Investigations on Polysaccharide Components of Cells of *Herpetomonas samuelpessoai* Grown on Various Media†

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ABSTRACT: *Herpetomonas samuelpessoai*, when cultured in various media, forms a linear β-D-(1→2)-mannopyranan and a branched-chain glucuronoxylan containing D-glucopyranosyluronic acid nonreducing end units connected with α- and β-linked D-xylopyranose units, as depicted in fragmental structures I, II, and III. Traces of amylose and α-linked mannopyranose moieties are also present. Mannose-containing materials predominate over glucuronoxylan in cells grown with proline as the carbon source (medium C). In a medium with sucrose as the carbon source and complex supplements (medium A) the proportion of glucuronoxylan is greater, and when the supplements were all chemically defined (medium B), a galactose-containing component is also formed. Glucuronoxylan is liberated from cells with hot aqueous alkali and it could be freed from lower molecular weight mannan by fractional precipitation. Mannan was obtained as the only polymeric component, with lower molecular weight homologues, by extraction of cells with hot water. In terms of ratios of component xylose, mannose, and galactose, the flagella resemble whole cells. However, flagella contain polysaccharide with α-D-linked mannopyranose side chains rather than β-linked linear forms. These data are considered from standpoints of immunochemistry and electron microscopy.

The insect flagellate *Herpetomonas samuelpessoai*, when injected into mice, either as living cells (Souza et al., 1974; Takehara & da Silva, 1975) or as flagellar suspensions (Pereira et al., 1977), causes protection against infection by *Trypanosoma cruzi*. We believe that it is possible that this immunologic property is due to the polysaccharide or glycoproteins which are reported to exist at the surface of the cell membrane and flagella (de Souza et al., 1976).

In order to shed light on the protection phenomenon it is necessary to determine, at least as a first approach, the detailed chemical structure of purified polysaccharide components of *H. samuelpessoai* and *T. cruzi* and other organisms such as *Crithidia fasciculata* (Johnson et al., 1963; Takehara & da Silva, 1975; P. A. J. Gorin et al., to be published) and *Crithidia deanei* (Takehara & da Silva, 1975; Previato et al., 1979), whose protective properties against *T. cruzi* have also been studied. Furthermore, it is necessary to examine polysaccharides or glycoproteins from cells grown under a variety of conditions, since variations in polysaccharide contents occur in different preparations of *T. cruzi*. The polysaccharide extract of cells of the culture (epimastigote) form containing galactose, glucose, mannose, xylose, and glucosamine showed antigenic activity against a rabbit immune serum or a serum of a patient with Chagas' disease, even when the protein portion was removed (Gonçalves & Yamaha, 1969). However, when other antigenically active *T. cruzi* extracts were used to obtain immunosera from rabbits, this material was a polysaccharide complex containing only galactose and mannose (Gottlieb, 1977). Also, a complex polymer containing galactose, mannose, and a trace of glucose (de Lederkremer et al., 1976) has been obtained from epimastigote forms. Cell membrane surface carbohydrates contain units which show structural variation (de Souza & Meyer, 1975), since trypo- and epimastigote forms have been reported to react with concanavalin A, whereas in other experiments only epimastigote cells reacted (Alves & Colli, 1974).

In other examples, *C. fasciculata* gives mannan or arabinogalactan depending on culture conditions (P. A. J. Gorin et al., to be published), and *C. deanei* can also be manipulated to give a mannan or a polysaccharide mixture containing mannose, glucose, and rhamnose (Previato et al., 1979).

Materials and Methods

Culture Conditions. *H. samuelpessoai*, originally isolated from the gut of the reduviid *Zelus leucogrammus* (Galvão et al., 1970), was kindly supplied by Professor Isaac Roitman of the University of Brasilia. Cells were grown on three different media, including a chemically defined one containing sucrose, glycerophosphate, amino acids, vitamins, purine, and hemin (Roitman et al., 1972), which is referred to as medium B. The other media utilized sucrose (medium A) or proline (medium C) as the carbon source and contained (g/L) sucrose or proline (20), yeast extract (3), trypticase (3), KCl (20), folic acid (0.02), and hemin (0.01) (dissolved in 1 M NaOH), and the pH was finally adjusted to 7.0.

Large-scale runs were carried out as 20-L batches in a 25-L New Brunswick fermentor for 48 h at 28 or 37 °C with agitation (100 rpm) and aeration (8 L/min).

