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Immunological Characterization of Two Major Proteins Isolated from the Outer Membrane of *Proteus mirabilis*

FELIZITAS BUB,† PETRA BIEKER, HANS H. MARTIN, AND KATHRYN NIXDORFF*
Institut für Mikrobiologie, Technische Hochschule Darmstadt, D-6100 Darmstadt, West Germany

Two proteins with apparent molecular weights of 39,000 and 36,000 (M_r 39,000 and M_r 36,000, respectively) were isolated from the outer membrane of *Proteus mirabilis* 19. M_r 36,000 was shown to be free of detectable amounts of the M_r 39,000 protein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and free of lipopolysaccharide according to gas chromatographic analyses of 3-hydroxymyristic acid content. The M_r 39,000 protein contained no detectable amount of lipopolysaccharide and only a trace of M_r 36,000. Both isolated proteins gave strong reactions in antisera produced to purified *P. mirabilis* 19 cell walls (outer membrane proteins in the native state). This suggested that the proteins isolated by our methods essentially retained their native configuration upon resolubilization. Antisera produced in rabbits to the isolated proteins showed strongest reactions with the homologous antigen, but some cross-reactions with the heterologous protein and with *P. mirabilis* 19 lipopolysaccharide were observed. These cross-reactions could be attributed to specific responses to traces of the heterologous (contaminant) proteins present in the purified proteins used as immunizing antigens. The M_r 39,000 and M_r 36,000 proteins have no major antigenic determinants in common. Reactions with *P. mirabilis* 19 lipopolysaccharide in antisera to the outer membrane proteins could be completely removed by absorption of the antisera with the M_r 36,000 protein.

The outer membrane of gram-negative bacteria is unique in its structure and functional properties and accordingly has been the object of intense research in the past few years. In contrast to plasma membranes, the outer membrane contains a few major characteristic proteins that are present in larger amounts than the other components of this complex, namely phospholipid and lipopolysaccharide (13). Some species of these proteins apparently play a decisive role in permitting the nonspecific diffusion of hydrophilic molecules generally larger than those able to penetrate most plasma membranes (5, 11, 12, 15). These proteins may also be responsible to a great extent for the relative resistance of outer membranes to disaggregation by detergents (15).

The large amounts of major proteins and their distribution in the outer membranes are important considerations not only for structure-function studies but also for studies of defense mechanisms of hosts against gram-negative bacterial infections. Gram-negative bacteria are unfortunately the most frequent causative agents of hospital-acquired infections (1, 9). Because of the known resistance of these organisms to antibiotic therapy, experimental efforts have been made to control gram-negative infections through specific immunological responses. Outer membrane proteins represent potentially important antigens, and although there have been some indications in the literature that an immune response to cell surface proteins may play a role in protection against experimental gram-negative bacterial infections (10), the immunological properties of cell surface proteins of gram-negative bacteria have not been widely investigated. Problems encountered in the isolation and purification of proteins in an undenatured form from outer membranes have no doubt been largely responsible for the relatively few investigations in this area.

We described previously (15) the isolation of two major outer membrane proteins of *Proteus mirabilis* that were able to mediate the penetration of hydrophilic molecules through model membrane phospholipid vesicle bilayers and protect these model membranes from disaggregation by detergents. According to biochemical analyses, these proteins were isolated in highly purified form.

In the present report we have characterized these proteins immunologically to obtain further information concerning the purity of the isolated outer membrane proteins, the effectiveness of

† Present address: Institut für Medizinische Mikrobiologie, Johannes-Gutenberg Universität, D-6500 Mainz, West Germany.

the purification methods employed in preserving the native state of the isolated proteins, the immunological relationships of the two protein species, and their potential role as cell surface antigens.

MATERIALS AND METHODS

Bacterial strains. *P. mirabilis* strain 19 of this laboratory was cultivated on complex medium to the early stationary phase of growth by E. Merck AG, Darmstadt, Germany. The cells were stored at −25°C.

