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MEMBRANE LIPIDS OF MYCOPLASMAS

SHLOMO ROTTEM

Department of Membranes and Ultrastructure, The Hebrew University–Hadassah Medical School, Jerusalem (Israel)

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Contents

I. Introduction

Our current concept of membrane organization has emerged from intensive research on synthetic and biological membrane model systems. An extensively used biological membrane was the membrane of mycoplasmas. There are many advantages of using these organisms for membrane studies. Unlike other prokaryotic microorganisms, mycoplasmas

have no cell walls or intracellular membrane structures. The absence of a cell wall in mycoplasmas is a characteristic of outstanding importance to which the mycoplasmas owe many of their pecularities, for example, their morphological instability, osmotic sensitivity, resistance to antibiotics that interfere with cell wall biosynthesis, susceptibility to lysis by detergents, alcohols, etc. [1–4]. The fact that the mycoplasma cells contain only one membrane type, the plasma membrane, constitutes one of their most useful properties for membrane studies since it is certain that once the membrane is isolated, it is uncontaminated with other membrane types. This is also true for the erythrocyte. However, working with the mycoplasmas has the added advantage that these cells are capable of growth and reproduction. Another advantage in taking mycoplasmas as models for membrane studies stems from the fact that their membrane lipid composition can be altered in a controlled manner. This results from the partial or total inability of the mycoplasmas to synthesize long-chain fatty acids and cholesterol, making mycoplasmas dependent on the supply of fatty acids from the growth medium. The ability to introduce controlled alterations in the fatty acid composition and cholesterol content of mycoplasma membranes has been intensively utilized over the past decade to study the molecular organization and physical properties of biological membranes. The demonstration that the cytoplasmic membrane of *Acholeplasma laidlawii* underwent a reversible thermal transition similar to that observed in artificial bilayers [5–7] constitutes one of the arguments for the description of a membrane as a lipid bilayer with attached and embedded proteins.

Our detailed information concerning the role of phospholipid acyl moieties in membrane function, the regulatory mechanisms that control membrane fluidity and the transbilayer distribution of lipids between the outer and inner halves of the bilayer, stem from intensive studies carried out with mycoplasma cell membranes, primarily the cell membrane of *Acholeplasma laidlawii.* The present review will try not to overlap recent reviews on mycoplasma membranes but to update information and focus on some novel and unique lipids of the mycoplasmas. Readers seeking comprehensive and comparative reviews on the mycoplasma membrane, including detailed descriptions of the composition, distribution and biosynthesis of mycoplasma lipids, are referred also to 'The Mycoplasmas' (Barile, M.F., Razin, S., Tully, J.G. and Whitcomf, R.F., eds., Academic Press, 1979), a three volume series encompassing the various aspects of mycoplasmabiology, emphasizing outstanding developments made in the field during the past decade.

II. Lipid composition of mycoplasmas

Essentially all mycoplasma lipids are associated with the cell membrane. The lipid content of the membrane varies between the various species and depends on the growth phase and growth medium. During progression of mycoplasma cultures from the early logarithmic phase to the stationary phase of growth, the total lipid content of the cell membrane decreased [8–10]. This phenomenon is an expression of either a shutdown of lipid biosynthesis or an increase in membrane protein synthesis, since the ratios between the various classes of membrane lipids remain constant throughout the growth cycle. Substantial changes in the lipid content of the cell membrane of many *Mycoplasma* species were also noticed, by varying the serum concentration of the growth medium due to the massive incorporation of exogenous lipids from the growth medium [11–13]. Under optimal growth conditions, in a serum-free media, lipids from cells harvested in the mid-exponential phase of growth comprise 25–35% of the dry weight of the membrane [14].

The mycoplasmas have proven to be a veritable gold mine of new and unusual lipids.

For some the exact chemical structure has been resolved, while for others this information is still lacking. A compilation of our current knowledge of the lipids found in the various species can be found in other reviews [3,14,15]. The major membrane lipids of mycoplasmas are phospholipids, glycolipids and sterols. Neutral lipids are found in small amounts in all mycoplasmas. They consist mainly of glycerides and free fatty acids; the latter represent in some species the result of lipolysis which occurred during membrane isolation by the potent phospholipases and lipases [16,17]. In all the *Acholeplasma* species the cell membrane contains a de novo synthesized carotenoid pigment [18] that, except for *A. axanthum,* is easily visibly by its yellow color having an absorbance peak at 438 nm [19]. In *A. axanthum* the lack of absorption at 438 nm and the low incorporation of radioactive acetic acid into the nonpolar lipid fraction were taken as evidence for the absence of carotenoids [19]. This conclusion was recently challenged by showing that *A. axanthum* incorporates mevalonic acid into carotenoid pigments that due to being less unsaturated than those of other mycoplasma absorb light at shorter wavelengths [20]. The polyterpenoid structure of the carotenoid pigments was described previously [3] but their exact structural characterization is still obscure.

IIA. Phospholipids

The de novo synthesized phospholipids of mycoplasmas are rather simple, comprising primarily the acidic glycerophospholipids phosphatidylglycerol and diphosphatidylglycerol [15,21]. In most species tested so far, phosphatidylglycerol forms between 15 and 80% (by weight) of the de novo synthesized lipids. In *A. laidlawii* strains A and B phosphatidylglycerol is almost the only phospholipid but it comprises only 30% of the total de novo synthesized membrane lipids, the rest being glycolipids and phosphoglycolipids [22,23]. In the closely related *A. laidlawaii* oral strain both phosphatidylglycerol and diphosphatidylglycerol (10 and 20% of total lipids, respectively) are present [24]. Lyso and the more acylated derivatives of the acidic glycerophospholipids may also occur but in much lower amounts. Aminoacyl phosphatidylglycerols, in which an amino acid is

TABLE I

PHOSPHOLIPID AND CHOLESTEROL UPTAKE FROM GROWTH MEDIUM BY REPRESENTATIVE *ACHOLEPLASMA* AND *MYCOPLASMA* SPP.

The organisms were grown in a medium containing 4% horse serum (From Ref. 13).

Organism	Lipids in cells (μmol/g cell protein)			
	Phosphatidylcholine	Sphingomyelin	Cholesterol	
			Free	Esterified
A. laidlawii	0	0	10.2	0
A. axanthum	0	0	3.7	0
A. granularum	0	0	28.5	0
M. gallisepticum	37.8	15.5	76.0	4.7
M. hominis	20.3	32.7	76.4	30.7
M. arginini	31.9	37.8	58.2	27.5
M. pneumoniae	16.6	40.3	85.9	58.0
M. capricolum	40.1	20.8	67.2	67.7

esterified through its carboxyl group to one of the two free hydroxyl groups of the glycerol, were found in some mycoplasma. In *A. laidlawii* alanyl phosphatidylglycerol, with the alanine linked to C-3 of the terminal glycerol was described [25]. Although the alanyl form predominates, other amino acids can be covalently linked to the glycerol. Other unique phospholipids are found in some *Acholeplasma, Thermoplasma* and *Ureaplasma* species, and were described in detail elsewhere [15].

When grown in the ordinary mycoplasma media that contain horse serum, significant amounts of phosphatidylcholine and sphingomyelin as well as free and esterified cholesterol from the growth medium are incorporated into the cell membrane of many *Mycoplasma* species [12,13,20] (Table I). Phosphatidylcholine and sphingomyelin are uncommon lipids in wall-covered bacteria. These exogenous lipids are also not found in the *Acholeplasma* species [13]. In most *Mycoplasma* species the exogenous phospholipids are incorporated unchanged from the growth medium. In *M. gallisepticum* the phosphatidylcholine is a disaturated phosphatidylcholine differing from the 1-saturated 2-unsaturated phosphatidylcholine found in the growth medium [12]. The disaturated phosphatidylcholine is synthesized by the insertion of a saturated fatty acid at position 2 of lysophosphatidylcholine, derived from exogenous phosphatidylcholine of the growth medium, by what appears to be a deacylation-acylation enzymatic sequence.

