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Effects of Nonionic, Ionic, and Dipolar Ionic Detergents and EDTA on the *Brucella* Cell Envelope

IGNACIO MORIYON AND DAVID T. BERMAN*

Department of Bacteriology and Department of Veterinary Science, University of Wisconsin—Madison, Madison, Wisconsin 53706*

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Cell envelopes prepared from smooth and rough strains of *Brucella* were characterized on the basis of lipopolysaccharide and protein content. The action of three kinds of detergents on *Brucella* cell envelopes and *Escherichia coli* control cell envelopes was examined on the basis of the proteins and lipopolysaccharides that were extracted. As compared with those of *E. coli*, *Brucella* cell envelopes were resistant to nonionic detergents. Zwittergents 312 and 316 were most effective in extracting *E. coli* cell envelopes, and Zwittergent 316 was most effective in extracting *Brucella* cell envelopes. Sarkosyl extracted proteins but extracted only trace amounts of lipopolysaccharides from cell envelopes of both bacteria. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the Sarkosyl-resistant proteins revealed a composition similar to that of the proteins exposed on the surfaces of viable cells, as determined by the lactoperoxidase-^{125}I radioiodination method. EDTA, with either Tris-HCl or Tris-HCl–Triton X-100, did not have detectable effects on *Brucella* cell envelopes. Ultracentrifugation of purified lipopolysaccharides in detergents and EDTA demonstrated that, in contrast to that of *E. coli*, *Brucella* lipopolysaccharide was not stabilized by divalent cations. Sarkosyl was ineffective in dispersing lipopolysaccharides, whereas the action of Zwittergents was related to the length of their alkyl chains.

The cell envelopes of gram-negative bacteria are composed of two membranes separated by a periplasmic space and a rigid peptidoglycan layer. In contrast to the inner cytoplasmic membrane, the outer membrane contains a characteristic lipopolysaccharide (LPS), and its protein content is distributed among relatively few major proteins (40). The study of outer membrane proteins by general methods developed through work with *Enterobacteriaceae* and applied in investigations of a wide range of other gram-negative bacteria involves either the physical separation of inner and outer membranes (39) or their sequential extraction with different detergents (13, 42). With each of these general methods, the outer membrane is usually protected with 5 mM Ca^{2+} or Mg^{2+} or, conversely, is destabilized with EDTA (5, 7, 8, 17, 19, 21, 22, 25, 27, 29, 32, 35, 43, 48).

For a first approach to the investigation of outer membranes of *Brucella* spp., we thought it necessary to determine how various detergents and conditions of extraction known to be effective with other gram-negative bacteria would apply to *Brucella* cell envelopes. We found that *Brucella* cell envelopes differed significantly from the *Escherichia coli* cell envelopes used as controls throughout the experiments with respect to their stabilization by divalent cations and susceptibility to the action of nonionic detergents. The action of Sarkosyl and dipolar ionic detergents on the outer membrane was also examined. In this study, we describe experiments designed to test the hypothesis that the differential action of various detergents on outer membranes is a function of the interaction of the detergents with the LPSs.

We also extend our preliminary finding (I. Moriyon and D. T. Berman, Abstr., Annu. Meet. Am. Soc. Microbiol. 1981, K171, p. 166) that sequential extraction of *Brucella* cell envelopes with Sarkosyl and long-chain dipolar ionic detergents yields preparations containing mostly outer membrane proteins and LPS. We report here the observation of additional outer membrane proteins not extracted by this method.

MATERIALS AND METHODS

Cell disruption and fractionation. The characteristics of smooth, attenuated *Brucella abortus* 1119-3 and rough, avirulent *B. abortus* 45/20 and *B. melitensis* B115 have been described previously (20). Cells were grown in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, MD.) dialysate in flasks on a

rotatory shaker, harvested by centrifugation at 4°C, and washed once with cold 20 mM phosphate-buffered saline. The cells were suspended in 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.5), DNase and RNase were added at 100 μg/ml, and the cells were broken by ballistic disintegration in a colloid mill (Mini-mill; Greerco Corp., Hudson, N.H.; four 5-min periods at 22,500 rpm and 0°C). Disrupted cells were diluted in 10 mM HEPES–5 mM $MgCl_2$ (pH 7.5), glass beads were removed by low-speed centrifugation, and cell envelopes were collected by centrifugation for 60 min at 80,000 × *g* at 4°C. Cell envelopes were repeatedly washed and finally stored in the same buffer at −20°C. *E. coli* K-12 W 1485 F^+ cell envelopes were prepared with a French pressure cell as described previously (8) and stored as described above.

