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Characterization of *Brucella abortus* and *Brucella melitensis* Native Haptens as Outer Membrane O-Type Polysaccharides Independent from the Smooth Lipopolysaccharide

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***Brucella* native haptens (NHs) extracted with hot water from smooth (S)-type *B. abortus* and *B. melitensis* were purified to high levels of serological activity and compared with the polysaccharide obtained by acid hydrolysis (PS) of the S lipopolysaccharide (S-LPS). By ^{13}C nuclear magnetic resonance analysis, NHs showed the spectrum of a homopolymer of α-1,2- or α-1,2- plus α-1,3-linked 4-formamido-4,6-dideoxy-D-mannose (*N*-formylperosamine) previously reported for the LPS O chain. However, while PS contained up to 0.6% 3-deoxy-D-manno-2-octulosonate, this LPS-core marker was absent from NH. High performance liquid chromatography and thin-layer chromatography showed heterogeneity in NH purified from whole cells but not in PS. By immunoprecipitation, polysaccharides indistinguishable from NH were demonstrated in extracts obtained with phenol-water, saline at 60°C, and ether-water treatments, and none of these treatments caused S-LPS hydrolysis detectable with antibodies to the O chain and lipid A. Two lines of evidence showed that NH was in the cell surface. First, NH became biotinylated when *B. abortus* live cells were labelled with biotin-hydrazide, and the examination of cell fractions and electron microscopy sections with streptavidin-peroxidase and streptavidin-coloidal gold, respectively, showed that labelling was extrinsic. Moreover, whereas only traces of NH were found in cytosols, the amount of NH was enriched in cell envelopes and in the outer membrane blebs spontaneously released by brucellae during growth. Interactions between NH and S-LPS were observed in crude cell extracts, and such interactions could be reconstituted by using purified NH and LPS. The results demonstrate that NH is not a hydrolytic product of S-LPS and suggest a model in which LPS-independent O-type polysaccharides (NH) are intertwined with the O chain in the outer membrane of S-type brucellae.**

The members of the genus *Brucella* are gram-negative bacteria pathogenic for both animals and humans. Presently, several nomenspecies, which differ in host range, surface antigens, and other phenotypic and genotypic features, are recognized (1, 18). Three of the most common species, *B. melitensis*, *B. abortus*, and *B. suis*, bear the antigen characteristic of smooth (S)-type brucellae and elicit a strong immunological response in their natural hosts. The immunodominant molecules involved in the antibody response to S-type *Brucella* species are the outer membrane (OM) S lipopolysaccharide (S-LPS) (1, 9–13, 15, 27, 28, 32, 51) and the serologically related native hapten (NH) polysaccharide (14, 15, 19, 40, 45). The latter molecule has been also known as the fast-diffusing component (11), the first component (11), and the second polysaccharide, or polysaccharide B (10, 11, 13, 19, 28, 40). For the sake of clarity, the term NH has been maintained for those serologically reactive polysaccharides isolated from S-type *Brucella* strains, while the term polysaccharide B has been reserved for serologically reactive polysaccharides isolated from rough-type *B. melitensis* B115 (10, 13, 19, 28, 40).

Several independent studies have shown that the acid hydrolysis of S-LPS of *B. abortus* biotype 1 yields a specific polysaccharide (PS) constituted by an O chain (an unbranched homopolymer of α-1,2-linked 4-formamido-4,6-dideoxy-D-mannose [*N*-formylperosamine]) (8, 9, 46, 55) linked to a core oligosaccharide (8, 37, 40, 41). In *B. melitensis* biotype 1, the O chain consists of repeating blocks of five *N*-formylperosamine residues, four α-1,2-linked residues and one α-1,3-linked residue (7, 9, 33, 46). The *Brucella* LPS core oligosaccharide has been partially characterized and shown to be composed of glucose, mannose, 2-amino-2,6-dideoxy-D-glucose (quinovosamine), 2-amino,2-deoxy-D-glucose (glucosamine), 3-deoxy-D-manno-2-octulosonic acid (KDO), and unidentified sugars (8, 37, 40, 41, 56). Whereas there is agreement on the overall structure of the *Brucella* PS, there is controversy concerning the origin and chemical structures of NH and polysaccharide B. On one hand, it has been maintained that polysaccharide B is a cyclic glucan (7, 9, 32, 34, 45) similar to those found in the periplasmic space in members of the family *Rhizobiaceae* (6) and that NH is merely a PS resulting from a hypothetical hydrolysis caused by the procedures of extraction (54). On the other hand, in several chemical and immunochemical studies, NH has been reported to be related to polysaccharide B (13, 19, 40) and structurally similar to PS but in the presence of KDO, quinovosamine, and other core components (14, 38, 40). Because LPS and NH preparations have been used extensively in immunochemical, biological, and diagnostic studies, this controversy is not a trivial one and must be resolved. From the practical point of view, *B. abortus*- and *B. melitensis*-infected animals produce antibodies that react in immunoprecipitation tests against NH and polysaccharide B, whereas vaccinated animals do not, thereby allowing the differentiation of infected and vaccinated animals (10, 13–15, 27, 28, 32). From the point of view of their role in the biology of brucellae, a characterization of NH as an S-LPS distinct polysaccharide located in the

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OM of S-brucellae, similar to that described for some gram-negative bacteria (5, 23, 26, 29, 47), would place these molecules in a more interesting category it is known that surface molecules commonly are the main targets for the immune system and elements that play a relevant role in the virulence of pathogenic organisms. In this work, we describe purification protocols which yield a polysaccharide bearing all the serological properties of NH. We also demonstrate that NH is not a hydrolytic product of the S-LPS but rather a distinct O-chain-like polysaccharide and give strong experimental evidence supporting the hypothesis that NH is associated by noncovalent bonds to the S-LPS in the OM of S-brucellae.

MATERIALS AND METHODS

Bacterial strains and cultures. *B. abortus* 2308 (biotype 1 [A serotype]) and *B. melitensis* 16M (biotype 1 [M serotype]) are S virulent strains that have been used in previous works (11, 14, 31, 38). To ensure full expression of the S-type cell features, both strains were inoculated into either mice or guinea pigs and recovered from the spleens 2 weeks later. The isolates were tested for dissociation (1) and used to prepare stock seeds in skim milk that were kept at −80°C. Bacteria were propagated in 1.7% Casitone (E. Merck, Darmstadt, Germany)–0.3% Soytone (Difco Laboratories, Detroit, Mich.)–0.5% yeast extract (Merck)–0.25% K_2HPO_4–2% glucose–0.5% NaCl–0.01% antifoam A-butyl acetate (1:3) (Sigma Chemical Co., St. Louis, Mo.) in a 15-liter Biostat fermentor (B. Braun Mesulgen AG, Leinfelden, Germany) at 36°C and 35% O_2 saturation. After 36 h of incubation, bacteria were inactivated with phenol (0.5%, 36°C, 48 h), harvested by tangential flow filtration (Omega 100K filter; Filtron Technology Corp., Northborough, Mass.), and washed twice with saline. When live bacteria were used, they were grown in tryptic soy broth (Difco) in 2-liter flasks on an orbital shaker and harvested without inactivation by centrifugation in sealed cups at 7,000 × *g* for 20 min, and the initial steps of the extraction procedures described below were carried out in a safety hood.

Cell envelope and cytosolic fractions. A thick slurry of bacteria in 10 mM phosphate-buffered saline (pH 7.2) was passed twice trough a French press (Pressure Cell 40K, Aminco; SLM Instruments Inc., Urbana, Ill.) at an internal pressure of 35,000 lb/in^2. The homogenate was digested with nucleases (50 μg of DNase II type V and RNase A [Sigma] per ml) for 18 h at 37°C and fractionated by ultracentrifugation, and the cell envelope and cytoplasmic fractions were characterized as described previously (42). The OM blebs released spontaneously by exponentially growing S-brucellae were obtained by ultracentrifugation of spent broths as described previously (21). We have shown previously that this material represents OM fragments devoid of cytoplasmic or inner membrane markers (21).

S-LPS and NH extractions. The following procedures were used: (i) phenol-water, (ii) ether-water, (iii) saline, and (iv) hot water.

(i) Phenol-water method. *Brucella* crude S-LPS (fraction 5) was obtained by methanol precipitation of the phenol phase of a water-phenol extract exactly as described by Leong et al. (31). Fraction 5 is known to contain considerable amounts of NH in addition to S-LPS (11, 40). This fraction (10 mg/ml in 175 mM NaCl–0.05% NaN_3–0.1 M Tris-HCl [pH 7.0]) was partially purified by digestion with nucleases as described above and then digested three times with proteinase K (50 μg/ml) (Merck) for 1 h at 55°C and for 24 h at room temperature. Depending on the purpose, crude or purified S-LPS fractions were used in the experiments.

(ii) Ether-water method. The method described by Ribi et al. (49) for endotoxin extraction was used with minor modifications. Live bacteria were resuspended in water (25 g [wet weight] in 150 ml) and extracted by shaking with 0.3 volume of ethyl ether, and the water phase was decanted. After removal of cell debris by centrifugation, the water phase was mixed with 5 volumes of ethanol, and the precipitate was collected (5,000 × *g*, 10 min), dialyzed against distilled water, and freeze-dried.