IIB. Glycolipids

The term glycolipid is interpreted as lipids which are composed of carbohydrates in combination with long-chain fatty acids and which are readily extracted into organic solvents. It does neither include the phospholipids containing carbohydrate residues (phosphoglycolipids) found in *A. laidlawii* nor lipopolysaccharides that contain an oligosaccharide moiety and are not extracted into organic solvents. Glycolipids constitute a significant portion of membrane lipid in many mycoplasmas. The typical mycoplasmal glycolipids are the glycosyldiacylglycerols. The nature and number of sugar residues and their linkages provide for diversity of these lipids. The number of sugars varies from one to five. Usually the monoglycosyl compound predominates, in contrast to bacteria [27]. In *A. laidlawii* the glycolipids are mostly monoglucosyl diacylglycerol and diglucosyl diacylglycerol [22,28]. The molar ratio between the monoglycosyl diacylglycerol and the diglucosyl diacylglycerol is to a large extent determined by culture age [28,29] and membrane viscosity [22,30]. Increasing membrane viscosity by varying the fatty acid composition of membrane polar lipids, changing cholesterol content in the membrane or shifting down the growth temperature of the cells, stimulates the synthesis of monoglucosyl diacylglycerol at the expense of diglucosyl diacylglycerol. *A. modicum* contains as its predominant glycolipid a pentaglycosyl diacylglycerol [31] and in *M. pneumoniae* glycolipids containing both glucose and galactose were described [32]. A special type of glycolipid is found in the cell membrane of *Thermoplasma acidophilum*; this mycoplasma requires a combined high temperature and low pH for growth and reproduction. As might be expected, the membrane lipids of *T. acidophilum* contain ether lipids rather than typical fatty acid-derived acylglycerol residues. These lipids are comprised of two glycerol molecules bridged through ether linkages by two fully saturated, isoprenoid branched C_{40}-terminated diols [33–35] (Fig. 1). The alkyl chains may be either $C_{40}H_{82}$, $C_{40}H_{80}$ or $C_{40}H_{78}$. Though the tetraethers are unusual, they are not unique to *Thermoplasma* but also found in other thermoacidophilic bacteria [36]. It was suggested that the symmetrical diacylglycerol tetraether molecules provide these cells with the required structural

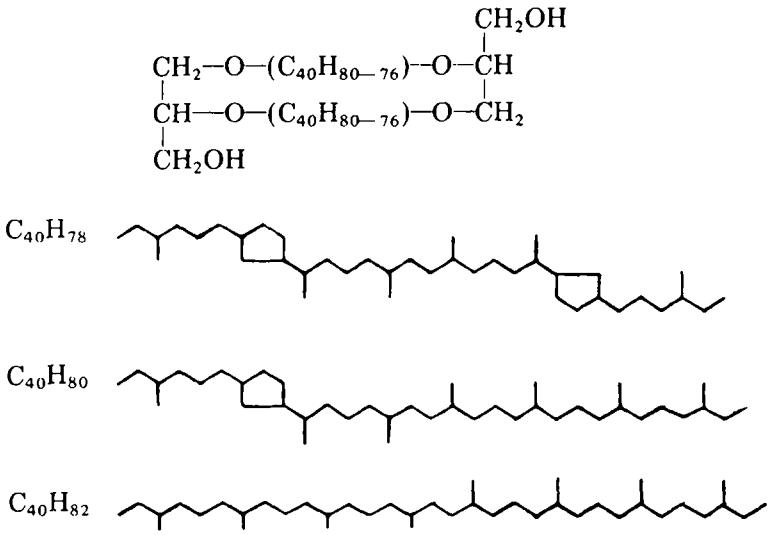

Fig. 1. Structure of the dialkyl diacylglycerol tetraethers from *Thermoplasma* [34].

stability [34]. The lipid backbone of these organisms is formed by a monolayer of the diacylglycerol tetraether molecules. Such monolayers have about the same thickness as that of a lipid bilayer and are much more stable. Therefore *Thermoplasma* membranes cannot be freeze-fractured [35], and the freedom of motion of its hydrocarbon chain is extremely low [37].

IIC. Lipopolysaccharides

A lipopolysaccharide, associated exclusively with the cell membrane, was recently found in mycoplasmas, mainly in the *Acholeplasma, Thermoplasma* and *Anaeroplasma* species. The lipopolysaccharide accounts for about 10% of the dry weight of the membranes. The molecular structure of the lipopolysaccharide monomer from *Thermoplasma*, *A. axanthum* and *A. modicum* were intensively studied by Smith and coworkers [15,31, 38–40]. The lipopolysaccharide of *Thermoplasma* behaves as monomer with an apparent molecular weight of 5300 and is composed of 24 mannose residues, all in the α-configuration, seven joined by 1→3-linkages, and 17 by 1→2-linkages. These mannose units are bounded through a α1→3-linkage to glucose which is glycosidically bounded to diacylglycerol tetraether [31]. A much larger monomeric unit approaching 100 000 dalton was described in *A. axanthum*, whereas in *A. modicum* the lipopolysaccharide fraction behaves as an heterogenous compound consisting of three components [15,39].

As expected from an oligosaccharide-containing molecule, the mycoplasma lipopolysaccharides are extractable in hot aqueous phenol and form long, ribbon-like structures in negatively-stained preparations [41]. However, the chemical composition of the mycoplasma lipopolysaccharide is distinct from the lipopolysaccharide found in Gram-negative bacteria. Thus, mycoplasma lipopolysaccharide lacks the typical sugar residues of Gram-negative lipopolysaccharide such as heptoses and ketodeoxyoctonate. It lacks phosphorylethanolamine and in most of the cases it consists of a linear oligosaccharide, glycosidically linked to diacylglycerol.

IID. Fatty acids

The fluidity of membrane lipids and the temperature range of the gel-to-liquid crystalline phase transition are primarily dependent on the chain length and chemical structure of the fatty acid residues of membrane phospho- and glycolipids. The fatty acid composition of mycoplasmas can be controlled to a large extent by the kind of fatty acids supplied to the growth medium. Such control is possible because mycoplasmas are defective in their fatty acid biosynthesis. *Acholeplasma* species are capable of synthesizing saturated but not unsaturated fatty acids from acetate [42-44]. *Mycoplasma* and *Spiroplasma* species thus far examined are incapable of fatty acid biosynthesis [2,4,15], while only the *Ureaplasma* species can synthesize both saturated and unsaturated fatty acids [45]. The synthesis of saturated fatty acid in *A. laidlawii*, the most widely studied mycoplasma, proceeds through the malonyl-coenzyme A pathway [46]. The block in the biosynthesis of unsaturated fatty acids by *A. laidlawii* has been identified at the level of β-hydroxydecanoyl thiolester dehydrase where branching of the pathway for the synthesis of unsaturated fatty acids occurs in bacteria [46]. Although incapable of synthesizing unsaturated fatty acids, *A. laidlawii* elongates both *cis* and *trans* unsaturated fatty acids to the corresponding hexadecenoic and octadecenoic acids [47,48]. The ability of *A. laidlawii* to synthesize saturated fatty acids interferes with attempts to obtain a controlled fatty acid composition of membrane polar lipids of this organism. In addition to the exogenous fatty acid incorporated from the growth medium, the organisms may contain as much as 50% of biosynthetic background containing mainly myristic and palmitic acids. By using antilipogenic compounds such as cerulenin, avidin or *N,N*-dimethyl-*trans*-4-oxo-2-dodecenamide that inhibit both de novo biosynthesis of long-chain fatty acids and the elongation of medium chain fatty acids by *A. laidlawii* [49–51], the biosynthetic background can be reduced. It was shown that growth of *A. laidlawii* cells cultured with avidin can be obtained by adding one or more exogenous fatty acids to the growth medium [50]. The extent of growth under these conditions depends primarily on the physical properties of the exogenous fatty acid(s) added. Fatty acids giving diacylglycerolipids of very high or very low viscosity are unsuitable growth substrates, while fatty acids giving diacylglycerolipids of intermediate viscosity support cell growth [50]. Using *N,N*-dimethyl-*trans*-4-oxo-2-dodecenamide it was also possible to abolish fatty acid heterogeneity without any obvious effects on cell growth, resulting in the sharpening of the thermotropic gel-to-liquid crystalline phase transition of membrane lipids [51].

Homogeneous fatty acid composition can be more easily obtained with the cholesterol-requiring *Mycoplasma* species. As these species are incapable of synthesizing either saturated or unsaturated fatty acid, or to alter the chain length of either, it is possible to control their fatty acid composition without the need to suppress the biosynthetic background. Thus *Mycoplasma* sp. strain Y grew well when elaidate (*trans*-octadecenoate) was the only fatty acid added to the growth medium, and this fatty acid was found to comprise over 97% of the fatty acid residues in the cells [52].