Isolation of Polysaccharides. Cells were isolated from a large-scale run by centrifugation and washed three times with physiological saline. They were extracted with 6% aqueous KOH (200 mL) for 6 h at 100 °C, the solution was neutralized (AcOH) and centrifuged, and the supernatant was added to EtOH (3 v/v). The precipitate was isolated, dissolved in H_2O, and passed through a mixed-bed resin column to remove nucleic acids. The eluate was concentrated to a small volume which was acidified with AcOH, the solution was centrifuged to remove the insoluble material, and polysaccharide was precipitated and isolated following addition of excess ethanol to the supernatant. Yield of dry cells and percentage yield of polysaccharide, based on cell weight, were as follows: proline medium (medium C) 1.5 g, 4%; and complex sucrose medium (medium A) 5.0 g, 3%. Values were not obtained for the chemically defined sucrose medium B.

To obtain mannose-containing components, cells isolated from 20 L of sucrose medium with complex additives were autoclaved at 120 °C for 30 min. The supernatant obtained on centrifugation was evaporated to a small volume and excess ethanol added. The resulting polysaccharide (80 mg) was

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isolated by filtration and the mother liquor evaporated to a syrup (30 mg).

Enrichment of Flagella (Piccini & Albergoni, 1973). Cells from chemically defined (medium B) and complex sucrose media (medium A) were shaken in cold 20% glycerol, the suspension being centrifuged at 1000*g* for 15 min, and the flagellar concentrate was isolated from the supernatant using 14000*g* for 15 min. The pellet appeared to contain, according to visual and electron microscopy, cellular flagellar fragments, and the process was repeated two more times to remove the cellular contaminants.

Detection and Analysis of Component Sugars of Polysaccharide. Monosaccharides arising from cells or KOH extracts were detected by paper chromatography following hydrolysis with 0.5 M H_2SO_4 for 18 h at 100 °C (Hough & Jones, 1962). Uronic acids were liberated using 4 M HCl at 100 °C for 6 h. Chromatograms were sprayed with *p*-anisidine hydrochloride following development with ethyl acetate–acetic acid–water (9:2:2 v/v/v; solvent A) and 1-butanol–ethanol–water (40:11:19 v/v/v; solvent B). Quantitative estimations were carried out by reduction of sugars with sodium borohydride, followed by acetylation and GLC of the resulting alditol acetates (Sawardeker et al., 1965).

D-Xylose was isolated from a cellulose column (eluant: acetone–water, 9:1 v/v) by fractionation of the polysaccharide hydrolysate (100 mg; cells from complex sucrose medium A).

Glucosamine was characterized and estimated in the polysaccharide by hydrolysis with 2 M HCl at 100 °C for 6 h and use of a Model 120 C Beckman amino acid analyzer.

Methylation of Polysaccharides. This was carried out by a method utilizing a column liquid phase of ECNSS-M (Supelco, Inc., Bellefonte, PA) in a GLC procedure. The resulting O-methylated alditol acetates were characterized by their retention times and distinctive EI mass spectral patterns (Jansson et al., 1976). In the case of glucuronoxylan from cells grown on medium A, it was methylated successively by Haworth and Kuhn procedures, and the product was converted to a mixture of methyl *O*-methylaldosides by successive treatments with refluxing 3% methanolic hydrogen chloride, aqueous 10% H_2SO_4 at 100 °C, and refluxing 3% methanolic hydrogen chloride (Gorin et al., 1969). The aldosides were characterized by their retention times on GLC (Jones, 1972) and quantitated approximately by the areas of the peaks. Methylated glucuronoxylan was treated successively with refluxing 3% methanolic hydrogen chloride and 10% aqueous H_2SO_4 at 100 °C (Gorin et al., 1969), and the resulting *O*-methylaldoses were examined on a paper chromatogram (solvent B; spray, *p*-anisidine hydrochloride).

Periodate Oxidation. Glucuronoxylan containing less than 8% of mannan (medium A) was oxidized with sodium metaperiodate, and the consumption of oxidant and the production of formic acid were measured over a period of time (Hay et al., 1965).

Carbon-13 Nuclear Magnetic Resonance Spectroscopy. ^{13}C NMR spectra were obtained from solutions of polysaccharide in D_2O at 70 °C under conditions previously described (Gorin et al., 1977). With a 200-mg sample, 80 000 transients were collected using full proton decoupling and the same operation parameters as indicated. Runs were carried out with coupling, or partial coupling when the decoupling frequency was the same as that of Me_4Si, and at a lower frequency of 400 Hz.

