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CHARACTERIZATION OF THE MYCOPLASMA MEMBRANE PROTEINS IV. DISPOSITION OF PROTEINS IN THE MEMBRANE

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SUMMARY

1. The disposition of proteins in membranes of *Acholeplasma laidlawii* and *Mycoplasma hominis* was studied by several techniques based on the use of proteolytic enzymes and labeling agents combined with electrophoretic analysis of membrane polypeptides in polyacrylamide gels containing sodium dodecylsulfate.

2. Over 15 different polypeptide bands were detected in electrophorograms of *A. laidlawii* and *M. hominis* membranes, ranging in molecular weight from 30 000 to over 200 000. The electrophoretic pattern of *M. hominis* was dominated by a polypeptide band (mol. wt 110 000) estimated according to staining intensity to account for over 50% of the total membrane protein.

3. The labeling intensity of isolated membranes by the lactoperoxidase-mediated iodination technique exceeded that of membranes of intact cells by a factor of 2.9–3.3 for *A. laidlawii* and 2.2–2.4 for *M. hominis*. Only 5–8% of the label was associated with cytoplasmic proteins. Electrophoretic analysis showed only a few of the membrane polypeptides to be labeled on treatment of intact cells as against the labeling of almost all the polypeptide bands on treatment of isolated membranes.

4. The use of the diazonium salt of [^{35}S]sulfanilic acid for the selective labeling of the proteins exposed on the outer membrane surface failed as the salt penetrated into the cells and labeled also the cytoplasmic proteins.

5. The results of the simultaneous digestion of intact cells and isolated membranes by trypsin and papain corroborated the iodination data in that only a few polypeptide bands, mainly of high molecular weight, disappeared on treatment of intact cells as against the disappearance of most of the polypeptide bands on treatment of isolated membranes. Pronase was found to be inadequate for disposition studies since it damaged membrane permeability and digested most effectively membrane polypeptides on both membrane sides when intact cells were treated. The peptides retained in the membrane after prolonged pronase treatment were poorly labeled by the lactoperoxidase-mediated iodination, indicating their localization within the membrane lipid matrix.

6. Our data point to an asymmetrical distribution of the proteins in mycoplasma membranes, with more proteins facing the cytoplasm than the exterior of the cell. The highest-molecular weight proteins in both membranes are apparently located

on the exterior surface while some of the major proteins may be embedded in the membrane, projecting into the cell interior, or even span the membrane, as may be the case with the major membrane protein of *M. hominis*.

INTRODUCTION

Mycoplasma membranes proved to be extremely useful tools in the elucidation of the molecular organization of lipids in biomembranes. Thus, biophysical analyses of mycoplasma membranes differing in fatty acid composition and cholesterol content [1–7] contributed much of the experimental support for the lipid-bilayer concept on which current membrane models are based [8, 9]. As against the extensive knowledge of the lipid component of mycoplasma membranes, little is known about the properties and mode of organization of the mycoplasma membrane proteins though they comprise about two-thirds of the total membrane dry weight [8]. The data available on the disposition of proteins in mycoplasma membranes are rather fragmentary. Thus, the ability to remove about 80% of the *Acholeplasma laidlawii* membrane polypeptides by pronase digestion was taken to suggest that the bulk of the membrane protein is exposed on the membrane surfaces while the pronase-resistant polypeptides are buried within the lipophilic membrane interior [10]. The finding of numerous particles, consisting apparently of protein, on the fracture faces of mycoplasma membranes [11–13] may also be taken in support of the idea that part of the protein is embedded in the membrane interior. Recent studies in our laboratory indicate that the ATPase and NADH oxidase of *A. laidlawii* are located on the inner surface of the membrane facing the cytoplasm [14].

The aim of the present study was to investigate more systematically the disposition of proteins in the *A. laidlawii* membrane and in the membrane of *Mycoplasma hominis*, which has been the subject of some of our more recent studies [15–17]. For this purpose we applied some of the newly developed techniques for the selective labeling or degradation of the proteins exposed on the outer membrane surface [18, 19]. The results obtained support the asymmetrical disposition of the proteins in mycoplasma membranes, with more proteins facing the cytoplasm than the exterior of the cell.

MATERIALS AND METHODS

Organisms and growth conditions

A. laidlawii (oral strain) and *M. hominis* (ATCC 15056) were grown statically in a modified Edward medium [20]. To grow *M. hominis*, the medium was supplemented with 20 mM L-arginine and adjusted to pH 6.5. The organisms were harvested after 16–18 h of incubation at 37 °C and washed twice in the cold with 0.25 M NaCl.

Assessment of leakiness of washed cells

For these experiments the growth medium was supplemented with 100 μCi [^{3}H]thymidine (13 Ci/mmole, Nuclear Research Center, Negev, Israel). The organisms were washed and suspended in the various media listed in Table I. 1-ml samples were withdrawn at various periods of time up to 120 min of incubation at 37 °C and cen-

trifuged at 12 000 $\times g$ for 20 min. The supernatant fluids were tested for radioactivity, and in the case of *A. laidlawii* also for hexokinase (glucokinase) activity, as previously described [21]. Radioactivity was also determined in the sedimented cells before and after extraction with cold 10% trichloroacetic acid [22] using a Packard Tri-Carb liquid scintillation spectrometer and a dioxane–toluene scintillation liquor [23].

Isolation of cell membranes

Cell membranes were isolated by osmotic lysis of the organisms [15,23]. The membranes were washed with deionized water, then with 0.05 M NaCl in 0.01 M phosphate buffer, pH 7.5, and again with deionized water. The washed membranes were resuspended in 0.25 M NaCl and the amount of protein in the suspension was determined according to Lowry et al. [24].

