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Identification and Extraction of *Pasteurella haemolytica* Membrane Proteins†

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The inner and outer membranes of *Pasteurella haemolytica* were separated by sucrose density gradient centrifugation after plasmolysis of the cells in 20% sucrose and fragmentation in a French pressure cell. Assays of the two membrane fractions for 2-keto-3-deoxyoctonate, succinate dehydrogenase, and NADH dehydrogenase and by sodium dodecyl sulfate-polyacrylamide gel electrophoresis indicated that each of the two membrane fractions was purified fivefold relative to the other. The outer membrane fraction contained two major proteins of molecular weights 30,000 and 42,000 (30K and 42K proteins), and the inner membrane fraction contained five proteins in approximately equal amounts. Intact bacteria as well as membrane fractions were extracted by procedures used by others for vaccine preparation to determine whether the outer membrane proteins were released. Extraction of the isolated membranes with 0.5 M potassium thiocyanate in 0.425 M NaCl with or without EDTA or with M sodium salicylate failed to release more than traces of the outer membrane proteins. Sodium dodecyl sulfate extracted essentially all of the proteins of both membranes, but the products of this procedure were of low solubility and presumably denatured. The inner membrane proteins were extracted with 0.5% Sarkosyl in 0.02 M sodium phosphate buffer (pH 7.5). The 42K outer membrane protein, most of the lipopolysaccharide, and some of the 30K outer membrane protein were extracted with 1% Zwittergent 3-16 in 0.25 M NaCl (pH 8), and the remaining 30K outer membrane protein was extracted with 1% deoxycholate in 0.25% NaCl (pH 8). Extraction of membranes in this sequence yielded partially purified membrane proteins that were soluble in dilute buffers.

Bovine pneumonic pasteurellosis, also known as shipping fever pneumonia, is the most serious cause of cattle losses in the United States and Canada (39). The complex etiology of the disease has been the subject of two reviews (5, 39). It seems to be generally accepted that the classic signs of the disease result from infection of the lungs by *Pasteurella haemolytica* or, less frequently, by *Pasteurella multocida*. The complex etiology arises from the fact that viruses and probably stress play important, perhaps essential roles in predisposing the bovine lung to *Pasteurella* infection. Due to the heavy economic impact of these cattle losses, there is considerable interest in the development of vaccines that will provide protection against the disease. Vaccines consisting of killed bacteria have been available and used for decades, but it is becoming generally recognized (5, 8, 37) that these products are ineffective and indeed may result in a higher rate of morbidity and mortality than observed in unvaccinated controls (8, 12). There are numerous reports of studies in which vaccines have been prepared by extracting intact pasteurellas, especially *P. multocida*, with various aqueous solvents, including 2.5% NaCl (20, 31), 0.5 M potassium thiocyanate (KSCN)–0.425 M NaCl (9), and M sodium salicylate (13). Capsular material has been previously reported (10) to be extracted with minimal cell destruction with 0.01 M phosphate-buffered saline solution at 41°C for 1 h. In many instances, these extracts have been reported to provide a degree of protective immunity (13, 27, 28). Due to the absence of analytical data, little is known regarding the extent to which the major macromolecular components of the cell envelope, proteins, polysaccharides, and lipopolysaccharides (LPS) may have been selectively extracted, and in some of these studies, the possibility that much of the macromolecular contents of the cytosol may have been released appears real and unanswered.

P. haemolytica secretes a cytotoxin (2) that kills alveolar macrophages of ruminants, and apparently only ruminants (17). Since this microorganism causes pneumonia only in ruminants, this correlation supports the concept that the cytotoxin may be a virulence factor. The cytotoxin has been purified and partially characterized previously (1, 15).

