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# VIRUS CAPPING ON MYCOPLASMA CELLS AND ITS EFFECT ON MEMBRANE STRUCTURE

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**The capping of mycoplasmavirus L3 on the surface of *Acholeplasma laidlawii* was investigated. In electron microscope studies we observed a reduced capping after treatment of the host cell with energy-blocking agents. Other drugs inhibiting ligand capping on eucariotic cells had no effect. Changes in membrane structure after virus adsorption were observed spectroscopically using the excimer fluorescence technique. The results are interpreted in terms of a lipid-protein phase separation in connection with virus capping.**

## Introduction

The mycoplasmas are a group of procaryotic microorganisms bounded only by a cell membrane. Since they do not contain any membraneous organelles, the cell membranes can be quite easily isolated after osmotic lysis of the cells. Further, mycoplasma membranes can be easily modified or labelled by additions to the growth medium. Due to these properties, mycoplasmas have been used for a large number of basic studies on biological membrane properties (reviewed in Ref. 1).

*Acholeplasma laidlawii* is the mycoplasma species which has been most frequently used in such membrane studies. It is also the host cell for three types of virus, designated MVL1, MVL2 and MVL3 [2]. The three types of virus differ considerably in structure and mode of replication.

In this paper we used the mycoplasma virus L3 group (MVL3), a non-enveloped complex virus exhibiting a cytocidal but not lytic infection cycle [3]. The adsorption of MVL3 to mycoplasmas follows biphasic adsorption kinetics. The reaction is partially reversible, with little dependence on the reaction temperature. It has some characteristics of an ionic reaction mechanism depending on cation concentration. More specific nature of MVL3 adsorption is indicated by the ten collisions needed for one successful adsorption. Moreover, there is specific requirement for $Ca^{2+}$. Cell surface receptors seem to be proteins.

An unusual virus-membrane interaction is the capping of adsorbed MVL3 virions on the surface of the *A. laidlawii* host cell [5]. Capping of adsorbed virions has not been described for any other virus. Possible reasons are that lateral motility of adsorbed virions is restricted on other procariotic cells by the presence of the bacterial cell wall. On the surface on eucariotic cells lateral motility of adsorbed virions should be possible, but due to the huge surface area of animal cells in comparison to any virion, very large numbers of adsorbed virions would be necessary to allow observation of the phenomenon in the electron microscope.

Capping of MVL3 on the surface of *A. laidlawii* in some properties seems analogous to capping of ligands on animal cell surfaces. It is temperature-dependent and requires active energy metabolism

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Abbreviations: MVL, mycoplasma virus laidlawii; CCCP, carbonylcyanide-*m*-chlorophenylhydrazone; DCCD, dicyclohexylcarbodiimide.

of the cell [6]. However, it is not clear whether MVL3 capping is dependent on a cytoskeletal system. In all procaryotes a structural organization comparable to the cytoskeleton of animal cells is hitherto unknown. Bacteria do not contain significant amounts of actin. Among the mycoplasmas, only a few species have been reported to contain actin-like properties [7,8]. These species, but not other mycoplasma or acholeplasma cells, have been reported to be inhibited by cytochalasin B. Other authors, however, were not able to inhibit the movements of motile mycoplasmas by this drug [9]. From *A. laidlawii* the isolation of a filamentous protein has been reported, which was, however, not actin-like [10]. Thus, if a microfibrillar system does exist in *A. laidlawii* it may not be based on actin-like proteins.

Recently we have described a further aspect of cell surface alteration during MVL3 infection. Whereas uninfected *A. laidlawii* cells have a coccoid appearance when prepared for scanning electron microscopic observation, infected cells showed numerous filamentous appendices [10]. This effect was observed only if the cells were fixed on poly(L-lysine)-coated carriers. It was, therefore, concluded that the distortion was a poly(L-lysine)-induced effect, made possibly by a structural alteration of the membrane due to the MVL3 infection.

In the present communication we report studies confirming the energy-dependence of MVL3 capping. Moreover, the effect of cytochalasin B and concanavalin A on MVL3 capping has been investigated.

Changes in the biophysical state of the *A. laidlawii* membrane caused by MVL3 adsorption were characterized by fluorescence spectroscopic techniques. These methods using fluorescence-labelled lipid molecules have been successfully applied to investigations of membrane structure in artificial as well as biological membranes [11,12]. We used the excimer formation technique together with fluorescence polarization measurements to characterize changes in the membrane structure of *A. laidlawii* membrane after virus adsorption.

