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MOTION OF FATTY ACID SPIN LABELS IN THE PLASMA MEMBRANE OF MYCOPLASMA

SHLOMO ROTTEM*, WAYNE L. HUBBELL**, LEONARD HAYFLICK* AND HARDEN M. McCONNELL**

*Department of Medical Microbiology, Stanford University School of Medicine and **Stauffer Laboratory for Physical Chemistry, Stanford, Calif. (U.S.A.)

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SUMMARY

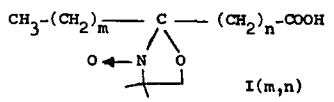
The electron paramagnetic resonance (EPR) spectra of spin labeled fatty acid derivatives (I (m,n)) in *Mycoplasma laidlawii* membranes showed a steep temperature dependence. The hyperfine splitting ($2T_m$) of these spectra decreased as the nitroxide radical was moved away from the polar head group of the fatty acid derivatives, demonstrating an increase in molecular motion of the nitroxide radical. The freedom of moti on of the spin label I (12,3) in *M. laidlawii* membranes was higher in membranes containing *cis*-Δ^9-octadecenoic acid than in membranes containing the corresponding *trans* isomer. A high freedom of motion was also observed in Mycoplasma membrane reaggregates having a high lipid to protein ratio. Upon increasing the relative amount of protein in the reaggregates a decrease in the freedom of motion of the nitroxide radical was found. The freedom of motion of the spin label I (12,3) in reaggregates formed at a high Mg^{2+} concentration (>20 mM) was similar to that of the native membranes ($2T_m = 62.0$ gauss). Throughout the growth cycle of *M. laidlawii* a high freedom of motion of spin label I (12,3) was found in cell membranes from the early growth phase ($2T_m = 58.0$ gauss). These membranes had a low density ($d = 1.165$) due to a high amount of oleic acid in membrane polar lipids. Decreasing the growth temperature of the cells resulted in an increase in the amount of [^{14}C]oleic acid and a decrease in the amount of [^{3}H]cholesterol incorporated into the cell membrane. The freedom of motion of spin label I (12,3) in membranes from *M. laidlawii* cells grown at 15° was much higher ($2T_m = 58.0$ gauss) than that in membranes from cells grown at 37° ($2T_m = 62.5$ gauss) when compared at the same temperature.

INTRODUCTION

Mycoplasma are bounded by a single lipoprotein membrane[1]. The mode of organization of protein and lipid in the membrane is still uncertain. The possibility of varying the lipid to protein ratio in membranes of *Mycoplasma laidlawii* by blocking protein synthesis demonstrated that synthesis of membrane lipids is uncoupled from that of membrane protein[2]. In addition, reaggregation of detergent-

solubilized membrane material from several Mycoplasma species occurred by the formation of a lipid backbone on which proteins are attached, indicating an associated lipid structure in the membrane[3,4]. Recently a thermal "phase transition" was observed in *M. laidlawii* membranes also suggesting that membrane lipids are organized in a bilayer conformation[5,6].

The use of electron paramagnetic resonance (EPR) techniques for studying problems of structure and function in mycoplasma membranes was stimulated by previous EPR studies of phospholipid dispersions and some biological membranes[7–10]. These studies showed that the EPR spectra of nitroxide spin labels, having the general formula



I(m,n)

yield information about the nature of the anisotropic motion of the spin label in the membranes and the physical state of the membrane lipids. The preferred orientation of the amphiphilic axis of spin labels I(m,n) in membranes was found to be perpendicular to the local membrane surface and the hyperfine splittings $2T$ maximum ($2T_m$) of these spectra were related to the freedom of motion of the nitroxide radical[8], greater freedom of motion being associated with smaller values of $2T_m$. In the present studies spin labels I(m,n) were utilized in investigating the lipid organization in the Mycoplasma membrane and changes occurring in the physical state of membrane lipids under varying physiological conditions.

