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Antigenic S-Type Lipopolysaccharide of *Brucella abortus* 1119-3†

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Antigenic phenol-phase soluble lipopolysaccharide isolated from *Brucella abortus* 1119-3 by hot phenol-water extraction was shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, controlled hydrolysis, periodate oxidation, methylation, and ^{1}H and ^{13}C nuclear magnetic resonance studies to be an S-type lipopolysaccharide which could be cleaved to yield a lipid A and an O-chain polysaccharide identified as an unbranched linear homopolymer of 1,2-linked 4,6-dideoxy-4-formamido-α-D-mannopyranosyl residues. The serological reactivity of bovine antiserum to *B. abortus* 1119-3 with the lipopolysaccharides of *Yersinia enterocolitica* serotype O:9 and *Vibrio cholerae* species has now been related to the occurrence of 1,2-linked *N*-acylated 4-amino-4,6-dideoxy-α-D-mannopyranosyl units in the O-chain polysaccharides of their lipopolysaccharides.

The diagnosis of brucellosis in humans and animals is frequently difficult to establish. Often the infection either is subclinical or yields varied responses in the host. Diagnosis, therefore, has frequently been based on the detection and quantification of *Brucella* antibodies in serum samples by agglutination, precipitin, complement fixation, and other methods (11). More recently, enzyme immunoassay tests with crude or purified antigens have been introduced (4, 20–22). False-negative and nonspecific reactions may be problems in these test systems. This report describes our work on the isolation, purification, and determination of the structure of a unique *Brucella* surface antigen which could be used both for diagnostic tests and for studies involving the production and use of monoclonal antibodies for the identification of *Brucella abortus*.

The present investigation led to the identification of the antigenic O-chain polysaccharide of *B. abortus* S-type lipopolysaccharide (LPS) as a linear homopolymer of 1,2-linked 4,6-dideoxy-4-formamido-α-D-mannopyranosyl units.

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MATERIALS AND METHODS

Cell production and LPS isolation. *B. abortus* 1119-3 (obtained as a lyophilized culture from R. D. Angus, Reagents Section, Scientific Services Laboratory, National Veterinary Services Laboratories, Ames, Iowa) was grown on potato infusion agar in Roux flasks for 48 h at 37°C as described in the National Animal Disease Laboratory Diagnostic Reagents Manual 65A (Agricultural Research Service, Animal Health Div., National Animal Disease Center, U.S. Department of Agriculture, Ames, Iowa). The growth from each flask (ca. 1.5 g [wet weight] per flask) was harvested in 40 ml of 0.85% (wt/vol) sodium chloride solution containing 0.5% (wt/vol) phenol, and after 20 h of incubation at 20°C, the killed cells were collected by low-speed centrifugation.

LPS was extracted by the hot phenol-water method of Westphal et al. (30). Wet cells of *B. abortus* 1119-3 (100 g) were suspended in water (400 ml) at 70°C, 95% aqueous phenol (400 ml) at 70°C was added to the vigorously stirred cells, and extraction was continued at 70°C for an additional 10 min. The mixture was cooled (4°C) and then centrifuged (5,000 × *g*) for 8 h at 4°C to give clear water and phenol layers. The collected water and phenol phases were dialyzed against running tap water and, when phenol free, were lyophilized. The aqueous- and phenol-phase extracts were taken up in 1% (wt/vol) sodium chloride (30 ml) and were ultracentrifuged (105,000 × *g*) at 4°C for 12 h to give crude LPS. After trypsin, RNase, and DNase digestion, the LPS was again collected under the same ultracentrifugation conditions, and the precipitated gels, dissolved in water, were lyophilized to yield LPS (contaminated by ca. 50% phospholipid).

Partial hydrolytic fission of LPS. A 0.5% (wt/vol) solution of LPS in 2% (vol/vol) acetic acid was heated in a boiling-water bath for 2 h. The precipitated lipid A material was removed from the cooled (4°C) solutions by low-speed centrifugation, and the clear aqueous solution was then lyophilized, redissolved in 3 ml of 0.05 M pyridinium acetate buffer (pH 4.7), and fractionated by gel filtration on a column of Sephadex G-50.

Analytical methods. Quantitative colorimetric methods used were the phenol-sulfuric acid method for neutral glycoses (10), the modified Elson-Morgan method for 2-amino-2-deoxyglycoses (12), the method of Chen et al. for phosphate (6), and the periodate oxidation-thiobarbituric acid method for 3-deoxy-2-octulosonate (1).

