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Structural Elucidation of the *Brucella melitensis* M Antigen by High-Resolution NMR at 500 MHz†

David R. Bundle,*,‡ John W. Cherwonogrodzky,§ and Malcolm B. Perry‡
Division of Biological Science, The National Research Council of Canada, Ottawa, Canada K1A 0R6, and Agriculture Canada, Animal Disease Research Institute, Nepean, Ontario, Canada K2H 8P9

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ABSTRACT: The *Brucella* M antigen from the species type strain *Brucella melitensis* 16M has been identified as a component of the cell wall lipopolysaccharide (LPS). O polysaccharide liberated from this LPS by mild acid hydrolysis exhibited M activity in serological tests and was shown to be a homopolymer of 4-formamido-4,6-dideoxy-α-D-mannopyranosyl residues arranged in an oligosaccharide repeating unit as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the native lipopolysaccharide. Structural analysis of the O polysaccharide by NMR methods was difficult due to apparent microheterogeneity of the repeating unit, which was in fact caused by the presence of rotational isomers of the *N*-formyl moiety. This problem was resolved by chemical modification of the polysaccharide to its amino and *N*-acetyl derivatives, the 500-MHz ^{1}H and 125-MHz ^{13}C NMR spectra of which could be analyzed in terms of a unique structure through application of pH-dependent β-shifts and two-dimensional techniques that included COSY, relayed COSY, and NOESY experiments together with heteronuclear C/H shift correlation spectroscopy. On the basis of these experiments and supported by methylation and periodate oxidation data, the structure of the M polysaccharide was determined as a linear polymer of unbranched pentasaccharide repeating units consisting of four 1,2-linked and one 1,3-linked 4,6-dideoxy-4-formamido-α-D-mannopyranosyl residues. The marked structural similarity of the M antigen and the A antigen, which is known to be a 1,2-linked homopolysaccharide of 4,6-dideoxy-4-formamido-α-D-mannopyranosyl units, accounts for cross-serological reactions of the two and the long-standing confusion surrounding the nature of their antigenic determinants. Structural and serological considerations in conjuction with the sodium dodecyl sulfate banding pattern of *Brucella* A LPS suggest that its biosynthesis differs appreciably from that of the M antigen, which appears to be synthesized by regulated assembly of preformed oligosaccharide repeating units. Temperate, lysogenic phage may be responsible for such biosynthetic and structural variations.

Serological detection plays a major role in the routine diagnosis of brucellosis, because it is not always possible to isolate the causative organism (Alton et al., 1975). Although antibodies directed against cell-surface polysaccharides of smooth *Brucella* form the basis of these serological tests, until recently little was known about their chemistry. Two cell wall antigens, A and M, have been recognized in smooth strains of *Brucella* (Wilson & Miles, 1932), and originally it was proposed that both antigens occurred as components of a single AP substance (aminopolyhydroxy compound) that also contained formic acid (Miles & Pirie, 1939; Ellwood et al., 1967). Although there has been ample evidence to suggest that the A and M antigens of *Brucella* correspond to the somatic antigens of the bacterial lipopolysaccharide, LPS[1] (Diaz et al., 1968; Moreno et al.,

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* Author to whom correspondence should be addressed.
‡ The National Research Council of Canada.
§ Animal Disease Research Institute.

[1] Abbreviations: NAc-PS, N-deformylated and N-acetylated M polysaccharide; COSY, ^{1}H–^{1}H shift-correlated spectroscopy; GC–MS, gas–liquid chromatography–mass spectroscopy; GLC, gas–liquid chromatography; LPS, lipopolysaccharide; *s*LPS, smooth LPS; NH_2-PS, N-deformylated M polysaccharide; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; Rha4NFo, 4,6-dideoxy-4-formamido-D-rhamnopyranose; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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1981, Lindberg et al., 1982), confusion has surrounded this issue, since other polysaccharides, polysaccharide B (Diaz et al., 1979), and native hapten (Moreno et al., 1981; Fernandez-Lago et al., 1982) were isolated from *Brucella* and found to possess serological activity. In large part this confusion occurred because of inadequate chemical characterization of the antigens, a task made very difficult by the consistent failure of available analytical tests to detect the monosaccharide component of the somatic antigen. The generally accepted view has, nevertheless, been to regard the *Brucella abortus* cell wall lipopolysaccharide (Diaz et al., 1968) as the A antigen. This conclusion was justified when it was demonstrated by NMR methods that the O antigen of *B. abortus* (Caroff et al., 1984b) was a homopolymer of 4,6-dideoxy-4-formamido-D-mannose (Figure 9), a rare sugar that was undetected by classical methods of sugar analysis but remarkably similar to the material described by Miles and Pirie in 1939. The precise nature of the M antigen and its relationship to the recently characterized A antigen remained unanswered, although it was assumed that like the A antigen it was the O polysaccharide component of a LPS.

Designation of *Brucella* strains as serologically A or M has been interpreted as the predominant expression of either antigen at the cell surface of *B. abortus*, *Brucella melitensis*, and *Brucella suis* (Wilson & Miles, 1933; Wilson, 1984). Since both antigens were considered to be present on all such smooth strains, the classification as either A or M positive was regarded as a difference in the quantitative distribution of A and M antigens as estimated by sera rendered monospecific by cross-absorption. Thus, the A antigen was said to be in excess in *B. abortus* and *B. suis* and the M in *B. melitensis*. Nevertheless, it is well documented that certain biotypes of *B. abortus* express the M antigen as the predominant serological marker, while biotype 2 of *B. melitensis* possesses the A antigen. Biotype 3 of this species and biotype 4 of *B. suis* are classified as positive for both A and M antigens (Wilson, 1984).

These observations could be explained by the simultaneous presence of two smooth LPSs differing in the chemical structure of their respective O polysaccharides, although in terms of our present understanding of enterobacterial LPS biochemistry it would be unusual to observe the presence of two structurally distinct lipopolysaccharides in pure cultures of a bacterium (Luderitz et al., 1971). A more likely explanation would involve the presence of a common antigenic determinant (Diaz et al., 1968). The resolution of these difficulties requires that the structural differences between the *Brucella* A and M antigens be firmly established.

In this paper application of NMR methods at 500 MHz to the structural analysis of the M antigen from *B. melitensis* 16M is reported, and the implications of these structural details for the serology and biosynthesis of the *Brucella* polysaccharides are discussed. The choice of *B. melitensis* strain was based on the proposal, founded upon DNA homology, that only one species, *B. melitensis*, be recognized in the genus *Brucella* and the species-type strain should be regarded as *B. melitensis* 16M (Verger et al., 1985).

Materials and Methods

Cell Production and LPS Isolation. B. melitensis 16M (supplied by Dr. M. Meyer, University of California at Davis) was grown on potato infusion agar in Roux flasks for 72 h at 35 °C. Bacteria (15 g, dry weight) were harvested in 0.1 M Tris-HCl buffer (pH 7.2) containing 1% (w/v) sodium chloride and 2% (w/v) phenol and were kept at 4 °C for 7 days. Following removal of cells by low-speed centrifugation, the supernatant was dialyzed against water, and the concentrated dialyzate was subjected to ultracentrifugation (105000*g*) for 12 h at 4 °C to yield the LPS as a deposited clear gel. After lyophilization, LPS (1.3 g) was obtained as a white powder. The supernatant was further dialyzed against distilled water, lyophilized, and assayed for LPS by the carbocyanine dye assay (Janda & Work, 1971). Although negative for LPS, this material, polysaccharide B, released glucose after acid hydrolysis and was purified by gel filtration chromatography on a 70 × 2.5 cm column of Sephadex G-50 and eluted with a K_{av} of 0.68. Cells were extracted by the phenol–water procedure as described for *B. abortus* (Caroff et al., 1984b) to yield 0.34 g of purified LPS. The total yield of purified LPS was 1.64 g and of polysaccharide B 0.36 g. It was observed over several batches that the amount of LPS that could be extracted from washed cells was variable.

