Biochimica et Biophysica Acta, 433 (1976) 229–239

BBA 77281

THE OUTER MEMBRANE OF *PROTEUS MIRABILIS*

III. SPECIFIC LABELING AND ENZYMIC HYDROLYSIS OF THE PROTEIN AND PHOSPHOLIPID COMPONENTS OF THE OUTER AND CYTOPLASMIC MEMBRANES

MIRIAM HASIN, SHMUEL RAZIN and SHLOMO ROTTEM

Biomembrane Research Laboratory, Department of Clinical Microbiology, The Hebrew University-Hadassah Medical School, Jerusalem (Israel)

(Received November 11th, 1975)

SUMMARY

1. The question of whether part of the outer membrane proteins and phospholipids are exposed on the external cell surface of *Proteus mirabilis* was approached by comparing the action of proteases, phospholipases and specific labeling reagents on intact cells and isolated outer membranes.

2. Pronase and trypsin degraded some of the outer membrane proteins in isolated membranes, but had no effect on intact cells. Likewise, the outer membrane proteins were intensely labeled by the lactoperoxidase-mediated iodination technique on treatment of isolated membranes, but were very poorly labeled on treatment of intact cells.

3. Phospholipase A from bee venom effectively hydrolyzed the outer membrane phospholipids in isolated membranes and in intact cells, whereas phospholipase C from *Bacillus cereus* hydrolyzed the phospholipids in isolated membranes only. An endogenous phospholipase A activity, triggered by cell rupture, was found to be associated mostly with the outer membrane.

4. Our results suggest that the protein and phospholipid components of the outer membrane of *P. mirabilis* are partially shielded on the external cell surface, most probably by the long carbohydrate chains of the lipopolysaccharide molecules.

INTRODUCTION

The envelope of Gram-negative bacteria consists of an outer membrane, an intermediate layer composed of peptidoglycan and an inner, cytoplasmic membrane [1]. The cytoplasmic membrane consists almost entirely of protein and phospholipids, the usual constituents of microbial cytoplasmic membranes [2], while the outer membrane contains, in addition to protein and phospholipids, large quantities of lipopolysaccharide. The presence of lipopolysaccharide as a major membrane component is a unique feature of the outer membrane of Gram-negative bacteria [1]. Elucidation of the molecular organization of this membrane is therefore of particular interest.

Previous publications in this series [3, 4] dealt with the isolation and the ultrastructural and chemical characterization of the outer and cytoplasmic membranes from *Proteus mirabilis*. The present communication is concerned with the localization of the various outer membrane components by specific labeling agents and hydrolytic enzymes. This was done by comparing the labeling and sensitivity of the proteins and phospholipids to proteolytic and lipolytic enzymes in intact cells and isolated outer membranes, working on the assumption that the labeling agent or enzyme will have access to proteins or phospholipids exposed on both membrane surfaces when isolated outer membranes are treated and only to proteins and phospholipids exposed on the external surface of the outer membrane when intact cells are treated. The data obtained suggest that the protein and phospholipid components of the outer membrane of intact cells are shielded to a marked extent from contact with exogenous macromolecules, most probably by the negatively charged carbohydrate chains of the lipopolysaccharide molecules located on the cell surface.

MATERIALS AND METHODS

Organism and membrane fractions. P. mirabilis strain 19, kindly provided by Professor H. H. Martin (Technische Hochschule, Darmstadt, Germany), was grown as described previously [3]. To label membrane phospholipids, 2 μCi of [1-^{14}C]oleic acid (59.7 Ci/mol, The Radiochemical Centre, Amersham, England) was added to each liter of the growth medium. The outer and cytoplasmic membrane fractions were isolated by sucrose density gradient centrifugation of the crude envelope fraction obtained by sonication of the cells in the presence of lysozyme as described in detail in the first communication of this series [3].

Iodination procedure. Lactoperoxidase-mediated iodination of membrane proteins was carried out by a modification of the procedure of Hubbard and Cohn [5] as described previously [6]. Some iodination experiments were also carried out by the procedure of Phillips and Morrison [7] as modified by Amar et al. [8]. In this case H_2O_2 was added directly to the reaction mixture to a final concentration of 0.05 %.