IIE. Positional distribution of fatty acids

Phospholipids derived from a variety of natural sources commonly show a nonrandom distribution of fatty acids; saturated fatty acids are located at position 1 of the glycerol while unsaturated, branched-chain or cyclopropane-containing fatty acids are usually found at position 2. Until recently, very little was known about the positional distribu-

TABLE II

POSITIONAL DISTRIBUTION OF PALMITATE AND OLEATE IN PHOSPHATIDYLGLYCEROL PREPARATIONS FROM REPRESENTATIVE *ACHOLEPLASMA* AND *MYCOPLASMA* SPECIES

Fatty acid distribution was determined after phospholipase A_2 treatment. The ratio in lysophosphatidylglycerol and in free fatty acids represent the ratios at position 1 and position 2 respectively [54].

Organism	Palmitate/oleate ratio			Position 1/Position 2 ratio of palmitate in phosphatidylglycerol
	Phosphatidylglycerol	Lyso-phosphatidylglycerol	Free fatty acid	
A. laidlawii	0.9	2.8	0.6	4.76
A. granularum	0.8	2.1	0.7	3.00
M. fermentans	2.5	0.4	10.2	0.04
M. mycoides subsp. *capri*	1.7	0.3	10.5	0.03
M. pneumoniae	1.4	0.2	4.9	0.04
M. capricolum	1.4	0.7	3.7	0.19
M. gallinarum	1.3	0.6	3.9	0.15
M. gallisepticum	1.1	0.2	7.9	0.03

tion of fatty acids in membrane lipids of mycoplasmas. Previous studies using *Acholeplasma laidlawii* B which requires no cholesterol showed that the fatty acid positional distribution in phospho- and glycolipids of this organism is in accord with that found elsewhere in nature [53]. On the other hand, an unusual positional distribution was recently found in the phosphatidylglycerol of many cholesterol-requiring *Mycoplasma* species [12, 54] (Table II) in which fatty acids having lower melting points were found more abundantly in position 1, while those with higher melting points were found in position 2.

The *Mycoplasma* species differ from *Acholeplasma* species in their growth requirement for cholesterol. Cholesterol reaches levels of about 50 mol% of total membrane lipids in *Mycoplasma* species as opposed to low cholesterol levels (up to 10 mol%) in the cholesterol non-requiring *Acholeplasma* species [55]. It was suggested that the requirement for cholesterol and its high content in the membrane of *Mycoplasma* species may be associated with the presence of phospholipids with unusual positional distribution [54]. One possibility is that the requirement for cholesterol is associated with different physical properties of the membrane imposed by the differences in the positional distribution of the fatty acids in membrane phospholipids. However, data obtained with artificial membrane systems [56,57] do not appear to support this suggestion. In these studies, force-area curves at the air-water interface of structural isomers of phosphatidylcholine with monounsaturated chains at position 1 or 2 were practically identical. The permeability of liposomes derived from these phosphatidylcholine preparations to glucose, erythritol and glycerol was also the same. Moreover, after mixing with cholesterol, the mean molecular area at the air-water interface, the permeability of the liposomes to nonionic substances and the energy content of the gel-to-liquid crystalline phase transition were decreased to about the same degree with both structural isomers. Another possibility is that the high cholesterol content in the membrane of *Mycoplasma* species affects the specificity of the acyltransferases located in the membrane. This possibility was recently excluded by showing that the positional distribution of palmitic and oleic acids in phos-

phatidylglycerol preparations from *M. capricolum* cells grown in a cholesterol-rich medium (native strain) and those adapted to grow in a cholesterol-poor medium (adapted strain) were very similar [54]. Since the native strain contained a 7-fold higher cholesterol concentration, it seems that in *M. capricolum* an unusual positional distribution of fatty acids exists regardless of the cholesterol content of the membrane.

IIF. Sterols

Mycoplasmas are the only prokaryotes showing a requirement for cholesterol for growth, thus making them unique models for studying the role of cholesterol in biological membranes [3,4,55]. Cholesterol in found in all species of *Mycoplasma, Spiroplasma* and *Ureaplasma* that require the sterol for growth, reaching levels comparable to those found in the plasma membrane of eukaryotic cells (25–30% by wt. of total membrane lipids). The *Acholeplasma* species not requiring sterol incorporate cholesterol into their membranes when this lipid is supplied in the growth medium. The cholesterol content of *Acholeplasma* membranes may reach levels of about 10% (by wt.) of total membrane lipids. None of the mycoplasmas tested so far, including the no sterol requiring species, is capable of cholesterol synthesis. Furthermore, the unesterified cholesterol incorporated from the growth medium is generally not esterified or changed in any other way. The cholesterol esters detected in membranes of most *Mycoplasma* species apparently originate from the growth medium, as their fatty acid composition resembles that of the esterified cholesterol fraction in the serum supplement of the growth medium [55,58].

Although any steroid containing an aliphatic side chain can be incorporated, only planar 3-hydroxy sterols permit growth and, hence, proper fit and function in the membrane [55,59,60]. Sterols which have been demonstrated to be functionally proper are cholesterol, β-sitosterol, ergosterol and cholestanol. For *M. capricolum* the requirement for sterol is met also by the methylcholestane derivatives lanosterol, cycloartenol, 4,4-dimethylcholesterol, 4β-methylcholestanol, cholesteryl methylether and 3α-methylcholestanol [61]. The unusual acceptance of diverse cholestane derivatives by *M. capricolum* contrasts with the structural attributes thought to be necessary for sterol function in mycoplasma and eukaryotic cells.

The uptake of the sterol was suggested to be a physical absorption process with an energy of activation of 6 kcal/mol [62]. Recent results showing that intact *M. capricolum* cells incorporate much less cholesterol under conditions where growth is inhibited [63] suggest that growing mycoplasmas may also possess a mechanism that catalyzes cholesterol incorporation into the membrane. The ordinary cholesterol donors in the growth media are serum lipoproteins. Using purified human-serum lipoproteins it was found that the amounts of cholesterol incorporated into the membranes from the low density lipoproteins (LDL) were much higher than those taken up from the high density lipoproteins (HDL) [64,65]. This result supports the notion that LDL is a far better cholesterol donor than HDL. The much higher molar ratio of unesterified cholesterol to phospholipid in LDL (about 0.8 : 1) as compared with that in HDL (about 0.15 : 1) may be responsible at least in part for the better performance of LDL as a cholesterol donor [65].

A model for the phospholipid-cholesterol complex in a membrane was recently presented by Huang [66] based on the structural properties of phospholipid and cholesterol molecules. Accordingly, the β-face of cholesterol is packed in close contact to the unsaturated fatty acyl chain esterified at position 2 of phosphatidylcholine while the α-face participates in strong van der Waal's interactions with the saturated acyl chains at position 1.

Since in the cholesterol-requiring *Mycoplasma* species unsaturated fatty acids seem to be commonly in position 1 of the de novo synthesized phospholipids [54], the β-face of the steroid nucleus will interact preferentially with the acyl chain esterified at position 1 of the phospholipids. Therefore the carbonyl oxygen of position 1, suggested by Huang [66] to be engaged in hydrogen bonding with the equatorial hydroxyl group of the cholesterol, is less common for such interaction in *Mycoplasma*.

III. Physical properties of mycoplasma membranes

The physical properties of mycoplasma membranes were intensively studied during the last decade by a variety of physical methods, such as differential scanning calorimetry [5,67 -69], differential thermal analysis [70,71], X-ray diffraction [6,7], EPR spectroscopy [72,73], NMR spectroscopy [74 -79], light scattering [80], and fluorescence polarization [81]. The results obtained were reviewed in detail previously [82,83]. These results firmly established that the bulk of membrane lipids in mycoplasmas constitutes a lipid bilayer. However, whereas differential scanning calorimetry and X-ray diffraction studies suggested that most lipid hydrocarbon chains associate with each other rather than with proteins, EPR spectroscopy [72,73] suggests that some membrane lipids interact with hydrophobic proteins which are partially embedded in, or traverse, the hydrocarbon core of the membrane [84]. The most striking event in the membrane is the gel-to-liquid-crystalline phase transition, with the bilayer conformation retained throughout the process. The entire temperature range of the phase transition can be detected calorimetrically or by X-ray diffraction. The phase transition is consistently dependent on the chain length and degree of unsaturation of the fatty acid residues of membrane lipids, and is undetectable in membranes containing high levels of cholesterol [81,85]. Unlike the narrow and well-defined thermotropic phase transition of dispersions of a specific phospholipid containing uniform fatty acid chains, the phase transition temperature range of isolated *A. laidlawii* membranes or their derived lipid dispersions is broad, and may range up to 30°C [71]. This broad range is apparently due to the fact that the bulk of the membrane lipids consists of a mixture of lipids with different transition temperatures, apparently due to the heterogeneity of the fatty acid chain and of the polar head groups [69, 86]. The fatty acid heterogeneity can be abolished by antilipogenic agents resulting in a marked sharpening of the thermotropic gel-to-liquid-crystalline phase transition [51]. Since under these conditions cell growth may not be affected, it seems that neither fatty acid heterogeneity nor a low-cooperatively lipid phase transition are essential for the proper functioning of the *A. laidlawii* cell membrane.

