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IS THE VERTICAL DISPOSITION OF MYCOPLASMA MEMBRANE PROTEINS AFFECTED BY MEMBRANE FLUIDITY?

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Summary

The influence of the physical state of the membrane lipid matrix on the vertical disposition of membrane proteins was studied with *Acholeplasma laidlawii*. Changes in membrane fluidity were brought about by altering the fatty acid composition of membrane lipids, by changing the growth temperature, by aging of cultures and by inducing changes in the membrane lipid-to-protein ratio through treatment with chloramphenicol. The lactoperoxidase-mediated iodination technique was used to label membrane proteins exposed to the aqueous surroundings. The degree of exposure of the iodine-binding sites of membrane proteins on the external surface of intact cells was found to undergo significant changes on varying growth conditions, but the changes could not be consistently correlated with changes in membrane fluidity, nor were they discernible on iodination of isolated membranes.

Introduction

The asymmetrical transbilayer distribution of membrane proteins and the dependence of their lateral and rotational mobility on the physical state of membrane lipids are well established properties of biomembranes, including those of mycoplasmas [2—5]. An interesting question is whether the physical state of the membrane lipid matrix also influences the vertical disposition of the proteins immersed in it. Borochov and Shinitzky [6] have recently postulated that the position of the amphipathic proteins in the membrane reflects an equilibrium state between the interactions of their hydrophobic parts with membrane lipids and their hydrophilic parts with the aqueous surroundings. Accordingly, with decreasing lipid fluidity, the interaction of the

hydrophobic parts of the proteins with the lipids will diminish, resulting in the squeezing-out of the proteins which will then occupy a new equilibrium position. Obviously the opposite vectorial displacement will occur on increasing the lipid fluidity. To support their thesis, Borochov and Shinitzky [6] brought evidence from experiments with human red blood cells in which membrane proteins on the cell surface were labeled with fluorescent sulfhydryl reagents. Changes in membrane fluidity introduced by altering their cholesterol content resulted in changes in the intensity of fluorescence, interpreted as reflecting changes in the vertical displacement of membrane proteins.

The ease of introducing controlled alterations in mycoplasma membrane fluidity [4,5] prompted us to study this problem with *Acholeplasma laidlawii.* Changes in membrane fluidity were brought about in several ways: by altering the fatty acid composition of membrane lipids [7,8], by changing the growth temperature [9,10], by aging of cultures [11] and by inducing changes in the membrane lipid-to-protein ratio by treatment with chloramphenicol [12]. The lactoperoxidase-mediated iodination technique was chosen to label the membrane proteins that are exposed to the aqueous surroundings. This technique has been widely used to study the disposition of membrane proteins in mycoplasmas [13—16]. The results reported in this communication indicate that the degree of exposure of the iodine-binding sites of membrane proteins on the cell surface changes on varying the growth conditions, but these changes could not be consistently correlated with changes in membrane fluidity.

Materials and Methods

Organisms and membranes. A. laidlawii (oral strain) was grown in a modified Edward medium containing 3% (v/v) horse serum [17]. To change the fatty acid composition of membrane lipids, the serum component of the medium was replaced by 0.5% (w/v) of lipid-depleted bovine serum albumin and 25 μg/ml of either oleic acid or elaidic acid [17]. The pH of the medium was adjusted to 8.5. Growth was followed by absorbance measurements at 640 nm. The organisms were harvested, washed once and resuspended in 0.25 M NaCl. Cell membranes were isolated by osmotic lysis, washed as previously described [17] and resuspended in 0.25 M NaCl. The density of the washed membranes was determined by sucrose density gradient centrifugation [11].

Iodination and proteolytic digestion of cells and membranes. Lactoperoxidase-mediated iodination of membrane proteins was carried out on washed cells and isolated membranes as previously described [15]. Proteolytic digestion of cells and membranes was carried out by applying 50 μg/ml trypsin (twice crystallized, Sigma, St. Louis, MO, U.S.A.) for 2 h at 37°C to suspensions of cells or membranes in 0.4 M sucrose [15].

