STRUCTURE AND FUNCTION OF ACHOLEPLASMA MEMBRANES-- EFFECTS OF LIPID CHAIN LENGTH AND CAROTENOID PIGMENTS

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STRUCTURE AND FUNCTION OF ACHOLEPLASMA MEMBRANES-- EFFECTS OF LIPID CHAIN LENGTH AND CAROTENOID PIGMENTS

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LEAF HUANG

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ABSTRACT

STRUCTURE AND FUNCTION OF ACHOLEPLASMA MEMBRANES--
EFFECTS OF LIPID CHAIN LENGTH
AND CAROTENOID PIGMENTS

By

Leaf Huang

The effects of lipid chain length and carotenoid pigment content on the structure and function of Acholeplasma laidlawii membranes were investigated.

Two membrane preparations from the cells were obtained by supplementing the growth media with either arachidic ($C_{20:0}$) or lauric ($C_{12:0}$) acid. The cells grown with arachidic acid supplementation yielded membrane lipids greatly enriched with the arachidoyl group and those supplemented with lauric acid yielded membrane lipids enriched with lauroyl, myristoyl, and palmitoyl groups. The cell size (0.1 - 0.7μ), the membrane thickness (70±14 Å), and the cell shape (coccoid) showed no difference for these two preparations. The arachidoyl enriched membrane had a greater buoyant density, a smaller permeability to glycerol, and a greater sensitivity to osmotic shock compared to the membrane enriched with shorter acyl groups. The spin label 12 NS is less mobile in the arachidoyl enriched membrane than in the shorter acyl groups enriched membrane. This difference in membrane fluidity can account for the differences observed for the membrane properties.

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Secondly, the carotenoid pigment content in the membrane could be varied by one order of magnitude by growing cells in media containing sodium acetate or propionate (5 g/l). This alteration influenced neither the fatty acyl composition nor the lipid/protein ratio in the membrane. However, spin-labeling experiments showed a greater lipid fluidity in carotenoid-poor membranes. Carotenoid-rich membranes were characterized by a higher buoyant density, higher osmotic fragility, and lower glycerol permeability. These results suggested that carotenoid pigments make the hydrophobic regions of Acholeplasma membranes more rigid.

Thirdly, when cells were grown in a medium containing 20 g/l of sodium acetate, the membrane carotenoid pigment content could be increased by 57-fold as compared to cells grown in a medium containing 20 g/l of sodium propionate. Although the same amount of arachidic acid was added to both kinds of medium, less arachidoyl groups were found in lipids of carotenoid-rich membranes than in carotenoid-poor ones. The relative amount of unsaturated acyl groups also increased. Spin-labeling experiments demonstrated only a slight difference in lipid fluidity between the two types of membrane. Cells grown at 28°C in a medium with acetate (5 g/l) contained significantly less arachidoyl groups and more unsaturated acyl chains in the membrane lipids than cells grown in the same medium at 37°C. Spin-labeling measurements revealed that cells grown at 28°C had more fluid membranes than those grown at 37°C. At the respective growth temperatures of these cells, however, the membrane lipid fluidity were rather similar.

It is concluded that Acholeplasma laidlawii cells are capable of adjusting their lipid composition in order to maintain the membrane fluidity within a narrow range.

STRUCTURE AND FUNCTION OF ACHOLEPLASMA MEMBRANES--
EFFECTS OF LIPID CHAIN LENGTH
AND CAROTENOID PIGMENTS

By

Leaf Huang

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Biophysics

1974

DEDICATION

to my wife, Shilling, and to my parents,

Professor and Mrs. P. C. Huang

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The author wishes to express his gratitude to Professor Alfred Haug for his constant enthusiasm and guidance throughout this dissertation work. The helpful advice from the committee members, Professors E. M. Eisenstein, A. El-Bayoumi, D. T. A. Lamport and H. T. Tien are also acknowledged. The author also owes thanks to Mr. D. D. Jaquet, Dr. D. Hoy and other members in the laboratory for many technical assistances and discussions. Financial support by Professor B. Rosenberg in author's first two years of graduate study is greatly appreciated. Finally, the author also wishes to thank Professor A. Lang for enabling me to carry out these investigations in the MSU/AEC Plant Research Laboratory.

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ORGANIZATION OF DISSERTATION

The main body of this dissertation, Chapters II, III and IV, is presented individually in the format of a scientific paper. The references are, however, combined at the end of the dissertation.

Materials in Chapters II, III and IV are to be submitted for publication.

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CHAPTER I

GENERAL INTRODUCTION

Biological membranes play an important role in cellular processes, such as neural signal transmission, cell and nuclear division, transport, energy metabolism, and macromolecular synthesis. During differentiation membranes participate in cell-cell interactions and play also a role in carcinogenesis. In the past several decades, many investigators worked to elucidate membrane structure and the relationship to physiological functions.

The exact three-dimensional arrangement of molecules in a biological membrane is far from clear at present time. However, several models describing the gross structure of membrane are available. They will be briefly reviewed here.

A. Models related to lipid bilayers:

1. Davson-Danielli-Robertson unit membrane model (1,2):
   In this model, lipids are arranged in two layers with hydrophobic chains interacting at the interior and hydrophilic head groups facing out. At both sides of the bilayer, proteins are distributed either in a globular or extended configuration.

2. Lipid-globular protein mosaic model (3): A basic lipid bilayer is assumed in the model. However, proteins are believed to be inserted into the bilayer in a mosaic fashion. Some proteins could merge into the bilayer, some could penetrate all the way through. An important feature of this model is that proteins can migrate laterally in the plane of membrane, provided the lipid bilayer is sufficiently fluid.
3. Lipid-Protein association model (4): In this model, lipids are present as a bilayer, but each phospholipid has one chain interacting with a hydrophobic bonding site on membrane protein, and the other chain directed into the non-polar membrane interior.

B. Models unrelated to lipid bilayer:

1. Spherical micelle model (5,6): Lipids are closely packed into globular micelles within the membrane, with hydrocarbon chains lying inside and polar head groups facing out. This configuration is perhaps essential for the fusion of two membranes.
2. Lipoprotein subunit model (7): In this model, lipids of membrane "subunits" are bound hydrophobically to the interior of proteins, with negatively charged groups on the surface. The membrane is the result of a two-dimensional aggregation of these lipoprotein subunits. The model was initially proposed for the chloroplast membrane.

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3. Repeating lipoprotein subunit model (8): The role of protein in constructing the membrane is strongly emphasized in this model. The membrane is essentially a continuous array of protein units, with lipids inserted in between. Many important functions such as energy transduction and protein transport were explained on the basis of this model.

All these models have their supporting evidence as well as some negative criticisms. However, it seems that the lipid-globular protein mosaic model has gained much attention in recent years. Biophysical, biochemical and cytological studies in a wide variety of biological systems lend their support to this model. A well-organized, thoroughly-discussed review on this model is available (9). Models proposed for one membrane system may not be suitable for other systems. One has to be careful when a given membrane structure is generalized.

In this thesis, attention has been focused on a particular membrane system, namely, membranes of Acholeplasma laidlawii. Before going into the main body of the thesis, a brief description about this organism seems necessary. Since recent reviews (10, 11), books (12) are available, individual references will not be cited in the following paragraphs.

Acholeplasma laidlawii belongs to the order Mycoplasmatales which are a group of procaryotic micro-organisms earlier called pleuro-pneumonia-like organisms, or PPLO. They are generally characterized by a small size (0.2 to few μ), devoid of cell wall, bound by

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the "unit membrane" structure, and lacking any intracellular membrane-bound organelles. A. laidlawii differs from other mycoplasmas by not requiring sterol for growth. Its morphology can be coccus, filamentous or amorphous, depending on species, growth medium, culture age and the methods employed to examine it. The genome of the organism is circular, of molecular weight $8\text{-}9\times10^8$ daltons, and have a low G+C content (30 to 36%). DNA replication appears to be semiconservative, and proceeds unidirectionally from at most a few growing points which are believed to be membrane-associated. The ribosomes and the mechanisms of transcription and translation are similar to those of the procaryotes. Phages infecting A. laidlawii cells have been discovered. Most of them are bullet-shaped particles and contain single-stranded DNA.

Acholeplasma laidlawii has been the subject of many membrane studies in recent years. One obvious reason is its relative simplicity in cellular structures which greatly eases membrane isolation and characterization. Electron microscopy reveals "unit membrane" structure both in cells and isolated membranes. The membrane contains 50 to 59% protein, 32 to 40% lipid, 0.5 to 2% carbohydrate, 2 to 5% RNA and about 1% DNA. The lipids are composed mainly of phospholipids and glycolipids, with varying amounts of carotenoids depending on the growth condition. Interestingly, the lipids can be highly enriched with a fatty acyl group whose corresponding fatty acid are supplied exogeneously. Polyacrylamide gel electrophoresis of the solubilized membrane exhibits at least 20 to 30 bands. Membrane-associated enzyme activities have been reported. K^+ and Na^+ transport are not coupled

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and are independent of the ATPase activity in the membrane. Active transport of K^+ ion, acetate, and perhaps D-glucose have been demonstrated. The generally reported membrane thickness is 75 to 100 Å. Particles of 75 to 125 Å in diameter have been found in the internal plane of the membrane. The cells have a negative surface charge and a very small surface conductivity. Membrane proteins contain 23 to 31% α-helices, 30 to 57% β-pleated sheets, and 13 to 45% random coils. Most lipids in the membrane appear to be arranged in a bilayer manner. Phase transitions of the hydrocarbon chains in the bilayer have been detected with differential thermal colorimetry, EPR, and X-ray diffraction techniques. At the growth temperature, the membrane has fluid lipid regions which influence important functions such as permeability and osmotic fragility. It is possible to solubilize the membrane by detergents into small "subunits," and form reaggregated membranes after removing the detergents. Lipid bilayer structure resumes in the reaggregated membrane. However, the proteins are incorrectly reassembled in these membranes.

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CHAPTER II

EFFECT OF FATTY ACYL CHAIN LENGTH ON SOME STRUCTURAL AND FUNCTIONAL PARAMETERS OF ACHOLEPLASMA MEMBRANES

Introduction

The limiting plasma membrane is the only membranous structure in the organism Acholeplasma laidlawii (previously named Mycoplasma laidlawii) (13). The lack of a cell wall structure (14) and the possibility of greatly varying the membrane fatty acyl group composition (15) make it an appropriate system for studying the role of lipids in the structure and function of biological membranes.

Extensive studies have investigated the fatty acyl group influences on the structure and function of Acholeplasma membranes. Increasing the quantity of unsaturation enhances non-electrolyte permeability (16), reduces osmotic fragility (17), and decreases the aggregation of particles on the membrane fracture face (18). However, the influence of the fatty acyl chain length on these membrane properties is apparently less understood (16). This influence will be most easily detected in membranes whose lipids differ greatly in the chain length of their acyl groups. This study investigated two types of Acholeplasma membranes highly enriched with either a 20-carbon saturated acyl group or a combination of acyl groups averaging

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14-carbon atoms. A pronounced dependence of physiological and physico-chemical properties upon chain length was found.

Materials and Methods

Organism and Growth

Acholeplasma laidlawii (oral strain) was kindly supplied by Dr. S. Rottem (The Hebrew University, Jerusalem, Israel). The basal growth medium consisted of 20g Bacto-tryptose (Difco, Detroit, Michigan) which had been lipid extracted (19), 5g D-glucose, 5g sodium acetate, and 5g tris(hydroxymethyl)aminomethane per liter. The pH of the medium was 8.2 to 8.4 without adjustment. Four g/l of bovine plasma albumin fraction V (Armour Pharmaceutical Co., Chicago, Illinois) was charcoal treated to remove fatty acids (20) and then sterilized by Millipore filtration together with 500 units/ml of penicillin G (Sigma, St. Louis, Missouri). The filtrate was then added to the basal medium. Either arachidic or lauric acid (5 mg/l) was introduced into the growth medium as a 70% aqueous ethanol solution. The final ethanol concentration in the medium never exceeded 0.5%.