The application of EPR spectrometry to mycoplasma membrane studies yielded information about the average lipid fluidity, the orientation of lipid components in the membrane and the phase properties of the membrane. The most intensive work was carried out with spin-labeled fatty acids that were incorporated into isolated membrane preparations [72], or were added to the growth medium and were utilized by the cells for the synthesis of spin-labeled phospho- and glycolipids [73]. The ease and realiability of determining the overall membrane fluidity using EPR spectrometry made it a useful technique for comparative fluidity studies [8,72,73,88]. Utilizing EPR studies, the effect of membrane proteins on the mobility of the hydrocarbon chains of *A. laidlawii* lipids was first noted [72]. The fluidity of the membranes was decreased upon increasing the protein/lipid ratio in membrane preparations. Further studies showed that proteolytic treatment and subsequent binding of cytochrome *c* or lysozyme resulted in an initial increase followed by a decrease in the fluidity of the lipid bilayer [87].

EPR spectrometry detects a discrete temperature within the gel-to-liquid-crystalline phase transition, which is related to the temperature of a phase transition of specific regions where the probe is concentrated rather to the transition of the bulk of the membrane lipids [89,90]. This temperature is determined from the inflection points observed in Arrhenius plots of the motion parameter (τ_0) of spin-labeled fatty acids that have a nitroxide group close to the methylene end groups of the molecule [91,92]. Such molecules, though far from being spherical, move in a nearly isotropic fashion, warranting the use of Kivelson's formula [93] for determining an empirical motion parameter (τ_0) related to the rotational correlation time (τ_c). EPR studies may also detect thermotropic phenomena other than the gel-to-liquid-crystalline phase transitions, such as cluster formation, liquid-liquid phase separation, transitions in the solvation shells of proteins, etc. [90,94–96].

A more detailed and quantitative understanding of the structure and organization of the hydrocarbon and polar regions of membrane lipids was provided by intensive NMR studies of *A. laidlawii* membranes [74,76,77]. Unlike the nitroxide-containing electron spin resonance probes, most NMR probes do not significantly perturb their environment. A detailed evaluation of NMR and other techniques was presented elsewhere [83].

IV. Control mechanisms for regulating membrane fluidity

The physical properties of membrane lipids are vitally important to mycoplasmas. *A. laidlawii* was not able to grow at temperatures at which its membrane lipids exist entirely in the gel state [71], confirming the notion that some of the lipid hydrocarbon chains must be in a fluid state to support proper membrane function [5]. *A. laidlawii* cells with up to about half of their membrane lipids in the gel state are able to function as well as cells whose membrane lipids are entirely in the fluid state, but growth rates decrease proportionately with the increase of membrane lipids in the gel state, and growth ceases entirely when more than 90% of membrane lipids are in the gel state [71]. The fact that growth and replication of mycoplasmas require a lipid bilayer in a fluid or partially fluid state is due, to a certain extent, to the findings that complete crystallization of membrane lipids results in a marked reduction in the activity of membrane transport systems [97,98]; the activity of membrane associated enzymes [92,99–110], the permeability of the cells to nonelectrolytes [70,99,100,102] and the elasticity of the membrane [103]. A regulatory mechanism is therefore necessary to maintain membrane lipids in an appropriate physical state when the growth temperature or the fatty acid composition of the medium are changed.

Several types of lipid change could alter the phase properties of mycoplasma membranes. An increase in the average chain length of the fatty acyl chains or a decrease in the ratio of unsaturated/saturated fatty acid moieties would raise the transition temperatures [5]. Altering the cholesterol and carotenoid content of mycoplasma cell membranes will modify the molecular packing of the phospholipid acyl chains [36,55,104]. Changes in the distribution of polar head groups of the negatively charged phospholipids of mycoplasma membranes, or allowing these groups to interact with divalent cations, could also markedly change transition temperatures [105].

IVA. Varying fatty acid composition

The fluidity of membrane lipids and the temperature range of the gel-to-liquid-crystalline phase transition are primarily dependent on the chain length and chemical structure

of the hydrocarbon chains of membrane lipids [106]. The cohesive forces between the hydrocarbon chains of the adjacent phospholipid molecules are predominantly London van der Waal's forces in membranes. These forces are additive and increase with a rise in the number of methylene groups in the interacting chains. Increasing the chain length of the fatty acids increases the attractive forces between the adjacent phospholipids and will result in a tightly-packed lipid bilayer where the freedom of motion of the hydrocarbon chains is low, and the temperature of the gel-to-liquid phase transition is high [107]. Since the attractive forces between the methylene groups decline rapidly with increasing distance between the groups, unsaturated fatty acids, branched-chain fatty acids, cyclopropane-containing fatty acids or any fatty acid containing a bulky side group that is incorporated into membrane phospholipids will increase membrane fluidity and decrease the gel-to-liquid-crystalline phase transition.

The regulation of the acyl chain length and the degree of unsaturation seems to play a major role in controlling membrane fluidity of the *Acholeplasma* species that require no cholesterol. A regulatory mechanism that senses temperature and maintains a constant membrane fluidity was demonstrated in *A. laidlawii* strain A, where decreasing the growth temperature to 15°C caused a significant increase in the amount of exogenous oleic acid incorporated into membrane lipids [72]. This resulted in higher fluidity of membranes from cells grown at 15 or 28°C than those at 37°C [30,72,108]. Accordingly, a direct downshift in transition temperature at lower growth temperatures was demonstrated by Melchior et al. [68] with the closely related *A. laidlawii* strain B using differential scanning calorimetry analyses. When cells were grown at 37°C, membrane lipids were fluid at 37°C but entirely in the gel state at 25°C, whereas when grown at 25°C, membrane lipids were mostly fluid even at 25°C. Temperature control of fatty acid composition in bacteria was first noted in *Escherichia coli* by Marr and Ingrahm [109]. The phenomenon was investigated in *E. coli* (for reviews see Refs. 110, 111), and the conclusion was reached that the mechanisms responsible for the temperature-induced fatty acid alteration operate at the level of both fatty acid biosynthesis, by changing the ratio of unsaturated to saturated fatty acids, and the acyltransferase-mediated incorporation of fatty acids into the glycerol backbone. In *A. laidlawii*, where saturated but no unsaturated fatty acids are synthesized [42,43], a thermal regulatory mechanism at the level of fatty acid biosynthesis is expected to affect the chain length of biosynthetic output by decreasing the chain length at lower growth temperatures and increasing it at higher growth temperatures. Yet, changes in the growth temperature produced only minor alterations in the pattern of the saturated fatty acids derived from de novo fatty acid biosynthesis [44] or from elongation of exogenous medium chain fatty acids [48]. In addition, the temperature range of the thermotropic phase transition of membranes derived from cells grown in fatty acid-free media at various temperatures varied only slightly with growth temperatures [71]. It seems, therefore, that the mechanism responsible for temperature-induced fatty acid alterations does not operate at the fatty acid biosynthesis level, but at the level of the transacylation reaction, where the membrane-bound transacylase is involved [8]. As the growth temperature is decreased, *A. laidlawii* adjusts the fatty acid composition of its complex lipids by incorporating exogenously supplied fatty acids with progressively lower melting points. Since the requirement for fatty acids with lower melting points can be fulfilled by *cis*-unsaturated, *trans*-unsaturated, branched-chain or cyclopropane-containing fatty acids, it seems that the physical properties rather than the chemical properties and the electronic configuration of the exogenous fatty acid added to the growth medium are important in determining the suitability of the fatty acid for the acyl trans-

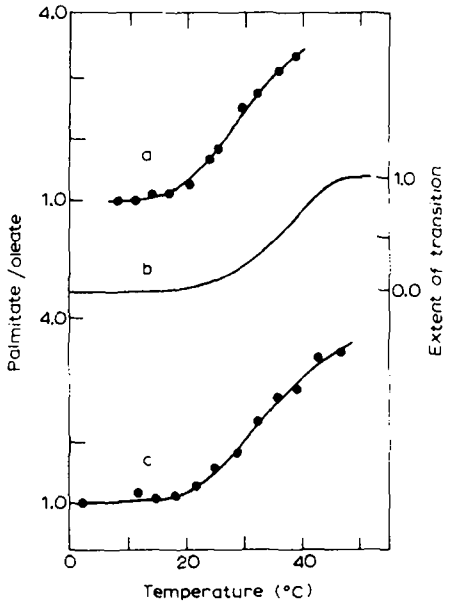

Fig. 2. Correlation between the palmitate/oleate ratio incorporated into *A. laidlawii* membrane lipids (a), the extent of transition in membranes (b), and the palmitate/oleate ratio of fatty acids physically bound to bilayers of extracted membrane lipids (c) [112].

fer [86,90]. It was therefore suggested that the temperature-sensing selection mechanism is thermodynamically determined and not dependent upon the acyltransferase specificity [112]. Thus, the control of membrane fluidity resides in the membrane itself, and the membrane-bound acyltransferase is not required to distinguish between different fatty acids. In fact, the pattern of esterification of palmitate and oleate at various temperatures closely parallels the physical state of the membrane bilayer and the physical binding of free fatty acids to liposomes made from membrane lipids (Fig. 2). As the temperature is lowered, an increased amount of oleate relative to palmitate is accepted by the bilayer where it is utilized by the transacylase for complex lipid biosynthesis.

