JOURNAL OF BACTERIOLOGY, May 1979, p. 361-369
0021-9193/79/05-0361/09$02.00/0

Vol. 138, No. 2

Purification and Characterization of Smooth and Rough Lipopolysaccharides from *Brucella abortus*

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Received for publication 27 December 1978

In an attempt to obtain pure and well characterized smooth lipopolysaccharide (S-LPS) and rough lipopolysaccharide (R-LPS), smooth and rough strains of *Brucella abortus* were extracted by two different modifications of the phenol-water method. S-LPS was obtained in the phenol phase, and R-LPS was obtained in the aqueous phase. Further purification was accomplished by treatment with enzymes, detergents, NaI as a chaotropic agent to separate non-covalently bound contaminants, and by gel filtration. The degree of purity of the molecules was determined by chemical and immunological analysis and by electrophoresis in sodium dodecyl sulfate-polyacrylamide gels. Lipid identification by gas-liquid chromatography showed seven major fatty acids. Palmitic acid accounts for about 50%, stearic acid accounts for about 10%, and hydroxylated fatty acids account for less than 5% of total fatty acids. 2-Keto-3-deoxyoctonate but not heptose was detected in the sugar analysis. Protein was found to be firmly bound to S-LPS but not to R-LPS.

Several investigators have shown that the crude endotoxin from smooth *Brucella* spp. is isolated from the phenol phase when cells are extracted with hot phenol-water (2, 3, 6, 18, 22, 23, 30). This fraction, containing 2-keto-3-deoxyoctonate (KDO), sugars, and lipids, presents both similarities and differences when compared with lipopolysaccharides (LPS) from *Enterobacteriaceae*.

Examples of such similarities and differences include observations on toxicity, pyrogenicity, and hybrid formation with *Escherichia coli* LPS. Baker and Wilson (1, 2) found that endotoxin preparations of smooth *Brucella abortus* were toxic for mice, but were less toxic than those from *E. coli*. Leong et al. (30) described qualitative differences in biological activity but not structural differences between brucella endotoxins and enterobacterial endotoxins. Jones et al. (22) reported that saline-extracted rough LPS (R-LPS) from *B. ovis* was toxic for mice and had limulus lysate gelation activity (LLGA) comparable to that of *E. coli* LPS. Munoz et al. (36) showed that an endotoxin preparation of smooth *B. abortus* had comparable LLGA to LPS from several different strains of *Salmonella* and *E. coli*, but did not increase sensitivity of mice to histamine.

Because these experiments were performed with crude brucella endotoxin preparations rather than with purified LPS, it was not possible to establish any relationship between chemical composition and biological activity that could help to explain the differences in behavior of these LPS molecules.

The work reported here describes preparation and analytical data for purified smooth LPS (S-LPS) and R-LPS from *B. abortus* which is essential to experiments on biological activity. These, in turn, may contribute to the understanding of the relationship of chemical composition to activity.

MATERIALS AND METHODS

Bacterial cultures. The bacterial strains used, their characteristics, and the conditions of culture were described previously (22).

Extraction of crude LPS. The crude S-LPS (f_5) was extracted from 60 g of acetone-dried cells of *B. abortus* strain 11-19 with hot phenol-water (Fig. 1) as described by M. S. Redfearn (Ph.D. thesis, University of Wisconsin-Madison, Madison, Wis., 1960) and Baker and Wilson (2).

The crude R-LPS (R_3) from *B. abortus* strain 45/20 was prepared by the following method (Fig. 2): 60 g of acetone-dried cells was extracted for protein with 2.5% NaCl at 4°C (23). The supernatant fluids were separated from the cells by centrifugation at 8,000 × *g* for 30 min at 4°C. The wet cells were frozen and stored for 4 weeks at −20°C. The cells were then washed and dried in acetone, and batches of 7 g were extracted with chloroform-petroleum ether-phenol as described by Galanos et al. (12). The batches of cell residue were kept for further extraction. The super-