The cells were originally grown in oleic acid supplemented medium. They could be adapted to supplementation with either arachidic or lauric acid after 6 - 10 daily transfers. The medium was inoculated (1% V/V) with a 24 hour-old cell culture. The cells were grown statically at 37°C, and harvested after 20-24 hours. They were collected by centrifugation at 9,000 xg for 20 minutes at 4°C. A one-day-old culture has a titre of approximately 10^9 c.f.u. Cell growth was monitored by either viable cell counting on plates

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or measuring turbidity. The plates contained either tryptose broth (not lipid extracted) with 1% agar and 5 mg/l oleic acid, or modified Edward's medium (21) with 1% agar. Turbidity measurements were made at 600 nm with a Coleman 44 linear spectrophotometer.

Membrane Buoyant Density

The harvested cells were washed twice in β-buffer (22) and lysed osmotically (23). After washing, the membranes were suspended in a small volume (about 2 ml) of a 1:20 dilution of β-buffer. This suspension (0.35 ml) was overlayed on 3.8 ml of a linear sucrose gradient (25-55% W/W in 1:20 β-buffer). Centrifugation was performed at 39,000rpm in a SW-56 rotor of a Beckman L2-B ultracentrifuge for 3 hours at 22°C. Fractions were collected after puncturing the bottom of the centrifuge tube and their refractive indices were measured with a Bausch & Lomb refractometer at 20°C. The refractive indices were converted to densitites from a standard table. Each fraction was also assayed for protein content.

Osmotic Fragility

The procedure used was essentially that of Rottem and Panos (24) with minor modifications. After harvest, the cells were washed once and resuspended in a small volume (about 1 ml) of 0.25 M NaCl and 0.01 M $MgCl_2$. An aliquot (o.2 ml) of this thick suspension was added to either 2 ml of 0.25 M NaCl or 2 ml of deionized distilled water, and mixed rapidly. Change in turbidity with time was monitored at 600 nm and room temperature with a Coleman 44 linear spectrophotometer. The fraction of turbidity left was defined as the ratio:

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$$\frac{\text{O.D.}_{600\ nm} \text{ of cells in water}}{\text{O.D.}_{600\ nm} \text{ of cells in 0.25 M NaCl}}$$

These ratios were normalized with respect to the zero time value (Fig. 4).

Osmotic Swelling

The osmotic swelling of cells in sucrose solutions at several concentrations was measured according to de Gier et al. (25). Osmotic equilibrium was achieved within one hour at 22°C and the turbidity of the cell suspension was read at 600 nm.

Glycerol Permeability

The glycerol transport was estimated according to a modification of the procedure of Bangham et al. (26). Cells were suspended in 200 mM sucrose, and 0.1 ml of this suspension was injected into 3 ml of isotonic glycerol solution in a round cuvette (diameter 1 cm) at the test temperature. The suspension in the cuvette was rapidly mixed with a Vortex-mixer and the measurements of the optical density were begun less than 3 sec after injection. The optical density (O.D.) at 600 nm as a function of time was traced by a Varian G-14A strip chart recorder. The initial O.D. change, $\frac{dA}{dt}$, was measured from the recorder chart and the initial change in reciprocal O.D., $\frac{d\frac{1}{A}}{dt}$, which is proportional to the initial swelling rate of the cell, was then calculated. The test temperature was maintained by a thermostated cuvette holder.

Electron Microscopy

After harvesting, the cells were fixed at room temperature with glutaraldehyde and osmium tetroxide, dehydrated, and embedded in Spur. Thin sections were made and stained with uranylacetate and lead citrate and examined with a Philips EM300 electron microscope. The osmolarity of all fixation solutions was closely matched to that of the culture medium to minimize alteration in apparent cell morphology (27). The membrane thickness was measured as the peak-to-peak distance from a densitometer scan of an enlarged photoprint of the micrograph.

Lipid Extraction and Determination of Fatty Acyl Group Composition

Plasma membranes were prepared from the cells by osmotic shock in deionized water, collected, and washed according to Razin *et al.* (17). Membrane lipids were extracted with chloroform-methanol (2:1, V/V), washed with salt solution, and dried under nitrogen according to Folch *et al.* (28). Methyl esters of fatty acids were prepared by reacting about 4 mg of lipids in 4 ml of 2% (V/V) concentrated H_2SO_4 in methanol at 40°C for 24 hours. The methyl esters were extracted with hexane and quantitatively analyzed by gas chromatography on a diethylene glycol succinate column at 170°C. A Hewlett-Packard 402 gas chromatograph equipped with a flame ionization detector was used. The esters were identified by comparison with standards obtained from Applied Science Lab, Inc., State College, Pennsylvania.

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Electron Paramagnetic Resonance Spectroscopy (EPR)

The spin label, 2-(10-carboxydecyl)-2-hexyl-4,4-dimethyl-3-oxazolidinyloxyl,(12NS), was synthesized in our laboratory (29). Approximately $2x10^{-7}$ moles of 12NS were dispersed in 1 ml of 0.25 M NaCl solution by Vortex agitation followed by brief sonication at room temperature. A membrane pellet which had been washed with 0.25 M NaCl was suspended in about 0.5 ml of the aqueous spin label dispersion. The final concentration in the spin-labeled membrane suspension was about 10 mg protein/ml and 0.1-0.2μ moles 12NS/ml. EPR spectra were recorded with a Varian EPR Spectrometer, model 4205-15, equipped with a variable temperature controller, model V4540.

Other Chemical Methods

The amount of total lipids in a membrane preparation was estimated colorimetrically according to the method of Saito and Sato (30), using cholesterol as standard. Protein was determined by the Folin phenol method of Lowry *et al.* (31), with bovine serum albumin as standard.

Chemicals

Oleic, arachidic and lauric acids were purchased from Sigma, and their purities were checked by gas chromatography of their methyl esters. All solvents were reagent grade and were distilled at least once prior to use.

Results

Cell Growth

Of the fatty acids with even-numbered chain lengths from C_{10} to C_{22}, only arachidic and lauric acids gave good growth after less than 14 daily transfers. The adaptation was more facile with arachidic than with lauric acid, with about 5 daily transfers needed for arachidic acid compared to 10 for lauric acid. In addition, the cell titre for lauric acid supplementation was at least a factor of five less than that for arachidic acid at the time of harvesting. The poor growth found for supplementation with medium length fatty acids is interesting but not explored further in this work.

Acholeplasma laidlawii (oral strain) is reported to have an absolute requirement for an octadecenoic acid (24). Attempts to grow cells on lipid extracted tryptose medium without any fatty acid supplementation failed. Nevertheless, it is possible that minute quantities of residual unsaturated fatty acids in the tryptose are sufficient to fulfill the growth requirement upon supplementation with arachidic or lauric acid. Cell growth depended upon the batch of Bacto-tryptose. Growth usually reached the late log phase in 20-24 hours (Fig. 1) but sometimes took as long as 48 hours.

Cell Morphology and Membrane Thickness

Cell morphology of strain B was reported to depend on the nature of fatty acid supplementation (32). However, cells of the oral strain had similar morphology and size distribution when the organism was grown in either arachidic or lauric acid supplemented media. Cells were all coccoid and 0.1 - 0.7μ in diameter as revealed

Figure 1. Growth curves of A. laidlawii (oral strain) cells in tryptose broth supplemented with 5 mg/l of arachidic acid (o), or lauric acid (Δ).

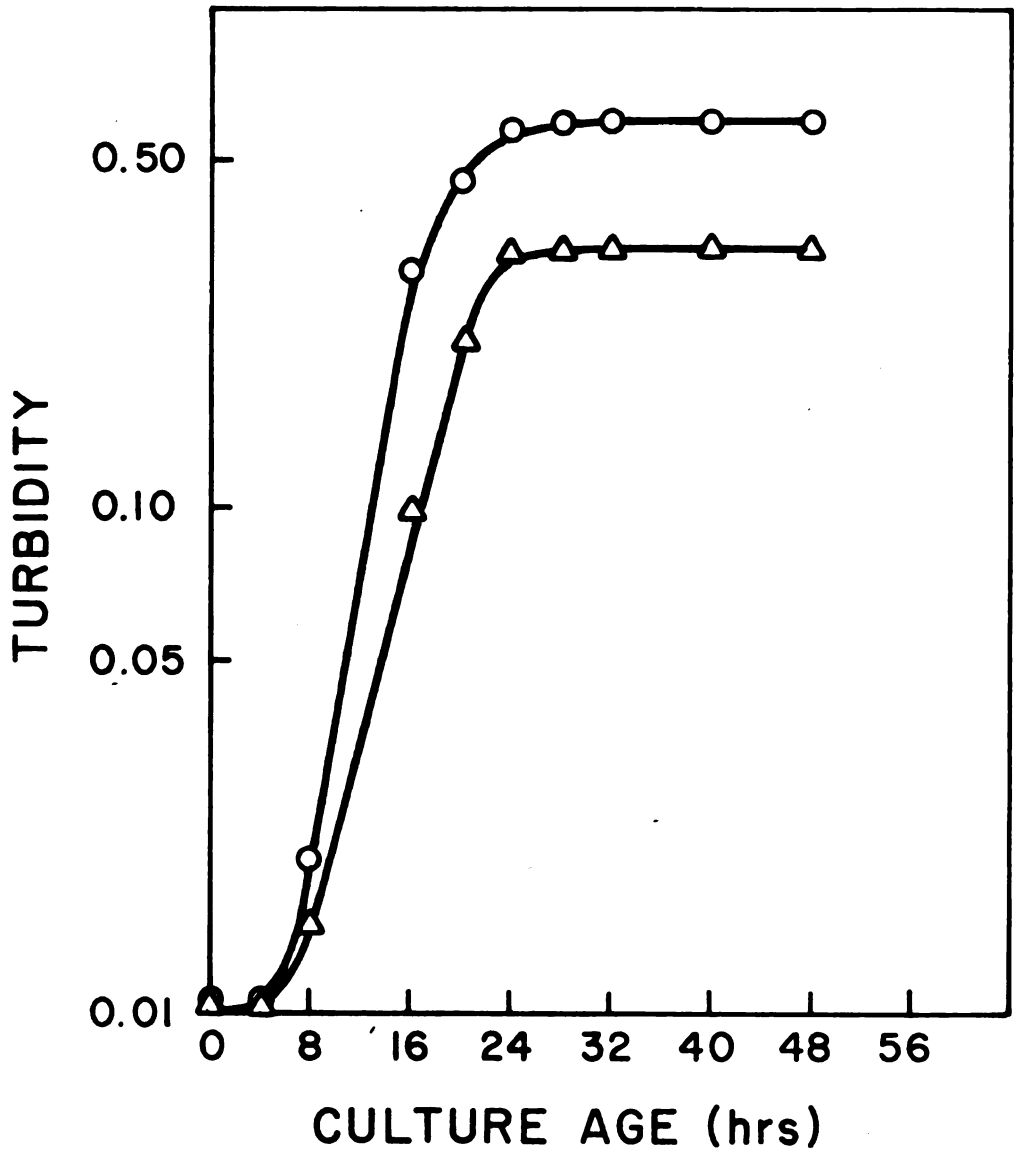


Figure 1

with thin section electron microscopy (Fig. 2). The membrane thickness was 70±14 Å for both types of cells.

Membrane Lipid Fatty Acyl Composition and Lipid/Protein Ratio

Table 1 lists the total fatty acyl compositions and the lipid/protein ratios for the membranes. The arachidoyl group was found only with arachidic acid supplementation and usually comprised about 55% of the total acyl groups. In one case it comprised 70%. With lauric acid supplementation, the lauroyl, myristoyl, and palmitoyl group percentages were 2, 8 and 4 times greater than with arachidic acid supplementation and the combined quantities of these three groups constituted about 3/4 of the total. The average chain length of the saturated fatty acyl group is about 18.4 carbon atoms for the arachidoyl enriched membranes compared to 14.4 for those enriched with the shorter acyl groups. For each fatty acid supplied, the amount of the oleoyl group was constant. Furthermore, unsaturated acyl groups comprised a total of about 1/4 of the total fatty acyl groups for both types of membranes, perhaps indicating that unsaturated acyl groups are required for membrane integrity. Thus, two types of Acholeplasma laidlawii membranes are available which differ greatly in the chain length of their saturated acyl groups.

The small quantity of lauroyl groups found with lauric acid supplementation suggests that the membrane structure requires a considerable quantity of longer chain acyl groups. The necessity for Acholeplasma to elongate the lauric acid supplied may be the reason why lauric acid supplementation gives poorer growth than

Figure 2. Electron micrograph of A. laidlawii (oral strain) cells grown in arachidic acid supplemented medium. Bar represents 0.5μ, cells grown in lauric acid supplemented medium gave similar morphology, size and membrane thickness.

