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Note

An approach towards the isolation and characterization of some polysaccharide components of the protozoan *Leishmania donovani*

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The protozoan parasite *Leishmania donovani* causes visceral leishmaniasis or the fatal disease kala-azar in humans. It has some antigenic relationships[1] with other *Leishmania* species, and, from serological studies, a considerable degree of cross-immunity in humans has been inferred[2]. Protozoan parasites are, in general, antigenically complex, and, possibly, a few antigens are required for the induction of immunity in infected hosts[3]. *Leishmania donovani* parasites exist in two forms, promastigote and amastigote; the presence of common antigenic polysaccharides in both forms has been established[4]. Several studies on the biochemical, physiological, and immunological aspects of the cell membrane of *L. donovani* have been reported by Dywer[5] *et al.*[6,7], and it was found that polysaccharide structures which bind specifically to concanavalin A (con A) and PHA lectins are present in the outer membrane of *L. donovani*.

Because the carbohydrates play a vital role in the shared antigenic properties of *Leishmania,* the carbohydrate-containing fractions of these protozoan parasites should be analyzed chemically. Our objective was to isolate some homogeneous polysaccharide–glycoconjugate fractions from the subcellular part of the promastigote form of *L. donovani* cells, and to establish, by a detailed structural investigation, a structure–activity relationship with respect to antigenicity.

Some definite chemical work had already been reported on the polysaccharide components of other protozoan parasites, namely, *L. tarentolae*[2] and *Trypanosoma cruzi*[8]. Very recently, the presence of an acidic glycoconjugate in *L. donovani* was reported[9], although it was only partially characterized. We now report the isolation and characterization of a neutral polysaccharide containing glucose, mannose, galactose, and arabinose.

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RESULTS AND DISCUSSION

Culture and maintenance of *Leishmania donovani* strain 81 cells were done at 22° on Ray's solid medium[10], and subcultures were made at 72-h intervals. After 3 days of growth, cells of *L. donovani* were collected, and were extracted with hot, aqueous phenol, following the method of Westphal and Jann[11]. Nucleic acid was separated by precipitation with Cetavlon. The macromolecular fraction was then precipitated with ethanol, dialyzed, and freeze-dried. To remove the lipid part of the lipopolysaccharide, if present, a portion was heated with acetic acid on a boiling-water bath for 3 h, and a small amount of lipid was precipitated, removed by centrifugation, and the supernatant, polysaccharide fraction freeze-dried. This was then subjected to fractionation on a column of Sephadex G-100, using sodium phosphate buffer (pH 7.4) as the eluant, and three fractions were collected. The polysaccharide fraction that had no appreciable u.v. absorption was freeze-dried after exhaustive dialysis. High-voltage paper electrophoresis revealed the fraction to be heterogeneous. This was again fractionated on a column of Sephadex G-150 with the same phosphate buffer as the eluant. The major portion of the material was eluted as a single peak, with two other small ones.

This major fraction was further purified by binding with con A according to the method of Yoshioka *et al.*[12], and the resulting major polysaccharide (98% carbohydrate) was found to be electrophoretically homogeneous, having $[\alpha]_{589.6}^{26}$ −19.6° (*c* 0.5, water). It does not give any color with iodine solution indicating the absence of glycogen. The same homogeneous polysaccharide fraction was isolated without the acetic acid treatment, and it proved that this major polysaccharide is not a carbohydrate part of a lipopolysaccharide.

TABLE I

METHYLATION STUDIES OF THE POLYSACCHARIDE (A) AND THE OLIGOSACCHARIDES (B)

Methyl sugars[a]	*T*[b]		*Approximate mol. %*					*Mode of linkage*
	1	*2*	*A*	*B*				
				I	*II*	*III*	*IV*	
2,3,5-Ara	0.48	0.44	20	—	—	—	—	Ara*f*-(1→
2,3,4,6-Glc	0.101	0.101	—	1	1	1	—	Glc*p*-(1→
2,3,4,6-Man	1.00	0.99	14'	—	—	—	1	Man*p*-(1→
2,4,6-Gal	2.29	2.03	20	—	—	—	1	→3)-Gal*p*-(1→
2,4,6-Glc	1.96	1.82	—	—	—	—	1	→3)-Glc*p*-(1→
2,3,6-Man	2.20	2.02	13	—	—	—	—	→4)-Man*p*-(1→
2,3,6-Glc	2.50	2.31	—	1	2	3	—	→4)-Glc*p*-(1→
2,6-Glc	3.83	3.37	32	—	—	—	—	→3,4)-Glc*p*-(1→

[a]2,3,5-Ara = 2,3,5-tri-*O*-methylarabinofuranose, *etc.* [b]T = retention times of the corresponding alditol acetates relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol as unity, on a column of (*1*) 3% of ECNSS-M and (*2*) 3% of OV-225.