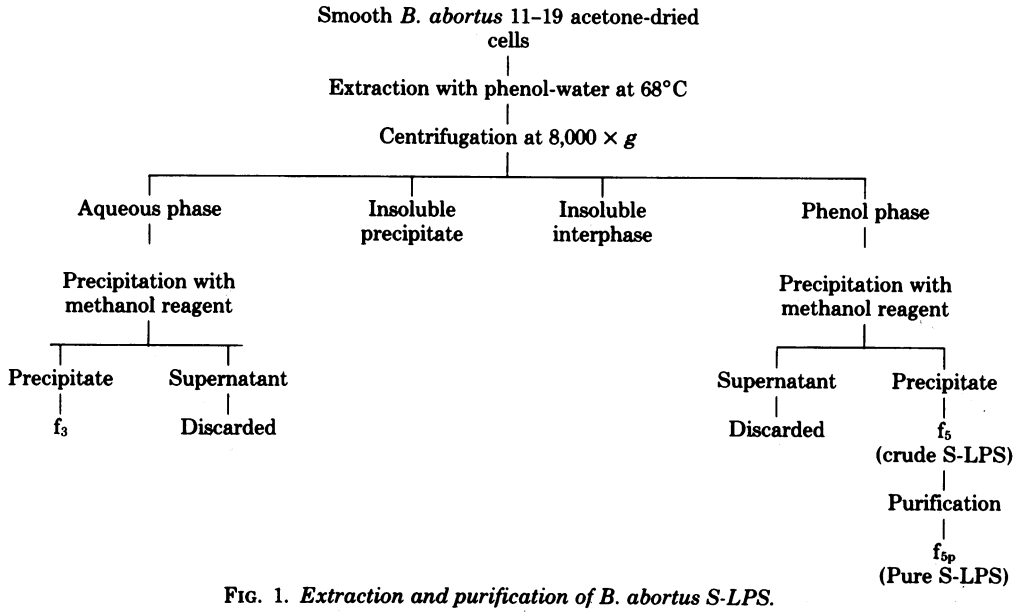

FIG. 1. *Extraction and purification of B. abortus S-LPS.*

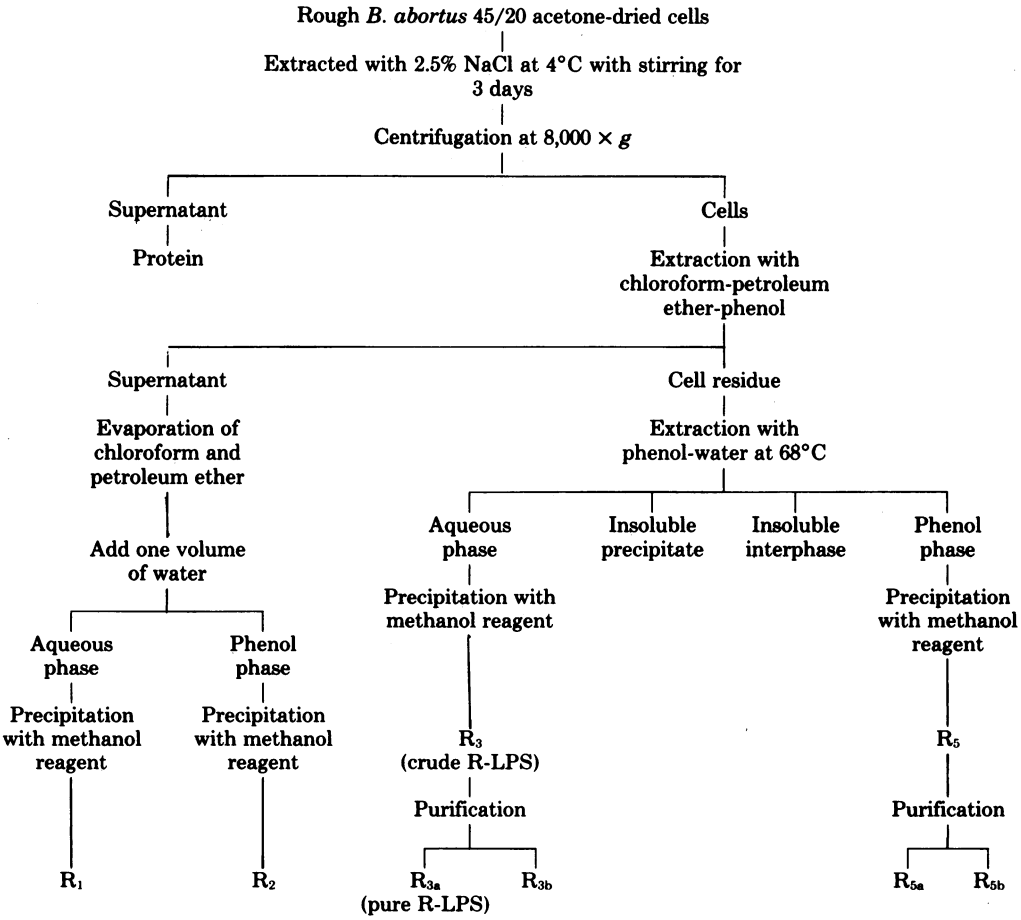

FIG. 2. *Extraction and purification of B. abortus R-LPS.*

natant fluids from all batches were pooled, and the chloroform and petroleum ether were evaporated under vacuum with rotation at 35°C. To the remaining liquid phenol, one volume of deionized distilled water (ddH_2O) was added. The mixture was agitated vigorously for 15 min and centrifuged at 8,000 × *g* for 30 min at 4°C, causing the formation of two layers: the upper layer consisted of phenol-saturated water (aqueous phase), and the lower layer consisted of water-saturated phenol (phenol phase). In contrast to similar preparations from *Enterobacteriaceae*, no precipitate was obtained at this step in the extraction of brucella R-LPS.