O Polysaccharide. The LPS (200 mg) in water (100 mL) containing acetic acid (5 mL) was heated on a boiling water bath for 2.5 h. The precipitated oily lipid A (16 mg) was removed, and the concentrated aqueous product was chromatographed on a 70 × 2.5 cm column of Sephadex G-50. The eluting buffer was 0.05 M pyridinium acetate, pH 4.7, and O polysaccharide (176 mg) was recovered in the peak eluting at the column void volume. The material was lyophilized and subjected to elemental analysis. Calcd for a homopolymer: C, 48.6; H, 6.4; N, 8.1. Found: C, 44.5; H, 6.3; N, 6.6; ash, 0.0. The optical rotation of this material was $[\alpha]^{20}_{D}$ +56.2° (*c* 1.0, water).

N-Deacylated and N-Acetylated O Polysaccharide Derivatives. Native O polysaccharide (100 mg) in 2 M sodium hydroxide (5 mL) containing sodium borohydride (4 mg) was heated in a sealed glass tube for 8 h at 100 °C. The diluted mixture was dialyzed against distilled water, and the amino derivative of the polysaccharide, NH_2-PS (87 mg), was obtained on lyophilization.

The N-acetylated form of the polysaccharide, NAc-PS, was obtained by treating NH_2-PS (60 mg) in water (12 mL) containing methanol (1 mL) and sodium carbonate (20 mg) with acetic anhydride (0.1 mL) at 20 °C for 30 h. The reaction mixture was treated with sufficient ammonium hydroxide solution to raise the pH to 11.0, and the temperature was raised to 40 °C for 10 min. The concentrated solution was fractionated by gel filtration chromatography on Sephadex G-50 as described for the native O polysaccharide, and the NAc-PS (60 mg) was recovered in the peak eluting at the column void volume.

Analytical Methods. Quantitative colorimetric methods were those described for the investigation of the *B. abortus* polysaccharide (Caroff et al., 1984b). The absolute configuration of glycoses was established by GLC of trimethylsilylated (−)-2-butylglycosides (Gerwig et al., 1978).

Gas–Liquid Chromatography and Mass Spectroscopy. GLC–MS analyses were made on a Hewlett-Packard 5985B GLC–MS system equipped with a capillary column (25 m) coated with OV-17 and a program temperature rise from 180 to 250 °C at 2 deg/min, and an ionization potential of 70 eV was employed. Retention times are quoted relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol (T_{GM}).

Methylation Analysis. Samples (2–3 mg) of the NAc-PS derivative were methylated by use of sodium methylsulfinylmethanide and methyl iodide in dimethyl sulfoxide according to the Hakomori procedure (Hakomori, 1964), and following dialysis of the product against running water, the lyophilized retentates were hydrolyzed with anhydrous hydrofluoric acid (1 mL) at 22 °C for 3 h. The methylated derivatives re-

maining after removal of the hydrogen fluoride in vacuo were treated in one of two ways: (a) conversion to alditol acetates by sodium borohydride reduction and acetylation as described in previous work (Caroff et al., 1984b) or (b) acetylation by treatment with acetic anhydride (1 mL) containing anhydrous sodium acetate (0.1 g) for 2 h at 125 °C. The products were directly examined by GLC–MS. Procedure a led to the detection of only one partially methylated alditol acetate, 1,2,5-tri-*O*-acetyl-4,6-dideoxy-3-*O*-methyl-4-(*N*-methylacetamido)-D-mannitol (T_{GM} 3.02). Procedure b showed the presence of two acetylated mannopyranose derivatives detected in the ratio 4:1: 1,2-di-*O*-acetyl-4,6-dideoxy-3-*O*-methyl-4-(*N*-methylacetamido)-α-D-mannopyranose (T_{GM} 1.86); 1,3-di-*O*-acetyl-4,6-dideoxy-2-*O*-methyl-4-(*N*-methylacetamido)-α-D-mannopyranose (T_{GM} 1.78).

NMR Spectroscopy. Polysaccharide samples were lyophilized once from deuterium oxide and dissolved at concentrations of 20 and 50 mg/500 μL. Samples of NH_2-PS in deuterium oxide solution were acidified by addition of deuterium chloride to lower the pD to ca. 2.0. Spectra were recorded for samples in 5-mm tubes at 500 MHz for ^1H and 125 MHz for ^{13}C on a Bruker AM-500 spectrometer. Proton spectra were recorded at 303 and 310 K, and ^{13}C spectra were recorded at 310 K employing ~1-W WALTZ decoupling (Shaka et al., 1983) as previously described (Bundle et al., 1986).

Homonuclear two-dimensional spectroscopy was performed with Bruker DISNMRP software. COSY (Bax et al., 1981) and NOESY (Kumar et al., 1980) experiments were performed with suppression of the HOD resonance as previously described. Mixing times of 100 and 150 ms were employed for NOESY experiments. Relayed COSY (Wagner, 1983) experiments were made with fixed delays of 30 and 80 ms in two separate experiments to optimize coherence transfer for large and small scalar couplings (Bax & Drobny, 1985). All homonuclear experiments were performed with quadrature detection in the F_1 dimension, and a total of $256t_1$ increments of 96 scans each (64 scans for COSY experiments) were recorded with a minimum delay between pulses of 1.2 s and a sweep width of 2500 Hz. The time domain data matrix was zero-filled to either 1024 × 1024 or 2048 × 2048 points, and following resolution enhancement in both dimensions by a nonshifted sine-bell function, the doubly transformed data were processed to give magnitude spectra. The symmetrical matrix was symmetrized about the diagonal for presentation and to reduce t_1 noise.

Heteronuclear shift correlation spectroscopy was performed with quadrature detection in F_1 (Bax & Morris, 1981), and a total of $256t_1$ increments of 320 scans each were recorded for a spectral width in F_2 of 7800 Hz and 1500 Hz in F_1. Data were processed to give after zero-filling a matrix ($t_1 \times t_2$) of 512 × 8192 points, and following resolution enhancement as previously described (Bundle et al., 1986), double transformation gave power spectra. Fixed delays of τ_1 = 3.4 ms and τ_2 = 1.7 ms were employed to select all multiplicities, and a recycle delay of ca. 1 s (one proton T_1) was used.

Potential Energy Calculations. The GESA algorithm (Paulsen et al., 1985) was used to estimate the preferred conformations of oligosaccharides composed of the *N*-acetyl and *N*-formyl derivatives of 4-amino-4,6-dideoxy-D-mannose, Rha4N, the coordinates of which were generated from neutron diffraction data of methyl α-D-mannopyranoside (Jeffrey et al., 1977) according to bond modification procedures described in the literature (Bock, 1983). The C4–N4 bond distance was taken as 0.14 nm, and the *N* atom was positioned along the bond vector defined by C4–O4 of α-D-mannopyranose. The C6 methyl group was generated with tetrahedral geometry and uniform C–H bond lengths of 0.11 nm. Calculations were done with this saccharide or those made by addition of acetyl or formyl groups to the amino function, in which case the acyl group was positioned such that the carbonyl C–O bond eclipsed the C4–H4 bond.