## Materials and Methods

*Organisms and growth conditions*

The host cell *A. laidlawii* K2 and mycoplasmavirus L3 have been described previously [3]. MVL3 was assayed as plaque-forming units on lawns of K2. K2 was assayed as colony-forming units on tryptose agar plates. Tryptose broth and tryptose agar plates were used to grow *A. laidlawii* at 37°C as described previously [3].

*Buffers and inhibitors*

Tris-buffered saline (2 mM Tris-HCl/150 mM NaCl (pH 7)) was used to wash the cells. Michaelis's buffer comprised 0.28 M sodium barbital/0.28 M sodium acetate/0.05 M NaCl (pH 5.9). The osmolality of this buffer was adjusted to be 400 mosmolal with sucrose.

Séchaud and Kellenberger's [13] fixative contained 0.1% uranyl acetate and 3.5% glutaraldehyde in Michaelis's buffer. Soft agar stock contained 0.7% agar in Tris-buffered saline. CCCP, concanavalin A and cytochalasin B were obtained from Sigma (Munith); DCCD was a product of Merck Schuchardt (Darmstadt, F.R.G.).

*Preparation for electron microscopy*

A K2 overnight culture was diluted 1:5 into fresh tryptose broth and incubated for 2 h at 37°C before each experiment. Inhibitors were added 30 min before virus infection to the following final concentrations: CCCP, 60 $\mu$g/ml; DCCD, 25 $\mu$g/ml; concanavalin A, 50, 100 or 200 $\mu$g/ml; or cytochalasin B, 40 $\mu$g/ml. Virus infection was at multiplicity of infection of about 500. After 20 min incubation at 37°C to allow virus adsorption, the infected cells from 1.5-ml samples were collected by centrifugation (4300 $\times g$, 10 min). The pellets were drained well and resuspended in 50 $\mu$l of soft agar stock kept liquid at 45°C. Small agar rods were formed by the technique of Séchaud, Kellenberger and Blondel [14]. These rods were washed in Michaelis's buffer, fixed in Séchaud and Kellenberger's fixative and postfixed in 1% tannic acid in Michaelis's buffer. Fixation was for 2 h at room temperature for both steps. Standard procedures were used for dehydration in an alcohol series and embedding in Epon. Epon blocks were then sectioned in a LKB Ultrotome II. The sections were examined in a Philips 301 electron microscope operating at 80 kV.

*Labelling with fluorescence probes*

Pyrene lecithin (Fig. 1) was synthesized in our

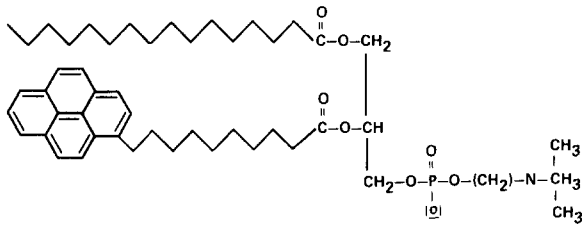

Fig. 1. Structure of the excimer-forming fluorescence probe pyrene lecithin.

laboratory as described previously [15]. Diphenylhexatriene was a product of Fluka (Neu-Ulm). Stock solutions were prepared in chloroform at a concentration of 1 mg/ml. 1 ml of one of such probe solution was transferred into a sterile Erlenmeyer flask and evaporated under a stream of nitrogen. The vessels were further dried in an evacuated oven at 70°C for 30 min. 5 ml of an *A. laidlawii* overnight culture containing about $4 \cdot 10^8$ colony-forming units per ml were diluted 10-fold into fresh tryptose broth, transferred to the prepared culture vessels and incubated for 6 h with constant shaking. After infection with MVL3 at a multiplicity of infection of about 500 the cultures were incubated at 37°C for another 30 min. The cells were then harvested by centrifugation (10 min, $4500 \times g$) and resuspended in Tris-buffered saline. Fluorescence spectra were taken with a Schöffel RRS 1000 fluorescence spectrometer. For temperature-shift studies the *A. laidlawii* culture was cooled in an ice-bath just before infection with MVL3.