Enzymatic and chemical analyses. Inner membrane markers succinic dehydrogenase (EC 1.3.99.1) and cytochrome *c* reductase (EC 1.6.99.3) and cytoplasmic marker malic dehydrogenase (EC 1.1.1.38) were assayed as described previously (19, 38, 45). Protein was determined by the method of Lowry et al. (28) as modified by Dulley and Grieve (11) with bovine serum albumin as the standard. The Coomassie blue G 250 binding protein assay of Bradford (2) was used for EDTA extracts. 2-keto-3-Deoxyoctulosonic acid (KDO) was used as an LPS marker and determined by the thiobarbituric acid method of Warren (49), with KDO as the standard after extraction of the chromogen with cyclohexanone as described by Osborn et al. (38). 2-Deoxyribose standards were included to correct for interference by 2-deoxysugars (49). Samples and standards were read at 536 nm (absorbance maximum of 2-deoxysugars) and at 552 nm (absorbance maximum of KDO) in a Gilford 252 spectrophotometer. *Brucella* LPS was estimated from the KDO values with the previously reported values of KDO in the LPSs of the three strains used (34).

Detergent and EDTA extractions. Cell envelopes from storage buffer were packed by centrifugation, washed once with distilled water, and suspended in detergent solutions in 10 mM Tris-hydrochloride (pH 8.5) at a concentration of 10 mg of cell envelope protein per ml. When specified, the extracting detergent-buffer solution contained 5 mM EDTA. Detergents were used at concentrations well above their critical micellar concentrations (16, 18). Cell envelopes were extracted for 60 min at room temperature with stirring and then pelleted by centrifugation for 30 min at 50,000 × *g*. The supernatant fluids were collected, and the pellet was reextracted as above. When cell envelopes were extracted sequentially with two different detergents, pellets were washed twice with distilled water before being extracted with the second detergent. For EDTA extraction, cell envelopes were suspended in 10 mM EDTA–10 mM Tris-hydrochloride (pH 7.5) at 5 mg of total cell envelope protein per ml. After being incubated for 15 min at room temperature, the extracted cell envelopes were pelleted by centrifugation for 30 min at 20,000 × *g*, and the supernatant fluids were collected.

LPS preparations and LPS centrifugation experiments. *B. abortus* 1119-3 S-LPS was extracted with phenol-water and purified with chaotropic salts and detergents as described by Moreno et al. (34). *E. coli* O128:B12 Westphal S-LPS was purchased from Difco Laboratories, Detroit, Mich. Both LPSs contained about 5% protein. In LPS centrifugation experiments, 5 to 10 mg of LPS was suspended in 4 ml of distilled water and dispersed by sonication (six 5-s bursts at 8 W with a Kontes microultrasonic apparatus). To this LPS suspension, 1 ml of a detergent solution was added to give final detergent concentrations of 1% Triton X-100, 0.5% Zwittergents 308 to 316, and 0.5% Sarkosyl. When EDTA was used, its final concentration was 5 mM. Controls contained distilled water and LPS, but no detergent. After being incubated for 15 min at room temperature, the mixture was centrifuged for 5 to 6 h at 100,000 × *g* at 15°C in a Beckman SW50.1 rotor. LPS sedimentation was followed by measuring the KDO concentration in the supernatant fluid.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method of Laemmli (24) in 12.5% polyacrylamide slabs, and proteins were stained as described by Fairbanks et al. (12). Molecular weight (MW) markers were phosphorylase *b* (MW, 94,000 [94K]), bovine serum albumin (MW, 67K), aldolase (MW, 40K), carbonic anhydrase (MW, 29K), hemoglobin (MW, 4 × 16K), and cytochrome *c* (MW, 12K). For SDS-PAGE, proteins in Zwittergent extracts were precipitated with 5 volumes of cold acetone and dissolved in a mixture, designated SDS-βM, containing 10 mM Tris-hydrochloride (pH 7.5), 2% SDS, 0.7 M 2-β-mercaptoethanol, and 10% glycerol.

