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LIPID MOBILITY IN *ACHOLEPLASMA* MEMBRANES USING DEUTERON MAGNETIC RESONANCE*

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Acholeplasma laidlawii B have been grown on media supplemented with perdeuterated lauric and palmitic acids. The lauric acid was shown to be elongated to myristic acid-d_{23} and palmitic acid-d_{23}, and these acids were incorporated into the membrane lipids.

Infrared measurements indicated the membrane proteins were predominantly α-helical.

Deuteron magnetic resonance spectroscopy of the deuterated lipids in intact membranes indicated that the lipids were in a state of mobility rather similar to that of the gel state of dimyristoyl lecithin. This is consistent with our interpretation of results obtained by differential scanning calorimetry and X-ray diffraction. The existence of rigid lipid regions in organisms lacking cholesterol may not be uncommon, and may occur in *E. coli* also.

I. Introduction

Nuclear magnetic resonance (NMR) spectroscopy is being increasingly used to study the nature of the rates of molecular motion in lipid and biomembrane systems[1,2]). We have recently extended the previous uses of NMR in membrane systems, which had been confined to proton nuclei, to carbon-13 nuclei in natural abundance[3]) (carbon magnetic resonance, CMR), and to selectively deuterium enriched molecules[4]) (deuteron magnetic resonance, DMR).

A feature of using either CMR or DMR is that H_2O can be used instead of D_2O (some proteins are known to change their conformation in D_2O[5])). The very low (0.0156%) natural abundance of ^2H means that DMR spectra in enriched systems will not have large overlapping water "solvent" peaks. Furthermore, by isotopic substitution particular parts of a membrane can

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be studied, the remaining proton nuclei being "transparent" because of their very different resonant frequencies. We have shown previously that by use of selectively deuterated molecules in a complex model membrane system (the lecithin-cholesterol system[4]) interesting information can be obtained about molecular mobility. Here, we extend the application to a biological membrane, the plasma membrane of *Acholeplasma laidlawii* B, into which we have biosynthetically incorporated large amounts of deuterated fatty acids. We compare the types of information obtained about hydrocarbon chain mobility in this system with that obtained using the techniques of X-ray diffraction and differential scanning calorimetry (DSC), and comment on the membrane lipid "fluidity" necessary for organism growth to occur.

II. Materials and methods

Acholeplasma laidlawii B (PG 9), was grown in tryptose broth at 30 °C or 37 °C, according to Razin et al.[6]). Tryptose (Difco Laboratories Inc., Detroit, Mich.) was fatty acid depleted according to Hendrikson and Panos[7]). Perdeuterated palmitic acid was the kind gift of Professor E. Stenhagen, and contained 98.2% ^{2}H. Further perdeuterated palmitic acid (purity 98% ^{2}H) and perdeuterated lauric acid (purity 97% ^{2}H) were synthesised according to Stenhagen and Dinh-Nguyên[8]). Purities were measured by mass spectrometry using a DCALC[9]) program kindly provided by Professor J. A. McCloskey. Fatty acids were added as sterile solutions in 70% ethanol (final ethanol concentration 0.2%) to a total concentration of 50 mg. litre^{-1}. Bovine serum albumin was "fatty acid poor" grade (Miles-Seravac, Kankakee, Ill.). Organisms were harvested in late log-stationary phase by Sharples centrifugation, washed twice in β-buffer (0.15 M NaCl, 0.05 M Tris, 0.01 M 2-mercaptoethanol, pH 7.4) and were then burst by osmotic lysis. Membranes were washed twice in dilute β-buffer (1:20 dilution) by centrifugation and resuspension, and were finally suspended in 0.15 M NaCl.

Potassium perdeuterolaurate was prepared by titration of a warm ethanolic solution of perdeuterated lauric acid with ethanolic potassium hydroxide, to a phenolphthalein endpoint. Crystals formed on cooling to −20 °C. The product was recrystallised once from 95% ethanol. The liquid crystalline sample was prepared by mixing 700 mg of the anhydrous salt with 300 mg H_2O, sealing in an NMR tube, then heating until isotropic. The sample was then cooled and the process repeated several times. 1,2-di(perdeutero)-myristoyl-L-lecithin was synthesised as described previously[4]).