Analytical procedures. Protein was determined according to Lowry et al. [18]. Lipids were extracted from cells with chloroform/methanol (2 : 1, v/v) for 2 h at 45°C. Lipid phosphorus was determined in the lipid extract according to Ames [19]. Radioactivity of ^{14}C-labeled cells and lipid extracts was determined in a Packard Tri-Carb liquid scintillation spectrometer using a toluene-Triton scintillation mixture [20]. The fatty acid composition of membrane lipids was determined by gas-liquid chromatography [21].

Electrophoresis of membrane proteins was performed in polyacrylamide gels

containing sodium dodecyl sulfate [15]. 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.

Electron paramagnetic resonance spectroscopy. Membranes were spin-labeled with a *N*-oxyl-4',4'-dimethyloxazolidine derivative of 12-ketostearic acid (Syva, Palo Alto, CA, U.S.A.) by exchange from bovine serum albumin [9]. Electron paramagnetic resonance spectra of the spin-labeled membranes were obtained by the use of a Varian E-4 spectrometer. The molecular motion was assessed from τ_0, an empirical motion parameter calculated according to Henry and Keith [22]. Greater freedom of motion, indicating higher fluidity, is associated with smaller values of τ_0.

Enzyme assays. Thioesterase (palmitoyl-CoA hydrolase, EC 3.1.2.2) activity was tested according to Rottem et al. [23] using palmitoyl-CoA as substrate. Activity was expressed as nmol CoA released/min per mg protein. Adenosine triphosphatase (EC 3.6.1.3) activity was measured by release of inorganic phosphate from ATP [24]. Results were expressed as μmol inorganic phosphate released/mg protein in 30 min.

Results

Effects of culture age

The progress of *A. laidlawii* cultures from the mid- to the late-logarithmic phase of growth was accompanied by changes in some of the properties of the cell membrane (Table I). The ratio of membrane lipid to protein decreased, leading to an increase in membrane density. Membrane fluidity decreased as indicated by increasing values of τ_0. The ATPase and thioesterase activities, known to be influenced by membrane fluidity [23,25] decreased on aging.

Electrophoretic patterns of membrane proteins did not discernibly change on aging, apart from a decrease in the staining intensity of one protein band (Fig. 1). Table II shows that the iodination values of membrane proteins exposed on the cell surface decreased on aging by about 25%, accompanied by

TABLE I

CHANGES IN MEMBRANE COMPOSITION IN AGING *A. LAIDLAWII* CULTURES *

Age of culture (h)	Absorbance of culture at 640 nm	Phospholipids (μg lipid phosphorus/mg protein)	Membrane density ** (g/cm^3)	Enzymic activities (specific activity units)		Membrane fluidity *** τ_0 (ns)
				ATPase	Thio-esterase	
18	0.12	9.2	1.172	3.1	0.7	7.46
19	0.17	8.0	1.175	2.4	0.6	7.31
20	0.24	6.4	1.177	2.1	0.6	8.48
22	0.34	5.1	1.182	2.0	0.5	10.50

* The growth medium contained 3% horse serum.

** Determined by sucrose density gradient centrifugation.

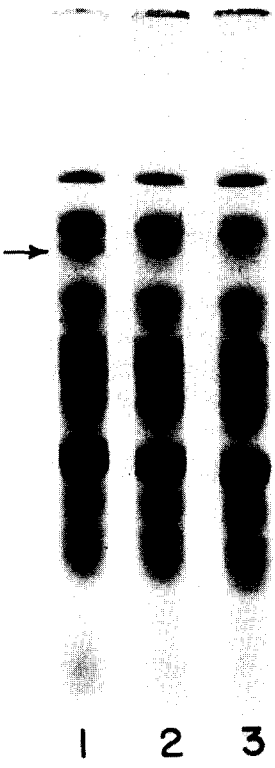
*** Calculated from the electron-paramagnetic resonance spectra of the spin-labeled 12-ketostearic acid at 37°C.

