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Isolation and Analysis of the Nucleic Acids and Polysaccharides from *Clostridium welchii*

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A method previously described for the use of bentonite in the isolation of the nucleic acids from two gram-positive organisms was applied to the isolation of the nucleic acids from two strains of *Clostridium welchii*. The nucleic acids were separated from polysaccharides by the fractional precipitation of their cetyltrimethylammonium salts from sodium chloride solution, and the base composition of the nucleic acids was determined. One strain of *C. welchii* investigated (NCTC 10578) was shown to produce considerable quantities of an acidic and also a weakly acidic or neutral polysaccharide; the other strain (ATCC 10543) gave very small quantities of the latter but none of the former polysaccharide. The monosaccharide composition of these polysaccharides was determined and the acidic polysaccharide was shown to resemble dermatan sulfate.

The isolation of nucleic acids from gram-positive microorganisms is more difficult than the corresponding isolation from gram-negative microorganisms, because of the difficulty encountered in rupturing the cell walls of the former without causing enzymatic and mechanical degradation of the nucleic acids. A procedure which can be used for many gram-positive bacteria is that described by Marmur (19) in which the cell wall is first ruptured by the use of lysozyme and the nucleic acids are extracted with sodium dodecyl sulfate. However, some gram-positive bacteria are resistant to the action of lysozyme. *Clostridium welchii* is susceptible to the action of lysozyme and the nucleic acids were obtained from this organism by the method of Marmur, but preliminary attempts at the isolation of nucleic acids from our strain of this bacterium by the use of lysozyme failed, partly because the deoxyribonucleic acid (DNA) was degraded to an extent which interfered with the separation of DNA from ribonucleic acid (RNA) by the fractionation of their cetyltrimethylammonium salts. We therefore applied to the isolation of the nucleic acids of this organism a procedure previously described (13), which involves the use of bentonite and mechanical breakage of the organisms followed by the isolation of RNA, DNA, and polysaccharides.

MATERIALS AND METHODS

Bentonite. A suspension of bentonite in 0.01 M acetate buffer, *p*H 6 was prepared as previously described (5).

Strains and conditions of growth. The following two strains of *C. welchii* were used: (i) a strain which has been in our department for over 10 years and has now been deposited in the National Collection of Type Cultures, NCTC 10578 type A (Central Public Health Laboratory, London); (ii) *C. welchii* type A, NCIB 8875 (also ATCC 10543). Both organisms were grown in Robertson cooked meat medium which had been heated to 100 C and subsequently cooled to 37 C before inoculation. The inoculum was incubated at 37 C for 18 hr and then used to inoculate 500 ml of a medium containing 0.1% Lab-Lemco beef extract, 0.2% Oxoid yeast extract, 0.5% Oxoid peptone, and 0.5% NaCl. The air was expelled from this medium by heating it in loosely closed screw-capped bottles in an autoclave for 15 min at 115 C. The medium was rapidly cooled; 0.5% sterile glucose solution was added, followed by 2 ml of inoculum; the bottle was filled to the top with more medium from which the air had been expelled, and the whole was incubated at 37 C for 18 hr.

Harvesting, cell breakage, and extraction of soluble cell components. A flow diagram of the extraction and fractionation procedures is shown in Fig. 1. The cells from 20 liters of medium were obtained by centrifugation of the medium at 0 C at 23,000 × *g*. The cells were washed with 0.14 M NaCl solution, and added to the vessel of a rotary ball mill (capacity 1 liter) with 200 ml of 0.01 M acetate buffer (*p*H 6). Ballotini beads (no. 5, 150 ml) and bentonite suspension (100 ml) were added, and the mixture was shaken at 4 C for periods of 1 hr. The mixture was then centrifuged at 23,000 × *g*, the supernatant fluid was removed, and the residue was shaken with fresh buffer. The amount of nucleic acid in the supernatant fluid could be determined directly from the absorbancy of the solution at 260 nm. In one case the initial extract of cells from 20 liters of culture of strain NCTC 10578 was examined separately. After shaking the cells for 1 hr at 4 C in a rotary ball mill as described above, the cell debris was removed and the quantity of nucleic acid in the super-