Although the de novo fatty acid biosynthesis and the chain elongation systems are not affected by a shift in the growth temperature, it seems as if both these mechanisms are involved in regulating the fatty acid composition of membrane lipids in response to variation in the fatty acid composition of the growth medium [48,113]. Thus, the spectrum of the end products of the de novo fatty acid biosynthetic pathway [113] as well as the products of the chain elongation system [48] are influenced by exogenous fatty acids added to the growth medium in a way that exogenous unsaturated or branched-chain fatty acids having low melting points tend to enhance the mean chain length of de novo biosynthesized fatty acids, and increase the extent of chain elongation, while exogenous long-chain saturated fatty acids having high melting points tend to exert an opposite influence. Hence, the de novo fatty acid biosynthesis and the chain elongation systems serve to buffer the physico-chemical effect of exogenous fatty acid incorporation by a compensatory shift in the average chain length of the product. A possible explanation for the mechanism of such a regulatory system was proposed by Silvius et al. [113] (Fig. 3). Since in *A. laidlawii* fatty acids with low melting points are preferentially directed to position 2 of the glycerol backbone, while fatty acids with high melting points are predominate in position 1 [53,114,115], an exogenous fatty acid with a low melting point

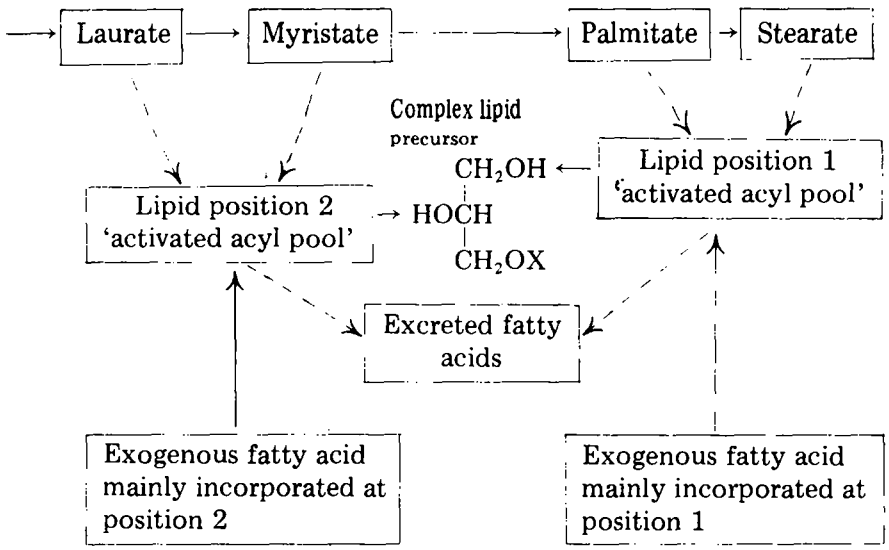

Fig. 3. Proposed mechanism by which an exogenous fatty acid may alter the metabolic fate of de novo biosynthesized fatty acids. Dashed lines represent steps in glycerolipid synthesis which do not lie directly along the fatty acyl chain-elongation pathway (top), but whose rates are directly influenced by the levels and nature of an exogenous fatty acid [113].

will complete with the short chain endogenously synthesized fatty acids for transfer to position 2 of the glycerol. If the termination of fatty acid chain elongation in *Acholeplasma* is determined by the competition between the fatty acid elongation and the transfer of the acyl groups to the glycerol backbone, an exogenous low-melting fatty acid specific for position 2 will decrease the rate of acyltransfer of the shorter chain biosynthetic products, enabling continued elongation of the fatty acyl thioesters.

IVB. The role of cholesterol

The fluidity and phase transition of membrane lipids is dependent on the sterol content of the membrane. Sterols must contain a planar nucleus, a free hydroxyl group at the 3β-position and a hydrocarbon side chain in order to exert an effect on artificial membrane systems [116] and mycoplasma membranes [55,59,60,102]. A sterol such as cholesterol, by virtue of its peculiar molecular shape, will be oriented in the membrane in such a way that its rigid ring system will be aligned parallel to the hydrocarbon chains of membrane phospholipids with the polar hydroxyl group anchoring one end of the cholesterol molecule to the polar surface of the bilayer. The planar hydrocarbon part of the cholesterol molecule that extends towards the center of the bilayer exerts a condensing effect on lipids at temperatures above the temperature of the phase transition, but at temperatures below the temperature of the phase transition, cholesterol will prevent the cooperative crystallization of the hydrocarbon chain and will therefore eliminate the phase transitions. By eliminating the phase transition at low temperatures, and decreasing membrane fluidity at high temperatures, cholesterol creates an intermediate fluid state of the lipid bilayer [104].

The idea that in biological membranes cholesterol regulates the fluidity of membrane phospholipids gained strong support from mycoplasma studies. The first of these studies

showed that the sterols required to promote the growth of *Mycoplasma* [117,118], *Ureaplasma* [60] and *Spiroplasma* [119], are those with molecular properties required to induce the condensing effect and to eliminate the thermal phase transition of artificial membranes [57,85,102]. The ability to alter the cholesterol content of *M. mycoides* subsp. *capri* by adapting the cells to grow with very little cholesterol [81] provided a most useful model for establishing the regulatory role of cholesterol in biological membranes. The cholesterol content of the adapted strain was less than 3% of the total membrane lipids as opposed to 22–26% in membranes of the native strain. The marked reduction in cholesterol levels produces profound changes in the fatty acid composition, ultrastructure, and biological and physical properties of the cell membrane of the adapted cells [81,99,120] but the most remarkable differences were the increased lipid fluidity in membranes of the cholesterol-depleted adapted strain and the detection of a thermotropic gel-to-liquid-crystalline phase transition in the adapted strain but not in the native strain. The differences in the phase behavior of the native and adapted strains are manifested in membrane ultrastructure. Freeze-fracture electron microscopy enables one to compare the hydrophobic membrane core of the native and adapted strains. Smooth-faced areas are believed to be mainly lipid domains while the particles are apparently of a protein nature [121]. Chilling the adapted strain to 4°C prior to the quick freezing causes the aggregation of particles, leaving over 66% of the fracture faces particle-free. No such phenomenon could be demonstrated with the native strain. The aggregation phenomenon is probably due to a thermotropic phase transition of membrane lipids [91]. The differences in phase properties of the native and adapted strains provided the first evidence to support the notion that cholesterol in a biological membrane functions as a regulator of membrane lipid fluidity by inducing an intermediate fluid state during changes in growth temperature or following alterations in the fatty acid composition of membrane lipids. Cholesterol, by preventing the crystallization of membrane lipids of the native *M. mycoides* subsp. *capri* cells at lower temperatures, keeps membrane lipids in a sufficiently fluid state to support the functions of key membrane enzymes such as the membrane-bound ATPase [99]. Thus, growth of the native cholesterol-rich strain continues at temperatures lower than 37°C, while in the adapted cholesterol-poor strain, growth was almost completely arrested at temperatures where most of the membrane lipids are in a gel state (25°C and below). The adapted cholesterol-poor strain was also successfully utilized to establish the relationships between cholesterol content and intracellular ion levels [120]. The decrease in potassium content, increase in sodium content and the reduced acidification of cholesterol-poor cells were taken to suggest that cholesterol affects the cation content via the increase in proton permeability which in turn controls the value of the $\Delta\psi$ responsible for intracellular K^+ equilibration. This hypothesis was substantiated by cholesterol recovery experiments where replenishing cholesterol to adapted cells resulted in an immediate increase of the intracellular K^+ content [120].