Degradation of Cellular Components with NaOH–$NaBH_4$. Cells (~3 g) derived from the proline medium C (4 L) were suspended in 4% sodium hydroxide (300 mL) containing sodium borohydride (11 g) at 100 °C for 5 h (Lee et al., 1972). The solution was neutralized with acetic acid and centrifuged, and the supernatant was deionized with mixed Amberlite 1R120 (H^+ form) and Dowex 1-X8 (HCO_3^- form). To the solution (0.5 L), ethanol (1.5 L) was added and the resulting precipitate (105 mg) isolated by filtration. The mother liquor was evaporated to a syrup which was triturated with methanol, and the mother liquor in turn was examined on a paper chromatogram (solvent B; spray, ammoniacal silver nitrate). The resulting white suspension was isolated, and salts were removed by deionization, and polysaccharide was precipitated from a small volume of water using excess ethanol to yield 50 mg. The mother liquor from the ethanol precipitation process was examined on a paper chromatogram (solvent: 1-butanol–ethanol–water, 1:1:1 v/v/v; spray, ammoniacal silver nitrate).

Characterization of Partial Hydrolysis Products of Glucuronoxylan. Glucuronoxylan (50 mg) containing less than 8% of mannan (medium A) was treated with 0.025 M H_2SO_4 (5 mL) for 18 h at 100 °C, and the solution was neutralized ($BaCO_3$), filtered, evaporated to dryness, and divided into two equal portions. One portion of residue and a product, obtained under identical conditions from the xylose-containing polysaccharide from *Tremella mesenterica* (Fraser et al., 1973), were compared on paper chromatograms (solvents A and B; spray, *p*-anisidine hydrochloride).

The other portion of the partial hydrolysate of *H. samuelpessoai* glucuronoxylan was dissolved in water (0.2 mL), and acetic acid (5 mL) was added, followed by lead tetraacetate (0.20 g) (Perlin, 1959). After 10 min, ethylene glycol (0.05 mL) was added, the solution was evaporated, the residue was dissolved in water, and the solution was deionized with mixed resins. Filtration and evaporation provided a residue which was examined and compared on paper chromatograms with the above partial hydrolysate of glucuronoxylan.

Glucuronoxylan (20 mg) was partially hydrolyzed in 0.5 M H_2SO_4 (2 mL) for 1 h at 100 °C, the solution was neutralized ($BaCO_3$), and the filtrate was treated with Amberlite IR-120 (H^+ form). Acidic components of the filtrate were absorbed on Dowex 1-X8 (HCO_3^- form) and following washing of the resin with water the uronic acid containing oligosaccharides were eluted with 5% aqueous formic acid. They were examined on a paper chromatogram (solvent: ethyl acetate–acetic acid–water, 3:1:1 v/v/v; spray, *p*-anisidine hydrochloride) and a portion was treated with lead tetraacetate, as above.

An acidic fraction was obtained in a similar manner following treatment of glucuronoxylan (20 mg) with 0.025 M H_2SO_4 (2 mL) for 3 h at 100 °C. The product was refluxed in 3% methanolic hydrogen chloride (3 mL) for 1 h, and the solution was neutralized (Ag_2CO_3), filtered, and evaporated. The residue was dissolved in 0.1 M sodium methoxide in methanol, sodium borohydride (20 mg) was added, and after 18 h at 0 °C followed by reflux, the solution was deionized with ion-exchange resins in water and the product hydrolyzed with 0.5 M H_2SO_4 for 18 h at 100 °C. The product was examined on paper chromatograms (solvents A and B; spray, *p*-anisidine hydrochloride). One portion was treated with D-glucose oxidase and another with sodium borohydride, followed by acetic anhydride–pyridine, to give alditol acetates, which were characterized by GLC.

Enzymic Degradations. The products from the following degradations were examined on paper chromatograms (solvents A and B; spray, *p*-anisidine hydrochloride). (a) Samples of glucuronoxylan (10 mg) containing mannan, obtained by KOH extraction of cells or flagella, were treated with exo-α-D-

Table I: Sugar Composition of Cells and Polysaccharide Extracts of *H. samuelpessoai*

medium[a]	% of component sugar			
	xylose	mannose	galactose	glucose
A, whole cells	72	28		tr[d]
A, KOH extract	85	15		tr
B, whole cells	53	24	22	tr
B, KOH extract	54	32	14	tr
C, whole cells	31	69		tr
C, KOH extract	83	17		
A, flagella-1[b]	40	18		42
flagella-2[c]	86	14		
A, flagellar KOH extract	52	19		29
B, flagella	37	6	26	31