Antibodies to the outer membrane (OM) proteins of gram-negative bacteria may also be expected to provide protection against infection by these microorganisms (14, 22, 38, 40). Such antibodies might promote phagocytosis and also might cause call death by impairing the function of these proteins. We here present what we believe to be the first complete report of the isolation of OMs and inner membranes (IMs) of *P. haemolytica* biotype A, serotype 1, the organism most commonly associated with shipping fever pneumonia in cattle, and identify the major proteins of these two membrane fractions. We also report analytical data characterizing extracts of intact bacteria by methods previously used for preparations designed to provide immunoprotection against infection by the pasteurellas, as well as extraction with several detergents.

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MATERIALS AND METHODS

Analytical methods. Protein was determined by the modified Lowry procedure of Markwell et al. (25), DNA was

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determined by the diphenylamine assay (11), and hexoses were determined by the method of Jermyn (16). Succinate dehydrogenase and NADH dehydrogenase activities were assayed by the method of Ellar et al. (6). Glucose-6-phosphate dehydrogenase was assayed by the method in the Worthington Manual, 1977, and LPS was estimated by the colorimetric determination of 2-keto-3-deoxyoctonate (KDO) (18). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 10 or 12.5% acrylamide slabs by the method of Laemmli (23). Staining was done with Coomassie blue R-250, and standards were from Sigma Chemical Co. (SDS-6H and SDS-7).

Organisms and culture conditions. *P. haemolytica* A-1 was isolated from the lungs of a calf that had died from shipping fever pneumonia, and the isolate was maintained in the lyophilized state. This isolate was identified as serotype 1, biotype A (see below). For each growth, lyophilized bacteria were reconstituted with 1 to 2 ml of sterile brain heart infusion broth (Difco Laboratories, Detroit, Mich.) and streaked for isolation onto blood agar plates. After incubation at 37°C for 20 to 24 h in a candle jar, a loopful of organisms was inoculated into 100 ml of brain heart infusion broth in a 250-ml Erlenmeyer flask. This flask was incubated at 37°C with shaking (150 rpm) for 12 to 13 h and was used to make a 1% (vol/vol) inoculum into fresh brain heart infusion broth (1 liter of medium in a 2-liter Erlenmeyer flask). Bacteria were grown as above until midlogarithmic phase (5 to 6 h) and harvested by centrifugation at 20,000 × *g* for 20 min at 4°C. Bacteria were washed once with phosphate-buffered saline (pH 7.2) and either used immediately or stored at −20°C.

Preparation of total membranes. Except as otherwise indicated, all procedures in this and the following section were performed at 4°C. Bacteria from 5 liters of growth medium were plasmolyzed by suspending them in 30 to 50 ml of cold 20% (wt/vol) sucrose in 0.01 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 7.4) (buffer I). Approximately 1 mg of DNase (type I; Sigma) and 1 mg of RNase (type I-A; Sigma) were added to the suspension, and the organisms were broken by one passage through a French pressure cell (Aminco Corp.) at 15,000 to 20,000 lb/in^2. After 20 min of incubation, the cells were removed by centrifugation at 6,000 × *g* for 15 min. the supernatant fluid was diluted with an equal volume of buffer I and centrifuged at 50,000 rpm in a 60 Ti rotor (Beckman Instruments, Inc., Fullerton, Calif.) for 90 min. The resulting pellets were washed three times with cold distilled water and then suspended in a few milliliters of cold distilled water with the aid of a Potter homogenizer. This preparation was used for extraction studies of total membranes.

Separation of OMs from IMs. The plasmolyzed and fragmented cells were diluted with an equal volume of buffer I and layered onto a sucrose step gradient which consisted of 8 ml of 2.02 M sucrose and 10 ml of 0.44 M sucrose in buffer I. The tubes were centrifuged in a 60 Ti rotor at 50,000 rpm for 90 min. This step concentrated the total membranes at the boundary between the 0.44 and 2.02 M sucrose solutions. After removing the upper layers with a pipette, the membrane layers were removed, pooled, and diluted with an equal volume of buffer I. Samples (3 to 4 ml) of diluted total membranes were then layered onto sucrose gradients consisting of 3 ml of 55% (wt/wt) sucrose and 6 ml each of 50, 45, 40, 35, and 30% sucrose all made in buffer I and centrifuged in an SW28 rotor (Beckman) at 26,000 rpm for 18 to 19 h. Tubes were fractionated with a gradient fractionator (ISCO, Lincoln, Nebr.), the contents of tubes containing corresponding fractions were pooled, and the absorbance was measured at 260 (A_{260}) and 280 nm. Fractions having a high A_{280} were diluted with cold distilled water and centrifuged in a 60 Ti rotor at 50,000 rpm for 90 min. Pellets were washed three times with cold distilled water and suspended in distilled water with the aid of a Potter homgenizer.