The polysaccharide contained galactose, mannose, arabinose, and glucose in the molar ratios of 1:1.3:1:1.6, as estimated by g.l.c. of their alditol acetates. The polysaccharide was completely methylated by the Hakomori method[13], followed by Purdie methylation[14]. After conversion into their alditol acetates, the methylated sugars obtained on hydrolysis thereof were analyzed by g.l.c., using columns *1* and *2*; the results are given in Table I. The absence of any amino sugar was confirmed by the modified Elson–Morgan method of estimation[15], and also by g.l.c. on column *3*.

The methylation data obtained are of immunological significance. Probably the most important structural features are the nonreducing end-groups, namely, arabino-furanosyl (20%) and mannopyranosyl (14%). In this polysaccharide, the mannopyranosyl residues as nonreducing end-groups have the α-D-configuration, as they are cleaved by α-D-mannosidase to yield free D-mannose, and, also, as the polysaccharide binds with con A. These nonreducing end-groups differ from those of the related reptilian parasite *Leishmania tarentolae,* which are[2], for polysaccharide *A,* mannopyranosyl (4%), and glucopyranosyl (13%), and for polysaccharide *B,* arabinofuranosyl (8%), mannopyranosyl (1%), and glucopyranosyl (25%).

The data so far obtained gave some insight into the structural details of the polysaccharide. Identification of 2,4,6-tri-*O*-methylgalactose (20%) shows that galactopyranose residues are substituted at O-3, and also, the presence of 2,3,6-tri-*O*-methylmannopyranose (13%) residues indicated that some mannose units are substituted at O-4. Finally, the characterization of 2,6-di-*O*-methylglucopyranose in a major proportion (32%) indicated that the polysaccharide is a highly branched, (1→4)-linked glucan.

On selective, partial hydrolysis of the polysaccharide with 0.1M TFA for 20 min at 95°, subsequent dialysis of the product afforded a nondialyzable polymeric material and a dialyzate. The dialyzate contained arabinose, as shown by paper chromatography, and g.l.c. as its alditol acetate confirmed the sole presence of arabinose. This indicated the presence of arabinofuranosyl groups as nonreducing ends.

On periodate oxidation, the polysaccharide consumed 0.65 mol of the oxidant per sugar unit in 12 h, and there was almost negligible liberation of formic acid as estimated by the method of Hirst *et al.*[16]. Thus, the periodate-oxidation results are in close agreement with those of the methylation studies. On graded hydrolysis with 0.5M TFA for 3 h at 100°, the polysaccharide afforded five oligosaccharides, along with arabinose, galactose, mannose, and glucose. Four of these oligosaccharides were isolated in homogeneous form by preparative paper-chromatography on Whatman No. 3 MM paper, using solvent systems *A* and *B*; it was not found possible to isolate the other one in pure form. The neutral oligosaccharides were confirmed as being *O*-α-D-glucosyl-(1→4)-α-D-glucose, *O*-α-D-glucosyl (1→4)-*O*-α-D-glucosyl-(1→4)-α-D-glucose, α-D-glucosyl-(1→4)-*O*-α-D-glucosyl-(1→4)-*O*-α-D-glucosyl-(1→4)-α-D-glucose, and *O*-α-D-mannosyl-(1→3)-*O*-D-galactosyl-(1→3)-α-D-glucose. They were characterized by their specific rotations,

