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Chemical Structure and Antigenic Aspects of Complexes Obtained from Epimastigotes of *Trypanosoma cruzi*†

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ABSTRACT: Cells of *Trypanosoma cruzi*, Y strain, were submitted to water extraction by successive freezing and thawing. Fractionation of soluble material on a P-10 column gave an antigenic glycoprotein (fraction I) whose carbohydrate portion (40%) contained galactose, mannose, glucose, and xylose in a molar ratio of 35:13:1:1. It was electrophoretically homogeneous (M_r ~25 000) and contained short chains of mannopyranosyl (23%) and galactopyranosyl (10%) nonreducing end units and 2-O-substituted mannopyranosyl units (19%). Extraction of the remaining cell fragments with phenol–water gave an antigenic $CHCl_3$–MeOH–H_2O-soluble fraction II (LPPG), which yielded a galactomannan (fraction III) (galactose and mannose in a molar ratio of 1:2.1) on degradation with hot aqueous NaOH–$NaBH_4$. It contained end units of galactofuranose (25%) and 2-O- (5%), 3-O- (32%), and 2,3-di-O- (19%) substituted mannopyranosyl units. Galactofuranose was directly linked (1→3) to mannopyranose and not via a phosphorodiester bridge. β-D-Gal*f*-(1→3)-Me-α-D-Man*p*, in contrast with Me-β-D-Gal*f*, was effective in inhibiting the precipitin reaction between LPPG and antiserum raised against LPPG. Fraction IV, insoluble in $CHCl_3$–MeOH–H_2O, contained galactose and mannose in a ratio of 1.4:1. After degradation with hot aqueous NaOH–$NaBH_4$, it gave a product (fraction V) containing galactose and mannose in a 1:2 ratio. Methylation analysis showed it to differ from fraction III since it contained a high proportion of nonreducing end units (41%) and 2-O-substituted units (16%) of mannopyranose.

Chagas' dissease is a complex clinical entity caused by the flagellate protoazoan *Trypanosoma cruzi*. This protozoan has a complex life cycle with developmental stages in different hosts (Brener, 1973). In the mammalian host, *T. cruzi* multiplies intracellularly as amastigotes and is subsequently released into the bloodstream as trypomastigotes. These are nondividing forms that can infect new host cells or be ingested by a triatomine bug. In the insect midgut lumen, trypomastigotes differentiate to epimastigotes, which are the invertebrate multiplying forms. In the insect rectum, epimastigotes differentiate to the nondividing metacyclic trypomastigotes, which are discharged in the feces and urine onto the vertebrate skin when the insect is feeding. Through skin discontinuities or through mucosal membranes, these forms can reach the blood circulation and thereafter penetrate cells, thus completing its biological cycle.

The clinical features of Chagas' disease are highly variable and depend on the interaction of several different host and/or parasite factors (Tafuri, 1979). Studies on the cellular membrane of *T. cruzi* have been performed with the aim of obtaining a better understanding of some of those complex interactions.

Surface glycoconjugates are the membrane components that are currently receiving special attention. Snary & Hudson (1979) isolated a surface glycoprotein of molecular weight (M_r) 90 000 that is present in epimastigote, trypomastigote, and amastigote forms and which was able to induce immunoprotection against experimental infections in mice (Scott & Snary, 1979). However, Nogueira et al. (1981) found that only bloodstream trypomastigotes contain a surface glycoprotein of M_r 90 000, whereas epimastigotes and trypomas-

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0006-2960/83/0422-4980$01.50/0 Published 1983 by the American Chemical Society

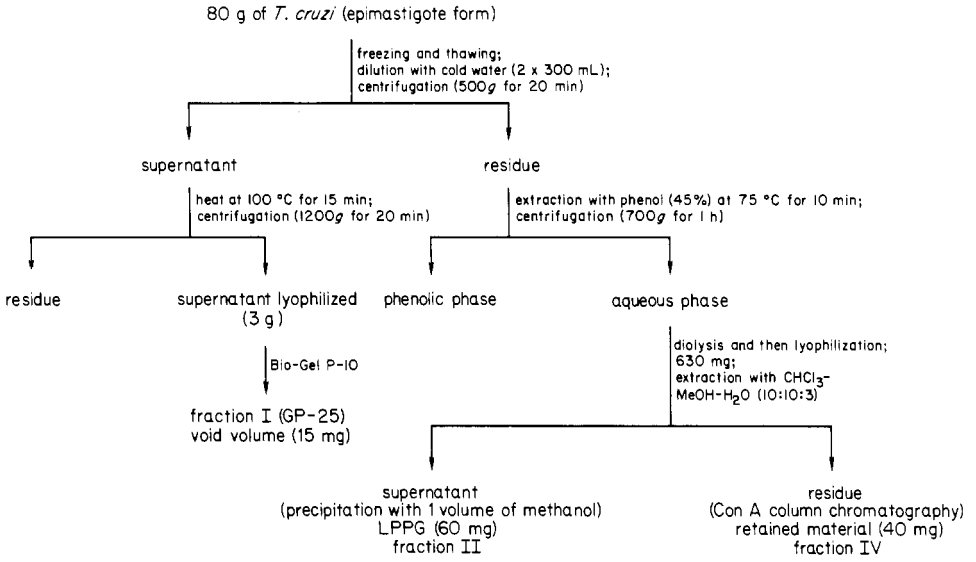

FIGURE 1: Fractionation scheme of methodology used in isolation of materials from cells of *Trypanosoma cruzi*.

tigotes from acellular cultures contain a glycoprotein with a molecular weight of 75 000.