*Excimer formation technique*

The formation of excited dimers (excimers) between a ground-state molecule and a molecule in the first excited singulet state has been used to determine the coefficient of the lateral diffusion of probe molecules such as pyrene, pyrene decanoic acid or pyrene lecithin in artificial and biological lipid bilayer membranes [16]. The method is described in detail elsewhere [15]. Here we will give only a short description. If a membrane preparation containing a given amount of pyrene (or pyrene derivative) is irradiated with light of wavelength 320 nm, a fluorescence spectrum can be observed. Beside the fluorescence of the monomer with intensity, $I$, at 390 nm (see, for example Fig. 5a) one can observe a broad emission at 473 nm which is the excimer fluorescence band with intensity $I'$. The intensities $I$ and $I'$ are proportional to the quantum yields of the monomer and excimer, respectively. Therefore the ratio $I'/I$ yields the information about the association constant, $k_a$, by the relation $I'/I \approx k_a \cdot C$, where $C$ is the probe concentration. Thus, from the fluorescence spectrum taken at a given concentration of probe molecules with respect to the lipid, the association constant, $k_a$, can be determined. The association constant is a measure of the lateral mobility of lipid molecules parallel to the plane of the membrane and is therefore a measure of membrane fluidity. On the other hand, if phase-separation phenomena under the influence of external substrates take place, the intensity ratio $I'/I$ increases if the pyrene molecules are squeezed out of rigidified membrane areas into fluid patches of a second type of lipid [17] because the local concentration, $C$, of the probe is increased. Thus an increase in the intensity ratio $I'/I$ will be observed even if there is no change in the association constant, $k_a$. That means changes in the fluorescence intensity ratio, $I'/I$, of excimer-forming probes can be caused either by changes in membrane fluidity or by phase-separation phenomena.

## Results

*Virus capping as determined by electron microscopy*

The typical appearance of thin sections of MVL3-infected *A. laidlawii* cells is shown in Fig. 2a. Virions adsorbed to the cells are clearly visible as electron-dense particles on the cell surface. Some empty virions probably have injected their DNA into the host cells. If actively growing cells were infected (Fig. 2a), most of the virions were found in aggregates over one pole of the cell, suggesting capped virus. The other extreme was seen in cells treated with DCCD prior to infection. Only few virus aggregates were visible, most virions being randomly distributed over the circumference of the sections (Fig. 2b). The occurrence of many empty cells indicates leakage of cell contents due to treatment with the drug.

To quantitate the observed images, for each experiment 250 cross-sections of infected cells were inspected and ordered into one of the six cate-

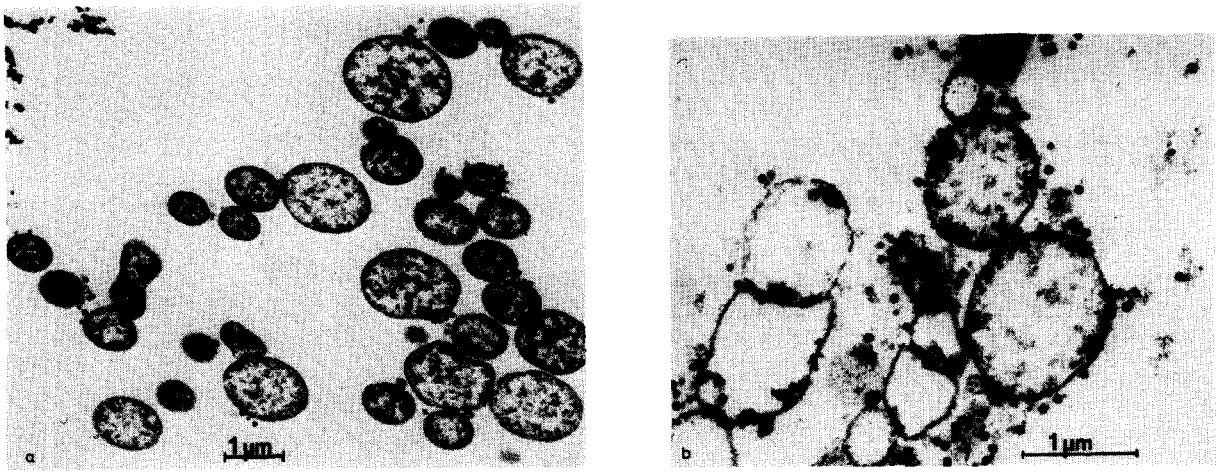


Fig. 2. Electron micrographs of *A. laidlawii* cells infected with MVL3 at multiplicity of infection 500. On actively growing cells, the adsorbed virions are mostly seen in aggregates over one pole of the cells (a), in cells treated with 25 µg/ml DCCD for 30 min prior to infection, capping of the virions is seen less frequently (b).