Potential energy calculations using the HSEA (Lemieux et al., 1980; Thorgersen et al., 1982) and GSEA (Paulsen et al., 1985) algorithms were performed for a hypothetical octasaccharide in which the first and sixth pyranose residues were α1,3-linked and the remaining residues were exclusively α1,2-linked. The position of 1,3 linkages was then varied within this structure and the calculation repeated. Two other derivatives were also used in separate calculations, and these corresponded to the 4-formamido and 4-acetamido derivatives of 4-amino-4,6-dideoxy-α-D-mannopyranose. The distance matrices derived from these calculations were then used to estimate the relative inter- and intraresidue NOE's (Noggle & Schirmer, 1971) observed for oligosaccharides representative of the proposed M antigen derivatized as the amino or *N*-acetyl compounds.

Molecular plots were produced by the PLUTO and VERSFAN (R. Norrestam, Department of Chemistry, Technical University of Denmark, Lyngby, personal communication) programs, and potential energy calculations were performed on an IBM 3080 computer.

General Methods. Optical rotations, immunodiffusion experiments, monoclonal antibodies, and general procedures used have been described in related publications from this laboratory (Caroff et al., 1984a,b; Bundle et al., 1984, 1987b).

RESULTS

Bacterial cells were grown on plates, and during the recovery of cells from Roux flasks by scraping, washing, and suspension in Tris–saline it was observed that LPS was eluted from the cell walls. On standing in Tris–saline buffer over a 1-week period at 4 °C, the *B. melitensis* cells released a second polysaccharide, and further quantities of LPS could be extracted from the washed cells by phenol–water extraction (Westphal et al., 1952). Whereas the LPS could be recovered by ultracentrifugation, the second polysaccharide was recovered by gel filtration chromatography. The latter component was in fact polysaccharide B (Diaz et al., 1979) previously characterized by Moreno and co-workers (Moreno et al., 1981) as having a high glucose content. NMR and methylation analysis showed this material to be a cyclic polymer of 1,2-linked β-D-glucopyranosyl residues (Bundle et al., 1987a). Examination of *B. melitensis* LPS by SDS–PAGE and silver staining (Tsai & Frasch, 1982) and comparison with that from *B. abortus* 1119-3 revealed O-chain banding, the spacing of which corresponded to a repeating unit composed of between four to six saccharide residues (Figure 1). The *B. abortus* LPS with its monomeric O polysaccharide repeating unit exhibits no resolvable banding but appears to have a molecular weight comparable to that of the M antigen.

O polysaccharide was released from the *s*LPS of *B. melitensis* by acetic acid hydrolysis and was purified by gel filtration. Serology established that O polysaccharide was precipitated by monoclonal antibodies specific for the M antigen (Bundle et al., 1987b) and that it was capable of inhibiting the binding of *B. melitensis* LPS to rabbit anti-M serum (Cherwonogrodzky et al., 1987). Elemental analyses for C, H, and N were similar to those obtained for the *B. abortus* O polysaccharide (Caroff et al., 1984a), a homopolymer of 4,6-dideoxy-4-formamido-D-mannose, although the

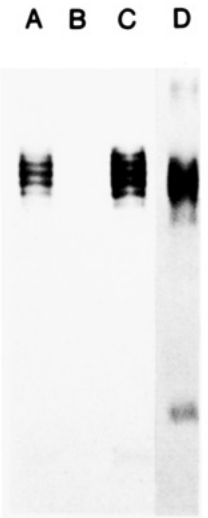

FIGURE 1: SDS–PAGE of *B. abortus* 1119-3 (lane D) and *B. melitensis* 16M (lanes A–C) LPS. Silver staining after periodate oxidation of the polysaccharide was employed. While banding indicative of an oligosaccharide repeating unit is to be seen with the *B. melitensis* LPS, the *B. abortus* LPS is an unresolved streak characteristic of an O polysaccharide repeating unit composed of a single monosaccharide residue. LPS of lanes A and C correspond with material released from the cell wall by Tris buffer and by extraction with phenol–water. Lane B is the aqueous phase of the phenol extracts.

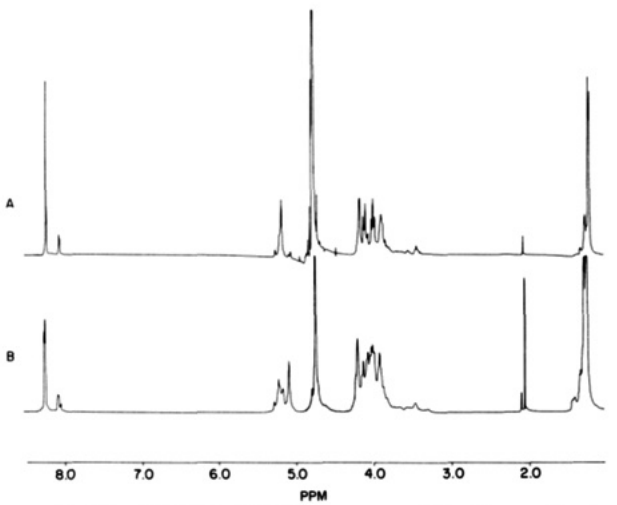

FIGURE 2: The 500-MHz ^1H NMR spectra of *B. abortus* O polysaccharide (A) and *B. melitensis* O polysaccharide (B).

optical rotation of the *B. melitensis* O polysaccharide was found to be higher, $[\alpha]^{20}_D$ +56.2° compared to $[\alpha]^{20}_D$ +37.7° (Caroff et al., 1984). Treatment of the N-deformylated and N-acetylated O polysaccharide (NAc-PS) by HF as previously described (Caroff et al., 1984b) gave 4-acetamido-4,6-dideoxy-D-mannose as the only monosaccharide.

Proton and ^{13}C NMR spectra of the native O polysaccharide showed signals characteristic of a polysaccharide composed of 4,6-dideoxy-4-formamido-α-D-mannose residues, but the spectra were more complex (Figure 2) than those of the previously identified 1,2-linked homopolymers of this monosaccharide (Caroff et al., 1984a,b). Although the additional spectral complexity was compounded by the rotational isomerism of the formamide moiety, it was immediately apparent from the number of anomeric, 6-deoxy, and formate resonances in both the ^1H and ^{13}C spectra (Figure 2 and 3A) that the M polysaccharide possessed a more elaborate structure than the A antigen. In particular, the ^{13}C spectrum (Figure 3A) revealed an additional anomeric resonance at 102.4 ppm and numerous lower intensity resonances throughout the spectrum.

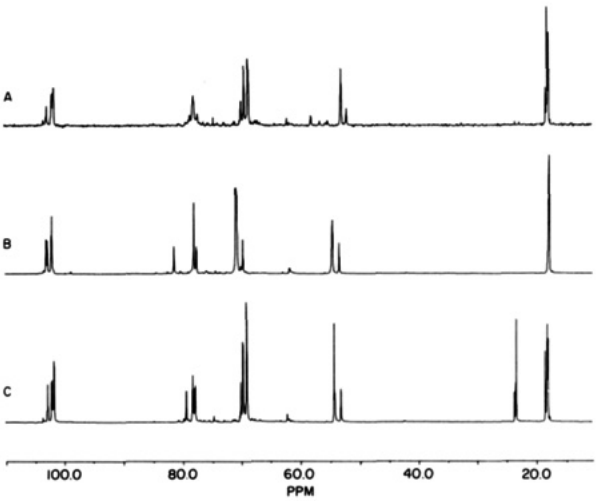

FIGURE 3: The 125-MHz ^{13}C NMR spectra of the *B. melitensis* O polysaccharide (A), which reveals multiple signals caused by the *E* and *Z* forms of the formamido group. Elimination of this apparent microheterogeneity is accomplished by conversion of the polysaccharide to the N-deformylated amino derivative NH_2-PS (B) and the *N*-acetyl derivative NAc-PS(C), both of which provide less complex spectra.