Proteolytic digestion

Suspensions of whole cells or isolated membranes prepared in 0.4 M sucrose (each containing 3 mg membrane protein/ml) were treated with varying concentrations of pronase (A grade, Calbiochem, Los Angeles, Calif.) or 50 μg/ml of either trypsin (2 times crystallized, Nutritional Biochemicals Corporation, Cleveland, Ohio) or papain (3 times crystallized, Mann Research Laboratories, New York, N.Y.) for periods ranging from 10 to 120 min at 37 °C. At the end of the incubation period the suspension was diluted with 20 vol. of cold 0.25 M NaCl and immediately centrifuged in the cold. The pellets were washed in 0.25 M NaCl and in the case of cells osmotic lysis was carried out at this stage to isolate the cell membranes. The degree of the proteolytic digestion was assessed by comparing the Lowry-reactive material in the treated and the native membranes.

Iodination procedure

The procedure of Phillips and Morrison [25] was followed with some modifications. The reaction mixture (2 ml) contained washed cells or isolated membranes (1.5 mg membrane protein/ml) suspended in a 0.25-M NaCl solution containing 0.05 M phosphate buffer, pH 7.5, 0.2 ml of a partially purified preparation of lactoperoxidase, 20 μCi K^{125}I in 10 μM KI. After 15 min incubation at 37 °C the reaction was terminated by dilution with cold 0.25 M NaCl and immediate centrifugation. The iodinated cells or membranes were washed with 0.25 M NaCl. The cells were osmotically lysed and the membranes were separated from the cytoplasmic fluid. Aliquots of the cell-membrane preparations and cytoplasmic fluid were taken for radioactivity measurements in a Packard Auto Gamma spectrometer.

Labeling by the diazonium salt of sulfanilic acid

The diazonium salt of [^{35}S]sulfanilic acid (10 Ci/mmole, The Radiochemical Centre, Amersham, England) was prepared immediately before use [26]. The diazonium salt was added to a final concentration of 3 mM to cell or membrane suspensions (containing 3 mg membrane protein/ml) in a 0.25-M NaCl solution containing 0.05 M phosphate buffer, pH 7.5. After 20 min of incubation at 37 °C the cells were sedimented by centrifugation. The sedimented cells were washed in 0.25 M NaCl, osmotically lysed and the cell membranes were separated by centrifugation. Radioactivity was determined in both membranes and cytoplasmic fluid.

Gel electrophoresis

The sodium dodecylsulfate gel electrophoresis system of Weber and Osborn [27] was employed with some variations. Membrane proteins were solubilized by boiling for 2 min in 0.01 M sodium phosphate buffer, pH 7.0, containing 1% sodium dodecylsulfate and 1% 2-mercaptoethanol. The solution was centrifuged at 27 000 $\times g$ for 15 min to remove non-solubilized material, the amount of which was usually negligible, in particular with *A. laidlawii* membranes. Samples (100–200 μg protein) were put on 5% or 7.5% polyacrylamide gels (0.6 mm $\times$ 80 mm) containing 0.1% sodium dodecylsulfate. The conditions of electrophoresis and staining were as described by Weber and Osborn [27]. For destaining, the gels were incubated for 48 h at 37 °C with shaking in a solution of 7.5% acetic acid and 5% methanol. The destaining solution was frequently changed during this period. Densitometer tracings of the stained gels were made in a Kipp and Zonen Densitometer model DD2. When radioactive iodine-labeled membrane proteins were analyzed, pairs of identical gels were prepared; one was stained and the other was sliced laterally into 2-mm sections which were counted in a Packard Auto Gamma spectrometer. The molecular weight of the membrane polypeptides was estimated by comparing to a calibration curve in which the logarithm of the molecular weights of standard proteins was plotted against the migration distance. The calibration curve prepared with bovine serum albumin (mol. wt 67 000), ovalbumin (mol. wt 45 000), chymotrypsinogen A (mol. wt 25 000) and lysozyme (mol. wt 14 400) showed all the points to fall on a straight line.

RESULTS

Leakiness of washed cells

The procedures used for studying the disposition of membrane proteins require agents which cannot penetrate the membrane of the intact cell. Hence, in order to obtain meaningful results, the permeability barrier of the cell should remain intact throughout the application of the labeling agent. Leakage of small solutes and macromolecules from mycoplasma cells suspended in the different labeling media was therefore examined. Release of hexokinase from the glycolytic *A. laidlawii* was taken to indicate the permeation of macromolecules through the cell membrane, and the release of thymidine-labeled components served as an indicator for the permeation of nucleotides and DNA. Table I shows that the release of hexokinase and thymidine-containing compounds from the cells suspended in the lactoperoxidase iodination medium was about the same as in the conventional suspension media for mycoplasmas, such as 0.25 M NaCl or 0.4 M sucrose. On the other hand, the presence of the diazonium salt caused a marked increase in the release of hexokinase and the thymidine-labeled compounds, indicating damage to the permeability barrier of the cell. Treatment with proteolytic enzymes, mainly pronase, increased leakage of thymidine-labeled compounds, but the values of hexokinase released were low. The low values reflect apparently the inactivation of the released enzyme by proteolysis. The values presented in Table I were obtained after 30 min of incubation at 37 °C. Lengthening the incubation period to 2 h did not significantly increase these values. Extraction of washed thymidine-labeled mycoplasma cells with cold 10% trichloroacetic acid released about 10% of the total radioactivity accounting apparently for

TABLE I

RELEASE OF [^{3}H]THYMIDINE-LABELED MATERIAL AND HEXOKINASE ACTIVITY FROM WASHED CELLS SUSPENDED IN DIFFERENT MEDIA

Organisms grown in the presence of [^{3}H]thymidine were harvested and washed in 0.25 M NaCl. Suspensions of the washed cells (3 mg cell protein/ml) were prepared in the different media, incubated at 37 °C for various periods of time and centrifuged. Hexokinase and radioactivity were determined in the supernatant fluids. The data presented in the table are those obtained after 30 min incubation at 37 °C.