Samples for infrared spectroscopy were prepared by spreading membranes over an AgCl plate. Surplus water was then removed under vacuum over

P_4O_{10} for 20 min. Samples were run on a Perkin-Elmer 257 double-beam grating infrared spectrophotometer. $CHCl_3$ and $CHCl_3$–MeOH (2:1) extractions were performed, in situ, by washing lipids off the plate and collecting the extract.

Total lipids were hydrolysed with 1:1 MeOH–2N aqueous KOH at room temperature for 10 hr. The mixture was acidified with concentrated hydrochloric acid, and the fatty acid ether extracted. The ether extracts were washed with water, dried over Na_2SO_4, and then methylated with diazomethane.

Gas-chromatography mass-spectrometry (GCMS) was carried out using a Pye gas chromatograph, on $6' \times \frac{1}{4}''$ stainless steel columns, coated with OV-17 stationary phase, on runs programmed 80–200 °C, He flow rate 40 ml min^{-1}. A Biemann molecular separator on a MS-12 mass spectrometer was used, and spectra were recorded at 70 eV.

DMR spectra were obtained in 10 mm Bruker NMR tubes, at 8 MHz, on a modified Varian wide-line spectrometer. Signals were accumulated in a Nuclear Measurements type 546C signal averager.

III. Results

A. Infrared spectroscopy of membranes

The infrared spectra of the perdeuterolaurate supplemented membranes (fig. 1) indicate that a large (≥ 50 mole % lipid) quantity of perdeuterated acid has been incorporated into the membrane structure. The carbonyl absorption appears at 1738 ± 2 cm^{-1}, both in 'intact' membranes (fig. 1a) and in the $CHCl_3$ (fig. 1b) and $CHCl_3$–MeOH (2:1) (fig. 1d) lipid extracts. This indicates that the fatty acid has been esterified, and that the ester is probably aliphatic. We show later that chain elongation has also occurred. The bands at 2193 cm^{-1} and 2092 cm^{-1} correspond to the CD_2 ν_{as} and ν_s modes. The 2193 cm^{-1} band also has a contribution from $\nu_{as}CD_3$ and the 2092 cm^{-1} band from ν_sCD_3. The principal C–H absorptions occur in the membrane at 2922 cm^{-1} ($\nu_{as}CH_2$) and 2850 cm^{-1} (ν_sCH_2). The appearance of absorption maxima at 1650 cm^{-1} and 1539 cm^{-1} indicates that a considerable percentage of the membrane protein is in an α-helical conformation (typically 1650 cm^{-1}) together with some random coil (1535 cm^{-1} is characteristic of random coil conformation and 1546 cm^{-1} of α-helix[10]), consistent with the results on protonated *Acholeplasma* of Steim[11]). No evidence of β-structure is apparent in the intact membrane. However, after $CHCl_3$–MeOH 2:1 extraction, a pronounced shoulder on the 1650 cm^{-1} band appears, at 1628 cm^{-1}, characteristic of β-structure. This lack of β-structure is interesting, since in the related *Mycoplasma laidlawii* S, Choules and Bjorklund[12]) have