(iii) Saline extraction. S-LPS-NH complexes (Fraction SALT-1; see Results) were obtained and fractionated as summarized in Fig. 1. Live bacteria were resuspended in 175 mM NaCl (20 g [wet weight] in 100 ml) and heated at 60°C for 2 h. The cell debris was sedimented (8,000 × *g*, 30 min, 4°C), and the supernatant was dialyzed and freeze-dried (fraction SALT-1). Fraction SALT-1 was digested first with nucleases and then with proteinase K as described above and ultracentrifuged (200,000 × *g*, 6 h, 10°C), and the sediment (fraction SALT-2) and the supernatant (fraction SALT-3) were dialyzed and freeze-dried.

(iv) Hot water extraction. This protocol is summarized in Fig. 2. Either inactivated or live bacteria were resuspended in distilled water (20 g [wet weight] in 100 ml) and heated at 120°C for 30 min. The cell debris was removed (8,000 × *g*, 30 min, 4°C), and the extract was precipitated with 3 volumes of absolute ethanol at −20°C overnight. The resulting precipitate was collected by centrifugation at 5,000 × *g* for 10 min (fraction HW-1), and the supernatant was mixed with 2 additional volumes of ethanol. The new precipitate was sedimented at 5,000 × *g* for 10 min (fraction HW-2), digested with nucleases and proteinase K as described above for fraction 5, and ultracentrifuged (200,000 × *g*, 6 h at 10°C).

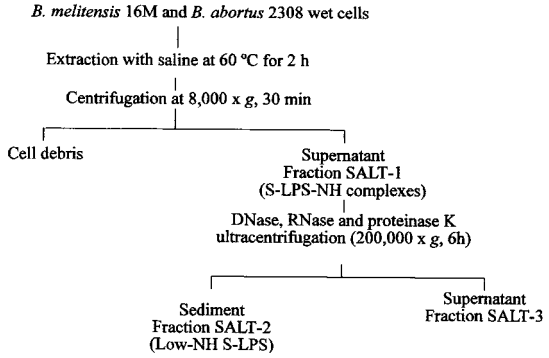

FIG. 1. Saline extraction and fractionation of S-LPS–NH complexes.

The supernatant (fraction HW-3) was extracted with a volume of phenol at 70°C for 30 min, the mixture was chilled and centrifuged (8,000 × *g*, 0°C, 15 min), and the aqueous phase was collected (fraction HW-4). The phenol phase was precipitated first with 3 volumes of ethanol (fraction HW-5) and then with 4 additional volumes of ethanol (fraction HW-6) at −20°C overnight. All the above-described fractions were dialyzed against distilled water and freeze-dried. In addition to bacteria, cell envelopes and OM blebs were used to obtain fractions HW-1 and HW-2.

Acid hydrolysis of S-LPS. Since SALT-2 contained S-LPS and only small quantities of NH (see Results), this fraction was suitable for obtaining PS. Hydrolysis of SALT-2 (10 mg/ml) in 2% acetic acid was performed at 100°C for 30 min. After ultracentrifugation at 200,000 × *g* for 6 h at 4°C (41), the supernatant containing the PS was dialyzed and freeze-dried.

Immunoassays. (i) Antibodies. Immune sera were obtained from *B. abortus* (biotype 1) field-infected or *B. abortus* strain 19-vaccinated cows (14) or from rabbits either infected intravenously with 10^9 CFU of *B. melitensis* 16M and bled 15 days later or immunized intramuscularly with acetone-killed cells of the same strain (11). Absorptions with whole cells were done as described previously (11). Monoclonal antibodies (MAbs) to the *Brucella-Yersinia* common epitope (Baps-C/Y), to group-3 OM proteins (BaOmp-3), and to *Brucella* lipid A (BaLa1) were produced and characterized as described previously (51).

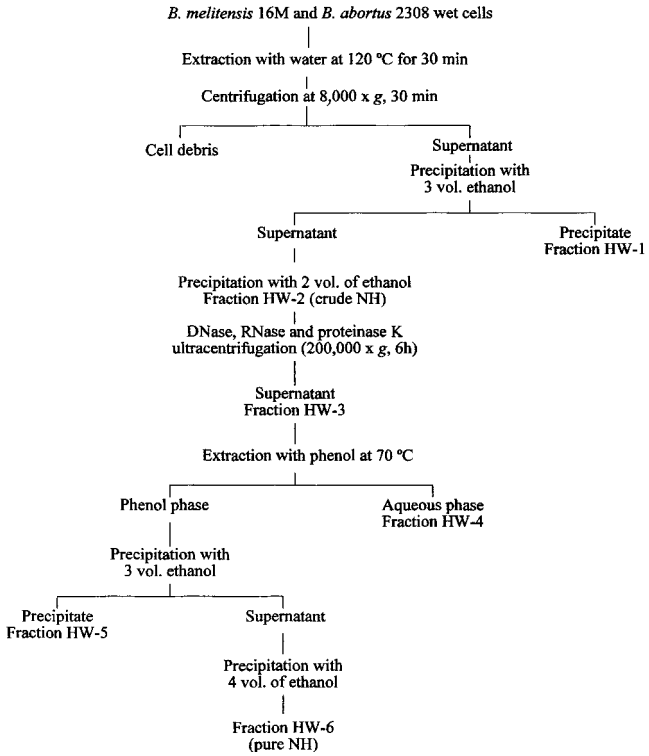

FIG. 2. Extraction and purification of NH from S-brucellae.

(ii) Gel immunoprecipitations. The agar gel immunodiffusion test (AGID) was carried out in 1.1% Nobel agar (Difco) in 100 mM borate buffer (pH 8.3) containing 10% NaCl. We have shown previously that diffusion and immunoprecipitation of both NH and PS are optimal under those conditions (14). The reverse radial immunodiffusion test was performed as described before (10, 14, 28). Immunoelectrophoresis was carried out in 1.0% Indubiose A37 (Sepracor/IBF s.a., Villeneuve la Garenne, France) in 0.04 M sodium barbital-HCl buffer (pH 8.6) at 5 to 10 V/cm for 1 h. After electrophoresis, troughs were cut and filled with cattle sera not requiring 10% NaCl for immunoprecipitation of NH (10) or with the sera from experimentally infected or immunized rabbits, and the slides were incubated at 25°C.

(iii) Immunoblots. Electroblotting on Immobilon-P (Millipore Corp., Bedford, Mass.) was performed in a Trans-Blot Semi-Dry apparatus (Bio-Rad Laboratories, Richmond, Calif.) according to the instructions of the manufacturers. After incubation with the above-described MAbs, blots were developed with peroxidase-conjugated rabbit anti-mouse immunoglobulin G (Nordic Immunological Laboratories, Tilburg, the Netherlands) and a solution of 4-chloro-1-naphthol-hydrogen peroxide (24). Alternatively, when electroblotting was done with biotinylated materials (see below), the electroblots were incubated with avidin-peroxidase (Sigma) and developed with the above-described peroxidase substrate.

AGID immunoprecipitates from biotinylated materials (see below) were capillary transferred to Immobilon-P (Millipore) for biotin detection as described by Sittenfeld and Moreno (52), with the following modifications. After 3 h of transfer to remove nonimmunoprecipitated components, the membrane was replaced by a clean one and transference was carried out for an additional 20 h. The immunocomplexes containing the biotin marker were then visualized on the membrane as described above.

Chromatographic procedures. (i) Gel filtration chromatography. Conventional gel filtration of fractions HW-3 was performed on a Sephacryl S-300 HR (Pharmacia, Uppsala, Sweden) column (90 by 2.6 cm) with 175 mM NaCl as the eluent, at a flow rate of 8 ml min^{-1}. Fractions were examined by AGID with a pool of sera from *B. abortus*-infected cattle.

(ii) HPLC. High performance liquid chromatography (HPLC) was performed with TSK4000SW, TSK3000SW, or TSK2000SW glass columns (30.0 by 0.8 cm) and an SW precolumn (TosoHaas GmbH, Stuggart, Germany) fitted to a model 625 liquid chromatography unit with a model 486 absorbance detector (Waters Associates, Inc., Milford, Mass.) set at 200 to 210 nm and/or a Sedex-45 light-scattering detector (SEDERE, Alfortville, France). The mobile phases were water or 80 to 350 mM NaCl. Samples were dissolved at 1 mg/ml in the mobile phase, sonicated briefly, and filtrated through a 0.22-μm-pore-size filter, and 50-μl aliquots were chromatographed at 0.25 or 0.5 ml min^{-1}. The relative molecular weight markers (853,000; 380,000; 186,000; 100,000; 84,000; 23,700; 12,200; and 5,800) from the Pullulan Standards Kit P-82 (Showa Denko K.K., Tokyo, Japan) were used for linear polysaccharides. Since these standards did not absorb UV light above 190 nm, detection was achieved with the light-scattering detector and confirmed by microcolorimetry (20). To test the identity of some components, samples were digested with β-D-glucoside glucohydrolase (EC 3.2.1.21; Sigma) as described previously (14). For plotting, the chromatograms were exported as ASCII files to a conventional personal computer drawing program (SigmaPlot Scientific Graphing System; Jandel Scientific Corporation, Erkrath, Germany).

(iii) HPTLC. High performance thin-layer chromatography (HPTLC) was performed on HPTLC precoated silica gel 60 plates (Merck) as described by Touchstone and Dobbins (53) with pyridine-ethyl acetate-acetic acid-water (6:2:1:3 [vol/vol]) as the mobile phase. Bands were visualized by spraying the plates with sulfuric acid-methanol (1:1) followed by heating at 100°C. To test the antibody reactivity of the bands, plates were dried, and a vertical strip was stained after protecting the rest of the plate with a clean glass; by using the stained strip as a reference, unstained bands were marked and scraped off, and the silica was extracted by stirring in the above-described solvent mixture for 4 h at 25°C. Silica was removed (2,000 × *g*, 10 min), the solvent was evaporated to dryness, and the isolated materials were tested by AGID with the serum from an infected cattle.