The two layers were separated carefully with a Pasteur pipette, and the aqueous phase was dialyzed at 4°C for 1 week against tap water and 1 day against ddH_2O. It was then concentrated 10-fold by pervaporation at 37°C. Three volumes of cold methanol reagent (99 parts of methanol and 1 part of methanol saturated with sodium acetate) were added, and the precipitate which formed was collected by centrifugation at 8,000 × *g* for 20 min at 4°C, suspended in 50 ml of ddH_2O, dialyzed for 1 week, and finally lyophilized (R_1). The supernatant fluid was discarded.

To the phenol phase, three volumes of cold methanol reagent were added. The solution was stirred for 5 h at 4°C. The precipitated material was collected, suspended in water, and lyophilized as described above (R_2).

The batches of cell residue were extracted with hot phenol–water, using the method described above for the crude S-LPS. The pooled fractions obtained from the aqueous phase (R_3) and the phenol phase (R_5) were lyophilized and kept for analysis and further purification.

Purification of crude S-LPS. The crude S-LPS (f_5), isolated in the phenol phase, contained other bacterial components such as proteins, nucleic acids, and polysaccharides, apparently associated with S-LPS by strong noncovalent bonds. Batches of 12.5 mg each of f_5 were dissolved in 2.5 ml of dimethyl sulfoxide and stirred for 20 min at room temperature. To disrupt the noncovalent associations between the LPS and other bacterial components, 2.5 ml of 4 M NaI was then added as a chaotropic agent, with slow stirring. The solution was held at room temperature for an additional 20 min, and the S-LPS was then precipitated with 15 ml of cold methanol reagent and stirred for 1 h at 4°C. The precipitate was redissolved in 2.5 ml of dimethyl sulfoxide. NaI was added, and S-LPS was precipitated with methanol reagent. The procedure was repeated six more times. After the last precipitation with methanol reagent, the S-LPS was redissolved in 10 ml of ddH_2O, dialyzed at 4°C for 2 days against several changes of ddH_2O, and lyophilized. A 44-mg amount of this material was dissolved in 2.5 ml of 0.05 M Tris-hydrochloride buffer (pH 8.1), 2 M NaI, and 5% Tween 40 and chromatographed on Sephadex G-200. The purified S-LPS (f_{5p}) obtained in the exclusion volume was dialyzed and lyophilized.

Purification of crude R-LPS. A suspension of 75 mg of lyophilized fraction R_3 in 9 ml of 0.01 M phosphate-buffered saline (pH 7.2) was treated with 0.75 mg of ribonuclease (Sigma) and 0.75 mg of deoxyribonuclease (Sigma), and the mixture was stirred for 18 h at room temperature. The precipitate obtained was washed with ddH_2O until the absorbance of the supernatant fluid at 260 nm was less than 0.05. The precipitate was resuspended in ddH_2O, dialyzed, and lyophilized (R_{3a}). The supernatant fluid after removal of R_{3a} was dialyzed and lyophilized (R_{3b}).

Fraction R_5 was purified in the same manner as fraction R_3 except that additional digestion with 0.75 mg of Pronase (Sigma) was done after treatment with nucleases. The two fractions obtained (R_{5a} and R_{5b}) were dialyzed and lyophilized as described above.

Solubilization of R-LPS. *B. abortus* strain 45/20 R-LPS was solubilized by adsorbing it to bovine serum albumin (R_{3a}-BSA) as described by Galanos et al. (13) for lipid-A-BSA preparations.

Antigens for comparative purposes. Crude R-LPS from *B. ovis* was extracted with 0.15 M NaCl at 80°C for 2 h (37). This material contained 52.67% protein, 2.95% KDO, 33% fatty acid (FA), and LLGA at 0.1 ng/ml.

Sonic extracts from *B. abortus* strain 45/20 were prepared as described by Schurig et al. (44).

E. coli LPS from serotype O128:B12 was purchased from Difco and purified by three cycles of centrifugation at 106,000 × *g* for 4 h as described by Westphal and Jann (51).

Immune sera. Antisera against crude *B. ovis* R-LPS, *B. abortus* 45/20 cells, and *B. abortus* 45/20 protein antigen were produced in rabbits as described previously (5, 23, 44).

Chemical analysis. Total carbohydrates were estimated by the tryptophan sulfuric acid method (9), with a mixture of equal amounts of D-galactose, L-rhamnose, D-mannose, and D-glucose (Sigma) as standard (30). Heptose was determined by the cysteine-sulfuric acid reaction (8) with L-glycero-D-mannoheptose (Sigma) as standard. KDO was determined by a modification of the method described by Warren (49) with correction for 2-deoxyribose, using appropriate mixtures of KDO and 2-deoxyribose (Sigma) as standard. Total protein was estimated by the Lowry method (33) with bovine serum albumin (Sigma) as standard. Fatty acid amide and fatty acid ester were determined colorimetrically (15) with tripalmitin (Sigma) as standard. Nucleic acids were estimated by the ratio of absorbance at 280/260 nm after hydrolysis of the material with 0.01 N NaOH, with purified RNA (Sigma) as standard (25).