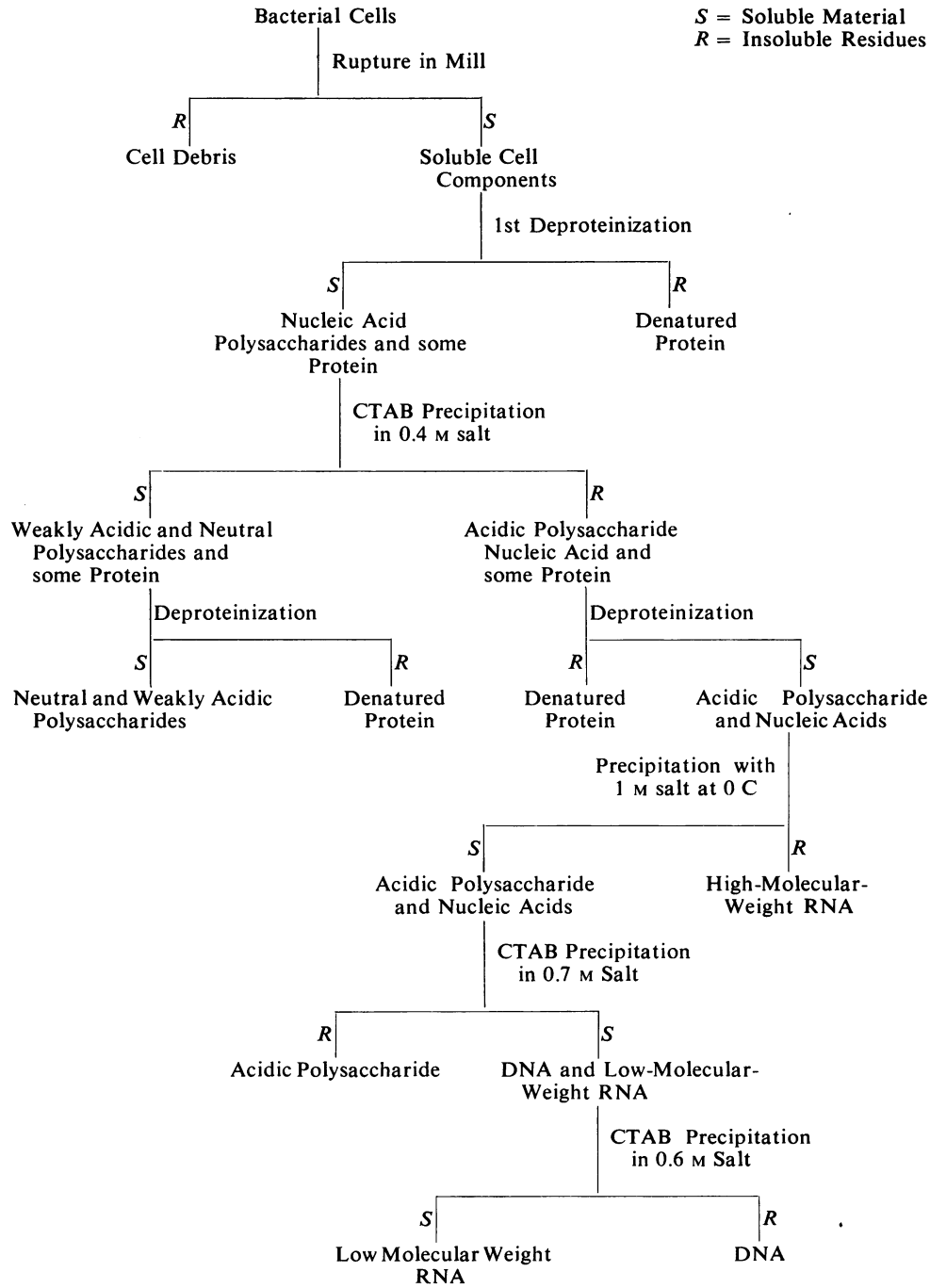

FIG. 1. *Flow diagram for extraction of nucleic acids and polysaccharides.*

natant fluid was determined. Ethanol (3 volumes) was added, and a fibrous precipitate was formed which was removed by centrifugation. It was dissolved in water (150 ml), and the protein was removed by shaking the solution with chloroform–*n*-amyl alcohol (9:1) until no denatured protein was evident as a layer at the interface. The crude polysaccharide/nucleic acid mixture was then dialyzed and freeze-dried. In other preparations, the residue from the initial extraction was reextracted until no more ultraviolet-absorbing

material could be obtained (usually five or six extractions were required); the material was precipitated from the combined extracts with ethanol and deproteinized as described above.