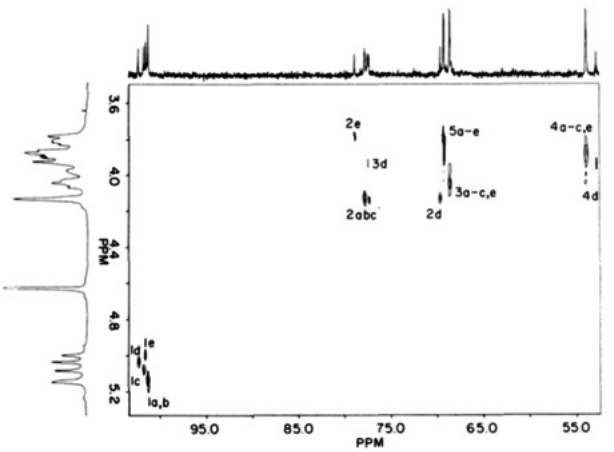

FIGURE 4: C/H shift-correlated map for the NAc-PS derivative. The spectra were obtained at 310 K, and individual ^1H and ^{13}C spectra are displayed along the F_1 and F_2 axes.

Table I: Chemical Shifts[a] (ppm) and Assignment of NAc-PS of *B. melitensis*

	a	b	c	d	e
H1	5.16	5.15	5.095	5.046	5.012
H2	4.158	4.13	4.15	4.145	3.795
H3	4.07	4.04	4.058	3.945	4.00
H4			3.96	3.86	
H5			3.88	3.82	
H6			1.23	1.17	

[a] Measured relative to internal acetone, 2.225 ppm. Chemical shifts are first-order values, measured at 310 K.

N-Deformylated polysaccharide (NH_2-PS) and its N-acetylated derivative (NAc-PS) gave less complex ^{13}C NMR spectra (Figure 3B,C), both of which were amenable to structural analysis.

The anomeric region of the NAc-PS sample showed four discrete C1 signals, one of which possessed a relative intensity of 2 (displayed as the F_2 projection of the C/H correlation map, Figure 4), indicative of a pentameric repeating unit. In support of this five discrete signals due to the C6 carbon atoms of the 6-deoxyhexoses were observed (Table I). ^1H spectra also indicated five anomeric protons (Figures 4 and 5), and

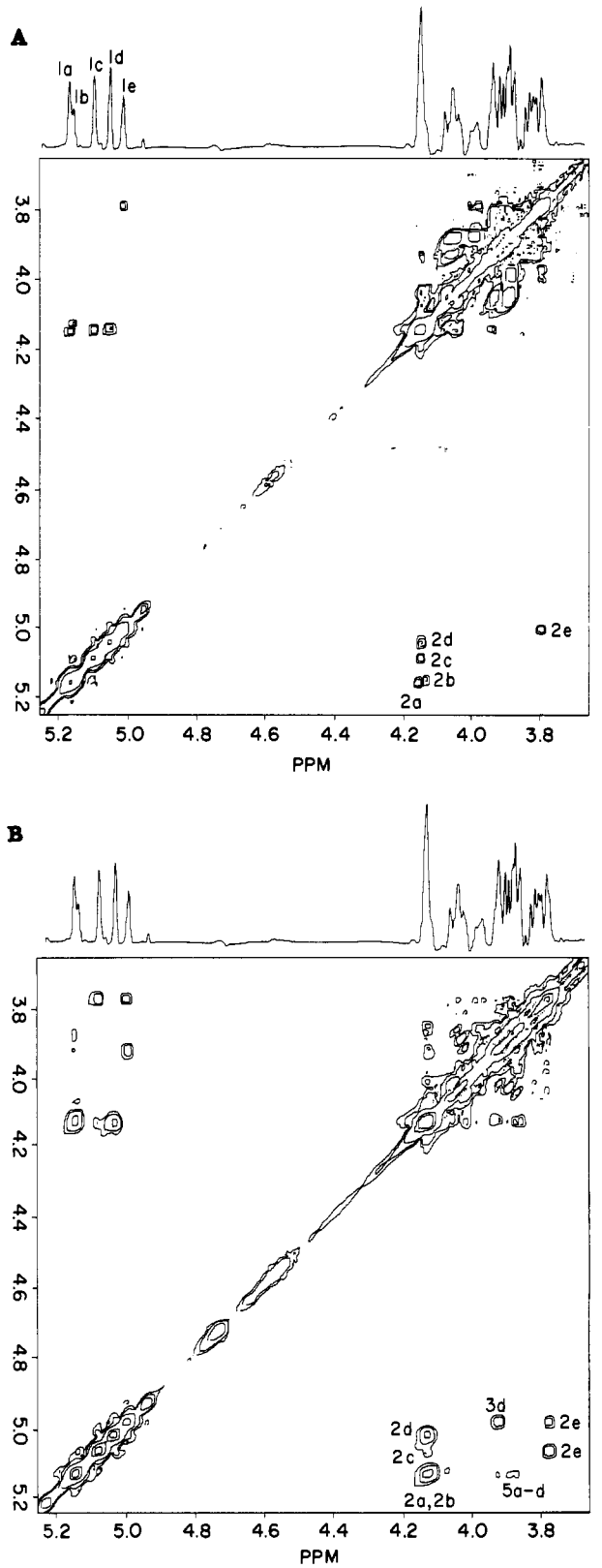

FIGURE 5: (A) COSY spectrum of the NAc-PS derivative. Resonance assignments for the five H2 protons can be made in the normal manner, and although connectivities may be followed to establish broad regions associated with H3 and H4 resonances, intense overlap of resonances and cross-peaks precludes a rigorous assignment of the subspectra of each pyranose residue. (B) NOESY spectrum of the NAc-PS derivative. Cross-peaks between H1 resonances and protons on contiguous pyranose rings enable the linkage site to be established. Since the majority of linkages are α1,2, cross-peak overlap occurs for three of the five linkages. Additional cross-peaks between H5 and H1a/b resonances are consistent with expectations for 1,2 linkages between pyranosides with the *manno* configuration.

Table II: Chemical Shifts[a] (ppm) and Assignment of NH_2-PS *B. melitensis*

	a	b	c	d	e
H1	5.08	5.08	5.065	4.94	5.00
H2	4.00	4.00	3.98	4.01	4.01
H3	3.78	3.78	3.78	3.72	3.82
H4	2.78	2.78	2.78	2.84	2.78
H5	3.78		3.65	3.80	
H6	1.26				1.32

[a] Measured relative to internal acetone, 2.225 ppm. Chemical shifts are first-order values measured at pD 8.0 and 310 K.