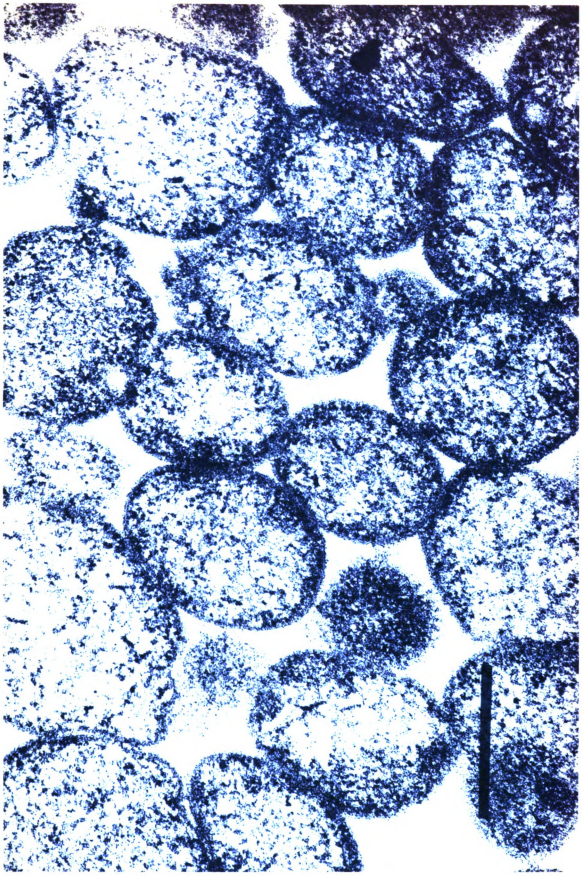


Figure 2

Table 1. Fatty Acyl Compositions of Total Membrane Lipids and Membrane Lipid/Protein Ratio of *Acholeplasma Laidlawii* Cells Supplemented With Two Different Saturated Fatty Acids.[a]

Fatty Acid Supplemented	Fatty Acyl Composition (Mole %)								Saturated/ Unsaturated Fatty Acyl Groups (mole/mole)	Membrane Lipid/Protein Ratio (mg/mg)
	12:0[b]	14:0	16:0	16:1	18:0	18:1[c]	18:2	20:0		
Arachidic	7.9	4.4	5.8	1.8	1.8	17.4	6.9	54.0	2.8	0.41
Lauric	15.2	35.0	23.2	2.7	3.0	17.0	3.8	none	3.3	0.42

[a]Cells were grown in lipid pre-extracted trytose medium supplemented with fatty-acid-poor BSA and fatty acid indicated. For details see Materials and Methods.

[b]Number to the left of the colon refers to number of carbon atoms; number to the right refers to number of double bonds.

[c]Our column condition could not distinguish geometric isomers. However, an infrared spectroscopic analysis of the total fatty acid methyl esters indicated only methyl oleate was present.

arachidic acid supplementation. Acholeplasma laidlawii B cells are also known to elongate lauric acid to myristic and palmitic acids (33).

Membrane Buoyant Density

Fig. 3 shows the results of isopycnic centrifugation of the two membrane types. At 20°C the peak densities are 1.195 g/ml for membranes enriched with long chain and 1.189 g/ml for membranes enriched with short chain fatty acyl groups. Membranes isolated from cells grown in lauric acid supplemented medium are about 0.5% less dense. We believe the membrane buoyant density is mainly determined by the lipid/protein ratio and the packing of the membrane lipids. In our studies the lipid/protein ratio was found to be independent of chain length. Therefore the density difference between the two membrane types apparently results from different lipid packing.

Kahane and Razin (34) have shown the lipid/protein ratio and the buoyant density of membranes for Acholeplasma cells grown in modified Edward's medium are pH dependent. Their values for the lipid/protein ratio and buoyant density interpolated from their data to pH 8.2, the pH of our experiment, are 0.51 and 1.177 g/ml respectively. The greater densities for our membranes are probably due to the value of 0.4 found for the lipid/protein ratio.

Osmotic Fragility of Membranes

The rate of osmotic lysis of the cells is shown in Fig. 4. Cells grown with lauric acid supplementation proved more resistant to osmotic shock than those grown with arachidic acid. After one hour at

Figure 3. Isopycnic centrifugation of A. laidlawii (oral strain) membranes in a linear sucrose density gradient (25 - 55%, W/W in 1:20 β-buffer).

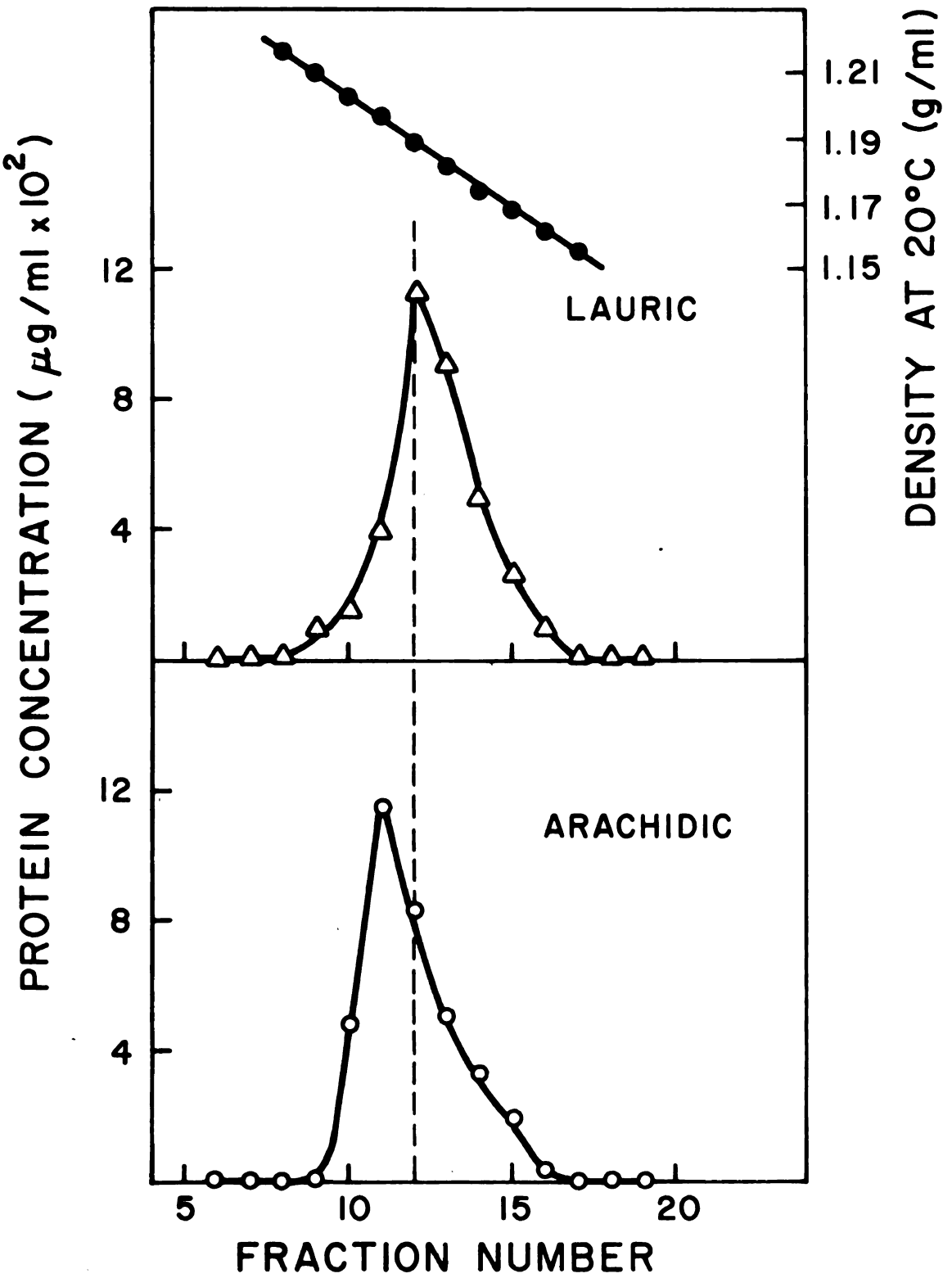

PROTEIN CONCENTRATION (μg/ml x 10²)
DENSITY AT 20°C (g/ml)
1.21
1.19
1.17
1.15
12
8
4
LAURIC
12
8
4
ARACHIDIC
5
10
15
20
FRACTION NUMBER

Figure 3

Figure 4. Osmotic lysis of A. laidlawii (oral strain) cells grown in arachidic acid (o) or lauric acid (Δ) supplemented medium.

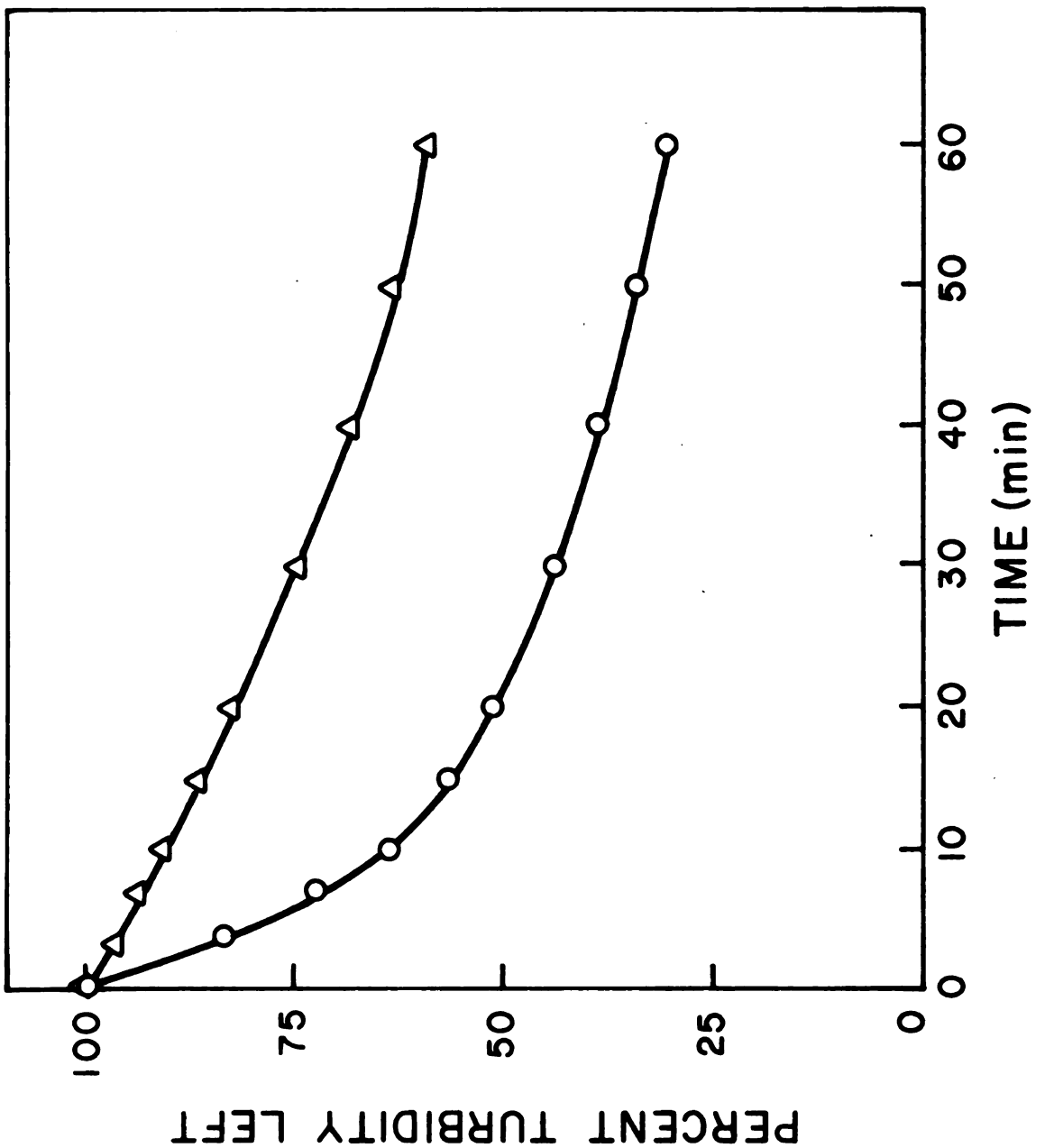

PERCENT TURBIDITY LEFT
100
75
50
25
0
0
10
20
30
40
50
60
TIME (min)

Figure 4

room temperature, the percent turbidity remaining differed by approximately a factor of two.