Employing specific monoclonal antibodies raised against epimastigotes, Snary et al. (1981) isolated a minor glycoprotein (M_r 72 000) present in epimastigotes and metacyclic trypomastigotes. This glycoprotein induced immunoprotection in mice challenged with metacyclic trypomastigotes but did not protect when challenged with bloodstream forms (Snary & Ferguson, 1982). Of the complex, 52% consisted of carbohydrate whose components were glucosamine, glucose, galactose, mannose, ribose, and the previously unobserved fucose. The protein component was rich in serine (Snary et al., 1981).

In other studies, direct chemical analysis was used to ascertain the chemical structure of carbohydrate-containing material obtained from *T. cruzi*. Aqueous chloral extracts of epimastigotes have been examined by Gonçalves & Yamaha (1969), who isolated an antigenic material containing glucose, galactose, mannose, glucosamine, and xylose. Aqueous alkali extracts of epimastigotes (Gorin et al., 1981) contained nonreducing end units of α-D-mannopyranose, D-galactopyranose, and β-D-galactofuranose with short chains of 1→2-linked α-D-mannopyranosyl residues. Galactofuranosyl units were linked (1→3) to α-D-mannopyranosyl residues. Alves & Colli (1975) obtained, after phenol extraction, four different components with distinct electrophoretic mobilities. The three glycoprotein components contained, collectively, galactopyranosyl residues (Alves et al., 1979) and a lipopeptidophosphoglycan (LPPG) consisting of galactose, mannose, and glucose in a molar ratio of 22:35:1, respectively, and also traces of glucosamine (Lederkremer et al., 1976). Galactosyl units were shown to be in the furanosyl form (Lederkremer et al., 1980).

In the present investigation, we report the partial chemical structures and the antigenic properties of the carbohydrate moieties of two molecules, isolated from epimastigote forms of *T. cruzi*, which are present in all developmental stages of the parasite. Another complex was also isolated and investigated.

Materials and Methods

Culture Conditions. Epimastigote forms of *T. cruzi* (Y strain) were grown in 500-mL flasks containing agar plus rabbit blood as the solid phase and Brain-Heart Infusion (BHI, Difco) as the liquid phase. The latter (100 mL) was used to inoculate media (1 L) in 3-L Erlenmeyer flasks containing the following components (in units of grams per liter): BHI, 37; folic acid, 0.02; hemin, 0.01, dissolved in M NaOH (1.0 mL). After 7 days at 25 °C with slow agitation (80 rpm), the cells were harvested by centrifugation, washed 3 times with 0.9% aqueous NaCl, and frozen at −20 °C.

Extraction and Purification of Glycoconjugates. Frozen cells were rapidly thawed, cold water was added, and the resulting suspension was removed by centrifugation. The resulting pellet was dispersed in cold water and the suspension recentrifuged. The combined supernatants were heated to 100 °C and lyophilized, and the residue was fractionated on a column (2 × 120 cm) of Bio-Gel P-10, giving fraction I. This and further procedures are summarized in Figure 1. The pellet remaining after centrifugation was extracted with aqueous phenol at 75 °C (Osborn, 1966) and the aqueous layer lyophilized. The residue was extracted by the method of Lederkremer et al. (1977) with $CHCl_3$–MeOH–H_2O (10:10:3 v/v/v) to provide soluble fraction II. Insoluble material was dissolved in 0.018 M sodium phosphate buffer, pH 7.2 in aqueous 0.9% NaCl, and fractionated on a column (1 × 10 cm) of concanavalin A (Con A)–Sepharose 4B according to the method of Lloyd (1970). Bound material was eluted with aqueous 0.1 M methyl α-D-mannopyranoside in the same buffer to yield fraction IV.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE). Electrophoresis was performed in a discontinuous buffer system (Laemmli, 1970) with a 7.5–15% gradient in the concentration of the polyacrylamide gel. Gels were stained with Coomassie Brilliant Blue, specific for protein. The carbohydrate-containing bands were detected with the periodate–Schiff (PAS) reagent (Fairbanks et al., 1971).

Gel Column Chromatography. Columns of Bio-Gel P-4 (−400 mesh) and P-10 (200–400 mesh) were equilibrated and eluted with distilled water (for details, see figures).

Detection and Analysis of Sugar Components of Carbohydrate-Containing Materials. These were hydrolyzed by using 3 M trifluoroacetic acid at 100 °C for 3 h. Following evaporation of the hydrolysate, the residue was successively reduced with aqueous sodium borohydride and acetylated to yield alditol acetates, which were analyzed by gas–liquid chromatography (GLC) by the method of Sawardeker et al. (1965).

Methylation Analysis of Carbohydrate-Containing Materials. These were methylated successively by the methods of Haworth (1915) and Kuhn et al. (1955). Per-O-methylated products were degraded to partially O-methylated alditol

acetates, which were identified and quantitated (Jansson et al., 1976). In the present investigation, GLC–mass spectrometry (MS) was carried out with a Finnigan Model 4000 GLC–MS unit interfaced with an Incos 2300 data system. Electron-impact spectra were obtained respectively every 2 s, scanning from m/z 40 to m/z 420. GLC was performed on capillary columns (0.25-mm diameter × 30 m), one coated with OV-17 (Barreto-Bergter et al., 1981) and the other with OV-17 blended with OV-225 (1:3 ratio by weight) according to Gorin et al. (1982). Products were identified by their electron-impact MS spectra and characteristic retention times.