Extrinsic labelling of polysaccharides. As a test for the presence of NH in the cell surface, the following method was developed. Exponentially growing *B. abortus* 2308 cells were harvested alive and resuspended in 100 mM sodium acetate (pH 5.5)–0.05% NaN_3 (0.70 g [wet weight] in 1 ml), and a 0.5 volume of 30 mM sodium periodate was added. The mixture was incubated at 25°C with constant stirring in the dark, and after 1 h, oxidation was blocked by the addition of a 0.5 volume of 80 mM sodium sulfite and incubation for 15 min. Labelling was achieved by the addition of a 0.5 volume of 5 mM biotin-hydrazide (Pierce Chemical Co, Rockford, Ill.) in 100 mM sodium acetate (pH 5.5)–0.05% NaN_3 and incubation for 1.5 h at 25°C. The cells were collected by centrifugation and washed three times with 175 mM NaCl to remove unbound biotin. Milder labelling conditions (periodate and biotin-hydrazide incubations of 15 min at 10°C) gave similar results.

As a first control for extrinsic labelling, both biotinylated and fresh bacteria were disrupted by sonication (15 1-min pulses of 100 W at 30-s intervals) (Sonifier-450; Branson Ultrasonic Co., Danbury, Conn.), unbroken cells were removed (10,000 × *g*, 20 min), and cell envelopes and cytosol were separated by ultracentrifugation (100,000 × *g*, 4 h, 10°C). To demonstrate that cytosol components would become biotinylated if labelling were not extrinsic, fresh cells were first disrupted by sonication and then treated with periodate and biotin-hydrazide as described for the intact cells, and the corresponding envelopes and cytosol (soluble fraction) were separated (100,000 × *g*, 4 h, 10°C). All the above-described materials were dialyzed and freeze-dried for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotting (see above). Contamination of the cytosol with OM components was tested with the appropriate MAbs. As an additional control for extrinsic labelling, freeze-dried biotinylated cells were fixed in 10% glutaraldehyde (Merck) in double-distilled water, included in a low-viscosity multicomponent epoxy resin (Spurr kit; Sigma), and thin sections (Sorvall MT2 Microtome; Du Pont Company, Newtown, Conn.) were collected over gold grids and dried. The resin was permeabilized by soaking in double-distilled water (5 min) and treatment with 10% H_2O_2 for 10 min at 25°C in the dark (3). After three washings with water, the sections were incubated with a 1/100 dilution of streptavidin-gold (10-nm-diameter bead) conjugate (Sigma) (4 h, 25°C) and washed four times with double-distilled water. Finally, the sections were stained with 4% uranyl acetate (Sigma) in water and 0.4% lead citrate (Sigma) in 0.1 M NaOH and examined with an electron microscope (Hitachi Scientific Instruments, Mountain View, Calif.).

NH-LPS interactions. Fraction HW-3 from *B. melitensis* 16M (see above) was dissolved (2 mg/ml) in 100 mM sodium acetate (pH 5.5)–0.05% NaN_3 and treated with 0.5 volume of 30 mM sodium periodate (15 min, 25°C) with constant stirring in the dark. After the addition of 0.5 volume of 80 mM sodium sulfite and incubation for 5 min, a 0.5 volume of 5 mM biotin hydrazide in 100 mM sodium acetate (pH 5.5)–0.05% NaN_3 was added, and the mixture was incubated for 30 min at 25°C. The reagents were then removed by dialysis against distilled water, and the remaining materials were freeze-dried. NH-LPS interactions were studied by mixing 1 volume of the biotin-labelled fraction (0.8 mg/ml in water) with 1 or 2 volumes of *B. melitensis* S-LPS (5 mg/ml). The mixture was sonicated (12 s at 10 to 20 W) and analyzed immediately by AGID followed by capillary transfer of the immunoprecipitates to Immobilon-P (see above). Alternatively, the mixture was analyzed by HPLC (TSK4000SW, in distilled H_2O and 175 mM NaCl), and fractions were collected on microtiter plates. After incubation at 4°C overnight for adherence to polystyrene, biotinylated NH was detected with avidin-peroxidase and incubation for 15 min at 20°C with 0.1% 2,2′-azinobis(3-ethylbenzo-thiazoline)sulfonic acid diammonium salt (Sigma) in 0.05 M citrate buffer (pH 4) with 0.004% H_2O_2. The development of color was determined at 405 nm in a Titertek Multiskan enzyme-linked immunosorbent assay reader (Flow Laboratories Inc., McLean, Va.).

Other analytical methods. SDS-PAGE was carried out by using the Tris-glycine buffer system described by Laemmli (30). The gels were either silver stained for LPS (54) or proteins (43) or electrotransferred to Immobilon P (Millipore). The sensitivity of the silver staining for *Brucella* LPS was from 60 ng (fraction SALT-2) to 200 ng (fraction 5). Total protein was determined by the method of Markwell et al. (35) with bovine serum albumin used as a standard. The thiobarbituric acid method was used to measure KDO, with the modifications described previously (14), and with a sensitivity threshold of 10 μg of S-LPS (fraction SALT-2), equivalent to approximately 0.1% KDO in the samples. For routine analysis of fractions by ^{13}C nuclear magnetic resonance (NMR), 15 to 30 mg of sample was dissolved in distilled water with tetramethylsilane as the internal reference, and the spectra were recorded at room temperature with a model AC-200E spectrometer (Bruker Analytische Messtechnik, Silberstreifen, Germany) at 50.33 MHz. In addition, ^{13}C- and ^{1}H-NMR spectra were recorded at 125 and 500 MHz, respectively, with fractions HW-5 and HW-6 from whole cells and with fraction HW-2 from OM blebs by using a Bruker FT at 23 and 36°C.

RESULTS

Presence of NH in hot water extracts. Figure 3A shows the results of immunoelectrophoretic analysis of hot water fractions of *B. melitensis* 16M. When fraction HW-1 was developed with the serum from an infected rabbit, two major immunoreactive components were detected: a slightly cathodic component close to the serum trough and a slowly diffusing component precipitating closer to the antigen well. In previous studies, it has been unequivocally shown that the later precipitin line corresponds to the S-LPS (10, 11, 13, 39, 40), and the name NH has been used for the fast-diffusing component (14, 15, 40, 45). In contrast, the S-LPS, but not the NH, was detected with the sera from animals immunized with killed cells (Fig. 3A). Consistent with previous reports (10, 13, 14, 32, 40), the sera from infected cattle developed both the S-LPS and NH precipitin lines, and the sera from vaccinated cattle either did not precipitate with the extract or developed only the S-LPS line. Absorption of the sera with whole S cells completely removed the antibodies to both the NH and the S-LPS, as observed previously (11, 37, 40).

As shown in Table 1, fraction HW-2 contained amounts of

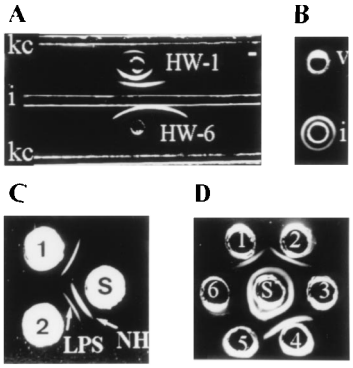

FIG. 3. Serological analysis of *Brucella* fractions. (A) Immunoelectrophoresis of *B. melitensis* fraction HW-1 (5,000 μg/ml) and HW-6 (500 μg/ml) with sera from infected rabbits (i) or rabbits immunized with killed cells (kc). (B) reverse radial immunodiffusion test performed with the serum from an infected (i) or a vaccinated (v) cow and *B. melitensis* HW-6 (10 μg/ml) included in the gel. (C) AGID analysis of *B. melitensis* fraction SALT-1 (20,000 μg/ml) before (circle 1) or after (circle 2) treatment with ether-water or heat. (D) AGID analysis of *B. melitensis* phenol-water fraction 5 (5,000 μg/ml), ether-water extract (5,000 μg/ml), HW-2 from OM blebs (50 μg/ml), HW-1 from whole cells (5,000 μg/ml), and HW-6 from whole cells (50 μg/ml) (circles 1 to 4 and 6, respectively), HW-6 from *B. abortus* (50 μg/ml) (circle 5), and serum from an infected cattle (S).