[a] Medium A, sucrose carbon source, complex additives; medium B, sucrose carbon source, chemically defined additives; medium C, proline carbon source, complex additives. [b] Flagella prepared with one fractionation step. [c] Flagella prepared with three fractionation steps. [d] tr = trace.

mannosidase (2 mg; Jones & Ballou, 1969) in water (0.1 mL) for 18 h. The enzyme was active against bakers' yeast mannan and a α-D-(1→2)-linked mannotriose under similar conditions. (b) The KOH extract of a flagellar preparation contaminated with other cell debris after one centrifugation (see above) was treated with glucoamylase, which caused degradation to glucose (Pazur, 1964). The component was characterized as amylose by virtue of the intensity of its reaction with iodine (McReady & Hassid, 1943).

Results

The sugar composition of cells of *H. samuelpessoai* varied considerably depending on the growth medium. For example, in those with sucrose as the carbon source the proportion of xylose compared with mannose, obtained on hydrolysis of whole cells, was high, but in a chemically defined medium (B) galactose was formed, whereas it was not detected when complex additives were used. In some experiments, sucrose was substituted with proline as the carbon source (medium C) since this is a component of the contents of the insect gut, its natural habitat (Bursell, 1966; Raghupathi & Campbell, 1969). In such cases mannose predominated over xylose (Table I), and as in each of the other hydrolyses, ribose was detected, arising from the RNA cell component.

Treatment of cells from medium A with 6% aqueous KOH at 100 °C destroyed protein, leaving polysaccharide and traces of RNA. The precipitate obtained with excess ethanol, following neutralization with AcOH, was freed of residual RNA by a deionization procedure. Xylose and mannose were components of each polysaccharide, but the proportion of the latter was diminished compared with whole cells (Table I). In order to determine whether this difference is due to a partial fractionation on ethanol precipitation of components of differing molecular weight, the following experiment was carried out. Cells of *H. samuelpessoai* obtained from the proline medium (medium C), containing xylose and mannose in a 35:65 ratio, were treated with aqueous sodium hydroxide containing sodium borohydride at 100 °C. Ethanol precipitation gave a fraction with a xylose–mannose ratio of 83:17. The mother liquor was evaporated to a syrup which was triturated with methanol, the insoluble portion was precipitated from water with ethanol, and the product was examined by ^{13}C NMR spectroscopy. The spectrum (Figure 1B) corresponded to that of a β-D-(1→2)-linked mannopyranan obtained from *C. deanei*. This structure was confirmed by a methylation

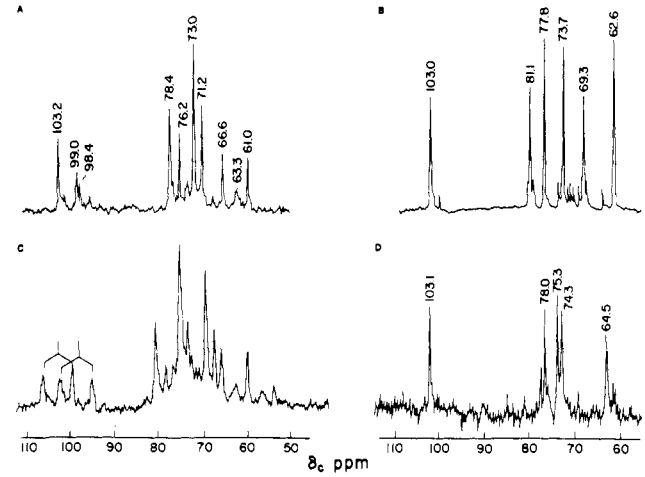

FIGURE 1: Carbon-13 NMR spectra of (A) glucuronoxylan from cells of *H. samuelpessoai* obtained from proline (medium C) and complex sucrose media A; (B) mannan of *H. samuelpessoai*; (C) above glucuronoxylan, obtained without proton decoupling; (D) β-D-(1→4)-linked xylopyranan of wood.

experiment in which 3,4,6-tri-*O*-methylmannitol triacetate was obtained and characterized by GLC–MS, in agreement with a (1→2)-pyranose linkage. The polysaccharide's $[\alpha]^{25}_D$ of −59° corresponded to −50° for the mannan of *C. deanei* (Previato et al., 1979), which has (1→2)-glycosidic linkages with the β configuration. Other components of the above methanol-soluble fraction were detected by paper chromatography and GLC of their acetates. They were glycerol, *myo*-inositol, and rhamnitol, the latter apparently existing as single units attached to an alkaline-labile link.