Gel electrophoresis. The sodium dodecyl sulfate gel electrophoresis system of Fairbanks et al. [9] was used with some modifications outlined in detail by Amar et al. [6]. Densitometer tracings of the stained gels were made in a Kipp and Zonen densitometer model DD2. When radioactive iodine-labeled outer membrane proteins were analyzed, pairs of identical gels were prepared, one was stained and the other sliced laterally into 2-mm sections, which were counted in a Packard Auto Gamma spectrometer.

Proteolytic digestion. Suspensions of cytoplasmic and outer membrane preparations (1 mg protein/ml) or whole cells (5 mg protein/ml) in 10 mM Tris · HCl buffer, pH 8.0 (or pH 6.0, when papain was used as the digestive enzyme) containing 0.25 M sucrose, were treated with 50 μg/ml suspension of either pronase (A grade, Calbiochem, Los Angeles, Calif.), trypsin (twice crystallized, Nutritional Biochemicals Corp., Cleveland, Ohio) or papain (three times crystallized, Mann Research Laboratories, New York, N.Y.) for 2 h at 37 °C. The reaction was stopped by the addition of EDTA to a final concentration of 5 mM when pronase was used, and by the addition of 50 μg/ml of *N*-α-*p*-tosyl-L-lysine chloromethyl ketone HCl when trypsin was

used as a digestive enzyme. 2 min after the addition of the specific inhibitor, the reaction mixtures were diluted with 9 vols. of cold water. With papain, where no specific inhibitor was used, this dilution served to stop the reaction. The diluted suspensions of treated membranes were centrifuged at 37 000 $\times g$ in the cold for 30 min and the pellets were suspended in water. The diluted suspensions of treated cells were centrifuged at 10 000 $\times g$ for 10 min. The sedimented cells were washed twice with water and the cytoplasmic and outer membrane fractions were isolated as described before [3] and resuspended in water. It should be noted that, unlike with untreated cells, a quantitative isolation could not be obtained of the cytoplasmic and outer membrane fractions from treated cells since about 30 % of the total envelope fraction was sedimented by the sucrose density gradient centrifugation. Aliquots of these suspensions were taken for protein determination [10] and for electrophoretic analysis. The degree of the proteolytic digestion was assessed by comparing the Lowry-reactive material in the treated and native membranes.

Lipid extraction. Lipids were extracted from ^{14}C-labeled cytoplasmic and outer membrane preparations by two successive extractions with chloroform/methanol (2 : 1, v/v), the first at 45 °C for 2 h and the second at room temperature overnight. The extracts were combined and dried under a stream of N_2.

Measurement of phospholipase C activity. Hydrolysis of *P. mirabilis* phospholipids by a partially purified preparation of phospholipase C (EC 3.1.4.3) from *Bacillus cereus* (10 units/mg protein, kindly provided by A. Loyter, The Hebrew University, Jerusalem) was determined as previously described [11] except that the reaction was stopped by the addition of 9 ml of chloroform/methanol (2 : 1, v/v). The samples were then shaken for 1 min, centrifuged at 12 000 $\times g$ for 10 min and the lower chloroform layer was collected, dried and subjected to chromatography on silica gel G plates [12]. The lipid spots were scraped off the plate into scintillation vials containing 10 ml of a dioxane/toluene scintillation liquor [13] and radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer. Phospholipase C activity was expressed as the percentage of radioactivity in the diglyceride fraction of the total radioactivity in membrane lipids. When lipid extracts from isolated membranes were used as substrates for phospholipase C activity, the dried lipids were dispersed in the buffered salt solution by sonication for 15–30 s in an M.S.E. ultrasonic disintegrator (60 W, 20 kHz) at 1.5 A. Phospholipase C from *Clostridium perfringens* (9 units/mg protein, Worthington Biochemical Corp.) was tested as described for the phospholipase C from *B. cereus* [11], but the reaction mixture contained, in addition to membranes or cells, 30–200 μg enzyme, 5 mM $CaCl_2$, 10 mg bovine serum albumin in 1 ml of 100 mM Tris/maleate buffer, pH 7.3.