Osmotic Swelling and Glycerol Permeability

Both types of cells studied exhibit similar osmotic behavior. The increase in volume is linearly proportional to the reciprocal value of the absorbance (26). In our case (Fig. 5) a linear relationship exists between reciprocal absorbance and reciprocal sucrose concentration, indicating the osmotic swelling of our cells follows the Boyle-Van't Hoff law for an ideal osmometer. These results are in agreement with those reported for strain B (16).

Once the ideal osmometer behavior of cells was established, the method of Bangham et al. (26) to estimate the passive glycerol permeability of cells should be valid. Glycerol permeation depends on temperature and on the fatty acyl group composition of the membrane (Fig. 6). The results show the membranes enriched with shorter chain saturated acyl groups to be more permeable to glycerol than those with longer chains. For lecithin liposomes (25) and strain B cells (16) the glycerol permeability also depends on chain length.

An arrhenius plot of the permeability data gave straight parallel lines. The activation energy was 17.5±1.0 Kcal/mole for both cell types, in close agreement with that reported for strain B cells (16).

Spin Labeling of Membranes

The dependence of membrane fluidity on the length of the fatty acyl groups was investigated with the fatty acid spin label 12NS. At

Figure 5. Osmotic swelling of A. laidlawii (oral strain) cells grown in arachidic acid (o) or lauric acid (Δ) supplemented medium. Absorbances were measured one hour after cells swelled to equilibrium at room temperature in sucrose solutions of various concentration.

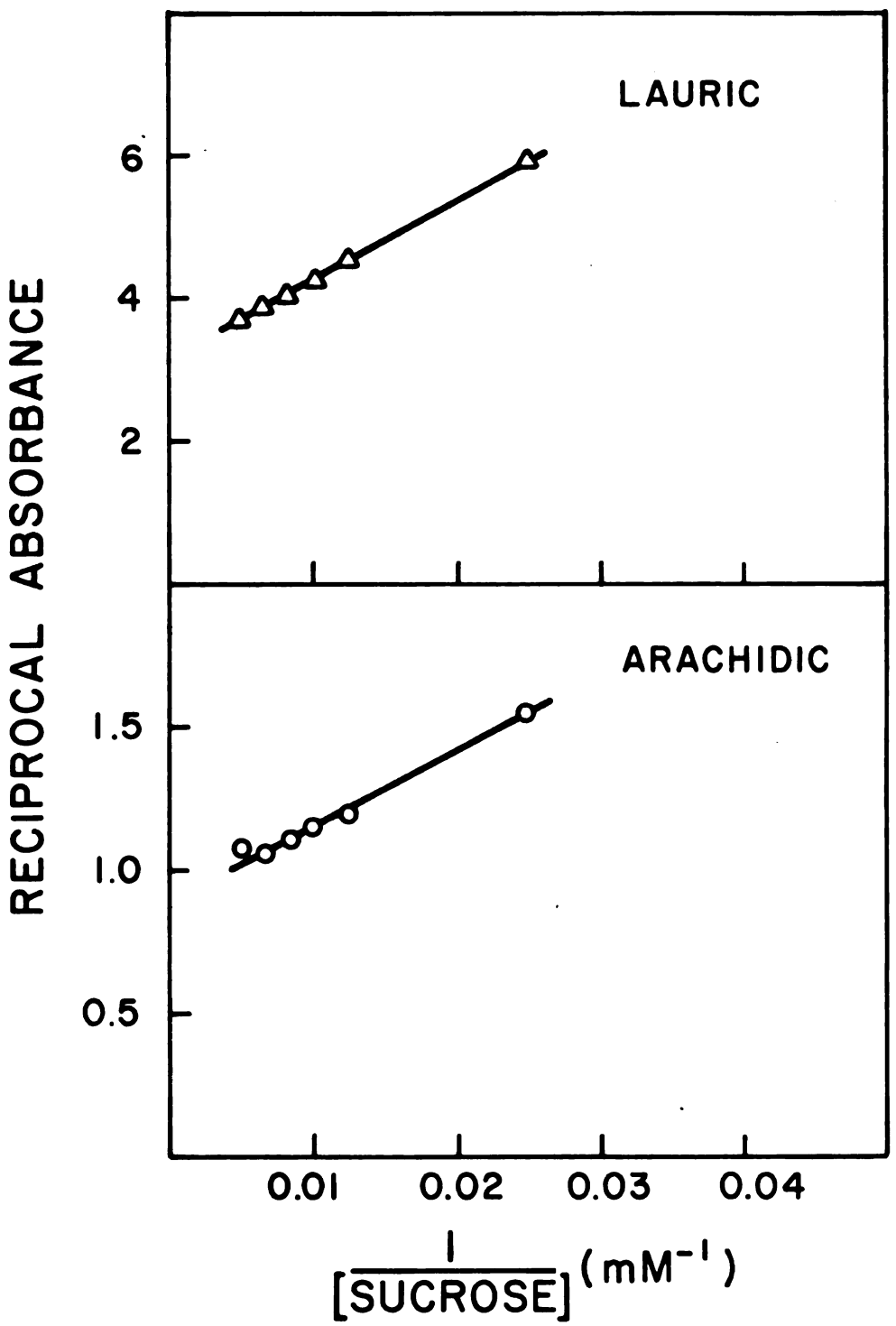

LAURIC
ARACHIDIC
RECIPROCAL ABSORBANCE
6
4
2
1.5
1.0
0.5
0.01
0.02
0.03
0.04
1/[SUCROSE] (mM⁻¹)

Figure 5

Figure 6. Temperature dependence of glycerol permeability in A. laidlawii (oral strain) cells grown in arachidic (o), or lauric acid (Δ) supplemented medium. The initial swelling rate of cells in isotonic glycerol solution is proportional to the passive permeability of glycerol to the cell membranes.

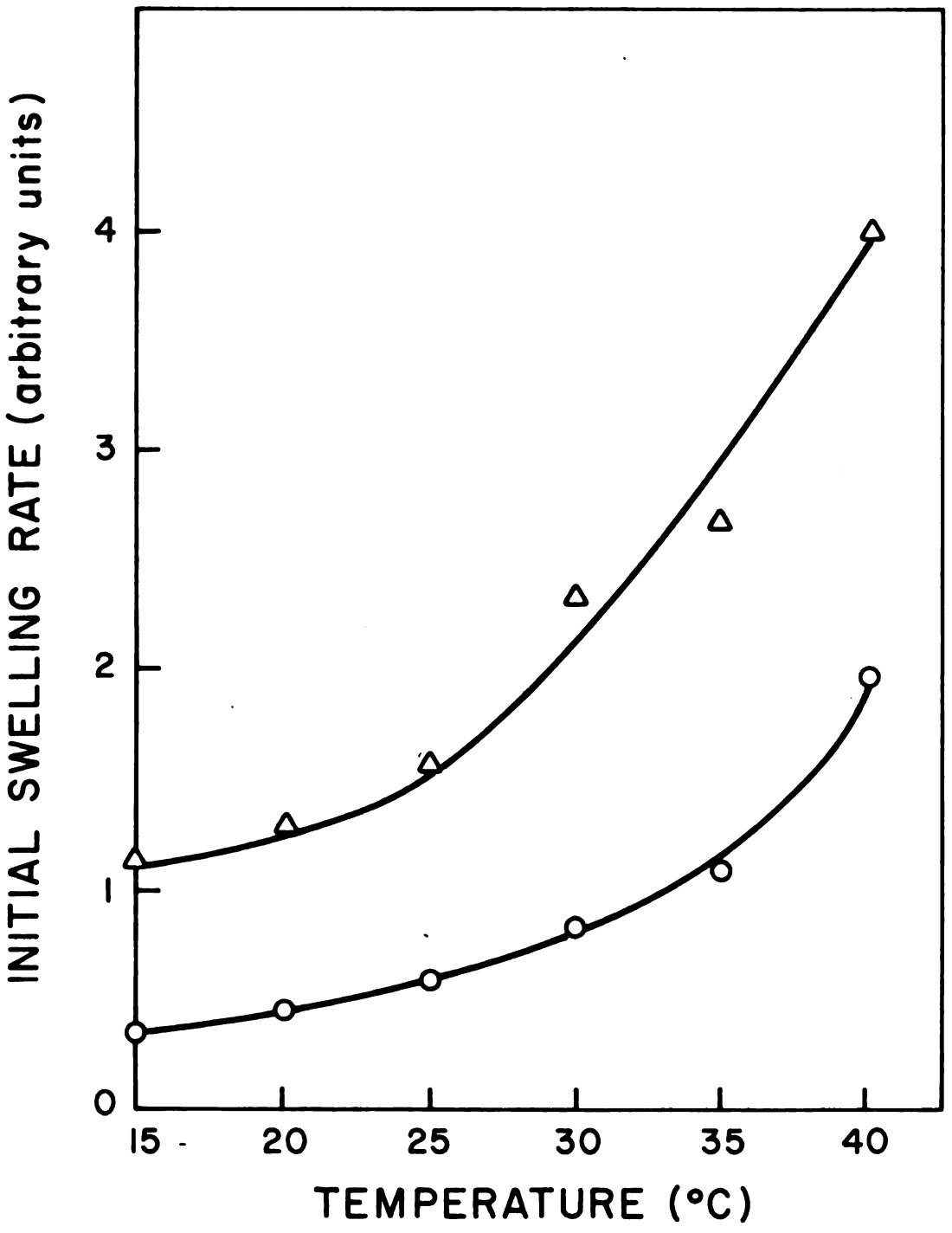

INITIAL SWELLING RATE (arbitrary units)
0
1
2
3
4
15
20
25
30
35
40
TEMPERATURE (°C)

Figure 6

22°C, the spin label has greater mobility in membranes enriched with the shorter fatty acyl groups than those enriched with the arachidoyl group (Fig. 7). The rotational correlation time τ_c of the spin label was calculated with the following formula (35):

$$\tau_c = 6.5 \times 10^{-10} \times W_o \times \left(\sqrt{\frac{h_o}{h_{-1}}} - 1 \right) \text{ sec.} \qquad (1)$$

where W_o is the peak-to-peak width (gauss) of the central resonance peak; h_o and h_{-1} are the peak heights of the central and high-field peaks respectively. The value of τ_c is 10 nsec for arachidoyl enriched membranes and 6 nsec for membranes enriched with shorter chain acyl groups. This indicated that the hydrophobic region of the former membrane is relatively more "viscous" than that of the latter one, i.e., the hydrocarbon chains are packed somewhat tighter. Signals from small amounts of free label appear in both spectra, as indicated by arrows in Fig. 7, but do not significantly interfere with the quantitative measurements.

Discussion

The biological functions of a membrane depend significantly on the fluidity of the membrane (16,17,24). Research progress in correlating membrane fluidity with function is rather difficult since in vivo variation of fluidity can only be achieved for few organisms. The strains of Acholeplasma laidlawii are organisms suitable for such studies since the membrane lipid composition, and hence its fluidity, depends upon the growth medium. By growing Acholeplasma on a medium

Figure 7. Electron paramagnetic resonance spectra of a fatty acid spin label, 12NS, incorporated into membranes of A. laidlawii (oral strain) cells grown in the medium supplemented with the fatty acid indicated. Arrows indicate signals from unincorporated spin labels.