Carbon-13 Nuclear Magnetic Resonance (^{13}C NMR) Spectroscopy. NMR spectra were obtained from carbohydrate-containing materials (25 mg of fraction III and 10 mg of fraction V) by using a Varian XL-100-15 spectrometer with Fourier transform from D_2O solutions (0.85 mL) of a compound contained in a coaxial glass cylinder fitting snugly within a 12-mm diameter × 20.3-cm tube maintained at 70 °C. The spectral width was 5000 Hz, the acquisition time 0.8 s, the pulse width 9.5 μs, and the number of transients 150 000 (Gorin et al., 1977). Chemical shifts are expressed in δ relative to the resonance of tetramethylsilane (Me_4Si), obtained in a separate experiment.

Amino Acid Analyses. Protein (0.1 mg) was hydrolyzed in 0.5 mL of 6 M HCl at 110 °C for 18 h. The resulting amino acids were analyzed by using a Beckman Model 21 amino acid analyzer.

β-Elimination Reaction of Fraction I (GP-25). Alkali-catalyzed β-elimination of serine- and threonine-linked carbohydrates was performed on GP-25 (5 mg) in 0.1 M NaOH in the presence of 0.1 M $NaBH_4$ and a trace of $NaBT_4$ at 25 °C for 18 h. The solution was neutralized with acetic acid and concentrated to a small volume, and the products were fractionated on a column (0.8 × 50 cm) of Bio-Gel P-10. The resulting fractions were assayed for protein (Lowry et al., 1951), carbohydrate (Dubois et al., 1956), and radioactivity.

Alkaline Borohydride Treatment of Fractions II (LPPG) and IV. This was carried out by using 0.1 M $NaBH_4$ in 0.1 M NaOH for 5 h at 100 °C (Lee & Scocca, 1972). The reaction mixture was neutralized with acetic acid, and the products of degradation were fractionated by column chromatography on Bio-Gel P-4.

Successive Sodium Periodate Oxidation and Sodium Borohydride Reduction of Fraction II. This fraction (LPPG) was treated with an excess of aqueous sodium periodate for 20 min and the reaction terminated by the addition of ethylene glycol. The product was reduced with sodium borohydride, and after 1 h, acetic acid was added and the solution dialyzed (Gorin & Spencer, 1959). The product contained arabinofuranosyl in place of galactofuranosyl units.

Other Analytical Methods. Phosphate was determined by the method of Ames (1966) and hexosamine by a modification of the Elson–Morgan reaction (Belcher et al., 1954). Sialic acid was assayed by the thiobarbituric acid method (Warren, 1959).

Antisera. Anti *T. cruzi* sera were obtained by immunizing rabbits with 10^8 epimastigote forms emulsified in complete Freund's adjuvant and injected twice, with an interval of 7 days by the intramuscular route. One week after the second injection, the rabbits were boosted 3 times (every other day) by the intravenous route using 10^7 epimastigotes. The animals were bled 5–7 days after the last intravenous injection.

Ouchterlony immunodiffusion tests on agarose gel plates (Kabat & Mayer, 1961) were performed at 5 °C overnight, whereas quantitative immunoprecipitation was carried out according to Raschke & Ballou (1971). For precipitin inhibition studies, serum and inhibitor were incubated at 37 °C for 2 h, after which the antigen was added and the incubation continued at 4 °C for 48 h.

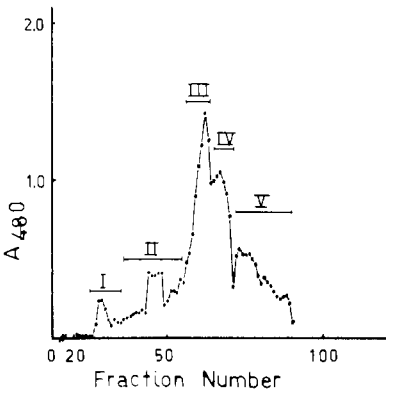

FIGURE 2: Gel filtration on a 2 × 120 cm column of Bio-Gel P-10 of aqueous extract of epimastigotes from *T. cruzi*. Fractions were assayed for carbohydrate by using the phenol–sulfuric acid method. Fraction size, 5.0 mL.

Table I: Sugar Analyses (in Percent) of Fractions Obtained after P-10 Column Chromatography of the Aqueous Extract of *T. cruzi*

	fraction				
sugar	I (PG-25)	II	III	IV	V
ribose				17.0	21.2
xylose	1.9				
mannose	29.3	41.7	48.8	9.8	tr
galactose	67.1	14.8	tr[a]	10.6	tr
glucose	1.6	43.5	51.0	tr	tr
inositol				75.8	78.8

[a] Trace.

Radiolabeling and Radioimmunoprecipitation Assay of GP-25. GP-25 was radioiodinated by the iodogen method (Fraker & Speck, 1978) to a specific activity of 2 × 10^5 cpm/μg. The radiolabeled preparation was specifically immunoprecipitated by chronic Chagas' disease human serum, and complexes were isolated by absorption on *Staphylococcus* A particles (New England Enzyme Center, Boston, MA) by described procedures (Kessler, 1975).

Results

Aqueous Extraction of Cells. Epimastigote forms of *T. cruzi* were obtained by growth on a BHI-based medium. Glycoconjugates were extracted by thawing frozen cells, followed by addition of cold water to form a cell suspension. The procedure was carried out at low temperature to minimize possible proteolytic degradation. After centrifugation, the solution was heated at 100 °C for 15 min, and the precipitate was centrifuged off. The supernatant was lyophilized and the residue fractionated by column chromatography on Bio-Gel P-10. Elution with water provided five fractions (Figure 2) whose sugar composition is presented in Table I.

