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EFFECTS OF ENERGIZATION ON MEMBRANE ORGANIZATION IN MYCOPLASMA

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Fluorescence polarization and ESR experiments using various probes demonstrated that addition of glucose to resting *Mycoplasma capricolum* and *Mycoplasma mycoides* subs *capri* had, if any, a very limited effect on the physical state of their membrane lipids. Under the same conditions the degree of exposure of primary amino groups of membrane proteins to the aqueous surrounding, estimated from fluorescence labeling by fluorescamine and the cycloheptaamylose-fluorescamine complex was significantly increased. This energy dependent increase was blocked by dicyclohexylcarbodiimide (DCCD), an inhibitor of the membrane bound Mg^{2+} stimulated ATPase of mycoplasma and by carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) which, in mycoplasma, only affects the chemical component of the proton-motive force. Variations in the proton activity gradient across the membrane induced by changing the pH of the labeling medium resulted in parallel variations in the ratio of relative intensities of labeling of energized to resting cells. The values taken by this ratio were up to two for a maximal proton gradient of 0.9 pH unit and tended to unity when the intracellular and extracellular pH tended to equalize. It is concluded that, upon mycoplasma cell energization, membrane proteins undergo a conformational change resulting in the exposure of new free amino groups. This conformational change is primarily dependent on the existence of a ΔpH across the membrane and occurs in the absence of important modifications in the physical state of membrane lipids.

Introduction

Modifications in membrane organization as a function of the energy state occur in a variety of cells or organelles and may involve lipid proteins or both membrane components [1–5]. In *Acholeplasma laidlawii* the difference between metabolizing and resting cells in the availability of phosphatidylglycerol hydrolysis by exogenous phospholipase A_2 [6] and in the degree of exposure of membrane proteins [7] also strongly suggests the existence, in mycoplasma, of an energy-dependent process of membrane reorganization. Glycolysing mycoplasma develops an electro-chemical potential gradient for protons ($\Delta\tilde{\mu}H^+$) created via the activity of a membrane bound Mg^{2+}-dependent ATPase [8,9]. In *Mycoplasma mycoides* subs *capri*, a sterol requiring mycoplasma, inhibition of the Mg^{2+}-ATPase activity by DCCD collapses both the electrical and the chemical components ($\Delta\psi$ and ΔpH, respectively) of the $\Delta\tilde{\mu}H^+$, resulting in a complete inhibition of K^+ active influx. On the other hand, proton conductors like FCCP or CCCP when used alone inhibit only the ΔpH, leaving the

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Abbreviations used: $\Delta\psi$, electrical potential; ΔpH, transmembrane proton gradient; $\Delta\tilde{\mu}H^+$, electrochemical proton gradient; DCCD, dicyclohexylcarbodiimide; FCCP, carbonyl-cyanide *p*-trifluoromethoxyphenylhydrazone; CFC, cycloheptaamylose-fluorescamine complex; NPN, *N*-phenyl-1-naphthylamine; DPH, 1,6-diphenyl-1,3,5-hexatriene.

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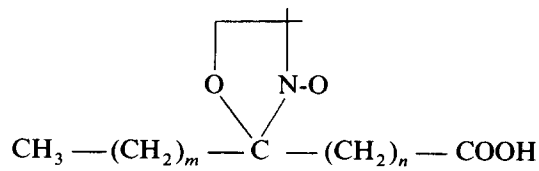
transmembrane potential and the active transport of K^+ nearly intact [8,9]. This specificity led us to examine the effects of energy state, ΔpH and $\Delta\psi$ on membrane organization in *M. mycoides* var *capri* and the related *M. capricolum*. The first set of experiments was conducted at a constant pH using resting and metabolizing cells as such or in the presence of either DCCD, valinomycin, FCCP, and gramicidin. The external pH was varied from 5.8 to 8.2 in the second experimental series. The physical state of membrane lipids was estimated by ESR and fluorescence polarization techniques. Five different nitroxyde fatty acid derivatives, I(10,5), I(1,14), I(12,3), I(12,3) methyl ester and I(12,3) alcohol, and four fluorescent probes, 1,6-diphenyl-1,3,5-hexatriene, *trans*-parinaric acid, *N*-phenyl-1-naphthylamine, and 16-anthroylstearate were used. The absence of lipids containing primary amino groups in mycoplasma [10,11] allows the use of fluorescamine to specifically label the exposed primary amino groups of membrane proteins. Because some controversy concerning the impermeability of biological membrane towards fluorescamine still exists [4,12–14], experiments were performed with both fluorescamine and the impermeant cycloheptaamylose-fluorescamine complex [15–17]. The results show that in *Mycoplasma* var *capri* and *M. capricolum* energization has, if at all, a very limited effect on the physical state of membrane lipids. On the other hand, the existence of $\Delta\tilde{\mu}H^+$ is associated with a comparable increase in labeling by fluorescamine and CFC suggesting that energization of mycoplasma exposed more primarily amino groups of membrane proteins to the aqueous medium. The effects of ionophore and of variations in external pH further suggest that this process is principally dependent on the existence of a pH gradient across the membrane. A brief account of the results was presented at the Third Conference of the International Organization of Mycoplasmology in Custer, SD, September 1980.