Measurement of phospholipase A activity. Hydrolysis of the *P. mirabilis*-labeled phospholipids by phospholipase A from bee venom (EC 3.1.1.4, 1180 units/mg solid, Sigma) or by phospholipase A from *Crotalus terrificus terrificus* (EC 3.1.1.4, 210 units/mg protein, Sigma) was measured by the release of radioactive fatty acids in a reaction mixture (1 ml) containing labeled membranes (1 mg protein) or cells (10 mg protein), 25–50 μg enzyme, 2.5 mM $CaCl_2$ in 100 mM Tris · HCl buffer, pH 7.5. The reaction was carried out at 37 °C for 2 h and was then stopped by the addition of chloroform/methanol (2 : 1, v/v) as described for phospholipase C. The extracted lipids were chromatographed on silica gel G plates and radioactivity in the various lipid spots was determined as described above. Phospholipase A activity was expressed

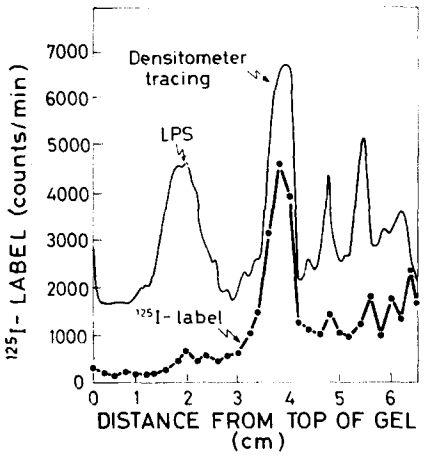

Fig. 1. Distribution of iodine label in the outer membrane proteins. The iodination was carried out on isolated outer membranes. LPS, lipopolysaccharide.

as the percentage of radioactivity in the free fatty acid fraction of the total radioactivity in membrane lipids.

The endogenous phospholipase A activity in *P. mirabilis* membranes was measured by the release of radioactive fatty acids from labeled membrane phospholipids as described above, or from an exogenous labeled substrate. The labeled substrate, 1-[9,10-$^3H_2$]palmitoyl phosphatidylcholine (kindly provided by Dr. Z. Ben Gershon, The Hebrew University, Jerusalem) was dispersed in 100 mM Tris · HCl buffer (pH 7.5) by sonication for 15 min in an M.S.E. ultrasonic disintegrator (60 W 20 kHz) at 1.5 A. The reaction mixture (1.5 ml) contained 0.2 ml of the sonicated phospholipid suspension, 2.5 mM $CaCl_2$, 0.15 ml of 5 % Triton X-100 and washed outer or cytoplasmic membranes (0.25 mg protein) in 100 mM Tris · HCl buffer (pH 7.5). The reaction was stopped after 2 h at 37 °C by the addition of chloroform/methanol (2 : 1, v/v). The percentage of radioactivity in the free fatty acid fraction of the lipid extract was determined as described above.

RESULTS

Lactoperoxidase-mediated iodination of the outer membrane proteins

Table I shows that labeling by ^{125}I is most intensive in the case of isolated outer membranes of *P. mirabilis* as against the very low labeling of intact cells, treated

TABLE I

COMPARISON OF LACTOPEROXIDASE-MEDIATED ^{125}I LABELING OF INTACT CELLS AND ISOLATED OUTER MEMBRANES BY TWO DIFFERENT TECHNIQUES

Reaction mixture	Radioactivity (cpm/mg protein)			
	Glucose oxidase technique [5]		H_2O_2 technique [7]	
	Cells	Outer membrane	Cells	Outer membrane
+Lactoperoxidase	2 709	709 677	3 085	396 367
−Lactoperoxidase	2 170	3 893	2 537	4 748