Medium	*A. laidlawii*		*M. hominis*
	Radioactivity released (% of total)*	Hexokinase released (% of total)	Radioactivity released (% of total)*
NaCl (0.25 M)	8	4.9	12
NaCl (0.25 M)+phosphate (0.05 M)	8	4.8	9
NaCl (0.25 M)+phosphate (0.05 M)+ lactoperoxidase	8	5.3	8
NaCl (0.25 M)+phosphate (0.05 M)+ diazonium salt of sulfanilic acid (0.003 M)	25	14.0	28
Sucrose (0.4 M)	9	4.5	9
Sucrose (0.4 M)+trypsin (50 μg/ml)	14	3.5	16
Sucrose (0.4 M)+papain (50 μg/ml)	15	7.0	18
Sucrose (0.4 M)+pronase (20 μg/ml)	22	2.0	31
Deionized water	90	100.0	76

* Calculated from the values of total radioactivity in washed cells.

the intracellular pool of nucleotides. The higher values of radioactivity released from cells suspended in media containing the diazonium salt or proteolytic enzymes, in particular pronase (Table I) indicate the release of some high-molecular weight DNA from the cells in addition to nucleotides.

Electrophoretic characterization of membrane proteins

Fig. 1 shows the electrophoretic pattern of membrane polypeptides of *M. hominis* and *A. laidlawii* in polyacrylamide gels containing sodium dodecylsulfate. The pattern of *M. hominis* was consistently found to be more clearly resolved than that of *A. laidlawii*. Visual inspection of the gels revealed about 15 polypeptide bands with *A. laidlawii* membranes and over 20 bands with *M. hominis* membranes. The electrophoretic patterns were highly reproducible in different runs and with different batches of membranes. The designation of the polypeptide bands, their estimated molecular weight, and their relative quantity, assessed by their staining intensity, are listed in Table II. It can be seen that the molecular weights of the polypeptides range from 30 000 to over 200 000. The *M. hominis* pattern is dominated by a band (Band E, mol. wt 110 000) which according to its staining intensity accounts for approx. 55% of the total membrane protein. The electrophoretic pattern of the *A. laidlawii* membrane polypeptides showed a more even distribution of the stain, with five major bands each accounting for 10–20% of the total protein.

As membrane lipids were not removed prior to electrophoresis, their migration in the gels could be determined. For this purpose, membrane lipids were labeled by

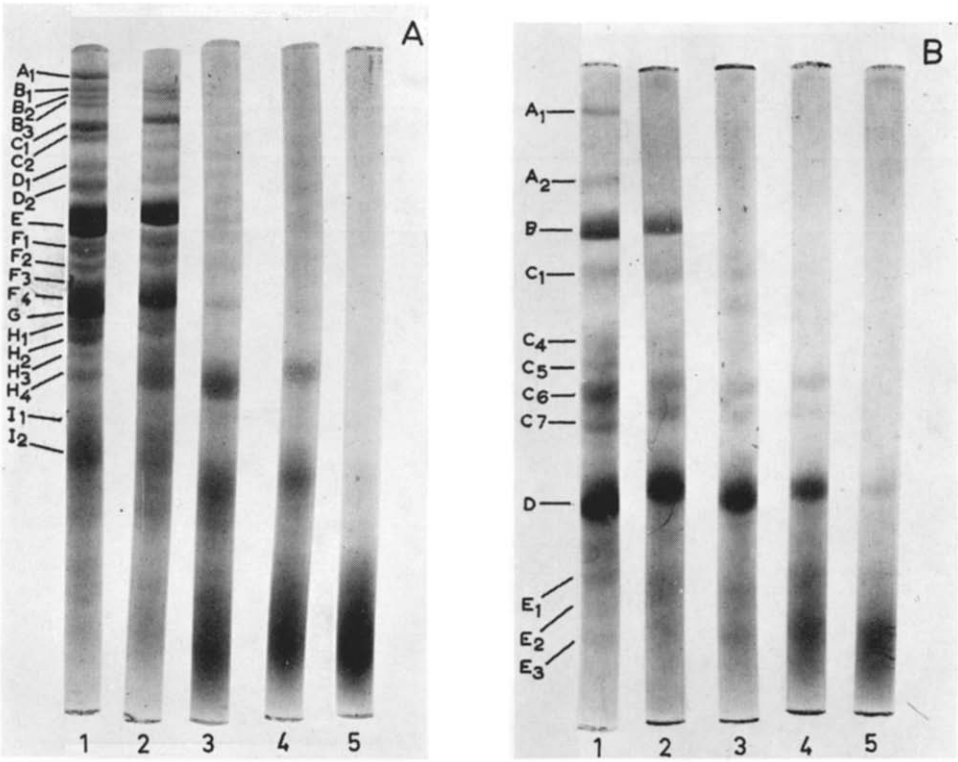

Fig. 1. Electrophoretic patterns of membrane polypeptides of *M. hominis* (A) and *A. laidlawii* (B) digested with pronase at 37 °C. 1, untreated membranes; 2, membranes treated with 2 μg pronase/ml for 10 min; 3, membranes treated with 2 μg pronase/ml for 2 h; 4, membranes treated with 20 μg pronase/ml for 10 min; 5, membranes treated with 20 μg pronase/ml for 2 h.

growing the organisms in the presence of radioactive oleic or palmitic acids [16, 23]. Electrophoretic analysis showed that over 90% of the labeled lipid migrated very close to the stain marking the front.