Materials and Methods

Materials. Fluorescamine and cycloheptaamylose were purchased from Pierce Company. The cycloheptaamylose complex was prepared according to Nakaya et al. [15]. *N*-Phenyl-1-naphthylamine, *trans*-parinaric acid, 16-anthroyl stearic acid were obtained from Molecular Probes Inc. (Plano, TX). 1,6-Diphenyl-1,3,5-hexatriene, 9-aminoacridine, and merocyanine 540 were purchased from Aldrich Chemical Co. (Milwaukee, WI), and Eastman Organic Chemical (Rochester, NY), respectively. The stearic acid nitroxide probes (I(m, n)), I(12,3), I(10,5), I(1,14)



and the methyl ester derivative of I(12,3) were purchased from Synvar Associates (Palo Alto, CA). The fatty alcohol I(12,3)Alc was kindly prepared by Dr. M. Dvolaitsky (College de France) by selective reduction of I(12,3). FCCP, gramicidin, and valinomycin were obtained from Boehringer. [^{3}H]Acetate, [^{14}C]butyrate, and [^{14}C]methylamine were purchased from C.E.A. Saclay.

Organisms and growth conditions. Mycoplasma mycoides subs *capri* and *M. capricolum* (California Kid Strain 14, ATC 27342) were cultivated on a modified Edward medium [18] as previously described [19]. Fatty acid poor bovine serum albumin (Miles Laboratories Inc., IL) was used to replace the PPLO serum fraction. Sodium salts of palmitic acid plus oleic acid (5 μg/ml each) were added as the source of fatty acid together with cholesterol (10 μg/ml). Organisms were collected during the early to mid log phase ($A_{640nm} \leq 0.3$) by centrifugation (8000 $\times$ g, 15 min) and washed once with cold 0.25 M NaCl solution containing 0.01 M $MgCl_2$. When membranes were prepared by osmotic lysis [20], $MgCl_2$ was omitted from the washing solution.

Characterization of the physical state of membrane lipids. Fluorescence polarization measurements were performed on a SLM 4000 apparatus equipped with a four-cell thermostatically controlled compartment and a magnetic stirrer (SLM Inc., Urbana, IL). A Nesslab temperature programmable circulatory bath was connected to the spectrofluorometer and the temperature was moni-

tored with a thermolinear probe directly placed in the cell compartment. Light scattering was reduced to very low levels by the use of cut-off filters. In all conditions, the individual values obtained were the mean of at least four successive measurements which by themselves were the average of ten determinations. Results of steady-state depolarization experiments are expressed in terms of fluorescence anisotropy r, with

$$r = \frac{I_{\parallel} - I_{\perp} G}{I_{\parallel} - 2 I_{\perp} G}$$

where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensity observed with the analyzing polarizer parallel and perpendicular to the polarized excitation beam. A correction factor, G, equal to $I'_{\parallel}/I'_{\perp}$, the primes indicating excitation polarized in a perpendicular direction was used to correct for the unequal transmission of differently polarized light. For measurements, organisms were resuspended (final concentration 150–250 μg protein/ml) in 0.1 M phosphate buffer (0.1 M phosphate/35 mM NaCl/1 mM KCl/2 mM $MgCl_2$) adjusted at different pH. Labeling was achieved by preincubating the suspension with either *trans*-parinaric acid, 1,6-diphenyl-1,3,5-hexatriene, *N*-phenyl-1-naphthylamine, and 16-anthroylstearic acid as previously described [21]. Excitation wavelengths were respectively 323, 362, 350, and 365 nm while emission was measured at 420, 430, and 480 nm, respectively. Polarization determinations were performed at 25°C.