MATERIALS AND METHODS

Organisms and growth conditions

Mycoplasma laidlawii (oral strain) and *Mycoplasma gallisepticum* (strain A5969) were grown in a modified Edward medium[11], or in a lipid pre-extracted tryptose medium[12] supplemented with an octadecenoic acid (5 μg/ml). For labeling membrane lipids 0.5 μC of [1-^{14}C]oleic acid (10 mC/mmole (ICN, Chemical Co., Radioisotope Division, Irvine, Calif.)) and/or 2 μC of [7-^{3}H]cholesterol (32 C/mmole (New England Nuclear, Boston, Mass.)) were added to each l of modified Edward medium containing unlabeled oleic acid (10 μg/ml). Growth was followed by measuring the absorbance at 640 nm with a Beckman DB spectrophotometer. The organisms were harvested after 16–24 h at 15°, 22° or 37° and harvested at an absorbance of 0.17–0.19. The harvested cells were washed twice in the cold with 0.25 M NaCl.

Assessment of osmotic fragility

Osmotic fragility of the washed cells was determined as described before[13]. Results were expressed as percent lysis in 0.05 M NaCl.

Isolation of cell membranes

Cell membranes were obtained by osmotic lysis[3]. The membranes were washed twice with β-buffer (0.15 M NaCl, 0.05 M Tris, 0.01 M 2-mercaptoethanol in deionized water adjusted to pH 7.4 with HCl (ref. 14)) diluted 1:20 in deionized water (will

be referred to as "dilute β-buffer") and incubated for 1 h at 37° in dilute β-buffer containing 10 μmoles/ml $MgCl_2$ and 100 μg/ml ribonuclease A (5 × crystalline, Sigma Chemical Co., St. Louis, Mo.). The membranes were then collected by centrifugation at 37000 × *g* for 45 min and washed 5–10 times as previously described[3]. The washed membranes were resuspended in 0.25 M NaCl and kept at —20°. Only traces of nucleic acids could be found in the membranes.

Density gradient centrifugation

Samples (0.15 ml, 250 μg protein) of labeled membranes were layered over 4.2-ml linear 28–46% sucrose gradients. The gradients were centrifuged at 39000 rev./min for 2 h at 4° in a SW39 rotor of a Spinco model L-2 ultracentrifuge. Fractions (0.08 ml) were collected by puncturing the bottom of the tube and assayed for protein and/or radioactivity.

Extraction of lipids

Total lipids were extracted from lyophilized membranes with chloroform–methanol (2:1, v/v) and washed according to the method of FOLCH *et al.*[15]. Polar lipids were separated from neutral lipids by silicic acid chromatography as described previously[12] and the fatty acids were obtained after alkaline hydrolysis of the polar lipid fraction[16].

Solubilization and reaggregation of membranes

Labeled membranes (4–5 mg membrane protein per ml) were solubilized by incubating them in 0.01 M sodium dodecyl sulfate for 15 min at 37°. In order to remove the sodium dodecyl sulfate the solubilized membranes were passed through a Sephadex G-25 (Pharmacia AB, Uppsala, Sweden) column, as described by ROTTEM *et al.*[3]. The solubilized membranes were dialysed for 4 days at 4° against 1000 vol. of dilute β-buffer containing various concentrations of $MgCl_2$. The reaggregated material was collected by centrifugation at 37000 × *g* for 1 h, resuspended in 0.25 M NaCl and assayed for protein and radioactivity. The ratio of lipid to protein in the reaggregates was expressed as counts/min per mg protein.