FA analysis by GLC. The following FA methyl esters were used as reference compounds: GLC-80 (tridecanoic, tetradecanoic, pentadecanoic, hexadecanoic, heptadecanoic), GLC-50 (*cis*-9 hexadecenoic, *cis*-9 octadecenoic, *cis*-11 eicosenoic, *cis*-13 docosenoic, *cis*-15 tetracosenoic), and methyl linolenate (all from Supelco). β-Hydroxy myristic, β-hydroxy-palmitic, lauric acid (Applied Science Laboratories) and LPS samples were converted to their corresponding methyl esters by the esterification procedure described by Sweely and Moscatelli (47).

Trimethylsilyl derivatives were prepared by the method of Sweely et al. (46).

Hydrogenation of 10 to 20 μg of FA methyl esters was performed as described by O'Brien and Rosser (38).

A Varian 3700 gas chromatograph equipped with a Varian CDS-111 integrator was used for all gas-liquid chromatography (GLC) determinations. Two 200-cm

glass columns with 2-mm inner diameter were used for identification purposes. The polar column consisted of 10% SP-2300 on 100/120 Chromosorb W (Supelco), and the nonpolar column was packed with 3% SE-30 80/100 Chromosorb WHP (Applied Science Laboratories). The temperature program for the SP-2300 column began at 150°C and was increased at a rate of 6°C/min to a final temperature of 195°C and held for 5 min. The SE-30 column employed a temperature program starting at 210°C and increasing at a rate of 4°C/min to a final temperature of 235°C which was held for 10 min. Relative percentages of total FA were computed by the CDS-111.

Thin-layer chromatography in silica gel plates was used to determine the degree of extraneous lipid contamination in the various LPS preparations with lecithin and lysolecithin (Sigma) as standards.

Gel filtration chromatography. Sephadex G-200 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) was packed and developed in a column (2.5 by 96 cm) according to the manufacturer's instructions, with a 0.05 M Tris-hydrochloride buffer (pH 8.1), 2 N NaI, and 5% Tween 40. S-LPS was chromatographed, and the S-LPS-containing fractions eluted were precipitated with methanol reagent and redissolved to 1 ml in ddH_2O. Fractions were monitored for relative amounts of KDO and protein.

Immunological analysis. Immunoelectrophoresis was performed as described previously (44). The immunodiffusion technique was done as described by Ouchterlony and Nilsson (39).

SDS-PAGE. All LPS samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (29). Samples were solubilized by heating a suspension of LPS in a solution containing 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, and 0.01 M Tris-hydrochloride buffer (pH 6.8) for 3 min in a tube placed in a boiling water bath. The upper gel was 4% acrylamide, and the lower gel was either 10% or 15% acrylamide (13 by 10 cm, 0.15 cm thick). Electrophoresis was carried out at a constant current of 20 mA/gel and terminated when the bromophenol blue marker dye had traveled 8 cm. Immediately after electrophoresis, the gel was fixed in 25% isopropanol for 2 h. The samples were cut from the slab and stained by only one of the following techniques: periodic acid Schiff stain for carbohydrate (10), Coomassie brilliant blue R-250 for proteins (50), or Sudan black for lipids (39).

LLGA assay. The LLGA assay was carried out as described by Sullivan and Watson (45). Activity was expressed as the lowest concentration (in nanograms per milliliter) needed to form a solid gel.

RESULTS

We considered KDO, FA, and LLGA to be the most important markers for the presence of LPS. On this basis (Table 1), more S-LPS was present in the phenol phase (f_5) than in the aqueous phase (f_3). Fraction f_5 contained 24% protein and 8% nucleic acids. The pellet obtained after centrifugation of f_5 at 106,000 × *g* for 12 h had the same protein and nucleic acid content. Treatment of f_5 with Pronase destroyed the antigenicity of the proteins, as shown by immunoelectrophoresis with antiprotein serum, but the total amount of protein remained the same. When f_5 was purified by treatment with dimethyl sulf-

TABLE 1. *Chemical composition, LLGA, and relative amounts of each fraction obtained in the extraction and purification of S-LPS and R-LPS from B. abortus*[a]

Fraction	Heptose (%)	FA amide and FA ester (%)	Total carbohydrates (%)	KDO (%)	Protein (%)	Nucleic acids[b] (%)	LLGA (ng/ml)	Yield (%)
f_5 (crude S-LPS)	0	19	10.7	0.32	24	8	1	0.77
f_{5p} (pure S-LPS)	0	26.4	11.6	0.62	6.3	<1	0.1	0.05
f_3	0	7.7	8.4	0.26	1.7	80	500	0.4
R_1	ND[c]	ND	ND	0.49	3	ND	ND	0.007
R_2	ND	ND	ND	0	45	ND	ND	0.008
R_3 (crude R-LPS)	0[d]	10	13.6	0.25	1.3	82	10	0.4
R_{3a} (pure R-LPS)	0	27	6.5	0.74	1.5	<1	1[e]	0.04
R_{3b}	0	1.6	15.1	0	4.8	60	10,000	0.06
R_5	0	16.6	5.5	0.15	25	10	500	0.08
R_{5a}	0	18	9.3	0	10	<1	ND	0.04
R_{5b}	0	18	10	0.52	2.4	6	50	0.03
E. coli LPS, O128:B12	3.1	43	18.4	1.7	4.4	4	0.2	