Isolation of cell walls. *P. mirabilis* cell walls free of cytoplasmic membranes and cell contents were obtained as previously described (15) by shaking aqueous suspensions of bacteria with glass beads (diameter, 0.17 mm) in a cooled cell mill (E. Bühler, Tübingen, Germany) in the presence of 0.4% (wt/vol) sodium dodecyl sulfate (SDS) (Serva, Heidelberg, Germany). Extruded cell contents, solubilized cytoplasmic membranes, and SDS were removed from the cell walls by repeated washings with 0.02 M $NaHCO_3$ followed by distilled water in a centrifuge at 30,000 × *g* for 1 h at room temperature.

Extraction of lipopolysaccharide. Lipopolysaccharide I of *P. mirabilis* 19 was extracted with phenol-water and purified as described by Gmeiner (2).

Isolation of outer membrane proteins. (i) M_r 39,000. An outer membrane protein with an apparent molecular weight of 39,000 (M_r 39,000) was selectively extracted from *P. mirabilis* 19 cell walls as previously described (15) by using 1% (wt/vol) sodium deoxycholate (E. Merck AG) followed by gel filtration on Sephadex G-200 (Pharmacia, Uppsala, Sweden) in the presence of 0.25% sodium deoxycholate according to the method of Nakamura and Mizushima (13). After extensive dialysis (13), the product contained less than 0.002% detergent. The protein was stored in the lyophilized state.

(ii) M_r 36,000 method 1. We employed a modification of the method previously used to extract the protein with an apparent molecular weight of 36,000 (M_r 36,000), which is noncovalently associated with the peptidoglycan layer, from *P. mirabilis* 19 cell walls (15). Briefly, cell walls were extracted with 2% (wt/vol) SDS at 60°C (17). The M_r 36,000 protein remained associated with the peptidoglycan layer. The M_r 36,000 protein was then extracted from peptidoglycan with 90% (vol/vol) acetic acid (19, 20), and the supernatant after centrifugation in an ultracentrifuge (106,000 × *g* for 1 h at 4°C) was extensively dialyzed against distilled water. The modification involved resolubilization of the protein precipitated after dialysis in a buffer containing 0.01 M tris(hydroxymethyl)aminomethane-hydrochloride (pH 8.0), 0.1 M NaCl, 0.001 M ethylenediaminetetraacetate, 0.02% NaN_3, and 1% SDS at room temperature. The dissolved protein was then subjected to gel filtration on a Sephadex G-200 column (2.5 by 100 cm) in the same buffer containing 0.25% sodium deoxycholate instead of SDS. The protein fraction eluting with the void volume was collected and extensively dialyzed against 0.005 M tris(hydroxymethyl)aminomethane-hydrochloride (pH 8.0) and finally against distilled water. The protein was stored in the lyophilized state.

(iii) M_r 36,000 method 2. The M_r 36,000 protein was also isolated from cell walls by the method of Nakamura and Mizushima (14). This method involved a preextraction of cell walls with 2% SDS at 60°C as described above, followed by isolation of M_r 36,000 from the peptidoglycan sacculus at a high salt concentration (0.5 M NaCl) with 2% SDS at 50°C. The extraction mixture was concentrated to a volume of 6.0 to 8.0 ml by negative-pressure dialysis and subjected to gel filtration as described above.

Analytical methods. The protein contents of the isolated extracts were determined by the method of Lowry et al. (6), using the modification described by Markwell et al. (8). Fatty acids of phospholipids and lipopolysaccharide were analyzed by gas-liquid chromatography (3). SDS-polyacrylamide gel electrophoresis was carried out as previously described (15).

Immunization of rabbits. (i) Cell walls. Female chinchilla bastard rabbits weighing approximately 2,500 g (Wiga, Sulzfeld, Germany) were given four intravenous injections of 0.25, 0.25, 0.5 and 0.5 mg (dry weight) of *P. mirabilis* 19 cell walls on days 0, 6, 11, and 19, respectively. Blood was collected from ear veins on days 0, 6, 11, 14, and 25.