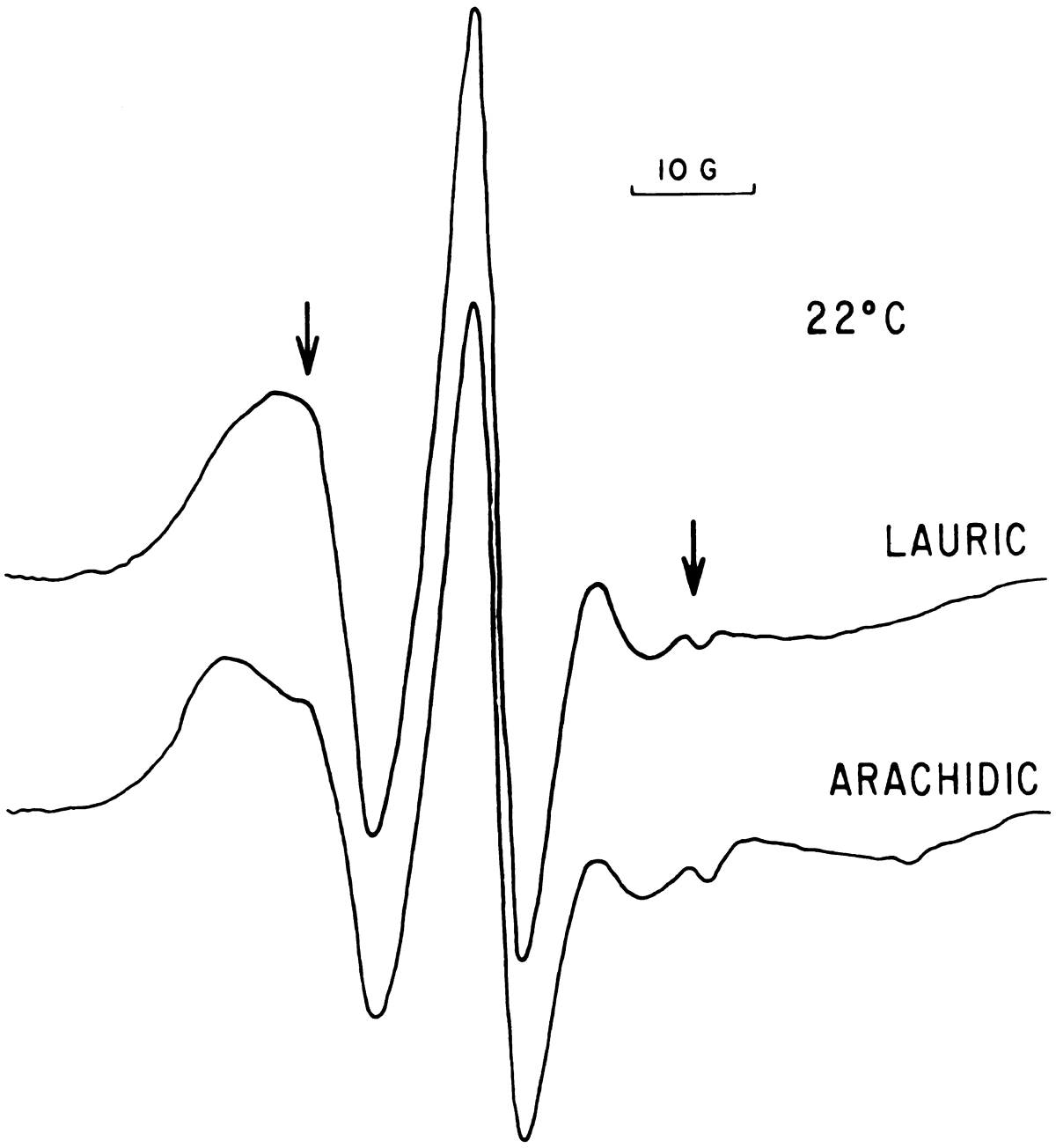
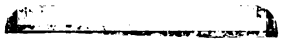


Figure 7



containing the shortest and longest saturated fatty acids that would support good growth, membrane preparations were obtained that represent the greatest difference in fluidity achieved by variation of the chain length of the saturated acyl groups yet reported. Compared to previous reports, the difference in length of the average saturated acyl group is about twice as large (16). Furthermore, a greater influence of the saturated acyl groups on membrane properties is expected than for previous preparations because the saturated/unsaturated acyl group ratio is 2.8 or 3.3 for our Acholeplasma membranes and about 1.0 for those of McElhaney et al. (16). Therefore, these membranes offer a better opportunity to elucidate the role of saturated acyl chains on membrane functions.

Recently it has been suggested that the Acholeplasma membrane consists of extensive lipid bilayer regions apparently penetrated by protein particles (36,37,38). The lipid regions in native membranes are found to be less fluid than the liposomes made from the respective lipid extracts (39). The liposomes may be more fluid either because of the absence of proteins or a change in the lipid packing. For Acholeplasma membranes it was found that the protein synthesis is uncoupled from lipid synthesis (40). Moreover, the majority of membrane polypeptides behave electrophoretically similar for various fatty acyl compositions of membrane lipids (41). Since the lipid/protein ratio and the SDS-polyacrylamide gel electrophoresis pattern of membrane proteins (unpublished results of L. Huang and D. D. Jaquet) for the two types of Acholeplasma membranes studied were very similar, we assume that the relative properties of the lipid regions

depend largely on the change in fatty acyl composition. The difference found in structural and functional parameters for the two types of membranes are therefore attributed to the change in lipid fluidity and its dependence upon acyl group composition.

The fluidity of the lipid regions seems to determine the results found for buoyant density, passive glycerol permeability, and osmotic fragility for the membrane preparations tested. The greater fluidity is associated with greater non-electrolyte permeability but lower density and osmotic fragility. The inverse relationship between chain length and fluidity is predicted since stronger intermolecular association results from longer chain length as melting point and viscosity data for pure lipids prove (42). The spin labeling experiment directly confirms this inverse relationship. However, the spin label mobility may not correctly reflect the fluidity of the lipid region because the label may be located only at the most fluid regions and/or perturb the lipid packing (43).

On the basis of our experimental findings a simple membrane model can be conceived. When the fatty acyl chain length increases and the membrane thickness remains constant, more matter has to be accommodated in a certain space. Therefore, the molecular motions of longer acyl chains are more restricted than those of shorter chains. Experimentally, it was found that the membrane thickness (peak-to-peak distance) was independent of the acyl chain length, within the experimental accuracy of the electron microscopy technique (44). These results disagree with those obtained by X-ray diffraction experiments (37,45), where the lipid bilayer thickness

was assumed to increase in proportion to the average fatty acyl chain length. However, in their studies the fatty acyl chain lengths were varied by enriching the Acholeplasma membranes with either erucic ($C_{22:1}$ cis) or palmitic ($C_{16:0}$) acid. The interpretation of bilayer thickness in terms of the fully extended hydrocarbon chains was weakened by the possibility that the cis double-bond in erucic acid may cause a change in average chain orientation relative to the plane of the membrane.

These speculations would be given a firmer basis if more precise techniques for measuring membrane thickness could be used.

CHAPTER III

CONTROL OF MEMBRANE LIPID FLUIDITY BY CAROTENOID PIGMENT CONTENT IN ACHOLEPLASMA LAIDLAWII

Introduction

Acholeplasma laidlawii cells contain carotenoid pigments in their limiting membranes (46). The content of these pigments can be increased by growing cells in an acetate-containing medium or decreased by growing them in a medium containing propionate, diphenylamine, or thallium acetate (47,48). The structure and function of these pigments in Acholeplasma cells has been the subject of several studies. These pigments may function as a transport carrier for glucose and acetate, since some carotenoid pigments isolated from the cell are covalently linked to glucose and/or acetate (46). This hypothesis was later refuted because A. laidlawii cells could be grown under such conditions that neither carotenoids nor cholesterol were accumulated in the membrane (48). It has also been reported that the carotenoid pigments in these cells can protect the membranous adenosine triphosphatase against photodynamic inactivation (49).

The membranes of all sterol-requiring Mycoplasmas examined to date contain large amounts of sterol, but no carotenoids; and

those of sterol-nonrequiring species contain carotenoids instead of sterols. This fact suggests a common function of carotenoids and sterols such as cholesterol (50). Since cholesterol reduces the lipid fluidity by interacting with membrane phospholipids (51,52,25), it is probable that carotenoids in Acholeplasma cells function similarly. For cells other than Acholeplasma, it has been suggested that carotenoids are perhaps involved in stabilizing membranes (53). In this article, we present direct proof that carotenoids indeed play a role in controlling the membrane lipid fluidity.

Methods

Organism and Growth

A. laidlawii (oral strain) was a gift of Dr. S. Rottem (The Hebrew University, Jerusalem, Israel). The growth medium consisted of 20g/l of lipid-extracted tryptose (19), 5g/l of sodium acetate or sodium propionate, 5g/l of D-glucose, and 5g/l of tris (hydroxy-methyl) aminomethane, 4g/l of fatty-acid-free bovine plasma albumin, 500 units/ml of penicillin G and 5mg/l of arachidic acid as described in Chapter II. The complete culture medium was inoculated (1% v/v) with a 24-hour old cell culture which has already been adapted to arachidic acid supplementation. The culture was grown statically in the dark at 37°C, and harvested at late log growth phase (about 24 hours).

Plasma Membrane Preparation

The plasma membranes of A. laidlawii cells were prepared according to Razin et al. (17). The membranes were washed twice in

deionized distilled water and used freshly or stored at -20°C under nitrogen in the dark for at most 24 hours.

Determination of Fatty Acyl Composition and the Carotenoid Content in the Membrane Lipids

Lipids were extracted from isolated membranes by the methods of Folch et al. (28). The optical absorption of the "Folch lower phase" was measured at 450nm against chloroform-methanol 2:1,v/v). The amount of carotenoid pigments in the membrane lipids are expressed as OD_{450nm}/mg membrane protein. The trans-methylation of membrane lipids, the extraction of the methyl esters of fatty acids, and the subsequent analysis of these esters by gas chromatography were carried out as described before. The amount of lipids was determined colorimetrically (30), using cholesterol as standards.

Buoyant Density of the Membrane

The procedure of the isopycnic centrifugation was the same as before, except that both the membrane suspension and the sucrose density gradient were prepared in deionized water instead of 1:20 β-buffer (22). The membrane buoyant density was defined as that of the peak fraction collected from the centrifuge tube. Membrane proteins were assayed by the method of Lowry et al. (31).

Spin-Labeling of the Membranes

A. laidlawii membranes were labeled in vitro with a fatty acid spin label, 2-(10-carboxydecyl)-2-hexyl-4,4-dimethyl-3-oxazolidinyloxyl, (12NS), as described before. However, deionized

water was used to wash the membranes and to disperse the spin label. The spin-labeled membrane suspension contained about 10mg protein/ml and 0.1-0.2 μmoles 12NS/ml. Electron paramagnetic resonance (EPR) spectra were recorded with a Varian EPR spectrometer, model 4502-15, equipped with a variable temperature controller, model 4540.

Osmotic Swelling, Relative Glycerol Permeability and Osmotic Fragility of the Cells

All procedures were the same as described in Chapter II. Swelling of the cells in hypotonic sucrose solutions was monitored by the absorbance at 600nm. The passive glycerol permeability was estimated by measuring the initial swelling rate of cells in the isotonic glycerol solution at various temperatures. This method only yields the relative permeability (26). The osmotic fragility was recorded kinetically by comparing the turbidity loss of the cell suspension in water or 0.25M NaCl. The fraction of turbidity (OD_{600nm}) left was defined as the ratio:

$$\frac{OD_{600nm} \text{ of cells in water}}{OD_{600nm} \text{ of cells in 0.25M NaCl}}$$

These ratios were normalized with respect to the zero time value.

Results

Carotenoid Content in A. laidlawii Membranes

Membranes from cells grown in medium with 5g/l sodium acetate contained a high amount of carotenoid pigments and were bright yellow.

In contrast, when grown in sodium propionate (5g/l), cells contained practically no carotenoid pigments and were rather pale. The carotenoid content of membranes from these two types of cultures is shown in Table 2. The ratio of pigment content of these cultures was about 10-fold. This result is in agreement with those reported earlier (49,17). Sodium acetate is known to be a precursor in the biosynthesis of carotenoids (12). Sodium propionate probably inhibits the formation of these pigments by interfering with the transport of acetate across the cell membrane (54).

Lipid Fatty Acyl Composition and Membrane Lipid/Protein Ratio

Although small amounts of $C_{13:0}$ and $C_{15:0}$ acyl groups were present in lipids of cells grown with propionate, the overall acyl group composition remained practically unchanged (Table 2) when the pigment content was lowered by a factor of ten. Whether the cells were grown in acetate or propionate, membrane lipids were equally enriched with the arachidoyl group (about 70%,w/w). The membrane lipid-protein ratio was also identical in both cases. Therefore, any difference in physico-chemical and physiological properties between these two types of membrane seems to result from the 10-fold difference in carotenoid content.