Paramagnetic resonance spectroscopy techniques

The *N*-oxyl-4′,4′-dimethyloxazolidine derivatives of 5-ketopalmitic acid, 8-ketopalmitic acid, 5-ketostearic acid and 12-ketostearic acid were synthesized according to KEANA *et al.*[17]. The keto methyl esters were prepared according to JONES[18]. Aqueous dispersions of *M. laidlawii* membrane lipids were prepared by agitation of the lipid in deionized water[19]. The lipid dispersions were spin labeled as described by HUBBELL AND MCCONNELL[7]. *M. laidlawii* membranes were spin labeled by exchange from bovine serum albumin[8]. For the exchange 0.2 ml of membrane suspension in 0.25 M NaCl (2–3 mg protein) was incubated for 5 min at 4° with 0.5 ml of approx. 2.5 mM spin label in 5% bovine serum albumin fraction V (fatty acid Poor, Pentex, Kankakee, Ill.). The membranes were then sedimented for 30 min at 37000 × *g* in the cold, washed once with 5 ml of an ice cold solution of 0.25 M NaCl and resuspended in 0.2 ml of the same solution. EPR spectra were obtained using a Varian E-4 spectrometer. Samples were held in a sealed Pasteur

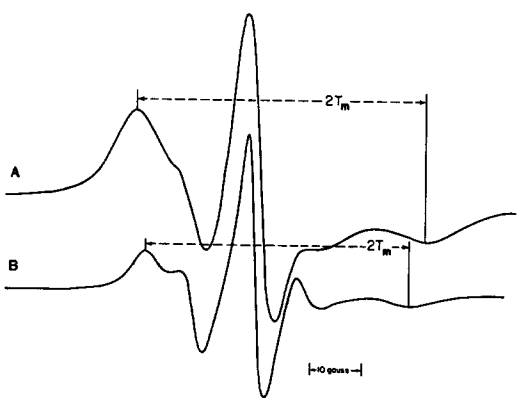
pipette. Unless otherwise stated all spectra were recorded at 15°. The hyperfine splitting ($2T_m$) was measured to within $\pm$ 0.5 gauss.

Assay procedures

Protein was determined by the Folin phenol method of LOWRY *et al.*[20] with crystalline bovine plasma albumin as a standard. Glutaraldehyde fixation of membranes was carried out according to SABATINI *et al.*[21]. For radioactivity measurements, samples (0.05–0.1 ml) of membranes (0.5–1.0 mg protein) or lipid fractions were transferred to scintillation vials. 1-ml portions of soluene 100 (Packard Instrument Co., Downers Grove, Ill.) were added to the vials and the vials were incubated for 1 h at 45°. Each vial then received 10 ml of toluene scintillation liquor (5 g 2,5-diphenyloxazole, 500 mg 1,4-bis-(5-phenyloxazolyl-2) benzene in 1 l of toluene) and radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer.

RESULTS

Fig. 1 shows the similar EPR spectra of *N*-oxyl-4',4'-dimethyloxazolidine derivative of 5-ketostearic acid (spin label I (12,3)) in *Mycoplasma laidlawii* membranes (A) and in aqueous dispersions of *M. laidlawii* membrane lipids. Although there is a great similarity in the spectra, the large hyperfine splitting ($2T_m$) is different, indicating a higher freedom of motion of spin label I (12,3) in the lipid dispersion. The hyperfine splitting ($2T_m$) of *N*-oxyl-4',4'-dimethyloxazolidine derivatives of 5-ketopalmitic acid (spin label I (10,3)), 8-ketopalmitic acid (spin label I (7,6)), 5-ketostearic acid (spin label I (12,3)) and 12-ketostearic acid (spin label I (5,10)) in *M. laidlawii* membranes showed a pronounced temperature dependence (Fig. 2).



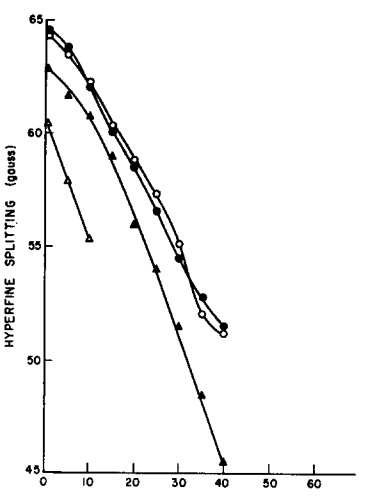


Fig. 1. The EPR spectra of *N*-oxyl-4',4'-dimethyloxazolidine derivative of 5-ketopalmitic acid (spin label I (10,3)) in *M. laidlawii* membranes (A) and in aqueous dispersion of *M. laidlawii* membrane lipids (B).