Fractionation of the macromolecular constituents. The deproteinized solution obtained above was made 0.4 M with respect to sodium chloride, and cetyltrimethylammonium bromide (CTAB) was added until precipitation was complete. The precipitate of the cetyltrimethylammonium salt of the nucleic acids and any highly acidic polysaccharide were removed by centrifugation, and the neutral and weakly acidic material was precipitated from the supernatant fluid by the addition of ethanol (3 volumes). This latter precipitate was dissolved in water to give a viscous solution; it was dialyzed against water and freeze-dried.

The cetyltrimethylammonium salt of the acidic material was dissolved in 1 M NaCl, and the sodium salt was precipitated by the addition of ethanol (3 volumes). The ribosomal RNA was precipitated as the sodium salt from 1 M NaCl solution as previously described (13). The salt concentration was then lowered to 0.7 M, and CTAB was added so that the final CTAB concentration was 1%. The precipitate of the cetyltrimethylammonium salt of the acidic polysaccharides was removed by centrifugation, converted into the sodium salt in the normal way, dialyzed against water, and freeze-dried. The sodium chloride concentration of the solution was then reduced to 0.6 M, and the cetyltrimethylammonium salt of the DNA was removed by centrifugation and recovered in the usual way (13). Ethanol was added to the supernatant fraction from the 0.6 M NaCl-CTAB precipitation, and the sodium salts of the RNA soluble in 1 M sodium chloride was recovered.

Analysis of nucleic acids. The total phosphorus content was estimated by the method of Jones et al. (11).

The purine and pyrimidine contents of the nucleic acids were determined as follows. DNA samples were hydrolyzed as described by Wyatt and Cohen (31) and the bases were determined as described by Wyatt (30). RNA samples were hydrolyzed and the bases were determined as described by Markham and Smith (18) after separating the bases in the solvent system described by Kirby (15).

Analysis of polysaccharides. Polysaccharide preparations were hydrolyzed for component identification by dissolving them in 2 N HCl (3 mg/ml) and heating at 100 C for 3 hr. The hydrolysates were evaporated to dryness under reduced pressure and stored in a vacuum desiccator over solid sodium hydroxide to remove final traces of HCl. Residues were separated by ascending cellulose thin-layer chromatography in pyridine–ethyl acetate–acetic acid–water (5:5:3:1). Paper electrophoretograms of the residues were developed on Whatman no. 2 paper, 0.05 M formate buffer (*p*H 3.5), with a potential of 20 v/cm. Components were revealed by the following methods: ultraviolet-absorbing materials by photography or visual examination in ultraviolet light; hexoses and hexuronic acids by silver nitrate or aniline hydrogen phthalate sprays; and 2-amino-2-deoxyhexoses by the ninhydrin spray.

The crude mixture containing polysaccharide and nucleic acid from cells of strain NCTC 10578 was examined to determine its content of amino acid and 2-amino-2-deoxyhexose. Amino acids and 2-amino-2-deoxyhexoses were liberated from the polysaccharide/nucleic acid mixture by the method of Spackman et al. (26)—hydrolysis in 6 N HCl at 110 C for 22 hr, with norleucine used as an internal control for the purpose of correcting for destruction during hydrolysis and transference loss. The composition of the hydrolysates was determined by using a Technicon Automatic Amino Acid Analyzer calibrated with a standard mixture containing 20 naturally occurring amino acids, norleucine, 2-amino-2-deoxy-D-galactose, and 2-amino-2-deoxy-D-glucose (0.05 μmole of each).

The polysaccharide fractions were hydrolyzed in 3.9 N HCl at 100 C for 9 hr, and the liberated 2-amino-2-deoxy-hexoses were determined by using the Svennerholm (27) modification of the Elson-Morgan reaction (8). 2-Amino-2-deoxy-D-glucose hydrochloride was used as a standard.