Membrane Lipid Fluidity

Membrane lipid fluidities of the two types of membrane were detected by the spin-labeling technique. The fatty acid spin label 12NS was introduced into the isolated membranes and intercalated into the hydrophobic region (55). A typical EPR spectrum of these

Table 2. Total Lipid Fatty Acyl Composition, Carotenoid Content, and Lipid/Protein Ratio of A. laidawii Membrane.[a]

Medium ingredient (5g/l)	Fatty acyl composition (% w/w)[b]																Carotenoid content (OD_{450}/mg membrane protein)	Lipid/ Protein Ratio (mg/mg)
	UI[c]	$C_{12:0}$	$C_{12:1}$	$C_{13:0}$	$C_{14:0}$	$C_{14:1}$	$C_{15:0}$	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{20:0}$	UI[c]	UI[c]	UI[c]		
Sodium propionate	0.2	5.5	-	2.1	2.7	-	0.5	4.4	0.9	2.0	4.8	1.1	72.3	1.9	-	0.8	0.065	0.49
Sodium acetate	-	6.1	0.5	-	2.4	0.7	-	4.9	2.0	3.0	5.0	2.1	70.9	0.7	0.8	0.9	0.661	0.51

[a]Cells were grown in medium supplemented with arachidic acid (5mg/l).

[b]Fatty acyl groups are listed according to their retention time in the chromatographic column. The retention increases from left to right on the table.

[c]Unidentified.

spin-labeled membranes is shown in Fig. 8. The hyperfine splitting $2T_{\parallel}$ in the spectrum is related to the rotational mobility of the spin label and therefore reports the local fluidity of membrane lipids (55). A high value of $2T_{\parallel}$ reflects a low fluidity and vice versa. At high temperatures, the high-field "dip" in the spectrum became so shallow that an accurate measurement of $2T_{\parallel}$ was not possible. In these cases, an empirical motion parameter, τ_c, was calculated according to formula (1). A large value of τ_c indicates a rigid micro-environment around the label. Table 3 lists the values of these motion parameters of the two types of membrane at different temperatures. Membranes with higher levels of carotenoids gave higher values of motion parameters at all temperatures tested. This implied that the hydrophobic regions of the carotenoid-rich membrane were more fluid as compared to those of the carotenoid-poor membrane. In other words, the presence of carotenoid pigments somehow made the membrane more rigid.

Membrane Buoyant Density and Relative Glycerol Permeability

The buoyant densities of both membrane preparations are shown in Table 4. Those membranes with greater amounts of carotenoid pigments were denser. Since the two types of membrane had nearly identical lipid/protein ratios and fatty acyl compositions, the difference in density likely resulted from a modification in lipid packing. The results of the spin-labeling experiment directly supports this assumption. Since both types of cell behaved like an ideal osmometer, the initial swelling rate in the isotonic glycerol

Figure 8. Typical electron paramagnetic resonance spectrum of the spin label 12NS incorporated into A. laidlawii membrane sample at 8°C. Arrows indicate signals from unincorporated spin labels. Magnetic field strength increases from left to right.

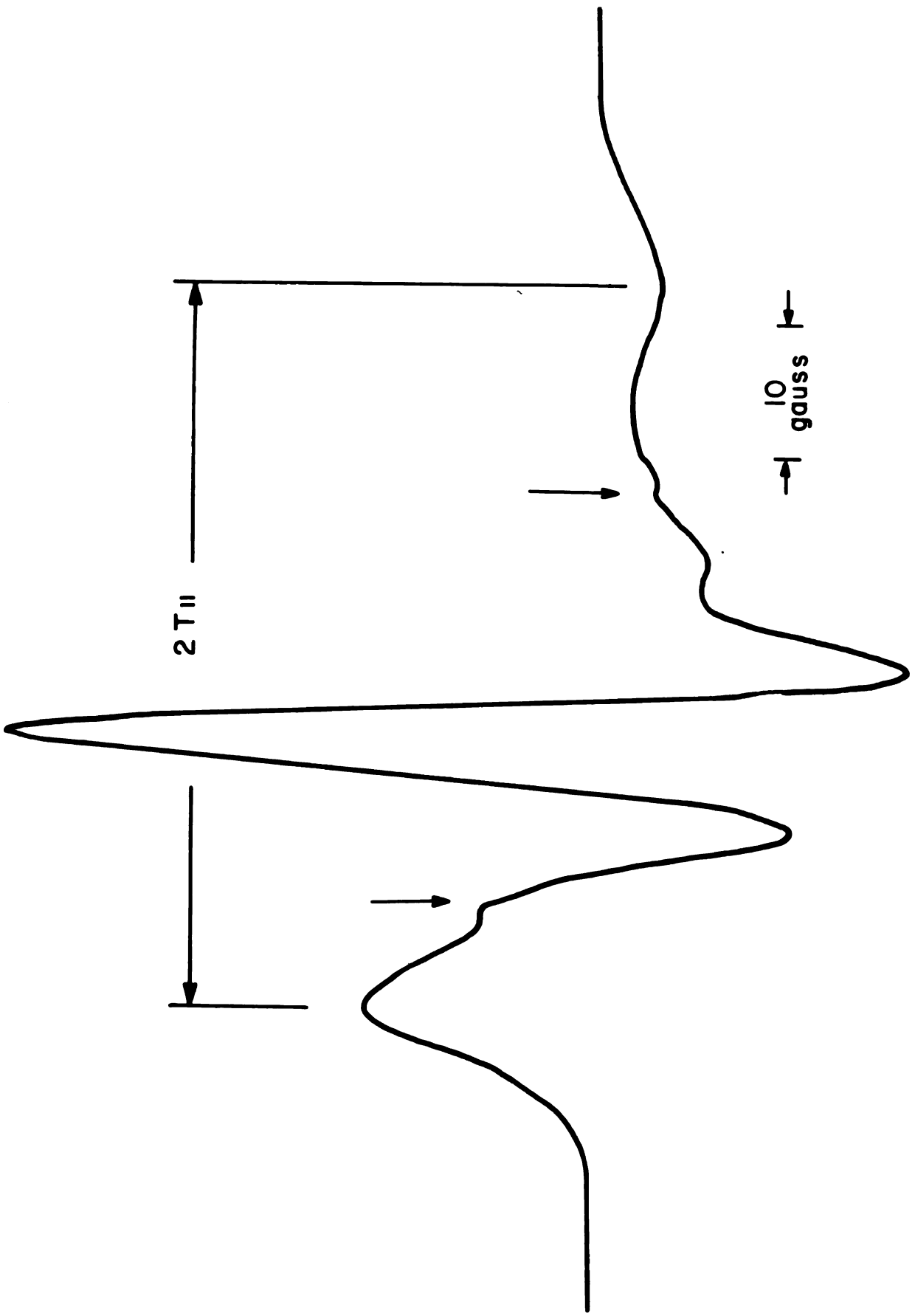


Figure 8

Table 3. Motion Parameters of the Spin Label 12NS Incorporated into Two Types of Membrane of A. laidlawii.

Experiment number	Medium Ingredient (5g/l)	$2T_{\parallel}$ (gauss)[a]		τ_c (nsec)[a]
		8°C	22°C	37°C
I	Sodium propionate	55.17	44.82	1.2
	Sodium acetate	56.72	48.27	2.9
II	Sodium propionate	56.20	46.55	2.6
	Sodium acetate	57.24	48.96	3.1

[a]The hyperfine splitting $2T_{\parallel}$ was measured to within ±0.4 gauss; and τ_c to within ±0.2 nsec.

Table 4. Buoyant Densities and Relative Glycerol Permeabilities of A. laidlawii Membranes.[a]

Medium ingredient (5g/1)	Buoyant density (g/ml at 20°C)[b]	Initial swelling rate in isotonic glycerol solution (arbitrary units)[c]		
		15°C	22°C	38°C
Sodium propionate	1.160±0.005	0.21±0.005	0.51±0.03	1.76±0.31
Sodium acetate	1.173±0.005	0.16±0.02	0.43±0.02	1.55±0.20

[a]Cells were grown in medium supplemented with arachidic acid (5mg/1).

[b]Results came from 3 different experiments. Data expressed as mean ± standard deviation.

[c]Results came from 4 different experiments. Data expressed as mean ± standard deviation.

solution was proportional to the glycerol permeability. Cells grown in propionate were significantly more permeable to glycerol than cells grown in acetate at all three temperatures tested (Table 4). In both cases, the initial swelling rate increased with temperature. At temperatures higher than 45°C, the cells swelled so rapidly that the initial swelling rate became difficult to measure. When a non-electrolyte molecule such as glycerol permeates a lipid barrier, the rate of penetration depends on the packing of lipids. Therefore, the slower permeation in the carotenoid-rich cells suggested a more viscous lipid region in the membrane. This conclusion is consistent with results of the spin-labeling experiments.

Osmotic Fragility of Cells

The resistance of the cell to the osmotic lysis was measured kinetically. The results showed that propionate-grown cells were more resistant to osmotic lysis than those grown in acetate (Fig. 9). After one hour at room temperature the propionate-grown cells had about 10% more turbidity left than the acetate-grown ones. Thus, cells having more fluid membranes are tougher to lyse. This results agrees with the hypothesis that lipid fluidity enhances the membrane tensile strength against osmotic shock (17,56).

Discussion

The results presented here demonstrate that the hydrophobic regions of the carotenoid-rich membrane are less fluid than the corresponding regions of the carotenoid-poor ones. Consequently, carotenoid-rich membranes are characterized by higher osmotic

Figure 9. Osmotic fragility of A. laidlawii cells. Cells were grown in an arachidic acid supplemented medium containing sodium propionate or acetate (5 g/l).

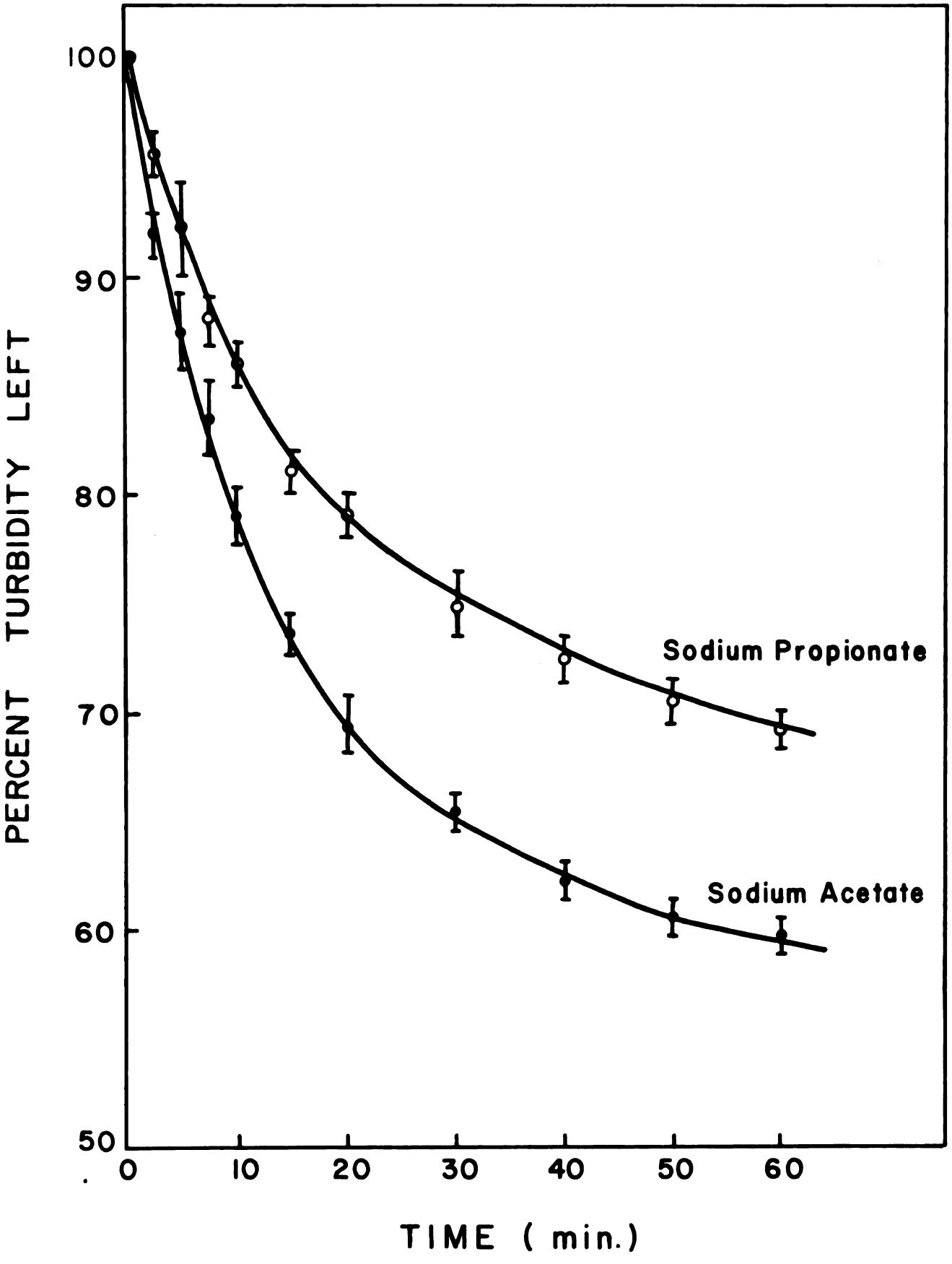

100
90
80
70
60
50
PERCENT TURBIDITY LEFT
0
10
20
30
40
50
60
TIME (min.)
Sodium Propionate
Sodium Acetate