Extraction of intact bacteria. Freshly harvested (unwashed) bacteria were extracted with 10 volumes of 0.5 M KSCN–0.425 M NaCl (pH 6.3) for 5 h at 37°C in a shaking water bath by the method of Gaunt et al. (9) or with 10 volumes of 0.5 M KSCN–0.425 NaCl–0.01 M EDTA (pH 6.3). Bacteria were also extracted with 10 volumes of 2.5% NaCl (wt/vol) in 0.02 M sodium phosphate buffer (pH 7.2) for 1 h at 41°C.

RESULTS

Isolation and characterization of membranes. Attempts were made to isolate IMs and OMs by the method of Koplow and Goldfine (21). Although two membrane bands were resolved by sucrose gradient centrifugation, the analytical results (enzymic and KDO) showed that the IM and OM bands were highly cross-contaminated. Kelley and Parker (19) reported that plasmolysis of organisms with sucrose before breakage in the French pressure cell and the use of a step density gradient for isolating total membranes from cytosol resulted in improved separation of IMs from OMs. These results were confirmed in this study. Our best results were obtained with a linear 30 to 55% (wt/wt) sucrose gradient in buffer I (Fig. 1). The two bands, designated IM and OM, appeared to be well separated. The IM-enriched band, centered at a density of 1.15 g/ml, was yellow-orange, and the OM-enriched band, centered at 1.21 g/ml, appeared white.

The results of chemical and enzymatic analyses of the two membrane fractions (Table 1) show that the separation of the two membranes was not as complete as one might expect from the appearance of Fig. 1. Although KDO was presumably a constituent only of the OM, its concentration was rather high in the IM fraction. Of the two dehydrogenases, used as markers of the IM, we noted that the succinate dehydrogenase activity was quite low in the OM fractions, and the NADH dehydrogenase activity was ca. 1/5 as great

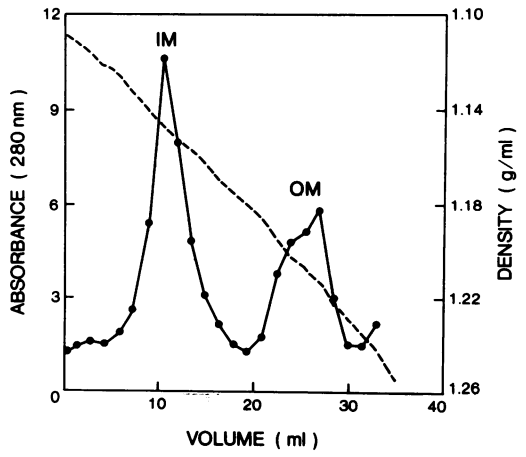

FIG. 1. Sucrose gradient centrifugation of the cushioned total membrane fraction from *P. haemolytica*. Membranes were prepared and analyzed by sucrose density gradient centrifugation as described in the text. The top of the gradient is at the left. The IM- and OM-enriched fractions are shown.

TABLE 1. Composition of the membrane fractions

Fraction	Buoyant density (g/ml)	SDH activity[a]	NADH DeHase activity[b]	Amt of KDO (nmol/mg of protein)
Total membranes		155	532	ND[c]
IM	1.154	214	790	12.4
OM	1.214	12	172	40.9

[a] SDH, Succinate dehydrogenase. Activity is expressed as micromoles of dichloroindophenol reduced per minute per milligram of protein.
[b] NADH DeHase, NADH dehydrogenase. Activity is expressed as for SDH.
[c] ND, Not determined.

as in the IM fraction. From these data, it seemed likely that we could expect about a fivefold relative enrichment of the characteristic proteins of the IMs and OMs, and this would be sufficient to permit their identification.