Chemical Structure of Fraction I. On SDS–PAGE, fraction I (GP-25) gave a single band of apparent M_r ~25 000, detected by Coomassie Brilliant Blue and giving an intense color with PAS (Figure 3). Fraction I contained 60% protein, and the remaining carbohydrate component yielded, on hydrolysis, galactose, mannose, glucose, and xylose in a molar ratio of 35:13:1:1. The glycosidic linkage positions contained in fraction I were determined by methylation analysis. The per-O-methylated derivative of fraction I was degraded to partially O-methylated alditol acetates, which were identified

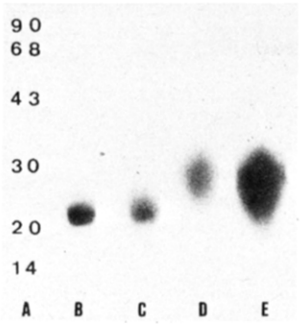

FIGURE 3: Polyacrylamide gel electrophoresis of *T. cruzi* preparations: Lane A is a molecular weight standard consisting of phosphorylase *b* (M_r 94 000), bovine serum albumin (M_r 68 000), ovalbumin (M_r 43 000), carbonic anhydrase (M_r 30 000), soybean trypsin inhibitor (M_r 20 000), and lysozyme (M_r 14 000); lanes B and D are stained with Coomassie Blue and show fraction I (GP-25) and fraction II (LPPG), respectively; lanes C and E show PAS staining of fraction I (GP-25) and fraction II (LPPG), respectively.

Table II: *O*-Methylalditol Acetates Formed on Methylation Analysis of Carbohydrate-Containing Materials Isolated from Epimastigote Forms of *T. cruzi*

		% of *O*-methylalditol acetates in terms of peak area		
O-methyl derivative	RRT[a]	fraction I	fraction III	fraction V
2,3,4-Me_3Xyl	0.65	1		
2,3-Me_2Fuc	0.81			2
2,3,4,6-Me_4Man	1.00[b]	23.5	7	40
2,3,4,6-Me_4Glc	1.00[b]	1		2
2,3,5,6-Me_4Gal	1.04	2	25	6
2,3,4,6-Me_4Gal	1.10	10	6	6
3,4,6-Me_3Man	1.50	19	5	16
2,4,6-Me_3Man	1.54	4	32	
2,4,6-Me_3Gal	1.62	8		
2,3,4-Me_3Man	1.70	8		
2,3,6-Me_3Glc	1.72	8		18
2,3,4-Me_3Gal	2.02	8.5		
4,6-Me_2Man	2.35		19	5
4,6-Me_2Gal	2.47	1	6	
3,4-Me_2Man	2.75	2		
2,4-Me_2Gal	2.92	4		5

[a] Retention time of peak relative to that of 2,3,4,6-tetra-*O*-methylmannitol diacetate. [b] Separable on a blended OV-17–OV-225 column.

and quantitated by using GLC–MS (Jansson et al., 1976). These data, summarized in Table II, indicate the presence, as principal structures, of nonreducing and units of mannopyranose and galactopyranose, 2-O-, 3-O-, and 6-O-substituted mannopyranosyl units, and 3-O- and 6-O-substituted galactopyranosyl residues. The proportion of di-O-substituted units (2,3-di-*O*-Gal*p*, 3,6- and 2,6-di-*O*-Man*p*) was much lower than that of nonreducing end units, thus indicating that the carbohydrate portion of fraction I consists mainly of short chains.

In terms of minor components, fraction I contains xylose as nonreducing pyranosyl end units and traces of a 1→4-linked glucopyranosyl moiety.

The galactopyranosyl units of fraction I are mainly in the β-D-configuration. It is already known that galactosyl units, isolated from extracts of cells of *T. cruzi*, are in the D form (Gorin et al., 1981). Since fraction I has a galactose content of 28% and a relatively low specific rotation of +16°, the presence of many galactopyranosyl units with the α-D-configuration is unlikely. Such units would have a strongly positive rotational contribution. For example, an analogous

Table III: Amino Acid Composition of GP-25 before and after β-Elimination

amino acid[a]	before	after
aspartic acid	3.86	5.10
threonine	9.72	6.30
serine	13.24	6.98
glutamic acid	7.89	7.97
proline	16.66	14.24
glycine	9.90	9.22
alanine	5.55	18.31
half-cystine	2.61	1.99
α-aminobutyric acid	0.00	tr[b]
valine	6.80	6.31
methionine	3.52	3.76
isoleucine	2.45	2.06
leucine	5.52	5.33
tyrosine	1.21	1.67
phenylalanine	2.41	3.92
histidine	1.64	1.52
lysine	3.77	2.79
arginine	3.22	2.61

[a] Residues per 100 residues. [b] Trace.

