm mark. A radiochromatoscan was done and is located above the reproduction of the paper. Conditions of scanning are given in the legend to figure 3. Migration was from right to left and details of the procedure are given in the text.

was applied in a 5 cm streak to a 14″ × 2″ strip of Whatman No. 1 filter paper and gently wetted with 0.1 M borate buffer of pH 8.7. Electrophoresis was carried out at 40 volts/2.5 cm for 1 hour with the ends of the paper strip immersed in borate buffer containing the electrodes. After drying, the paper was passed through a Packard radiochromatogram scanner at a rate of 1 cm/min. The paper was also placed on x-ray film for 2 weeks after which it was cut in half, longitudinally. One half was treated with the reagent of Trevelyan (10) for reducing groups and the other half was cut at 2.5 cm intervals and the papers eluted with H_2O. The tomato leaf assay was used to determine where biological activity was located. It was noted that the spot on the x-ray film coincided with the weakly reducing spot on the paper strip which corresponded to the area containing biological activity as depicted in figure 6. The polysaccharide borate complex had a mobility of 1.5×10^{-4} cm volt^{-1} sec^{-1}.

The intrinsic viscosity of the polysaccharide was 0.125 deciliters/g which favorable compares with a value of 0.148 for a dextran with a molecular weight of 22,500 obtained from Pharmacia Inc.

The sedimentation constant of a 1% aqueous solution the polysaccharide was 0.76 at 20°. The Schlerien diagrams of the migration of the polysaccharide in the centrifugal field are illustrated in figure 7.

Small droplets of a 1% solution of the polysaccharide were placed on the surface of a collodion membrane on a copper grid, dried and then observed in a Zeiss EM-9 electron microscope. Figure 8 shows a typical field of polysaccharide crystals (C); amorphous globules (G) are also seen. The globules burst into crystals during the viewing process which was probably due to the high vacuum of the column of the electron microscope or heating due to exposure to the beam. The crystals were 20 mμ × 40 mμ in size.

That the polysaccharide was obtained in a purified state is satisfied by several physical interia of purity, namely, 1 peak when subjected to electrophoresis, 1 peak by ultracentrifugation, 1 evenly shaped peak after elution from a column of Sephadex G-200, and the appearance of homogenity in preparations viewed under the electron microscope.

Nature of Bonding and Degree of Branching. Proton nuclear magnetic resonance was done on 1 ml of a 10% D_2O solution of the polysaccharide. The spectrum is shown in figure 9. Peak 1 is attributed to the C_5 and C_6 protons and peak to the C_2C_3 and C_4 protons according to Pasika and Cragg (7). Peak 4 may be attributed to the proton on the C_1 carbon at the acetal linkage. The ratio of the area of peaks 1+2 to the area under peak 4 is 8.3:1 indicating that the polysaccharide is a branched structure. If it were a linear chain of residues a ratio of 6:1 would be expected. The glycosidic anomeric proton absorption occurs at 5.1 ppm (peak 4) and has a coupling constant of 4.0 count/sec. A coupling constant of 3.2 ± 0.6 count/sec is reported by van der Veen (11) to occur in oligosaccharides with an α linkage and a coupling constant of 7 ± 0.7 count/sec to occur in oligosaccharides with a β linkage. Therefore, a predominance of α linkages is suggested to be present in this polysaccharide. Peak 3 in figure 8 is due to the resonance line of D_2O.

Discussion

Acid hydrolysis followed by chromatography of the purified ^{14}C labeled polysaccharide revealed 2 unidentified reducing compounds and 1 unidentified non-reducing compound. Glucose and mannose were in a ratio of 1.5:1.0 and 6 amino acids were identified. The molecular weight, emperical formula, and amino acid analysis would allow for 2 residues of each amino acid, except 4 for methionine, per molecule of toxin. Since it has a peptide mioety, this toxin might more correctly be termed a glycopeptide. This is in contrast to the work of Spencer and Gorin whose data indicated that galactose and fucose were also present in the polysaccharide, but it should be pointed out that their work was done on the non-purified toxin preparation. Unpublished data from this laboratory suggested that the chemical and physical environment of the bacterial culture affected the sugar ratio in the toxin. That more than 1 polysaccharide was present in crude toxin preparations was pointed out by Rai (unpublished, Ph.D. thesis) who followed the incorporation of mannose-^{14}C into the components of the crude toxin preparation of *C. michiganense*. He also showed that mannose, rather than glucose, served as the better precursor to labeled toxin. That more than 1 polysaccharide was present in the crude preparations was verified as shown in figure 1. Peak 1 contained a polysaccharide that had a molecular weight greater than 2×10^5. It was water soluble and was taken up by the plant, but did not possess physiological activity. This suggested that there is some