(ii) Isolated outer membrane proteins. A 3.0-mg amount of lyophilized protein was suspended in 1.0 ml of a buffer containing 0.1 M tris(hydroxymethyl)-aminomethane-hydrochloride (pH 7.1) plus 0.9% NaCl and sonicated for 90 s in an ice bath by using a Branson apparatus (model S-125; Branson Instruments Co., Danbury, Conn.) at power level 4 with a microtip. The suspension was brought to 1.5 ml with the buffer and mixed with 1.5 ml of Freund complete adjuvant (Difco, August Hedinger, Stuttgart, W. Germany) by using an Ultraturrax mixer (Janke & Kunkel, Staufen, W. Germany). Rabbits received 1.0 ml (1.0 mg) injected intradermally in 10 sites on the back on day 0. For the second injection animals were given 2.0 mg of the protein in the same manner on day 40. Blood was collected from ear veins on days 0, 40, and 50.

Alkali treatment of lipopolysaccharide. A 0.4-ml amount of 1.0 N NaOH was added to 5.0 mg of lipopolysaccharide suspended in 2.0 ml of distilled water, and the mixture was heated at 56°C for 1 h. The mixture was then neutralized with 1.0 N acetic acid and brought to 10 ml with distilled water.

Coating of erythrocytes with lipopolysaccharide. Sheep erythrocytes (Deutsche bioMerieux, Nürtingen, W. Germany) were coated with alkali-treated lipopolysaccharide by the method of Schlecht and Westphal (18).

Coating of erythrocytes with outer membrane proteins. Tannin-treated sheep erythrocytes were coated with outer membrane proteins by the method of Boyden as described by Nowotny (16).

Hemagglutinin assays of antisera. Hemagglutinin titers of antisera were determined by using either lipopolysaccharide-coated or protein-coated sheep erythrocytes in a microtiter system (Cooke Engineering Co., Alexandria, Va.), as described by Takatsky (21).

Quantitative precipitin test. Amounts of antibody in antisera were determined by a microprecipitation method (7), using either alkali-treated lipopolysaccharide or outer membrane proteins. To ensure

complete solubilization of the outer membrane proteins, samples containing 0.5 mg of protein per ml in 0.9% NaCl were sonicated as described above for the preparation of proteins for immunization. After sonication, SDS was added to a final concentration of 0.2%. The amount of protein in precipitates was determined by the method of Lowry et al. (6). In the case of the protein antigens, the amount of outer membrane protein added at equivalence was subtracted from the total precipitated protein to obtain the amount of antibody protein precipitated.

Absorption of antisera with isolated outer membrane proteins. A 2.0-mg amount of lyophilized outer membrane protein was added to 500 μl of heat-inactivated, undiluted serum and allowed to incubate for 30 min at 37°C. The mixtures were then left at 4°C for 2 days (mixed daily) and finally centrifuged for 20 min at 1,000 × *g* in the cold to remove precipitated complexes.

Absorption of antiserum with alkali-treated lipopolysaccharide. A 20-mg amount of alkali-treated *P. mirabilis* 19 lipopolysaccharide was mixed with 1.0 ml of heat-inactivated, undiluted antiserum and allowed to incubate for 30 min at 37°C. The mixtures were kept at 4°C overnight and subsequently centrifuged at 1,000 × *g* for 20 min to remove precipitated complexes.

RESULTS AND DISCUSSION

Isolation and purity of outer membrane proteins. The purity of the M_r 39,000 protein isolated with 1% sodium deoxycholate by the method of Nakamura and Mizushima (13) varied slightly from preparation to preparation. In the most pure form, which was used for this investigation, the protein contained no detectable amount of 3-hydroxymyristic acid by gas-liquid chromatography analyses (less than 0.085% lipopolysaccharide) and 0.45% phospholipid. SDS-polyacrylamide gel electrophoresis showed no trace of the major outer membrane protein with an apparent molecular weight of 17,000 (M_r 17,000) and only a slight trace of the M_r 36,000 protein when large amounts of the M_r 39,000 protein were applied to the gel slabs (Fig. 1, lanes B and D).

Two methods for isolation of the M_r 36,000 protein in the native state were employed. The M_r 36,000 protein isolated by method 1 contained no detectable amount of 3-hydroxymyristic acid, which is characteristic of lipopolysaccharide, and between 0.48 and 1.4% phospholipid, depending on the batch extracted. No traces of the M_r 39,000 and M_r 17,000 proteins were detectable on SDS-polyacrylamide gel electrophoresis (Fig. 2, lanes C and D). The final gel filtration step, which was a modification of the procedure previously used (15), gave preparations which were consistently free of lipopolysaccharide, M_r 39,000, and M_r 17,000.