Further studies were made on the highly acidic polysaccharide. With D-glucurono-6,3-lactone used as a standard, its uronic acid content was determined by the carbazole method of Dische (6) and the modification introduced by Bitter and Muir (3). Inorganic sulfate was determined by the method of Jones and Letham (12) after hydrolysis in 2 N HCl at 100 C for 3 hr. A standard solution of sodium sulfate was used as a reference. *N*-acetyl was estimated after de-*N*-acetylation in 2 N HCl in methanol: the methyl acetate produced was determined spectrophotometrically as the hydroxamic-ferric complex (17).

The nature of the uronic acid moiety in the highly acidic polysaccharide was determined, after hydrolysis of the polysaccharide in N HCl (10 mg/ml) at 100 C for 3 hr, by gas-phase chromatography. The hydrolysate was evaporated to dryness by rotary evaporation in vacuo (bath temperature 30 C), and the residue was dissolved in redistilled analytical reagent grade dry pyridine (200 μliters). Trimethylsilylation was accomplished by the addition of hexamethyldisilazane (50 μliters) and trimethylchlorosilane (25 μliters) and warming to 37 C for 10 min. Samples (10 μliters) of the solution were analyzed by gas-phase chromatography with a Pye 104 double-column gas chromatograph. The stationary phase was 10% silicone ester 30 on celite as the solid support packed into glass columns (152 by 0.6 cm diameter), and the carrier gas was nitrogen at a flow rate of 40 ml/min. Analyses were made isothermally at 180 C: peaks were detected by a flame ionization detector and recorded on a Honeywell 0-1 mv recorder. Standards of 1,2-isopropylidene-L-idofuranurono-6,3-lactone and dermatan sulfate were hydrolyzed, and derivatives were made under identical conditions and analyzed by gas-phase chromatography. Standards of D-glucurono-6,3-lactone and D-galacturonic acid were dissolved in N HCl (5 mg/ml), rotary-evaporated to dryness, and chromatographed as their trimethylsilyl derivatives.

The highly acidic polysaccharide was also analyzed for the acidic mucopolysaccharides, hyaluronic acid,

chondroitin 4- and 6-sulfates, and dermatan sulfate, by the microscale method established by Barker, Kennedy, and Somers (1). The polysaccharides were incubated with chondroitinase, extracted from *Proteus vulgaris* in 0.025 M tris(hydroxymethyl)aminomethane buffer (*p*H 8.0) for 24 hr, and the products were subjected to periodate oxidation studies. Oxidation was carried out with 0.2 M sodium metaperiodate at 20 C for 20 min. The periodate oxidation products were analyzed for malondialdehyde and β-formyl pyruvic acid by the Warren (29) spectrophotometric assay. The absorption spectrum of the chromophore was determined (malondialdehyde, λmax 532 nm; β-formyl pyruvic acid, λmax 549 nm).

RESULTS

Isolation and composition of the nucleic acids. The nucleic acids were isolated and analyzed by the methods described. The results are given in Table 1. The samples of RNA contained no detectable DNA. (The procedure used would detect only 5% DNA because of the small amounts available.) The DNA samples gave 100% reaction with the Dische Diphenylamine Reagent (7), and the absence of uracil in the base analyses showed that RNA was virtually absent.

Composition of the crude polysaccharide/nucleic acid mixture. The crude mixture of polysaccharide and nucleic acid which was extracted from cells of strain NCTC 10578 was shown to contain 14% nucleic acid. As the material had been rigorously extracted with chloroform *n*-amylalcohol, it was suspected that the remaining 86% of this material was polysaccharide and associated protein. The mixture was examined by hydrolysis and chromatography, and the following major monosaccharide components were identified: D-galactose, 2-amino-2-deoxy-D-galactose, and D-galacturonic acid. Amino acid analysis with the autoanalyser demonstrated the presence of the following amino acids, the quantities being proportions of the total amino acids detected: 18.8% glutamic acid, 14.8% aspartic acid, 23.1% serine, 8.4% threonine, 5.8% histidine, 11.5% alanine, 5.0% isoleucine, 4.5% valine, and 3.1% of other amino acids. By this technique both 2-amino-2-deoxy-D-galactose and 2-amino-2-deoxy-D-glucose were also identified and shown to be present in the ratio 3:1. The molar ratio of 2-amino-2-deoxyhexoses to amino acids was shown to be 3.7:1.