gories shown in Fig. 3. The percentage of sections in each category was then calculated. Histograms showing the average of all experiments done are presented in Fig. 4. Untreated cells (Fig. 4a) showed most adsorbed virions to be in categories 1 and 6. Only minor changes were found in cytochalasin B- or concanavalin A-treated cells (Fig. 4d, e). In contrast, treatment with DCCD resulted in less cell sections in categories 1 and 6, with the majority now in category 4 (Fig. 4b). Cells treated with CCCP showed intermediate results, with less cells in category 4 compared with DCCD-treated cells but significantly more compared with untreated cells (Fig. 4c).

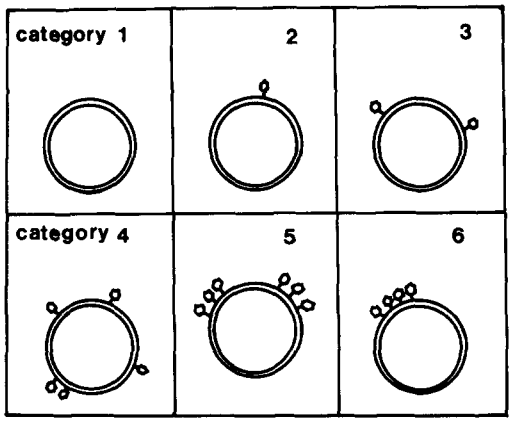


Fig. 3. Categories of cross-sections of MVL3-infected cells. Category 1 was defined as sections with no virion visible on the circumference, category 2 as sections with one and category 3 as sections with two virions. The sections in category 4 had three or more virions adsorbed which were distributed on the circumference. Sections with two groups of at least three adsorbed virions were ordered in category 5 if each group did not cover more than a quarter of the circumference. Only one group of adsorbed virions which did not cover more than a quarter of the circumference was the definition for a section in category 6.

*Virus-induced change in membrane structure as determined by fluorescence spectroscopy*

Fluorescence spectra of pyrene lecithin incorporated into mycoplasmic cells *A. laidlawii* before and after addition of MVL3 were measured. Typical spectra taken at 37°C are shown in Fig. 5b. In this example the intensity ratio, $I'/I$, decreased from 0.7 to 0.55 after addition of virus. Within one sample we had good reproducibility. However, the amount of incorporated pyrene lecithin changed for different cell preparations. The same was true for the effect of virus addition. We repeated the measurements with a number of cell preparations. The results obtained at 37°C are given as a histogram in Fig. 6, where the number of probes refers to different cell preparations. The change in $I'/I$ after virus addition is given as a percentage with respect to the uninfected cell suspension. We observed an average decrease in $I'/I$ to about 87% of the original excimer-to-monomer ratio after addition of MVL3. However, there seems to be also a