Figure 9

fragility, lower glycerol permeability, and higher buoyant density. However, no significant change in membrane osmotic fragility was observed when the carotenoid content of strain B cells is increased ten-fold (17). Also, protoplasts of Sarcina lutea prepared from a colorless mutant or from a wild type strain grown in diphenylamine do not exhibit different membrane osmotic fragility as compared to those from wild type cells (57). Both reports did not present any data about the membrane lipid fluidity and the fatty acyl composition. Nevertheless, it is possible that the fatty acyl composition is modified upon a drastic change in carotenoid content such that the cell can maintain a proper membrane fluidity necessary for normal growth. Consequently, one expects no significant change in osmotic fragility as long as the membrane lipid fluidity stays essentially constant. This argument is further supported by preliminary results from this laboratory that A. laidlawii cells indeed modify their membrane fatty acyl composition and maintain the lipid fluidity within a narrow range when the carotenoid content is altered by about 60-fold.

Numerous reports demonstrated that cholesterol and a number of its derivatives condense the packing of phospholipids in various biological membranes (58,59), and in model lipid membranes (51,52,25). Our experiments showed that carotenoid molecules may have similar functions. These findings are consistent with the hypothesis that carotenoids and sterols play a similar role in the membranes of Mycoplasmas. However, it is presently unclear how these two types of lipid perform a similar function with entirely different chemical

structures. There are at least four major types of carotenoid in A. laidlawii membranes (60). Whether one or several of these pigments is responsible for the control of membrane fluidity remains to be investigated.

The nature of interaction between carotenoids and membrane lipids is unknown. If the forces are mainly hydrophobic, one expects that the interaction would depend on the characteristics of hydrocarbon chains of adjacent lipids, such as the degree of unsaturation, branching, and steric configuration. Experiments elucidating such aspects may be carried out by enriching membranes with suitable fatty acyl groups other than the arachidoyl one.

CHAPTER IV

REGULATION OF MEMBRANE LIPID FLUIDITY IN ACHOLEPLASMA LAIDLAWII

Introduction

The lipid fatty acyl composition of Acholeplasma laidlawii can be drastically changed (61). Normally its membrane does not contain sterols; however, if offered in the growth medium sterols can be incorporated into the membrane up to 3-4% (62). The membrane carotenoid content can also be altered to a large extent by appropriately feeding the organism (47,48). All these biochemical alterations influence the membrane lipid fluidity (39,56,59) which plays a crucial role in many membrane functions, such as permeability (59), membrane-bound enzyme activity (35), transport of certain nutrients (63), and osmotic stability (56). Therefore, it is reasonable to raise the question whether and how the membrane fluidity of Acholeplasma cells is regulated in response to certain stresses. For this purpose the cells were grown at a reduced temperature or the plasma membrane was enriched with an extreme amount of carotenoid pigments.

Methods

Organism and Growth

Acholeplasma laidlawii (oral strain) was originally from Dr. S. Rottem (Hebrew University, Jerusalem, Israel). The growth medium contained lipid-extracted tryptose broth supplemented with arachidic acid (5mg/l). The concentration of sodium acetate, however, was raised (20 g/l) to obtain highly pigmented cells, and sodium propionate (20 g/l) was substituted for acetate when pigment-depleted cells were needed. The high concentration of acetate or propionate did not change the pH of the strongly buffered medium (pH 8.4). For 37°C-grown cultures, the inoculum (1%,v/v) was a 24-hour old culture which had already been adapted to an arachidic acid and sodium acetate (5 g/l) supplemented medium. The inoculum for 28°C cultures was prepared identically except that it had been adapted to 28°C. Cells were grown statically in the dark and harvested by centrifugation at the late log phase.

Preparation of Plasma Membranes

The procedures of Razin *et al.* (17) were followed.

Analysis of Fatty Acyl Composition and Determination of Membrane Carotenoid Content

Lipids were extracted from isolated plasma membranes according to Folch *et al.* (28). The optical absorption at 450 nm of the "Folch lower phase" was measured with chloroform: methanol (2 :1,v/v) as reference. The membrane carotenoid content was expressed as OD_{450}/mg membrane protein. The methyl esters of lipid fatty acyl groups were

prepared, extracted and analyzed gas-chromatographically as described before. Membrane proteins were assayed by the method of Lowry et al. (31).

Spin-Labeling of Membranes

The general methods to introduce spin-labeled fatty acids into isolated membranes have been described in Chapter III. Two fatty acid spin labels were employed, namely, 2-(10-carboxydecyl)-2-hexyl-4,4-dimethyl-3-oxazolidinyloxyl, (12 NS), and 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl, (5 NS), which were obtained from Synvar, Palo Alto, California. These fatty acid spin labels intercalate into the hydrophobic region of the membrane (55). The hyperfine splitting $2T_{\parallel}$ from the EPR spectrum was chosen to determine the mobility of the spin label in the membrane (Fig. 10) (55). A large splitting indicates a high degree of orientation of the spin label and therefore a rigid micro-environment around the label. At temperatures above 25°C, accurate measurements of $2T_{\parallel}$ became rather difficult when spectra of 12NS-labeled membrane were recorded. In these cases, the relative rotational correlation time τ_c of the spin label was calculated according to formula (1). A large value of τ_c results from a slow rotational motion of the spin label, and therefore indicates a viscous micro-environment.

Results

Carotenoid Content of Membranes

When cells were grown in a high-acetate medium (20 g/l), large quantities of yellow pigments accumulated in the membrane. On

the other hand, cells looked pale if they were obtained from a high-propionate medium (20 g/l). The carotenoid content in these two types of membrane differed by about 57-fold (Table 5). Recently, we have observed a 10-fold difference in membrane carotenoid content when the growth medium contained a lower concentration of sodium acetate or propionate (5 g/l). If the concentration of acetate was raised to 20 g/l, the membrane pigment content became appreciably higher (OD_{450}/mg membrane protein increased from 0.66 to 2.3). However, a similar four-fold increase in propionate concentration only yielded a slightly lower carotenoid content (OD_{450}/mg membrane protein decreased from 0.06 to 0.04). Therefore, propionate at a concentration of 5 g/l was already sufficient to block virtually all the carotenoid pigment synthesis. On the other hand, cells were highly capable of synthesizing and accumulating carotenoid pigments when large quantities of acetate were available in the medium.

Fatty Acyl Composition of Total Membrane Lipids in Carotenoid-Rich and Carotenoid-Poor Cells

Although the same amount of arachidic acid was present in the medium, the lipids of carotenoid-poor cells became enriched with the arachidoyl group up to 56%. The carotenoid-rich cells, however, only had 35 % (Table 5). The average chain length of the saturated fatty acyl groups was 18.7 and 17.1 carbon atoms for carotenoid-poor and -rich cells, respectively. Moreover, lipids of acetate-grown cells also contained more unsaturated fatty acyl groups than those of cells cultured in propionate. Small amounts (less than 1%) of saturated

Table 5. Total Lipid Fatty Acyl Composition and Carotenoid Content of A. laidlawii Membranes.[a]

Medium ingredient (20g/l)	Fatty acyl composition (mole %)														Saturated/ unsaturated acyl groups (mole/mole)	Carotenoid content (OD_{450}/mg membrane protein)
	$C_{10:0}$	$C_{11:0}$	$C_{12:0}$	$C_{13:0}$	$C_{14:0}$	$C_{14:1}$	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{20:0}$	$C_{20:1}$	$C_{20:2}$		
Sodium propionate	1.3	0.4	2.7	0.2	1.8	0.6	10.4	1.2	5.7	9.9	3.8	55.5	2.2	4.3	3.6	0.04
Sodium acetate	2.5	--	10.9	-	5.8	-	14.0	5.3	5.3	10.7	1.9	34.5	3.9	5.2	2.7	2.29

[a]Cells were grown in medium supplemented with arachidic acid (5mg/l).

fatty acyl groups with odd-numbered carbon atoms were found in the lipids of propionate-grown cells. In conclusion, cells seemed to modify their fatty acyl composition towards shorter chains and higher unsaturation in response to a large accumulation of carotenoid pigments.

Membrane Lipid Fluidity of Carotenoid-Rich and Carotenoid-Poor Cells

The membrane lipid fluidity was measured with the spin-labeling technique. Typical EPR spectra of membranes labeled with 12NS are shown in Fig. 10. Table 6 lists the values of the two motion parameters measured at various temperatures. Both parameters of 12NS in carotenoid-poor membrane were smaller at all temperatures tested, e.g., τ_c at 37°C was about 10% lower. Thus the carotenoid-poor membranes were slightly more fluid than the carotenoid-rich ones. This result was unexpected in view of our previous report where a ten-fold alteration in carotenoid content led to 20-60% difference in τ_c at 37°C. An interpretation is provided by the findings in the present studies that the pronounced increase in carotenoid content was accompanied by a concomitant modification of the fatty acyl composition. An increase in carotenoid content made membrane lipid regions more rigid. On the other hand, shorter acyl chains and more unsaturated acyl groups led to a more fluid membrane. As a net result, the membrane lipid fluidity was maintained within a narrow range.

Figure 10. Electron paramagnetic resonance spectra of the spin label 12NS incorporated into isolated membranes from A. laidlawii. Cells were grown in a medium containing either sodium acetate or sodium propionate (20 g/l). Spectra were recorded at 20°C. Arrows indicate signals from unincorporated spin labels. Magnetic field strength increases from left to right.