The protein composition of the membrane fractions was analyzed by SDS-PAGE (Fig. 2). The OM-enriched fraction contained two major proteins with molecular weights of 42,000 and 30,000 (42K and 30K proteins). Bands that were more intense in lane 3 than in lane 4 were assumed to be IM proteins. These included 31K, 23K, 22K, 19K, and 15K proteins.

Extraction of total membranes. As a consequence of previous reports (7, 34) that the detergents Sarkosyl (sodium lauroyl sarcosinate) and Triton X-100 are capable of selectively solubilizing IM proteins, we examined the effects of these detergents (0.5% Sarkosyl, 2% Triton X-100) on *P. haemolytica* total membranes. Analyses of the insoluble and soluble detergent fractions by SDS-PAGE showed that Sarkosyl solubilized the IM proteins more completely than did Triton X-100 (data not shown). The Sarkosyl-insoluble total membrane fraction contained the two major OM proteins as well as the other OM proteins present in the isolated OM-enriched fraction (Fig. 3). In four separate experiments, it was found that Sarkosyl treatment of total membranes resulted in solubilization of 64% (average value) of the total membrane protein and only 11% of the KDO.

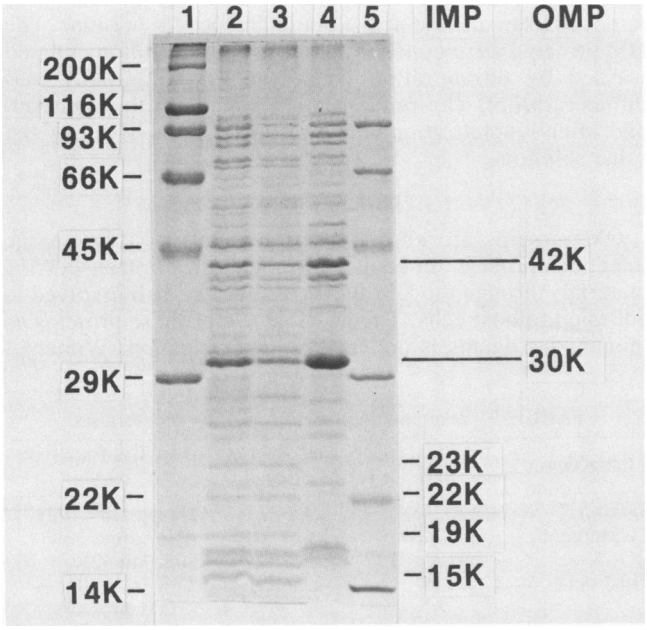

FIG. 2. SDS-PAGE analysis of the membrane fractions from *P. haemolytica*. Lanes: 1, molecular weight standards (in thousands); 2, cushioned total membranes; 3, IM-enriched fraction; 4, OM-enriched fraction; 5, molecular weight standards (in thousands). IMP refers to the IM protein, and OMP refers to the OM protein. Approximately 25 μg of protein was loaded per lane.