Gas-liquid chromatography (GLC) was done with a Hewlett-Packard model 5710A chromatograph fitted with a hydrogen flame detector and a model 3380A electronic integrator. The following conditions were used. Program A: glass column (2 mm by 120 cm) packed with 3% (wt/wt) SP2340 on Supelcoport (Supelco Inc., Bellefonte, Pa.); temperature program, start at 180°C (delay for 2 min) and then shifted to 240°C at 4°C/min. Program B: glass column (2 mm by 180 cm) packed with 3% (wt/wt) SP2340 on Supelcoport; temperature program, start at 200°C (delay for 2 min) and then shifted to 240°C at 1°C/min. Program C: glass column (2 mm

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by 180 cm) packed with 3% (wt/wt) butanediol succinate polyester on 80- to 100-mesh Chromosorb W run isothermally at 170°C. Program D: steel column (2 mm by 180 cm) packed with 10% (wt/wt) FFAP plus 1% phosphoric acid (Chromatographic Specialties, Brockville, Ontario, Canada) on 80- to 100-mesh Chromosorb W run isothermally at 120°C. Development was made with dry nitrogen at 30 ml/min, and retention times are quoted relative to that of D-glucitol hexaacetate (T_{GA}) or to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol (T_{GM}). Combined GLC and mass spectrometry (GLC-MS) was done with a Hewlett-Packard 5985 GC-MS system with program conditions A, B and C and an ionizing potential of 70 eV. The identity of glycose derivatives was established in each case by comparison of retention times and mass spectra with those of authentic reference samples.

Lipids were analyzed by GLC-MS (program C) of their methyl esters derived by sealed glass tube methanolysis of samples (1 mg) with 3% (wt/vol) methanolic hydrogen chloride (1 ml) for 4 h at 100°C, followed by neutralization (Ag_2CO_3).

Glycoses were determined by GLC (program A) of their alditol acetate derivatives (14, 15) with inositol as an internal standard. Their configurations were established by GLC of their trimethylsilylated (−)−2-butyl glycoside derivatives by the method of Gerwig et al. (13).

Paper chromatography was done on water-washed Whatman no. 1 filter paper with pyridine-ethyl acetate-water (2:5:5 [vol/vol]; top layer) (solvent A) or 1-butanol–pyridine–0.1 M HCl (5:3:2 [vol/vol]) (solvent B) as the mobile phases. Glycoses were detected with 2% (wt/vol) *p*-anisidine HCl in ethanol (17), with the periodate oxidation-alkaline silver nitrate reagents (29) or the periodate oxidation-thiobarbituric acid spray reagents (for 3-deoxy-2-octulosonate) (1). Mobilities are quoted relative to D-galactose.

Gel filtration was done on a column (2 by 40 cm) of Sephadex G-50 (Pharmacia Fine Chemicals) with 0.05 M pyridinium acetate as the eluant buffer.

NMR. Proton-coupled and -decoupled carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectra were recorded on samples in D_2O contained in tubes (diameter, 10 mm) at 30°C with a Varian CFT-20 spectrometer operating at 20 MHz. Chemical shifts are reported downfield from external tetramethylsilane. The 125-MHz ^{13}C spectrum was recorded at 37°C on a Bruker AM-500 spectrometer. The spectrometer conditions were 8 μs for a 90° pulse, 32,000 data points, and 25-kHz spectral width, and a gated 1-W broad-band decoupling to establish nuclear overhauser enhancement during a relaxation delay of 0.5 s, followed by 1-W composite pulse decoupling during the 0.65-s acquisition time. Proton magnetic resonance (^{1}H-NMR) spectra were recorded with a Varian CFT-20 spectrometer operating at 79.9 MHz, the HOD signal being suppressed by selective saturation with an inversion recovery pulse sequence. Samples in D_2O were examined at 75°C, and chemical shifts are quoted downfield from internal 4,4-dimethyl-sila(2,2,3,3-$^2H_4$) pentanonate. Coupling constants are reported in hertz.

Methylation analysis. Polysaccharide (2 mg) was methylated with sodium methylsulfanylmethanide and methyl iodide in methyl sulfoxide according to the directions of Hakomori (16). The product, recovered by chloroform extraction, was hydrolyzed by treatment with anhydrous hydrogen fluoride (HF) (1 ml) for 4 h at 20°C. After the removal of the HF in vacuo, the residue was examined by paper chromatography. The major portion of the hydrolysis product was converted to alditol acetate derivatives by treating its aqueous solution (1 ml) with sodium borodeuteride (25 mg) for 18 h at 20°C. After acidification with acetic acid, concentration, evaporation from methanol (5× 10 ml) to remove borate, and treatment of the residue to acetic anhydride (0.5 ml) at 115°C for 2 h, the product was examined directly by GLC-MS (program B).