The mother liquor obtained following precipitation of the mannan with ethanol was also examined on paper chromatograms. The R_f values of the resulting spots, compared with that of *myo*-inositol which was also present, were 0.41, 0.50, 0.59, 0.71, 0.84, 1.20, and 1.47, the last two corresponding to mannopyranosylmannitol and mannitol, respectively. It appeared possible that each of the spots corresponds to a reduced mannose-containing oligosaccharide that is formed by degradation of alkali-labile linkages or by direct extraction (see below). Their low molecular weight would thus explain the diminution of the mannose component during the preparation of polysaccharide from cells by hot aqueous KOH extraction, since they would be removed preferentially from glucuronoxylan by fractional precipitation.

Cells obtained from the complex medium A provided β-D-(1→2)-linked mannopyranan on autoclaving in addition to a series of reducing oligosaccharides, detected by paper chromatography (rhamnose, inositol, and glycerol were not present). These ranged in degree of polymerization down to 2, and mannose was absent. The mannan, obtained by ethanol precipitation, was of low molecular weight since nonreducing and reducing end units were detected by ^{13}C NMR spectroscopy. These gave C1 signals at δ_C 101.0 and at δ_C 95.2 and 93.8 (β and α anomers), respectively, were each of approximately one-tenth the size of the major C1 signal at δ_C 103.0, and corresponded in chemical shift to analogous carbons in a β-D-(1→2)-linked mannopyranose tetrasaccharide (Gorin, 1973). The reduced mannan obtained from NaOH–$NaBH_4$ treatment of cells gave as the only minor C1 signal that of the nonreducing end units at δ_C 101.1 (Figure 1B).

In order to determine the detailed structure of the glucuronoxylan, a sample was obtained from cells grown on the complex sucrose medium A. It contained xylose and mannose in a ratio of 12:1, each component being the D enantiomer since

the former was converted to D-xylose phenylosazone with $[\alpha]^{25}_D$ −39° (Ehrenstein, 1926) and mannose was susceptible to oxidation with crude glucose oxidase. Also present was 1.6% of glucosamine.

The polysaccharide was titrated against 0.1 M NaOH and found to have an acid equivalent of 923 in terms of molecular weight, indicating the presence of one acidic component in approximately every seven sugar units. Following partial hydrolysis with 0.025 M H_2SO_4 at 100 °C acidic components were isolated by absorption on ion-exchange resin and elution with dilute formic acid. The product was refluxed in methanolic hydrogen chloride, the resulting methyl esters were reduced by sodium borohydride to hydroxymethyl groups, and the product was hydrolyzed with acid. Paper chromatography showed glucose and xylose, the former being distinguishable from gulose by its slower R_f (Levy, 1954), and its susceptibility to D-glucose oxidase showed it to be the D isomer. Its identity was confirmed by sodium borohydride reduction, followed by acetylation, and detection of glucitol hexaacetate by GLC. The acidic component is therefore D-glucuronic acid.

A branched structure was indicated for the glucuronoxylan since, on sodium periodate oxidation for 18, 48, and 78 h, consumption of 1.16, 1.17, and 1.23 mol of oxidant per mol of anhydroaldose unit was observed, respectively, with concomitant formation of 0.44, 0.47, and 0.52 mol/mol of formic acid. The presence of ramifications was confirmed by methylation experiments. Conversion of the methylated glucuronoxylan to *O*-alditol acetates, which were examined by GLC–MS, showed the presence of traces of 3,4,6-tri-*O*-methylmannitol triacetate corresponding to β-D-(1→2)-linked mannopyranan, with 2,3-di-*O*-methylxylitol triacetate (main peak), a smaller proportion of 3-*O*-methylxylitol tetraacetate, and a trace of 2,3,4-tri-*O*-methylxylitol diacetate. The formation of the 2,3-di-*O*-methyl derivative could indicate the presence of 2-O- and/or 4-O-substituted xylopyranose and/or 5-O-substituted xylofuranose structures, possibilities confirmed by conversion of the methylated polysaccharide to a methyl glycosidic mixture containing methyl 2,3-di-*O*- and 3,4-di-*O*-methylxylosides. These were detected by GLC as two peaks apiece and were apparently present in approximately equal proportions. The glucuronic acid component of the polysaccharide was shown to exist as glucopyranosyluronic acid nonreducing end units, since on conversion of the methylated polysaccharide to a mixture of *O*-methylaldoses 2,3,4-tri-*O*-methylglucuronic acid was identified by virtue of its R_f on paper and bright pink color formed with *p*-anisidine hydrochloride.