NH similar to those in HW-1 but only trace amounts of S-LPS. Because of this, and also because fractions equivalent to HW-2 have been used in serological studies (13–15, 19, 27), NH was purified from HW-2 by monitoring serological activity as the main criterion (Table 1). The only fraction devoid of serological activity was HW-4, and ^{13}C-NMR analysis showed signals at 103.70 (C-1), 83.99 to 83.29 (C-2), 77.86 (C-5), 77.00 (C-3), 69.83 (C-4), and 62.23 (C-6) ppm, which, on the basis of previous studies (6, 7, 34), were identified as corresponding to a pure cyclic β-(1,2)-glucan. All the remaining fractions showed the presence of NH by AGID, with a serological activity that increased from 500 μg/ml (*B. melitensis*) or 5,000 μg/ml (*B. abortus*) for HW-2 to 5 μg/ml for HW-6. In agreement with these results, the immunoelectrophoretic analysis of fraction HW-6 with sera from infected rabbits (Fig. 3A) showed only the NH line, and HW-6 was not precipitated with sera from animals immunized with killed cells (Fig. 3A). Concurrently with the serological criterion for NH purification, the KDO contents decreased from 0.70% (*B. melitensis*) or 0.50% (*B. abortus*) in fraction HW-1 to beyond detection in fraction HW-6. Moreover, ^{13}C-NMR analysis did not reveal the presence of β-glucans, and by SDS-PAGE followed by periodate-silver staining no materials were detected in HW-6. This proved both the absence of S-LPS and the inability of this NH fraction to migrate in SDS-PAGE. Although KDO was detected, fraction HW-5 was negative for LPS by SDS-PAGE, suggesting enrichment by the phenol extraction of some KDO-containing material which, however, was not detected by SDS-PAGE. The 500-MHz ^{1}H-NMR and 50.33- and 125-MHz ^{13}C-NMR analyses of *B. abortus* 2308 and *B. melitensis* HW-5 and HW-6 fractions showed that both preparations were polysaccharide molecules composed of *N*-formylperosamine sugars. The characteristic coupling constant (J, C-1, H1 = 173 Hz) observed for the anomeric carbon shows that the *N*-formylperosamine residues of both bacterial fractions are α linked. The *B. abortus* HW-5 and HW-6 fractions were built of a homopolymer constructed of α-1,2-linked *N*-formylperosamine units as demonstrated by sharp ^{13}C-NMR signals at 101.65 ppm (C-1), 78.05 ppm (C-2), 69.35 ppm (C-3), 52.96 ppm (C-4), 68.72 ppm (C-5), and 17.99 ppm (C-6). The *B. melitensis* HW-5 and HW-6 fractions demonstrated two sets of signals. The first set corresponded to α-1,2-linked *N*-formylperosamine units as demonstrated by the corresponding ^{13}C-NMR signals for the six carbon atoms (101.57 ppm [C′-1], 78.07 ppm [C′-2], 69.31 ppm [C′-3], 53.01 ppm [C′-4], 68.71 [C′-5], and 18.00 [C′-6]). The second set of peaks with ^{13}C-NMR signals at 102.66 ppm (C′-1), 77.22 ppm (C′-2), 69.86 ppm (C′-3), 51.97 ppm (C′-4), overlapping 68.71 ppm (C′-5), and 18.29 (C′-6) corresponded to the presence of α-1,3-linked *N*-formylperosamine as demonstrated by the 102.72-ppm shift of the anomeric carbon (40, 48). The close 4-to-1 proportion (3.77 ± 0.43) of the carbon atoms (Z/Z′, 4.6; C-1/C′-1, 3.4; C-2/C′-2, 3.6; C-3/C′-3, 3.3; C-4/C′-4, 3.9; and C-6/C′-6, 3.8) between the 1,2- and 1,3-linked sugars indicates that the *B. melitensis* HW-5 and HW-6 fractions are built of four α-1,2-linked residues intermixed with one α-1,3-linked *N*-formylperosamine residue, in a manner similar to that demonstrated by the O-chain polysaccharide of

TABLE 1. Immunochemical characterization of *B. melitensis* 16M and *B. abortus* 2308 hot water-extractable fractions

Fraction	Serological activity of NH[a]	% Yield[b]	% Protein[c]	% Kdo[c]	% LPS[c]	Carbohydrate residue(s) and linkage(s)[d]
B. melitensis 16M						
HW-1	600	14	40	0.70	40	
HW-2	500	15	10	0.09	<0.1	
HW-3	40	11	6	0.09	<0.1	Cyclic β-1,2 glucan, α-1,2 *N*-formylperosamine, α-1,3 *N*-formylperosamine
HW-4	>5,000	7	0	0.02	<0.1	Cyclic β-1,2 glucan
HW-5	10	0.6	13	0.27	<0.1	α-1,2 *N*-formylperosamine, α-1,3 *N*-formylperosamine
HW-6	5	0.2	0	0.07	<0.1	α-1,2 *N*-formylperosamine, α-1,3 *N*-formylperosamine
B. abortus 2308						
HW-1	1,250	30	50	0.50	40	
HW-2	5,000	5	30	0.10	<0.1	
HW-3	80		13	0.04	<0.1	Cyclic β-1,2 glucan, α-1,2 *N*-formylperosamine
HW-4	>5,000	2.4	4	0.03	<0.1	Cyclic β-1,2 glucan
HW-5			30	0.24	<0.1	α-1,2 *N*-formylperosamine
HW-6	5	0.33	8	0.03	<0.1	α-1,2 *N*-formylperosamine

[a] Minimal concentration (in micrograms per milliliter) yielding the NH precipiting line by AGID with a pool of sera from *B. abortus*-infected cattle.
[b] Expressed as the percentage of the cell (dry weight).
[c] Expressed as the percentage of the fraction (dry weight).
[d] Determined by ^{13}C-NMR.

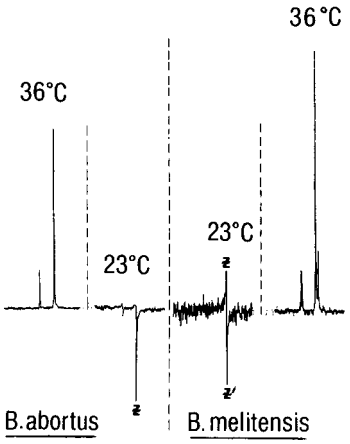

FIG. 4. ^{13}C-NMR spectra of *B. melitensis* and *B. abortus* pure NH at 125 MHz recorded at 23 and 36°C (only the section corresponding to the *N*-formyl group is presented).

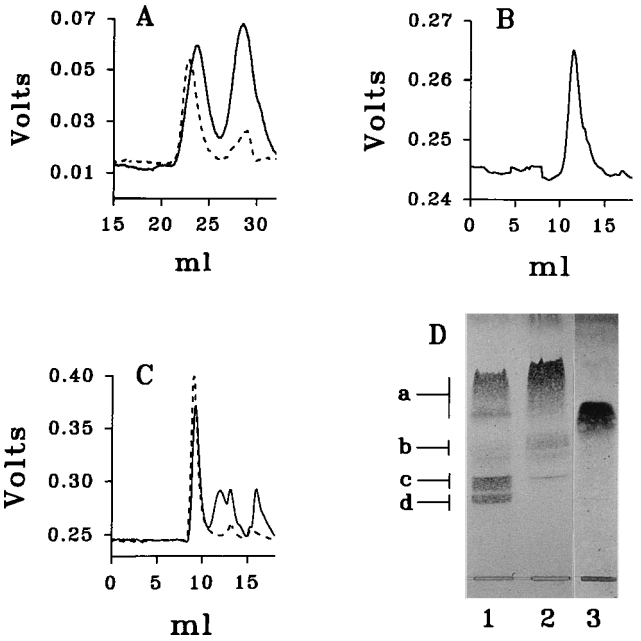

FIG. 5. HPLC and HPTLC analyses of *B. melitensis* hot-water fractions. (A) HPLC (TSK 4000SW-3000SW tandem in 175 mM NaCl) of fraction HW-3 crude extract (——) or treated with β-glucosidase (-----). (B) HPLC (TSK 2000SW in 175 mM NaCl) of fraction HW-4. (C) HPLC (TSK 2000SW in 175 mM NaCl) of fraction HW-5 (-----) and HW-6 (——). (D) HPTLC of HW-5 (lane 1), HW-6 (lane 2), and HW-4 (lane 3). Components detected by HPTLC were arbitrarily labelled a to d.

the same strain (8, 38). Two conformations, E (^{13}C-NMR signal sets of 169.00 to 168.60 ppm) and Z (^{13}C-NMR signal sets of 166.08 to 165.48 ppm), of the *N*-formyl group were seen in both polysaccharides. The Z configuration predominates over the E conformation. The formamidocarbonyl resonance that occurred at the Z′ conformation (165.48 ppm) clearly distinguished the α-1,3-substituted sugar from the Z conformation of the α-1,2-linked residue (168.89 ppm). Under the present conditions of analysis we did not detect 1,3 linkages in *B. abortus* preparations, despite the sensitive ^{13}C-NMR spectrum inversions carried out at 23°C of the Z′ configuration of the formyl group corresponding to the 1,3-linked perosamine sugars (Fig. 4). Significantly, when used in the reverse radial immunodiffusion test for the differential diagnosis of cattle brucellosis, fractions HW-5 and HW-6 showed the characteristic property of precipitating with sera from infected cattle but not from vaccinated cattle (Fig. 3B).

Fractions HW-3 to HW-6 were further characterized by HPLC and HPTLC. Two major peaks were detected in fraction HW-3 by HPLC, the second of which was sensitive to β-glucosidase (Fig. 5A). Fraction HW-4 contained a single peak which was also sensitive to β-glucosidase and had a molecular weight of 4,300 for *B. melitensis* (Fig. 5B) and 6,100 for *B. abortus* (data not shown), and the presence of a single component, which stained dark blue in contrast to the yellow and brown colors of the bands in HW-5 and HW-6, was also confirmed by HPTLC (Fig. 5D).