Isolation of M_r 36,000 by method 2 gave preparations which were free of M_r 39,000 and M_r 17,000 (Fig. 1, lanes C and E) but contained 3 to 9% lipopolysaccharide and 0.5 to 0.7% phospholipid, even after the final gel filtration step.

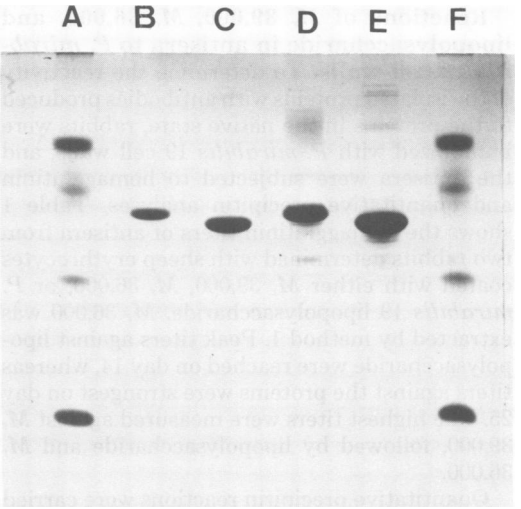

FIG. 1. *SDS-polyacrylamide gel electrophoresis of M_r 39,000 and M_r 36,000 outer membrane proteins of P. mirabilis 19. Lanes A and F, Standard proteins: bovine serum albumin (M_r, 67,000), ovalbumin (M_r, 45,000), chymotrypsinogen (M_r, 25,000), and cytochrome c (M_r, 12,500). Lane B, M_r 39,000 (15.5 μg); lane C, M_r 36,000 (20 μg); lane D, M_r 39,000 (35 μg); lane E, M_r 36,000 (40 μg). M_r 36,000 was isolated by method 2.*

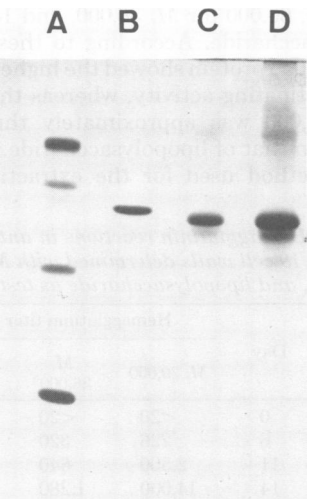

FIG. 2. *SDS-polyacrylamide gel electrophoresis of M_r 36,000 outer membrane protein of P. mirabilis 19, isolated by method 1. Lane A, Standard proteins (see legend to Fig. 1); lane B, M_r 39,000 (15.5 μg); lane C, M_r 36,000 (20 μg); lane D, M_r 36,000 (40 μg).*

Reactions of M_r 39,000, M_r 36,000, and lipopolysaccharide in antisera to *P. mirabilis* 19 cell walls. To determine the reactivity of the isolated proteins with antibodies produced to the proteins in the native state, rabbits were immunized with *P. mirabilis* 19 cell walls, and the antisera were subjected to hemagglutinin and quantitative precipitin analyses. Table 1 shows the hemagglutinin titers of antisera from two rabbits determined with sheep erythrocytes coated with either M_r 39,000, M_r 36,000, or *P. mirabilis* 19 lipopolysaccharide. M_r 36,000 was extracted by method 1. Peak titers against lipopolysaccharide were reached on day 14, whereas titers against the proteins were strongest on day 25. The highest titers were measured against M_r 39,000, followed by lipopolysaccharide and M_r 36.000.

Quantitative precipitin reactions were carried out on these antisera to determine the actual amounts of antibodies produced to the three antigens (Table 2). The largest amounts of antibodies were produced to lipopolysaccharide and M_r 36,000. The amounts of antibodies produced to the M_r 39,000 protein antigen contained in cell walls were smaller, but considerable. The specific hemagglutinating activities (calculated as hemagglutinin titer per milligram of antibody) in the hemagglutination test were 1,632,000 for M_r 39,000, 60,000 for M_r 36,000, and 183,000 for lipopolysaccharide. According to these figures, the M_r 39,000 protein showed the highest specific hemagglutinating activity, whereas the activity of M_r 36,000 was approximately three times lower than that of lipopolysaccharide.