TABLE 1. *Yields[a] and analyses of the nucleic acids isolated from two strains of Clostridium welchii*

Yields	NCTC 10578	ATCC 10543	ATCC 10543[b]
Soluble RNA			
Weight obtained	7.6 mg	6.8 mg	
Ribosomal RNA			
Weight obtained	22 mg	21 mg	
Phosphorus content	8.9%	8.9%	
Base content[c]			
Guanine	29.4	29.6	
Adenine	28.3	28.4	
Cytosine	21.9	21.6	
Uracil	20.4	20.4	
DNA			
Weight obtained	4.1 mg	3.7 mg	
Phosphorus content	8.4%	9.0%	8.8%
Base content[c]			
Guanine (G)	15.5	15.4	15.5
Adenine	34.7	34.7	34.8
Cytosine (C)	15.5	15.6	15.5
Thymine	34.3	34.3	34.2
GC content	31%	31%	31%

[a] Micrograms of freeze-dried cells per gram.
[b] This sample of DNA, isolated by Marmur's method (19), was obtained from the Worthington Biochemical Corp., Freehold, N.J.
[c] Expressed as moles per 100 nucleotides.

Composition of weakly acidic/neutral polysaccharides. The yield of weakly acidic or neutral polysaccharides was 7.5 mg of freeze-dried cells per g from strain NCTC 10578 and 1.3 mg of freeze-dried cells per g from strain ATCC 10543. Hydrolysis and chromatographic analysis showed that polysaccharides from both sources contained the same monosaccharides. D-Galactose, 2-amino-2-deoxy-D-galactose, and D-galacturonic acid were identified along with smaller amounts of D-glucose and 2-amino-2-deoxy-D-glucose. Because of the limitations imposed by sample size, a further analysis was made only of the polysaccharides from the first strain. Optical rotation studies showed an $[\alpha]_D^{28}$ of $+83 \pm 4°$ (0.4% water).

Composition of acidic polysaccharide. The yield of acidic polysaccharide from strain NCTC 10578 was 1.0 mg of freeze-dried cells per g, but there was no detectable yield from strain ATCC 10543. Although the polysaccharide contained 8% nucleic acid, the small amount of material precluded further fractionation.

The polysaccharide had an $[\alpha]_D^{28}$ of $-43 \pm 13°$ (0.29% water). Acidic hydrolysis gave 2-amino-2-deoxy-D-galactose, which was identified chromatographically and electrophoretically, and two other components. One of these had a chromatographic and electrophoretic mobility similar to that of a uronic acid, but was not glucuronic or galacturonic acid, whereas the other behaved as a neutral disaccharide. As the acidic hydrolysis time was reduced, the proportion of this overall neutral compound increased. No neutral monosaccharides were detected.

When uronic acid was determined by the Dische (6) method, the uronic acid to sulfate molar ratio

was 0.5:1. A ratio of 1.6:1 was obtained by using the Bitter and Muir (3) modification of the Dische (6) method. These results are consistent with the uronic acid having the L-*ido*-configuration rather than the D-*gluco*-configuration, since the relative color yields of D-glucuronic acid and L-iduronic acid in the Dische (6) and Bitter and Muir (3) methods are 0.50:1 and 0.28:1 (sample 0.32:1). The molar ratio of 2-amino-2-deoxyhexose to sulfate was 1.12:1, and the molar ratio of *N*-acetyl to sulfate was 1.24:1.

Gas-phase chromatography of the trimethylsilyl derivatives of the acidic hydrolysis products gave a peak which corresponded exactly with the single peak obtained under identical conditions for the standard L-idurono-6,3-lactone. Apart from the minor peaks attributable to 2-amino-2-deoxy-D-galactose, the hydrolysis products of dermatan sulfate gave a single peak which corresponded exactly with the peak obtained for the L-idurono-6,3-lactone standard and the acidic polysaccharide. Similar analysis of D-glucurono-6,3-lactone and of D-galacturonic acid gave no peaks in the vicinity of the one attributable to L-idurono-6,3-lactone.

Assay (29) of the periodate oxidation products of the chondroitinase-treated polysaccharide gave a sharp chromophore absorption maximum of 549 nm. This liberation of β-formyl pyruvic acid indicated hydrolysis of the parent polysaccharide by chondroitinase to give 4-deoxy-α-L-*threo*-hex-4-enosyluronic acids.