Extrinsic radioiodination of live cells. Extrinsic ^{125}I labeling was performed by the method of Marchalonis (30). The reaction mixture (200 μl of phosphate-buffered saline [pH 7.2]) contained 10^8 to 10^{10} mid-log phase cells, 500 μCi of carrier-free $Na^{125}I$ (New England Nuclear Corp., Boston, Mass.), 5 μg of lactoperoxidase, and 8×10^{-5} M H_2O_2. The reaction was stopped after 30 min with 10 μl of 1 M 2-β-mercaptoethanol, and four times the number of cold cells in 20 mM NaI–phosphate-buffered saline were added. After repeated washings with the same solution, the cells were extracted with SDS-βM for 5 min at 100°C and centrifuged, and the supernatants were analyzed by SDS-PAGE. Coomassie blue-stained, dried gels were autoradiographed on Kodak XAR-5 film.

Reagents. Nonionic detergents, Sarkosyl, 2-deoxyribose, KDO, lactoperoxidase, and SDS-PAGE standards, as well as the reagents used in the enzymatic analysis, were from Sigma Chemical Co., St. Louis, Mo. Zwittergents were from Calbiochem, La Jolla, Calif., and SDS-PAGE reagents were from BioRad Laboratories, Richmond, Calif.

RESULTS

Cell envelope characterization. The *Brucella* cell envelopes obtained as described above had a typical gram-negative structure as judged by electron microscopy of thin sections (data not shown). They contained 97 to 99% of the cytochrome *c* reductase and succinic dehydrogenase activities but no malic dehydrogenase activity. The LPS/protein ratios of the cell envelopes were found to be 0.35 for *B. melitensis* B115, 0.26 for *B. abortus* 45/20, and 0.58 for *B. abortus*

TABLE 1. Protein extracted by detergents and EDTA from *E. coli* and *Brucella* cell envelopes[a]

Treatment	HLBN[b]	Percentage of total cell envelope protein extracted from:			
		E. coli	*B. melitensis* B115	*B. abortus* 45/20	*B. abortus* 1119-3
Triton ×-45–5 mM EDTA	10.4	36	11.5	16	
Triton ×-100–5 mM EDTA	13.5	75	16.5	17	15
Triton ×-100	13.5	41	12.5	15.5	16.3
Triton ×-165–5 mM EDTA	15.8	31.5	16	15	
Tween 20–5 mM EDTA	16.7	16	8.5	6.5	
Zwittergent 308		12	8	10.5	
Zwittergent 312		51	25	35	
Zwittergent 316		58	56	60	45
Sarkosyl		49	55	57	50
10 mM EDTA		9.2	<1	<1	<1

[a] Cell envelopes were extracted in 4% (nonionic) and 1% (dipolar ionic and Sarkosyl) detergent concentrations.

[b] HLBN, Hydrophilic-lipophilic balance numbers (see reference 18).

1119-3. This is in good agreement with the LPS/protein ratios reported for cell envelopes of deep rough (0.20) and smooth (0.66) variants of *Salmonella typhimurium* (38).

Action of detergents and EDTA. Cell envelopes of all three *Brucella* strains were, as compared with the *E. coli* cell envelope control, resistant to the action of nonionic detergents (Tables 1 and 2). Likewise, EDTA had no detectable effect on *Brucella* cell envelopes, but it removed both LPS and protein from *E. coli* cell envelopes. Moreover, EDTA in combination with Triton X-100 increased the amount of protein and LPS extracted by this detergent from *E. coli* cell envelopes but not that extracted from any of the *Brucella* cell envelope preparations (Tables 1 and 2). The effectiveness of the dipolar ionic detergents was related to the length of their respective alkyl chains; best results were obtained with Zwittergents 312 and 316 for *E. coli* cell envelopes and with Zwittergent 316 for *Brucella* cell envelopes. The ionic detergent Sarkosyl extracted about 50% of the cell envelope proteins of both *E. coli* and *Brucella* spp., but in contrast with Zwittergents, little LPS was solubilized.

Dispersal of LPS by detergents and EDTA. LPSs are amphiphilic molecules that form large micelles in aqueous solutions that can be sedimented by ultracentrifugation (44). We took advantage of these properties to study the action of detergents and EDTA on LPS, since dispersion of the micelles should render the LPS nonsedimentable. Figure 1 shows that, either with or without detergent, the amount of LPS remaining in the supernatant fluids reached an equilibrium after 5 to 6 h of centrifugation. It also shows that detergents and EDTA influenced the amount of LPS that could be sedimented by centrifugation. These experiments established