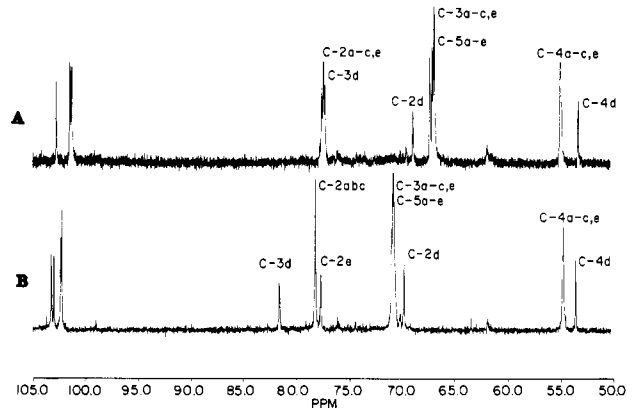

FIGURE 6: ^{13}C spectra of the NH_2-PS derivative recorded at 310 K in (A) basic solution, pD ca. 8.0, and (B) acid solution, pD ca. 2.0. Resonances β to the C4 amino group, e.g., C5 and C3, experience ca. 4.0-ppm upfield shifts upon protonation. C2 and C4 resonances show less than 1-ppm shift changes.

the acetamido methyl group resonances at ~2.0 ppm were resolved into five separate signals following resolution enhancement. The chemical shift values of the anomeric protons (Table II) indicated that the anomeric configuration of all linkages was α, and this conclusion was in agreement with the heteronuclear one-bond C/H coupling constants (Bock & Pedersen, 1974) of ca. 170 Hz (Table I) recorded for each anomeric carbon atom.

Structural determination by NMR methods demands that the assignment of the largest possible number of ^1H and ^{13}C resonances be unambigously established, and to facilitate this, a system of residue notation was adopted on the basis of the sequence of anomeric protons in the spectra of NAc-PS. In order of descending chemical shifts, these and the associated spin systems of each pyranose residue were designated a–e. In the case of the NH_2-PS derivative where chemical shift ordering of the anomeric protons changed, the notation of the spin systems agrees with that used for NAc-PS. Assignment of ^1H resonances of both polymer derivatives (Tables I and II) was accomplished by two-dimensional homonuclear COSY (Figure 5A) (Nagayama et al., 1980) and relayed coherence transfer spectroscopy, not shown (Wagner, 1983). Severe overlap precluded the observation of discrete cross-peaks for most H4 and H5 resonances, although NH_2-PS provided H4 signals well removed from the other ring protons at ~2.8 ppm (Figures 6 and 7), thereby confirming the position of the H3 and H5 protons from the position of H3/H4 and H4/H5 cross-peaks. Heteronuclear C/H correlation spectroscopy (Bax & Morris, 1981) was used to establish the assignments of ^{13}C resonances corresponding to the well-resolved ^1H resonances of both O polysaccharide derivatives, NAc-PS (Figures 4 and 5) and NH_2-PS (Figures 7 and 8) (Tables III and IV), although substantial overlap of most C3, C4, and C5 resonances precluded the identification of either individual ^{13}C or individual ^1H resonances in these instances.

Table III: ^{13}C Chemical Shifts (ppm) of NAc-PS of *B. melitensis* at 310 K

	(1→2)a	(1→2)b	(1→2)c	(1→3)d	(1→2)e	*B. abortus* A polysaccharide
C1	101.20 (172 Hz)[a]	101.2 (172 Hz)[a]	101.7 (174 Hz)[a]	102.3 (176 Hz)[a]	101.5 (175 Hz)[a]	101.3
C2	77.7	77.7	77.3	69.7	78.9	77.8
C3	68.5	68.5	68.5	77.5	68.5	68.5
C4	53.8	53.8	53.8	52.7	53.8	53.8
C5	69.5	69.5	69.5	69.2	69.5	69.3
C6	17.6[b]	17.54[b]	17.50[b]	17.9[b]	17.43[b]	17.54
C=O	175.45[b]	175.0[b]	175.45[b]	175.4[b]	175.4[b]	
CH_3CONH	22.8[b]	23.1[b]	22.8[b]	22.8[b]	22.8[b]	22.9

[a] $^1J_{C,H}$ coupling constant measured by gated decoupling. [b] Chemical shifts are quoted relative to internal 1% 1,4-dioxane at 67.4 ppm.

Table IV: ^{13}C Chemical Shift (ppm) Assignment for NH_2-PS of *B. melitensis* at 310 K and pD 8.0 (Values in Parentheses Recorded at pD ~2.0)

	b	d	a	c	e
C1	102.0	102.9	102.0	102.1	102.7
C2	78.0 (77.4)	69.6 (68.7)	78.0 (77.4)	78.0 (77.4)	77.4 (77.2)
C3	70.8 (67.0)[a]	81.3 (77.0)	70.7 (67.0)[a]	70.7 (67.2)[a]	70.7 (67.2)[a]
C4	54.5[a] (54.8)	53.3 (53.0)	54.5[a] (54.8)	54.5[a] (54.8)	54.5[a] (54.8)
C5	70.6 (66.6)	70.6 (66.6)	70.6 (66.6)	70.6 (66.6)	70.6 (66.6)
C6	17.86[a]	17.70[a]	17.81[a]	17.74[a]	17.70[a]

[a] Assignments uncorroborated by correlation data.

The ^{13}C chemical shift data of the NH_2-PS derivative showed significant β-shifts of ca. 4 ppm (Table IV) when these data were recorded for samples in acidic as compared to basic solution. Only carbon atoms β to the aminated carbon, i.e., C3 and C5, of each pyranose ring system should experience significant protonation shifts (Kotowycz & Lemieux, 1973). Consequently, this observation was used to establish two structural features, the existence of 1,3 glycosidic linkages and the absence of branching. The spectral region between 105 and 50 ppm is displayed for spectra recorded under basic (Figure 6A) and acidic conditions (Figure 6B). Only one resonance (81.3 ppm) of the group of signals situated between 82 and 77 ppm showed a substantial β-shift. All of the poorly resolved resonances centered around 70.5 ppm by comparison experienced β-shifts, while a single resonance at 69.6 ppm was essentially unaffected by protonation. The C4 signals can be seen to be unaffected by β-shifts as would be expected. Since only the β-carbon atoms C3 and C5 of each ring system experienced protonation effects, it can immediately be concluded that the resonance at 81.3 ppm is due to a C3 atom while the unaffected resonance at 69.6 ppm may be assigned to a C2 atom, most likely of the same ring. That this is the case is to be seen from the sensitivity to protonation shifts of all other resonances in the 71–69-ppm portion of the spectrum (Figure 6), thereby requiring that these signals are due to either C3 or C5 atoms. The resonances at 77.0 ppm by virtue of their downfield shifts and absence of β-shifts are assigned to C2 atoms of residues glycosylated at this position. Integration of these resonances relative to the signal at 81.3 ppm, the other glycosylation site, indicates a 4:1 ratio again suggestive of a pentameric repeating unit, previously suggested by consideration of the anomeric regions of ^1H and ^{13}C spectra. Branching may be excluded as a structural feature provided the two carbon resonances, 81.3 and 69.6 ppm, may be unambiguously assigned to the C3 and C2 ring carbon atoms of a single pyranose residue.