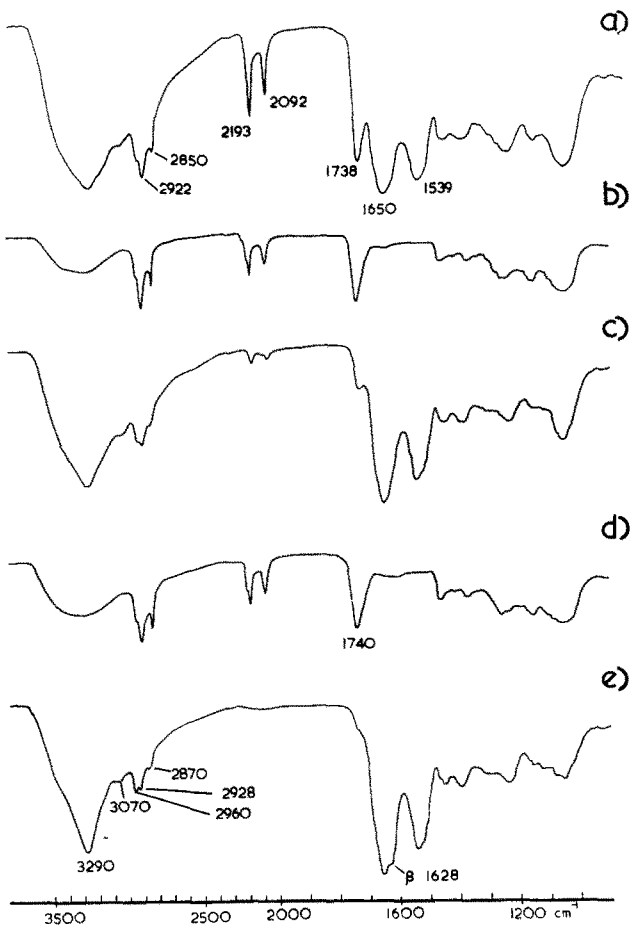

Fig. 1. (a) Infrared spectrum of perdeutero-laurate supplemented *Acholeplasma laidlawii* B membranes, dried on AgCl plate, 30°C. – (b) $CHCl_3$ extract. – (c) Residue after $CHCl_3$ extraction. – (d) $CHCl_3$–MeOH (2:1) extract. – (e) Residue after $CHCl_3$–MeOH (2:1) extraction.

found evidence (from optical rotatory dispersion, circular dichroism and infrared spectroscopy) that 56% of the membrane proteins have β-structure, 30.7% α and only 13.2% coil. We only find evidence of extensive β-structure on protein denaturation.

In fig. 1b the infrared spectrum of the $CHCl_3$ extract is shown, and in fig. 1c that of the membrane protein residue, after $CHCl_3$ extraction. It is apparent that some of the lipids are bound more strongly than others to the protein matrix. However, all lipid, as indicated by loss of the 2193 cm^{-1}, 2092 cm^{-1} and 1738 cm^{-1} bands is removed on $CHCl_3$–MeOH (2:1) ex-

traction. The $CHCl_3$–MeOH (2:1) total lipid extract (fig. 1d) is similar to a "normal" protonated extract. No C–D absorption is detetable in the protein residue (fig. 1e). The 3070 cm^{-1} and 3290 cm^{-1} bands in the protein residue (fig. 1e) arise from the peptide NH group.

B. Gas chromatography-Mass spectrometry of methyl esters

GCMS results on the fatty acid methyl esters obtained by saponification of the perdeuterolaurate supplemented membrane lipids indicated that chain-elongation had occurred. Hendrikson and Panos[7]) showed that in complex but exhaustively defatted growth media, the predominant fatty acids present in *A. laidlawii* B membranes were myristic (57.28%) and palmitic (22.37%). Our results with laurate supplemented membranes likewise indicated predominance of myristic and palmitic acids, with myristic acid the largest single species. It has previously been shown[13]) that *A. laidlawii* B supplemented with *n*-C_6, C_8 or C_{10} fatty acids increase production of palmitic acid, i.e. that chain-elongation can occur.

The presence of alkyl chain ^1H–^2H "hybrid" fatty acids is apparent from the mass spectra of the methyl esters, obtained on their elution from the gas chromatogram. Deuterated isotopomeric species are known to have shorter retention times than their protonated counterparts[14]). On rapidly scanning the leading edge of the peak corresponding to the approximate retention time of methyl myristate, it is seen that the molecular ion occurs at $m/e=265$. This accompanied by a characteristic low mass number isotopic substitution pattern, that is also seen in the mass spectrum of the perdeutero lauric acid supplement (fig. 2a and b), which is here indicative of some species containing 1, 2 or more protons. It is thus clear that the ion of $m/e=265$ corresponds to $[CD_3(CD_2)_{10}CH_2CH_2CO_2Me]^+$ (methyl myristate-d_{23}). This shows that lauric acid has been elongated by a protonated C_2-unit. The molecular ion corresponding to the leading edge of the methyl palmitate peak (fig. 2c) occurs at $m/e=293$. It is accompanied by the same isotope pattern, and thus corresponds to $[CD_3(CD_2)_{10}CH_2CH_2CH_2CH_2CO_2Me]^+$ (methyl palmitate-d_{23}). The lauric acid has thus been elongated by 2 protonated C_2-units.

The base peak in the mass spectrum of methyl myristate-d_{23} occurs at $m/e=75$, and in methyl palmitate-d_{23} at $m/e=74$. This is consistent with the above proposed structures in which a McLafferty rearrangement[15]) (fig. 3) with transfer of a γ-^2H(methyl myristate-d_{23}) or γ-^1H(methyl palmitate-d_{23}) occurs. Ions of the general formula $[CH_2)_nCOOCH_3]^+$, with $n=2$, 6, 10, are apparent in both spectra, e.g. in fig. 2b $m/e=151$ corresponds to $[(CH_2)_2(CD_2)_4COOMe]^+$, and in fig. 2c $m/e=147$ corresponds to $[CH_2)_4(CD_2)_2COOMe]^+(n=6)$.

