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Membrane surface potential of *Spiroplasma floricola*

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Anionic charges, cytochemically identified as lipid phosphate groups, cover the outer membrane surface of *Spiroplasma floricola*. They induce a negative membrane surface potential which affects the distribution of ions, including protons. Accordingly, the pH at the interface differs from the bulk pH. By using the fluorescent lipoid pH indicator 4-heptadecyl-7-hydroxycoumarin, the pH at the membrane surface was determined. From the difference of the bulk and the interfacial pH the membrane surface potential of *S. floricola* was calculated to be $\varphi = -118$ mV.

Membrane charge; Membrane surface potential; (*Spiroplasma floricola*)

1. INTRODUCTION

Spiroplasmas are helical, motile microorganisms devoid of a rigid cell wall, but endowed with a cytoplasmic membrane similar to, but more stable than that of eucaryotic organisms [1,2]. By using polycationized ferritin as a probe [3] a dense and rather homogeneous layer of anionic sites, i.e. lipid phosphate groups, on the membrane surface of *S. floricola* was detected by electron microscopy. These negative charges induce a negative surface potential ϕ at the membrane/aqueous phase interface. This membrane surface potential affects the distribution of ions, including protons. Accordingly, the pH at the interface differs from the bulk pH. Some important biomembrane processes, such as cell-cell adhesion, membrane fusion, and ion transport, are assumed to depend significantly upon the membrane surface potential [4,5].

Fromherz and colleagues developed a theory and a method to calculate the membrane surface potential of lipid monolayers and liposomes from the difference between the values of the bulk and interfacial pH. The pH at the membrane surface was determined by measuring the apparent pK of the fluorescent lipoid pH indicator, 4-heptadecyl-7-hydroxycoumarin (henceforth referred to as dye), which, due to its hydrophobic side chain, is anchored in the membrane, the fluorophore being located at the membrane/aqueous medium interface. Both the excitation spectra and the quantum yield of the dye differ for both the undissociated and dissociated forms, respectively, thus allowing one to determine the degree of dissociation (α) and hence the pK of the dye [6].

We applied this method to the determination of the membrane surface potential of *S. floricola*.

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2. MATERIALS AND METHODS

4-Heptadecyl-7-hydroxycoumarin (dye) was a generous gift from P. Fromherz (Ulm). A 5 mM solution was prepared in ethanol.

S. floricola BNR1 [7] (kindly provided by J.C. Vignault and J.M. Bové, Pont-de-la-Maye, France) was grown in Hepes-buffered DSM 4 medium which contained (per 100 ml) 1.5 g PPLO broth base (Difco), 8 g sucrose (Merck), 2.5 mg phenol red, 1.32 g Hepes (pH 7.5) and 10 ml inactivated (30 min at 56°C) horse serum (Flow).

Spiroplasmas were grown at 34°C with the pH being maintained constant at 7.5 by automatic titration. The shape and motility of the spiroplasmas were regularly monitored under dark-field microscopy. After cooling in an ice bath, the spiroplasmas were harvested by centrifugation (Sorvall, SS-34, 10000 rpm, 4°C, 10 min). The wet weight was determined.

For labelling, spiroplasmas were resuspended in cold incubation medium (spiroplasmal concentration: 20 mg wet wt/ml) which contained (per 100 ml) 1.5 g PPLO broth base (Difco),

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8.0 g sucrose (Merck), and 1.32 g Hepes (pH 7.5) and further incubated at 34°C for 10 min with the pH being kept constant at 7.5. The dye was then added to the suspension (final concentration: 1 μg dye/1 mg wet wt), which was incubated for a further 15 min to allow for incorporation and distribution of the dye.

Finally, spiroplasmas were sedimented as described above, and the sediment was resuspended in ice-cold NaCl/KCl/Hepes medium (final concentration: 30 mg wet wt/ml) which consisted of 25 mM Hepes + 115 mM NaCl + 13 mM KCl (pH 7.5). Again the spiroplasmas were incubated for 15 min at 34°C with the pH being kept constant at 7.5, then cooled and kept at 0°C.