Susceptibility of membrane proteins to proteolytic enzymes

Table III shows the results of treatment of intact cells and isolated membranes with proteolytic enzymes. Of the enzymes tested, pronase was the most effective and papain the least. The percentage of membrane protein digested and released was consistently much higher when isolated membranes were treated. The susceptibility of the various membrane proteins to proteolytic digestion could be assessed by comparing the electrophorograms of treated and untreated membranes. In order to make the comparison of the band-staining intensity meaningful, the samples put on the gels were derived from the same quantity of membranes without adjusting the protein to correct for the loss of peptides by proteolytic digestion. In this way the staining intensity of the polypeptides which resisted digestion was not artificially increased. Fig. 1 shows that treatment of isolated membranes of both organisms with 20 μg pronase/ml for periods as brief as 10 min at 37 °C resulted in the disappearance of

TABLE II

POLYPEPTIDES OF *A. LAIDLAWII* AND *M. HOMINIS* MEMBRANES DETECTED BY POLYACRYLAMIDE GEL ELECTROPHORESIS

A. laidlawii			*M. hominis*		
Polypeptide band	Tentative mol. wt*	Percent of total**	Polypeptide band	Tentative mol. wt*	Percent of total**
A_1	215 000	1.7	A_1	200 000	1.8
A_2	145 000	2.1	B_1	187 000	0.7
B	120 000	19.3	B_2	185 000	0.7
C_1	98 000	10.0	B_3	180 000	0.7
C_2	88 000	1.9	C_1	167 000	4.0
C_3	85 000	2.4	C_2	157 000	2.2
C_4	78 000	5.9	D_1	140 000	1.7
C_5	71 000	4.1	D_2	128 000	4.0
C_6	68 000	11.2	E	110 000	55.4
C_7	57 000	10.6	F_1	105 000	0.5
C_8	50 000	1.3	F_2	95 000	1.3
D	44 000	20.1	F_3	91 000	2.4
E_1	42 000	2.9	F_4	83 000	2.1
E_2	33 000	2.1	G	77 000	10.3
E_3	31 000	3.6	H_1	70 000	0.5
			H_2	66 000	0.4
			I_1	44 000	3.6
			I_2	35 500	7.5

* Estimated according to a calibration curve prepared with standard proteins. Major polypeptides are underlined.

** Determined according to the peak areas on the densitometer tracings.

TABLE III

SUSCEPTIBILITY TO PROTEOLYTIC ENZYMES OF MEMBRANE PROTEINS OF INTACT CELLS AND ISOLATED MEMBRANES

Enzyme	Percent protein digested and released from membrane			
	A. laidlawii		*M. hominis*	
	Intact cells	Isolated membranes	Intact cells	Isolated membranes
Pronase (20 μg/ml; 2 h)	40	61	37	55
Trypsin (50 μg/ml; 2 h)	18	54	25	44
Papain (50 μg/ml; 2 h)	5	16	16	24

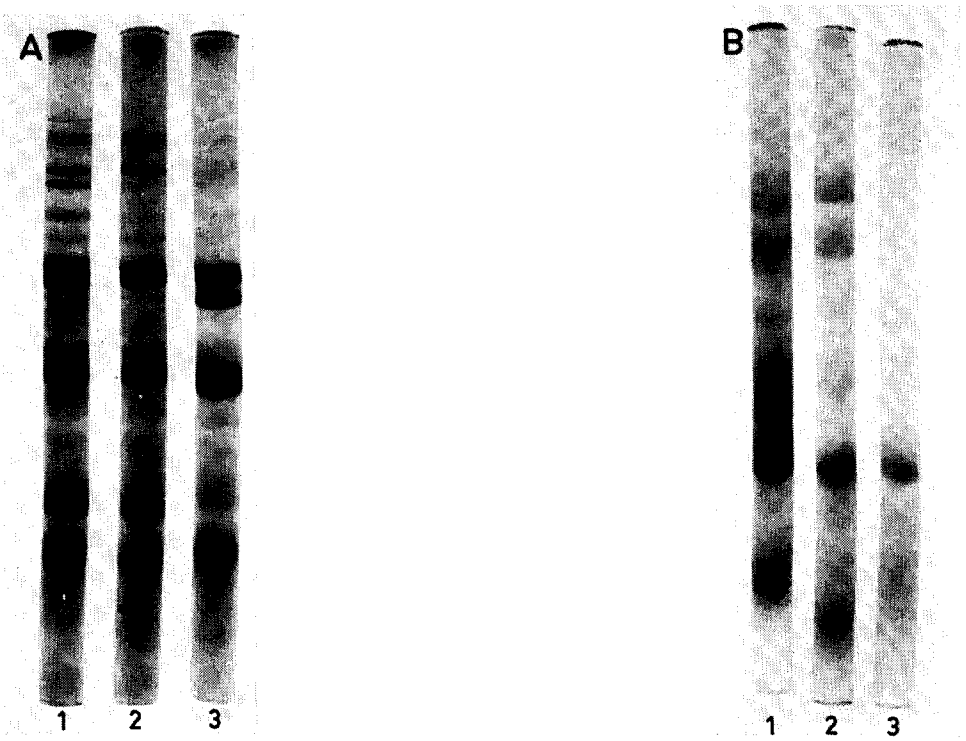

Fig. 2. Electrophoretic patterns of membrane polypeptides of *M. hominis* (A) and *A. laidlawii* (B) digested by 50 μg trypsin/ml for 2 h at 37 °C. 1, undigested membranes; 2, digestion carried out on intact cells; 3, digestion carried out on isolated membranes.

almost all the polypeptide bands which characterize the native membranes. The disappearance of the bands was accompanied by the accumulation of fast-moving, low-molecular weight peptides which are still associated with the membrane. It is of interest to note the relative resistance of Band D of *A. laidlawii* to proteolytic digestion (Fig. 1). Although a difference existed between the membrane-polypeptide patterns of pronase-treated cells and the isolated membranes, it could be detected only after short incubation periods with very low enzyme concentrations.