ESR. Methanolic solutions of spin labels were dried as a thin film at the bottom of a test tube. Suspensions of mycoplasma (approx. 6 mg cell protein/ml) were added to the tube and gently mixed. The ratio of spin label to membrane protein was about 5 μg spin label/mg membrane protein. Samples were transferred to 50 μl capillary tubes for ESR measurements. X band ESR spectra were recorded with a Bruker ER 200 ESR spectrometer equipped with a variable temperture accessory. Spectrum recording (200 s) started 60–90 s after the introduction of the sample in the capillary. ESR signals from spin-labeled intact mycoplasma underwent a time-dependent decay. Aeration of the cell suspension or exposure to $K_3Fe(CN)_6$ (< 1 mM) were effective means of restoring the ESR signals.

Labeling with fluorescamine and CFC. For fluorescamine experiments cells were resuspended (0.25 to 1 mg cell protein/ml) in the phosphate buffer solution as such or containing the different ionophores and inhibitors. Energization was achieved by adding 10 mM glucose to the cell suspension. When the effect of an inhibitor or ionophore had to be tested it was added at the same concentration in the buffer which did not contain glucose. Experiments were generally conducted at 25°C but some were performed at 37°C. After a 10-min equilibration period 10 μl of a solution of fluorescamine in acetone was rapidly injected through a microsyringe in 3 ml of the reaction mixture. The suspension was vortexed during the injection to insure rapid mixing. Because of its limited solubility CFC was directly dissolved in the phosphate buffer and the reaction was started by adding an aliquot of a thick cell suspension to obtain a final concentration of 0.5 mg/ml CFC and 0.25–1 mg/ml cell protein.

Fluorescence intensities obtained after reaction between free amino groups and fluorescamine or CFC were measured on an Aminco Bowman (American Instrument Corp., Silver Springs, MD) or a Perkin Elmer MPF-44A spectrofluorometer at an emisson wavelength of 486 nm ($\lambda_{exc} = 405$ nm).

Measurements of ΔpH. The pH gradient across the cell membrane was determined from the equilibrium distribution of [^{3}H]acetate, [^{14}C]butyrate, and [^{14}C]methylamine as previously described [9,22]. Qualitative variations of ΔpH were also estimated from changes in the fluorescence intensity of 9-aminoacridine [22].

Determination of $\Delta\psi$. Variations in transmembrane potential were followed as previously described [8,9] by measuring the relative fluorescence intensity of the mycoplasma suspension labeled by merocyanine 540. This method whose results agree fairly well with those obtained by the Rb^+ equilibrium method permitted the rapid estimation of the $\Delta\psi$ [22].

Protein determination. Proteins were measured as described by Lowry et al. [23] using bovine serum albumin as standard.

Results

Physical state of membrane lipids (pH 7.2)

Addition of glucose to resting *M. capricolum* or *M. mycoides* subs *capri* cells had no effect on the fluorescence anisotropy of *trans*-parinaric acid and 16-anthroylstearic acid and induced only a very limited decrease in the anisotropy of 1,6-diphenyl-1,3,5-hexatriene (from 0.247 ± 0.003 to 0.242 ± 0.004) and *N*-phenylnaphthylamine (from 0.070 ± 0.002 to 0.065 ± 0.002) (Fig. 1). Comparison with measurements of r as function of temperature indicated that the possible increase in membrane fluidity corresponding to such anisotropy variations was less than that produced by a 3–4°C rise in temperature. As shown in Fig. 1, this effect was reversed upon addition of FCCP. The hyperfine splitting values of the spin-labeled fatty acid I(12,3), I(10,5), and I(1,14), in resting cells, were respectively 61.9 ± 0.2 G, 54 ± 0.2 G, and 40.7 ± 0.2 G. No modification of the ESR spectra and of the $2T_{\|}$ was detected upon mycoplasma cell energization. This holds true for the I(12,3) alcohol and methyl ester derivatives whose $2T_{\|}$ were respectively 60 ± 0.3 G and 59.5 ± 0.2 G. Finally, passive efflux of [^{14}C]erythritol from preloaded resting cells was unaffected by glucose addition (data not shown).