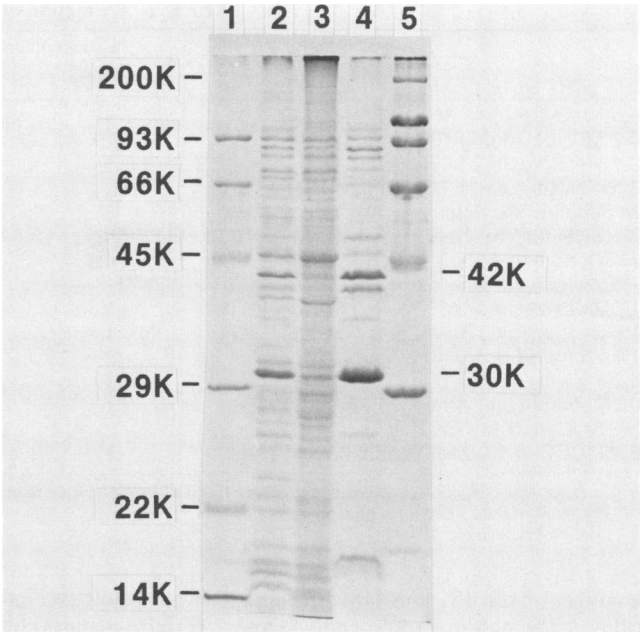

FIG. 3. SDS-PAGE analysis of fractions obtained during Sarkosyl treatment of total membranes. Lanes: 1, molecular weight standards (in thousands); 2, protein extracted from total membranes with SDS; 3, protein extracted from total membranes with Sarkosyl; 4, proteins not extracted from total membranes with Sarkosyl but subsequently extracted with SDS; 5, molecular weight standards (in thousands). Approximately 25 μg of protein was loaded per lane.

The total membranes were also extracted with 1 M NaCl–0.1 M citrate (pH 8.8) (35) at 6, 25, and 50°C. Only ca. 12 to 15% of the total membrane protein was released, including none of the major OM proteins and very little KDO.

Extraction of Sarkosyl-insoluble membranes. After extracting the IM proteins with Sarkosyl, we investigated the extent to which the two major OM proteins and the LPS were solubilized by various detergents and chaotropic agents. The results are presented in Table 2. Under a variety of conditions of ionic strength and temperature, Zwittergent 3-16 (Calbiochem-Behring) selectively extracted the 42K OM protein, and deoxycholate (DOC) selectively extracted the 30K OM protein. SDS extracted both proteins, but subsequent studies showed that in contrast to proteins extracted with Zwittergent and DOC, those extracted with SDS were of limited solubility and presumably denatured. We also noted that extraction of either OM protein was accompanied by solubilization of most of the LPS as judged from the solubilization of KDO. Although the usefulness of SDS for preparative purposes was limited by denaturation of the membrane proteins, it was nevertheless a useful reagent for analytical purposes, since any protein remaining after extraction with other extractants was solubilized by SDS and could be examined by SDS-PAGE. This information was useful in calculating percent recovery.

Extraction of intact bacteria. Since we had now developed the necessary analytical procedures for the quantitative

TABLE 2. Extraction of Sarkosyl-insoluble membranes

Extractant	Extraction conditions		% Protein soluble	% KDO soluble	OM proteins extracted[a]	
	h	°C			30K	42K
0.5 M KSCN–0.08 M NaCl (pH 6.3)	5	37	19	0	–	–
0.2 M LiCl–10 mM EDTA (pH 6.0)	2	45	11	0	–	–
2.5% NaCl–0.05 M Tris (pH 7.4)	2	45	2	0	–	–
2.5% NaCl–0.05 M Tris–10 mM EDTA	2	45	4	0	–	–
M Sodium salicylate	3	37	0	1	–	–
2% SDS–0.05 M Tris (pH 7.4)	0.5	70	98	71	+	+
1% DOC–0.05 M Tris (pH 7.4)	0.5	25	24	50	+	–
1% Zwittergent 3-16[b]	1	25	44	76	–	+
1% Zwittergent 3-16[b]	1	37	53	77	–	+
1% Zwittergent 3-16–0.25 M NaCl[b]	1	37	84	100	–	+
1% Zwittergent 3-16–0.25 M NaCl[b]	2	45	77	93	–	+
0.2% Zwittergent 3-16[b]	1	37	55	ND[c]	–	+
0.2% Zwittergent 3-16–0.25 M NaCl[b]	1	37	62	ND	–	+
1% DOC–0.25 M NaCl[b]	2	45	57	ND	+	–
0.5% DOC–0.5% Zwittergent–0.25 M NaCl[b]	2	45	38	ND	+	–