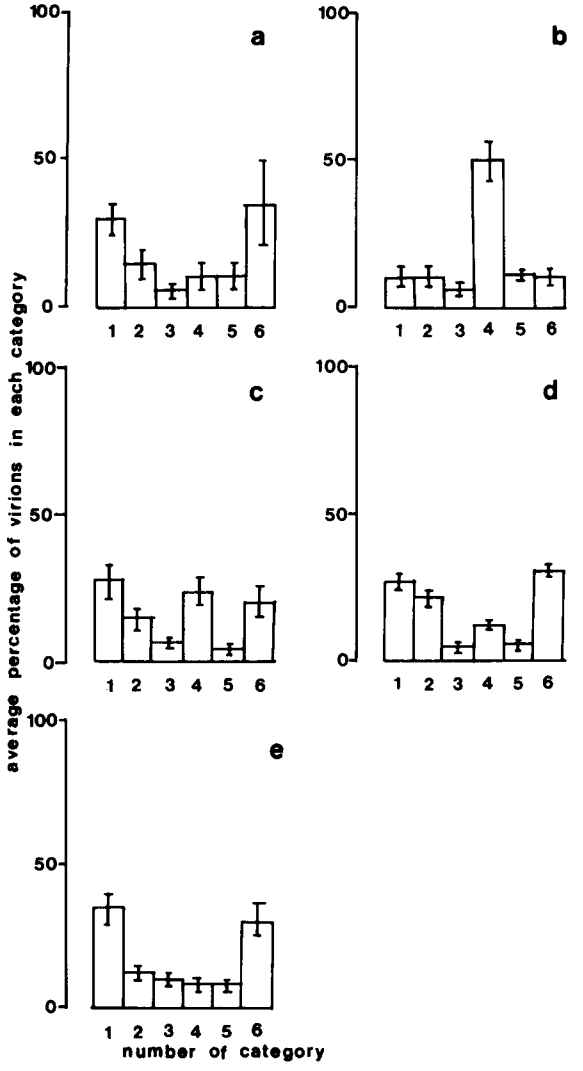


Fig. 4. Histograms showing the average percentage of virions in each of the categories as defined in Fig. 3. For each experiment 250 cell sections were counted. Distribution of adsorbed virions on: (a) untreated, actively growing cells (average from five experiments); (b) cells pretreated with 25 $\mu$g/ml DCCD (average from five experiments); (c) cells pretreated with 60 $\mu$g/ml CCCP (average from five experiments); (d) cells pretreated with 40 $\mu$g/ml cytochalasin B (average from two experiments); (e) cells pretreated with 100 $\mu$g/ml concanavalin A (average from three experiments).

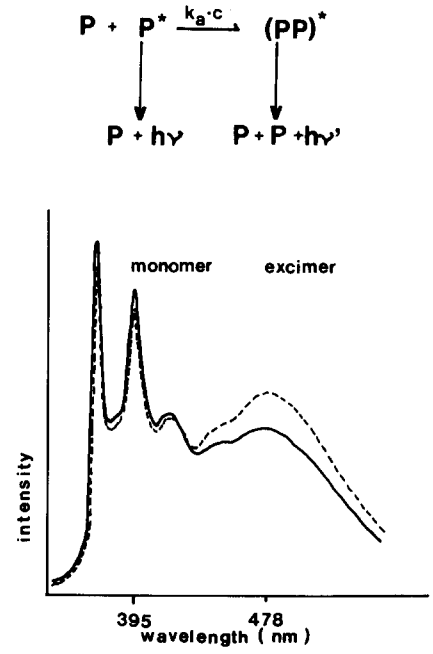


Fig. 5. (a) Reaction scheme for the excimer formation. An excimer (PP) * is formed if a ground-state pyrene lecithin molecule, P, collides with a molecule in the first excited singulet state, P *. The second-order rate constant $k_a \cdot C$ characterizes the frequency of collisions and is thus viscosity-dependent. Excimer and monomer will decay to ground-state molecules under emission of fluorescence with the frequency $\nu'$ or $\nu$. (b) Typical fluorescence spectra of pyrene lecithin incorporated into *A. laidlawii* cell membranes before (- - - - - -) and after (——) addition of MVL3. The excimer fluorescence band with the intensity $I'$ at 478 nm is clearly visible in addition to the monomer fluorescence with the intensity $I$ at 395 nm. Note the decrease in the intensity ratio $I'/I$ after virus adsorption.

fraction of samples that did not respond to virus addition, leading to a second maximum in the histogram at about 100%. The measurements were repeated at 4°C. The histogram is shown in Fig. 6b. Only a minor decrease in the fluorescence intensity ratio could be observed. In addition we performed fluorescence polarization experiments using diphenylhexatriene as optical probe. No change in polarization degree was observed after virus adsorption.