This was accomplished most easily by a relayed COSY experiment, not shown (Wagner, 1983), that correlated the two protons H2d and H3d as components of the spin system of a single pyranose ring. The NAc-PS derivative possessed a resolved set of anomeric resonances, each of which provided, via scalar connectivities, the identity of the H2 and H3 resonances of the five pyranose residues. However, the signal of

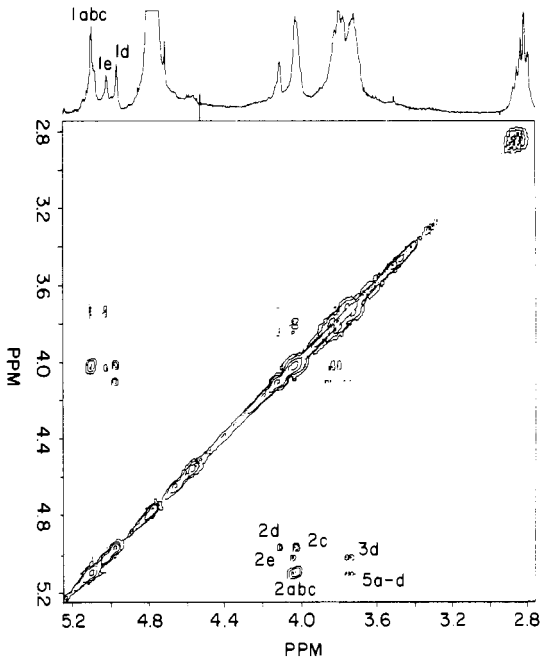

FIGURE 7: NOESY spectrum of the NH_2-PS derivative which shows similar cross-peak patterns to that of the NAc-PS derivative (Figure 5B) except that the relative positions of the H1d and H1e protons are reversed, and since H2d is separated from the other four H2 resonances, the cross-peaks between H1d/H2d and H1d/H2c establish that residue d is linked to c. Cross-peaks between H1a/b and H5 protons are again evident in accordance with the proximity of H1 and H5 resonances in α1,2-linked *manno* saccharides.

H2d could not be unambiguously correlated with that of C2d because the five H2 resonances a–e overlapped. Although examination of the COSY cross-peaks (Figure 5A) showed that each of these protons may in fact be assigned a discrete chemical shift, the resolution of the C/H shift correlation experiment will not support a comparable level of discrimination especially in the F_1 dimension. Examination of the analogous experiments for the NH_2-PS derivative (Figure 8) allowed this problem to be solved, since its ^1H spectrum provided H2d well removed from the other H2 resonances. Thus, correlation of H3d and H2d with carbon resonances at 81.3 and 69.6 ppm is unequivocal, and the polymer is established to be linear.

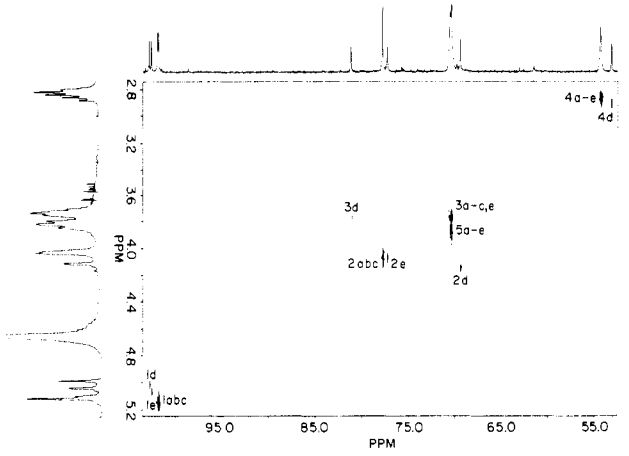

FIGURE 8: C/H shift-correlated spectrum of the NH_2-PS derivative used to correlate the C3d resonance at 81.3 ppm with its proton H3d and the unsubstituted C2d resonance at 69.6 ppm with the H2d proton. Relayed COSY subsequently correlate H2d with H3d via the H1d/H2d/H3d spin network.

Table V: Torsional Angles, Interproton Distances, and NOE Values

	structural element		
	(1→2)α-D-Rha4NR[a](1→2) (a–b–c or d–a–b)	(1→2)α-D-Rha4NR[a]-(1→3) (c–e–d)	(1→3)α-D-Rha4NR[a]-(1→2) (e–d–a)
torsional angles ϕ and ψ (deg)	49, 21	52, 25; 56, 18	52, 25; 42, 15
interproton distance (nm)			
intra-ring	H1b/H2b 0.26	H1e/H2e 0.26	H1d/H2d 0.26
inter-ring	H1b/H2c 0.26	H1e/H3d 0.26	H1d/H2a 0.24
	H1b/H5a 0.22	H5e/H2d 0.23	H1d/H5d 0.23
calcd relative NOE (%)[b]	H2b{H1b} 11	H2e{H1e} 12	H2d{H1d} 10
	H2c{H1b} 12	H3d{H1e} 10	H2a{H1d} 16
	H5a{H1b} 14	H2d{H5e} 19	H5d{H1d} 14
obsd NOE (cross-peaks)	H2b{H1b} +	H2e{H1e} +	H2d{H1d} +
	H2c{H1b} +	H3d{H1e} +	H2a{H1d} +
	H5a{H1b} +	H2d{H5e} +	H5d{H1d} nd[c]

[a] R = CH_3CO or H (cf. NOESY data of Figures 5B and 7). [b] Calculated as described by Thogersen et al. (1982) and Noggle and Schirmer (1971). [c] nd, not observed.

The structural arguments based upon chemical shift data were corroborated by NOE measurements, which permitted both the site and sequence of glycosidic linkages to be established (Dabrowski et al., 1981; Prestegard et al., 1982). Sequence information for a linear homopolymer would normally be degenerate, but since the M polysaccharide contains two linkage types, the nonequivalence of each residue may be used as an aid in resonance assignment and to provide independent verification of the identity of the spin networks of the pyranose residues within the repeating unit of the two polysaccharide derivatives. Two-dimensional NOESY experiments (Kumar et al., 1980) were well suited to polymers such as NAc-PS and NH_2-PS since the poor separation of anomeric resonances prohibited the effective use of NOE difference spectroscopy (Richarz & Wuthrich, 1978). As expected for α-pyranosides with the D-*manno* configuration, two principal cross-peaks correlated with each discrete anomeric proton (Figure 5B). The scalar correlation of H1/H2 available from COSY experiments (Figure 5A) allowed the intraresidue H1/H2 cross-peak originating from dipolar coupling to be identified. The second cross-peak due to dipolar coupling was the interresidue NOE and located the site of glycosylation. It is immediately obvious from the NOESY experiment performed on the NAc-PS derivative (Figure 5B) that the anomeric proton H1e correlates via two cross-peaks with H2e and H3d, which indicates that residue e is linked α1,3 to residue d. Similarly, H1c correlates with H2c and H2e, which requires that unit c is linked through an α1,2 bond to residue e. Since the signals H2a, H2b, and H2c are poorly resolved, the deduction of further linkage sequence could not be made, although the absence of cross-peaks other than those for H1/H2 dipolar correlations indicates that the remaining linkages are exclusively α1,2. Thus for the NAc-PS derivative NOE data were consistent with either sequence c–e–d–b–a or sequence c–e–d–a–b in which residue e was 1,3 linked to d and all other linkages were 1,2. Similar conclusions were reached from NOESY experiments performed with the NH_2-PS derivative (Figure 7). Here, however, other features of the linkage pattern are more clearly seen since the relative ordering of the anomeric resonances of residues d and e was reversed and the H2d signal is clearly resolved to the low-field side of the overlapping resonances of the other four H2 protons (Figure 7). Consequently, the cross-peaks between H1d, H2d, and either H2a or H2b are readily seen, and in the case of H1e cross-peaks with H2e and H3d are unambiguously established, since the latter resonance is easily correlated with H1d and H2d via scalar coupling. Although it is without structural significance, the sequence d–b–a could not be distinguished from the alternate sequence d–a–b in either set of NOESY data.