[a] The numbers represent percent of total dry weight of fraction. Yield is percent of dry weight of cells.
[b] Estimate of nucleic acid content on basis of 280/260 ratio (25).
[c] ND, Not determined.
[d] Interference in the quantitative analysis.
[e] As R_{3a}-BSA.

oxide and NaI, followed by gel filtration, the amount of protein decreased by 74% and the nucleic acids almost disappeared, whereas the lipids increased by 39%, KDO increased by 94%, and total carbohydrates remained about the same. The LLGA increased 10-fold (Table 1).

Three separate batches of R-LPS were extracted from *B. abortus* strain 45/20, and in each batch R_3 contained the R-LPS as shown by immunoelectrophoresis, and analysis for LLGA, KDO, and lipid. The data shown in Table 1 represent the analysis of four fractions (R_1, R_2, R_3, R_5) obtained from a single batch. The quantities of R_1 and R_2 obtained were too small to permit analysis for all components. All fractions except R_2 contained KDO. Fraction R_3 was centrifuged at 106,000 × *g* for 6 h, and a pellet was not obtained. Fraction R_5 was insoluble. The purification of R_3 and R_5 (Fig. 2) resulted in four additional fractions, designated R_{3a}, R_{3b}, R_{5a}, and R_{5b}. When R_{3a} was compared with crude R_3 (Table 1), it contained approximately three times more lipid and KDO and less than half the amount of carbohydrate. There was little change in the protein content, but the nucleic acids decreased to less than 1%. The LLGA of R_{3a} was 10 times greater than that of R_3; however, it was only one-tenth as potent as f_5. Because of these characteristics, R_{3a} was considered to be composed mainly of R-LPS.

Heptose was not detected in any of the fractions analyzed. To establish the possible presence of interfering components, a known amount of D-glycero-mannoheptose or of *E. coli* LPS was mixed with a known amount of each of the different fractions. Only R_3 but not R_{3a} was found to interfere with the quantitative determination of heptose.

Fatty acid analysis. Table 2 shows the GLC lipid analysis of S-LPS (f_5 and f_{5p}) and R-LPS (R_3 and R_{3a}) fractions. All four fractions contained the same seven major FA, which account for an average of 85% of the total FA. Several other FA were also detected, but no single one of these accounted for more than 2% of the total, although when added together, they accounted for about 15% of the total FA. These minor FA were observed consistently among different preparations.

Two of the major FA were identified as methyl palmitate and methyl stearate (equivalent chain length [ECL] values of 16.0 and 18.0, respectively). Neither was affected by hydrogenation or trimethylsilyl derivatizing procedures, and they comigrated with methyl palmitate and methyl stearate standards respectively on GLC columns of different polarity.

Only one of the major species (ECL 18.5) exhibited a change in retention behavior after hydrogenation, indicating the presence of one or more unsaturations. Untreated, it migrated in the vicinity of methyl oleate. When hydrogenated, it did not comigrate with methyl stearate, leaving its identity unknown. The peak occurring at ECL 21.9 was the only trimethylsilyl-sensitive species of the seven major FA, suggesting that it was a hydroxylated methyl ester. This FA was distinct from β-OH-myristic acid and β-OH-palmitic acid.

TABLE 2. *Relative amounts of fatty acids in S-LPS (f_5 and f_{5p}) and R-LPS (R_3 and R_{3a}) as determined by GLC*

ECL	LPS fractions				FA
	S-LPS		R-LPS		
	f_5	f_{5p}	R_3	R_{3a}	
16.0	36.7[a]	52.0	44.1	58.8	Palmitic acid
18.0	12.2	6.7	10.0	12.0	Stearic acid
18.5	8.8	9.0	6.0	6.6	Unsaturated unknown
19.3	13.6	2.8	5.8	5.7	Saturated unknown
20.8	9.9	1.3	4.5	3.3	Saturated unknown
21.9	4.3	5.7	4.9	2.7	Hydroxylated unknown
23.0	tr	6.1	1.1	1.9	Saturated unknown
Others	11.9 (12)[b]	14.3 (15)	17.8 (13)	8.3 (13)	

[a] Numbers are percentage of total fatty acids.
[b] Numbers in parentheses represent number of peaks.

LPS from *E. coli* O128:B12 was used as control for the GLC analysis. This preparation consistently presented the four major FA (lauric, myristic, palmitic, and β-OH-myristic) reported in the literature for *E. coli* LPS (4, 34). Figure 3 shows representative chromatograms of S-LPS from *E. coli* O128:B12 and smooth *B. abortus* strain 11-19.