[a] –, Specified OM proteins were not detected by SDS-PAGE with Coomassie blue stain; +, specified OM proteins were detected.
[b] Prepared in 0.02 M sodium phosphate buffer (pH 7.2).
[c] ND, Not determined.

analysis of the IM and OM proteins as well a polysaccharides, LPS, and macromolecules expected to be released by lysis of the cells, it was of interest to determine which macromolecules had likely been released in procedures used by others (9, 31) for the preparation of vaccines by extraction of intact members of the family *Pasteurelleae* with various chelating and chaotropic agents. Freshly harvested (unfrozen) bacteria were extracted under the following conditions: (i) 0.5 M KSCN–0.425 M NaCl (pH 6.3) for 5 h at 37°C; (ii) 0.5 M KSCN–0.425 M NaCl–0.01 M EDTA (pH 6.3) for 5 h at 37°C; and (iii) 2.5% NaCl in 0.02 M sodium phosphate (pH 7.2) for 1 h at 41°C. The two KSCN extracts contained 60 to 70% protein, 5% hexose, KDO, and DNA. SDS-PAGE analysis showed many proteins (40 to 50 bands). It was evident that extensive cellular lysis had resulted from exposure to these solutions. The A_{660} value was found to decrease slowly after suspension of the organisms and throughout the extractions, and after 5 h of incubation, the solutions were highly viscous and no growth occurred after plating on blood agar. DNA was also detected in the extract. Attempts to detect bacterial lysis by the release of glucose-6-phosphate dehydrogenase activity failed due to the fact that the activity of this enzyme was destroyed by KSCN under incubation conditions i and ii. The 2.5% NaCl extract, condition iii, contained 50% protein, 5% hexose, KDO, and DNA. This extraction probably also resulted in some cellular lysis, as DNA was detected and 40 to 50 bands were observed by SDS-PAGE. A slight decrease in A_{660} was observed after 1 h of incubation, although no glucose-6-phosphate dehydrogenase activity was detected. Again, it appeared that none or only a small amount of the two major OM proteins was extracted in any of these extraction procedures.

Sequential extraction and purification of OM proteins. The results recorded in Table 2 suggest that essentially all of the membrane proteins might be selectively extracted in native form by sequential extraction with Sarkosyl, followed by Zwittergent and then DOC. We have tested this procedure in a series of experiments in which ionic strength and other parameters were varied. Data from the experiment yielding the best results, presumably representing near optimum conditions for the extraction, are reported in Table 3.

Since most of the LPS (60% in Table 3) was released in the Zwittergent extraction, a desirable first step in the further purification of the 42K protein would be the removal of the LPS from this fraction. Since DOC is known to dissociate highly aggregated forms of LPS from many gram-negative bacteria, size exclusion chromatography on Sephadex G-100 appeared to be a promising method for accomplishing this purpose. This step resulted in two well-resolved peaks, with K_{av} values of 0.06 and 0.56 (Fig. 4). $K_{av} = (V_e - V_t)/(V_0 - V_t)$, where V_e is the elution of a peak and V_0 and V_t are the void volume and total volume of the column, respectively. The first peak contained the OM proteins, and the second peak consisted largely of LPS and Zwittergent. Tube-by-tube assays for KDO demonstrated that most of the LPS eluted just before the second A_{280} peak and had a K_{av} of 0.51. Analysis of the chromatography fractions (KDO, protein, SDS-PAGE) confirmed that 75 to 80% of the KDO was removed from the first peak containing the OM proteins. The OM proteins were concentrated in an ultrafiltration cell and washed by ultrafiltration with 1 mM $NaHCO_3$–0.1 mM dithioerythritol. The proteins were soluble in this solution, and after lyophilization, they could be redissolved in the same solution.