TABLE II

EFFECT OF CULTURE AGE ON THE IODINATION OF MEMBRANE PROTEINS AND THEIR SENSITIVITY TO TRYPSIN

Lactoperoxidase-mediated iodination and trypsin digestion were carried out on intact cells and isolated membranes as described in Materials and Methods.

Age of culture (h)	^{125}I label (cpm $\times 10^{-5}$/mg membrane protein)		Labeling ratio (membranes/cells)	Percent protein released by digestion	
	Membranes	Cells		Membranes	Cells
18	34.5	9.3	3.7	59.7	20.9
19	35.1	8.2	4.3	60.2	18.7
20	34.3	7.5	4.6	58.8	16.3
22	33.7	6.8	5.0	58.8	14.7



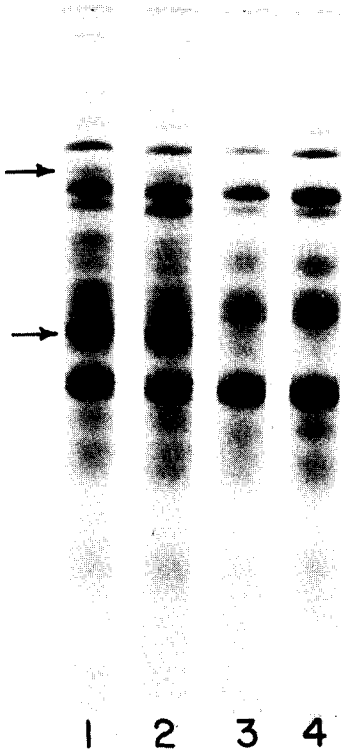


Fig. 1. Effects of culture age on the electrophoretic patterns of *A. laidlawii* membrane proteins. Cultures were harvested after: (1) 18 h, (2) 20 h and (3) 22 h of incubation at 37° C. Arrow points to a protein band which decreases on aging.

Fig. 2. Effects of alterations in growth temperature and fatty acid composition of membrane lipids on the electrophoretic patterns of *A. laidlawii* membrane proteins. Membranes from cells grown with: (1) oleic acid at 35° C, (2) elaidic acid at 35° C, (3) oleic acid at 25° C and (4) elaidic acid at 25° C. Arrows point to protein bands present at 35° C but absent at 25° C.

a similar decrease in their sensitivity to digestion by trypsin. The iodination values and sensitivity to proteolysis of isolated membranes were not affected by aging (Table II). Electrophoresis of membranes from iodinated cells showed that the decrease in labeling on aging was not due to a decrease in the labeling of a specific protein, but rather to a decrease of about 25% in the labeling of all the proteins exposed on the cell surface.

Effects of growth temperature and fatty acid composition

Oleic acid comprised over 81 mol% of total fatty acids in membranes of *A. laidlawii* grown in the albumin-containing medium supplemented with oleic acid. When the medium was supplemented with elaidic acid, this acid comprised almost 92 mol% of the fatty acids in the membranes. As expected, the oleate-enriched membranes were more fluid than the elaidate-enriched membranes. When measured at 25°C the τ_0 values were 7.1 ns for the oleate-enriched membranes and 8.5 ns for the elaidate-enriched membranes.

The changes in fatty acid composition and membrane fluidity had little effect, if any, on membrane protein composition. However, a shift-down in the growth temperature from 35 to 25°C caused some changes in the electrophoretic profile of membrane proteins. At least two protein bands were not present in the electrophoretic pattern of membranes isolated from cells grown at 25°C with either oleic or elaidic acid (Fig. 2). The iodination values of oleate-enriched cells and membranes differed very little from those of elaidate-