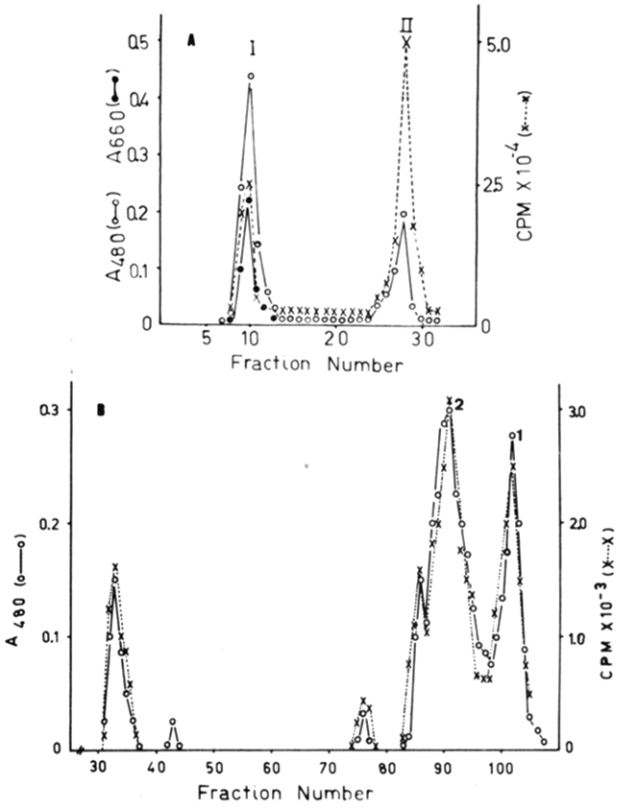

FIGURE 4: (A) Gel filtration of GP-25 after β-elimination on a 1 × 50 cm column of Bio-Gel P-10. Fractions were assayed for carbohydrate (○), protein (●), and ^3H radioactivity (×). Fraction size, 1.0 mL. (B) Gel filtration of peak II from Figure 4A on a 1 × 100 cm column of Bio-Gel P-4. Fractions were assayed for carbohydrate (○) and ^3H radioactivity (×). Fraction size, 1.0 mL.

substance, methyl α-D-galactopyranoside, has a specific rotation of +179°.

The presence of short carbohydrate chains in fraction I was confirmed by their release on β-elimination with aqueous $NaOH–NaBH_4–NaBT_4$. They are attached to serine and threonine since the reaction product contained far fewer residues of these amino acids (6.98% and 6.30%) than fraction I (13.24% and 9.72%), respectively, as indicated in Table III. Gel filtration chromatography of the β-elimination product on P-10 gave rise to two radioactive peaks (Figure 4A). Peak I (like fraction I) was in the excluded volume and contained

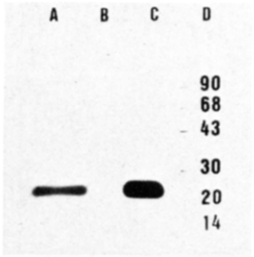

FIGURE 5: Immunoprecipitation profiles of ^{125}I-GP-25. (Lane A) ^{125}I-GP-25 alone; (lanes B and C) ^{125}I-GP-25 mixed with normal human serum and serum for patients with chronic Chagas' disease, respectively; (lane D) molecular weight markers.

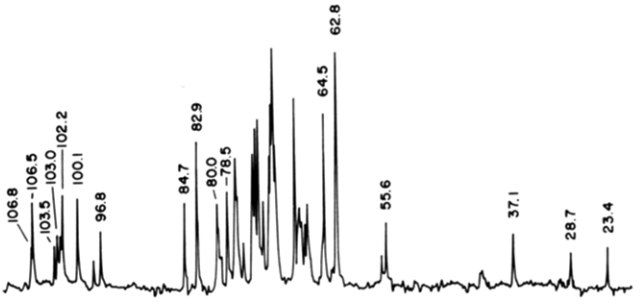

FIGURE 6: Carbon-13 NMR spectrum of fraction III isolated from epimastigotes of *T. cruzi*.

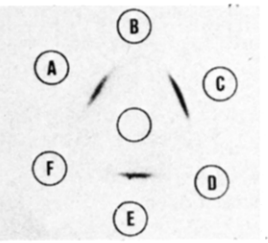

FIGURE 7: Stained immunodiffusion plate demonstrating reaction between rabbit hyperimmune serum in the center well and the following: A/C/E, fraction II (LPPG) intact; B/D/F, fraction II (LPPG) after sodium periodate oxidation.

carbohydrate and protein. However, this does not necessarily signify that it represents undegraded material. Peak II, which had a lower molecular weight, consisted of carbohydrate only, and when it was subjected to further column chromatography on P-4, two main fractions were obtained with elution characteristics corresponding to di- and tetrasaccharides (Figure 4B).

As can be seen from Table III, the predominant amino acid of fraction I is proline followed by successively smaller proportions of serine, glycine, threonine, glutamic acid, and valine.

Antigenic Properties of Fraction I. Fraction I was found to be specifically precipitated by chronic Chagas' disease serum (Scharfstein et al., 1982a,b). Analysis of the corresponding immune complexes on SDS–PAGE confirmed the glycoprotein of M_r ~25 000 as the isolated antigen (Figure 5).

Isolation and Chemical Characterization of Fraction II (LPPG). Hot phenol–water extraction of insoluble cell debris, obtained after the aqueous extraction procedure described above, provided material which was retained in the aqueous phase. Extraction of this material with $CHCl_3$–MeOH–H_2O provided soluble fraction II (LPPG), which contained carbohydrate (62%), protein (10%), hexosamine (1.5%), phosphate (7.8%), and lipid, but no sialic acid. The carbohydrate portion contained galactose and mannose in a molar ratio of 1:2 and traces of glucose and inositol, as determined by examination of the product obtained on successive hydrolysis, sodium borohydride reduction, and acetylation. Fraction II gave on SDS–PAGE (Figure 3) a single band that was positive for protein (Coomassie Blue), carbohydrate (PAS), and lipid (Sudan Black; result not shown). In terms of these analytical data, fraction II corresponds to the LPPG of Lederkremer et al. (1976, 1977, 1978) despite the difference in extraction procedure.