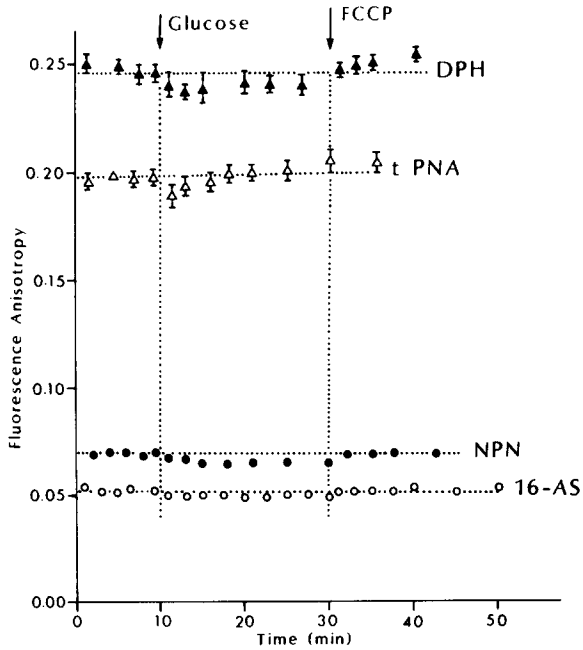


Fig. 1. Effect of glucose addition on the physical state of membrane lipids. Organisms were resuspended (250 μg cell protein/ml) in 0.1 M phosphate buffer (pH 7.20) containing the fluorescent probes 1,6-diphenyl-1,3,5-hexatriene (▲, DPH, 2 μM), *trans*-parinaric acid (△, tPNA), *N*-phenyl-1-naphthylamine (●, NPN), and 16-anthroylstearic acid (○, 16-AS), (0.5–1 μg/ml). Glucose was added (5 mM) at least 10 min following the time needed by each probe to reach their maximal level of fluorescence intensity. FCCP was added as ethanolic solution to a final concentration of $5 \cdot 10^{-6}$ M to $5 \cdot 10^{-7}$ M. The amount of ethanol added was always less than 0.5%. For NPN and 16-anthroylstearic acid, the size of dots was of the same order that the errors on the measurements.

Accessibility of primary amino groups (pH 7.2)

For cells resuspended in phosphate buffer at pH 7.2 and 25°C, the labeling of protein primary amino groups by fluorescamine was achieved within 30 s, independently of the energy state of the membrane (Fig. 2). The fluorescence intensity levels reached were always significantly higher in energized than in resting organisms. According the cell batch the ratios energized/non energized of fluorescence intensities ($I(+)/I(o)$) ranged from

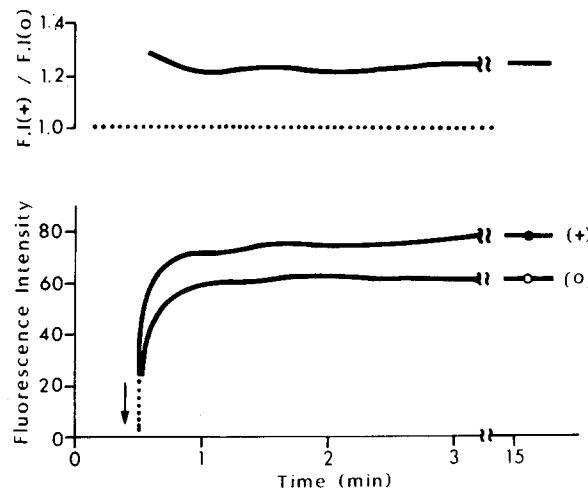


Fig. 2. Time course of the reaction of fluorescamine with mycoplasma cells. Fluorescamine (50 μg/ml) was rapidly injected in the cell suspension (0.25 mg cell protein/ml, phosphate buffer, pH 7.2) and the development of fluorescence was recorded as function of time. In lower part of the figure, ordinates give the relative intensity in absence (○) and in presence (+) of glucose. The upper part of the figure represents the evolution of the ratio of relative intensity of energized (F.I(+)) to non-energized (F.I(o)) organisms during the same period.

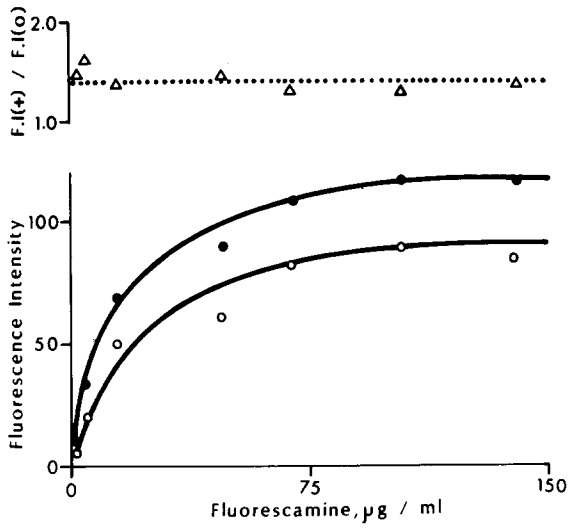


Fig. 3. Extents of labeling of mycoplasma cells as function of fluorescamine concentration. Cells were resuspended (0.25 mg cell protein/ml) in phosphate buffer (pH 7.2) and the relative fluorescence intensity corresponding to various amounts of fluorescamine added from identical values of acetone recorded (lower part). Upper part of the figure as in Fig. 2. ○, resting cells. ●, energized organisms.