The S-LPS (f_5 and f_{5p}) and R-LPS (R_3 and R_{3a}) fractions did not reveal any non-covalently bound neutral lipid or phospholipid when tested by thin-layer chromatography.

Immunoelectrophoretic analysis. When tested in immunoelectrophoresis against serum of a rabbit hyperimmunized with *B. abortus* 45/20 protein antigens (Fig. 4), f_5 (well B) showed several precipitin lines, whereas after purification there were no lines even at a twofold concentration of f_{5p} (well A). When tested against serum of a cow infected with smooth *B. abortus*, both f_5 and f_{5p} showed the characteristic precipitin line of S-LPS (31). Fraction 5 showed an additional precipitin line which is believed to be composed of a polysaccharide antibody complex (6).

All fractions were tested against sera of rabbits immunized with the rough antigens. Only R_3 and

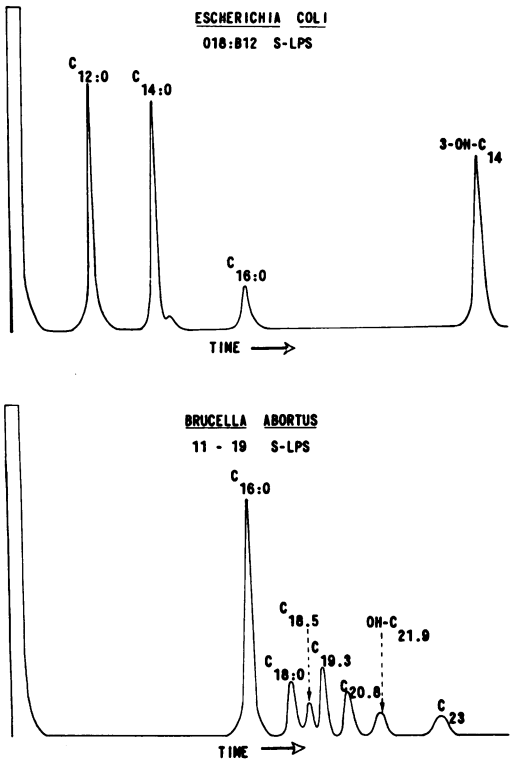

FIG. 3. *Comparison of gas-liquid chromatograms on 10% SP-2330 100/120 Chromosorb W of S-LPS from E. coli O128:B12 and smooth B. abortus strain 11-19.*

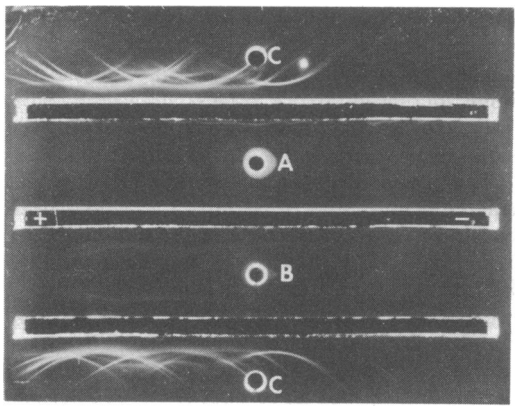

FIG. 4. *Immunoelectrophoretic analysis in 0.8% agarose. (A) 20 mg of f_{5p} per ml, (B) 10 mg of f_5 per ml, and (C) 10 mg of protein antigen from B. abortus strain 45/20 per ml were tested against serum from a rabbit immunized with protein antigen from B. abortus strain 45/20.*

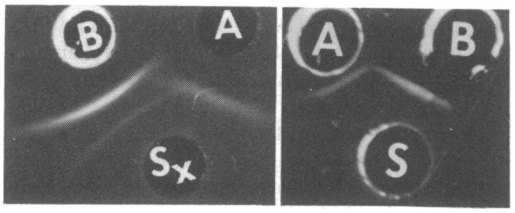

FIG. 5. *Immunodiffusion in 0.8% agarose. (A) 10 mg of R_{3a}-BSA per ml and (B) 10 mg of B. ovis crude R-LPS per ml were tested against serum from a rabbit immunized with B. ovis crude R-LPS antigen (S) and against serum from rabbit immunized with B. abortus 45/20 whole cells (Sx).*

R_{3a}-BSA showed the precipitation line characteristic of the R-LPS antigens (5).

Immunodiffusion. Two patterns were observed when fraction R_{3a}-BSA and crude *B. ovis* R-LPS were examined by immunodiffusion. Figure 5 shows a reaction of identity between fraction R_{3a}-BSA and crude *B. ovis* R-LPS tested against serum from a rabbit immunized with crude *B. ovis* R-LPS antigen. However, when these antigens were tested against sera from rabbits hyperimmunized with *B. abortus* 45/20 whole cells, two lines of precipitation, one with partial identity and the other with no identity, appeared on the side of crude *B. ovis* R-LPS.