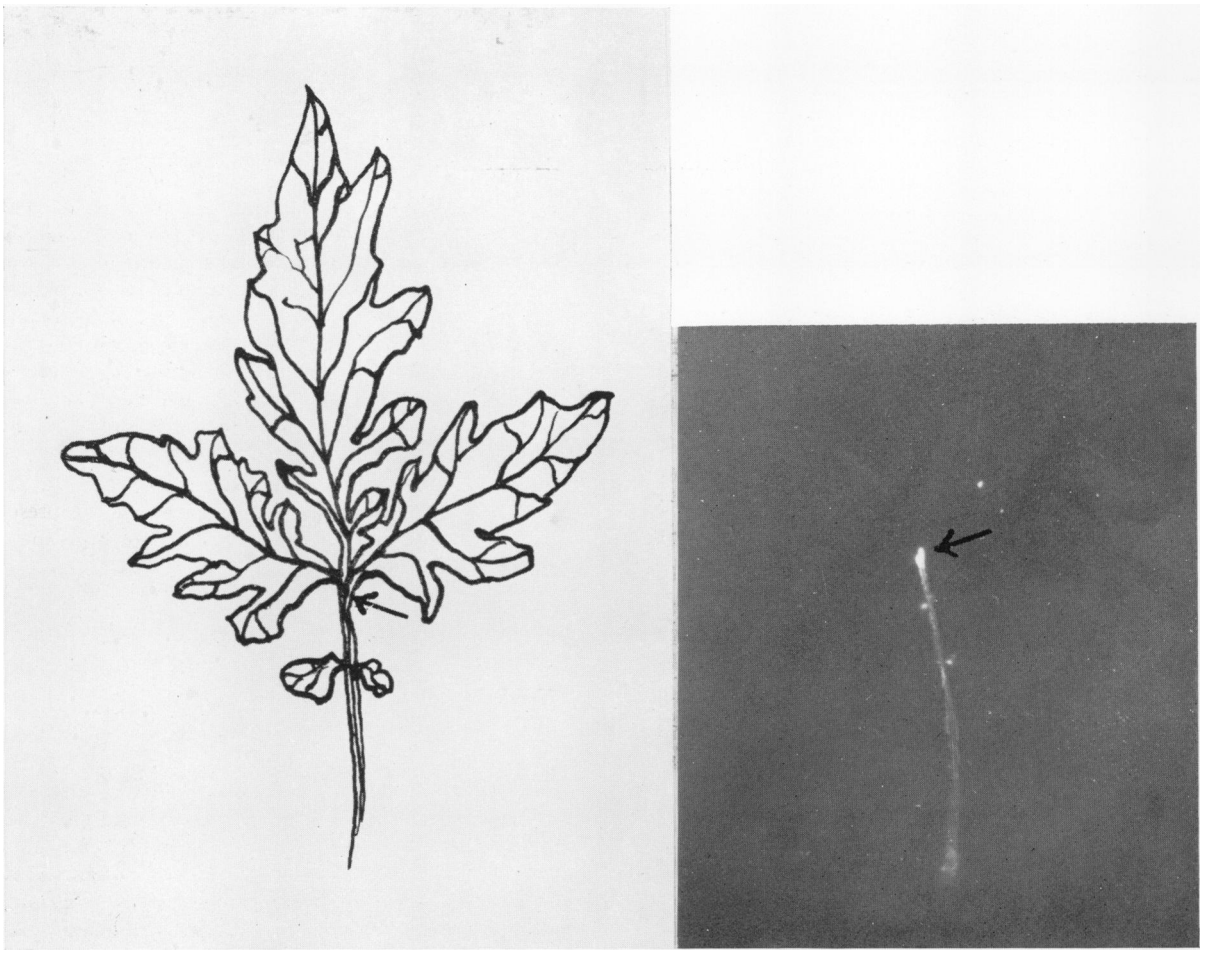

FIG. 5. A reproduction of a plant cutting (left) which was placed in a 0.2 % solution of purified polysaccharide (sp. act. of 27,000 dpm/mg) for 2 hours and then exposed to a Kodak No-Screen X-ray film for 2 weeks (right). Initial wilting was observed after 30 minutes figure 4. The location of the toxin in the plant is represented by the exposed spot on the x-ray film which is the juncture of the tomato leaflet to the petiole. Reisolation of the toxin from this leaf showed that antigenicity and toxicity were retained.

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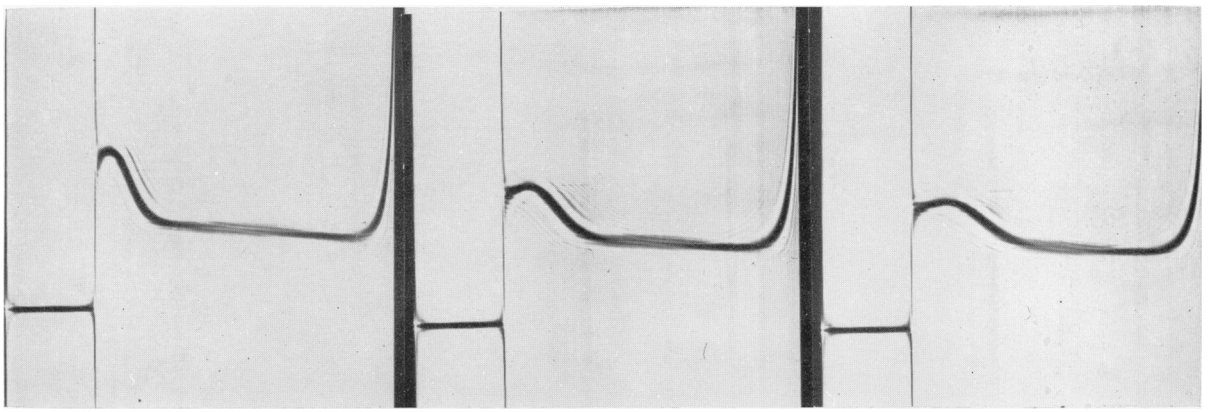