The possibility of xylofuranose structures in the glucuronoxylan was excluded by ^{13}C NMR spectroscopy experiments. The proton-decoupled spectrum (Figure 1A) contains C1 signals at δ_C 103.2 and from δ_C 98.4 to 99.0, a region from δ_C 78.4 to 71.2 which could arise from a number of structures, signals at δ_C 66.0 and at δ_C 63.3 corresponding to C5's of pyranose or furanose units, and a signal at δ_C 61.0 with an unusually low chemical shift for a sugar carbon. According to methylation results the only possible furanose structure could be from 5-*O*- and/or 2,5-di-*O*-substituted structures. These would give rise to C5 signals in the δ_C 78.4–71.2 region which, on removing[1] or reducing the proton decoupling power, would give triplets corresponding to CH_2's interspersed with doublets of CH's. However, no triplets could be detected over a range of decoupling power (for example, see Figure 1C) and 5-O-substituted furanose moieties are therefore absent.

The configuration of the glycosidic linkages of xylopyranose units in the glucuronoxylan appears, from the ^{13}C NMR spectrum, in the absence of furanose units, to be mixed with α and β of the pyranose form. The C1 signal at δ_C 103.2 should arise from β units, by analogy with that of β-D-(1→4)-linked xylopyranan (Figure 1D) which is at δ_C 103.1 and/or from β-D-glucopyranosyluronic acid units. These signals at δ_C 98.4–99.0 should arise from α units since the C1 of methyl α-D-xylopyranoside is at higher field (δ_C 101.3) than the C1 of its β anomer (δ_C 105.7). Here too, minor α-D-glucopyranosyluronic acid units could contribute to the signals. The concept of a mixture of α- and β-linked units is supported by the specific rotation of +69° ($[M]_D$ +9300°) of the glucuronoxylan in view of the molecular rotations of +25260 and −10750° for the α and β anomers of methyl D-xylopyranoside, respectively (Hudson, 1925).

Paper chromatography indicated that, on partial acid hydrolysis, the glucuronoxylan gave three oligosaccharides, one with a mobility corresponding to that of 4-*O*-β-D-xylopyranosyl-D-xylose and others with comparative mobilities, R_{X2} of 1.8 (which is equal to the R_f of galactose) and 0.45. On treatment of the mixture with lead tetraacetate in acetic acid, xylose and 4-*O*-β-D-xylopyranosyl-D-xylose disappeared, being susceptible to oxidation, leaving the spots with R_{X2} 0.45 and 1.8, which should be a tri- and a disaccharide with 2-O-substituted reducing end units because of their stability to oxidation (Perlin, 1959). Since 2-*O*-β-D-xylopyranosyl-D-xylose, obtained from partial hydrolysis of the polysaccharide of *T. mesenterica*, has R_{X2} 1.1, the spot with R_{X2} 1.8 should be 2-*O*-α-D-xylopyranosyl-D-xylose. Significantly, no β-(1→4)-linked xylotriose was detected in the partial hydrolysis product, thus precluding the presence of consecutive (1→4) links, as in a main chain.

Glucuronic acid containing oligosaccharides were obtained from glucuronoxylan by partial acid hydrolysis under more vigorous conditions and absorption on ion-exchange resin. Elution with dilute formic acid provided a mixture which gave, on paper chromatography, pink spots with mobilities compared with that of a standard 2-*O*-(β-glucopyranosyluronic acid)-D-mannose (R_{GM} 1.0) of 1.35, 0.76, 0.55, and 0.36. On treatment with lead tetraacetate only the spot with R_{GM} 0.55 disappeared. Thus, the fastest spot, by virtue of its R_{GM} and oxidation characteristics, should be 2-*O*-(glucopyranosyluronic acid)xylose, and because of its resistance to oxidation and the isolation of 2-*O*-α-D-xylopyranosyl-D-xylose in other experiments, the spot with R_{GM} 0.76 should be D-GlcA*p*-(1→2)-α-D-Xyl*p*-(1→2)-D-Xyl (I). The slightly slower mobility of

D-GlcA*p*-(1→2)-α-D-Xyl*p*-(1→2)-D-Xyl
I

D-GlcA*p*-(1→2)-β-D-Xyl*p*-(1→4)-D-Xyl
II

-(1→4)-α-D-Xyl
III

the lead tetraacetate susceptible spot (R_{GM} 0.55) can be explained by the presence of a β-D-Xyl*p*-(1→4)-D-Xyl component (in structure II) in place of an α-D-Xyl*p*-(1→2)-D-Xyl structure in trisaccharide I. Such components would be expected to make differing contributions in chromatographic mobility by analogy with 2-*O*-α-D-xylopyranosyl-D-xylose and its slower 4-O-β isomer.