The NOE measurements used to support the structural analysis and to deduce sequence are conformationally dependent, and it is, therefore, important to check the pattern of observed NOE's against those predicted for the proposed structure. This is particularly important since the largest interresidue NOE does not necessarily occur for the anomeric/aglyconic proton (Dua et al., 1986). The intrinsic stereochemistry of the component monosaccharides as well as the conformation about the glycosidic linkage accounts for such observations. By employing the potential energy algorithms HSEA (Lemieux et al., 1980; Thorgeson et al., 1982) and GSEA (Paulsen et al., 1985), which have been successfully used to describe the solution conformation of oligosaccharides (Thorgeson et al., 1982; Brisson & Carver, 1983; Bock, 1983; Yu et al., 1986), both the stereochemistry and conformational preference are taken into account when a set of calculated NOE's is derived. The interproton distances obtained from the distance matrix of the minimum energy conformation were used to calculate the relative NOE's (Table V). Although this discrete conformation may be virtual (Jardetzky, 1980), it is assumed that the most heavily populated conformational states lie close to the energy minimum and that for the purposes of ranking relative NOE's this assumption holds. It must be stated, however, that although calculated and observed NOE's may agree, the energy minimum is only one of several conformational states that satisfy the constraints imposed by interproton distances. The model does not exclude other solutions, and the conformation of Figure 10 should be viewed as a working model consistent with NOE measurements and offering an explanation of serological characteristics associated with the A and M antigens.

The M polysaccharide was methylated by the Hakomori procedure, and the partially methylated monosaccharides were obtained by HF hydrolysis. After reduction and acetylation, the only partially methylated alditol derivative that could be detected by GC–mass spectroscopy was the 3-*O*-methyl ether. No evidence for the presence of the corresponding and possibly coincident 2-*O*-methyl ether could be obtained from the ion fragmentation pattern. When the monosaccharides liberated by HF hydrolysis were acetylated under basic conditions and

the resulting peracetates subjected to GC–mass spectroscopy, both methyl ethers were detected in the ratio 3-*O*-methyl:2-*O*-methyl 4:1.

Periodate oxidation of the O polysaccharide gave an unchanged polymer, as judged by ^1H and ^{13}C NMR, in support of the earlier conclusions with regard to the absence of branching. When the NH_2-PS derivative was treated in the same manner, the polymer was degraded.

Discussion

The *Brucella* M antigen was extracted from the *B. melitensis* strain 16M, since this is regarded as the species type M strain. LPS detached from the cell wall by being allowed to stand in Tris buffer was identical with the LPS that remained attached to cells and that was subsequently recovered from the phenol phase of a typical water–phenol extraction procedure (Westphal et al., 1952). Since LPS was readily detached from the cell wall, cells prior to phenol extraction were left suspended in Tris buffer for 7 days, and the supernatant after centrifugation was analyzed for carbohydrate after the carbocyanin dye assay indicated the absence of LPS. The supernatant was found to contain significant amounts of a polysaccharide that eluted as a sharp peak on Sephadex G-50 but contained glucose as the only monosaccharide. This material has subsequently been shown (Bundle et al., 1987a) to be a cyclic 1,2-β-glucan that corresponds to the material previously described as polysaccharide B (Diaz et al., 1979; Moreno et al., 1981). Both the M LPS and its O polysaccharide were precipitated by bovine serum from cattle naturally infected with *B. melitensis*, demonstrating that the O antigen component of LPS is the antigenic portion of the classical *Brucella* M antigen.

Mild acid hydrolysis released the O polysaccharide from the LPS, and after conversion of the polysaccharide to its *N*-acetyl derivative followed by hydrogen fluoride hydrolysis, 4-acetamido-4,6-dideoxy-D-mannose was identified as the monosaccharide component. Elemental analysis was in agreement with an O polysaccharide composed exclusively of 4,6-dideoxy-4-formamido-D-mannose. While the specific rotation of the M polysaccharide was markedly different from that of the A polysaccharide, methylation analysis initially gave only one methylated product, the 3-*O*-methyl ether of 4-amino-4,6-dideoxy-D-mannose, which would require that the M antigen was identical with the A polysaccharide. Since this was inconsistent with the serological activity (Cherwonogrodzky et al., 1987), optical rotation, and NMR structural data, the workup of the methylated products was eventually modified to facilitate recovery of the 2-*O*-methyl ether. The ratio of this derivative to the 3-*O*-methyl ether then agreed with the conclusions based upon NMR experiments, although the apparent resistance of the monosaccharide 2-*O*-methyl ether, liberated from the polysaccharide by HF treatment, to subsequent derivatization as an alditol acetate was unexplained.

Although the M antigen was a homopolymer of the same monosaccharide as the A antigen, the more complex NMR spectra of the native O polysaccharide (Figures 2 and 3) indicate the presence of other linkage types. However, the microheterogeneity that arose within the polymer as a consequence of configurational isomerism of the formate group precluded a structural elucidation by NMR methods even at 500 MHz with either simple one-dimensional methods as in the case of the A antigen (Caroff et al., 1984b) or elaborate two-dimensional methods based upon a rigorous assignment of all resonances such as those employed for the serologically related O polysaccharides of *Salmonella landau* and *Escherichia coli* 0157:H7 that also contained the rare sugar

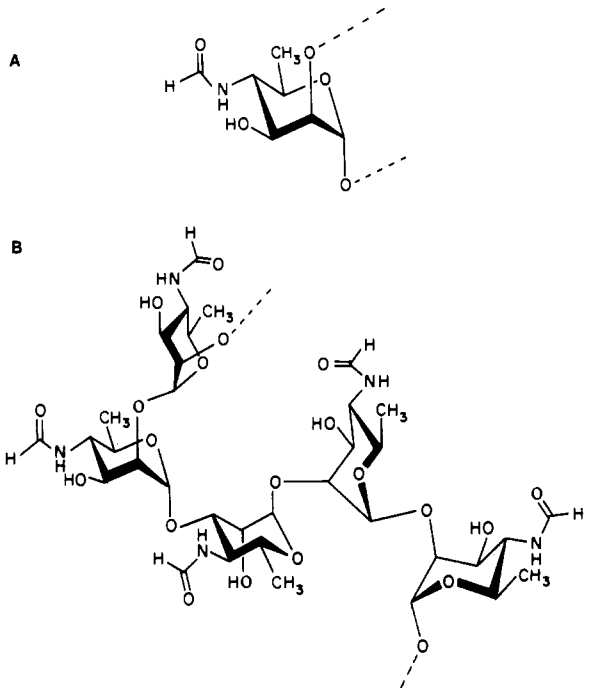

FIGURE 9: (A) *B. abortus* A antigen repeating unit structure. (B) Structure of *B. melitensis* M antigen, represented as a chemical repeating unit of sequence [2)α-D-Rha4NFo*p*(1→2)α-D-Rha4NFo*p*(1→3)α-D-Rha4NFo*p*(1→2)α-D-Rha4NFo*p*(1→2)α-D-Rha4NFo*p*(1→].

4-amino-4,6-dideoxy-α-D-mannose (Bundle et al., 1986). After chemical modification of the M antigen, the derivatives NAc-PS and NH_2-PS provided spectra that were tractable, and structural analysis was performed on these derivatives.

Severe overlap of resonances occurred in the ^1H and ^{13}C spectra of both derivatives. The structural elucidation was based, therefore, not upon a complete labeling of spin systems of individual pyranose residues and their subsequent "reconnection" via networks of observed NOE's [cf. Bundle et al. (1986)]. Rather an examination of the number of 1,2 and 1,3 linkages present in the repeating unit was made followed by the exclusion of alternate structures after the possibility of branching had been eliminated.