Despite having identical ^{13}C-NMR spectra, *B. melitensis* fractions HW-5 and HW-6 had different profiles by HPLC (Fig. 5C) and HPTLC (Fig. 5D). In particular, HW-6 was enriched in a single component (the major HPLC peak, Fig. 5C; band a, Fig. 5D). This sort of heterogeneity was also observed in the corresponding *B. abortus* 2308 fractions, although the proportion of bands c and d was comparatively reduced (data not shown). This heterogeneity did not reflect the presence of contaminants because when bands a to d were obtained by preparative HPTLC and tested by AGID, all of them developed the NH precipitin line. Finally, by HPLC, the molecular weight of the major component in HW-5 and HW-6 was 14,500 (20,500 to 7,000) for *B. melitensis* and 10,000 (17,200 to 5,000) for *B. abortus.*

The heterogeneity of NH was also observed when HW-3 was fractionated by gel filtration on Sephacryl S-300HR. HPTLC demonstrated that bands a to d successively eluted from 250 to 290 ml, and these materials were free of protein or S-LPS by SDS-PAGE, had no KDO, and showed serological activity at 2.5 μg/ml. Glucans and KDO-positive (0.2%) material, which was not characterized further, eluted after 300 ml. The results were similar for *B. melitensis* and *B. abortus.*

Presence of NH in other extracts. Although the extract obtained with saline at 60°C (fraction SALT-1) showed only a precipitin line identical to that of the S-LPS (Fig. 3C), after treatment with ether-water or brief heating in water at 100°C the S-LPS and NH precipitin lines were clearly observed (Fig. 3C). Accordingly, fractionation of SALT-1 (Fig. 1) after enzyme digestion yielded two fractions, SALT-2 and SALT-3. The sedimented fraction, SALT-2, contained 1.1 and 1.65% KDO (*B. melitensis* and *B. abortus*, respectively), was indistinguishable from the classical phenol-water fraction 5 S-LPS by SDS-PAGE (data not shown), and showed the NH precipitin line only at concentrations above 2,500 μg/ml. In contrast, fraction SALT-3 showed only traces of LPS by either SDS-PAGE or KDO measurement and contained NH (by AGID) and β-glucans (by HPTLC). In addition to the presence of S-LPS, a component serologically indistinguishable from HW-6 (pure NH) was also observed by AGID analysis of the phenol-water (fraction 5) and ether-water (Fig. 3D) extracts.

Presence of NH in cell envelopes, OM blebs, and cytosolic fractions. The concentration of NH in the cytosolic fraction was determined by AGID after removal of heat-sensitive (120°C, 30 min) materials and lyophilization. For *B. melitensis* 16M, this cytosolic concentrate developed the NH precipitin line at concentrations above 1,800 μg/ml (equivalent to 2,500 μg of crude cytosol per ml), but the S-LPS precipitin line was also observed at the same concentration. For *B. abortus* 2308, the NH precipitin line was only observed at above 15,000 μg/ml (or 45 mg of crude cytosol per ml). In contrast, NH was de-

tected in fraction HW-2 from *B. melitensis* and *B. abortus* cell envelopes at 50 and 200 μg/ml, respectively. Furthermore, NH was detected in fraction HW-2 from *B. melitensis* and *B. abortus* OM blebs at 2.5 and 5 μg/ml, respectively. The KDO content of fraction HW-2 from OM blebs was less than 0.1%, and by HPTLC (data not shown) and HPLC (Fig. 6A) it showed an overall profile similar to that of HW-6 from whole cells, with which it gave an immunological reaction of total identity (Fig. 3D). Finally, the ^{13}C-NMR spectrum of the NH from OM blebs (Fig. 6B) was identical to that of fraction HW-6 from whole cells.

Effect of the extraction procedures and acid hydrolysis on S-LPS depolymerization. Treatment of *Brucella* SALT-2 (S-LPS fraction with low NH content) with water (at either 100 or 120°C), ether-water, phenol-water, or prolonged exposure to phenol at 60°C or trichloroacetic acid at 4°C did not cause any S-LPS hydrolysis detectable as either PS or lipid A release (Table 2). In contrast, acid hydrolysis of SALT-2 from *B. abortus* yielded a PS containing 0.62% KDO and with serological activity of 300 μg/ml. By HPLC, the PS showed a single peak corresponding to a molecular weight of 13,200 (21,300 to 4,200). Hydrolysis of other S-LPS preparations (phenol-water fraction 5, HW-1, or ether-water extracts) released materials with lower KDO contents, consistent with the presence of NH in these preparations.

Extrinsic labelling of NH. NH was extracted with hot water from biotinylated cells and precipitated with 5 volumes of ethanol. When examined by AGID at the adequate concentration, this material developed a precipitin line showing a reaction of total identity with NH from unlabeled cells (Fig. 7A). After immunoprecipitates were transferred to Immobilon-P, the precipitin line from biotinylated cells, but not that from unlabelled cells, reacted with the avidin-peroxidase conjugate (Fig. 7B). Despite the fact that cells became swollen by the labelling procedure, electron microscopy of biotinylated cells showed streptavidin-colloidal gold located on the cell surface but not in the cytosolic area (Fig. 7C). By electroblotting and avidin-peroxidase detection, most of the biotin was detected in the cell envelope fraction (Fig. 8A, lane b). Although the cytosolic (soluble) fraction also showed biotinylated material smearing after a protein with a size of approximately 28,000 (Fig. 8A, lane c), both the MAb Baps-C/Y (Fig. 8B, lane c) and the MAb Omp-3 (not shown) reacted with the material in the smear. The controls confirmed the lack of migration of NH in SDS-PAGE (Fig. 8, lane i). Finally, this localized labelling of the cytosolic fraction from whole cells contrasted with the intense labelling of the cytosolic fraction from cells that disintegrated before biotinylation (Fig. 8A, lane e).

LPS-NH interactions. As described above, and depending on the starting material and subsequent treatment with organic solvents or temperature, S-LPS and NH migrated either as a single band (Fig. 3C, well 1) or as separate bands (Fig. 3C, well 2) in AGID. The S-LPS–NH interaction was also observed by HPLC when this analysis was performed with water as the eluent, and the interaction was almost eliminated when the analysis was performed with 175 mM NaCl (Fig. 9A). In addition, when NH-low S-LPS fractions (SALT-2) and biotinylated-NH mixtures were mixed, sonicated briefly, and analyzed by HPLC, or by AGID followed by capillary transfer on Immobilon P, biotinylated NH was detected in both the HPLC peak (Fig. 9B) and the AGID precipitin line (data not shown) corresponding to the S-LPS. A marked tendency to form self-aggregates with high molecular weights (400,000 for *B. melitensis* NH and 113,000 for *B. abortus* NH) was observed with pure NH (fraction HW-6 or HW-2 from OM blebs) when HPLC was performed at low ionic strength (Fig. 9C).

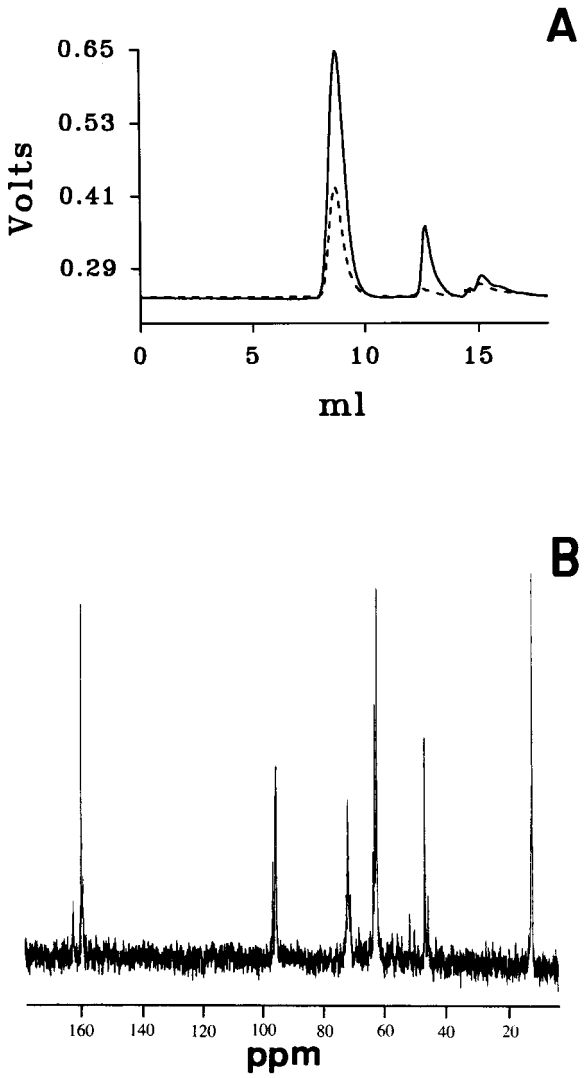

FIG. 6. Characterization of *B. melitensis* HW-2 from OM blebs by HPLC on TSK 2000SW in 175 mM NaCl (fraction HW-6 from whole cells [-----] was included as a control) (A) and ^{13}C-NMR (10 mg at 50.33 MHz) (B).

DISCUSSION

In this work, we have demonstrated by serological, chemical, physical, and cytological analyses that NH is an S-LPS independent polysaccharide located on the surface of S-brucellae, possibly anchored by noncovalent interactions with the O polysaccharide chain of the S-LPS.