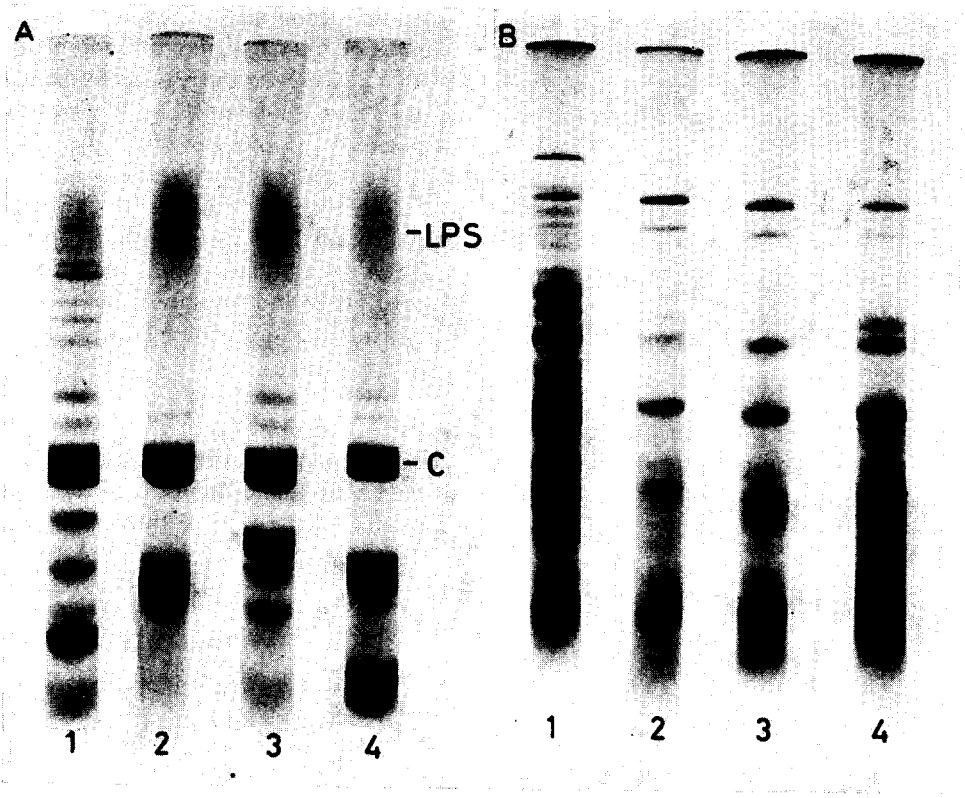

Fig. 2. Electrophoretic patterns of outer membrane preparations (A) and cytoplasmic membrane preparations (B) treated with 50 μg/ml of different proteolytic enzymes for 2 h at 37 °C. (1) untreated membranes; (2) membranes treated with pronase; (3) membranes treated with trypsin; (4) membranes treated with papain; LPS, the diffuse band consisting of lipopolysaccharide [14]. It is stained purple by Coomassie blue, whereas the protein bands are stained blue. C, the major protein band dominating the electrophoretic pattern of the outer membrane [3].

TABLE II

SUSCEPTIBILITY OF THE OUTER AND CYTOPLASMIC MEMBRANE PROTEINS TO PROTEOLYTIC DIGESTION

Proteolytic enzyme*	Percent protein released from the membrane	
	Outer membrane	Cytoplasmic membrane
Pronase	29.5	48.6
Trypsin	24.1	34.9
Papain	25.6	25.1

* Digestion carried out by 50 μg enzyme/ml at 37 °C for 2 h.

under the same conditions by two different iodination techniques. The iodination of isolated outer membranes resulted in the labeling of all the major protein bands detected by polyacrylamide gel electrophoresis (Fig. 1). However, the region of the wide diffuse band at the upper part of the gel (marked LPS on the figure) was only weakly labeled. This diffuse band, which was stained purple by Coomassie blue (Fig. 2A), has been identified as lipopolysaccharide [14]. The membrane phospholipids were also poorly labeled as indicated by the weak labeling at the running front of the gel, where the outer membrane phospholipids are migrating [14]. In fact, the lipopolysaccharide fraction extracted from iodinated outer membranes with phenol/water [15] contained only 5.3 % of the total iodine label, while the phospholipid fraction extracted from the outer membrane with chloroform/methanol contained only 1.2% of the label.

Susceptibility of the outer and cytoplasmic membrane proteins to proteolytic digestion

Treatment of isolated outer and cytoplasmic membranes with pronase, trypsin or papain resulted in the digestion of some of the membrane proteins as seen by the loss of Lowry-reactive material from the membranes (Table II) and by the changes