The method used for the extraction of M_r 39,000 was chosen to produce the purest preparations in an undenatured form (13). The strong reactivity of this protein with antibodies formed against the protein in the native state suggests that this method of isolation was indeed successful in preserving the natural state of the molecule.

TABLE 1. *Hemagglutinin reactions in antisera to P. mirabilis 19 cell walls determined with M_r 39,000, M_r 36,000, and lipopolysaccharide as test antigens*

Antiserum	Day	Hemagglutinin titer with[a]:		
		M_r 39,000	M_r 36,000[b]	Lipopolysaccharide
1	0	<20	<20	<20
	6	226	320	453
	11	2,500	640	10,000
	14	14,000	1,280	20,000
	25	40,000	5,000	7,000
2	0	<20	<20	<20
	6	226	160	20
	11	2,500	453	10,000
	14	10,000	1,280	20,000
	25	40,000	5,000	5,000

[a] Hemagglutinin titers are reported as geometrical mean values of two parallel determinations. Titers represent the reciprocal of the last serum dilution after the addition of antigen to show hemagglutination.
[b] M_r 36,000 was isolated by method 1.

TABLE 2. *Amounts of antibody in antisera to P. mirabilis 19 cell walls measured with M_r 39,000, M_r 36,000, and alkali-treated lipopolysaccharide as test antigens in quantitative precipitation tests*

Antiserum[a]	Amt of antibody (mg) per ml of serum determined with[b]:		
	M_r 39,000	M_r 36,000[c]	Alkali-treated lipopolysaccharide
1	0.82	4.08	4.64
2	1.13	3.78	4.08

[a] Antisera from day 25 for the protein antigens and day 14 for the alkali-treated lipopolysaccharide.
[b] Values represent the means of two separate determinations.
[c] M_r 36,000 was isolated by method 1.

The specific hemagglutinating activity of the M_r 36,000 protein isolated with acetic acid (method 1) was considerably lower than that of M_r 39,000. Extraction with acetic acid should not irreversibly denature the protein (19, 20); however, another method of extraction of M_r 36,000 (method 2), which is also designed to preserve the native state of the protein, was used for comparison. No difference in reactivity of the above antisera with the M_r 36,000 proteins isolated by methods 1 and 2 could be detected (data not shown).

The stronger reactivity of M_r 39,000 over M_r 36,000 might be explained by the greater solubility of M_r 39,000 in the absence of detergent. M_r 39,000, in the concentrations employed, was completely soluble after a short ultrasonic treatment in buffer, whereas M_r 36,000 was still slightly turbid and completely soluble only after addition of detergent. This could have a profound effect on the efficiency of coating of sheep erythrocytes for the hemagglutination tests and the resulting titers measured, as the procedure was carried out in buffer without detergent.

For the quantitative precipitation tests both proteins were completely solubilized in the presence of 0.2% SDS. The precipitin curves showed the normal zones of antibody excess, equivalence, and antigen excess typical of soluble antigens.

Reactions of M_r 39,000, M_r 36,000, and lipopolysaccharide in antisera to the isolated outer membrane proteins. To test the purity and immunological relation of the isolated

proteins, antibodies to M_r 39,000 and M_r 36,000 (isolated by method 1) were produced in rabbits by using a standard immunization procedure for protein antigens. Results of hemagglutinin and quantitative precipitin reactions of the antisera with M_r 39,000, M_r 36,000, and lipopolysaccharide are presented in Tables 3 and 4.

Although the strongest reactions generally occurred in response to the homologous antigen, considerable cross-reactions with the heterologous protein and with lipopolysaccharide were observed. In the isolated form, M_r 39,000 appeared to be slightly more immunogenic than M_r 36,000 (Table 4).

Specificity of the cross-reactions to isolated outer membrane proteins. To determine whether the cross-reactions observed with M_r 39,000 in M_r 36,000 antisera or with M_r 36,000 in M_r 39,000 antisera were specific for the respective heterologous antigens, absorptions of these antisera with both antigens were carried out (Table 5).