It thus appears that the deuterated lauric acid is incorporated only into

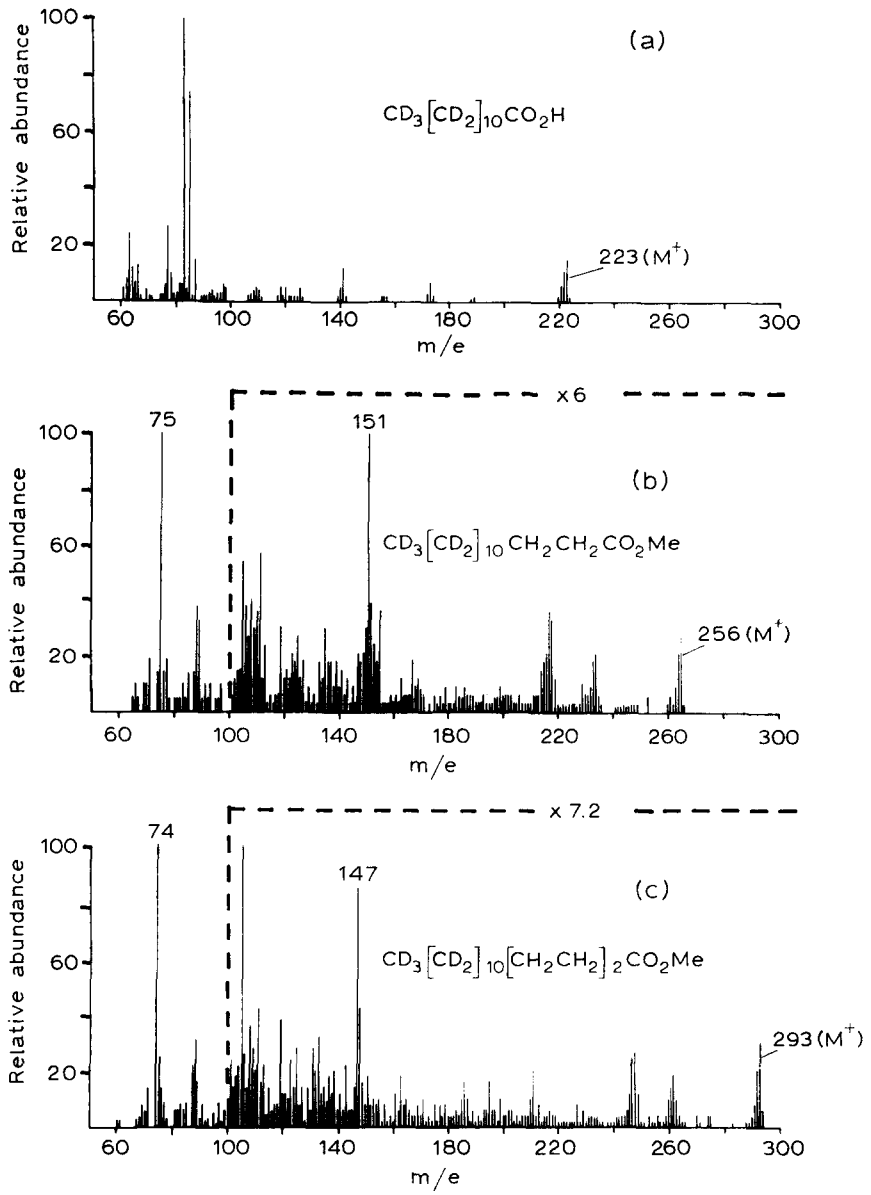

Fig. 2. (a) 70 eV mass spectrum of perdeutero-lauric acid. – (b) 70 eV mass spectrum of methyl myristate-d_{23} on elution from gas chromatogram. – (c) 70 eV mass spectrum of methyl palmitate-d_{23} on elution from gas chromatogram.

the membrane lipids, and that some elongation occurs. Engelman[16]) has shown that palmitic acid is, in fact, directly incorporated into the membranes of *Acholeplasma laidlawii* B, so the palmitate enriched lipids were not further investigated.