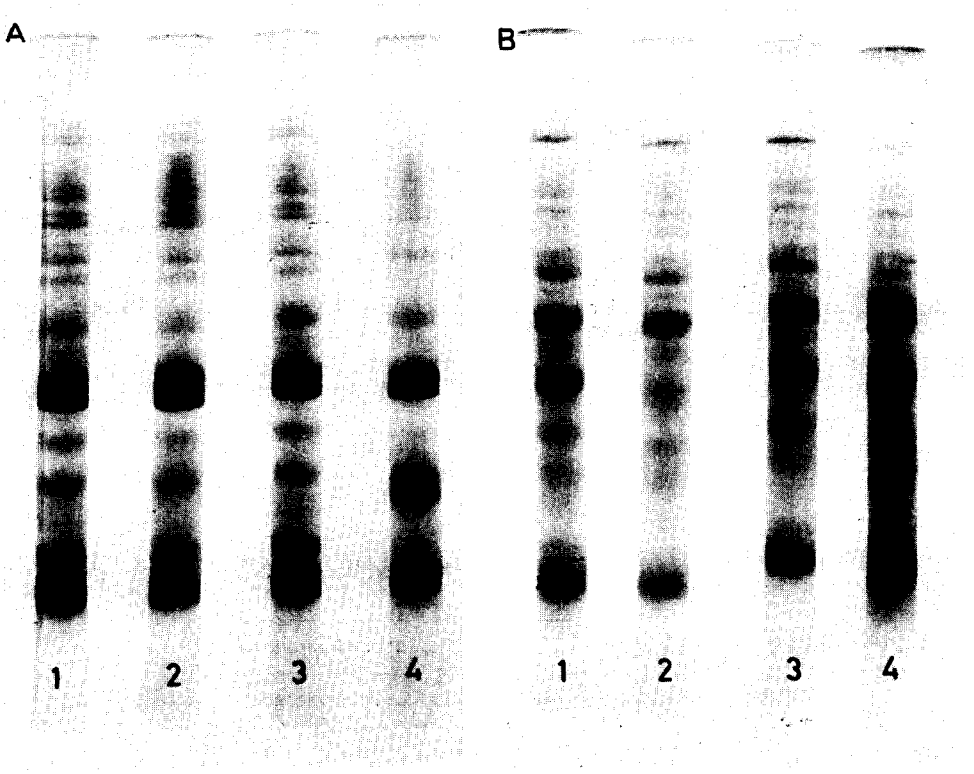

Fig. 3. Electrophoretic patterns of outer membranes (A) and cytoplasmic membranes (B) isolated from cells treated with 50 μg/ml of different proteolytic enzymes. (1) membranes from untreated cells; (2) membranes from cells treated with pronase; (3) membranes from cells treated with trypsin; (4) membranes from cells treated with papain.

in the electrophoretic profiles of membrane proteins (Figs 2 and 3). Comparison of the capacity of the three proteolytic enzymes to release Lowry-reactive material from isolated outer membranes showed it to be about equivalent (Table II). However, electrophoretic analysis showed the enzymes to differ somewhat with respect to the protein species attacked and the size of the polypeptide fragments which were produced during digestion and which remained attached to the membrane (Fig. 2A). In general, the enzymes attacked most of the high molecular weight proteins of the outer membrane. Because of its very intense staining it is difficult to tell whether the major outer membrane protein band (Band C, ref. 3) was attacked by pronase and trypsin, but it does seem to be attacked by papain. The diffuse lipopolysaccharide band at the upper part of the gel was not affected by any of the proteolytic treatments (Fig. 2A). In fact, the degradation of the outer membrane proteins was not accompanied by any significant release of lipopolysaccharide, as measured by the loss of radioactivity from membranes in which the lipopolysaccharide was labeled by [^{14}C]galactose [14].

Pronase and trypsin were more effective than papain in releasing peptides from isolated cytoplasmic membranes. In addition, the percentage of Lowry-reactive material released by pronase and trypsin from the cytoplasmic membrane was markedly higher than that released from the outer membrane (Table II). Electrophoretic analysis of the digested cytoplasmic membranes revealed that the proteolytic enzymes act selectively with respect to the species of membrane proteins attacked. Thus, some of the proteins resisted digestion by all of the three enzymes tested (Fig. 2B).

Since the outer membrane could not be quantitatively recovered from lysed cells, it was impossible to measure with any accuracy the percentage of the outer membrane protein released on treatment of intact cells by the proteolytic enzymes. Nevertheless, electrophoretic analysis of the outer and cytoplasmic membranes isolated from treated cells indicated that pronase and trypsin had little or no effect on the proteins of both membranes, while papain attacked the outer membrane proteins of intact cells in a way resembling its action on isolated outer membranes (Fig. 3).

Susceptibility of the outer and cytoplasmic membrane phospholipids to hydrolysis by phospholipases

The phospholipids in isolated outer and cytoplasmic membranes were highly susceptible to attack by phospholipase A preparations from bee venom and *Cr. terrificus*. However, as can be seen in Table III the enzyme from bee venom acted also on phospholipids of intact cells, whereas the enzyme from *Cr. terrificus* did not. The activity of the exogenous phospholipases was superimposed on that of a potent endogenous phospholipase A, mostly associated with the outer membrane. The initial rate of the endogenous phospholipase A activity of an isolated outer membrane preparation was about six times higher than that of a cytoplasmic membrane preparation when measured on sonicated dispersions of labeled phosphatidylethanolamine. Intact cells exhibited very low endogenous phospholipase A activity when measured on the cell-bound phospholipids (Table III) or on labeled phosphatidylethanolamine dispersions. Treatment of intact cells with high concentrations of pronase (up to 400 μg/ml) did not affect their endogenous phospholipase A activity, but similar treatment of isolated outer membranes decreased this activity by 60 %.