SDS-PAGE. SDS-PAGE patterns of f_5, f_{5p}, R_3, R_{3a} from *B. abortus* and LPS from *E. coli* stained for carbohydrates, protein, and lipid are shown in Fig. 6. When stained for carbohydrates, five diffuse bands with different electrophoretic mobilities were observed in *E. coli* LPS (F, G, H), and there was only a single diffuse band with the two smooth *B. abortus* fractions (C, D, E, and I). Both f_5 and f_{5p} showed similar diffuse bands when stained for carbohydrates, lipid, or protein; however, f_5 (C) was stained more strongly by Coomassie blue than f_{5p} (I) and showed additional bands in the middle and lower parts of the gel. The band in the bottom of the gel charged with f_5 also stained for lipids but not for carbohydrates. This lipid band disappeared after the purification of f_5 to f_{5p}.

The migration of S-LPS molecules in the gel depended on the acrylamide concentration. Fractions R_3 and R_{3a} migrated to the bottom of the gel in both 10 and 15% acrylamide gels. This band stained for carbohydrates and lipids but not for proteins.

Gel filtration chromatography. When f_5 was chromatographed in Sephadex G-200 in the presence of Tween 40 and NaI, only the fraction excluded in the void volume contained both KDO and protein.

DISCUSSION

In this work, R-LPS from *B. abortus* strain 45/20 has been isolated and characterized for

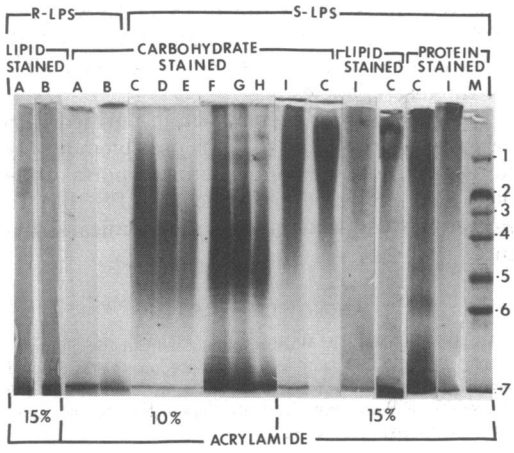

FIG. 6. *Scale reconstructed patterns of fractions f_5, f_{5p}, R_3, R_{3a}, and E. coli LPS in SDS-PAGE. The gel concentrations, marker proteins, and staining procedure are indicated in the figure. Concentrations and identification of fractions are as follows: R_{3a}, 0.3 mg (A); R_3, 0.3 mg (B); f_5, 0.6 mg (C); f_5, 0.4 mg (D); f_5, 0.2 mg (E); E. coli LPS, 0.6 mg (F); E. coli LPS, 0.4 mg (G); E. coli LPS, 0.2 mg (H); f_{5p}, 0.6 mg (I); and marker proteins, 5 μg of each one (M). Molecular weights were as follows: phosphorylase, 94,000 (1); BSA, 68,000 (2); catalase, 60,000 (3); fumarase, 49,000 (4); aldolase, 40,000 (5); carbonic anhydrase, 29,000 (6); and hemoglobin, 16,000 (7).*

the first time. The method of extraction consisted of three consecutive steps: a preliminary extraction of the readily soluble components with 2.5% NaCl solution, a second extraction with chloroform–petroleum ether–phenol, and a final extraction with phenol–water. From the crude fractions analyzed, only fraction R_3 had all the characteristics of R-LPS. This fraction (R_3) contained less than 1.5% protein but relatively large quantities of nucleic acids that may contribute to its preferential partitioning into the aqueous phase when extracted with phenol–water. This hypothesis is supported by the fact that removal of nucleic acids during purification resulted in an insoluble product which was composed of R-LPS (R_{3a}) that was solubilized by complexing with BSA (R_{3a}-BSA). This extraction method also has been succesfully employed for the extraction of R-LPS from rough strain *B. melitensis* B115 and from *B. canis* (unpublished data). Due to the small amounts of fractions R_1 and R_2 obtained, we decided to omit the second extraction step with chloroform–petroleum ether–phenol in later attempts to isolate R-LPS from rough brucellae. The omission of this step did not alter the composition or yield of fraction R_3 or subsequent fraction R_{3a} to any significant extent. The method differs from the extraction with hot saline which we previously used to obtain R-LPS from *B. ovis*, but which did not extract R-LPS from *B. melitensis* B115, *B. abortus* 45/20, or *B. canis* (22).

Consistent with the previous reports, the crude endotoxin (f_5) was obtained in the phenol phase from smooth brucella extracted by the Redfearn method. This is not a unique characteristic of brucella S-LPS, since LPS from other bacteria have also been isolated in this phase (7, 16, 17, 40).

Fraction 3 (f_3) obtained in the aqueous phase has been studied before. Hurvell and Lindberg (19) confirmed Redfearn's observation that this fraction had antigenic similarities with the crude S-LPS (f_5), which they attributed to the carbohydrate moiety. There are several reports on the presence of small amounts of lipid (1, 27, 30) and variable amounts of KDO (18, 27, 30, 41) but no one to date has demonstrated significant endotoxic activity in f_3 (1, 18, 22, 30, 41). We also found a relatively low content of lipid and KDO and very little LLGA.