NH was consistently extracted and purified by nonhydrolytic procedures to obtain fractions (HW-6 and Sephacryl gel filtration fractions from whole cells and HW-2 from OM blebs) which showed the characteristic NH precipitin line with sera from infected animals at a concentration as low as 2.5 μg/ml. The variety of sera and precipitation conditions used unequivocally prove that the molecule purified is, in fact, the NH described in serological diagnosis works (13–16, 28, 32). Obviously, the high level of serological activity of purified NH shows the high purity of the preparations obtained and is in keeping with the high density of epitopes of these *N*-formylperosamine homopolymers (9, 46, 51), which also explains the sensitivity and efficacy of the serological criterion used in the purification. Germane to the validity of the serological crite-

TABLE 2. Effects of different treatments on depolymerization of NH-free *B. abortus* S-LPS

Treatment	Detection by[a]:		
	AGID[b]		Dot blot[c] (lipid A)
	PS	S-LPS	
None	−	++++	−
Water			
5 h, 100°C	−	++++	−
4 h, 120°C	−	++++	−
Reextraction			
ether-water	−	++++	−
phenol-water at 6°C	−	++++	−
90% Phenol, 72 h, 25°C	−	++++	−
1% Trichloroacetic acid, 24 h, 4°C	−	++++	−
2% acetic acid			
5 h at 25°C	−	++++	−
15 min at 100°C	+	+++	−
30 min at 100°C	++	++	+
60 min at 100°C	+++	+	++
120 min at 100°C	++++	−	++++

[a] Results are expressed as relative levels of detection: undetectable at 5,000 μg/ml (−) to high levels detectable at 300 μg/ml (++++). Similar results were obtained with *B. melitensis.*

[b] Performed with a pool of sera from *B. abortus*-infected cattle.

[c] Performed with anti-lipid A MAb BaLa1.

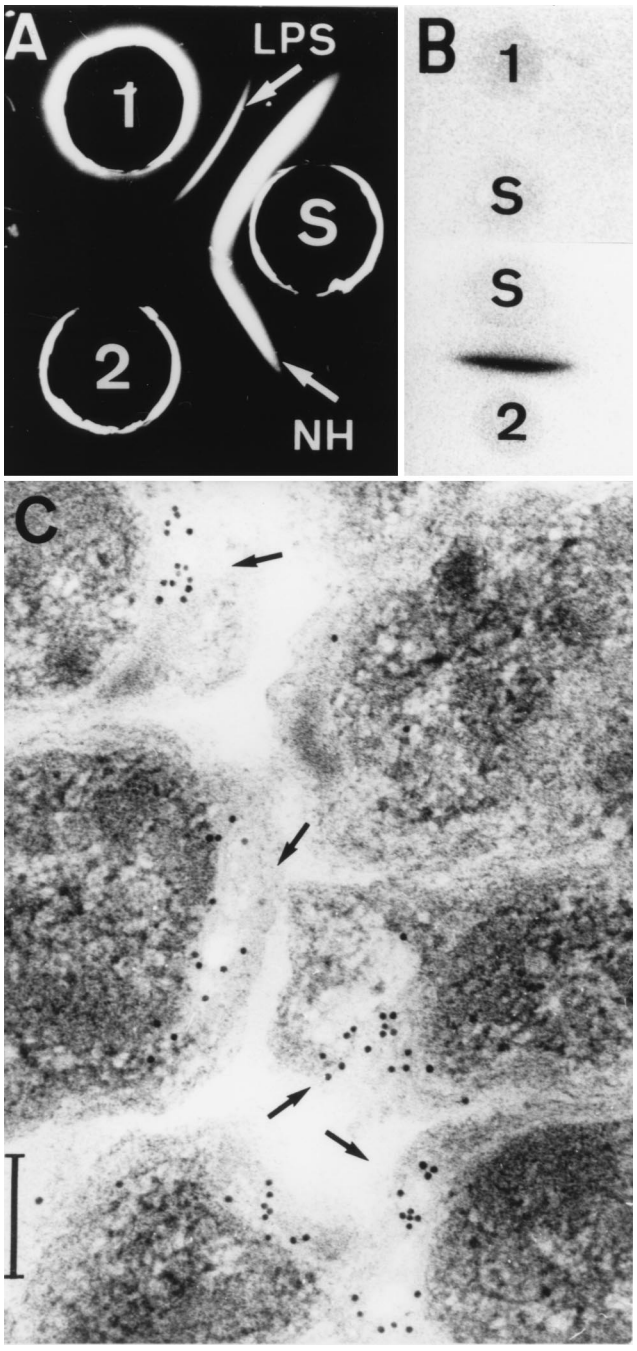

FIG. 7. Biotinylation of NH on surface of *B. abortus* live cells. (A) AGID analysis and (B) avidin-peroxidase blots of ethanol precipitates from unlabelled (circle 1) and biotin-labelled (circle 2) cell extracts. (C) Biotin detection (arrows) on a thin section of labelled cells. Bar, 0.25 mm.

rion and to the nature of NH is the nature of polysaccharide B present in the rough mutant *B. melitensis* 115. We have previously reported on the serological identity between NH and polysaccharide B (19, 40). On the other hand, several investigators have observed that *B. melitensis* extracts contain high amounts of a serologically inactive cyclic β-glucan (7, 34) and, on the basis of this quantitative criterion, have claimed that polysaccharide B is this cyclic β-glucan. The results presented here, however, illustrate the basic fact that biological criteria must prevail over chemical or simple quantitative criteria when molecules with previously defined biological activity are being purified. Therefore, polysaccharide B must be considered an NH-related molecule whose exact structure is yet to be determined and not a serologically inactive β-glucan.

The detailed NMR analysis of the NH from OM blebs or from whole cells showed spectra similar to those previously reported for the *Brucella* O-chain S-LPS (7–9, 33, 46, 55, 56), allowing the conclusion that perosamine sugars in *B. melitensis* 16M NH are both α-1,2 and α-1,3 linked, while those in *B. abortus* 2308 are α-1,2 linked. However, since about 1% of α-1,3 linkages have been reported for the *B. abortus* O chain (36), it is noteworthy that we failed to detect α-1,3 linkages in *B. abortus* NH despite sensitive ^{13}C-NMR spectrum inversions (at 23°C) of the Z′ conformation of the formyl group corresponding to the 1,3-linked perosamine sugars. This absence of α-1,3 linkages in *B. abortus* NH polysaccharides is consistent with the results of studies with MAbs (51) and with our interpretation that the cross-reactivity in serological tests with *B. abortus* and *B. melitensis* S-antigens is due to the presence of the common epitope C/Y and not to different proportions of the A and M epitopes in the same molecule (36).

Further analysis showed that NH was not identical to the PS obtained by acid hydrolysis of an S-LPS fraction with a low NH content (SALT-2). First, NH preparations which were pure by both the serological and ^{13}C-NMR criteria were more heterogeneous (see below) than the PS by HPLC and also had higher levels of serological activity (2.5 to 5.0 versus 300 μg/ml). More significantly, while PS contained up to 0.62% KDO, this sugar was not detected in NH, thus confirming previous reports on the absence of LPS core sugars (KDO and quinovosamine) in NHs (14, 38, 40, 45) or NH-like polysaccharides (25). Moreover, molecules serologically indistinguishable from the NH were demonstrated in extracts obtained under conditions (hot water, saline at 60°C, ether-water, or phenol water) which were shown not to cause hydrolysis of *Brucella* S-LPS. Therefore, on the basis of the present knowledge of the structure of the S-LPS (50), our results do not bear out the conclusion (56) that

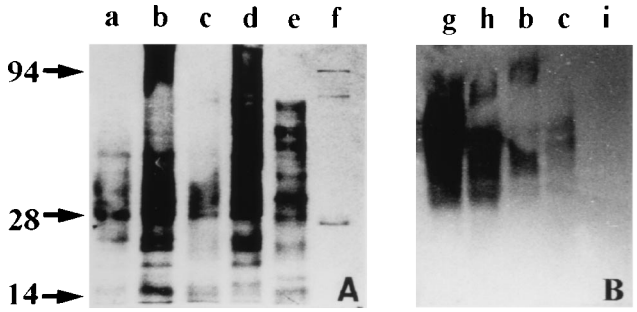

FIG. 8. Electroblots of cell fractions from biotin-labelled *B. abortus* live cells developed with avidin-peroxidase (A) and MAb Baps-C/Y (B). Lanes: a, whole biotinylated cells, b, cell envelope fraction from biotinylated cells; and c, cytosolic fraction from biotinylated cells. Controls (lanes d to i, respectively) were cell envelopes, cytosolic fraction from cells that were disrupted before biotinylation, nonbiotinylated whole cells, phenol-water S-LPS (fraction 5), OM blebs, and fraction HW-6.

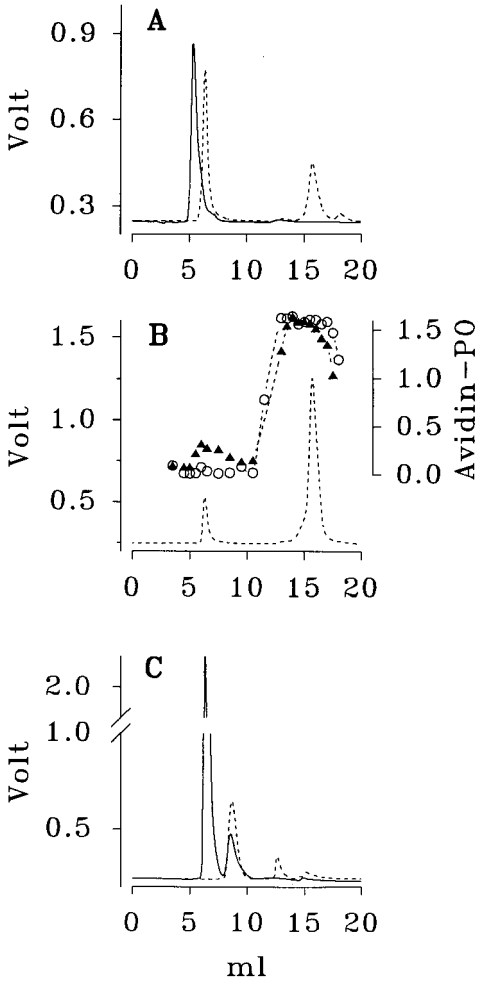

FIG. 9. Demonstration of NH-LPS and NH-NH interactions by HPLC. (A) *B. melitensis* HW-1; (B) a mixture of *B. melitensis* biotinylated HW-3 and fraction SALT-2; (C) HW-6 from *B. melitensis*. The analysis was done with TSK4000SW (A and B) or TSK2000SW (C) in either distilled H_2O (——) or 175 mM NaCl (-----), and detection was carried out by using a UV detector (Volt) or with avidin-peroxidase (Avidin-PO) for biotin detection in fractions from the biotinylated HW-3–SALT-2 mixture (▲) or from biotinylated HW-3 (○).