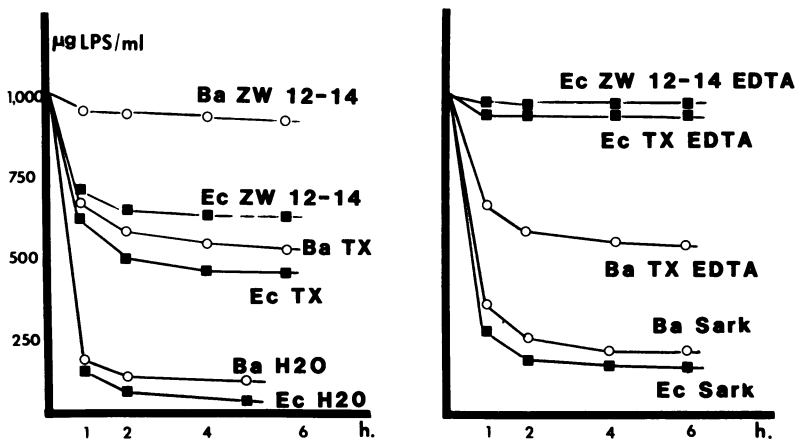

FIG. 1. Effect of detergents and EDTA on the sedimentation by centrifugation of *B. abortus* (Ba) and *E. coli* (Ec) S-LPSs. The numbers on the abscissa represent the concentration of LPS in supernatants; those on the ordinate represent the time of centrifugation. ZW 12-14, Zwittergents 312 and 314; TX, Triton X-100; Sark, Sarkosyl; and H_2O, controls without detergent.

TABLE 2. KDO extracted by detergents and EDTA from *E. coli* and *Brucella* cell envelopes[a]

Treatment	Percentage of total cell envelope KDO extracted from:			
	E. coli	*B. melitensis* B115	*B. abortus* 45/20	*B. abortus* 1119-3
Triton ×-100	10	19.5	13.5	16.3
Triton ×-100–5 mM EDTA	69	18	11.5	16
Zwittergent 316	31	43	40	36.5
Sarkosyl	5.5	10.5	10	9
10 mM EDTA	8.5	<1	<1	<1

[a] Concentrations are the same as in Table 1.

through two lines of evidence that, in contrast with *E. coli* LPS, *Brucella* LPS was not stabilized by divalent cations that could be chelated by EDTA. First, the amount of LPS dispersed by Triton X-100 with and without EDTA was essentially the same for *B. abortus* (40 to 60%) but very different for *E. coli* (40% versus 90 to 97%, respectively). Second, Zwittergents 312 and 314 dispersed >90% of *Brucella* LPS but only about 60% of *E. coli* LPS when EDTA was not present. The addition of EDTA increased the amount of dispersed *E. coli* LPS to >90%. On the other hand, Zwittergents 308 and 310 (data not shown) and Sarkosyl were minimally effective in dispersing *E. coli* and *B. abortus* LPSs.

Sequential detergent extractions and extrinsic ^{125}I labeling. Filip et al. (13) demonstrated that Sarkosyl preferentially extracted the inner membranes of *E. coli*. This effect is not, however, absolutely specific (3). The results presented above suggested that, in contrast to Triton X-100 and Triton X-100–EDTA, Sarkosyl might also have a preferential action on *Brucella* cell envelopes. As an independent test of this hypothesis, we compared the protein composition of the Sarkosyl-resistant fraction of cell envelopes with the proteins accessible to extrinsic labeling in intact cells. As can be seen in Fig. 2, most of the Sarkosyl-resistant cell envelope proteins subsequently extracted with SDS-βM at 100°C had their counterparts in SDS-β-M (100°C) extracts of extrinsically labeled cells, as examined by autoradiography. SDS-PAGE analysis (data not shown) of the Sarkosyl-extractable fractions of both *E. coli* and *B. abortus* cell envelopes showed them to contain a large number of proteins, among which the 88K, 68K, and 40K proteins and other proteins were noteworthy by their absence. These results, along with the protein/KDO ratios (Tables 1 and 2) in Sarkosyl extracts, support the proposition that Sarkosyl has a preferential, but not completely selective, action on the inner membranes of *Brucella* cell envelopes.

Zwittergent 316 extracted both protein and LPS efficiently, as well as some of the Sarkosyl-resistant cell envelope proteins (Fig. 2). Its action was not, however, as complete as the extraction with SDS-βM at 100°C, and only protein 88K and the groups of proteins at about 38K and 30K were extracted by Zwittergent 316. Particularly conspicuous was the almost total lack of proteins with MWs of <29K, of which several were present in SDS-βM (100°C) extracts of both Sarkosyl-extracted cell envelopes and ^{125}I-lactoperoxidase-labeled cells. Comparison of the Sarkosyl-resistant cell envelope proteins of *E. coli* extracted by SDS-βM at 100°C and by Zwittergent 316 also led to the conclusion that Zwittergent 316 has a limited action on these membranes as well. These *E. coli* extracts lacked several proteins, most notably the OmpA protein.