The ^{13}C NMR spectrum of the glucuronoxylan (Figure 1A) is useful when considering structural possibilities offered by other data. For example, the signal at δ_C 61.0 is at higher field

[1] Under these conditions the ^{13}C signal at δ_C 103.2 becomes a doublet with J = 169 Hz (Figure 1C). However, this coupling constant does not distinguish between α-furanose and β-pyranose units since the C1–H1 coupling values of methyl α-D-xylofuranoside and methyl β-D-xylopyranoside are 164 and 170 Hz, respectively.

than any signals of ring carbons present in the methyl pyranoside derivatives of xylose or glucuronic acid (Gorin & Mazurek, 1975). However, methyl α-D-xylopyranoside gives a C5 signal at δ_C 62.6, and since O-substitution often results in small upfield displacements (~1 ppm) of signals of adjacent carbons (Gorin, 1975), the signal at δ_C 61.0 must arise from 4-O-substituted α-D-xylopyranose residues (III). A similar argument leads to the conclusion that the majority of β-D-xylopyranose units are not substituted in the 4 positions since a C5 signal at δ_C 66.6 corresponds closely with that of unsubstituted methyl β-D-xylopyranoside, which is at δ_C 66.9 (Gorin & Mazurek, 1975).

The methylation data outlined above leave doubt as to the configuration of the D-xylopyranosyl branch points, which are 2,4-di-O-substituted. It would be expected that α-D-xylopyranosyl units would also give a signal at δ_C 61.0, whereas β units would give a C4 signal at δ_c 64.5, like the β-D-(1→4)-linked xylopyranan (Figure 1D). The minor signal at δ_C 63.3 does not correspond closely to this value and is nearer to the C5 signal of methyl α-D-xylopyranoside (δ_C 62.6) and thus may arise from 2-O-substituted α-D-xylopyranose units. However, such arguments may not be regarded as conclusive.

Thus far the configuration of the D-glucopyranosyluronic acid units is unknown, as is the structure of the main chain of the glucuronosylxylan, except that it is not a β-D-(1→4)-linked xylopyranan.

In terms of chemical structure the above glucuronoxylan resembles those obtained from cells grown on the proline medium C at 28 °C and the complex sucrose medium A at 37 °C since the ^{13}C NMR spectra are similar. When a chemically defined sucrose medium, B, was used, the spectrum contained additional signals at δ_C 103.7, 68.8, and 62.8, presumably arising from a galactose-containing component. The minor mannose components of glucuronoxylan preparations are apparently β-D-(1→2)-linked mannopyranans since they give on methylation and degradation 3,4,6-tri-*O*-methylmannitol acetate and are not degraded by exo-α-D-mannosidase.

Concentrates of flagella were prepared from cells of *H. samuelpessoai* grown on the complex sucrose medium A by cleavage from the cells, followed by differential centrifugation. The preparation, on hydrolysis, gave xylose, mannose, and glucose in a ratio of 40:18:42 (Table I), the glucose component representing amylose, which was characterized by degradation to glucose by glucoamylase and the degree of intensity of the blue color formed with iodine. The mannan obtained by KOH extraction (sugar ratio 52:19:29) was different from that of the cell since it contained α-D-mannopyranose side-chain units by virtue of its susceptibility to exo-α-D-mannosidase. Two further centrifugal fractionations of the flagellar preparation freed it from amylose, giving a product containing xylose and mannose in the ratio 86:14.

Enriched flagellar preparations from cells grown on the chemically defined sucrose medium, B, contained, after one centrifugal fractionation, xylose, mannose, galactose, and glucose (from amylose), thus also paralleling the components of the whole cells. In this case further purification was not carried out in an attempt to remove amylose.

Discussion

The differences in composition of polysaccharides occurring in cells of *H. samuelpessoai* grown on three different media, A, B, and C, were considerable (Table I). Xylose predominated over mannose in cells from chemically defined (B) and complex sucrose medium (A). With the chemically defined medium there was a galactose-containing polysaccharide which was absent in cells from the complex medium A. With the proline medium C, the mannose content of the cells was much greater than that of xylose. Such variations of polysaccharides indicate that great care must be taken in comparing structural and immunogenic data of other protozoans.