The endogenous phospholipase A activity was found to be extremely thermostable, so that boiling, used in our early experiments to stop hydrolysis of membrane

TABLE III

HYDROLYSIS OF THE OUTER AND CYTOPLASMIC MEMBRANE PHOSPHOLIPIDS BY EXOGENOUS AND ENDOGENOUS PHOSPHOLIPASES A

Membrane phospholipids were labeled during growth with [1-^{14}C]oleic acid. The reaction mixture contained 25 μg/ml of either one of the exogenous phospholipases, or none when endogenous phospholipase A activity was tested. Incubation was at 37 °C for 2 h.

Preparation treated	Phospholipid hydrolysis (radioactivity in free fatty acid fraction, percent of total)		
	Phospholipase A from bee venom	Phospholipase A from *Cr. terrificus*	Endogenous phospholipase A activity
Outer membranes	94.4	91.9	74.6
Cytoplasmic membranes	82.3	80.4	22.0
Intact cells	39.7	7.4	5.1

phospholipids by exogenous phospholipases, resulted in a most pronounced hydrolysis of the membrane phospholipids. The enzymic reactions were therefore stopped by the addition of chloroform/methanol to the treated membrane suspensions.

Phospholipase C from *B. cereus* was quite effective in the hydrolysis of phospholipids in isolated outer and cytoplasmic membranes, but acted very poorly on phospholipids of intact cells (Table IV). The endogenous phospholipase A apparently competes with the exogenous phospholipase C on the same substrate. Thus, in the outer membrane, where the endogenous phospholipase A activity is more pronounced, the radioactivity detected in the free fatty acid fraction was higher than that found in the diglyceride fraction, the reverse being true for the cytoplasmic membrane. In fact, the extracted phospholipids from both membranes were hydrolyzed to the same degree by phospholipase C (Table IV) supporting the above suggestion that the differences in the hydrolysis data between the two membranes result from differences in their

TABLE IV

HYDROLYSIS OF THE OUTER AND CYTOPLASMIC MEMBRANE PHOSPHOLIPIDS BY PHOSPHOLIPASE C FROM *BACILLUS CEREUS*

Membrane phospholipids were labeled during growth with [1-^{14}C]oleic acid. The reaction mixtures contained 30 μg phospholipase C/ml. Incubation was at 37 °C for 2 h.

Preparation treated	Phospholipase C activity (radioactivity in diglyceride fraction, percent of total)	Endogenous phospholipase A activity (radioactivity in free fatty acid fraction, percent of total)
Outer membranes	34.4	43.5
Cytoplasmic membranes	59.6	12.6
Intact cells	5.7	2.1
Extracted phospholipids		
Outer membrane	73.0	13.3
Cytoplasmic membrane	72.1	8.7

endogenous phospholipase A activity. Phospholipase C from *Cl. perfringens* showed no activity on phospholipids of *P. mirabilis* whether they were part of cells, isolated membranes or lipid dispersions. The enzyme was weakly active on dispersions of labeled phosphatidylethanolamine, releasing only 17 % of the label in the diglyceride fraction in a reaction mixture containing 200 μg/ml of the enzyme preparation.

None of the different phospholipases tested in our study produced any detectable changes in the electrophoretic patterns of the outer or cytoplasmic membrane proteins, indicating the absence of any significant protease activity from the enzyme preparations. Moreover, the extensive degradation of the outer membrane phospholipids was not accompanied by a significant release of lipopolysaccharide, as measured by the loss of radioactivity from membranes in which the lipopolysaccharide was labeled by [^{14}C]galactose [14].