Absorption of antisera to M_r 39,000 with M_r 36,000 removed all reactivity with M_r 36,000 but did not affect the strength of the reaction with M_r 39,000. Similarly, absorption of M_r 36,000 antisera with M_r 39,000 removed all reactivity with M_r 39,000 but left the reaction with M_r 36,000 intact.

These results suggest that the two proteins are not related immunologically, i.e., that they have no major antigenic determinants in common. This indicates that the reactions with the heterologous protein antigen are specific and that the antibodies were produced against trace amounts of that antigen as a contaminant of the pure protein preparation, despite the fact that not even a trace of the M_r 39,000 protein was detectable in SDS-polyacrylamide gel electrophoresis of M_r 36,000 extracts. The contaminating proteins were present in amounts large enough to induce an immune response but too small to react significantly with specific antibodies (no removal of antibodies to M_r 39,000 by absorption with M_r 36,000 or of antibodies to M_r 36,000 by absorption with M_r 39,000).

Specificity of the hemagglutinin reactions in antisera to isolated outer membrane proteins with lipopolysaccharide. Although no trace of lipopolysaccharide could be detected in the isolated outer membrane protein preparations by means of very sensitive gas chromatographic analysis for 3-hydroxymyristic acid, antisera to these proteins showed consid-

TABLE 3. *Hemagglutinin reactions in antisera to either M_r 39,000 or M_r 36,000 measured with M_r 39,000, M_r 36,000, and lipopolysaccharide as test antigens*

Antiserum[a]	Immunogen	Hemagglutinin titer with[b]:		
		M_r 39,000	M_r 36,000[c]	Lipopolysaccharide
3	M_r 39,000	10,000	320	160
4	M_r 39,000	10,000	320	320
5	M_r 36,000[c]	640	2,500	450
6	M_r 36,000[c]	2,500	5,000	960

[a] Antisera were collected on day 50.
[b] Hemagglutinin titers are reported as geometric means of two parallel determinations. Titers represent the reciprocal value of the last serum dilution after addition of antigen to show hemagglutination.
[c] M_r 36,000 was isolated by method 1.

TABLE 4. *Amounts of antibody in antisera to either M_r 39,000 or M_r 36,000 measured with M_r 39,000, M_r 36,000, and alkali-treated lipopolysaccharide as test antigens in quantitative precipitation tests*

Antiserum[a]	Immunogen	Amt of antibody (mg) per ml of serum with[b]:		
		M_r 39,000	M_r 36,000[c]	Alkali-treated lipopolysaccharide
3	M_r 39,000	1.54	0.50	0.36
4	M_r 39,000	1.80	0.56	0.39
5	M_r 36,000[c]	0.36	0.76	0.43
6	M_r 36,000[c]	0.25	1.16	0.67

[a] Antisera from day 50.
[b] Values represent means of two separate determinations.
[c] M_r 36,000 was isolated by method 1.

TABLE 5. *Hemagglutinin reactions in antisera to isolated outer membrane proteins of P. mirabilis 19 after absorption with either M_r 39,000 or M_r 36,000*

Immunogen	Antiserum[a]	Treatment of antisera	Test antigen	Hemagglutinin titer[b]
M_r 39,000	3	No absorption	M_r 39,000	10,000
	3	No absorption	M_r 36,000[c]	250
	3	Absorbed with M_r 36,000[c]	M_r 39,000	10,000
	3	Absorbed with M_r 36,000[c]	M_r 36,000[c]	<2
M_r 36,000[c]	6	No absorption	M_r 36,000[c]	5,000
	6	No absorption	M_r 39,000	2,500
	6	Absorbed with M_r 39,000	M_r 36,000[c]	5,000
	6	Absorbed with M_r 39,000	M_r 39,000	<2

[a] Antisera from day 50.
[b] Hemagglutinin titers are reported as geometrical mean values of two parallel determinations. Titers represent the reciprocal of the last serum dilution after the addition of antigen to show hemagglutination.
[c] M_r 36,000 was isolated by method 1.

erable reactions with *P. mirabilis* 19 lipopolysaccharide. To determine whether these reactions were specific for lipopolysaccharide, hemagglutinin reactions were also measured against sheep erythrocytes coated with lipopolysaccharide I from *P. mirabilis* D52. This lipopolysaccharide has a sugar composition different than that of *P. mirabilis* 19 lipopolysaccharide (2). Ribitol plays a decisive role in the serological specificity of *P. mirabilis* D52 lipopolysaccharide (4), whereas lysine in *P. mirabilis* 19 lipopolysaccharide (2) is most likely an important determinant of antigenic specificity.