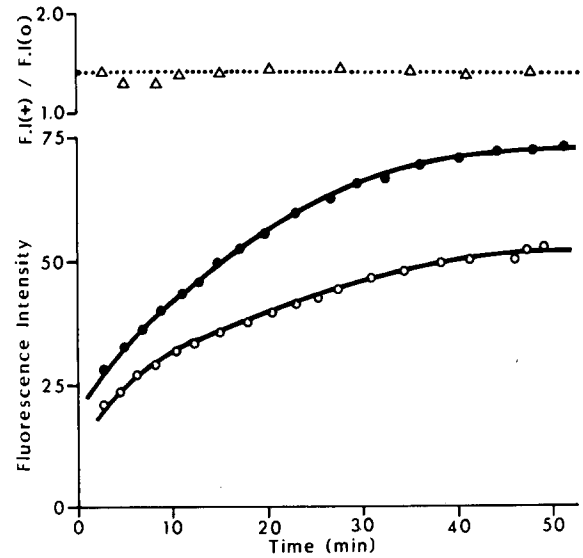


Fig. 4. Time course of the reaction of CFC with mycoplasma. Organisms were resuspended (0.25 mg cell protein/ml) in phosphate buffer (pH 7.2) containing 0.5 mg/ml CFC and no glucose (○) or glucose (●) added. Upper part of the figure as in Figs. 2 and 3.

1.1 to 1.5. A hundred time variation in the concentration of the fluorescamine added to the cell suspension did not significantly modify the value taken by this ratio (Fig. 3). Addition of triton X-100 (0.5 mg/ml) prior to labeling increased the fluorescence intensity by 2.3 times. On the other hand, the fluorescence intensity recovered from the cell soluble fraction prepared from labeled organisms after washing and alternate freeze thaw cycles in isotonic phosphate buffer was generally less than 5% for both energized and non-energized cells.

Using the cycloheptaamylose-fluorescamine complex (CFC) the time needed for the completion of the reaction was, in accordance with literature [15], increased but the results were essentially identical, i.e., more primary amino group being exposed upon energization (Fig. 4). The range of variation of the corresponding $I(+)/I(o)$ ratios was comparable to that obtained with fluorescamine. While the addition of DCCD ($5 \cdot 10^{-5}$ M), an inhibitor of the membrane bound ATPase of mycoplasma [8], abolished the increase in fluorescence intensity (Table I), the extent of labeling by fluorescamine of isolated membranes was unaffected by the addition of ATP, under conditions of ATP hydrolysis (presence of Mg^{2+}). This suggests the process is dependent on the activity of the ATPase but also requires the integrity of the cellular membrane.

Effect of ionophores

Addition of valinomycin ($5 \cdot 10^{-7}$ M) to resting and metabolizing cells produced only a limited decrease in the $I(+)/I(o)$ ratios obtained with fluorescamine or CFC. On the other hand, the $I(+)/I(o)$ ratios tended to unity when either FCCP ($5 \cdot 10^{-7}$ to $5 \cdot 10^{-6}$ M) or gramicidin were added (Table I).

Merocyanine 540 can be used to monitor the relative changes in transmembrane potential ($\Delta\psi$) in mycoplasma [8,9,22]. Concurring with our previous data (Fig. 4, Ref. 8) FCCP at identical concentrations to those used in fluorescamine or CFC experiments had nearly no effect on the fluorescence intensity of merocyanine in metabolizing cells while the addition of both FCCP and valinomycin collapsed the fluorescence quenching induced by glucose. On the other hand, ΔpH the second component of the proton motive force

TABLE I

EFFECT OF ENERGIZATION ON PROTEIN LABELING

Organisms were resuspended (0.25 mg cell protein/ml) in 0.1 M phosphate buffer, pH 7.2. For labeling fluorescamine a final concentration of 75 μg/ml was used. For CFC the concentration used was 0.5 mg/ml. Glucose was added at a final concentration of 10 mM; valinomycin, FCCP, gramicidin, and DCCD were added both in the energized and non-energized cell batches at final concentration of $5 \cdot 10^{-7}$ M, $5 \cdot 10^{-7}$ M, 0.1 μg/ml, $5 \cdot 10^{-5}$ M, respectively. (+) = labeling in presence of 10 mM glucose. (o) = labeling in absence of added glucose. n.d., not determined.