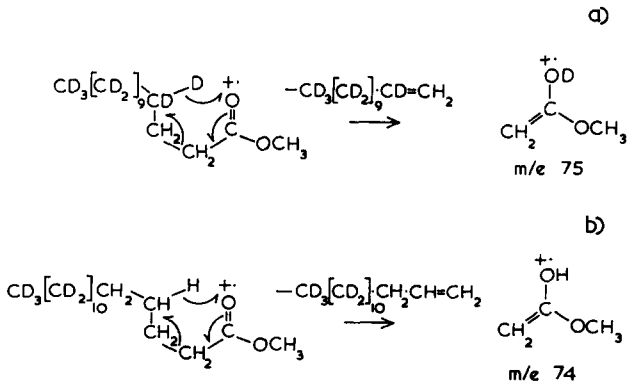

Fig. 3. (a) McLafferty rearrangement of methyl myristate-d_{23}, showing formation of base peak *m/e* 75 by γ-^{2}H transfer. – (b) McLafferty rearrangement of methyl palmitate-d_{23}, showing formation of base peak *m/e* 74 by γ-^{1}H transfer.

C. Deuteron resonance

Deuteron NMR spectra are invariably dominated by the interaction between the electric quadrupole moment of the deuteron nucleus and the electric field gradient at the nuclear site. The magnitude of the electric field gradient is described by a tensor, V_{jk}. This normally has five components since it is symmetric and has vanishing trace. This symmetric tensor can be reduced to diagonal form by choosing a new set of orthogonal axes, x', y', z'. With respect to these axes the components $V_{x'x'}$, $V_{y'y'}$, $V_{z'z'}$ do not vanish. Generally we define $|V_{z'z'}| \geqslant |V_{y'y'}| \geqslant |V_{x'x'}|$. Then, the lineshape of the DMR spectrum is described[17]) by an asymmetry parameter η, such that

$$\eta = \frac{(V_{x'x'} - V_{y'y'})}{V_{z'z'}}.$$

In single crystal specimens the DMR spectrum is a doublet with a frequency splitting $\Delta\nu$, where

$$\Delta\nu = \frac{3}{2}\frac{eQ}{h}[V_{x'x'}l^2 + V_{y'y'}m^2 + V_{z'z'}n^2].$$

eQ is the electric quadrupole moment of the deuteron nucleus and l, m and n are the direction cosines of the magnetic field direction with respect to the

principal axis system of the electric field gradient tensor. The factor 3/2 is a scaling factor appropriate to nuclei with a nuclear spin of 1[17]).

In non-crystalline specimens all magnetic field orientations occur. The overall spectral *width* is determined by the value of e^2Qq ($eq = V_{z'z'}$), which is 170 KHz for deuterons in static CD bonds[18]), and the *shape* by the value of the asymmetry factor η. Residual magnetic dipolar broadening has the effect of blurring the sharp features, typical examples for different η values are shown in figs. 4a and 4b.

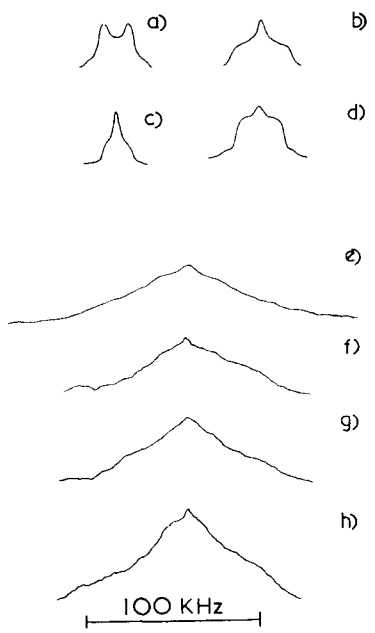

Fig. 4. Theoretical and experimental deuteron resonance spectral lineshapes. (a) Theoretical powder lineshape for $\eta = 0$. – (b) Theoretical powder lineshape for $\eta = 1.0$. – (c) DMR spectrum of potassium perdeuterolaurate (70 wt.%)-H_2O, 30°C (smectic liquid crystalline). – (d) DMR spectrum of di(perdeutero)myristoyl-Lα-lecithin (5 wt.%)-H_2O at 30°C (smectic liquid crystalline). – (e) DMR spectrum of di(perdeutero)myristoyl-Lα-lecithin (5 wt.%)-H_2O at 10°C (gel state). – (f) DMR spectrum of *Acholeplasma laidlawii* B membranes, supplemented with perdeutero-lauric acid at 30°C, spectrum recorded at 30°C. – (g) DMR spectrum of *Acholeplasma laidlawii* B membranes, supplemented with perdeutero-palmitic acid at 30°C, spectrum recorded at 30°C. – (h) DMR spectrum of *Acholeplasma laidlawii* B membranes, supplemented with perdeutero-palmitic acid at 37°C, spectrum recorded at 37°C.