## Discussion

The results presented here confirm our preliminary data, that capping of MVL3 on the surface of *A. laidlawii* is a partially energy-dependent process. Although the three-dimensional distribution of virus on the cell surface was estimated from two-dimensional sections, there can be no doubt that energy-blocking agents inhibit the redistribution of adsorbed virus particles. For statistical purposes the sections were classified into six cate-

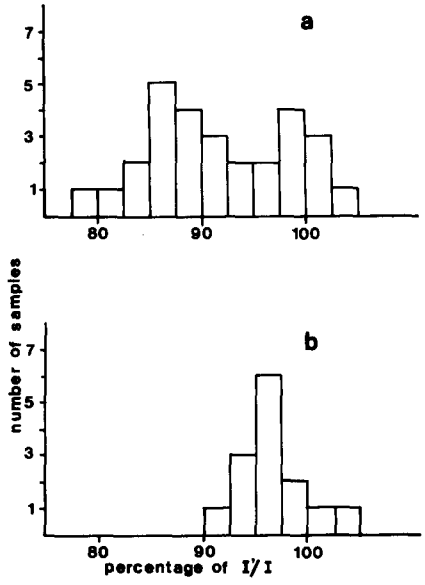


Fig. 6. Histograms showing the relative change in the excimer-to-monomer intensity ratio, $I'/I$, of pyrene lecithin in *A. laidlawii* cell membrane after virus adsorption. The percentage of $I'/I$ is given with respect to the value obtained in uninfected cells, which is taken as 100%. (a) Incubation with MVL3 and fluorescence measurement at 37°C; (b) incubation with MVL3 and fluorescence measurement at 4°C.

gories as defined in the Results section. Besides class 6, where a virus cap was visible, sections without any visible virus were considered indicative of virus capping: since virus capping leads to a large surface area void of adsorbed virions, cell sections with no virus will be more frequent in cells with capped virus. Predominance of distributed virions (class 4), is indicative of no virus capping. If only one or two virus particles were found on one cell section, no conclusion was drawn about the three-dimensional virus distribution. Virions patched in two groups (class 5) are possibly an intermediate state in MVL3 capping. The histograms presented in Fig. 4 clearly demonstrate the predominance of class 4 in DCCD-treated cells. DCCD is an ATPase-blocking agent causing irreversible inactivation of *A. laidlawii* as colony-forming units in the concentration used in these experiments. The second drug used to block energy metabolism was CCCP, an uncoupling agent of oxidative phosphorylation which, however, in *A. laidlawii* seems to act via a breakdown of the membrane potential [18]. The inhibition by CCCP is reversible. It is interesting to note that treatment with CCCP partially inhibits MVL3 capping. The effect is less pronounced than in DCCD-treated cells. However, in which way a breakdown of membrane potential may influence MVL3 capping cannot be answered at present.

Cytochalasin B, a drug which disintegrates actin filaments in eucaryotic cells, had no pronounced effect on MVL3 capping at 40 $\mu$g/ml. Some possible, small influence seen in Fig. 4d may still be within the experimental error. Since similar concentrations of cytochalasin B have been reported to inhibit the growth of motile mycoplasma species, it can be concluded that microfilaments which could be disaggregated by cytochalasin B are not involved in MVL3 capping.

Drugs like vinblastin or colcemid which act on the microtubular system of the cytoskeleton were not tested, since microtubules have never been reported in any procaryotic cell. Nor do these drugs inhibit procaryotic cell division.

Concanavalin A, a lectin from *Concanavalis ensiformis* which also inhibits ligand capping on eucaryotic cells [19], also has no effect on MVL3 capping (Fig. 4e). The mechanism of concanavalin A action on eucaryotic cells is still unknown. However, the lack of effect on MVL3 capping is a further indication that virus capping on the *A. laidlawii* surface is controlled in a manner different to the ligand capping on eucaryotic cells.

To achieve a better understanding of the process of MVL3 capping, we investigated alterations in the membrane structure as induced by MVL3 infection. Such alterations had been predicted from the results of scanning electron microscopic experiments [10].

Our spectroscopic approach clearly demonstrates that there is a change in membrane structure after addition of virus. We observe a decrease in the intensity ratio, $I'/I$, of the excimer to the monomer fluorescence of pyrene lecithin in the cell membrane of *A. laidlawii*. A decrease in $I'/I$ which is equivalent to a decrease in the association rate $k_a \cdot C$ (see, for example, Fig. 5a) could originate from a rigidification of the cell membrane or from an increase in the area of membrane lipid that is able to incorporate the fluorescence probe. To discriminate between these two possibil-

ities we have performed fluorescence polarization measurements using diphenylhexatriene as optical probe. No change in fluorescence polarization was observed after virus addition. Thus membrane fluidity, at least in the vicinity of the probe, is not altered after virus addition.