Because of its much slower and more selective activity trypsin was found to be preferable to pronase in this type of experiment. Fig. 2 shows the marked difference in the polypeptide patterns of membranes treated with trypsin before and after isolation from the cells. Treatment of *M. hominis* cells resulted in the disappearance of only a few bands in the high-molecular weight region (A_1, C_2, D_1, F_2 and B_1 in most cases), while the major E and G bands became weaker. Trypsin treatment of isolated membranes resulted in the disappearance of more bands. The stain intensity of Band E was weakened as occurred with treated cells but, unlike the findings with treated cells, Band G became more accentuated and a prominant band appeared in the region of F_1. The differences between the polypeptide patterns of membranes treated before or after their isolation from the cells were even more pronounced with *A. laidlawii* (Fig. 2). Band D, which resisted best pronase digestion (Fig. 1), showed also

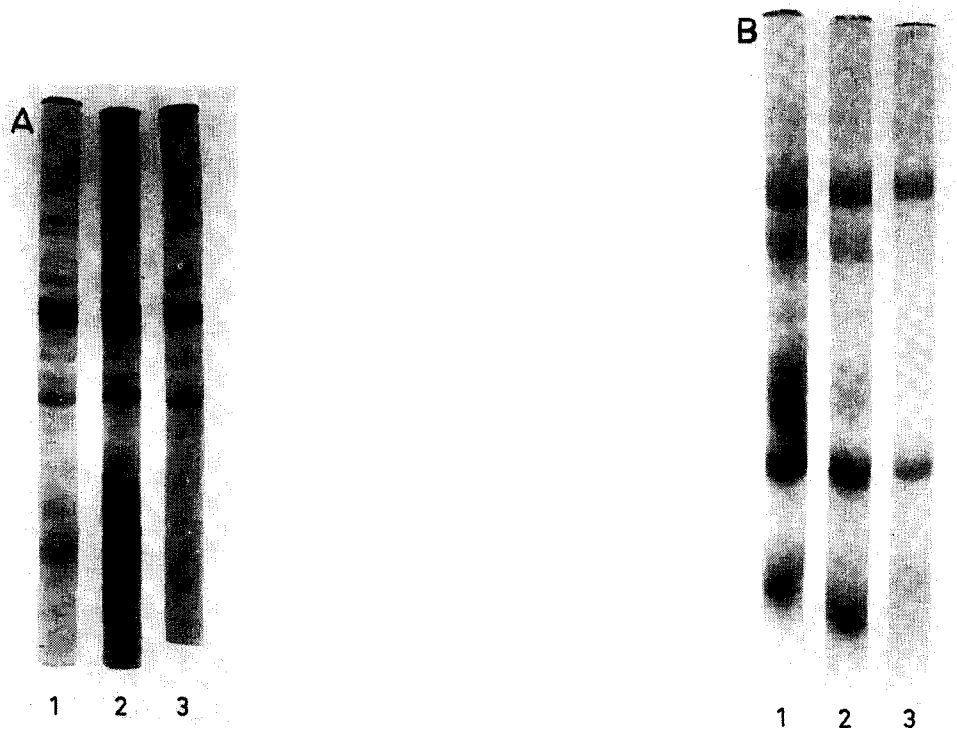

Fig. 3. Electrophoretic patterns of membrane polypeptides of *M. hominis* (A) and *A. laidlawii* (B) digested by 50 μg papain/ml for 2 h at 37 °C. 1, undigested membranes; 2, digestion carried out on intact cells; 3, digestion carried out on isolated membranes.

a marked resistance to trypsin.

Papain, which showed the lowest proteolytic activity on the mycoplasma membrane proteins (Table III) gave the results shown in Fig. 3. Again, more polypeptide bands disappeared from isolated membranes than from membranes of intact cells. However, the patterns obtained are different than those obtained with trypsin. There was no increase in the staining intensity of Band G and no appearance of any band in the region of F_1 in the electrophorogram of treated *M. hominis* membranes, and, unlike the results obtained with trypsin, Band B of the *A. laidlawii* pattern resisted digestion by papain.

Lactoperoxidase-mediated iodination and diazotized sulfanilic acid labeling of membrane proteins

The leakage experiments (Table I) indicated no significant damage to the permeability barrier of the cells incubated in the lactoperoxidase iodination mixture, supporting the use of this technique for the selective iodination of the proteins exposed on the outer membrane surface of mycoplasmas. When intact mycoplasma cells were iodinated by this technique, only a small fraction of the label (5–8%) was detected in the cytoplasmic-protein fraction (Table IV). On the other hand, the diazonium salt of sulfanilic acid damaged considerably the permeability barrier of the cells as

TABLE IV

LABELING OF MYCOPLASMA CELL FRACTIONS BY THE DIAZONIUM SALT OF [^{35}S]SULFANILIC ACID AND BY THE LACTOPEROXIDASE-MEDIATED IODINATION

Treatment with	Radioactivity in cell fractions (cpm/mg protein)					
	A. laidlawii			*M. hominis*		
	Whole cells	Cytoplasmic fluid	Membranes	Whole cells	Cytoplasmic fluid	Membranes
Lactoperoxidase + $K^{125}I$	90 100	6630	80 200	99 170	7580	87 800
Diazonium salt of [^{33}S]sulfanilic acid	96 100	38 400	58 200	138 400	48 200	91 900

evidenced by the marked release of cytoplasmic constituents (Table I) and by the high percentage of label found in the cytoplasmic proteins (Table IV).