Fig. 2. Temperature dependence of the hyperfine splitting ($2T_m$) of *N*-oxyl-4',4'-dimethyloxazolidine derivatives of 5-ketostearic acid, spin label I (12,3) (○—○); 12-ketostearic acid, spin label I (5,10) (△—△); 5-ketopalmitic acid, spin label I (10,3) (●—●); and 8-ketopalmitic acid, spin label I (7,6) (▲—▲); in *M. laidlawii* membranes.

When the nitroxide group occupied the same position on the fatty acid derivative chain relative to the carboxyl group, the hyperfine splitting ($2T_m$) was found to be the same and independent of the total chain length. Thus a palmitic acid derivative spin label (I (10,3)) and a stearic acid derivative spin label (I (12,3)) showed the same hyperfine splitting (Fig. 2). However, the hyperfine splittings ($2T_m$) were found to be sensitive to changes in the position of the nitroxide group on the fatty acid derivative chain decreasing as the nitroxide group is moved away from the polar end of the molecule (Fig. 2). This positional dependence demonstrated an increase in the molecular motion of the nitroxide radical with the increasing of distance from the polar head group.

Fig. 3 shows the EPR spectra of spin label I (12,3) in membranes containing *cis*-Δ^9-octadecenoic (oleic) acid or the corresponding *trans* isomer as the major fatty acid. Both *cis* and *trans* isomers were incorporated into membrane polar lipids to about the same degree (60–66 % of total fatty acids). However, the EPR spectra revealed a higher freedom of motion of spin label I (12,3) in membranes containing the *cis*-octadecenoic acid isomer. This difference was most pronounced when the spectra were obtained between 5 and 20° (Fig. 4).

The effect of membrane protein on the freedom of motion of spin labels in membrane lipids is demonstrated in Table I. At low Mg^{2+} concentrations a membrane-like structure having a high lipid to protein ratio was formed. The freedom of motion of spin label I (12,3) in this reaggregated membrane was higher than that found for reaggregates formed at higher Mg^{2+} concentrations having a low lipid to protein ratio. The stabilization of membrane lipids by proteins was more pronounced in reaggregated membranes from *M. gallisepticum* than in reaggregates of *M. laidlawii*.

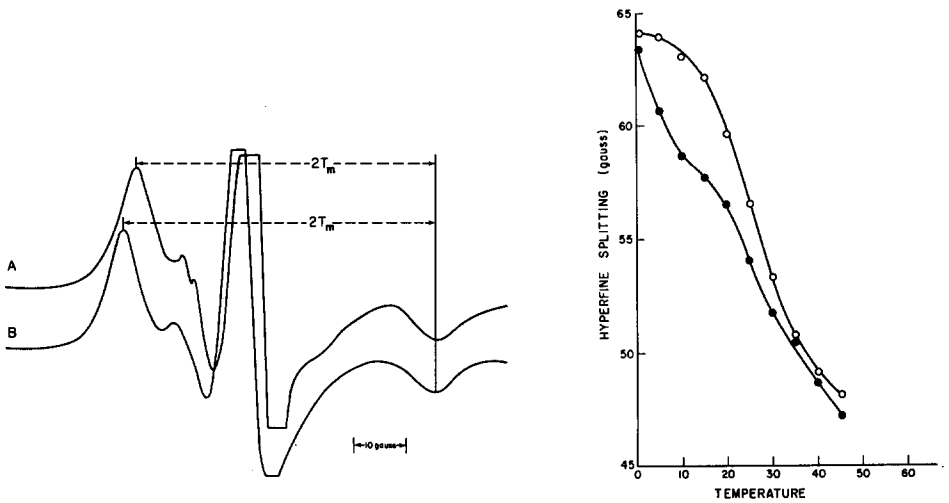


Fig. 3. The EPR spectra of *N*-oxyl-4′4′-dimethyloxazolidine derivative of 5-ketostearic acid (spin label I (12,3)) in membranes of *M. laidlawii* grown in a lipid pre-extracted tryptose medium[12] supplemented with *cis*-Δ^9-octadecenoic acid (A), or *trans*-Δ^9-octadecenoic acid (B).