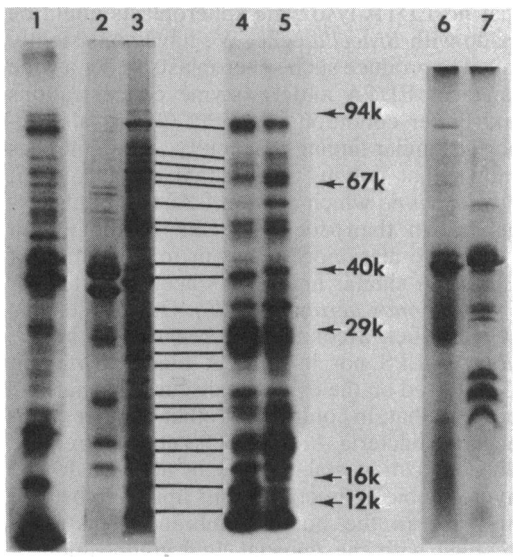

FIG. 2. SDS-PAGE analysis of Sarkosyl-resistant cell envelope proteins and extrinsically ^{125}I-lactoperoxidase-labeled surface proteins. Lane 1, autoradiograph of 100°C SDS extracts of ^{125}I-labeled *E. coli*; lanes 2 and 3, Coomassie blue-stained, 100°C SDS-extracted, Sarkosyl-resistant proteins of *E. coli* and *B. abortus* 45/20, respectively; lanes 4 and 5, autoradiograph of ^{125}I-labeled *B. abortus* 45/20 and *B. abortus* 1119-3, respectively; lanes 6 and 7, Coomassie blue-stained, Zwittergent 316-extracted, Sarkosyl-resistant proteins of *B. abortus* and *E. coli*, respectively.

DISCUSSION

In *E. coli* and other-gram negative bacteria (5, 7, 14, 19, 21–23, 31, 32, 35, 43, 48), divalent cations stabilize the outer membrane by interacting with the hydrophilic moiety of the LPS (41). Thus, treatment with EDTA releases significant amounts of protein and LPS from the outer membrane and allows the preparation of EDTA-lysozyme spheroplasts (26, 38). The selective solubilization of the inner membrane components in the presence of Mg^{2+} with Triton X-100 has a similar basis (42). We have found that EDTA, in combination with Tris (15), does not affect the stability of *Brucella* cell envelopes or enhance the action of Triton X-100 on them. That this difference between *Brucella* spp. and *E. coli* is related to differences in their LPSs was shown by centrifugation of LPS in dipolar ionic detergents and Triton X-100 in the presence and absence of EDTA. Schindler and Osborn (41) demonstrated that the high-affinity Ca^{2+}-binding sites of *S. typhimurium* LPS are the KDO residues of the molecule. Moreno et al. (34) found that the LPS of *B. abortus* 1119-3 contains 2.7 times less KDO than does the *E. coli* O128:B12 LPS used in our work. Obviously, this might result in significantly less binding of divalent cations to *Brucella* LPS. We have also tested some of the implications of these findings, i.e., that no EDTA-lysozyme spheroplasts could be made with *Brucella* cells. We have consistently failed to produce such spheroplasts under a wide range of EDTA and lysozyme concentrations and buffer conditions. Dubray (9) has also reported similar findings. Recently, Vaara (46) has shown that polymyxin-resistant mutants of *S. typhimurium*, which contain LPS that binds less polymyxin than does the LPS of the parent strain, are also more resistant to the action of EDTA. A similar finding has been reported for *Pseudomonas aeruginosa* (36). Our results also suggest such a connection, because neither *B. abortus* LPS nor its lipid A binds polymyxin (33). Based on the evidence discussed so far, we propose that, in contrast with that of other gram-negative bacteria, *Brucella* LPS does not require divalent cation stabilization in addition to the hydrophobic interactions of its lipid A to be held in place in the outer membrane. This is in keeping with the unusual lipid composition of this LPS that, in contrast to *E. coli* LPS, does not contain β-hydroxymyristic acid or fatty acids of less than 16 carbons and has large amounts of stearic and palmitic acids, as well as others of more than 18 carbons (34).