Any motion that is rapid compared with the spectral width will, as is customary in NMR, produce a narrowed spectrum. In the limit of a rapid isotropic motion the quadrupolar interaction will be averaged towards zero and a single DMR line will be observed. Any motion which is not isotropic,

for example the rotation of a CD_3 group about an axis perpendicular to the D_3 plane of the group, will not average the interaction to zero. Instead a reduced interaction showing axial symmetry about the rotation axis will be observed. The size of the reduction and the symmetry of the reduced tensor can often be used to identify the type of averaging motion that is occurring.

C–D bonds are known to have very low (0–0.2) asymmetry parameters[19]). Consequently the theoretical line shape, taking into account dipolar broadening, resembles that in fig. 4a. In the smectic liquid crystalline phases of potassium perdeuterolaurate-H_2O (fig. 4c) and 1, 2-di(perdeutero)myristoyl-L-lecithin-H_2O (fig. 4d) we have line shapes similar to those expected from a high asymmetry parameter system. It is known that ^1H absorption lines of these systems are "super-Lorentzian"[20]), i.e., a wide correlation time distribution along the alkyl chain, exists. Groups (CD_3 or CD_2) that are very near the methyl end of the alkyl chain will undergo sufficiently rapid and isotropic motion, that the observed interaction will tend to zero. They are thus expected to give rise to a relatively narrow central line in the DMR spectrum, which may or may not have a small residual splitting. Groups which are "immobile", i.e. groups near the polar/apolar interface, will be undergoing slow anisotropic motion[21]), hence the observed interaction is non-zero, and a quadrupole splitting, with an overall lineshape characteristic of a $\eta = 0$–0.2, will be obtained. When these two (extreme) lineshapes are superimposed, the "high asymmetry parameter" lineshape is obtained.

In the gel state, the lecithin spectrum (fig. 4e) is very broad. The DMR spectra of laurate supplemented membranes (in which extensive chain elongation has occurred), grown and examined at 30 °C is similarly broad (fig. 4f), as are the spectra obtained from perdeuteropalmitate supplemented membranes grown at 30 °C and 37 °C (fig. 4g and 4h).

IV. Discussion

In our model systems deuterated lecithin-H_2O and deuterated potassium laurate-H_2O, well-defined quadrupole splittings are apparent in the liquid crystalline phases. In the lecithin the splitting characteristic of the most immobile CD_2 group, is 27 ± 1 KHz at 30 °C[4]). In potassium laurate the maximum splitting is 20 ± 1 KHz. It is clear that the membrane spectra are considerably broader than these two model liquid crystalline samples, and are rather similar in structure to the gel phase spectrum of the lecithin. The half-height linewidths of the membrane spectra (which are composed of a wide range of overlapping doublets) are approximately 65 KHz, the same as the lecithin gel spectrum.

It has been shown previously that in the gel phase of lecithin, that the

hydrocarbon chains are relatively rigid, and have proton linewidths of 4 gauss[22]). Nevertheless, there is considerably more motion in the gel than in the (anhydrous) crystalline material, where proton linewidths at liquid nitrogen temperatures are ~16 gauss[23]).

We have shown that with the laurate supplemented membranes, we are dealing principally with a (terminally) deuterated myristate/palmitate mixture This mixture accounts for the relatively high thermal phase transition temperatures observed with these membranes, similar to those of unsupplemented or palmitate supplemented membranes, and the consequent similarly of the 30 °C laurate and palmitate supplemented membrane spectra. With the palmitate supplemented membranes grown and examined at 37 °C, it is apparent that the lipids are more mobile than with the membranes grown and examined at 30 °C, since there is a rather more narrow central component apparent in the spectrum (fig. 4h), resembling that obtained with the liquid crystalline phase of dimyristoyl lecithin at 30 °C. We would suggest that in the 37 °C palmitate supplemented membranes that more lipids are in a liquid crystalline state than with the 30 °C palmitate supplemented membranes.