Fig. 4. The effect of temperature on the hyperfine splitting ($2T_m$) of *N*-oxyl-4′,4′-dimethyl-oxazolidine derivative of 5-ketostearic acid (spin label I (12,3), in membranes of *M. laidlawii* grown in a lipid pre-extracted tryptose medium[12] supplemented with octadecenoic acid isomers (5 μg/ml); ○—○, *trans*-Δ^9-octadecenoic acid; ●—●, *cis*-Δ^9-octadecenoic acid.

TABLE I

HYPERFINE SPLITTING OF *N*-OXYL-4',4'-DIMETHYLOXAZOLIDINE DERIVATIVE OF SODIUM 5-KETOSTEARATE IN NATIVE MEMBRANES AND IN MEMBRANE REAGGREGATES

For reaggregation of membrane material, membranes containing labelled lipids were solubilized in 10 mM sodium dodecyl sulfate, and reaggregated by dialysis against dilute buffer containing various Mg^{2+} concentrations for 4 days at 4°. Paramagnetic spectral analysis and the hyperfine splittings were determined as described under MATERIALS AND METHODS.

Preparation	*Mg^{2+} in dialysis buffer (mM)*	*M. gallisepticum*		*M. laidlawii*	
		Lipid/protein (counts/min per mg protein)	*Hyperfine splitting (gauss)*	*Lipid/protein (counts/min per mg protein)*	*Hyperfine splitting (gauss)*
Native membranes	—	12 736	61.75	5 125	59.50
Membrane reaggregates	5	44 527	58.50	14 030	58.25
	10	17 440	61.25	7 170	58.75
	20	12 460	62.00	5 454	59.50

In both membranes at 20 mM $MgCl_2$ the reaggregates resembled the original membranes both in their chemical composition and in their fluidity. The composition of the reaggregates as well as their fluidity were not affected by the removal of the sodium dodecyl sulfate from the solubilized membrane material prior to the reaggregation. The freedom of motion of spin label I (12,3) in native membranes of *M. laidlawii* or *M. gallisepticum* was not affected by glutaraldehyde fixation of the membranes, by incubation of the membranes for 2 h at 4° in 20 mM $MgCl_2$, or by prolonged (2–6 days) dialysis of the membrane suspensions against dilute β-buffer containing EDTA (up to 0.05 M).

The increase in osmotic fragility of *M. laidlawii* cells grown in Edward medium supplemented with oleic acid (10 μg/ml) from 41% in cells from early growth phase (absorbance 0.07) to 74% in cells from late growth phase (absorbance 0.48) indicated structural changes in the cell membrane with age. Fig. 5 shows that the amount of [^{14}C]oleic acid incorporated into cell membranes was higher in cells harvested at an early phase of growth. These membranes possessed a highly fluid membrane as judged by the freedom of motion of spin label I (12,3) and a much lower density (d = 1.166) than that of membranes from cells harvested at a late phase of growth (d = 1.184). The distribution of [^{14}C]oleic acid in membranes and membrane fractions of cells harvested at various growth phases is shown in Table II indicating that the major change with age is the degree of unsaturation of membrane fatty acids rather than the lipid to protein ratio.