***N*-Deacylation and *N*-acetylation of polysaccharide.** O-chain (80 mg) in 1 M sodium hydroxide (4 ml) containing sodium borohydride (2 mg) was heated at 100°C in a sealed glass tube for 90 min. After slow neutralization in the cold (4°C) with 2 M hydrochloric acid (2 ml), the *N*-deacylated product was recovered as the void volume eluate obtained on Sephadex G-50 column gel filtration.

N-Acetylation of the above *N*-deacylated O-chain (70 mg) was effected by treatment of its solution in water (10 ml) containing methanol (0.5 ml) with acetic anhydride (0.2 ml) at room temperature for 3 h. After the solution was concentrated, the residue in water (2 ml) was treated with ammonia (100 μl), and the *N*-acetylated polysaccharide (66 mg) was recovered by Sephadex G-50 gel filtration.

General methods. Concentrations were made under reduced pressure and below 40°C. Optical rotations were determined at 20°C in 10-cm microtubes with a Perkin-Elmer 243 polarimeter. For immunodiffusion studies, 1% solutions of glycans in saline were used. LPSs were examined as *O*-deacylated products by treatment of LPS (1 mg) with 0.2 M NaOH (0.2 ml) at 70°C for 1 h, followed by neutralization with 0.2 M HCl (0.2 ml) and dilution to 1 ml. Diffusion studies were made at 4°C in 1% agarose gels containing 0.8 or 8% NaCl, and precipitin lines were allowed to develop over 2 days.

RESULTS AND DISCUSSION

Extraction of *B. abortus* 1119-3 cells was done by the hot phenol-water method (30), followed by collection of the precipitated LPS gels obtained by ultracentrifugation of the concentrated dialyzed water and phenol phases. The R-type LPS found in the water phase of the phenol-water extract was not examined further since it did not react in immunodiffusion with bovine antiserum prepared against whole *B. abortus* cells. The crude LPS recovered in a 1.2% yield from the phenol phase showed strong serological reactivity with the bovine *B. abortus* antiserum, as recorded by other workers (8, 9, 23, 25), and this material was subjected to further study.

The crude phenol-phase LPS, which gave a single strong precipitin line in immunodiffusion against *B. abortus* bovine antiserum contained ca. 50% (wt/wt) of a phospholipid material which was selectively removed from the LPS by several extractions with chloroform and with methanol. The residual LPS was judged to be pure by the fact that it gave a single peak at 467 nm in the carbocyanine dye assay (18), showed absence of adsorption in the 210- to 300-nm region of its UV spectrum, yielded no amino acids after 2 M HCl (100°C) hydrolysis, and did not contain carboxylic acids characteristic of those present in the phospholipid contaminant of the initial crude phenol-phase LPS product.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the phenol-phase LPS, followed by silver nitrate staining (28), showed a typical S-type LPS profile, the fine separation of the bands in the high-molecular-weight region being characteristic of the spacing expected of an LPS having a O-chain polysaccharide of a single, uniform, repeating glycoside residue (25).

Partial hydrolysis of the LPS (200 mg) with hot dilute acetic acid gave an oily insoluble lipid A (66 mg), whereas

Sephadex G-50 gel filtration of the concentrated water-soluble products gave a broad elution peak (K_{av}, 0.07) (102 mg) of the O-chain polysaccharide. No products were seen in the elution range between the O-chain and monosaccharide region, indicating the absence of material characteristic of a core oligosaccharide. The fraction eluting in the monosaccharide region (60 mg) contained essentially phosphate but was negative by colorimetric analysis for 3-deoxy-2-octulosonate.