Labeling agent	Relative intensity ratio ($I(+)/I(o)$)				
	Control	+Valinomycin	FCCP	+Gramicidin	+DCCD
Fluorescamine	1.50	1.38	1.04	1.00	1.00
CFC	1.31	1.26	1.06	1.01	n.d.

estimated by the flow dialysis technique or by a fluorescence technique using 9-aminoacridine as a ΔpH probe was inhibited by FCCP alone (Table II, Fig. 5).

Effect of external pH

Variations of external pH from 5.8–6.2 to 8.2, values within the range of what is obtained in the growth medium during mycoplasma growth, resulted in a decrease of the $I(+)/I(o)$ ratio from approximately 2 to unity (Fig. 6, Table III).

No univocal changes in the physical state of membrane lipids were observed when cells were resuspended at various pH. Thus by ESR the hyperfine splitting of I(12,3) acid slightly de-

Fig. 5. Variations of ΔpH induced by glucose and ionophores. Cells were incubated at 0.25 mg cell protein/ml into buffered saline solution containing 5 mM phosphate (pH 7.2) and 1.25 μM 9-aminoacridine. Ordinate: relative changes in fluorescamine intensity ($\Delta F/F$) brought on by glucose (5 mM), and FCCP ($5 \cdot 10^{-7}$ M). $\lambda_{excitation} = 400$ nm. $\lambda_{emission} = 455$ nm.

TABLE II

EFFECT OF FCCP AND VALINOMYCIN ON ΔpH

Intracellular pH (pH_i) was determined by the flow dialysis technique as described in Materials and Methods. pH_{ex}, pH of the suspending media. n.d., not determined.

pH_{ex}	pH_i			
	Without glucose	Glucose	Glucose +FCCP	Glucose +valinomycin
5.7	5.5	6.6	5.6	6.7
6.4	6.4	7.0	6.4	n.d.
7.2	7.1	7.7	7.1	7.7
7.7	7.7	7.9	7.6	7.9

TABLE III

VARIATION OF THE $I(+)/I(o)$ RATIO AS FUNCTION OF EXTERNAL pH

Cells were resuspended (0.25 mg cell protein/ml) in 0.1 M phosphate buffer and labeled by either 0.75 μg/ml fluorescamine or 0.5 mg/ml CFC. FCCP ($5 \cdot 10^{-7}$ M) was used to block the effect of glucose addition on the fluorescence intensity.

Labeling condition	External pH		
	5.8	6.8	8.2
Fluorescamine	1.75	1.35	0.98
CFC	2.04	1.38	0.98
CFC+FCCP	0.83	0.95	0.93

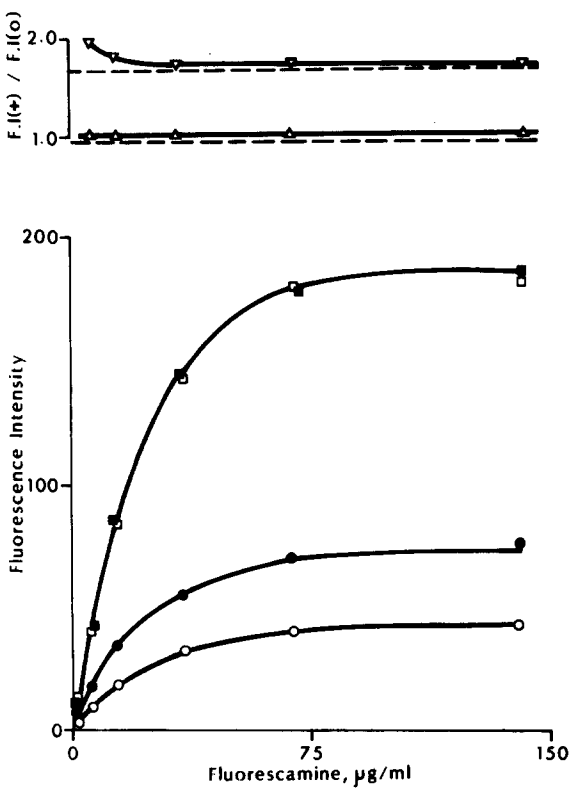


Fig. 6. Relative labeling as function of external pH. Experiments were conducted as in Fig. 3 but using a 0.1 M phosphate buffer adjusted to 6.2 (●, ○, ▽) or to 8.2 (■, □, △). Filled symbols correspond to measurements obtained in energized cells. The dashed line in the upper part of the figure corresponds to the F.I(+)/F.I(o) values obtained in membranes isolated from corresponding organism labeled by CFC.