DISCUSSION

OM proteins are known to play important roles in the infectious process. In addition to their roles in transporting materials through the OM (porins), they are also involved in adhesion to host cells. The possible use of these proteins as immunizing agents is under active investigation. Winter et

TABLE 3. Sequential extraction of total membranes

Extraction	Amt of protein (mg)	Amt of KDO (μg)	SDS-PAGE results
Sarkosyl[a]	540 (68)[b]	453 (17)	IM proteins only
Zwittergent[c]	145 (18)	1,623 (60)	42K, some 30K
DOC[d]	41 (5)	368 (14)	30K, no 42K
Final pellet	72 (9)	256 (9)	Traces of all OM proteins

[a] Total membranes (6.14 g [wet weight]) were extracted with 150 ml of 0.5% Sarkosyl in 0.02 M sodium phosphate buffer (pH 7.2) for 20 min at 23°C.
[b] Values in parentheses indicate percentage of total.
[c] Residue from *a* was extracted with 75 ml of 1% Zwittergent 3-16 in 0.25 M NaCl–0.01 M Tris (pH 8.0) for 1 h at 37°C.
[d] Residue from *c* was extracted with 30 ml of 1% DOC–0.25 M NaCl–0.05 M Tris (pH 8.0) for 1 h at 37°C.

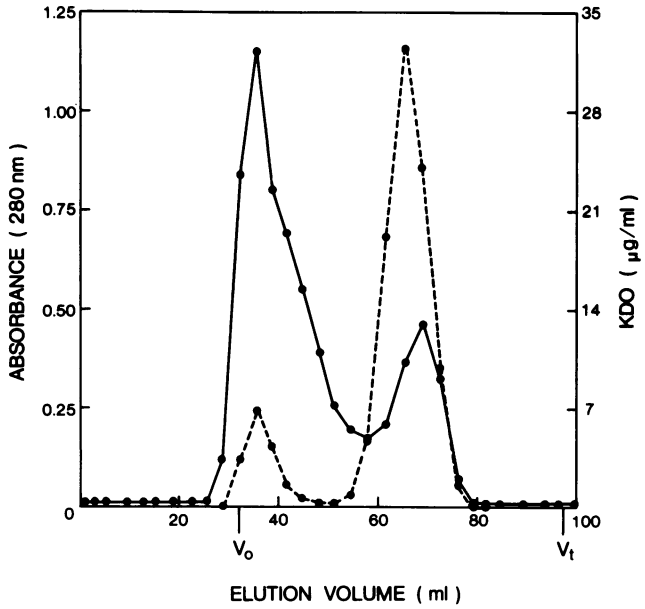

FIG. 4. Removal of LPS from the Zwittergent-soluble fraction by Sephadex G-100 gel filtration. The column (1.6 by 48.4 cm) was equilibrated with 1% DOC-Tris-NaCl buffer, and elution was performed with the same buffer. V_o, Void volume; V_t, total column volume.

al. (38) have reported studies of the immune response in cattle to an OM protein, known to be a porin, from *Brucella abortus*. Immunization with 5 mg in a novel adjuvant yielded antibody titers that were higher than those in animals that had recovered from the disease. Hedstrom et al. (14) reported that mice, after infection with *Pseudomonas aeruginosa*, have high titers to the OM proteins of this microorganism. The B polysaccharide in association with the OM proteins of *Neisseria meningitidis* was found by Zollinger et al. (40) to stimulate the formation of antibodies that were bacteriocidal. Immunization with a similar preparation from *Salmonella* sp. (22) provided protective immunity in mice.

One of the objectives of this research was to identify the macromolecules that are released from *P. haemolytica* by extraction methods that have previously been used for the preparation of vaccines. In this study, we found that extraction of whole cells with 0.5 M KSCN–0.425 M NaCl with and without added 10 mM EDTA resulted in extensive cellular lysis. After treatment for 1 h at 41°C with 2.5% NaCl in 0.02 M sodium phosphate (pH 7.2), conditions used by Penn and Nagy (31), some cellular lysis was observed but not to the extent of the other procedures. None of these procedures resulted in the release of significant amounts of the two major OM proteins. Although the efficacy of a proposed vaccine for cattle must ultimately be based on field trials with calves rather than theoretical considerations, we believe these considerations should be taken into account in the design of a vaccine for hosts as expensive as cattle. Because of the reasons stated above, based both on precedent and rationale, we have chosen to prepare the OM proteins in a form suitable for field testing. The sequential extraction procedure that we have developed appears to have accomplished this purpose. Antibodies to specific surface structures (capsular polysaccharide, pili, OM proteins, etc.) might be expected to be effective immunogens by preventing bacterial adherance, by promoting phagocytosis, and by causing death of the bacteria due to impaired membrane function.