The O-chain fraction was a readily soluble white powder which had $[\alpha]_D$ +37.7° (*c* 1.2 in water). Analysis determined the following: C, 44.50; H, 6.55; N, 6.2; ash, 0.04%. The polysaccharide gave a ^{13}C-NMR spectrum (30°C; 20 MHz), showing signals at 166.16, 101.81, 78.26, 69.52, 68.84, 53.15, and 18.07 ppm which were subsequently assigned to the carbon atoms of 1,2-linked 4,6-dideoxy-4-formamido-α-D-mannospyranosyl units (Fig. 1). The proton-coupled ^{13}C-NMR spectrum (30°C; 20 MHz) showed signals at 166.16 (d, 1C, $^1J_{C,H}$ = 197.3 Hz, N-CHO), 101.73 (d, 1C, $^1J_{C,H}$ = 173.3 Hz, C-1), 53.15 (d, 1C, $^1J_{C,H}$ = 144.6 Hz, C-4), and 18.07 (q, 1C, $^1J_{C,H}$ = 127.4 Hz, C-6) ppm, for which the indicated assignments were made. The partial ^{1}H-NMR (75°C; 79.9 MHz) spectrum of the O-chain showed signals at 8.18 (s, 1H, HNC*H*O), 5.13 (bd, 1H, $J_{1,2}$ = ~3.8 Hz, H-Cl), and 1.19 (bd, 3H, $J_{5,6}$ = 8 Hz, H_3C6) ppm, for which the indicated assignments were made. The combined NMR data suggested that the O-chain was composed of a single aminohexose repeating unit, probably containing a 6-deoxy function and a *N*-formylated amino group. The presence of the *N*-formylated group was verified by the fact that the alkaline hydrolysis of the O-chain yielded 1 mol of formic acid per anhydrohexose residue, as determined by GLC (program D) analysis.

Both the native O-chain polysaccharide and its *N*-acetyl derivative were almost completely degraded by hydrolysis with 2 M hydrochloric acid or 1 M sulfuric acid or by methanolysis. The *N*-acetylated O-chain had $[\alpha]_D$ + 32.0° (*c* 1.3 in water), and its ^{13}C-NMR spectrum (30°C; 20 MHz) showed signals at 177.66 (NH*C*OCH$_3$), 103.43 (C-1), 79.91 (C-2), 70.97 (C-3), 70.70 (C-5), 55.91 (C-4), and 25.03 (NHCO*C*H$_3$) ppm, to which the indicated assignments were made, subsequent to its chemical characterization.

The *N*-acetylated O-chain (40 mg) treated with anhydrous HF (2 ml) at 20°C for 12 h yielded a single chromatographically pure glycose (14 mg). After purification by preparative paper chromatography, this was found to have $[\alpha]_D$ + 13.3°C (*c* 2.0 in water) and gave a single spot on paper chromatography having an mobility relative to that of D-galactose of 2.40 (solvent A), the same as that of authentic 4-acetamido-4,6-dideoxy-D-mannose. The identity of the glycose as 4-acetamido-4,6-dideoxy-D-mannose was further established by the facts that its ^{13}C-NMR spectrum (30°C, 20 MHz) was identical with that given by an authentic standard, that the reduced ($NaBD_4$) and acetylated glycose on GLC-MS (program A) gave a single peak with a T_{GA} value of 1.33 whose mass spectrum and GLC retention time were identical to those given by a reference sample of 1,2,3,5-tetra-*O*-acetyl-4-acetamido-4,6-dideoxy-D-[1-^{2}H]-mannitol (5).

Once the identity of the glycose unit of the O-chain had been established as the *N*-acetyl derivative of 4-amino-4,6-dideoxy-D-mannose, its mode of linkage was determined by methylation analysis. The methylated O-chain (16) on hydrolysis with anhydrous HF afforded a single glycose. After reduction ($NaBD_4$) and acetylation, this glycose gave an alditol derivative which on GLC-MS analysis (program B) gave a single peak (T_{GM}, 8.22) which had the same retention time and mass spectrum as 1,2,5-tri-*O*-acetyl-4,6-dideoxy-3-*O*-methyl-4-(*N*-methylformamido)-D-[1-^{2}H]-mannitol (5). The above findings lead to the conclusion that the polysaccharide is linked throughout by 1,2 glycosidic bonds.

The accumulated evidence indicates that the O-chain was composed of 1,2-linked 4,6-dideoxy-4-formamido-α-D-mannopyranosyl residues. The O-chain did not consume perio-