It thus appears that large amounts of the membrane lipids, at their growth temperature, are in a state of mobility similar to that observed in the gel state of dimyristoyl lecithin.

At present it is not possible to ascertain the extent of lipid-protein interaction. However, using X-ray techniques, Engelman[16]) has shown that *both* 4.15 Å and 4.6 Å high angle Bragg spacings, corresponding to gel and liquid crystalline regions, are present in palmitate supplemented membranes, at their growth temperature of 37 °C. It was also shown that the high temperature end of the phase transition, which can be measured with considerable accuracy, since it is evidenced by the disappearance of a *sharp* 4.15 Å line, occurred at 44 °C, i.e. *well above the growth temperature*. It was also suggested that extensive hydrophobic lipid-protein interaction was not a major structural feature.

Steim et al.[24]) have shown that in stearate supplemented *Acholeplasma laidlawii* B, that the thermotropic gel-liquid crystalline phase transition occurs in the temperature range ~37–73 °C. This is further evidence for a *predominantly gel state of the membrane lipids* occurring at the growth temperature.

It has been shown that membranes from organisms grown on unsupplemented tryptose are also situated on a broad thermal phase transition[24–26]), and our own DSC measurements on the 30 °C grown deuterated palmitate and laurate supplemented membrane lipids indicate that they are likewise situated on a broad thermal phase transition. (For a discussion of the significance of broad thermal phase transitions in heterogeneous systems see Phillips et al.[27]). Since our DMR results have detected large quantities of *rigid* hydro-

carbon chains, *at the growth temperature*, it seems that perhaps limited amounts of the membrane lipids need to be in a "fluid" state for organism growth. This general rigidity could be an explanation of, for example, the extremely low rate of turnover of the polar lipids in *Acholeplasma laidlawii* B membranes[28]).

Biophysical significance

The fact that other micro-organisms, e.g. *Escherichia coli*[29]), have been shown to grow, in normal laboratory media, with their lipids situated *on* a broad phase transition and also to exhibit both 4.2 Å and 4.6 Å high angle diffractions from their membranes at their growth temperature[30]), suggests that the presence of gel and liquid crystalline regions may not be a particularly rare occurrence in membranes. It may be significant that these organisms specifically lack cholesterol, and may thus regulate permeability by having both gel and liquid crystalline regions in their membranes.

In this context, it would appear that too much fluidity in membrane lipids is in some instances physiologically harmful. We have demonstrated that lauric acid is in fact elongated to myristic and palmitic acids, which will result in decreased mobility of the membrane lipids.

Similarly, Esfahani et al.[30]) showed that when supplemented with linolenate or oleate, *E. coli* would tend to regulate membrane lipid fluidity by production of predominantly palmitate containing phospholipids. However, when supplemented with elaidate, this was incorporated into the phospholipids (phosphatidylethanolamines) to the extent of 75% total fatty acids. Dielaidoyl phosphatidylethanolamines are expected to have similar fluidities to dipalmitoyl phosphatidylethanolamines[31]), and indeed the X-ray detected thermal phase transition was shown to extend from 30 °C to 40 °C for organisms grown at 37 °C.

That biologically relevant transport processes can in fact occur in rigid systems has recently been demonstrated by Krasne et al.[32]) where it was shown that the ion transporting antibiotic, gramicidin, was able to mediate potassium ion movement across "solid" rather than "liquid" black lipid membranes.

V. Conclusions

Acholeplasma laidlawii B elongate perdeuterated lauric acid to myristic and palmitic acids.

The conformation of the membrane proteins in *Acholeplasma laidlawii* B membranes containing extensively deuterated lipids, is predominantly α-helical.

The membrane lipids of *Acholeplasma laidlawii* B membranes which have been grown in media supplemented with perdeuterated lauric and perdeuterated palmitic acids, are relatively immobile, compared to the liquid crystalline model systems lecithin-water and potassium laurate-water, and are rather similar in mobility to the *gel state* of lecithins.

Whilst it has been previously considered that a liquid crystalline state of the membrane lipids is necessary for normal growth to occur, we suggest that in some cell systems, especially those lacking cholesterol, that the membranes may be heterogeneous, containing rigid gel lipid areas together with fluid liquid crystalline areas.

Acknowledgements

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