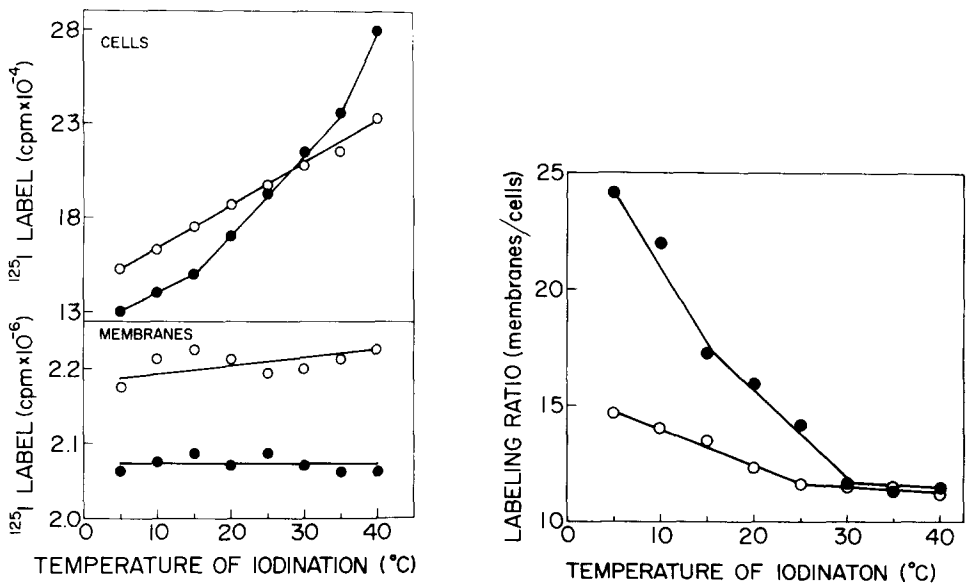


Fig. 3. Effect of alterations in the temperature of the lactoperoxidase-mediated iodination on the labeling of intact cells and isolated membranes of *A. laidlawii* enriched with oleic acid (●———●) or elaidic acid (○———○).

Fig. 4. Effect of growth temperature and temperature of the lactoperoxidase-mediated iodination on the labeling ratio of isolated membranes to membranes of intact *A. laidlawii* cells enriched with elaidic acid and grown at 25°C (●———●) or at 35°C (○———○).

enriched cells and membranes (Fig. 3). It should be noted that the labeling ratios of membranes to cells in these experiments were much higher than those obtained in the aging experiments where cells were grown with horse serum instead of bovine serum albumin (compare results in Figs. 3 and 4 with those in Table II). Fig. 3 also shows that the iodination values of intact cells increased on raising the iodination temperature, whereas those of isolated membranes showed little change. Since the labeling of the isolated membranes was not influenced by temperature, the increased labeling of intact cells does not appear to be the result of a more efficient iodination by the lactoperoxidase at higher temperatures, but rather indicates the exposure of more iodine-binding sites on the cell surface. Another explanation might be based on the leakiness and fragility of the wall-less mycoplasmas. Accordingly, the increase in the labeling intensity of intact cells with higher temperature arises from the greater permeability of the cells to lactoperoxidase or from the lysis of some of the cells. The addition to the iodination mixture of 20 mM Mg^{2+}, known to stabilize membranes and to protect mycoplasma cells from lysis [26], did not affect the labeling intensity of the cells. Only a minor fraction of the iodine label (about 5.5% of the total) was found in the cytoplasmic fluid of the treated cells at 5° C as well as at 40° C, indicating that no significant changes in the permeability of the cells to the lactoperoxidase took place on increasing the iodination temperature. Changes in the exposure of the iodine-binding sites of membrane proteins on the cell surface of *A. laidlawii* cells grown at different temperatures can be seen in Fig. 4. As was shown above, the relative labeling of

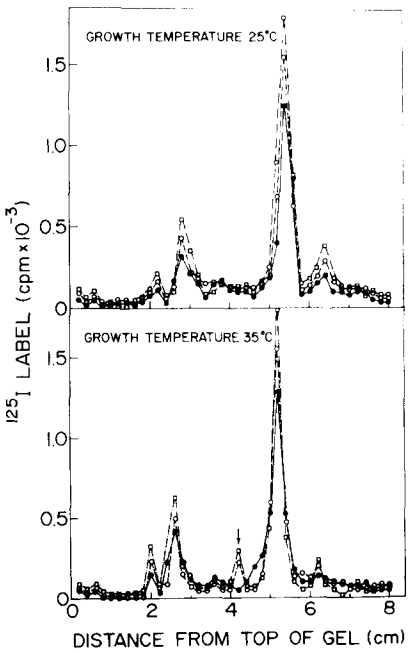


Fig. 5. Effect of growth temperature and temperature of the lactoperoxidase-mediated iodination on the distribution of the iodine label in membrane proteins of *A. laidlawii* enriched with elaidic acid. Iodination was carried out on intact cells. Temperature of iodination: 15° C (●———●), 25° C (○———○), or 35° C (□———□). The arrow points to a protein band which appears only in membranes of cells grown at 35° C.