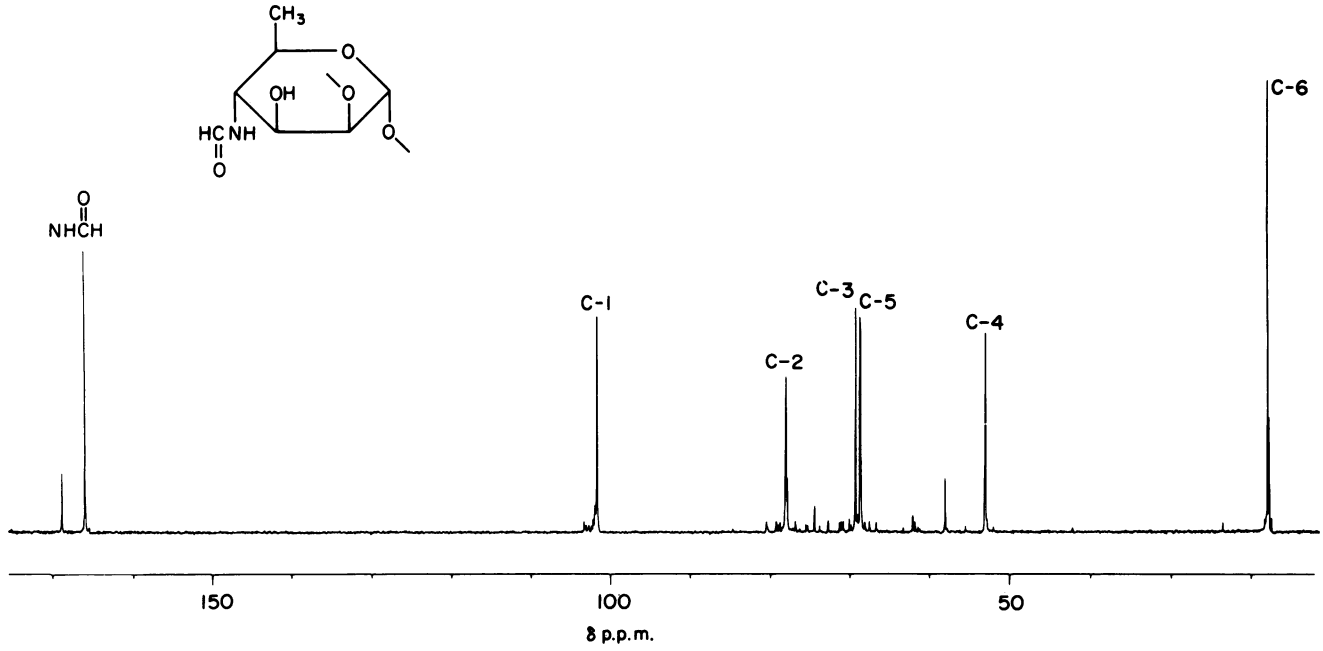

FIG. 1. Proton-decoupled ^{13}C-NMR spectrum (37°C; 125 MHz) of the native O-chain polysaccharide of *B. abortus* 1119-3 S-type LPS. Microheterogeneity is apparent and is caused by the presence of both E and Z conformations of the *N*-formyl group.

date, a result consistent with the proposed structure if all the potential amino groups were *N*-formylated. The presence of the *N*-formyl group already indicated by the release of formic acid on alkaline hydrolysis of the native O-chain was further confirmed from the observed doublet arising from the carbonyl carbon (166.16 ppm, $^1J_{C,H}$ = 197.3 Hz) in the coupled ^{13}C-NMR spectrum and the low field singlet at 8.18 ppm seen in the ^{1}H-NMR spectrum.

The positive specific optical rotation of the O-chain, the characteristic α-D-glycopyranosyl coupling constant ($^1J_{C,H}$ = 173.3 Hz) (2, 3) observed for the anomeric carbon atom (101.73 ppm) in the proton-coupled ^{13}C-NMR spectrum, and the anomeric proton signal (5.13 ppm), characteristic of α-D-mannopyranosyl residues (7), seen in the ^{1}H-NMR spectrum, indicate that the native O-chain is a linear polymer of only 1,2-linked 4,6-dideoxy-4-formamido-α-D-mannopyranosyl units.

Hydrolysis of the O-chain with hot 2 M HCl or 1 M H_2SO_4 caused complete destruction of the 4-aminoglycose component. This hydrolytic procedure did not destroy D-mannose (1.51%), D-glucose (1.01%), and 2-amino-2,6-dideoxy-D-glucose (1.0%) (quinovosamine), identified by GLC of their trimethylsilylated (−)−2-butyl glycoside derivatives and quantitated by GLC (program A) analysis of the derived acetate derivatives of mannitol (T_{GA}, 0.90), glucitol (T_{GA}, 1.00), and 2-amino-2,6-dideoxyglucitol (T_{GA}, 1.40) (26). It is probable that D-glucose, D-mannose, and D-quinovosamine arose from the terminal core oligosaccharide, and their total (3.56%) is compatible with an average O-chain length of ca. 96 aminoglycose units. The ^{13}C-NMR results (Fig. 1) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis molecular weight estimations were also consistent with the proposition that the O-chain is essentially a homopolymer with an average chain length of 96 to 100 glycosyl units. It is evident that the core region forms a minor component of the structure. Aldoheptose, which is an LPS common core constituent, was not detected, and although free 3-deoxy-2-octulosonate could not be detected in the hot dilute acetic acid hydrolysate of the LPS, free 3-deoxy-2-octulosonate (1.82%) was liberated from the LPS by 2 M HCl (2 h at 90°C) hydrolysis (M. Caroff, D. Sc. thesis, Université de Paris-Sud, Orsay, France, 1982).