Degradation of fraction II with hot aqueous NaOH–$NaBH_4$ gave rise to a carbohydrate, which was isolated as a single peak by column chromatography on P-4. Its elution characteristics were those of a moiety having approximately 10 sugar units, when a 1→4-linked series of α-D-glucopyranosyl oligosaccharides were used as standards. It was designated fraction III and was found to contain galactose and mannose in a ratio of 1:2.1, hexosamine (2%), phosphate (5%), and traces of glucose and inositol. Its ^{13}C NMR spectrum (Figure 6) contained a complex C-1 region indicating the presence of many carbohydrate structures. This region extended from δ 96.8 to 106.8. One of the predominant signals at δ 106.5 is at unusually low field and corresponds to β-D-galactofuranosyl units (Gorin et al., 1981). Its sharpness, having a line width of 2 Hz, showed that the units are glycosidically linked to an adjacent sugar unit, and although phosphate is present, it is not, as previously suggested (Lederkremer et al., 1980), present in a phosphorodiester bridge between the units. If this was so, the signal would appear as a doublet due to ^{13}C–O–^{31}P coupling of 3–6 Hz (Lapper et al., 1972, 1973). Examples of line splitting in polysaccharide spectra have been summarized (Gorin, 1981).

Methylation analysis of fraction III, using the methodology described above, gave rise to a complex mixture of partly O-methylated alditol acetates (Table II). Their structures and proportions indicated that most of the nonreducing end units were galactofuranose (25%) with smaller proportions of mannopyranose (7%) and galactopyranose (6%). Since the mannopyranosyl units were 2-O- (5%), 3-O- (32%), and 2,3-di-O- (19%) substituted, the nonreducing end units of β-D-galactofuranose are mainly linked 1→3 to adjacent D-mannopyranosyl residues. These have the α-configuration since the specific rotation of fraction III is +31°. Since this value is much higher than that (−84°) of a β-D-galactofuranan (Haworth et al., 1937), the rotational contribution of the D-mannopyranosyl units must be strongly positive and thus α. The presence of the β-D-Gal*f*-(1→3)-α-D-Man*p*- structure is confirmed by the presence in the ^{13}C NMR spectrum of the signal at δ 106.5, typical for this structure (Gorin et al., 1981).

Another interesting feature of the ^{13}C NMR spectrum are two C-1 signals at high field (δ 97.6 and 96.8). Such resonances have only been encountered rarely (Gorin, 1981), one example being from 2-acetamido-2-deoxy-α-D-glucopyranosyl units, which would also give a C-2 signal at δ 55.8 and a CH_3–CO–NH signal at δ 23.4. However, fraction III only contains 2% hexosamine, and such units would have to give rise to signals that are disproportionately large.

Antigenic Properties of Fraction II. Fraction II (LPPG) gave a precipitation line in immunodiffusion plates with rabbit immune serum against epimastigote forms (Figure 7). After rapid oxidation of fraction II with aqueous sodium periodate followed by sodium borohydride reduction, a process that converts nonreducing end units of β-D-galactofuranose to α-L-arabinofuranose, without affecting other units appreciably, the modified complex became nonimmunoprecipitable by the same antiserum. The precipitin curve of LPPG antiserum against intact LPPG shows an equivalence point at 20 μg of antigen with 100 μL of antiserum in a total volume of 500 μL. The precipitate contained about 40 μg of protein. For hapten

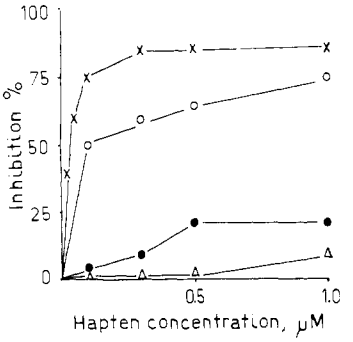

FIGURE 8: Hapten inhibition of the precipitin reaction between *T. cruzi* LPPG and antiserum raised against intact LPPG: (×) oligosaccharide obtained after alkaline degradation of LPPG (fraction III); (○) β-D-Gal*f*-(1→3)-Me-α-D-Man*p*; (●) methyl β-D-galactofuranoside; (△) methyl α-L-arabinofuranoside.

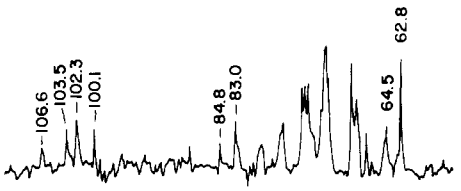

FIGURE 9: Carbon-13 NMR spectrum of fraction V isolated from epimastigotes of *T. cruzi*.

inhibition studies, we used oligosaccharide obtained after alkaline degradation of LPPG, β-D-Gal*f*-(1→3)-Me-α-D-Man*p*, methyl β-D-galactofuranoside, and methyl α-L-arabinofuranoside. The results are shown in Figure 8.

Chemical Characteristics of Fractions IV and V. The material that was insoluble in $CHCl_3$–MeOH–H_2O in the above extraction process was further processed. Its yield was lower than that of the soluble fraction II. It was fractionated by selective absorption on a column of Con A–Sepharose, and fraction IV was obtained by elution with aqueous 0.1 M methyl α-D-mannopyranoside. It contained units of galactose and mannose, in a molar ratio of 1.4:1, hexosamine (6%), and traces of glucose and fucose.

Fraction IV was degraded to fraction V with hot aqueous NaOH–$NaBH_4$. It contained galactose and mannose in a ratio of 1:2 and traces of glucose and fucose. The hexosamine content was not determined. One interesting feature, which is not yet explained, is the reduction of the galactose content on alkaline degradation.

The ^{13}C NMR spectrum of fraction V (Figure 9), although having similarities to that of fraction III, has one important difference in that a signal is present at δ 103.5. This corresponds to nonreducing end units of α-D-mannopyranose linked 1→2 to α-D-mannopyranosyl units (Gorin, 1973), conforming with the methylation data (Table II). This indicated nonreducing end units (41%) and 2-O-substituted units (16%) of mannopyranose. Also characterized were nonreducing end units of galactopyranose (6%) and β-D-galactofuranose (6%; see typical ^{13}C NMR signal at δ 106.6) and a trace of 4-O-substituted fucopyranosyl (or 5-O-substituted furanosyl) residues (2%). Fucose has been recently identified as a component of a minor epimastigote glycoprotein (Snary et al., 1981). A trace of a short-chain 1→4-linked glucopyranosyl component is also apparently present in fraction V.