The low amounts of cholesterol that can be incorporated into *A. laidlawii*, which does not require sterols, were not sufficient to eliminate the phase transition of membrane lipids but only to reduce the energy content of the phase transition [85]. The incorporation of cholesterol into *A. laidlawii* membranes reduced, however, the permeability of the cells to nonelectrolytes, an observation that can be satisfactorily rationalized by postulating a condensing effect of cholesterol upon the lipid bilayer of *A. laidlawii* [70,85,102]. The requirement of mycoplasmas for cholesterol as a regulator that helps to maintain a constant membrane fluidity is probably due to the necessity of overcoming the inability of these cells to operate mechanisms for controlling membrane fluidity at the levels of

fatty acid and complex lipid biosynthesis. Such mechanisms operate efficiently in bacteria [110], and to a certain extent in the *Acholeplasma* species that are capable of varying the chain length of the fatty acid synthesized [48] or selectively incorporating exogenous fatty acids from the growth medium [112]. However, all *Mycoplasma* and *Spiroplasma* species tested to date are incapable of synthesizing or elongating fatty acids. Furthermore, it is not clear if the sterol-requiring mycoplasmas possess the ability to adjust incorporation of fatty acids from the growth medium in response to variations in growth conditions, since studies carried out so far with *M. hominis* [58] and *S. citri* [119] showed that these organisms preferentially incorporated palmitic acid into their membrane phospholipids. The incorporation of large quantities of sterol into the membrane of the cholesterol-requiring parasitic mycoplasmas may be necessary to prevent membrane lipids from crystallization at their optimal growth temperatures.

IVC. Carotenoids as regulators of membrane fluidity

The idea that in *Acholeplasma* species, carotenoids with a planar hydrocarbon structure may fulfill functions analogous to those of sterols in *Mycoplasma* species was put forward by Smith [3]. This idea recently gained considerable support from studies [122, 123] showing that the carotenoids, preferentially oriented with their amphiphilic axis parallel to the hydrocarbon chain of membrane polar lipids, are acting as reinforcers of the lipid bilayer. Thus the inhibition of carotenoid biosynthesis or the selective removal of carotenoids from *A. laidlawii* membranes result in a decrease in membrane viscosity, whereas the incorporation of the carotenoids into membranes increases membrane viscosity [123]. These observations are in support of the hypothesis that a series of polyterpenoids C_{30}–C_{50} - acyclic or polycyclic, conformationally mobile or rendered rigid by conjugation or by polycyclization – can play a role as membrane stabilizers instead of sterols [36]. The regulatory role of carotenoids in controlling the fluidity of *A. laidlawii* cell membrane was recently suggested by showing that the carotenoids biosynthesis system senses membrane fluidity [123].

V. Structural and functional properties of mycoplasma membranes are affected by the physical state of membrane lipids

The most important conclusion from studies on the gel-to-liquid-crystalline phase transition in *A. laidlawii* membranes is that under physiological conditions these membranes are at least in part in the liquid crystalline phase. Evidence that pointed to a correlation between the lipid phase properties, membrane structure and function were accumulated during the past few years. The studies on the effect of membrane viscosity on the permeability and transport processes were recently reviewed [98] and therefore are not dealt with in this review.

VA. Physiology and morphology of the cells

A. laidlawii shows changes in cell morphology and growth rate depending on the fatty acid supplemented [124]; thus, when grown on oleate, the growth rate is high and the cells are filamentous, while poor growth was obtained when cells were grown on palmitate, and they are spherical. A more precise correlation between growth and the thermotropic transition of membrane lipids revealed that the physical state of membrane lipids

has a marked effect on both the temperature range within which *A. laidlawii* cells can grow, and on growth rates within the permissible temperature range [71]. The minimum growth temperature is defined by the lower boundary of the gel-to-liquid-crystalline phase transition of the membrane lipids. The optimum and maximum growth temperature are also influenced by the boundaries of the phase transition but to a much smaller extent. The cells will not grow at temperatures below their transition [5,71], but some cell growth will continue at temperatures within the temperature range of the transition, where most of the membrane lipids are in the gel state. Growth does not stop until only about 10% of membrane lipids remain fluid [71].

The integrity of mycoplasma cells is primarily determined by their osmotic fragility. The precise mechanism of osmotic lysis is still unknown, but there is no doubt that it is determined, at least in part, by the physical state of the membrane lipids [125]. The idea that unsaturated fatty acids may allow for a looser packing of mycoplasma membrane lipids resulting in greater eleasticity of the membrane was advanced some time ago [124]. Furthermore, it was shown [126] that the replacement of oleate (C-18 : 1 *cis*) by elaidate (C-18 : 1 *trans*) resulted in a decreased osmotic fragility, suggesting that an optimal fluid state is required to maintain osmotically stable cells. When part of the membrane lipids are in the fluid phase, the elasticity of the cell membrane enables the cells to swell and behave as a good osmometer. However, when membrane lipids are in the gel phase, the cells are unable to swell and will lyse in a slightly hypotonic medium [103].

The fatty acid composition and physical properties of membrane lipids also have an effect on the thickness of the *A. laidlawii* membranes. X-ray diffraction studies with *A. laidlawii* membranes enriched with either erucic (C-22 : 1 *cis*) or palmitic (C-16 : 0) acid showed that the lipid bilayer thickness increased in proportion to the average fatty acyl chain length [7]. Thus, below the phase transition a thickness of 52 Å was measured in the erucate enriched membranes as against 47 Å in palmitate enriched membranes. As the chain becomes more fluid, the dimensions of the bilayer might decrease, and in the fluid phase, the thickness of erucate or palmitate enriched membranes were 41 and 38 Å, respectively.

VB. Distribution of membrane proteins

The asymmetrical transbilayer distribution of membrane protein and the dependence of their lateral and rotational mobility on the physical state of membrane lipids are well established properties of biomembranes, including those of mycoplasmas [4,127,128]. Freeze-fracture electron microscopy studies provide evidence that the physical properties of mycoplasma membrane lipids may change the distribution of the intramembranous protein particles in the membrane. In general, *A. laidlawii* membranes frozen from temperatures above the phase transition have a random distribution of intramembrane particles whereas these particles are aggregated or patched below the transition temperature as a result of 'squeezing out' of particles by the lipids as they undergo phase separation [81,91]. The correlation between the physical state of membrane lipids and particle aggregation was best manifested in the sterol-requiring *M. mycoides* subsp. *capri*, where in the native strain containing high levels of cholesterol that are sufficient to eliminate the liquid-crystalline-to-gel phase transition, no aggregation of particles was obtained, but in the cholesterol-depleted adapted strain, maintaining the cells at 4°C prior to freezing caused the formation of aggregated arrays of particles [81]. Data that temperature induced changes in the physical state of membrane lipids can also cause changes in the

location of surface proteins were presented by Wallace et al. [129,130] using an avidin-ferritin label that was specifically bound to amino groups of membrane protein that are exposed to labeling at the membrane surface. By visualizing the distribution of the complex on the surface of *A. laidlawii* cells, they concluded that below or above the gel-to-liquid-crystalline lipid phase transition the labeled sites were dispersed but in membranes labeled at an intermediate temperature where lipids are in a mixture of the two phases, patches of low and high density label were found. It therefore appears that both intramembranous protein particles and surface proteins of mycoplasma are mobile and their relative positions are affected by the physical state of membrane lipids, but whereas intramembranous particles are completely aggregated at the completion of the phase transition and the aggregates are not dispersed as the temperature is lowered further [91], surface proteins seem to be aggregated at temperatures within the boundaries of the phase transition but dispersed at lower temperatures [129,130].

A most intriguing question is whether the physical state of mycoplasma membrane lipids also influence the vertical disposition of proteins immersed in the lipid bilayer. Borochov and Shinitzky [131] have recently postulated that the position of the amphipathic proteins in the membranes reflects the equilibrium state between the interactions of their hydrophobic parts with membrane lipids and their hydrophilic parts with the aqueous surroundings.

Accordingly, with decreasing lipid fluidity, the interaction of hydrophobic parts of the proteins with the lipids will diminish resulting in the squeezing out of the proteins which will then occupy a new equilibrium position. The opposite vectorial displacement will occur on increasing the lipid fluidity. However, in *A. laidlawii* changes in the degree of exposure of the iodine-binding sites of membrane proteins could not be consistently correlated with changes in membrane fluidity brought about by altering the fatty acid composition of membrane lipids, by changing growth temperature, by aging of cultures and by inducing changes in the membrane lipid/protein ratio through treatment with chloramphenicol [10]. It was therefore suggested that changes in the exposure of proteins on the surface of intact cells are associated with alterations in the energized state of the membrane rather than with changes in the physical state of membrane lipids [10,132].