Solutions were prepared, consisting of NaCl/KCl/Hepes adjusted to pH 7.5; 8, 8.5; ...; 13. Measurements at pH 14 were carried out in 0.1 M NaOH.

In our experiments with 50 mM $CaCl_2$, the NaCl content was reduced to 50 mM; in those with 100 mM $CaCl_2$ no NaCl was present.

Neutral (Triton X-114) micelles were prepared and labelled as in [8].

Quartz cuvettes were filled with 3 ml of the pH-adjusted solutions, and 100 μl spiroplasma suspension was added immediately before the excitation spectrum was recorded over the range 250–420 nm with a Hitachi-Perkin-Elmer 204 fluorescence spectrophotometer with the analyzer wavelength set at 450 nm. For peak analysis the excitation wavelength was set at 370 nm.

The membrane surface potential was calculated according to the formula derived by Fromherz and colleagues [6]:

$$\phi = -(pK_{ch} - pK_0) \times 2.3RT/F \quad (1)$$

where pK_{ch} and pK_0 denote the apparent dissociation constants of the dye at a charged and a neutral (Triton X-114) interface, respectively; R is the molar gas constant; T the absolute temperature and F represents Faraday's constant. pK_{ch} and pK_0 can be obtained by measuring the dependence of the dye's degree of dissociation (α) upon the pH of the aqueous bulk medium (pH_b), and calculated according to

$$pK = pH_b - \log[\alpha/(1 - \alpha)] \quad (2)$$

The degree of dissociation α was calculated from the fluorescence intensity (I) at the excitation wavelength of 370 nm, according to

$$\alpha = I/I_{max} \quad (3)$$

where I_{max} denotes the maximally obtainable fluorescence intensity at 370 nm, when the dye is completely dissociated.

3. RESULTS AND DISCUSSION

We observed rapid and complete incorporation of the dye into the spiroplasmal membranes. A noxious effect on spiroplasmal metabolism and motility was not detected. The experimental results were independent of the dye concentration used.

The membrane-bound dye readily responded to the varied pH in the surrounding media. However, in Ca^{2+}-free media at bulk pH > 11.5 a rapid increase in fluorescence intensity with time could be observed due to lysis of the spiroplasmas, as observed under dark-field microscopy. Therefore, for determination of the value of α, the fluorescence intensity at λ_{ex} = 370 nm was recorded rather than the whole excitation spectrum. In media containing Ca^{2+}, the spiroplasmas were considerably more stable, allowing the whole spectrum to be recorded. A set of excitation spectra recorded from spiroplasmas suspended in media containing 50 mM $CaCl_2$ are shown in fig.1.

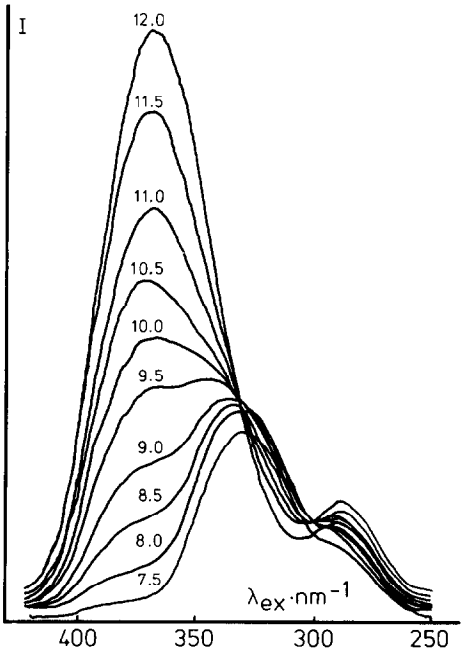

Fig.1. Fluorescence excitation spectra of the lipoid pH indicator 4-heptadecyl-7-hydroxycoumarin incorporated into *Spiroplasma floricola* membranes, at different pH (as indicated) of the aqueous suspension medium containing 50 mM $CaCl_2$. Spectra were recorded between 250 nm and 420 nm with the analyzer wavelength set at 450 nm.