In attempting to explain the preferential partitioning of the brucella S-LPS into the phenol phase, we previously proposed that it might be bound covalently to a hydrophobic protein (21). In the present work, we found a protein (or proteins) tightly bound to the purified S-LPS. This protein could not be removed from the S-LPS by gel filtration in the presence of detergent and the chaotropic agent NaI, by ultracentrifugation, or in SDS-PAGE after boiling in the presence of 2-mercaptoethanol (diffuse protein band I, Fig. 6). Even though these experiments are not absolute proof of a covalent linkage between the protein and S-LPS, they strongly suggest it. In addition to this tightly bound protein, the crude S-LPS contained several noncovalently bound proteins demonstrable by immunoelectrophoresis and by SDS-PAGE. These could be dissociated by the action of the chaotropic agent NaI and then removed by the methanol precipitation steps, followed by gel filtration in the presence of detergent and NaI.

In contrast to the S-LPS, we could not demonstrate a protein bound to the purified R-LPS. Whether the R-LPS inserted in the outer membrane of the brucella cell lacks this protein, or whether the method of extraction used removes this protein from the R-LPS cannot be determined at the present time.

Our observation that *B. abortus* S-LPS migrates in SDS-PAGE depending on the gel concentration is consistent with the report of Russel and Johnson (42). They examined the effect of acrylamide concentration on the migration of neisseria LPS and showed that the basis of the

separation was molecular size and not intrinsic charge. Jann et al. (20) suggested that the association of SDS with LPS was most probably through the lipid A moiety of LPS since it is the hydrophobic region of the amphipathic molecule.

The antigenic specificity of R-LPS from *B. abortus* and *B. ovis* was compared by immunodiffusion, and the result varied depending on the immune serum used. With serum prepared against crude *B. ovis* R-LPS, the two preparations showed identity; however, when anti-*B. abortus* 45/20 whole cell serum was used, crude *B. ovis* R-LPS showed a precipitin line and a spur that was absent with *B. abortus* R-LPS. The apparent contradiction can be explained by the fact that the hot saline extraction method used to isolate R-LPS gives a preparation highly contaminated with proteins that cannot be removed totally with Pronase treatment (24). In contrast, the R-LPS from *B. abortus* obtained by the extraction procedure described here gives a preparation almost devoid of proteins. Since the antiserum produced against *B. abortus* 45/20 whole cells has antibodies against other components in addition to the R-LPS, it is not surprising to find precipitin lines corresponding to proteins of crude *B. ovis* R-LPS preparations.

There are conflicting reports on the presence of heptose in brucella S-LPS. Although some investigators (26–28, 41) reported heptose, Hurvell and Lindberg (19) did not detect it on analysis by GLC of S-LPS (f_5) extracted by the Redfearn method. We also could not detect this sugar in the S-LPS and R-LPS of *B. abortus*. Moreover, analysis of R-LPS from *B. melitensis* and *B. canis* (unpublished data) did not reveal heptose. The absence of heptose is not unique to brucella LPS, since other bacterial lipopolysaccharides also lack this sugar (32, 43).

The FA composition of *B. abortus* LPS was markedly different from that of enterobacterial LPS. *B. abortus* LPS does not contain lauric or myristic acid or large amounts of hydroxylated FA. Palmitic acid ($C_{16:0}$), stearic acid ($C_{18:0}$), an unsaturated FA (ECL of 18.5), and relatively small amounts of an unknown hydroxylated FA (ECL of 21.9) were identified.

Our findings of the FA composition in LPS from *B. abortus* differ considerably from those reported by Lacave et al. for crude extracts of *B. melitensis* (27, 28) and Marx et al. for *B. abortus* (35). The overall FA composition has been reported to be very similar in both *B. abortus* and *B. melitensis* (48). Moreover, with a few exceptions (14), intraspecies LPS lipid composition for various bacteria has been shown to be relatively constant (11, 34). Therefore, we do not believe that the difference in species can account for the reported disparity in the brucella LPS lipids. Differences in extraction method, fractions studied, and method of analysis could be responsible for the discrepancies.

The methods for preparation of purified LPS described here should make it possible not only to resolve the often contradictory reports of the biological properties of brucella LPS, but to relate these properties to specific chemical structure.

ACKNOWLEDGMENTS

We thank Bambi L. Wilson for her help in the chemical assays, David L. Nelson for his advice on the fatty acid analysis, Mary Hayes for her technical assistance, and the University of Wisconsin Graduate School for the gas-liquid chromatography apparatus.

This investigation was supported by the College of Agricultural and Life Sciences, University of Wisconsin, by Cooperative Agreements with the United States Department of Agriculture, Animal and Plant Health Inspection Service, and the Science and Educational Administration, and by a grant from the Consejo Nacional de Investigaciones Cientificas y Tecnologicas (CONICIT) de Costa Rica.

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