The addition of H_2O_2 to the reaction mixture was not required for the iodination of the mycoplasma membrane proteins, as against its requirement for the iodination of the erythrocyte membrane proteins. Nevertheless, an increase in the H_2O_2 concentration above a certain level decreased iodination as it did with erythrocytes (see also ref. 25).

Table V compares the iodination of membrane proteins of intact cells and isolated membranes. The iodination values of the isolated membranes exceeded those of membranes of intact cells by a factor of 2.9–3.3 for *A. laidlawii* and by 2.2–2.4 for *M. hominis*. The label associated with membrane lipids only amounted to about 5% of the label bound to the proteins. It is of interest to note here that the amount of label bound to the lipids of isolated membranes was about twice as much as that bound to the lipids of intact cells (Table V).

Figs 4 and 5 show that only part of the membrane polypeptides were labeled when iodination was carried out on intact cells, whereas almost all the polypeptides were labeled when isolated membranes were treated. Of the polypeptides labeled in intact cells, those of highest molecular weight were labeled in both organisms.

TABLE V

LABELING OF MEMBRANES OF INTACT CELLS AND ISOLATED MEMBRANES BY THE LACTOPEROXIDASE-MEDIATED ^{125}I IODINATION

Preparation	Radioactivity in membrane components (cpm/mg protein)			
	A. laidlawii		*M. hominis*	
	Membrane protein	Membrane lipid*	Membrane protein	Membrane lipid*
Membranes of intact cells	223 000	14 700	176 600	11 800
Isolated membranes	689 200	30 300	413 300	21 500

* Extract from membranes containing 1 mg protein.

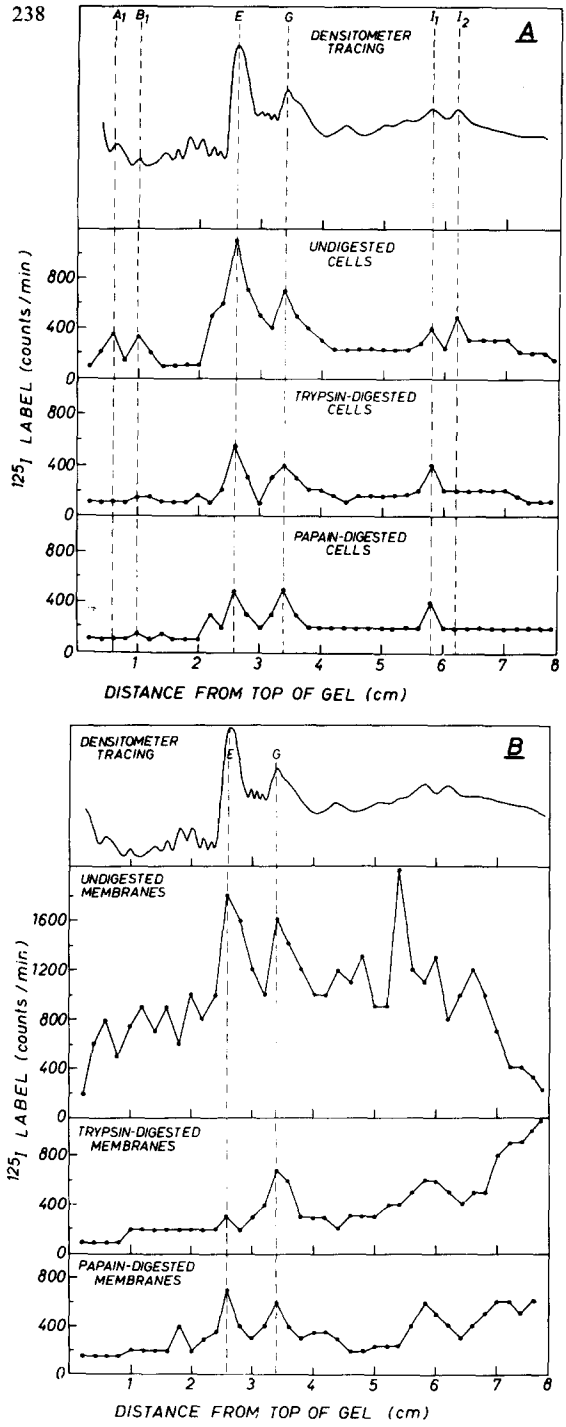

Fig. 4. Distribution of iodine label in *M. hominis* membrane polypeptides. (A) Intact cells were subjected to lactoperoxidase-catalyzed iodination followed by digestion with 50 μg trypsin/ml or 50 μg papain/ml for 2 h at 37 °C. The membranes were then isolated and solubilized in 1 % sodium dodecylsulfate and electrophoresed on 5 % polyacrylamide gels containing 0.1 % sodium dodecylsulfate. A densitometer tracing of the polypeptide pattern of a stained gel, including the designation of some bands of interest, is given at the top of the figure. (B) The same legend as for A but the iodination and proteolytic digestion were carried out on isolated membranes.