intact cells increased on increasing the iodination temperature, causing a decrease in the labeling ratio of membranes to cells. The labeling ratio was higher for cells grown at 25° C than for cells grown at 35° C when iodination was carried out below 30° C (Fig. 4).

The distribution of label in membranes of lactoperoxidase-treated cells grown and iodinated at different temperatures is shown in Fig. 5. As was previously reported [14], only some of the membrane proteins were labeled on treatment of intact cells. The labeling intensity of these proteins was found to increase on increasing the iodination temperature. Another feature of Fig. 5 is that one of the protein bands marked with an arrow, of those that only appear in membranes of cells grown at 35° C (see Fig. 2), became labeled on iodination of intact cells, suggesting that it is located on the outer surface of the membrane. The intensity and distribution of the label in isolated membranes were not significantly affected by the growth and iodination temperature.

Effects of chloramphenicol treatment

The addition of chloramphenicol to an actively growing culture of *A. laidlawii* inhibited protein synthesis, as measured by the incorporation of [^{14}C]-phenylalanine into the trichloroacetic acid-insoluble fraction of the cells, while the incorporation of [^{14}C]acetate by the cells was significantly stimulated (Fig. 6). Extraction of the [^{14}C]acetate-labeled cells with chloroform/methanol (2 : 1, v/v) showed about 80% of the radioactivity to be associated with the lipid fraction, in accord with previous findings [27]. Table III summarizes some of the changes which took place in membranes of the treated cell. The continued synthesis of membrane lipid while membrane protein synthesis was inhibited led to an increase in the ratio of membrane lipid to protein, and to

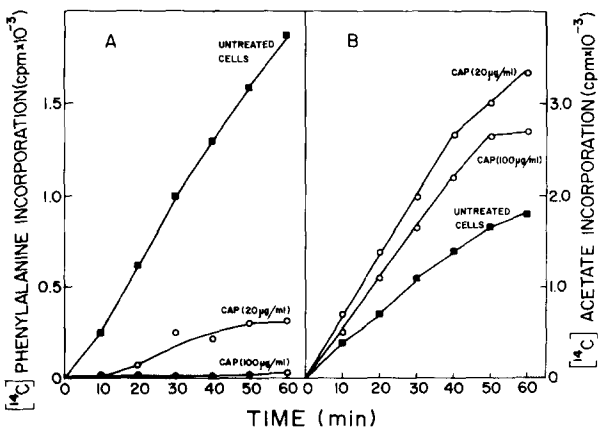


Fig. 6. Effect of chloramphenicol (CAP) on the incorporation of [^{14}C]phenylalanine (A) and [^{14}C]-acetate (B) into the trichloroacetic acid-insoluble fraction of growing *A. laidlawii* cells. The growth medium was supplemented with 0.5 µCi [^{14}C]phenylalanine or 0.5 µCi [^{14}C]acetate per ml, and the inhibitor was added when the absorbance of the culture at 640 nm reached 0.20. Samples (1 ml) were withdrawn at various time intervals and the organisms were precipitated with 10% cold trichloroacetic acid and collected by filtration through 0.45 µm Millipore filters pre-soaked in solutions of unlabeled phenylalanine or acetate as required. The filters were washed twice with cold trichloroacetic acid and their radioactivity was determined.