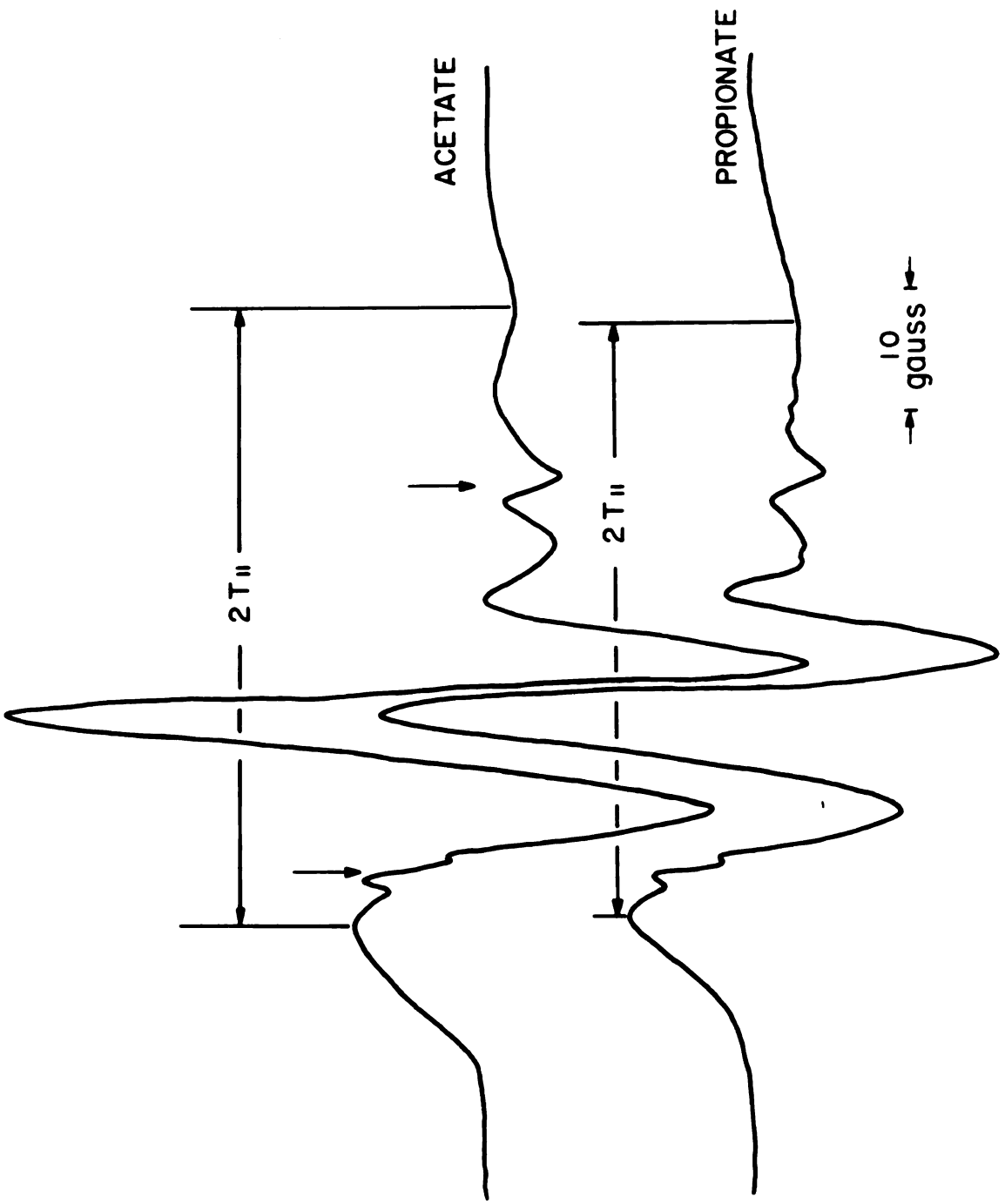


Figure 10

Table 6. Temperature Dependence of Motion Parameters of the Spin Label 12NS Incorporated into A. laidlawii Membranes.[a]

Medium ingredient (20g/1)	$2T_{\parallel}$ (gauss)[b]						τ_c (nsec)[b]
	0°C	5°C	10°C	15°C	20°C	25°C	37°C
Sodium propionate	57.6	57.2	56.2	51.7	49.9	47.2	2.8
Sodium acetate	58.6	57.6	56.2	53.4	51.4	48.3	3.1

[a]Cells were grown in medium supplemented with arachidic acid (5mg/1).

[b]The hyperfine splitting $2T_{\parallel}$ was measured to within ±0.4 gauss; and the rotational correlation time τ_c to within ±0.2 nsec.

Fatty Acyl Composition of Total Membrane Lipids and the Carotenoid Content in Cells Grown at Different Temperatures

To test the effect of lowering the growth temperature on the membrane lipid fluidity, A. laidlawii cells were cultured at 37°C and 28°C. The medium contained sodium acetate (5 g/l) and arachidic acid (5 mg/l). The fatty acyl composition of cells grown at these two temperatures is shown in Table 7. At 28°C, the cell reduced the arachidoyl group content appreciably and enhanced the number of shorter chains. Therefore, the average length of saturated fatty acyl chains was reduced from 17.9 to 16.8 carbon atoms. At the same time, more unsaturated fatty acyl groups appeared in cells grown at 28°C. The ratio of saturated/unsaturated fatty acyl groups was higher for cells grown at 37°C (Table 7). Although the fatty acyl composition was modified appreciably, the carotenoid content of the membrane remained unchanged (Table 7).

Membrane Lipid Fluidity of Cells Grown at Different Temperatures

The lipid fluidity of the membrane was determined with the spin-labeling technique. In order to detect possible membrane phase changes, the spin label 5 NS was chosen because its hyperfine splitting $2T_{\parallel}$ can be measured over the entire temperature range studied (0-60°C). The value of $2T_{\parallel}$ was temperature dependent. A plot of $2T_{\parallel}$ vs. temperature for both types of membrane (Fig. 11) shows the following features: (a) $2T_{\parallel}$ decreased linearly with increasing temperature. However, the slopes of the straight lines abruptly changed at certain temperatures, perhaps indicating phase transitions

Table 7. Fatty Acyl Composition of Total Lipids and Membrane Carotenoid Content of A. laidlawii Cells Grown at Two Different Temperatures.[a]

Growth temperature (°C)	Fatty acyl composition (mole %)										Saturated/ unsaturated fatty acyl groups (mole/mole)	Carotenoid content (OD_{450}/mg membrane protein)
	$C_{12:0}$	$C_{12:1}$	$C_{14:0}$	$C_{14:1}$	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{20:0}$		
37	9.6	0.8	12.1	--	7.6	4.8	1.7	4.2	2.2	57.0	7.3	0.46
28	9.0	1.1	19.4	2.4	16.8	8.0	1.9	5.1	3.2	33.2	4.1	0.43

[a]Cells were grown in media supplemented with arachidic acid (5mg/l).

Figure 11. Temperature dependence of the hyperfine splitting $2T_{\parallel}$ of the spin label 5NS incorporated into A. laidlawii membranes. Cells were grown either at 37°C (▲), or at 28°C (●). Arrows indicate the growth temperatures.

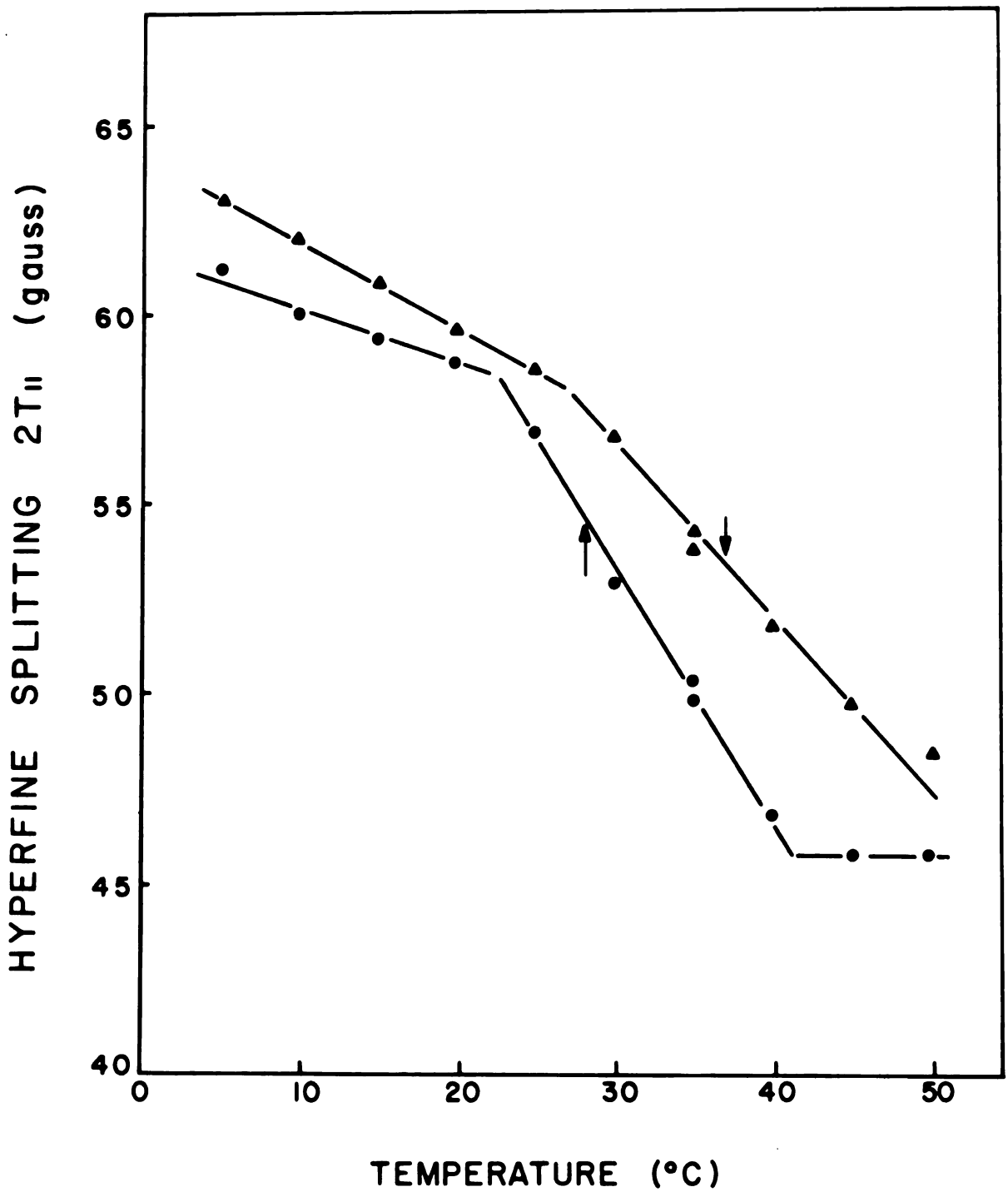

HYPERFINE SPLITTING 2T∥ (gauss)
65
60
55
50
45
40
0
10
20
30
40
50
TEMPERATURE (°C)

Figure 11

of membrane lipids. Transitions occurred at 23° and 41°C for membranes from cells grown at 28°C, and at 27°C for cells grown at 37°C. (b) Generally, 28°C-grown cells were characterized by smaller $2T_{\parallel}$'s, compared with 37°C-grown ones. This indicated more fluid lipid regions in membranes from cells grown at 28°C. (c) Most importantly, for 28°C-grown cells the hyperfine splitting measured at 28°C differed only slightly from that measured at 37°C for 37°C-grown cells (marked with across in Fig. 11). Therefore, A. laidlawii cells were able to maintain their membrane lipid fluidities within a narrow range when the growth temperature was altered.

Discussion

In the previous chapter, we observed that the membrane lipid fluidity decreases without modification of the fatty acyl composition, when the membranes of A. laidlawii cells contained a low level of carotenoid pigments. The cells can apparently tolerate such a decrease in fluidity. When large quantities of carotenoids appear in the membrane, however, the cell has to modify its fatty acyl composition to compensate for the otherwise drastic decrease in fluidity. This is achieved by a decrease of the average acyl chain length and by a simultaneous increase in the amount of unsaturated acyl groups. The result of the spin-labeling experiment supports this inference from the biochemical observations.

When the growth temperature of A. laidlawii is altered, the cell also modifies certain membrane constituents in order to maintain a proper lipid fluidity at the new growth temperature. This is

achieved by a modification of the fatty acyl composition instead of a change in the carotenoid content of the membrane. Compared with cells grown at 37°C, the fatty acyl composition of cells cultured at 28°C is characterized by shorter chains and more unsaturated acyl groups. Upon lowering the growth temperature, Acholeplasma cells incorporate more exogeneous oleic acid into the membrane lipids and thus increase the lipid fluidity (56). Acholeplasma cells are also known to adjust themselves such that the growth temperature always lies within the range of their lipid phase transitions (64). Other calorimetric studies suggested a liquid-crystalline state of Acholeplasma lipids at the growth temperature (36).

In other micro-organisms, larger amounts of unsaturated fatty acyl group in the membrane lipids are observed when the growth temperature is lowered (65-68). For Escherichia coli, a regulatory mechanism has been proposed which controls the composition of saturated vs. unsaturated fatty acyl groups in order to maintain the physical properties of phospholipids within narrow limits (66). A similar regulatory mechanism must also exist in A. laidlawii, since the membrane lipid fluidity is maintained within a narrow range when the cells are subject to environmental stresses. The regulation system responding to the change in growth temperature may not necessarily differ from that sensing the level of carotenoid pigments in the membrane. This regulatory system may be triggered by the change in the membrane lipid fluidity produced by these two parameters. A membrane-bound enzyme involved in lipid biosynthesis may play a crucial role in such a control system. Reduction of fluidity may

enhance the substrate affinity towards shorter or unsaturated acyl groups. The acyl-coA: glycerol-3-phosphate transacylase was thought to be the enzyme responsible for the temperature control of phospholipid biosynthesis in E. coli (69).

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