FIG. 7. Ultracentrifugal pattern of a 1 % aqueous solution of the purified polysaccharide. Analysis was performed at 60,000 rpm with a bar angle of 60°. The pictures were taken 35, 67, and 99 minutes after 60,000 rpm had been reached. The sedimentation is toward the right.

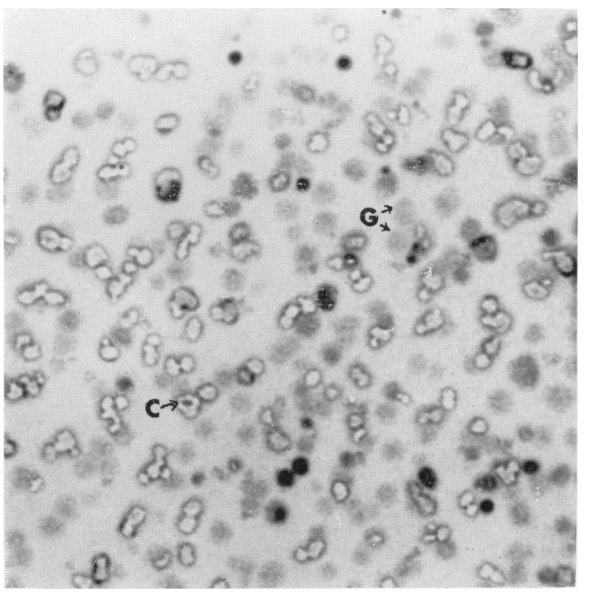

FIG. 8. Electron micrograph of crystalline and amorphous polysaccharide toxin. During viewing the amorphous electron transparent particles were seen to burst into crystals approximately 20 mμ × 40 mμ in size. The field is magnified × 60,000. C, crystal; G, globule.