Protonation shifts established that only one of five linkage carbon atoms experienced a substantial β shift (Kotowycz & Lemieux, 1973), indicating the presence of a single α1,3 linkage in a pentasaccharide repeating unit. This observation was then carried one step further to eliminate the possibility of a branched structure by correlating the corresponding unsubstituted C2d resonance with the glycosylated center, C3d. This was achieved by relayed coherence transfer experiments *J* tuned so that the H2d and H3d resonances were correlated via their anomeric proton H1d and finally with their directly bonded carbon atoms by heteronuclear shift correlation maps (Figure 8).

The linear pentasaccharide structure (Figure 9) deduced from these data was supported by NOE experiments performed on both modified polysaccharides. Further, the potential energy calculations used as a check of the consistency of observed NOE's with the proposed structure furnished additional information that accounted for the unexpected, 0.35 ppm upfield shift of the H2e resonance relative to the near identical shifts of all other H2 resonances, H2a/d (Table I). In the disaccharide fragment e–d involving the 1,3 linkage, calculation places H2e at ca. 0.28 nm from the carbonyl oxygen of the acetamido moiety of residue d. Since this anomalous shift is not observed in the ^1H spectra of the NH_2-PS

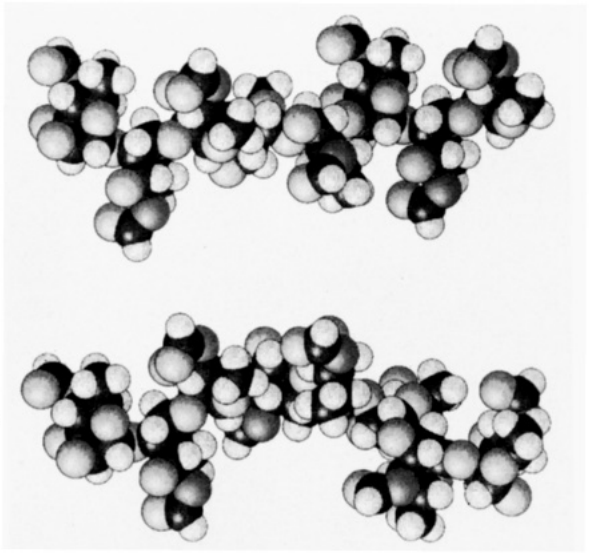

FIGURE 10: (A) Space-filling model of the A antigen displayed as a hexasaccharide fragment in the preferred conformation predicted by potential energy calculations. (B) Space-filling model of the preferred conformation of the *Brucella* M antigen presented as a hexasaccharide in which the third glycosidic linkage is the single 1,3 in an otherwise 1,2-linked oligosaccharide. The first three residues of each antigen can be seen to be of near identical topography, and the insertion of the 1,3 linkage clearly alters the topography and direction of chain propagation.

derivative, it is concluded that the most highly populated conformers adopted by the polysaccharide place H2e not only in close proximity to the carbonyl group but in a relative orientation such that it experiences anisotropic shielding due to the electron density associated with the carbon–oxygen double bond.

The structure proposed for the M antigen differs from that of the A antigen by a single 1,3 linkage every fifth residue (Figures 9 and 10). While it is clear that this difference may explain quite well the long-standing problems of *Brucella* serology, this apparently small structural difference raises several questions regarding the biochemistry and genetics of the *Brucella* LPS.

Models for linear hexasaccharide fragments of the A and M antigens are shown in Figure 10 and demonstrate how the introduction of a 1,3 linkage alters the direction of chain propagation and hence the topography presented by the polysaccharide to specific antibodies. The nearly identical surface presented by those tetrasaccharide segments of the M antigen that involve residues exclusively 1,2 linked is clearly capable of reacting with a large population of antibodies that are generated in response to the A antigen. Conversely, antibody populations generated by the M antigen would include substantial numbers of antibodies that cross-react with the A antigen in addition to a significant proportion that would be specific for the unique structural feature of the M antigen. In polyclonal responses, the relative proportion of these various specificities could vary with circumstances. We have observed that monoclonal antibodies generated to *Brucella* A (Bundle et al., 1984) and M (Bundle et al., 1987) antigens do in fact show this pattern of reactivities, and these considerations are also borne out by literature observations relating to cross-absorption of A and M antisera to render them monospecific. Thus, it is reported that it is easier to remove heterologous A antibodies from anti-M sera than to remove heterologous M antibodies from anti-A sera. Further, antisera apparently monospecific for the A antigen as judged by immunodiffusion still contained low-titre agglutinins for the M antigen (Diaz et al., 1968). The structural details and model of the A and M antigens provide a reasoned account of documented *Brucella* serology without the necessity of invoking the concept of two distinct LPS molecules for a single bacterium.

However, considerations of the manner in which the A and M antigens are biosynthesized point to major differences between strains expressing these two antigens. The banding pattern observed for the *Brucella* A LPS on SDS–PAGE indicates a unique single repeating unit inferring biosynthetic assembly by addition of monosaccharide units to the growing O polysaccharide rather than by block assembly of larger oligomers. The M antigen by comparison appears to be built from pentasaccharide blocks in the manner normally associated with prior assembly on antigen carrier lipid (Robbins et al., 1964), since it shows the banding pattern characteristic of such smooth LPS (Palvia & Makela, 1980; Goldman & Leive, 1980). If *B. melitensis* 16M is indeed the species-type strain of *Brucella* (Vergeret et al., 1985), then the transition from a strain that synthesizes the M antigen O polysaccharide to one making the A antigen must involve a series of genetic changes. By analogy with *Salmonella* group B organisms (Straub & Bagdian, 1966) where the action of a lysogenic phage, P27, alters the linkage site generated by polymerase action, the conversion of M serotypes to an A serotype could result from the action of a lysogenic phage. Presumably the action of such a phage could interfere with controlled assembly of the O polysaccharide in a number of ways. Suppression of a chromosomal polymerase could, if this were a 1,3 glycosyl transferase, result in O polysaccharide biosynthesis dependent not upon the *rfc* but rather the *rfe* gene locus, as is the case for the *E. coli* 09 mannan (Kopmann & Jann, 1975). The biological repeating unit of the M antigen in this case would correspond to [3)αRha4NFo*p*(1→2)αRha4NFo*p*(1→2)-αRha4NFo*p*(1→2)αRha4NFo*p*(1→2)αRhaNFo*p*(1→]. Alternatively, the addition of a 1,3-linked residue to the growing repeating unit may serve as the recognition signal for action by a 1,2 polymerase, in which case the biological repeating unit would be [2)αRha4NFo*p*(1→3)αRha4NFo*p*-(1→2)αRha4NFo*p*(1→2)αRha4NFo*p*(1→2)αRha4NFo*p*-(1→]. If M polysaccharide assembly is repressed by phage and the repression is unstable as happens for the P27 phage in *Salmonella bredney* (Lindberg et al., 1978), then O polysaccharide with both the A and M determinants could result. Strains of *Brucella* with such dual serological properties are known. Progress toward a better understanding of *Brucella* O polysaccharide biosynthesis is the subject of ongoing studies.

In summary, the *Brucella* A and M antigens although structurally distinct have been shown to be homopolymers of the same monosaccharide that differ in respect of every fifth glycosidic linkage. This accounts for the long-standing cross-serological reactivities of the two antigens. However, the question as to whether the antigenic determinants of both antigens may sometimes be carried on a single molecule cannot be unequivocally answered, because of the likelihood that phage conversion accounts for the altered structure of the A relative to the M antigen. If unstable phage conversion of the *B. melitensis* species-type strain has given rise to the A antigen, it is possible that both A and M determinants may be expressed on a single LPS or by different LPS "molecules" on one bacterium.

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Registry No. M antigen, 111114-38-6.

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