by permethylation studies, by enzymic hydrolysis, and by comparison with the data reported for glucose oligomers. These studies showed that the gluco-oligosaccharides are identical with those isolated from starch. This result also proves that the polysaccharide contains a α-D-(1→4)-linked glucose as the main chain. The ^{1}H-n.m.r. spectrum (D_2O, 60°) of the polysaccharide permitted some primary structural assignments. The spectral patterns were essentially invariant in the temperature range of 25–60°, except for line broadening of all resonances at lower temperature and upfield shifting of the HOD signal at higher temperatures. The spectral region of δ 3.2–5.4 contains resonances for the protons of the CH and CH_2 in the polysaccharide, and by comparison with the known chemical shifts[17–20] of different sugars in D_2O, the region δ 4.90–5.4 may be assigned to anomeric protons. The major anomeric signal appeared at δ 4.99 as a doublet (J 3.0 Hz), in addition to a multiplet centered at δ 5.35. From the downfield chemical-shift values and the low coupling constant of the main signal at δ 4.99 (J 3.0 Hz), it may be assigned to the anomeric proton of the α-glycosidic linkage of the major sugar residue, namely, glucose. Furthermore, the anomeric signals of the sugar residues which appeared as a multiplet centered at δ 5.35 also suggested the major α-glycosidic linkages in the polysaccharide.

On considering all of the foregoing results, the tentative structure of the average repeating unit of the polysaccharide was assigned to be as depicted.

→4)-α-Glc-(1→	4)-α-Glc-(1→	4)-α-Glc-(1→	4)-α-Glc-(1→	4)-α-Glc-(1→
3	3	3	3	3
↑	↑	↑	↑	↑
1	1	1	1	1
Gal	Gal	Gal	Man	Man
3	3	3	4	4
↑	↑	↑	↑	↑
1	1	1	1	1
α-Man	α-Man	Ara	Ara	Ara

This structure explains the linkages of the different monosaccharide units, and also those of various oligosaccharides identified, but it does not necessarily depict the correct sequence of branches.

The most interesting feature of the polysaccharide is the occurrence of all four sugar units, glucose, galactose, mannose and arabinose, reported[2] to be present in polysaccharide B of *L. tarentolae,* although in different relative proportions. The presence of α-D-mannopyranosyl as the nonreducing end-group in the polysaccharides of both *Leishmania* species, as also in that of *Trypanosome cruzi,* indicates the structural significance for the serological cross-reactions occurring between them. The protozoan *Crithidia fasciculata*[21] was also found to contain an arabinogalactan having a main chain of (1→3)-linked β-D-galactopyranosyl units, partially substituted at O-2 by D-arabinofuranosyl residues. Furthermore, the

presence of α-D-glucopyranosyl residues and a nonreducing end-group of arabinofuranose in both of the *Leishmania* species has been established. All of the foregoing structural features have great chemical significance regarding the indistinguishability between these lizard and human *Leishmanias*, and also, the evolutionary hypothesis[22] which suggests the evolution of *Leishmanias* from a purely reptilian to a mammalian parasite.

EXPERIMENTAL

General methods. — Evaporations were conducted in a rotary evaporator below 40° (bath temperature). The paper-chromatographic separations were performed by the descending technique on Whatman No. 1 and No. 3 MM papers. The solvent systems employed were *A*, 6:4:3 1-butanol–pyridine–water; *B*, 5:5:1:3 ethyl acetate–pyridine–acetic acid–water. Aniline oxalate and alkaline silver nitrate were used for detecting the spots. Gel-filtration chromatography was conducted with Sephadex G-100 and G-150. Elutions were monitored with both a KNAUER differential refractometer and an LKB UVIcord SII at 278 nm, and also by the phenol–sulfuric acid method. A Shandon high-voltage electrophoresis apparatus, model L-24, was used for testing the homogeneity of the materials. I.r. spectra were recorded with a Perkin–Elmer 177 spectrophotometer. For g.l.c., a Hewlett–Packard Model 5730A gas chromatograph fitted with an f.i.d. and a t.c.d. detector, and a glass column (1.83 m × 6 mm) containing (*1*) 3% of ECNSS-M on Gas Chrom Q (100–120 mesh), at 190° (for alditol acetates of sugars) and at 170° (for partially methylated alditol acetates), (*2*) 3% of OV-225 at 155°, and (*3*) 3% of Poly A 103 at 190° were used. ^{1}H-N.m.r. spectra of the polysaccharide were recorded with a JEOL FX-100 spectrometer operated at 100 MHz. Samples were subjected to deuterium exchange by repeated dissolution in D_2O and lyophilization. Sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) was used as an internal standard.

Cell culture. — Promastigotes of *L. donovani*, strain 81, were kindly supplied by Mr. B. B. Sanyamat, Immunobiological Laboratory, Calcutta, India. Cells were grown on slightly modified Ray's solid media[7] as follows.