Changing the growth temperature of *M. laidlawii* cells resulted in changes in the chemical composition of the cell membrane as well as in the freedom of motion of spin labels in the membrane. Table III shows the increase in the amount of [^{14}C]-oleic acid and a decrease in the amount of [^{3}H]cholesterol incorporated into *M. laidlawii* membranes when growth temperature was lowered to 15°. The EPR spectra of spin label I (12,3) in these membranes showed an increase in the freedom of motion of the nitroxide radical with the decrease in the growth temperature of the cells. The differences in the hyperfine splitting ($2T_m$) of the spin label in membranes

TABLE II

DISTRIBUTION OF [^{14}C]OLEIC ACID IN MEMBRANE FRACTIONS OF *M. laidlawii* CELLS HARVESTED AT DIFFERENT AGES OF CULTURE

The cells were grown in Edward medium containing [^{14}C]oleic acid. Membranes were isolated and their lipids extracted and saponified as described under MATERIALS AND METHODS.

Age of Culture (h)	*Absorbance of culture (640 nm)*	*[^{14}C]Oleic acid in membrane fractions*		
		Whole membranes (counts/min per mg protein)	*Membrane lipids (counts/min per mg lipid)*	*Membrane fatty acids (counts/min per mg fatty acid)*
15	0.175	16 465	16 968	22 500
18	0.380	13 020	12 400	16 850
21	0.520	10 600	8 690	11 620

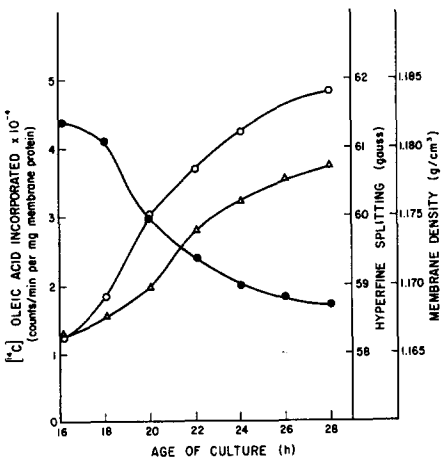


Fig. 5. The effect of the age of the culture on the density of *M. laidlawii* membranes (○—○); the hyperfine splitting ($2T_m$) of *N*-oxyl-4′,4′-dimethyloxazolidine derivative of 5-ketostearic acid (spin label I (12,3)) in the membranes (△—△); and the amount of [^{14}C]oleic acid incorporated into the membranes (●—●). The cells were grown in Edward medium containing [^{14}C]oleic acid and unlabeled oleic acid (10 μg/ml) and cell membranes were isolated as described in detail under MATERIALS AND METHODS. Membrane densities were determined using a linear sucrose density gradient (28–46%).

TABLE III

INCORPORATION OF [^{14}C]OLEIC ACID AND [^{3}H]CHOLESTEROL INTO MEMBRANES OF *M. laidlawii* CELLS GROWN AT DIFFERENT TEMPERATURES

The cells were grown in Edward medium containing [^{14}C]oleic acid and [^{3}H]cholesterol. Cell membranes were isolated as described under MATERIALS AND METHODS.

Temperature of growth	*Radioactivity (counts/min per mg membrane protein)*	
	^{14}C	^{3}H
15°	63 216	3 403
22°	58 676	4 548
37°	31 954	9 495

from cells grown at 15°, 22° and 37° were most pronounced when the spectra were taken between 5 and 20° (Fig. 6).

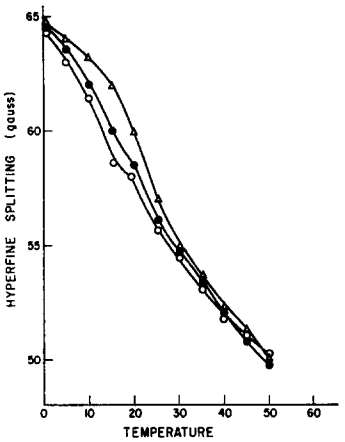


Fig. 6. The effect of temperature on the hyperfine splitting ($2T_m$) of *N*-oxyl-4',4'-dimethyloxazolidine derivative of 5-ketostearic acid (spin label I (12,3)) in membranes of *M. laidlawii* grown in Edward medium at 15° (○—○); 22° (●—●); and 37° (△—△).