DISCUSSION

The isolation of pure nucleic acids from *C. welchii* NCTC 10578 was complicated by the presence of polysaccharides which precipitated with the DNA throughout the fractionation procedure and which also caused the solutions to have a high viscosity which interfered profoundly with the separation of DNA from RNA by the fractionation of their cetyltrimethylammonium salts in the manner previously described (10, 13). When the RNA was destroyed by treatment with ribonuclease, the DNA samples so obtained contained about 50% polysaccharide. The modification of the fractionation procedure described here gave, however, essentially pure samples of nucleic acids and polysaccharides, although the phosphorus content of the DNA was slightly low. In this procedure the weakly acidic or neutral polysaccharide was separated from the remainder of the acidic material by precipitation of the latter from a solution of 0.4 M NaCl by CTAB. All highly acidic material, such as the nucleic acids and sulfated polysaccharides, is precipitated at this concentration of salt. The preliminary removal of the neutral polysaccharide resulted in a considerable decrease in the viscosity of the solution and facilitated the subsequent fractionation of the acidic material. The microsomal RNA was separated by precipitation from 1 M NaCl solution, and the sulfated polysaccharide was separated from the DNA and soluble RNA by precipitation at 0.7 M NaCl with CTAB. Scott (24) has shown that sulfated polysaccharides can be precipitated by CTAB from 0.7 M NaCl, whereas polysaccharides containing only carboxylic acid functions are precipitated at a much lower concentration. The DNA and soluble RNA were separated in the usual way (10).

The molecular weight of 3×10^6 obtained for the DNA showed that, although some degradation had occurred, the DNA was sufficiently undegraded to be of use in cross hybridization experiments with high-molecular-weight DNA. The base composition of the DNA found here [31% guanine plus cytosine (GC) content] lies within the range (26.5 to 32.0% GC) previously reported (20, 23).

There was a marked difference in the results obtained from the isolations from the two strains NCTC 10578 and ATCC 10543. The latter contained hardly any neutral polysaccharide fraction and no detectable acidic polysaccharide. Furthermore, a sample of DNA obtained from the Worthington Biochemical Corp. and isolated from *C. welchii* ATCC 10543 by the method of Marmur (19) was pure. Had the bacterium originally contained an acidic polysaccharide, the DNA would have contained this as an impurity since the method fails to separate the two. Observation of the two strains, after growth under identical conditions by phase-contrast microscopy, showed that NCTC 10578 had a clearly defined capsule whereas ATCC 10543 possessed no visible capsule. It was shown by Smith (25) that continuous subculturing of smooth varieties of *C. welchii* in meat broth often gives rise to mucoid variants which are covered with a slime layer. This was further investigated by Izumi (9), who showed that a polysaccharide was excreted into the medium by these variants. He separated the polysaccharide into two fractions consisting of a neutral polysaccharide and an acidic mucopolysaccharide-like substance. An attempt to isolate a polysaccharide by the method of Izumi (9) from the culture fluids in which the two strains used here had been grown gave no comparable amount of material. (He obtained 470 mg from 2 liters of culture after a preliminary purification procedure.) Thus it would appear that strain ATCC 10543 does not produce a polysaccharide in any quantity, and that NCTC 10578 has a capsule which is more rigidly attached to the cell than is the slime layer of the mucoid variants.

A crude sample of the polysaccharide material from the capsule of the strain NCTC 10578 was shown to contain a number of amino acids and, since the material had been deproteinized, it is unlikely that they arose from nonassociated protein. Many polysaccharides exist as protein complexes, and it has been shown that the hydroxyl group of serine can be involved in the carbohydrate to protein link (16). The high value for serine obtained in the amino acid analysis of this crude material may therefore be significant.

The results obtained for the base content of the nucleic acids are similar for the two strains, and for DNA, to the results obtained for the commercially available sample of *C. welchii* DNA.

The weakly acidic or neutral polysaccharide obtained from the NCTC 10578 contained the same monosaccharides as the polysaccharide from ATCC 10543. The polysaccharide(s) contained 2-amino-2-deoxy-D-galactose, 2-amino-2-deoxy-D-glucose, D-galactose, D-glucose, and D-galacturonic acid. It is unlikely that all these monosaccharides were derived from one polysaccharide, and presumably the carbohydrate material contained a mixture of polysaccharides which had the same general characteristics.