The polysaccharide, obtained following alkaline extraction and ethanol precipitation from cells, contained a lower proportion of mannose to xylose than was present in cells. The preferential precipitation of xylose over mannose components was marked in the case of proline-grown cells and appeared to be due to the presence of lower molecular weight mannose moieties. Sodium borohydride–sodium hydroxide treatment of cells gave a glucuronoxylan which was isolated free from mannose by ethanol precipitation. The mother liquor contained glycerol, rhamnitol, and *myo*-inositol in addition to mannan whose molecular weight was low by virtue of a minor ^{13}C NMR signal at δ_C 101.0 that corresponded to nonreducing end units. A series of five low molecular weight oligosaccharides that may consist of mannose units attached to mannitol was also formed. A parallel experiment, in which cells grown on a complex sucrose medium A were extracted with hot water, gave a low molecular weight β-D-(1→2)-linked mannopyranan as evidenced by a ^{13}C NMR spectrum and oligosaccharides, the mixture being free of protein and glucuronoxylan. The mannose-containing moieties thus appear to exist in a chemically unbound state in the cells, in contrast to glucuronoxylan which requires the action of alkali for solubilization and is probably bound to protein. If so, glucosamine, detected in small amounts, may serve as a bridge between these two components. However, further work is required in order to weigh this possibility.

A β-D-(1→2)-linked mannopyranan has been previously recognized in cells of *C. deanei* (Previato et al., 1979), and a glucuronoxylan, represented by structures I, II, and III, has not been previously recognized in Nature.

Partly purified flagellar preparations from cells grown on both sucrose media A and B contained cellular amylose, glucuronoxylan, and branched-chain mannans with α-D-mannopyranose units in the side chains, in contrast to the β-D-mannan of whole cells. The amylose component was removed on further purification of the flagella (from medium A) and may be associated with a cytoplasmic body.

This information on chemical structure makes it possible to interpret cytochemical experiments carried out on *H. samuelpessoai* promastigotes, grown on a synthetic medium B containing sucrose, and examined by microscopy (de Souza et al., 1976). It was found that the cell membrane contained a periodate-positive layer (Thiery's method) which reacts somewhat with ConA (the ConA horseradish peroxidase diaminobenzidine method). Certainly, the glucuronoxylan, with structures I, II, and III, and β-D-(1→2)-linked mannopyranan react with periodate, but reaction would not be expected with ConA, except for the α-reducing end units of the β-D-(1→2)-linked oligosaccharides. In view of this result it may be necessary to look elsewhere for cell components reacting with ConA. However, the flagellar surface reacts strongly with ConA and horseradish peroxidase, and, with ConA alone, flagellar–flagellar agglutination was observed using visual microscopy, both observations agreeing with the degradation of flagellar mannan with exo-α-D-mannosidase. The position in the cell of amylose, which reacts with periodate but not ConA, is not clear and neither is the site of the galactose-containing component, which may be in the form of β-D-pyranose units by virtue of a low-field C1 signal at δ_C 103.7 in the ^{13}C NMR spectrum. Clearly, more specific

cytochemical techniques are required to determine the relative location of glucuronoxylan, amylose, α- and β-linked mannans, and galactose components in the cells.

In view of the protection given by *H. samuelpessoai* against infection by *T. cruzi* in terms of parasitemia and mortality, it is of interest to speculate on the possible role of certain polysaccharides in immunogenic terms. For example, *C. fasciculata* and *C. deanei* have been recently reported not to protect mice against *T. cruzi* infection (Takehara & da Silva, 1975), and since both protozoans also contain β-D-(1→2)-linked mannopyranan, this component of *H. samuelpessoai* is probably not immunogenically active. However, the glucuronoxylan is of interest because of the presence of xylose in the hydrolysate of a polysaccharide-containing extract of *T. cruzi* (Goncalves & Yamaha, 1969). It would therefore be pertinent to determine the chemical structure of this component and to test the *H. samuelpessoai* glucuronoxylan with sera of patients infected with Chagas' disease. Also of interest is the finding that the flagella of epimastigote forms of *T. cruzi* give rise to protective activity against challenge doses of *T. cruzi* in mice (Segura et al., 1977), a property also noted with flagellar suspensions of *H. samuelpessoai* (Pereira et al., 1977). Since the flagella of *H. samuelpessoai* contain short-chain α-D-mannopyranosyl structures by virtue of the reactions with exo-α-D-mannosidase and ConA, they contain a chemical structure in common with the epi- and trypomastigote forms and flagella (of epimastigotes) of *T. cruzi*, which react with ConA. It is therefore possible that these flagellar components of *H. samuelpessoai* contain material active in the protection experiments.

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