NH is derived from the S-LPS as a hydrolytic artifact caused by the conditions of extraction. On the other hand, NH must be regarded as an independent polysaccharide chemically similar to that PS section which bears the common epitopes (C and C/Y) and the A or M epitope (9, 46, 51).

Perusal of the literature reveals that NH-like polysaccharides have been observed often (4, 10, 11, 13, 14, 16, 25, 40, 45) but not always (8) in immunoprecipitation analysis of *Brucella* extracts obtained by several protocols. Since LPS hydrolysis cannot explain the presence of NH in the extracts, the failure to observe NH in some studies could be caused by inappropriate conditions for immunoprecipitation, differences in extraction protocols, or both. The first possibility is suggested by the fact that the NH precipitin line is not developed unless hypertonic gels (13, 25, 40, 45), an adequate buffer (14), and sera from infected animals are used. In this respect, it is important to emphasize that antibodies from vaccinated animals or animals immunized with killed cells which react with the O chain can precipitate the S-LPS while not developing the NH line (this work and references 10, 32, 40). In addition, in some modifications of the original (31) phenol-water protocol, the alcohol precipitation steps have been substituted by extensive dialysis and sedimentation of the S-LPS micelles by ultracentrifugation (7, 8), and this results in the loss of a high proportion of NH in the supernatants (2, 38). Finally, some attenuated S *B. abortus* strains produce only reduced amounts of NH (14), and production of NH is also reduced upon repeated laboratory subculture (2). Thus, the use of bacteria recently isolated from either naturally or experimentally infected animals greatly favors the detection of NH.

It has been proposed before that NH is a biosynthetic precursor of the LPS O chain (40), and this hypothesis would be compatible with the results of the chemical characterization. However, we found that while NH preparations from whole cells were heterogeneous by HPLC and HPTLC criteria, NH preparations from OM blebs were enriched in a single component (Fig. 5, band *a*). This strongly suggests that the major component could be located in the OM (see below) and that the minor ones could be biosynthetic precursors. The reason for the heterogeneity observed in extracts that showed the ^{13}C-NMR spectrum of the pure N-formylperosamine homopolymer is not known, but it is possible that it could result from variations in the degree of polymerization or in the degree of formylation of the perosamine amino group, which would be difficult to detect by NMR.

The presence of NH in the OM is supported by two independent lines of evidence. First, compared with the cytosolic and cell envelope fractions, NH was enriched in the OM blebs from which, significantly, it was obtained essentially free of periplasmic β-1,2 glucans by a simple two-step protocol. In addition, the experiments in which live cells were treated with periodate-biotin-hydrazide showed biotinylation of serologically identified NH. Although localized labelling was detected in the cytosolic (soluble) fraction, the use of MAbs demonstrated that this was due to contamination with group 3 OM proteins and its tightly-linked S-LPS (22, 51), probably as a result of the extensive ultrasonic treatment necessary to disrupt the *Brucella* cells. Moreover, despite the fact that the procedure created some artifacts in the bacteria, labelling was detected only on the cell surface by electron microscopy of thin sections. Therefore, the early proposal by Dubray (17) on the hypothetical presence in the OM of S-brucellae of a polysaccharide different from the LPS O chain can be reformulated to include the NHs as one of the surface polysaccharides. The existence of NH-type polysaccharides is not a unique phenomenon of S-brucellae, and it could be a common event in gram-

negative bacteria. For instance, LPS independent polysaccharides carrying O-chain antigenic determinants have been described for *Proteus mirabilis* (5), *Yersinia enterocolitica* O:9 (14, 19), and *Escherichia coli* strains of the serotypes O111 (23, 47), O55:B5 and O127:B8 (47), O100:K?(B) (26), and O104 (29). Although the mechanism by which those polysaccharides remain in the OM is not known, the NH could be anchored in the OM of *Brucella* cells through interactions with the LPS O chain. This hypothesis is supported by the consistent coextraction of NH and S-LPS under a variety of conditions (this work and references 4, 10, 11, 13, 14, 16, 25, 40, 45), by the NH-LPS tight interactions observed and by the fact that such interactions were restored with LPS and pure NH. That the interaction would happen between *N*-formylperosamine polysaccharides (O chain and NH) is also suggested by the fact that NH manifested a marked self-aggregation tendency. Obviously, such an interaction would also explain the mechanism of extraction by hot water, as heat would break noncovalent bonds between the polysaccharides which, during cooling, would self-aggregate separatedly because of the different physicochemical features of S-LPS and NH. If the model is correct, the similar chemical compositions and molecular weights of the PS and of the OM NH suggest that the latter could be embedded among the PS moieties of the S-LPS. Although a definite capsular polysaccharide has seldom been observed in *Brucella* cells (44), an O-type polysaccharide intertwined with the LPS would be difficult to discriminate by electron microscopy.

The characterization of NHs as OM polysaccharides chemically similar to the O chain but lacking the core and lipid A moieties of LPS raises the obvious question of their role in the biology of S-brucellae. Research is in progress to study possible functions of NH in the interaction of these bacteria with the host immune defenses.