DISCUSSION

Whether any of the outer membrane proteins is exposed on the external cell surface of *P. mirabilis* in a form enabling its interaction with exogenous macromolecules is still uncertain. Our finding that pronase and trypsin had little or no effect on the outer membrane proteins when treatment was carried out on intact cells, coupled with the insusceptibility of intact cells to iodination by the lactoperoxidase-mediated iodination technique, suggests either that none of the outer membrane proteins is localized on the external cell surface, or that proteins which are located on this surface are shielded and protected from interaction with macromolecules by the 0-antigenic side chains of the lipopolysaccharide molecules known to be present on the external surface of Gram-negative bacteria [1, 16]. Once the outer membrane of *P. mirabilis* was isolated, essentially all of its major proteins became labeled by lactoperoxidase, and most of them could be digested by the proteolytic enzyme. Papain differed from pronase and trypsin in its ability to digest the outer membrane proteins in intact cells. A smaller molecular size cannot explain this difference since the molecular weights of papain and trypsin are very similar [17].

Proteolytic digestion of isolated membranes revealed selectivity in the protein species attacked, even when the potent and non-specific pronase was used. While pronase could release 80 % of the total protein from mycoplasma membranes, the remaining 20 % being peptides too small to be retained in polyacrylamide gels [8, 18], this did not occur on the digestion of the P. *mirabilis* membranes. The percentage of peptides released, even from the cytoplasmic membrane, was definitely smaller than that from the mycoplasma membranes. Moreover, some protein bands in the *P. mirabilis* membranes were not attacked at all, even by prolonged pronase treatment. It appears that these proteins are protected from the proteolytic enzyme, probably due to their close association with other membrane components. Furthermore, those proteins that are attacked appear to be split into polypeptide fragments large enough to be retained in the gels. In this respect, our results with *P. mirabilis* membranes correspond with the data on the proteolytic digestion of *Escherichia coli* membranes [19, 20].

The use of phospholipases has provided some information on the disposition of the outer membrane phospholipids. Phospholipase A from bee venom degraded phospholipids of intact cells which suggests that at least part of the phospholipid

molecules of the outer membrane are exposed on the external cell surface. On the other hand, phospholipase C from *B. cereus* acted very weakly on intact cells, though it effectively hydrolyzed the phospholipids of isolated outer and cytoplasmic membranes. Very similar results were reported for human erythrocytes. Phospholipase C from *B. cereus* did not degrade phospholipids in intact erythrocytes, but was very active on phospholipids of ghosts, while phospholipase A_2 from bee venom, sea snake and *Naja naja* acted on phosphatidylcholine in intact erythrocytes, as well as in ghosts [21]. Zwaal [22] suggests that the ability of the different phospholipases to exert their action depends strongly on the packing of the lipid in the native membrane. Accordingly it can be suggested that the phospholipids in the outer membrane of intact *P. mirabilis* cells are too tightly packed to be attacked by phospholipase C and that the isolation of the membrane may cause the relaxation of this tight packing, thus enabling the action of this enzyme. One cannot rule out, however, the possibility that the susceptibility of intact *P. mirabilis* cells to bee venom phospholipase A was due to the presence of the lytic polypeptide, melittin, shown previously [23] to induce a disruption of the phospholipid matrix.

Our study shows that *P. mirabilis* resembles other Gram-negative bacteria in possessing a very potent endogenous phospholipase A activity, associated with the outer membrane. Fortunately, this enzymic activity is triggered only when the structure of the cell envelope is affected [24, 25], so that it hardly interferes with the determination of the activity of exogenous phospholipases on intact cells. However, the endogenous activity became quite pronounced on the isolation of the membranes, somewhat blurring the results of the treatment of these membranes by exogenous phospholipases. Although the endogenous phospholipase A activity of *P. mirabilis* has not been investigated in detail, our data suffice to indicate its resemblance to the endogenous phospholipase A activity of *E. coli* and *Salmonella typhimurium* in being highly heat-stable and in being compartmentalized in the outer membrane [26–29].

In conclusion, our data suggest that the protein and phospholipid components of the outer membrane are partially shielded on the external cell surface of *P. mirabilis* and are thus protected from degradation by certain proteolytic and lipolytic enzymes. This shield very probably consists of the long polar carbohydrate chains of the lipopolysaccharide molecules assumed to produce a "picket fence" on the external cell surface [1]. The high content of uronic acids in the *P. mirabilis* lipopolysaccharides [30, 31] may be expected to increase the effectiveness of this "picket fence" barrier, by contributing to it a pronounced negative charge. Our efforts to test the validity of this hypothesis by the removal of the carbohydrate chains on the cell surface with commercially available carbohydrases have been unsuccessful so far. Another approach to test this hypothesis can be based on the use of rough (R) mutants in which the carbohydrate chains of the lipopolysaccharide molecules are partially missing. Kotelko et al. [30] recently succeeded in isolating such R-mutants from *P. mirabilis*. Comparison of the susceptibility to proteolytic and lipolytic digestion of intact cells from these mutants with that of cells from the smooth *P. mirabilis* strain employed in our study may be expected to resolve the question of whether or not the negatively charged carbohydrate chains of the lipopolysaccharide on the *P. mirabilis* cell surface are responsible for the shielding effect.