The acidic polysaccharide was detected only in strain NCTC 10578. Per mole of sulfate, the polysaccharide contained 1.12 moles of 2-amino-2-deoxyhexose, 1.24 moles of *N*-acetyl, and 1.6 moles of uronic acid when determined by the Bitter and Muir (3) method. The 2-amino-2-deoxyhexose was identified as 2-amino-2-deoxy-D-galactose, whereas thin-layer chromatographic evidence indicated the presence of L-iduronic acid. This configurational assignment to the uronic acid was substantiated by the uronic acid determinations. Any protein present would have tended to give a high value for uronic acid.

Proof that the uronic acid was in fact iduronic acid was obtained by gas-phase chromatography as the trimethylsilyl derivative. Although several peaks might be expected from the lactones and free acid forms of a uronic acid, it has been shown (submitted for publication) that a single peak is obtainable under certain conditions as in the present investigation. The mobility of the derivative was identical to that obtained from an L-idurono-6,3-lactone standard and from a dermatan sulfate standard.

Periodate oxidation of the chondroitinase-hydrolyzed polysaccharide showed that β-formyl pyruvic acid was formed by periodate oxidation. This demonstrated that the chondroitinase had hydrolyzed the polysaccharide to give 4-deoxy-α-L-*threo*-hex-4-enosyluronic acids. No malondialdehyde, which would have arisen from 2-deoxy-D-ribose, was detected. The enzymatic action therefore demonstrates that the polysaccharide contains a β-(1 → 4)-2-acetamido-2-deoxy-D-hexose to uronic acid linkage, and that the uronic acid has the D-*gluco*- or L-*ido*-configuration—the enzyme is specific for this linkage and these uronic acids (see reference 1). Combining this result with the results of gas-phase chromatographic identification of the uronic acid and electrophoretic and paper chromatographic identification of the 2-amino-2-deoxyhexose, it would appear that the polysaccharide contains a β-(1 → 4)-2-acetamido-2-deoxy-D-galactose to L-iduronic acid linkage.

Since the polysaccharide also contained, within experimental limits, equimolar proportions of sulfate, uronic acid, 2-amino-2-deoxy-hexose, and *N*-acetyl groups, the results indicate that it possessed the repeating structure of the acidic mucopolysaccharide, dermatan sulfate, i.e., (1 → 4)-*O*-α-L-idopyranosyluronic acid-(1 → 3)-2-acetamido-2-deoxy-4-*O*-sulfo-β-D-galactopyranose. The existence of a repeating unit was further substantiated by the increased yield of an overall neutral disaccharide with decreased hydrolysis time. The approximate value for the specific rotation was of the same order as that quoted for dermatan sulfate (−59°) by Meyer et al. (21).

Mucopolysaccharides have been detected previously in bacterial capsules. Hyaluronic acid has been found in *Aerobacter aerogenes* (28), *Streptococcus pyogenes* (22), *Pseudomonas aeruginosa* (4), and encapsulated strains of Group A streptococci (14). Although dermatan sulfate is usually isolated from mammalian tissues (1, 21), it appears to exist in the capsule of some strains of *C. welchii*.

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LITERATURE CITED

1. Barker, S. A., J. F. Kennedy, and P. J. Somers. 1968. Methods for the microscale identification of some acidic mucopolysaccharides. Carbohyd. Res. 8:482–490.
2. Barker, S. A., J. F. Kennedy, and P. J. Somers. 1969. The acidic mucopolysaccharides of human skin. Carbohyd. Res. 10:57–63.
3. Bitter, T., and H. Muir. 1962. A modified uronic acid carbazole reaction. Anal. Biochem. 4:330–334.
4. Bonde, G. J., F. E. Carlsen, and C. E. Jensen. 1957. Production of hyaluronic acid by *Pseudomonas aeruginosa*. Acta Pharmacol. Toxicol. 13:205–212.
5. Brownhill, T. J., A. S. Jones, and M. Stacey. 1959. The inactivitation of ribonuclease during the isolation of ribonucleic acid and ribonucleoproteins from yeast. Biochem. J. 73:434–438.
6. Dische, Z. 1947. A new specific color reaction of hexuronic acids. J. Biol. Chem. 167:189–198.
7. Dische, Z. 1955. Color reactions of nucleic acid components,