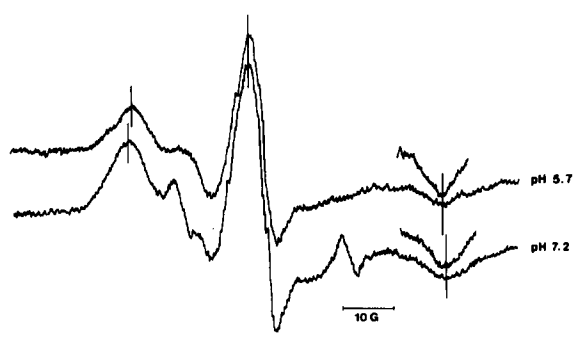


Fig. 7. ESR spectra of intact mycoplasma cells. Spectra was recorded at a 100 G field sweep and outer hyperfine splitting ($2T_{\parallel}$) were magnified by increasing the receiver gain.

creased from 62 ± 0.2 G to 61 ± 0.2 G when pH varied from 7.7 to 5.7 (Fig. 7) but no significant difference were observed for the I(12,3) alcohol and methyl ester derivatives. This can be simply explained by the fact that the anchoring of stearic acid spin label depends on the protonation state of the carboxylic group [24]. Using DPH, fluorescence polarization was barely affected varying between 0.253 ± 0.004 at pH 6.2 and 0.244 ± 0.004 at pH 8.2, i.e., in the reverse direction of what was expected from I(12,3) spin-labeled fatty acid data.

Similarly to the results described for pH 7.20, addition of glucose to resting cells at pH 5.8 or 8.2 had, if any, a very limited effect on the physical state of membrane lipids.

Discussion

The aim of the present work was to analyze the changes in the membrane organization that follows energization in mycoplasma cells. Data demonstrate that, in intact cells, the degree of accessibility of primary amino groups to CFC or fluorescamine was enhanced upon energization while no significant changes in membrane fluidity was detectable by ESR, *trans*-parinaric acid, or 16-anthroylstearic acid fluorescent probes. Using DPH and NPN a limited decreased in fluorescent anisotropy was noted. It could correspond to either a small increase in the fluidity of the local environment viewed by these probes or reflect an effect of energization at the level of membrane proteins, as DPH can bind to their hydrophobic portions [25] and NPN can lie at the lipid-protein interfaces [26]. Data from CFC, fluorescamine and other probes of membrane fluidity favor this last hypothesis, but even if the first possibility holds, it can be safely said that changes in membrane fluidity following energization, if they exist, are of very limited importance. In addition, the absence of univocal variations in the physical state of membrane lipids when pH is varied within a large range (5.6 to 8.0) or when the osmolarity of the medium is modified (unpublished results) suggests that the phospholipid matrix of *M. capricolum* and *M. mycoides* subs *capri* is very stable over a large range of physicochemical conditions. This property could have been related to the high level of cholesterol within the membrane of these

organisms [27]. Some experiments were performed on low-cholesterol adapted cells and led to the same results indicating that this is not true.The suitability of CFC and fluorescamine as a probe for membrane proteins in mycoplasma is due to the fact that these organisms have no amino phospholipids leading to a specific labeling of primary amino groups of proteins. Because some controversy exists about the impermeability of various plasma membrane to fluorescamine we used both fluorescamine and the cycloheptaamylose fluorescamine complex, well recognized as impermeant labeling agent [15–17]. Evidence for the impermeability of *Mycoplasma capricolum* and *M. mycoides* subs *capri* membranes to fluorescamine was obtained from many observations. (a) Results with fluorescamine were identical to those obtained with CFC used as labeling agent. (b) Cells disrupted by Triton X-100 and then reacted with fluorescamine were labeled to an extend up to three times more than in intact organisms. (c) When cells were labeled by fluorescamine and then subjected to osmotic lysis, generally less than 5% of the total fluorescence was recovered in the soluble fractions. This fluorescence could have originated from peripheral proteins released from the membrane during the lysis procedure. (d) In contrast to liposomes [14], a hundred time variation in fluorescamine concentration does not significantly modify the labeling ratio energized/non-energized cells. Pooling all these experimental results it can be concluded that fluorescamine only labeled the external surface of *M. capricolum* and *M. mycoides* subs *capri* membranes. The relative degree of permeability in various cells and organelle membranes may correspond to differences in membrane composition and/or physical state.