When measuring the fluorescence intensity at λ_{em} = 450 nm, we obtained maximal intensity for the dye's undissociated form at λ_{ex} = 330 nm, and for the dissociated form at λ_{ex} = 370 nm. The excitation spectra overlapped in a minimal and negligible way, i.e. when the dye is in the undissociated form, then $I_{370nm} \approx 0$. When the fluorescence intensity of the completely dissociated form at λ_{ex} = 370 nm is I_{max}, then the degree of dissociation α corresponds to $\alpha = I_{370nm}/I_{max}$. For α = 0.5, $pK = pH_b$. For calculation of the mem-

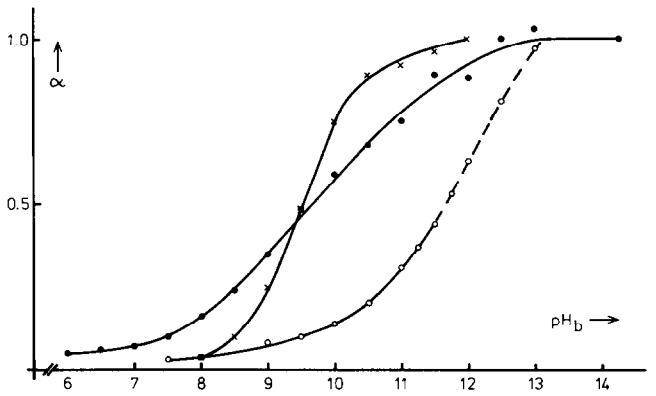

Fig.2. Dissociation degree (α) of the lipoid pH indicator 4-heptadecyl-7-hydroxycoumarin incorporated into *Spiroplasma floricola* membranes suspended in 25 mM Hepes + 115 mM NaCl + 13 mM KCl (—○—), or 25 mM Hepes + 100 mM $CaCl_2$ + 13 mM KCl (—●—), respectively, and neutral (Triton X-114) micelles in 25 mM Hepes + 115 mM NaCl + 13 mM KCl (—×—), vs pH of the aqueous suspension medium.

brane surface potential, one has to determine the pH of the suspension medium leading to $\alpha = 0.5$.

3.1. *Determination of* I_{max}

I_{max} is obtained when the dye is completely dissociated, i.e. when the fluorescence intensity does not increase on increasing the pH of the suspension medium. With neutral (Triton X-114) micelles this point is reached at pH ≥ 11, and for the determination of α, I_{max} is taken as I_{370nm} at pH 12. This simple procedure failed with *S. floricola*. When I_{370nm} is plotted vs pH of the suspension medium, one can see that I_{max} is not reached even at pH 14 (fig.2). It proved necessary to try a different approach based on the following considerations and observations. For a given preparation of *S. floricola* the proportion of the fluorescence observed at 450 nm of the dissociated and undissociated forms, i.e.

$\beta = I_{mx}$(dissociated,370 nm)/I_{mx}(undissociated, 330 nm)

is constant. If one succeeds, e.g. by addition of cations, in reducing the negative surface charges, at least partially, complete dissociation of the dye should be achieved at lower pH. Then, after determination of β, $I_{max} = \beta I_{mx}$ (undissociated, 330 nm).

The negative membrane surface charges could readily be neutralized by substituting NaCl in the suspension media by 100 mM $CaCl_2$. Then I = constant = I_{max} was obtained at pH ≥ 12 of the suspension media. For different preparations of spiroplasmas, β was found to vary only between 2.8 and 3.1.

Thus I_{max} could be determined for Ca^{2+}-free suspension media, and the degree of dissociation α was calculated. For *S. floricola* in NaCl/KCl/Hepes we obtained $\alpha = 0.5$ at pH 11.6, i.e. $pK_{ch} = 11.6$.

For neutral (Triton X-114) micelles we obtained: $pK_0 = 9.6$. Hence, the membrane surface potential of *S. floricola* was calculated to be $\phi = -(11.6 - 9.6) \times 2.3RT/F = -2 \times 59$ mV $= -118$ mV. The measurements in suspension media containing Ca^{2+} gave $pK_{ch} = 10.1$ (at 50 mM) and 9.6 (at 100 mM), the corresponding membrane surface potentials being $\phi = -30$ and 0 mV, respectively.

Varying the cholesterol content of *S. floricola* and treating the organisms with pronase did not influence the membrane surface potential.

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