Discussion

The knowledge of the structure and function of cell-surface glycoconjugates of *T cruzi* may be of importance for the understanding of the relationship between parasites and host cells. These glycoconjugates have been prepared, in most cases, from detergent extracts of radioiodinated parasites by a process of immunoprecipitation (Snary & Hudson, 1979; Snary, 1980; Araujo & Remington, 1981; Nogueira et al., 1981). However, such preparations have not yet been subjected to analysis that would give an insight into their detailed chemical structures.

In the present study, we have investigated the chemical structures of the carbohydrate components of three fractions, designated I, II, and IV, isolated from epimastigote forms of *T. cruzi*.

The fractionation procedure, summarized in Figure 1, was as follows. Cells were disrupted and extracted by successive freezing and thawing, and liberated soluble material was fractionated on a column of P-10. Five fractions were obtained, but only the first one that was eluted, fraction I, was investigated further. The cell debris, isolated after extraction, was then extracted with hot phenol–water. The aqueous phase was lyophilized and the residue extracted with $CHCl_3$–MeOH–H_2O. Soluble material (fraction II) appears to correspond to the LPPG of Lederkremer et al. (1976, 1977, 1978), who extracted cells directly with phenol–water without a prior aqueous treatment. The preparations resemble each other closely in terms of chemical composition. Our other preparation, termed fraction IV, was obtained by fractionation of the $CHCl_3$–MeOH–H_2O-insoluble material on a column of Con A–Sepharose and consisted of the bound material.

Fraction I (GP-25) is a glycoprotein, which on SDS–PAGE electrophoresis gave rise to a single protein- and carbohydrate-positive band with an apparent molecular weight of ~25 000. The glycoprotein was radioiodinated and found to be specifically precipitated by serum obtained from chronic Chagas' disease patients. The antigenic role of the glycoprotein was confirmed by its recovery from the insoluble immune complex and examination by gel electrophoresis.

β-Elimination of fraction I with cold aqueous NaOH–$NaBH_4$–$NaBT_4$ indicated that the glycoprotein contained, at least to some extent, short carbohydrate chains. The reaction product was chromatographed on a column of P-10, and two labeled fractions were obtained. One was excluded from the column and could consist of an undegraded component of fraction I or a true β-elimination product. The other fraction was refractionated on a column of P-4 and gave rise to two fractions whose elution characteristics corresponded to those of a di- and tetrasaccharide. The oligosaccharides appear to be linked to serine and threonine, since these amino acids were decomposed during the β-elimination reaction.

Methylation analysis of fraction I supported the suggested existence of short sugar chains. This resulted in the formation of many *O*-methylalditol acetate fragments (Table II) which show the presence of predominant nonreducing end units of mannopyranose and galactopyranose and 2-O-substituted mannopyranosyl residues. The mannosyl units could exist as α-D-Man*p*-[(1→2)-α-D-Man*p*]$_n$- with n = 0–2, as in the polysaccharide isolated after vigorous alkaline extraction (Gorin et al., 1981). The detection of galactopyranosyl units agrees with the observation of Alves et al. (1979), who found them in their mixed glycoprotein cell-surface components. The proportion of tetra-*O*-methylalditol acetates arising from nonreducing end units of fraction I is much greater than di-*O*-methyl derivatives which would be products from branch points (present in equal amounts if it were a polysaccharide). Thus, the carbohydrates must be present as short chains linked to protein. The antigenic nature of GP-25 has been recently studied by Scharfstein et al. (1982a), who used affinity-purified human antibodies to demonstrate the presence of GP-25's antigenic sites at the surface of *T. cruzi* and in all stages of

parasite development (Scharfstein et al., 1982b).

A glycoprotein having a molecular weight of 90 000 which has been isolated from all forms of *T. cruzi* by Snary (1980) was found to protect mice against a challenge infection with *T. cruzi* (Scott & Snary, 1979). On the other hand, Nogueira et al. (1981) identified a protein with a molecular weight of 90 000 presumably implicated in the antiphagocytic activity of blood-form trypomastigotes. Glycoproteins with molecular weights of 72 000 and 75 000 were respectively detected by Snary et al. (1981) and Nogueira et al. (1981) exclusively in epimastigote and metacyclic forms. On the basis of presently available data, we cannot yet establish the structural relationship, if any, of these glycoproteins with GP-25.

Fraction II, which corresponds to LPPG, was also found to be antigenic. This was observed in an immunodiffusion gel using rabbit immune serum against epimastigote forms. It was found that the nonreducing end units of β-D-galactofuranose were involved in its antigenicity since on conversion of these units to α-L-arabinofuranose the antigenic activity disappeared. Also, when fraction III (see below) and β-D-Gal*f*-(1→3)-Me-α-D-Man*p* were used as competitive inhibitors, precipitation inhibitions of 85% and 75%, respectively, were observed. Methyl β-D-galactofuranoside was relatively ineffective. This is the first time in the *T. cruzi* system that specific sugar units have been demonstrated as being responsible for antigenic activity.

LPPG was only detectable in epimastigote forms, as indicated by using surface labeling techniques (Zingales et al., 1982). It was speculated that LPPG is involved in specific phenomena of epimastigote cells, for example, their lability to the alternative pathway of complement (Nogueira et al., 1975) and their capacity to divide in the midgut of the insect vector. However, using the indirect immunofluorescence technique, Previato et al. (1982) showed the presence of this substance in all stages of the life cycle of *T. cruzi*.