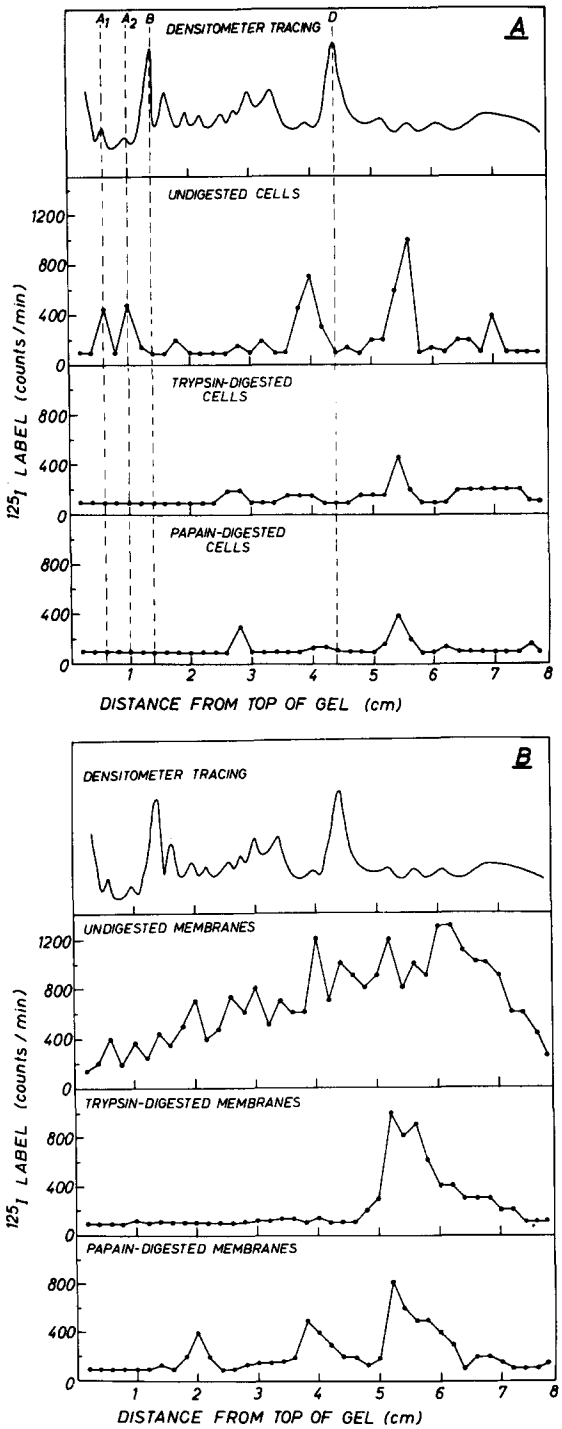

Fig. 5. Distribution of iodine label in *A. laidlawii* membrane polypeptides. Experimental details are the same as described under Fig. 4.

TABLE VI

COMPARISON OF LACTOPEROXIDASE-MEDIATED ^{125}I LABELING OF MEMBRANE PROTEINS DONE BEFORE AND AFTER PRONASE TREATMENT OF MEMBRANES

Treatment	*A. laidlawii*		*M. hominis*	
	Protein (mg)	Radioactivity (cpm/mg protein)	Protein (mg)	Radioactivity (cpm/mg protein)
Labeling with no digestion	3.05	1920	2.45	1315
Labeling followed by digestion*	0.72	440	0.26	300
Digestion followed by labeling	0.70	665	0.35	430

* Digestion by 200 μg pronase/ml at 45 °C for 18 h.

Iodine label was also found in the major polypeptide Bands E and G of *M. hominis* membranes (Fig. 4). However, in *A. laidlawii* the most intense labeling was not associated with the major polypeptide Bands B and D (Fig. 5).

When cells were labeled with iodine and then treated with trypsin or papain most of the label was removed from the membranes (Figs 4 and 5). The disappearance of label from the area of Bands A_1 and A_2 in *A. laidlawii* and A_1 and B_1 in *M. hominis* can be clearly seen. These are indeed the polypeptides which are digested by treatment of cells with the proteolytic enzymes (Figs 1 and 2). Nevertheless, part of the label is retained in other polypeptide bands suggesting their resistance to digestion by the enzymes.

The removal of label was more pronounced when isolated membranes were treated with trypsin or papain. Following digestion with trypsin, the residual label was found to be concentrated mainly in the area of the low-molecular weight polypeptides (Figs 4 and 5). The less effective papain left some bands labeled even at the region of the high-molecular weight polypeptides.

Table VI shows that the pronase-resistant Lowry-positive material, consisting apparently of low-molecular weight peptides (Fig. 1), is labeled to a much lesser degree than the total membrane protein. Essentially similar results were obtained when treatment with pronase preceded or followed iodination.

DISCUSSION

The membrane polypeptides of *A. laidlawii* and *M. hominis* exhibit a highly reproducible and species-specific pattern on electrophoresis in polyacrylamide gels containing sodium dodecylsulfate, in accord with our previous data [28] obtained with the acidic gel system of Takayama et al. [29]. Electrophoresis in the presence of sodium dodecylsulfate gives a fairly reliable estimate of the molecular weights of proteins [27]. According to our data the molecular weights of membrane polypeptides of *A. laidlawii* range from 31 000 to 215 000, differing from the range of 15 000 to 140 000 reported by Hjertén and Johansson [30]. The lower values of Hjertén and

Johansson may possibly be due to proteolytic digestion of membrane proteins during their preparation for electrophoresis by the endogenous peptidase(s) located in *A. laidlawii* membranes [31, 32]. The disappearance of shorter polypeptides due to endogenous proteolysis activated by the detergents used for membrane solubilization has been emphasized as a major cause for discrepancies in molecular-weight determinations by sodium dodecylsulfate gel electrophoresis [33]. The range of molecular weights of membrane polypeptides of *M. hominis* resembled that of *A. laidlawii*. However, the electrophoretic pattern of *M. hominis* was dominated by a band (Band E) estimated to comprise about one-half of the total membrane polypeptides. This is a very rough estimate based only on the relative amount of stain in the band and all the reservations raised against determining protein content in gels according to staining intensity [34, 35] are also valid in this case.