The solid-phase, blood-agar medium contained brain–heart infusion (Oxoid/Difco) 37 g, dextrose 10 g, and distilled water added to make to 1 L (pH 7.2). To each 250 mL of it in an Erlenmeyer flask was added 5.0 g of plain agar, and the mixture was heated in an autoclave for sterilization. Rabbit blood (5–6 mL) was added at 40–45°, and mixed in thoroughly, and this medium dispensed to pre-sterilized culture-tubes. Subculture and harvesting of the cells were done after 3 days. After 72-h growth at 22°, the cells were carefully scraped from the solid surface, suspended in, and twice washed with cold physiological saline (0.9%), and then centrifuged at 5000 r.p.m. The cell pellet was suspended in water, exhaustively dialyzed, and lyophilized; yield, 8–10 mg per culture tube.

Isolation of the macromolecular fraction. — Subcellular fractions in crude

form were isolated from the freeze-dried cells (8 g) by extraction with aqueous phenol at 68° following the method of Westphal and Jann[11]. The slurry was exhaustively dialyzed, and then centrifuged. The supernatant liquor was concentrated to 100 mL, and the nucleic acid was separated by precipitation with Cetavlon. The macromolecular fraction, including some lipopolysaccharides, was precipitated by adding 10 volumes of ethanol. The precipitate, in water, was dialyzed exhaustively, and then lyophilized; yield, 300 mg. This material was heated with 1% acetic acid for 3 h at 100°, and the lipid was precipitated, and centrifuged out. The supernatant liquor was lyophilized, to yield ~200 mg as a white, fluffy material.

Isolation of the polysaccharide. — The crude, water-soluble material (100 mg) was dissolved in sodium phosphate buffer (pH 7.4), and the solution was applied to a column (85 × 2.5 cm) of Sephadex G-100, which was then eluted by the same buffer. Three major fractions appeared as three peaks; only the middle one showed no appreciable u.v. absorbtion. This fraction was exhaustively dialyzed, and was found to be a heterogeneous polysaccharide fraction by high-voltage electrophoresis (35 V/cm) in borate buffer (pH 9.5) for 1.5 h, using glass-fiber paper (Whatman GF/A) and development by benzidine–periodate.

This material (50 mg) was applied to a column (80 × 2.2 cm) of Sephadex G-150, and the same phosphate buffer was used as the eluant. The major polysaccharide fraction, eluted as a single peak (with two other small ones), was dialyzed exhaustively and lyophilized (25 mg). It was further purified by binding with con A, according to the method of Yoshioka *et al.*[12]. A con A solution (25 mg/mL in 4M NaCl) was added to the polysaccharide solution (2 mg/mL) in M NaCl buffered at pH 6.8 with 18mM phosphate buffer. After precipitation was complete, the mxiture was centrifuged at 5,000*g* for 30 min. The bound polysaccharide was extracted with 10% TCA at 4°, the extract was centrifuged, and made neutral with NaOH; finally the free polysaccharide was precipitated with ethanol at 4°.

The small part of the material which did not bind with con A, and remained in the supernatant liquor, was not further investigated, due to paucity of the material.

The con-A-bound polysaccharide was recovered (20 mg), $[\alpha]_{589.6}^{26}$ −19.6° (*c* 0.5, water), and found to be electrophoretically homogeneous in high-voltage electrophoresis done as before.

Two portions of the polysaccharide (3 mg each) were hydrolyzed by two different methods, one by M sulfuric acid in a boiling-water bath for 16 h, and the other, by 2M TFA in a sealed tube for 3 h at 121°. After the usual treatments, each hydrolyzate was examined by p.c. in solvents *A* and *B*; the presence of glucose, galactose, mannose, and arabinose was revealed on spraying with alkaline silver nitrate. They were also analyzed by g.l.c. as their alditol acetates[23] in columns *1*, *2*, and *3*.

Estimation of sugars. — The polysaccharide (5.1 mg) was mixed with *myo*-inositol (1.2 mg), used as an internal standard, and hydrolyzed with 2M trifluoro-

acetic acid as already described. The alditol acetates were analyzed by g.l.c., using column *1*. The molar ratios of glucose, mannose, galactose, and arabinose were found to be 1.6:1.3:1:1.