Addition of glucose to resting cells resulted in a significant increase in the fluorescence intensity of the suspension. This indicates that following energization more primary amino groups of proteins become accessible to the aqueous surrounding. Such data agree with a previous report on *Acholeplasma laidlawii* [7] but also confirm the results obtained on mitoplast and chloroplasts [2,4], or *Rhodospirillum rubrum* [5] in which energization of membranes by illumination give clear stimulation of the fluorescence labeling. Taking the stability of the lipid core of the membrane into account it can be concluded that, in mycoplasma, the degree of exposure of fluorescamine reactive sites is not correlated with changes in membrane fluidity. This agrees with the recent findings of Amar et al. [7] in *Acholeplasma laidlawii* but argues against the concept of a vertical displacement of the membrane proteins upon changes in lipid fluidity [28].

The second important point raised by our study concerns the relative participation of the electrical ($\Delta\psi$) and chemical (ΔpH) components of the proton-motive force to the changes in membrane organization. Inhibition by DCCD of the effect of cell energization on the accessibility of primary amino groups implies that the membrane-bound Mg^{2+}-ATPase of mycoplasma plays a crucial role in the membrane reorganization process. The absence of a significant effect of ATP on the fluorescamine labeling of isolated membranes also indicates the neccessity of the cell integrity for a membrane reorganization to occur. Our data clearly demonstrate that FCCP or CCCP completely collapses the difference of labeling between metabolizing and resting cells. Similar results were obtained with proton conductors in mitoplast membranes under energized and non-energized states. In the mycoplasma strains used however, FCCP or CCCP when used alone inhibit only the ΔpH, leaving the $\Delta\psi$ nearly untouched [8]. Accordingly it can be concluded that the increased accessibility of primary amino groups is principally, if not exclusively, dependent on the existence of a ΔpH. Results from experiments at external pH of 5.8 and 8.2 support this conclusion. Thus, changing the external pH from 5.8 to 8.2 results in a decrease of the ΔpH from about one unit to a non-detectable value. This corresponds to a decrease in the $I(+)/I(o)$ ratio from about 2 to 1.0, i.e., the effect of energization on the accessibility of primary amino groups disappeared when the external pH was identical to the internal pH.

The observation that at pH 8.2 the effect of energization on exposure of primary amino groups vanished argues against a role of $\Delta\psi$ in the phenomenon as $\Delta\psi$ increases as external pH is raised [9]. Because we could not suppress the $\Delta\psi$ independently of the ΔpH it remains however possible that the $\Delta\psi$ must be present for the ΔpH effect to be effective.

As experiments were performed on intact cells both peripheral and integral proteins may be involved in the increased exposure of primary amino to the labeling agents. For integral proteins, it has already been shown that a transmembrane proton gradient can induce a conformational change resulting in the exposure of new free amino groups [2]. For peripheral proteins one can envisage the possibility that important changes in surface charges are brought on by the development of the ΔpH. This is turn would affect the conformation of some of those proteins leading to an increase in the accessibility of primary amines to fluorescamine or CFC. Changes in surface charge which are known to occur upon pH variation, modifications of divalent cation concentrations, energization have also been proposed to play a regulatory role for membrane-bound enzymes [29–31]. In the experimental conditions selected for the present study, i.e., high concentration of cations, 9-aminoacridine is believed to report mainly on ΔpH variations [32,33]. However, at low cation concentration, it also can be used to probe the surface potential (ψ_s) of membranes [34,35]. Relationships between ΔpH and ψ_s are however not well established and it is difficult to assess, from the present experiments what part of the fluorescence intensity variation is attributable to a possible change in ψ_s following energization. Correspondence in our conditions between ΔpH measurements by free-flow dialysis and qualitative results with 9-aminoacridine suggests either a minor contribution of ψ_s variations to the total fluorescence signal or that ψ_s is directly related to ΔpH. Further experiments are currently in progress to clarify the possible role of the changes in surface potential on the exposure of primary amino groups and on the protective effect of energization against the attack by phospholipase A_2 in mycoplasma.

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