This result favours our interpretation of the virus capping as a phase-separation phenomenon. MVL3 probably adsorbs to the proteins of the cell membrane [4]. The interaction may lead to a change in the protein organization of the cell membrane.

Some amount of the membrane protein may be concentrated in the capping region. The protein-depleted part of the membrane will now be able to take up label molecules from fluid patches that already existed in the absence of virions. An increase in the extent of these fluid patches after adsorption of virions to the cell surface will thus lead to a decrease in probe concentration per area of available fluid lipid membrane. Therefore the excimer-to-monomer ratio has to decrease. The lipid fluidity is not changed, as could be demonstrated by fluorescence polarization.

The results discussed so far were obtained at 37°C. At 4°C, no capping of virus on the cell surface was observed. This is consistent with our spectroscopic data showing no significant decrease in the excimer fluorescence intensity.

We postulate that virus infection of a cell leads to a change in membrane structure. Fluid patches of lipid regions with low protein content may be formed. Exvagination of the membrane that was observed in the scanning electron microscopy pictures [10] may originate from these fluid bilayer regions. Similar results were observed in erythrocyte membranes, where an increase in temperature leads to an increase in fluid areas in the cell membrane [12]. From our results we conclude that membrane structure and function in simple procaryotic cells are maintained through energy-dependent processes in a similar way as in eucaryotic cells. Since our data, as well as data from other groups, argue against a cytoskeleton of the type found in eucaryotic cells, mediators have to be postulated for the procaryotic cells which regulate lateral diffusion of membrane receptors in an energy-dependent way.

This is also to be seen in connection with the still unsolved question of cellular organization in procaryotic cells [20]. *A. laidlawii* seems to be a promising cell to be used in further studies in this subject.

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## References

1 Rottem, S. (1979) in The Mycoplasma, (Barile, M.F. and Razin, S., eds.), vol. 1, pp. 259–288, Academic Press, New York
2 Maniloff, J., Das, J., Putzrath, R.M. and Nowak, J.A. (1979) in The Mycoplasma (Barile, M.F. and Razin, S., eds.), vol. 1. pp. 411–430, Academic Press, New York
3 Haberer, K., Klotz, G., Maniloff, J. and Kleinschmidt, A.K. (1979) J. Virol. 32, 268–275
4 Haberer, K. and Maniloff, J. (1982) J. Virol. 41, 501–507
5 Haberer, K., Maniloff, J. and Gerlin, D. (1980) J. Virol. 36, 264–270
6 Haberer, K. and Maniloff, J. (1982) Rev. Infect. Dis., in the press
7 Neimark, H.C. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 4041–4045
8 Maniloff, J. and Chandhuri, U. (1979) J. Supramol. Struct. 12, 299–304
9 Radestock, U. and Bredt, W. (1977) J. Bacteriol. 129, 1495–1501
10 Haberer, K. and Pfisterer, M. (1981) Eur. J. Cell Biol. 25, 10–21
11 Galla, H.-J. and Hartmann, W. (1980) Chem. Phys. Lipids 27, 199–219
12 Galla, H.-J. and Luisetti, J. (1980) Biochim. Biophys. Acta 596, 108–117
13 Séchaud, J. and Kellenberger, E. (1972) J. Ultrastruct. Res. 39, 598–606
14 Séchaud, J. Kellenberger, E. and Blondel, B. (1972) J. Ultrastruct. Res. 39, 606–607
15 Galla, H.-J. and Hartmann, W. (1981) Methods Enzymol. (C), 71, 471–479
16 Galla, H.-J., Hartmann, W., Theilen, U. and Sackmann, E. (1979) J. Membrane Biol. 48, 215–236
17 Galla, H.-J. and Sackmann, E. (1975) J. Am. Chem. Soc. 97, 4114–4120
18 Amar, A., Rottem, S. and Razin, S. (1978) Biochem. Biophys. Res. Commun. 84, 306–312
19 Edelmann, G.M., Yahara, I. and Wang, J.L. (1973) Proc. Natl. Acad. Sci. U.S.A. 1442–1446
20 Kellenberger, E., Carlemalm, E., Staufer, E., Kellenberger, C. and Wunderli, H. (1981) Eur. J. Cell Biol. 125, 1–4