Methylation analysis. — Methylation was performed by the method of Hakomori[13]. The polysaccharide (5 mg, dried over P_2O_5) was dispersed in dry dimethyl sulfoxide (6 mL) in a vial. The vial was flushed with nitrogen and 2M methylsulfinyl sodium in dimethyl sulfoxide (6 mL) was added by using a syringe. The gelatinous solution was agitated in an ultrasonic bath for 30 min, and kept overnight at room temperature. Methyl iodide (4 mL) was added dropwise, with external cooling, and the resulting solution was agitated for 30 min in an ultrasonic bath. The product was then dialyzed and lyophilized.

This product showed a slight hydroxyl absorption in the i.r. spectrum, and was further subjected to two successive methylations by the Purdie method[14]. The product had no hydroxyl absorption in the i.r. spectrum; $[\alpha]_{589.6}^{26}$ −10.6° (*c* 0.23, $CHCl_3$); yield, 4.0 mg. The fully methylated polysaccharide (3 mg) was hydrolyzed with 2M H_2SO_4 after hydrolysis with 85% HCO_2H. After the usual work-up, the product was reduced by $NaBH_4$ (10 mg), followed by acetylation with 1:1 acetic anhydride–pyridine (0.5 mL). The resulting, partially methylated alditol acetates were examined by g.l.c. using columns *1* and *2*. The corrected g.l.c. data for the components are presented in Table I.

Selective, mild hydrolysis. — The polysaccharide (8 mg) was hydrolyzed with 0.1M TFA (2 mL) for 20 min at 95°, and the product was dialyzed against distilled water, affording nondialyzable, polymeric material and a dialyzate. The presence of only arabinose in the dialyzate was shown by p.c. in solvent *A*, and also by g.l.c. as its alditol acetate, using column *1*.

Periodate oxidation. — The polysaccharide (6 mg) was treated with 0.04M sodium metaperiodate at 4° in the dark; consumption of the oxidant became constant (monitored spectrophotometrically[24]) within 12 h, and the value was found to be 0.65 mol per sugar unit, with almost negligible liberation of formic acid.

Graded hydrolysis. — Aided by the results of a pilot experiment, the polysaccharide (35 mg) was heated with 0.5M TFA for 3 h at 100°. After the acid had been removed in a rotary evaporator, paper-chromatographic examination of the hydrolyzate indicated the presence of four oligosaccharides, namely, I, II, III, and IV (designated according to their mobility), together with the monomers arabinose, glucose, galactose, and mannose. Each oligomer was isolated by p.p.c. on Whatman No. 3 MM paper using solvents *A* and *B*, and, after elution, was then found to be homogeneous by p.c. On hydrolysis with 0.5M H_2SO_4 for 4 h at 100°, each of the first three oligosaccharides (I, II, and III) yielded only glucose, and the remaining one, IV, yielded glucose, galactose, and mannose.

The oligosaccharide I, $[\alpha]_{589.6}^{26}$ +130° (*c* 0.25, water), was methylated[10]; the hydrolyzed product showed the presence of 2,3,4,6-tetra-*O*-methylglucose and 2,3,6-tri-*O*-methylglucose in the molar ratio of 1:1 in g.l.c. as their alditol acetates. Similarly, on methylation and hydrolysis, oligosaccharide II, $[\alpha]_{589.6}^{26}$ +161° (*c* 0.21,

water), yielded 2,3,4,6-tetra-*O*-methylglucose and 2,3,6-tri-*O*-methylglucose in the molar ratio of 1:2, and oligosaccharide III, $[\alpha]_{589.6}^{27}$ +166° (*c* 0.20, water), yielded 2,3,4,6-tetra-*O*-methylglucose and 2,3,6-tri-*O*-methylglucose in the molar ratio of 1:3, as found in g.l.c. as their alditol acetates. The specific rotation values are similar to the literature values for maltose[25], maltotriose[26], and maltotetraose[27]. Oligosaccharide IV was similarly methylated, and, after hydrolysis, the methyl sugars were identified, by g.l.c. as their alditol acetates using columns *1* and *2* (see Table I), as 2,3,4,6-tetra-*O*-methylmannose, 2,4,6-tri-*O*-methylgalactose, and 2,4,6-tri-*O*-methylglucose in the molar ratios of 1:1:1. On treatment with α-D-glucosidase, each of the oligosaccharides I, II, and III yielded D-glucose, identified by p.c. and g.l.c.

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