VC. Lipid dependence of membrane-bound enzymes

The lipid dependence of some membrane-bound enzymes of mycoplasma was previously suggested [92,99- 101]. The lipid dependency of the Mg^{2+}-dependent ATPase of *A. laidlawii* was finally confirmed by showing that specific removal of phosphatidylglycerol from *A. laidlawii* membranes inactivates the enzyme [133]. Of all membrane lipids of this organism, phosphatidylglycerol was the only lipid species which could be used to reconstitute their enzymatic activity [133].

The requirement for a fluid lipid environment for optimal enzymatic activity of some membrane-bound enzymes was suggested by showing that within the temperature range of the lipid gel-to-liquid-crystalline phase transition the activation energy of the enzyme activity is increased. De Kruyff et al. [100] examined the rate-temperature profiles for the membrane-bound Mg^{2+}-dependent ATPase of *A. laidlawii* from cells supplemented with various fatty acids; they reported inflection points in the slopes of the Arrhenius plots of the activities that represented changes in the activation energy of the enzyme, with lower activation energy at temperatures above the inflection point and higher at temperatures below it. The inflection points were found to be dependent on the fatty

acid composition and seem to occur at the lower boundary of the gel-to-liquid-crystalline phase transition [100,101]. Inflection points were also noted in the Arrhenius plot of a membrane-bound thioesterase activity of *A. laidlawii* [92] but not in the Arrhenius plots of the NADH oxidase and *p*-nitrophenylphosphatase activities [92,100], although the membrane lipids underwent a phase transition within the temperature range where enzyme activities were determined.

The inflection 'points' observed with both Mg^{2+}-dependent ATPase and thioesterase were at a single temperature, close to the lower boundary of the lipid phase transition [92,100]. Thus, plots of thioesterase activity of elaidate or palmitate enriched membranes showed inflection points at 12 and 18°C, respectively. No inflection points were noted in linoleate or oleate enriched membranes which do not exhibit a lipid phase transition within the temperature range tested. The possibility brought up by de Kruyff et al. [100] that the ATPase of *A. laidlawii* is associated with a boundary lipid having a low transition temperature, thus showing inflection points at the lower end of the transition temperature, was recently challanged by Bevers et al. [133]. They demonstrated by reconstitution experiments that the fatty acid composition of both the boundary phospholipids, as well as that of the bulk phospholipids, determines the activation energy and the inflection temperature in the Arrhenius plot of the Mg^{2+}-dependent ATPase of mycoplasmas. Arrhenius plots of the ATPase activity of the cholesterol-depleted *M. mycoides* subsp. *capri* adapted strain showed inflection points at temperatures that corresponded well with the thermotropic membrane phase transition [99]. No inflection point could be detected, however, in Arrhenius plots of the cholesterol-containing native strain where a phase transition was eliminated. Cholesterol could also affect the Mg^{2+}-dependent ATPase activity of *A. laidlawii* membranes, as the incorporation of cholesterol into these membranes decreases the inflection temperature of the ATPase activity [100]. The cholesterol effect was reversed by filipin, a polyene antibiotic which interacts with cholesterol. Temperature dependent variations in both V and K_m values of the Mg^{2+}-dependent ATPase of *A. laidlawii*, which can produce a variety of Arrhenius plot artifacts, were recently described [134] suggesting that temperature variations in substrate binding affinity will have to be taken into account when determining the effect of temperature on the rate of a membrane-bound enzyme.

In *A. laidlawii* a correlation between membrane viscosity and the output of the membrane bound complex-lipid biosynthetic system was also described [30]. The two polar lipids most prone to membrane viscosity are the monoglucosyl diacylglycerol and diglucosyl diacylglycerol. It was suggested that since monoglucosyl diacylglycerol prefers a reversed hexagonal structure at all conditions, by the differential synthesis of these two glycolipids the cells maintain a balance between lipids in a lamellar and in an hexagonal phase [135].

VI. Transmembrane asymmetry of membrane lipids

The heterogeneity of mycoplasma membrane lipids consisting of carbohydrate-containing lipids (glycolipids and glycophospholipids), phospholipids and sterols requires the application of specific techniques for determining the transbilayer distribution of each lipid class. Only recently a somewhat more general procedure was described [24] where the lactoperoxidase-mediated iodination technique, widely used to determine polypeptide asymmetry in mycoplasma membranes [4], was successfully applied to the study of the transbilayer distribution of the various polar lipid species of *A. laidlawii* mem-

branes. The iodine was found to be bound to membrane lipids by an α-substitution process and gave results that were in accordance with those obtained by specific labeling techniques and phospholipases [23,136].

VIA. Glycolipids and phospholipids

Localization of carbohydrate-containing lipids in mycoplasmas can be achieved by using specific antibodies or lectins. In fact, immunological studies [137–139], agglutination experiments with lectins [140] and electron microscopic visualization of cytochemically-stained concanavalin A-surface carbohydrate complexes [141] indicated that carbohydrate moieties, presumably of glycolipids and glycophospholipids, are exposed on the external surfaces of several mycoplasma species. The concept of the asymmetrical distribution of the carbohydrate moieties was further promoted by Kahane and Tully [136] who showed that in all *Acholeplasma* and *Mycoplasma* species tested the amount of labeled lectins bound to intact cells was almost the same as that bound to isolated membranes, suggesting that the carbohydrate-containing membrane lipids are exposed on the cell surface.

Determination of phospholipid asymmetry in mycoplasmas is more difficult since the methodology developed so far for phospholipid distribution is based to a large extent on the use of agents that react with the primary amines of aminophospholipids and on the use of phospholipases [128]. Aminophospholipids, such as phosphatidylethanolamine or phosphatidylserine, are ubiquitous in eukaryotic and most prokaryotic cells but are almost completely absent from mycoplasmas. Phospholipases can be used only with organisms whose membrane phospholipids are accessible to the enzyme. Such accessibility is governed initially by the degree of shielding of membrane phospholipids by membrane proteins. It is conceivable that shielding might influence the rate of phospholipid hydrolysis or even completely prevent it. Phosphatidylglycerol, the major phospholipid of *M. hominis* membranes, is such a case, where hydrolysis by phospholipase C did not occur unless membrane proteins were removed by pronase digestion [17]. The masking of *M. hominis* membrane phospholipids by proteins was supported by showing that the phosphatidylglycerol in isolated *M. hominis* membranes fails to interact with its specific antiserum [142]. In contrast to *M. hominis* membrane phospholipids of *A. laidlawii* [23], *M. gallisepticum* and *M. capricolum* (Markowitz, O. and Gross, Z., unpublished data) are vulnerable to phospholipases that can be used for phospholipid localization studies in these organisms. The availability of the phosphatidylglycerol of *A. laidlawii,* which constitutes about 30% of membrane lipids, for hydrolysis by exogenous phospholipase A_2 depends on the state of membrane energization. In the presence of glucose, hydrolysis proceeds at a slower rate and is limited whereas in the non-energized state hydrolysis is completed quite rapidly [143]. The mechanism linking energy production and/or conservation to the disposition of phosphatidylglycerol is not yet known. The possibilities that in the energized state membrane lipids differ in their overall fluidity, binding to membrane protein, rate of translocation and/or lateral diffusion were brought up [143] but are open to further investigation. In non-energized *A. laidlawii* cells, the localization of phosphatidylglycerol was successfully studied by Bevers et al. [23] using pancreatic phospholipase A_2. Treating intact *A. laidlawii* cells with the enzyme led to the hydrolysis of 50% of the phosphatidylglycerol, whereas when isolated membranes of these cells were treated at 5°C, about 70% of the phosphatidylglycerol was hydrolyzed, suggesting the presence of three different phosphatidylglycerol pools: one (50% of the total) exposed

on the external surface; the second (20% of the total) exposed in the inner membrane surface; and the third (30% of the total) protected from the enzyme, probably by interaction with membrane proteins. Complete hydrolysis of phosphatidylglycerol obtained by phospholipase treatment of intact cells at 37°C was taken to indicate a translocation mechanism ('flip-flop') that enables the phosphatidylglycerol to move from the inner to the outer half of the lipid bilayer. The 'flip-flop' rate of phosphatidylglycerol in *A. laidlawii* suggested by phospholipase experiments seems to be much faster than that observed in liposomes [144], or biological membranes [145,146]. The fast rate may be due to the depletion of the phosphatidylglycerol in the outer half of the bilayer as a result of the phospholipase activity which would trigger the translocation of this compound from the inner half [23].