Results of hemagglutinin reactions in M_r 39,000 antisera and in M_r 36,000 antisera with these lipopolysaccharides are presented in Table 6. The reactions observed appeared to be specific for *P. mirabilis* 19 lipopolysaccharide (hemagglutinin titers of 128 and 500 for *P. mirabilis* 19 and 2 for *P. mirabilis* D52). However, absorption of anti-M_r 39,000 serum with M_r 36,000 protein removed all detectable antibodies reacting with *P. mirabilis* 19 lipopolysaccharide, whereas absorption of anti-M_r 36,000 serum with M_r 39,000 protein reduced the hemagglutinin reaction with *P. mirabilis* 19 lipopolysaccharide by only one dilution step. This result may or may not be significant. In any case, the greater part of the reactivity of *P. mirabilis* 19 lipopolysaccharide with the anti-protein sera may be attributed to cross-reactions with specific M_r 36,000 antibodies present in both antisera.

Earlier investigations from our laboratory suggested that M_r 36,000 has a cationic character (precipitation of phospholipids upon addition of large amounts of M_r 36,000 in model membrane systems). Antibodies produced to these basic groups on the protein could cross-react with lysine present as a component of *P. mirabilis* 19 lipopolysaccharide. We are therefore unable to determine in our system whether traces of lipopolysaccharide not detectable by gas chromatographic analyses were present in the protein preparations in amounts large enough to induce a specific immune response to this component.

Absorption of anti-cell wall sera (Table 1) with *P. mirabilis* 19 lipopolysaccharide removed all hemagglutinin activity against lipopolysaccharide and reduced hemagglutinin titers measured against M_r 36,000 by two to three dilution steps. Amounts of antibodies to M_r 36,000 were reduced from 4.08 and 3.78 mg/ml (Table 2) to 1.17 and 1.47 mg/ml, respectively. These results suggest that antigenic determinants on M_r 36,000 other than those that cross-react with *P. mirabilis* 19 lipopolysaccharide play a role in the serological specificity of the protein antigen. Absorption of these antisera with *P. mirabilis* 19 lipopolysaccharide did not reduce hemagglutinin titers or amounts of antibodies measured against the M_r 39,000 protein.

TABLE 6. *Hemagglutinin reactions in antisera to isolated outer membrane proteins of P. mirabilis 19 before or after absorption with either M_r 39,000 or M_r 36,000 measured against P. mirabilis 19 lipopolysaccharide and P. mirabilis D52 lipopolysaccharide as test antigens*

Antiserum[a]	Immunogen	Treatment of antisera	Hemagglutinin titer with[b]: *P. mirabilis* 19 lipopolysaccharide	Hemagglutinin titer with[b]: *P. mirabilis* D52 lipopolysaccharide
3	M_r 39,000	No absorption	128	2
6	M_r 36,000[c]	No absorption	500	2
3	M_r 39,000	Absorbed with M_r 36,000[c]	<2	ND[d]
6	M_r 36,000[c]	Absorbed with M_r 39,000	250	ND

[a] Antisera from day 50.
[b] Hemagglutinin titers are reported as geometrical means of two parallel determinations. Titers represent reciprocal value of the last serum dilution after the addition of antigen to show hemagglutination.
[c] M_r 36,000 was isolated by method 1.
[d] ND, Not determined.