TABLE III

EFFECTS OF CHLORAMPHENICOL TREATMENT ON *A. LAIDLAWII* MEMBRANE PROPERTIES

Duration of treatment *	Absorbance of culture at 640 nm **	Total membrane protein (mg)	[^{14}C]Acetate-labeled lipid (cpm/mg membrane protein)	Membrane density (g/cm^3)	Membrane fluidity τ_0 (ns)
0	0.12	9.1	11 145	1.172	7.5
1	0.14	9.5	19 410	1.170	6.4
2	0.14	9.7	27 040	1.165	6.3
4	0.15	10.4	33 090	1.163	6.0

* Chloramphenicol (100 μg/ml) was added to a 17 h culture. The growth medium contained 20 μCi of sodium [^{14}C]acetate per l.

** The absorbance of an untreated culture incubated simultaneously increased from 0.12 to 0.34 after 4 h.

decreased membrane density. Membrane fluidity increased as was indicated by lower values of τ_0 (Table III)

Electrophoretic analysis of membranes isolated from the chloramphenicol-treated cultures showed no visible changes in the protein profile. However, lactoperoxidase-mediated iodination of intact cells and isolated membranes from these cultures showed a gradual decrease in the iodination values of intact cells, as opposed to almost unchanged labeling values for isolated membranes (Table IV). That the decreased iodination of intact cells is due to a gradual decrease in the labeling of all the major proteins exposed on the cell surface is indicated in Fig. 7. The reduced labeling of the proteins on the cell surface following treatment of cultures with chloramphenicol was correlated with decreased sensitivity of these proteins to proteolytic digestion as was indicated by lower values of protein released from cells treated with trypsin. Resembling the iodination data, the percentage of the protein released by trypsin digestion of isolated membranes was not affected by the chloramphenicol treatment (Table IV). Electrophoretic analysis of membranes from cells treated with trypsin showed that most of the proteins that were susceptible to diges-

TABLE IV

EFFECT OF CHLORAMPHENICOL ON THE IODINATION OF MEMBRANE PROTEINS AND THEIR SENSITIVITY TO TRYPSIN

Chloramphenicol (100 μg/ml) was added to a 17 h culture. Lactoperoxidase-mediated iodination and trypsin digestion were carried out on intact cells and isolated membranes as described in Materials and Methods.

Duration of chloramphenicol treatment (h)	^{125}I label (cpm × 10^{-5}/mg membrane protein)		Labeling ratio (membranes/cells)	Percent protein released by digestion	
	Membranes	Cells		Membranes	Cells
0	34.5	9.3	3.7	64.0	20.9
1	34.4	8.8	3.9	64.0	19.5
2	35.0	7.5	4.7	65.5	16.7
4	34.5	7.0	4.9	64.7	14.3

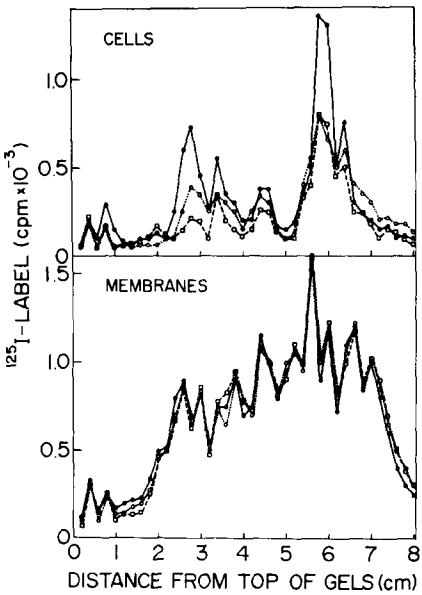


Fig. 7. Distribution of iodine label in membrane proteins of *A. laidlawii* treated with chloramphenicol. Intact cells and isolated membranes from an untreated 18 h culture (●), or from a culture treated with 100 μg/ml chloramphenicol for 2 h (○) or for 4 h (□), were subjected to lactoperoxidase-mediated iodination.

tion were of high-molecular weight. Chloramphenicol treatment did not significantly influence the protein species digested.