The lipid A obtained upon treatment of the LPS with hot dilute acetic acid was not examined in detail but was found to be composed of 2-amino-2-deoxy-D-glucose (10.1%), phosphate (5.9%), *n*-tetradecanoic acid (12%), *n*-hexadecanoic acid (33%), *n*-octadecanoic acid (15%), 3-hydroxytetradecanoic acid (27%), and 3-hydroxyhexadecanoic acid (4%).

In immunodiffusion, a single precipitin line was formed between bovine *B. abortus* antiserum and O-chain polysaccharide, phenol-phase LPS, *N*-acetylated O-chain, and O-chain *N*-acylated with *S*-2,4-dihydroxybutyric acid (the synthetic analog of the O-chain of *Vibrio cholerae* [19, 27]). The O-chain polysaccharides of *B. abortus* 1119-3 and *Yersinia enterocolitica* serotype O:9 (5) appear from our chemical evidence to be identical in structure and differ only in the minor reducing end terminal core regions. An examination of the molecular model (Fig. 2) of the *B. abortus* O-chain indicates that the *N*-acyl groups are exposed as potential epitopes, and monoclonal antibody can be prepared which recognizes different types of *N*-acylation in the basic aminoglycan structure. Polyclonal antisera which recognize the O-chain, irrespective of the form of *N*-acylation, must recognize common regions as structural features of the glycan backbone.

In addition to the serological cross-reactions between the antigens of *V. cholerae*, *B. abortus*, and *Y. enterocolitica* serotype O:9 being explainable in terms of their O-chain polysaccharides having a common feature in being homopolymers of 1,2-linked *N*-acylated derivatives of 4-amino-4,6-dideoxy-α-D-mannopyranosyl residues, we have recently found that some strains of *Escherichia coli* and *Salmonella* species showing serological reactivity with bovine *B. abortus* antiserum have O-chains in their LPSs in which single 4-acetamido-4,6-dideoxy-α-D-mannopyranosyl units are present in repeating tetrasaccharide units (M. B. Perry, L. MacLean, and D. W. Griffith, Can. J. Biochem., in press).

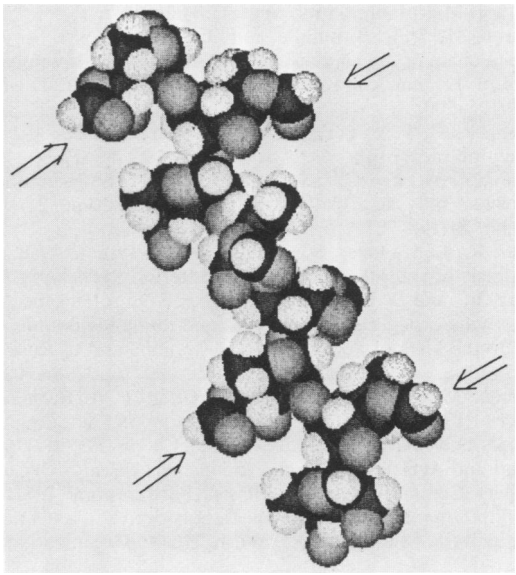

FIG. 2. *B. abortus* 1119-3 O-chain polysaccharide represented as an octasaccharide in the minimum energy conformation, showing (arrows) the exposure of the *N*-formyl groups on the oligosaccharide backbone.

The analytical data obtained for our antigenic *B. abortus* LPS are essentially similar to those of previously recorded preparations, and in view of our present finding that the main antigenic determinant is a labile 4-amino-4,6-dideoxyglycose, fortunately now easily recognized as a major constituent by use of NMR techniques and isolation with HF hydrolysis, it would be of interest to reexamine previous preparations by the NMR and HF procedures before making critical comparisons.

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