Loeb et al. (24), working with *Haemophilus influenzae*, and Kelley and Parker (19), working with *Vibrio cholerae*, have reported that plasmolysis of the bacterial cells before the use of the French press and the use of a step gradient for obtaining the total membranes (cushioning) resulted in improved separation of these two components. Our results confirm these observations and extend them to *P. haemolytica*.

The appearance of Fig. 1 might lead one to expect that the resolution of the two major components might be essentially complete. The buoyant densities (1.15 and 1.21) were similar to those of other gram-negative bacteria (4, 19, 24, 29, 33), and we observed no hybrid M band in any attempt at isolating the two membranes. The analytical results suggest that each of the membrane fractions was contaminated to the extent of ca. 20% with the other, based on the KDO and NADH dehydrogenase activities (Table 1). The succinate dehydrogenase assays suggest that the OM was contaminated only to the extent of ca. 5% with IM fragments. SDS-PAGE analysis of the two membrane fractions was consistent with 20% cross-contamination, and we therefore conclude that the succinate dehydrogenase data may be misleading, perhaps due to a loss of activity of this enzyme in the OM fraction. At any rate, the degree of purification was adequate for us to identify two major 30K and 42K OM proteins and several minor OM proteins as well as several IM proteins.

Cross-contamination of IM and OM fractions prepared in this way is a common observation. Zones of adhesion, also known as Bayer's junctions, provide channels for the transport of capsular polysaccharides and the components of the OM from their site of synthesis on the IM (30). These zones of adhesion could result in cross-contamination of the two membrane fractions. Plasmolysis (pretreatment of the cells by equilibration with 20% sucrose) results in shrinkage of the IM away from the peptidoglycan (32). This step improved the separation as judged from the assays used in Table 1 but did not eliminate the problem of cross-contamination.

Selective extraction and partial purification of the membrane proteins was accomplished by a sequential extraction procedure. Sarkosyl, a mild anionic detergent, was used first to remove the IM proteins. Similar results have been reported (7, 26, 36) with *Escherichia coli* as well as other bacteria. Triton X-100, on the other hand, which also extracts the IM proteins of *E. coli* (34), was considerably less effective for this purpose than Sarkosyl in our preliminary studies with *P. haemolytica*. Although Sarkosyl treatment of *E. coli* membranes also solubilized minor OM proteins (3), we found this method released little or none of the two major OM proteins. Our finding that Sarkosyl treatment of *P. haemolytica* total membranes released 64% of the protein and 11% of the KDO (LPS) is similar to that of Moriyon and Berman for *B. abortus* (26). Our finding that EDTA and Triton X-100 are relatively ineffective for extracting the membrane proteins is also similar to results with *B. abortus*.

The strong anionic (SDS), weak anionic (DOC), and long-chain dipolar (Zwittergent 3-16) detergents varied in their effectivenes to extract the Sarkosyl-insoluble OM proteins (Table 2). SDS was most effective, apparently extracting all the proteins, but it also appeared to denature the proteins, limiting the usefulness of this detergent for preparative purposes. The effectiveness of both Zwittergent 3-16 and DOC was increased with the addition of NaCl to a concentration of 0.25 M. The most remarkable observation relative

to these two detergents, however, was their selectivity, Zwittergent selectively extracting the 42K protein and DOC the 30K protein. Since Zwittergent solubilized essentially all of the LPS, it was not surprising that it should also release the 42K protein, but it was somewhat surprising that it released only a small fraction of the 30K protein.

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