VIC. Cholesterol

The mycoplasmas are a most useful tool for studying cholesterol localization and movement in the membrane, being the only prokaryotic cells that require cholesterol for growth. Two major approaches were used for cholesterol distribution studies in mycoplasma: (a) rapid kinetic measurements of filipin-cholesterol association, and (b) kinetic measurements of the exchange of cholesterol between cells or membrane preparations to high density lipoprotein (HDL). The filipin-cholesterol association studies are based on the observations that the binding of polyene antibiotics to sterol-containing membranes is easily monitored by absorbance or fluorescence intensity measurements [147,148]. Large changes in the fluorescence polarization [148] and circular dichroism [149] of filipin accompany its association with cholesterol and ergosterol. The major obstacle in utilizing filipin for membrane studies is the membrane perturbations caused by this probe. The extent of these perturbations depends on the experimental conditions such as temperature, period of exposure, antibiotic/sterol molar ratio etc. Stopped-flow kinetic measurements of filipin-cholesterol association represent a means by which filipin-induced membrane disruption can be minimized, especially if high cholesterol/filipin molar ratios and low temperatures are used together with the very short reaction times. The initial rates of filipin-cholesterol association were significantly lower with intact mycoplasma cells than with isolated membranes (Fig. 4). Since filipin-cholesterol association process follows second-order kinetics (first-order in filipin, first-order in cholesterol) [150], and the initial rate of interaction of filipin and cholesterol is sensitive to sterol accessibility and concentration [150,151], the ratio of the second-order rate constants in the unsealed isolated membrane relative to the intact cell is a measure of the cholesterol distribution [152]. These rate constants indicate a symmetrical distribution of cholesterol in the two halves of the bilayer of *M. gallisepticum* membranes whereas in *M. capricolum* about two-thirds of the free cholesterol is localized in the outer half of the lipid bilayer. Confirming the results obtained with filipin were the results obtained by exchange studies of [^{14}C]cholesterol between resting *M. gallisepticum* cells and HDL [88]. Cholesterol exists in *M. gallisepticum* cells in two different environments. One, representing about 50% of the total unesterified cholesterol, is readily exchanged with exogenous cholesterol, whereas cholesterol in the other environment interchanges at exceedingly slow rates (Fig. 5). Since over 90% of the cholesterol in isolated membranes was exchanged rapidly, it is likely that these environments represent the inner and outer halves of the lipid bilayer. Although exchange studies suggested that in resting *M. gallisepticum* cells the rate of 'flip-flop' of cholesterol from the inner to the outer half of the bilayer is exceedingly slow or non-

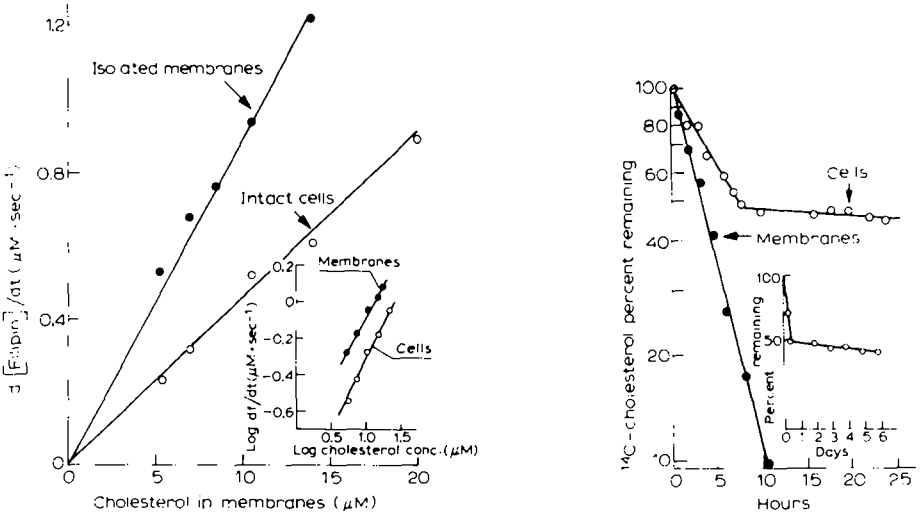

Fig. 4. Initial rate of disappearance of free filipin at 10°C on binding to varying concentrations of unesterified cholesterol in intact *M. gallisepticum* cells (○) and unsealed isolated membranes (●). Inset: A plot of the logarithm of the initial rate vs. the logarithm of cholesterol concentration, showing that the binding reaction is first-order with respect to cholesterol in both cells and membranes. The indicated cholesterol concentrations represent the final concentration of unesterified cholesterol after mixing of equal volumes of antibiotic with cells or membranes in the stopped-flow spectrophotometer in 0.4 M sucrose containing 10 mM sodium phosphate (pH 7.2) and 20 mM $MgCl_2$ [152].

Fig. 5. Exchange of [^{14}C]cholesterol from intact cells and unsealed isolated membranes of *M. gallisepticum* with unlabeled cholesterol of HDL. Radioactive cholesterol was added to the growth medium. Incubation of cells or membranes with a large excess of HDL (about 100 times more unesterified cholesterol than was present in the membranes) was carried out at 37°C in 0.4 M sucrose containing 10 mM sodium phosphate (pH 7.2) and 20 mM $MgCl_2$. Cholesterol in one environment of the intact cells, representing about 50% of the total unesterified cholesterol, is readily exchanged, with a half-time of about 4 h at 37°C [88].

existent [88]. One must assume that in growing cells, where cholesterol, taken up by mycoplasmas from an exogenous source, is first incorporated into the outer half of the lipid bilayer, the rate of translocation to the inner half has to occur within the 16–20 h growth period. Evidence that in growing *M. capricolum* cells rapid translocation of cholesterol occurred was recently presented [63]. Transfer of a cholesterol-poor adapted strain of *M. capricolum* to a cholesterol-rich medium resulted in an approx. 6-fold increase in the free cholesterol content of the membrane within 4 h of incubation. The second-order rate constants for filipin-cholesterol association indicate that the transbilayer distribution of cholesterol was essentially invariant throughout the growth period with 50% of the cholesterol located at the outer half and 50% at the inner half of the bilayer. However, when growth was inhibited translocation became much slower, and cholesterol accumulated in the outer half of the bilayer (Table III).

Information on the transbilayer distribution of lipids in a mycoplasma membrane is necessary in order to gain insight into the molecular organization of the membrane, but

TABLE III

EFFECT OF GROWTH INHIBITORS ON TRANSBILAYER DISTRIBUTION OF CHOLESTEROL IN ADAPTED *M. CAPRICOLUM* CELLS TRANSFERRED TO CHOLESTEROL-RICH MEDIUM

Cholesterol distribution was calculated from second-order rate constants for filipin binding to intact cells and isolated membranes [63].

Inhibitor	A_{640}	Free cholesterol (μg/mg membrane protein)	*K* cells/*K* membranes	Cholesterol distribution (μg/mg membrane protein)	
				Outer half	Inner half
None	0.26	127.0	0.45	57.2	69.8
Chloramphenicol	0.14	57.5	0.66	39.1	17.4
Valinomycin	0.17	79.7	0.90	71.5	8.2
Gramicidin	0.13	71.3	0.92	65.5	5.8

the functional significance of lipid asymmetry in mycoplasmas is still obscure. Asymmetry of the polar head-groups and variations in the fatty acid constituents of the various lipid classes might result in different fluidities of the inner and outer halves of the bilayer [153]. In fact, EPR spectroscopy of spin-labeled fatty acids incorporated in intact cells and isolated membrane preparations pointed to a higher fluidity of the outer half of the lipid bilayer of *M. hominis* and *A. laidlawii* cells [154]. Since the various lipid species of *A. laidlawii* may differ significantly in their melting temperatures [69], their asymmetrical transbilayer distribution could account for differences in the fluidity of the two membrane halves. However, the possible contribution of the membrane proteins located on the inner surface of mycoplasma membranes [155] has to be considered since membrane proteins may markedly affect membrane lipid fluidity [87]. An asymmetric distribution of lipid in specific areas of the cell membrane might influence the surface tensions of the inner and outer monolayer, thereby leading to the formation of areas with extreme curvature [128]. Mycoplasmas, which are devoid of cell walls might use such mechanisms to maintain their filamentous cell shape. A highly curved shape is typical of the cell membrane of the helical *Spiroplasma* cells which maintain their cell shape in the absence of any supportive structure. Although electron microscopy studies suggested the presence of fibrils in *Spiroplasma* [156,157] which may be responsible for the organism's helical shape, the possibility that lipid asymmetry plays a role in determining the helical shape cannot yet be excluded.

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