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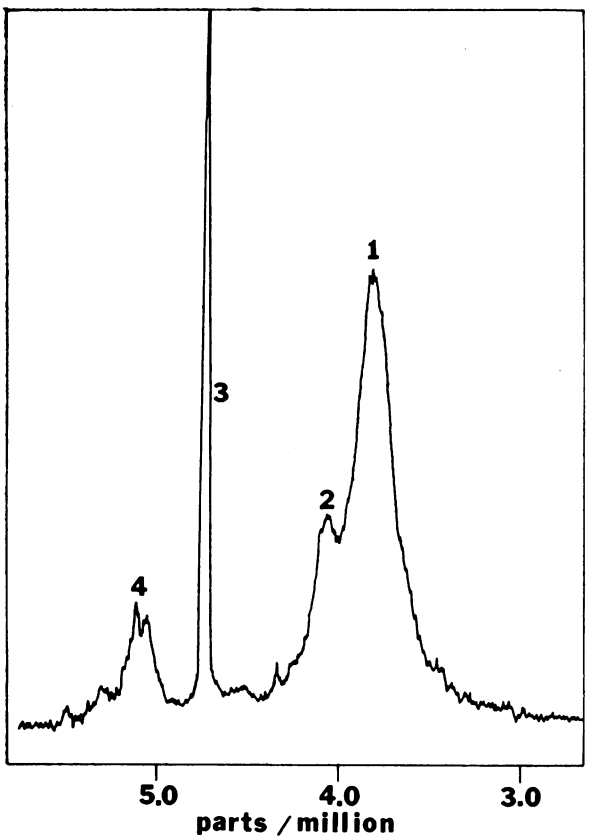

FIG. 9. Proton nuclear magnetic resonance of a 10 % solution of the polysaccharide. Peaks 1 and 2 are attributed to the C_5 and C_6 protons and the C_2, C_3 and C_4 protons respectively. Peak 4 is the proton on the C_1 involved in the acetal linkage. The D_2O peak is No. 3.

intrinsic property, other than mere size of a molecule, that may be involved in causing a plant to wilt. Both the work of Rai and the present work have shown that only 50 μg of the toxin, taken up by a 7.5 cm tomato leaf, is required for wilting to occur. The most commonly accepted theory for wilting caused by polysaccharides involves plugging of the vessels in the stem (1). It is difficult, however, to imagine how 50 μg of polysaccharide could block water flow in a tomato leaf with a diameter of 1 to 2 mm. Even though water movement in the stem is curtailed, an explanation other than plugging seems to be required. It may be suggested that the toxin affects the water relations of the cells in the area in which it becomes located, figure 5. At the present time electron microscope studies as well as ^3H_2O studies are in progress to determine the nature of the wilting phenomenon.

Approximately one-half of the biological activity of the polysaccharide was lost by refluxing for 1 minute in 0.5 N H_2SO_4 (fig 4). It is not likely that complete hydrolysis of the polysaccharide occurred in this time. The data may be interpreted as the loss of critical sugar or amino acid residues from the parent molecule resulting in loss of biological activity.

The serological reaction of the polysaccharide with an antiserum prepared to the crude toxin with a titer of 1:4 implies that high titers could be expected in antisera prepared to the purified polysaccharide. Since the serological technique was useful in detecting the polysaccharide in artificially treated leaves, it may eventually prove useful as a technique for the detection of *C. sepedonicum* in naturally infected plants in the field.

Acknowledgments

The author acknowledges the assistance of Dr. J. Robbins and G. Baker of the Department of Chemistry, Montana State University in the ultracentrifugation and NMR studies presented in this report. The assistance of Dr. T. Carroll of this department in the E.M. study is also appreciated.

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