Discussion

Altering the fatty acid composition of membrane lipids is the easiest and most direct method of introducing marked changes in mycoplasma membrane fluidity. Moreover, in accord with previous findings by Pisetsky and Terry [28], dramatic alterations in the fatty acid composition did not trigger any significant changes in the electrophoretic profile of membrane proteins, simplifying the interpretation of the iodination data. Our results show that the iodination values of oleate- and elaidate-enriched cells were about the same despite a marked difference in membrane fluidity between these two types of cells. Furthermore, the labeling values of both cell types were about the same even when iodination was carried out at temperatures below 10°C, where the elaidate-enriched membranes are in the gel state, while the membranes enriched with oleate are still in the liquid-crystalline state [8].

The process of aging in *A. laidlawii* cultures was accompanied by a decrease in membrane fluidity, corresponding to previous findings with aging *Mycoplasma hominis* cultures [11] and aging plant cells [29]. The decrease in the staining intensity of one membrane protein band on aging of *A. laidlawii* probably reflects a slower rate of biosynthesis or a faster turnover rate, as compared to other membrane proteins. Because it is a minor membrane protein, we could not determine its location in the membrane, but even if it were

located on the cell surface its disappearance cannot explain the marked decrease in the iodination values of intact cells, as the labeling of all the major proteins exposed on the cell surface was shown to decrease to about the same level.

Another system in which changes in the electrophoretic profile of membrane proteins complicate the interpretation of the iodination data is that based on alterations in the growth temperature. The decreased availability of the iodine-binding sites on the cell surface of cells grown at 25°C as compared to cells grown at 35°C is in line with the hypothesis of Borochov and Shinitzky [6], since membranes from *A. laidlawii* cells grown at low temperatures are more fluid [9,10]. However, one of the protein bands located on the cell surface was missing from membranes isolated from cells grown at 25°C (Figs. 2 and 5) so that part of the decrease in labeling can be attributed to the loss of this protein.

Treatment of *A. laidlawii* with chloramphenicol arrested protein synthesis, and as a result the electrophoretic profile of membrane proteins was 'frozen', showing no visible changes during the experimental period (4 h). Membrane lipid synthesis, on the other hand, continued, resulting in a higher membrane lipid-to-protein ratio and increased membrane fluidity. The higher fluidity can be attributed to the higher lipid-to-protein ratio, as suggested by Rottem and Greenberg [11]. Again, as in the other systems tested, the chloramphenicol-treated cells exhibited lower iodine-binding values as compared to untreated cells.

To facilitate discussion of our major issue, Table V correlates the changes in membrane fluidity with the changes in the iodine-labeling values of intact cells. The common feature of most of our systems is that the cells were exposed to unfavorable conditions (aging, low temperature of growth, chloramphenicol treatment). Table V shows that under these conditions there is a decrease in the labeling of the proteins exposed on the cell surface, a change which does not show any consistent correlation with the variations in membrane fluidity nor with the predicted changes in the exposure of membrane proteins according to the hypothesis of Borochov and Shinitzky [6]. Our recent finding that the

TABLE V

RELATIONSHIP OF CHANGES IN THE IODINE LABELING OF CELLS TO CHANGES IN MEMBRANE FLUIDITY

Cells	Change in membrane fluidity	Change in iodine labeling of cells	
		Actual results	Predicted results *
From aging cultures	↓	↓	↑
Grown at 25°C	↑	↓	↓
Grown with elaidate instead of oleate	↓	No change	↑
Iodinated at low temperatures	↓	↓	↑
Treated with chloramphenicol	↑	↓	↓

* Predicted from the hypothesis of Borochov and Shinitzky [6].
↑, increased.
↓, decreased.

dissipation of the electrochemical gradient across the membrane in *A. laidlawii* cells that were treated with ionophores is also accompanied by a decrease in the exposure of the iodine-binding sites on the cell surface [1] and the finding by Bevers et al. [30] that the availability of membrane phosphatidylglycerol for hydrolysis by phospholipase A_2 depends on the state of membrane energization in *A. laidlawii* cells leads us to suggest that the changes in the exposure of membrane proteins described in the present report are caused by changes in the energized state of the membrane of the intact cell, rather than from changes in membrane fluidity.

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