The main objective of our study was to test for an asymmetrical or vectorial arrangement of polypeptides in mycoplasma membranes. This was done by comparing the labeling and sensitivity of the polypeptides to proteolytic enzymes in intact cells and isolated membranes, on the assumption that the labeling agent or proteolytic enzyme will have access to polypeptides exposed on both membrane surfaces when isolated membranes are treated and only to polypeptides exposed on the outer membrane surface when intact cells are treated. The validity of this assumption depends on the fulfillment of several conditions: (a) that the labeling agent or enzyme is incapable of penetrating into the cells; (b) that the isolated membranes do not reseal; (c) that the disposition of polypeptides in isolated membranes does not significantly differ from that in membranes of intact cells. With regard to the first condition, the tendency of washed mycoplasmas to lose intracellular components and even lyse on prolonged incubation in non-nutrient media at 37 °C [36, 37] could invalidate the above assumption where mycoplasmas are concerned. Indeed, the experiments devised to test this point showed that some of the agents, in particular the diazonium salt of sulfanilic acid and pronase, markedly damage the permeability barrier of the cell, imposing serious restrictions on their use in disposition studies. The second condition appears to be fulfilled since mycoplasma membranes do not usually reseal after their isolation (see Fig. 4 in ref. 23). It is much more difficult, if not impossible at this stage, to state that no significant changes in protein disposition occur during the isolation of mycoplasma membranes.

Of the proteolytic enzymes used in our study, pronase appears to be the least suitable agent for investigating protein disposition because it caused the greatest damage to cell permeability (Table I). There is little doubt that even at low concentrations and rather short incubation periods this enzyme will penetrate into the cells and hydrolyze also the membrane polypeptides facing the cytoplasm. On the other hand, pronase has an advantage over the other proteolytic enzymes in being nonspecific in its activity, capable of hydrolyzing all membrane proteins. Thus, the varying rates of disappearance of certain membrane polypeptides during pronase treatment (Fig. 1) may indicate their different disposition rather than differences in their inherent resistance to digestion, as may well be the case with trypsin and papain. It is clear that digestion of the membrane polypeptides by pronase leads to their fragmentation to small peptides, a large portion of which is still associated with the membrane. Thus, the digested membrane may still retain about 50% of its Lowry-reactive material, while none of the original polypeptide bands can be seen in the gel.

Furthermore, prolonged pronase treatment results in clear gels, although the membranes still contain about 20% of the Lowry-reactive material. Hence, the peptides produced by prolonged pronase treatment are so small as not to be retained in the gels (see also ref. 38). The idea that these small fragments of the original polypeptide chains are buried within the lipid matrix of the membrane [10, 39, 40] is supported by their poor labeling by the lactoperoxidase iodination system (Table VI).

Although cell leakiness was somewhat increased by trypsin and papain, the consistent and reproducible differences in the electrophoretic patterns of membrane polypeptides of intact cells and isolated membranes treated with these two enzymes appear to exclude any significant permeation of the enzyme molecules into the cells. The small leakage values noted with trypsin and with papain may be attributed to the lysis of a small percentage of the cells rather than to increased permeability in all the cell population.

Of the two labeling procedures tested, the lactoperoxidase-mediated iodination procedure of Phillips and Morrison [25] was found to be suitable for mycoplasmas while labeling with the diazonium salt of sulfanilic acid [41] was found to be inappropriate due to either the fast penetration of the diazonium salt into the cells or to the induction of cell lysis, as was also found for erythrocytes [41]. Our finding that no H_2O_2 need be added for the iodination of mycoplasma membranes, as against the finding with erythrocytes is probably due to the production of H_2O_2 by the catalase-deficient electron-transport chain of mycoplasma membranes [8].

Although it is too early to classify all the detectable mycoplasma membrane-polypeptide bands as belonging to proteins located on the inner or outer membrane surfaces or within the membrane matrix, several tentative conclusions can already be made. The distribution of membrane polypeptides on the two membrane surfaces is asymmetrical as was also shown for erythrocytes [18, 19]. The fact that the iodination values of isolated membranes were between 2.5 and 3.0 times higher than in membranes of intact cells, depending on the mycoplasma species, indicates that a significantly higher percentage of membrane polypeptides are exposed on the inner membrane surface. Moreover, the different distribution of label among the polypeptide bands when iodination was carried out on cells or on isolated membranes, as well as the different susceptibility of membrane polypeptides to proteolytic digestion of intact cells or isolated membranes, also supports the asymmetry in polypeptide distribution.

The higher-molecular weight polypeptides (Bands A_1 and A_2 in *A. laidlawii* and A_1 and B_1 in *M. hominis*) are apparently located on the exterior membrane surface. This conclusion is based on their labeling by iodination of intact cells and on their susceptibility to hydrolysis on treatment of cells by trypsin or papain. The labeling and proteolysis data strongly suggest that none of the major membrane polypeptides of *A. laidlawii* (Bands B, C_1, C_6, C_7 and D) is located on the exterior cell surface. Yet, one cannot rule out the possibility that these proteins are indeed located on the outer membrane surface but are masked by the hexosamine polymer known to cover the cell surface of *A. laidlawii* [42, 43]. Band D of *A. laidlawii* apparently is partially or entirely embedded within the membrane since it resists digestion by trypsin and papain and even pronase digests it rather slowly (Fig. 1). The major Band E of *M. hominis* may constitute a protein which spans the membrane since it is only partially digested when intact cells or isolated membranes are treated

with trypsin or papain. Moreover, its labeling is more intense when isolated membranes are iodinated as compared to intact cells. The estimated molecular weight (110 000) of this polypeptide is large enough to account for a protein which extends through the cell membrane (see ref. 44).

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