DISCUSSION

A striking result of the present study is the remarkable similarity between the spectra of spin labels I (m,n) in the Mycoplasma membranes and aqueous dispersions of Mycoplasma phospholipids (Fig. 1). Especially noteworthy is the dramatic increase of nitroxide mobility as one goes from I (10,3) to I (5,10) in both the Mycoplasma membranes (Fig. 2) and phospholipid dispersions[8]. A similar effect has been noted with the I (12,3) and I (5,10) in the smectic liquid crystal system caprylic acid–decanol–water[22].

These observations lend support to the assumption that the local environment of spin labels I (m,n) in Mycoplasma membranes is an associated lipid structure whose properties are those of a bilayer.

It should be emphasized that the distribution of spin labels in the membrane is unknown and therefore we can draw no conclusion on the extent or arrangement of the assumed bilayer structure in the native membrane.

The fact that the freedom of motion of spin labels I (m,n) in the native Mycoplasma membrane was less than in dispersed mycoplasmal membrane lipids may indicate the influence of membrane proteins on the physical state of membrane lipids. The effect of protein was also shown in reaggregates of sodium dodecyl sulfate solubilized membrane material. Increasing the lipid to protein ratio in these reaggregates resulted in an increase in the freedom of motion of spin labels I (m,n). Glutaraldehyde fixation of native Mycoplasma membranes or changes in the Mg^{2+} concentration in the membranes did not affect the freedom of motion of spin labels I (m,n) in the membranes indicating that the physical state of membrane lipids is not critically sensitive to some conformational changes of membrane proteins.

The fatty acid composition of mycoplasma membrane lipids has a pronounced effect on the freedom of motion of spin label I (12,3). Thus, a higher 'fluidity' was

observed in membranes containing *cis*-Δ^9-octadecenoic acid than in membranes containing the corresponding *trans* isomer. It should be noted that these membranes contain only traces of cholesterol[23] and the amount of the *cis* and *trans* isomer incorporated were about the same. The high 'fluidity' of membranes containing the *cis* isomer corresponds to the lower melting point of this isomer. This is consistant with monolayer studies showing that a highly expended film is formed at the air–water interface by L-α-lecithin containing an unsaturated fatty acid in a *cis* configuration. A more condensed film was found with L-α-lecithin containing an unsaturated fatty acid in the *trans* configuration[24].

The loss of enzymatic activity as well as transport activities on aging is particularly pronounced in Mycoplasma[1]. The fact that a decrease in fluidity of the cell membrane lipid regions occurred on aging may indicate that the physical state of the membrane lipids is of importance to certain physiological activities of the cell[1,7,25]. This decreasing fluidity with cell age appears to be a result of a decrease in the ratio of unsaturated to saturated fatty acids that may be due to an increase in the rate of synthesis of saturated fatty acid with age [26] or to a selective incorporation of fatty acids from the media.

Parallel to the decrease in the fluidity of cell membranes with age, an increase in osmotic fragility of the cells was noticed, supporting previous assumptions that the conformation of membrane lipids has a determinative effect on the tensile strength of the membrane[23].

The change in the amount of oleic acid incorporated into *M. laidlawii* membrane lipids when the growth temperature was lowered can be understood simply as a process by which cells in a cold environment require unsaturated fatty acid of lower melting range to prevent a decrease in the "fluidity" of cellular membranes. This was supported by demonstrating a higher "fluidity" of membranes from cells grown at 15° than in membranes of cells grown at higher temperatures when compared at the same temperature. Changes in the content of unsaturated fatty acids in bacterial lipids upon dropping the growth temperature were described previously[27,28]. It is not known at present to what extent the cholesterol content affected these changes. The maximal amount of cholesterol in membranes of this non-sterol requiring Mycoplasma did not exceed 1 % of the membrane dry weight[29]. Such low levels of cholesterol have no effect on the osmotic fragility of *M. laidlawii* cells[23] and does not influence significantly the EPR spectra of the I (m,n) spin labels in phospholipid dispersions (W.L. HUBBELL, unpublished results).

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