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REFERENCES

1. **Alton, G. G., L. M. Jones, R. D. Angus, and J. M. Verger.** 1988. Techniques for the brucellosis laboratory. Institut de la Recherche Agronomique, Paris.
2. **Aragón, V., I. Moriyón, E. Moreno, and R. Díaz.** Unpublished results.
3. **Bendayan, M., and M. Zollinger.** 1983. Ultrastructural location of antigenic sites on osmium-fixed tissues applying the protein A-gold technique. J. Histochem. Cytochem. **31:**101–109.
4. **Berman, D. T., B. L. Wilson, E. Moreno, R. D. Angus, and L. M. Jones.** 1980. Characterization of *Brucella abortus* soluble antigen employed in immunoassay. J. Clin. Microbiol. **11:**355–362.
5. **Beynon, L. M., A. J. Dumanski, R. J. C. McLean, L. L. MacLean, J. C. Richards, and M. B. Perry.** 1992. Capsule structure of *Proteus mirabilis* (ATCC 49565). J. Bacteriol. **174:**2172–2177.
6. **Breedveld, M. W., and K. J. Miller.** 1994. Cyclic β-glucans of members of the family *Rhizobiaceae*. Microbiol. Rev. **58:**145–161.
7. **Bundle, D. R., J. W. Cherwonogrodzky, and M. B. Perry.** 1987. The structure of the lipopolysaccharide O-chain (M-antigen) and polysaccharide B produced by *Brucella melitensis* 16M. FEMS Microbiol. Lett. **216:**261–264.
8. **Caroff, M., D. R. Bundle, M. B. Perry, J. W. Cherwonogrodzky, and J. R. Duncan.** 1984. Antigenic S-type lipopolysaccharide of *Brucella abortus* 1119-3. Infect. Immun. **46:**384–389.
9. **Cherwonogrodzky, J. W., G. Dubray, E. Moreno, and H. Mayer.** 1990. Antigens of *Brucella*, p. 19–64. *In* K. Nielsen, and J. R. Duncan (ed.), Animal brucellosis. CRC Press, Inc., Boca Raton, Fla.
10. **Díaz, R., P. Garatea, L. M. Jones, and I. Moriyón.** 1979. Radial immunodiffusion test with a *Brucella* polysaccharide antigen for differentiating infected from vaccinated cattle. J. Clin. Microbiol. **10:**37–41.
11. **Díaz, R., L. M. Jones, D. Leong, and J. B. Wilson.** 1968. Surface antigens of smooth brucellae. J. Bacteriol. **96:**893–901.
12. **Díaz, R., and I. Moriyón.** 1989. Laboratory techniques in the diagnosis of human brucellosis, p. 73–83. *In* E. J. Young, and M. J. Corbel (ed.), Brucellosis: clinical and laboratory aspects of human infection. CRC Press, Inc., Boca Raton, Fla.
13. **Díaz, R., J. Toyos, M. D. Salvo, and M. L. Pardo.** 1981. A simple method for the extraction of polysaccharide B from *Brucella* cells for use in the radial immunodiffusion test diagnosis of bovine brucellosis. Ann. Rech. Vet. **12:** 35–39.
14. **Díaz-Aparicio, E., V. Aragón, C. Marín, B. Alonso, M. Font, E. Moreno, S. Pérez-Ortiz, J. M. Blasco, R. Díaz, and I. Moriyón.** 1993. Comparative analysis of *Brucella* serotype A and M serotype and *Yersinia enterocolitica* O:9 polysaccharides for the serological diagnosis of brucellosis in cattle, sheep, and goats. J. Clin. Microbiol. **31:**3136–3141.
15. **Díaz-Aparicio, E., C. M. Marín, B. Alonso-Urmeneta, V. Aragón, S. Pérez-Ortiz, M. Pardo, J. M. Blasco, R. Díaz, and I. Moriyón.** 1994. Evaluation of serological tests for the diagnosis of *Brucella melitensis* infection of goats. J. Clin. Microbiol. **32:**1159–1165.
16. **Dranovskaja, E. A., and P. A. Versilova.** 1971. Biochemical characteristics, antigenic structure and immunobiological properties of polysaccharide fractions of *Brucella* endotoxin. WHO document Bruc/71. 327. World Health Organization, Geneva.
17. **Dubray, G.** 1976. Localisation cellulaire des polyosides des bactéries des genres *Brucella* et *Escherichia* en phase lisse (S) or rugueuse (R). Ann. Inst. Pasteur Microbiol. **127B:**133–149.
18. **Fekete, A., J. A. Bantle, S. M. Halling, and R. W. Stich.** 1992. Amplification fragment length polymorphism in *Brucella* strains by use of polymerase chain reaction with arbitrary primers. J. Bacteriol. **174:**7778–7783.
19. **Fernández-Lago, L., I. Moriyón, J. Toyos, and R. Díaz.** 1982. Immunological identity of *Brucella* native hapten, polysaccharide B and *Yersinia enterocolitica* serotype 9 native hapten. Infect. Immun. **38:**778–780.
20. **Fox, J. D., and J. F. Robyt.** 1991. Miniaturization of three carbohydrate analyses using a microsample plate reader. Anal. Biochem. **195:**93–96.
21. **Gamazo, C., and I. Moriyón.** 1987. Release of outer membrane fragments by exponentially growing *Brucella melitensis* cells. Infect. Immun. **55:**609–615.
22. **Gamazo, C., A. I. Vitas, I. Moriyón, I. López-Goñi, and R. Díaz.** 1993. *Brucella* group 3 outer membrane proteins contain a heat-modifiable protein. FEMS Microbiol. Lett. **112:**141–146.
23. **Goldman, R. C., D. White, F. Ørskov, P. D. Rick, M. S. Lewis, A. K. Bhattacharjee, and L. Leive.** 1982. A surface polysaccharide of *Escherichia coli* O111 contains O-antigen and inhibits agglutination of cells by O-antiserum. J. Bacteriol. **151:**1210–1221.
24. **Hawkes, R., E. Niday, and J. Gordon.** 1982. A dot-immunobinding assay for monoclonal and other antibodies. Anal. Biochem. **119:**142–147.
25. **Hoffmann, E. M., and J. J. Houle.** 1986. Purification of nonlipopolysaccharide antigen from *Brucella abortus* during preparation of antigen used for indirect hemolysis test. J. Clin. Microbiol. **24:**779–784.
26. **Jann, B., K. Jann, and G. Schmidt.** 1970. Immunochemical studies of polysaccharide surface antigens of *Escherichia coli* O100:K?(B):H2. Eur. J. Biochem. **15:**29–39.
27. **Jiménez de Bagües, M. P., C. M. Marín, J. M. Blasco, I. Moriyón, and C. Gamazo.** 1991. An ELISA with *Brucella* lipopolysaccharide antigen for the diagnosis of *B. melitensis* infection in sheep and for the evaluation of serological responses following subcutaneous or conjunctival *B. melitensis* Rev 1 vaccination. Vet. Microbiol. **30:**233–241.
28. **Jones, L. M., D. T. Berman, E. Moreno, B. L. Deyoe, M. J. Gilsford, J. D. Huber, and P. Nicoletti.** 1980. Evaluation of a radial immunodiffusion test with polysaccharide B antigen for diagnosis of bovine brucellosis. J. Clin. Microbiol. **12:**753–760.
29. **Kogan, G., B. Jann, and K. Jann.** 1992. Structure of the *Escherichia coli* O:104 polysaccharide and its identity with the capsular K9 polysaccharide. FEMS Microbiol. Lett. **91:**135–140.
30. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
31. **Leong, D., R. Díaz, K. Milner, J. Rudbach, and J. B. Wilson.** 1970. Some structural and biological properties of *Brucella* endotoxin. Infect. Immun. **1:**174–182.
32. **Lord, V. R., and J. W. Cherwonogrodzky.** 1992. Evaluation of polysaccharide, lipopolysaccharide, and beta-glucan antigens in gel immunodiffusion tests for brucellosis in cattle. Am. J. Vet. Res. **53:**389–391.
33. **L'vov, V. L., V. E. Malikov, A. S. Shaskov, E. A. Dranovskaja, and B. A. Dmitriev.** 1985. Somatic antigens of the genus *Brucella*: structure of the O-specific polysaccharide chain of the lipopolysaccharide of *Brucella melitensis*. Bioorg. Khim. **11:**963–969.
34. **L'vov, V. L., G. N. Pluzhnikova, E. B. Lapina, A. S. Shashkov, S. A. Askerova, E. Malikov, E. A. Dranovskaya, and B. A. Dimitriev.** 1987. Molecular nature of the polysaccharide B antigen from *Brucella* (poly-B). Bioorg. Khim. **13:** 1070–1074.
35. **Markwell, M. A. K., S. M. Haas, L. L. Bieber, and N. E. Tolbert.** 1978. A modification of the Lowry procedure to simplify protein determination in

membrane lipoprotein samples. Anal. Biochem. **87:**206–210.
36. **Meikle, P. J., M. B. Perry, J. W. Cherwonogrodzky, and D. Bundle.** 1989. Fine structure of A and M antigens from *Brucella* biovars. Infect. Immun. **57:**2822–2828.
37. **Moreno, E., L. M. Jones, and D. T. Berman.** 1984. Immunochemical characterization of rough *Brucella* lipopolysaccharides. Infect. Immun. **43:**779–782.
38. **Moreno, E., H. Mayer, and I. Moriyón.** 1987. Characterization of a native polysaccharide hapten from *Brucella melitensis*. Infect. Immun. **55:**2850–2853.
39. **Moreno, E., M. W. Pitt, L. J. Jones, G. G. Schurig, and D. T. Berman.** 1979. Purification and characterization of smooth and rough lipopolysaccharides from *Brucella abortus*. J. Bacteriol. **138:**361–369.
40. **Moreno, E., S. L. Speth, L. M. Jones, and D. T. Berman.** 1981. Immunochemical characterization of *Brucella* lipopolysaccharide and polysaccharides. Infect. Immun. **31:**214–222.
41. **Moreno, E., E. Stackebrandt, M. Dorsch, J. Wolters, M. Busch, and H. Mayer.** 1990. *Brucella abortus* 16S rRNA and lipid A reveal a phylogenetic relationship with members of the alpha-2 subdivision of the class *Proteobacteria*. J. Bacteriol. **172:**3569–3576.
42. **Moriyón, I., and D. T. Berman.** 1982. Effects of nonionic, ionic and dipolar ionic detergents and EDTA on *Brucella* cell envelopes. J. Bacteriol. **152:**822–828.
43. **Oakley, B. R., D. R. Kirsch, and N. R. Morris.** 1980. A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. Anal. Biochem. **105:**361–363.
44. **Oberti, J., R. Caravano, and J. Roux.** 1982. Demonstration of an external layer in several species of the genus *Brucella*. Can. J. Microbiol. **28:**1300–1303.
45. **Perera, V. Y., A. J. Winter, and B. Ganem.** 1984. Evidence for covalent bonding of native hapten-protein complexes to smooth lipopolysaccharide of *Brucella abortus*. FEMS Microbiol. Lett. **21:**263–266.
46. **Perry, M. B., and D. R. Bundle.** 1990. Lipopolysaccharide antigens and carbohydrates of *Brucella*, p. 76–88. *In* L. G. Adams (ed.), Advances in brucellosis research. Texas A&M University Press, College Station.
47. **Peterson, A. A., and E. McGroarty.** 1985. High-molecular-weight components in lipopolysaccharides of *Salmonella typhimurium*, *Salmonella minnesota*, and *Escherichia coli*. J. Bacteriol. **162:**738–745.
48. **Redmond, J. W.** 1979. The structure of the O-antigenic side chain of lipopolysaccharide of *Vibrio cholerae* 569B (INABA). Biochim. Biophys. Acta **584:**346–352.
49. **Ribi, E., W. T. Haskins, M. Landy, and K. Milner.** 1961. Preparation and host-reactive properties of endotoxin with low content of nitrogen and lipid. J. Exp. Med. **114:**647–663.
50. **Rick, P. D.** 1987. Lipopolysaccharide biosynthesis, p. 648–662. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, vol. 1. American Society for Microbiology, Washington, D.C.
51. **Rojas, N., E. Freer, A. Weintraub, M. Ramírez, S. Lind, and E. Moreno.** 1994. Immunochemical identification of *Brucella abortus* lipopolysaccharide epitopes. Clin. Diag. Lab. Immunol. **1:**206–213.
52. **Sittenfeld, A., and E. Moreno.** 1988. A sensitive blotting system for detection of alpha-feto protein variants with monoclonal and polyclonal antibodies. J. Immunol. Methods **106:**19–26.
53. **Touchstone, J. C., and M. F. Dobbins.** 1983. Practice of thin layer chromatography, 2nd ed. John Wiley & Sons Inc., New York.
54. **Tsai, C., and C. E. Frasch.** 1982. A sensitive stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. **119:**115–119.
55. **Wu, A. M., and N. E. Mackenzie.** 1987. Structural and immunochemical characterization of the O-haptens of *Brucella abortus* lipopolysaccharides from strains 19 and 2308. Mol. Cell. Biochem. **75:**103–111.
56. **Zygmunt, M. S., G. Dubray, D. R. Bundle, and M. B. Perry.** 1988. Purified native haptens of *Brucella abortus* B19 and *B. melitensis* 16M reveal the lipopolysaccharide origin of the antigens. Ann. Inst. Pasteur/Microbiol. (Paris) **139:**421–423.