In the present investigation, the carbohydrate portion of fraction II was prepared by reductive cleavage with hot aqueous NaOH–$NaBH_4$ and purified by column chromatography on P-4. It was named fraction III, had a degree of polymerization (dp) in the range of 10, and had nonreducing end units of β-D-galactopyranose (25%) linked mainly 1→3 directly to α-D-mannopyranosyl units and not via a phosphorodiester bridge as previously suggested (Lederkremer et al., 1980). High proportions of 3-O- (32%) and 2,3-di-O-substituted (19%) α-D-mannopyranosyl units were also present. In general, the methylation and ^{13}C NMR spectral data are in agreement. The C-1 signals of β-D-galactofuranosyl units have already been mentioned under Results. In terms of the other main C-1 signals (Figure 6), which arose from α-D-mannopyranosyl units, the following assignments can be made. Those at δ 102.2 and 100.1 belong to C-1's of 3-O-substituted and 2,3-di-*O*-substituted D-mannopyranosyl units, respectively, since these major structures are indicated by the methylation data and the fact that the signal at high field of δ 100.1 has undergone a strong upfield β-shift due to 2-O-substitution. This has been observed in the case of the C-1's of 2,6-di-O-substituted α-D-mannopyranosyl residues of baker's yeast mannan, which give rise to a signal at δ 100.0 (Gorin, 1973).

Fraction IV was degraded to fraction V by using hot aqueous NaOH–$NaBH_4$. This carbohydrate had a chemical structure different from those of other fractions. It contained galactose and mannose in a molar ratio of 1:2 and traces of glucose and fucose and was structurally different from fraction III. According to the methylation analysis (Table II), it contained only a small proportion of nonreducing end units of galactofuranose (6%) and galactopyranose (6%) but consisted principally of nonreducing end units of mannopyranose (41%) some of which are likely linked 1→2 to internal mannopyranosyl units (16%). Such structures are consistent with the presence of C-1 signals in the ^{13}C NMR spectrum at δ 103.5 and 102.3, respectively, values that have been previously reported for yeast mannan, which has similar structures (Gorin, 1973) with the α-configuration. This structure is consistent with the specific rotation of galactomannan, which is +29°.

The present results may have interesting implications for the study of Chagas' disease. It is now possible to investigate the interaction of the host immune system with two cell-surface antigens which are present in all stages of development of *T. cruzi*. Some details of the chemical structures of the carbohydrate portions of these molecules have been determined. Remaining to be elucidated are the cellular function of the antigens and their roles in the infectious process.

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Functions of the 5′-Phosphoryl Group of Pyridoxal 5′-Phosphate in Phosphorylase: A Study Using Pyridoxal-Reconstituted Enzyme as a Model System†

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ABSTRACT: Pyridoxal-reconstituted phosphorylase was used as a model system to study the possible functions of the 5′-phosphoryl group of pyridoxal 5′-phosphate (PLP) in rabbit muscle glycogen phosphorylase. Kinetic study was conducted by using competitive inhibitors of phosphite, an activator, and α-D-glucopyranose 1-phosphate (glucose-1-P) to study the relationship between the PLP phosphate and the binding of glucose-1-P to phosphorylase. Fluorine-19 nuclear magnetic resonance (^{19}F NMR) spectroscopy of fluorophosphate bound to pyridoxal phosphorylase showed that its ionization state did not change during enzymatic catalysis. Evaluation of the apparent kinetic parameters for the activation of pyridoxal phosphorylase with different analogues having varied pK_{a2} values demonstrated a dependency of K_M on pK_{a2}. Molybdate, capable of binding as chelates in a trigonal-bipyramidal configuration, was tested for its inhibitory property with pyridoxal phosphorylase. On the basis of the results in this study, several conclusions may be drawn: (1) The bound phosphite in pyridoxal phosphorylase and, possibly, the 5′-phosphoryl group of PLP in native phosphorylase do not effect the glucose-1-P binding. (2) One likely function of the 5′-phosphoryl group of PLP in native phosphorylase is acting as an anchoring point to hold the PLP molecule and/or various amino acid side chains in a proper orientation for effective catalysis. (3) The force between the PLP phosphate and its binding site in phosphorylase is mainly electrostatic; a change of ionization state during catalysis is unlikely. (4) Properties of the central atoms of different anions are important for their effects as either activators or inhibitors of pyridoxal phosphorylase. (5) Our results with molybdate are consistent but do not prove that a trigonal-bipyramidal structure of PLP is involved in the catalytic mechanism of phosphorylase.

Various studies of α-glucan phosphorylases reconstituted with vitamin B_6 analogues suggest that the 5′-phosphoryl group of PLP[1] is likely to be involved in the catalytic process [see reviews by Helmreich & Klein (1980) and Graves & Wang (1972)]. Although phosphorylase reconstituted with pyridoxal is inactive (Illingworth et al., 1958), it was found some phosphate analogues could activate the enzyme (Parrish et al., 1977). These findings were explained by binding of phosphate and analogues at the site where the 5′-phosphoryl group of the coenzyme in native phosphorylase resides. Inorganic pyrophosphate was found a potent inhibitor to pyridoxal-re-

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[1] Abbreviations: glucose-1-P, α-D-glucopyranose 1-phosphate; PLP, pyridoxal 5′-phosphate; NMR, nuclear magnetic resonance; ppm, parts per million; EDTA, ethylenediaminetetraacetic acid.

0006-2960/83/0422-4987$01.50/0