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LITERATURE CITED

1. **Finland, M.** 1973. Excursions into epidemiology: selected studies during the past four decades at Boston City Hospital. J. Infect. Dis. **128**:76–124.
2. **Gmeiner, J.** 1975. The isolation of two different lipopolysaccharide fractions from various *Proteus mirabilis* strains. Eur. J. Biochem. **58**:621–626.
3. **Gmeiner, J., and H. H. Martin.** 1976. Phospholipid and lipopolysaccharide in *Proteus mirabilis* and its stable protoplast L-form. Difference in content and fatty acid composition. Eur. J. Biochem. **67**:487–494.
4. **Gmeiner, J., H. Mayer, I. Fromme, K. Kotelko, and K. Zych.** 1977. Ribitol-containing lipopolysaccharides from *Proteus mirabilis* and their serological realtionship. Eur. J. Biochem. **72**:35–40.
5. **Hancock, R. E. W., and H. Nikaido.** 1978. Outer membrane of gram-negative bacteria. XIX. Isolation from *Pseudomonas aeruginosa* PA01 and use in reconstitution and definition of the permeability barrier. J. Bacteriol. **136**:381–390.
6. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
7. **Lüderitz, O., H.-J. Risse, H. Schulte-Holthausen, J. L. Strominger, I. W. Sutherland, and O. Westphal.** 1965. Biochemical studies of the smooth-rough mutation in *Salmonella minnesota*. J. Bacteriol. **89**:343–354.

8. **Markwell, M. A. K., S. M. Haas, L. L. Bieber, and N. E. Tolbert.** 1978. A modification of the Lowry procedure to simplify protein determinations in membrane and lipoprotein samples. Anal. Biochem. **87**:206–210.
9. **Mauff, G., K. P. Schaal, and G. Pulverer.** 1979. Krankenhausinfektionen im Wandel der Zeiten. II. Erregerspektrum und Epidemiologie im Zeitalter der antimikrobiellen Chemotherapie. Forum Mikrobiol. **2**:66–72.
10. **Misfeldt, M. L., and W. Johnson.** 1979. Identification of protective surface proteins in ribosomal fractions from *Salmonella typhimurium*. Infect. Immun. **24**:808–816.
11. **Nakae, T.** 1976. Outer membrane of *Salmonella*. Isolation of protein complex that produces transmembrane channels. J. Biol. Chem. **251**:2176–2178.
12. **Nakae, T.** 1976. Identification of the outer membrane protein of *Escherichia coli* that produces transmembrane channels in reconstituted vesicle membranes. Biochem. Biophys. Res. Commun. **71**:877–884.
13. **Nakamura, K., and S. Mizushima.** 1975. In vitro reassembly of the membranous vesicle from *Escherichia coli* outer membrane components. Role of individual components and magnesium ions in reassembly. Biochim. Biophys. Acta **413**:371–339.
14. **Nakamura, K., and S. Mizushima.** 1976. Effects of heating in dodecyl sulfate solutions on the conformation and electrophoretic mobility of isolated major outer membrane proteins from *Escherichia coli* K-12. J. Biochem. **80**:1411–1422.
15. **Nixdorff, K., H. Fitzer, J. Gmeiner, and H. H. Martin.** 1977. Reconstitution of model membranes from phospholipid and outer membrane proteins of *Proteus mirabilis*. Role of proteins in the formation of hydrophilic pores and protection of membranes against detergents. Eur. J. Biochem. **81**:63–69.
16. **Nowotny, A.** 1969. Basic exercises in immunochemistry. Springer-Verlag, Berlin.
17. **Rosenbusch, J. P.** 1974. Characterization of the major envelope protein from *Escherichia coli*. Regular arrangement on the peptidoglycan and unusual dodecyl sulfate binding. J. Biol. Chem. **249**:8019–8029.
18. **Schlecht, S., and O. Westphal.** 1967. Über die Herstellung von Antiserum gegen die somatischen (O-) Antigene von Salmonellen. II. Mitteilung: Untersuchungen über Hämagglutinintiter. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. **205**:487–501.
19. **Schubert, D.** 1973. Association of protein fractions and lipids from human erythrocyte membranes. II. Studies on a loosely bound protein fraction. Hoppe-Seyler's Z. Physiol. Chem. **354**:781–790.
20. **Schubert, D., J. Poensgen, and G. Werner.** 1972. Association of protein fractions and lipids from human erythrocyte membranes. I. Studies on a strongly bound protein fraction. Hoppe-Seyler's Z. Physiol. Chem. **353**: 1034–1042.
21. **Takatsky, S.** 1956. The use of spiral loops in serological and virological micro-methods. Acta Microbiol. Acad. Sci. Hung. **3**:191–202.