Nonionic detergents were much less effective in extracting *Brucella* cell envelopes than *E. coli* cell envelopes. Results obtained with liposomes (18) suggest that the effectiveness of nonionic detergents is determined by their ability to disperse the membrane lipid. Values ranging from 63 to 89% fatty acids of 18 carbons or longer have been reported for *Brucella* cell envelopes (1, 6) and, to the best of our knowledge, these high percentages of long-chain fatty acids are unusual among gram-negative bacteria. It is possible that the greater hydrophobicity of the *Brucella* cell envelopes compared with those of *E. coli* accounts for their resistance to nonionic detergents. There were no significant differences between the amounts of protein solubilized from *E. coli* cell envelopes by Zwittergents 312 and 316 (alkyl chains of 12 and 16 carbons, respectively), but with *Brucella* cell envelopes, Zwittergent 316 extracted about twice as much protein as did Zwittergent 312. These data are also consistent with the hypothesis that the *Brucella* cell envelope is more hydrophobic than that of *E. coli*. The fact that nonionic detergents with increasing hydrophilic-lipophilic balance numbers are not more effective than Triton X-100 is not in disagreement with this interpretation. Studies done with membranes of different origins have shown that all of the effective nonionic detergents have hydrophilic-lipophilic balance numbers in the range 12.5 to 14.5 (4, 18). On the basis of experiments which showed that LPS from *Proteus mirabilis* or *Salmonella minnesota* protected phospholipid vesicles from disruption by detergents, Nixdorff et al. (37) suggested that LPS is involved in the protection of the outer membrane from detergents. It is likely that *Brucella* LPS plays such a role in the resistance of *Brucella* cell envelopes to nonionic detergents since, in contrast to *E. coli* LPS, Triton X-100 in the presence of EDTA was not able to disperse more than 60% of *Brucella* LPS. Moreover, only the long-alkyl-chain dipolar ionic detergents which were effective in dispersing *Brucella* LPS were also effective in extracting cell envelope proteins.

The properties of *Brucella* cell envelopes discussed above are germane to the problem of isolation of their outer membrane proteins. Insensitivity to EDTA and resistance to nonionic detergents preclude the use of EDTA and nonionic detergents either to obtain spheroplasts or to apply the Schnaitman method (42, 43) to *Brucella* spp. However, we found that, as with *E. coli* cell envelopes, Sarkosyl solubilized large amounts of protein without removing similar proportions of LPS or the proteins exposed on the outer surface. The inability of Sarkosyl to disperse isolated LPSs suggests that this may also be the basis for its inability to extract outer membrane proteins from cell envelopes of various gram-negative bacteria. Moreover, Zwittergents 308 and 310 (alkyl chains of 8 and 10 carbons, respectively) were, as was Sarkosyl (11-carbon-long chain outside its polar head),

not effective in dispersing *B. abortus* or *E. coli* LPSs. Since Zwittergents 312 and 314 were 95% efficient, this suggests that a minimal length of 12 carbons in the alkyl chain is necessary for dispersing the LPSs.

Dubray and Bezard (10) have recently isolated three proteins with MWs of 38K, 30K, and 13K from SDS-prepared peptidoglycan sacculi of *B. abortus* 45/20 that were protective when used as antigens in a mouse model for brucellosis (10). Since Zwittergents are not strong denaturing agents, the Sarkosyl-Zwittergent extraction should offer a better alternative for studying the biological activity of the 38K and 30K proteins.

Recently, Verstreate et al. (47) reported enrichment of the outer membrane fractions of *B. abortus* cell envelopes by density gradient centrifugation, as shown by an increase of the KDO/protein ratio. When either unfractionated or enriched cell envelope fractions were extracted with Sarkosyl or Triton X-100, digested with lysozyme, and reextracted with Triton X-100–EDTA or Zwittergent 314, the final extracts contained three principle clusters of proteins when analyzed by SDS-PAGE. The differences in the action of Triton X-100–EDTA observed by us and by Verstreate et al. (47) may be attributable to the fact that, presumably due to Formalin inactivation of the cells (47), Verstreate et al. had to resort to lysozyme digestion of the cell envelopes. Our data also show conclusively that there are additional proteins accessible to extrinsic labeling in the Sarkosyl-resistant fraction that are not extracted by Zwittergents and that the outer membrane protein profile of *B. abortus* is more complex than that of *E. coli*.

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