ACKNOWLEDGEMENTS

We thank Dr. I. Kahane for his valuable advice. This work was supported by a grant from the Stiftung Volkswagenwerk.

REFERENCES

1 Costerton, J. W., Ingram, J. M. and Cheng, K. J. (1974) Bacteriol. Rev. 38, 87–110
2 Salton, M. R. J. (1967) Annu. Rev. Microbiol. 21, 417–442
3 Hasin, M., Rottem, S. and Razin, S. (1975) Biochim. Biophys. Acta 375, 381–394
4 Rottem, S., Hasin, M. and Razin, S. (1975) Biochim. Biophys. Acta 375, 395–405
5 Hubbard, A. L. and Cohn, Z. A. (1972) J. Cell Biol. 55, 390–405
6 Amar, A., Rottem, S., Kahane, I. and Razin, S. (1976) Biochim. Biophys. Acta, 426, 258–270
7 Phillips, D. R. and Morrison, M. (1970) Biochem. Biophys. Res. Commun. 40, 284–289
8 Amar, A., Rottem, S. and Razin, S. (1974) Biochim. Biophys. Acta 352, 228–244
9 Fairbanks, G., Steck, T. L. and Wallach, D. F. H. (1971) Biochemistry 10, 2606–2617
10 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
11 Rottem, S., Hasin, M. and Razin, S. (1973) Biochim. Biophys. Acta 323, 520–531
12 Freeman, C. P. and West, D. (1966) J. Lipid Res. 7, 324–327
13 Rottem, S., Stein, O. and Razin, S. (1968) Arch. Biochem. Biophys. 125, 46–56
14 Razin, S., Markowitz, O., Hasin, M. and Rottem, S. (1976) Biochim. Biophys. Acta 433, 240–251
15 Westphal, O., Lüderitz, O. and Bister, F. (1952) Z. Naturforsch. 7b, 148–155
16 Mühlradt, P. F. and Menzel, J. (1974) Eur. J. Biochem. 43, 533–539
17 Altman, P. L. and Dittmer, D. S. (1964) Biology Data Book, pp. 284–286, Fed. Am. Soc. Exp. Biol., Washington
18 Morowitz, H. J. and Terry, T. M. (1969) Biochim. Biophys. Acta 183, 276–294
19 Bragg, P. and Hou, C. (1972) Biochim. Biophys. Acta 274, 478–488
20 Inouye, M. and Yee, M. (1972) J. Bacteriol. 112, 585–592
21 Zwaal, R. F. A., Roelofsen, B. and Colly, C. M. (1973) Biochim. Biophys. Acta 300, 159–182
22 Zwaal, R. F. A. (1974) Biochem. Soc. Trans. 2, 821–825
23 Williams, J. C. and Bell, R. M. (1972) Biochim. Biophys. Acta 288, 255–262
24 Duckworth, D. H., Bevers, E. M., Verkleij, A. J., Op Den Kamp, J. A. F. and van Deenen, L. L. M. (1974) Arch. Biochem. Biophys. 165, 379–387
25 Audet, A., Nantel, G. and Proulx, P. (1974) Biochim. Biophys. Acta 348, 334–343
26 Bell, R. M., Mavis, R. D., Osborn, M. J. and Vagelos, P. R. (1971) Biochim. Biophys. Acta 249, 628–635
27 Scandella, C. J. and Kornberg, A. (1971) Biochemistry 10, 4447–4456
28 Osborn, M. J., Gander, J. E., Parisi, E. and Carson, J. (1972) J. Biol. Chem. 247, 3962–3972
29 Albright, F. R., White, D. A. and Lennarz, W. J. (1973) J. Biol. Chem. 248, 3968–3977
30 Kotelko, K., Gromska, W., Papierz, M., Szer, K., Krajewska, D. and Sidorczyk, Z. (1974) J. Hyg. Epidemiol. Microbiol. Immunol. 18, 405–410
31 Gmeiner, J. (1975) Eur. J. Biochem. 58, 621–626