p. 285–305. *In* E. Chargaff and J. N. Davidson (ed.), The nucleic acids. Academic Press Inc., New York.
8. Elson, L. A., and W. T. J. Morgan. 1933. A colorimetric method for the determination of glucosamine and chondrosamine. Biochem. J. **27**:1824–1828.
9. Izumi, K. 1962. Mucopolysaccharides produced by a strain of *Clostridium perfingens*. J. Bacteriol. **83**:956–959.
10. Jones, A. S. 1963. The use of alkyltrimethylammonium bromides for the isolation of ribo- and deoxyribonucleic acids. Nature (London) **199**:280–282.
11. Jones, A. S., W. A. Lee, and A. R. Peacocke. 1951. The determination of phosphorus in deoxypentose nucleic acids. J. Chem. Soc. 623–624.
12. Jones, A. S., and D. S. Letham. 1956. A spectrophotometric method for the determination of sub-micro quantities of sulphur with 4-amino-4-chlorodiphenyl. Analyst **81**:15–18.
13. Jones, A. S., and R. T. Walker. 1968. The isolation of nucleic acids from gram-positive micro-organisms. Arch. Biochem. Biophys. **128**:579–582.
14. Kendall, F. E., M. Heidelberger, and M. H. Dawson. 1937. A serologically inactive polysaccharide elaborated by mucoid strains of group A hemolytic streptococcus. J. Biol. Chem. **118**:61–69.
15. Kirby, K. S. 1955. Some new solvent systems for the paper chromatography of nucleic acid degradation products. Biochim. Biophys. Acta **18**:575–576.
16. Lindahl, U., and L. Roden. 1966. The chondroitin 4-sulphate-protein linkage. J. Biol. Chem. **241**:2113–2119.
17. Ludowieg, J., and A. Dorfmann. 1960. A micromethod for the colorimetric determination of *N*-acetyl groups in acid mucopolysaccharides. Biochim. Biophys. Acta **38**:212–218.
18. Markham, R., and J. D. Smith. 1951. Chromatographic studies of nucleic acids. 4. The nucleic acids of the tobacco yellow mosaic virus including a note on the nucleic acids of the tomato bushy stunt virus. Biochem. J. **49**:401–407.
19. Marmur, J. 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. J. Mol. Biol. **3**:208–218.
20. Marmur, J., and P. Doty. 1962. Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. J. Mol. Biol. **5**:109–118.
21. Meyer, K., D. Davidson, A. Linker, and P. Hoffman. 1956. The acidic mucopolysaccharides of connective tissue. Biochim. Biophys. Acta **21**:506–518.
22. Pierce, A. W., and A. G. C. White. 1954. Hyaluronic acid formation by *Streptococcus pyogenes*. Proc. Soc. Exp. Biol. Med. **87**:50–54.
23. Schildkraut, C. L., J. Marmur, and P. Doty. 1962. Determination of the base composition of deoxyribonucleic acid from its buoyant density in CsCl. J. Mol. Biol. **4**:430–443.
24. Scott, J. E. 1962. The precipitation of polyanions by long-chain aliphatic ammonium salts. 6. The affinity of substituted ammonium cations for the anionic groups of some biological polymers. Biochem. J. **84**:270–275.
25. Smith, H. W. 1959. The bacteriophages of *Clostridium perfringens*. J. Gen. Microbiol. **21**:622–630.
26. Spackman, D. H., W. H. Stein, and S. Moore. 1958. Automatic recording apparatus for use in the chromatography of amino acids. Anal. Chem. **30**:1190–1206.
27. Svennerholm, L. 1957. The determination of hexosamines with special reference to nervous tissue. Acta Soc. Med. Upsal. **61**:287–306.
28. Warren, G. H. 1950. The isolation of a mucopolysaccharide from *Aerobacter aerogenes*. Science **111**:473–474.
29. Warren, L. 1959. The thiobarbituric acid assay of sialic acids. J. Biol. Chem. **234**:1971–1975.
30. Wyatt, G. R. 1951. The purine and pyrimidine composition of deoxypentose nucleic acids. Biochem. J. **48**:584–590.
31. Wyatt, G. R., and S. S. Cohen. 1953. The bases of the nucleic acids of some bacterial and animal viruses: the occurrence of 5-hydroxymethyl cytosine. Biochem. J. **55**:774–782.