Evidence for Two Pools of Cholesterol in the *Acholeplasma laidlawii* Strain B Membrane: A Deuterium NMR and DSC Study†

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ABSTRACT: Recent investigations have indicated that there exists a well-defined range of membrane hydrocarbon order compatible with good growth of the microorganism *Acholeplasma laidlawii* B [Monck, M., Bloom, M., Lafleur, M., Lewis, R. N. A. H., McElhaney, R. N., & Cullis, P. R. (1992) *Biochemistry 31*, 10037–10043]. Since cholesterol increases hydrocarbon order in membranes, it was of interest to examine the effect of cholesterol on the hydrocarbon order and growth characteristics of *A. laidlawii* B. Cholesterol is normally absent from *A. laidlawii* membranes since it is neither biosynthesized nor required for the growth or survival of the microorganism. However, cholesterol will be incorporated into the membrane if exogenously supplied to the *A. laidlawii* culture. For membranes prepared from cells grown in the presence of cholesterol, chemical determinations indicated cholesterol represented as much as 40 mol % of the total membrane lipid. However, ^{2}H NMR order parameter measurements and DSC studies of the same membrane preparation suggested that cholesterol was present at significantly lower levels (≈10–15 mol %) in the membrane lipid bilayer. Further incorporation of cholesterol into the *A. laidlawii* lipid bilayer was found to occur with an increase in temperature or by lyophilization and rehydration at high temperatures, suggesting that sterol present in a separate pool in the membrane preparation could then gain access to the bilayer. ^{2}H NMR spectra of *A. laidlawii* membrane preparations containing deuterium-labeled cholesterol indicate that the bulk of the cholesterol present in this separate pool is in a solid form.

Cholesterol and related sterols are major components of at least the plasma membranes of eukaryotic cells. In contrast prokaryotes rarely synthesize or exhibit a growth requirement for sterols (Nes & McKean, 1977; Rohmer et al., 1979), a finding of considerable evolutionary significance (Bloch, 1976, 1983; Nes & Nes, 1980; Bloom et al., 1991). Many members of the mycoplasmas, a diverse but evolutionarily related group of cell wall-less eubacteria, require exogenous cholesterol or a closely related sterol for cell growth (Razin, 1982; Rottem, 1979). It has been found that even mycoplasmas which do not require sterol will incorporate variable but significant amounts of exogenous cholesterol into their plasma membranes (McElhaney, 1984, 1989). Since these simple organisms offer many natural advantages in studies of membrane structure and function (Rottem, 1979), both sterol-requiring and sterol-nonrequiring mycoplasma species have contributed greatly to our understanding of the role of cholesterol and related sterols in the membrane (Razin, 1982; Rottem, 1979; McElhaney, 1984, 1989).

A number of studies on the effect of cholesterol incorporation on the structure and function of the membranes of the sterol-nonrequiring mycoplasma *Acholeplasma laidlawii* B in particular have been carried out using a wide variety of techniques. For example, differential scanning calorimetry (DSC)[1] studies have shown that the incorporation of cholesterol reduces the temperature, enthalpy, and cooperativity of the lipid gel to liquid-crystalline phase transition (deKruijff et al., 1972, 1973). ^{2}H NMR and ESR spectroscopic studies have demonstrated that cholesterol incorporation substantially increases the degree of hydrocarbon order for membranes in the liquid-crystalline state (Davis et al., 1980; Rance et al., 1982; Jarrell et al., 1983; Butler et al., 1978; Koblin & Wang, 1981). In addition, the incorporation of cholesterol has been shown to reduce the nonelectrolyte permeability (McElhaney et al., 1970, 1973; de Kruijff et al., 1972, 1973), the valinomycin-mediated K^+ permeability (van der Neut-Kok et al., 1974), and the rates of glucose uptake (Read & McElhaney, 1975) in *A. laidlawii* cells and to reduce the ATPase activity in isolated membranes (de Kruijff et al., 1973). In most of the above studies, cholesterol levels of 14–28 mol % were obtained, consistent with the general finding that the sterol-nonrequiring mycoplasmas incorporate substantially less exogenous cholesterol into their membranes than the sterol-requiring mycoplasmas, which typically incorporate cholesterol to levels approaching 50 mol % (Razin, 1982; Rottem, 1979). Moreover, as indicated by the qualitative and quantitative effects of cholesterol on membrane lipid bilayer organization and on membrane function, it appears from most of the above studies that the majority of the exogenous cholesterol associated with the *A. laidlawii* B membranes is located in the lipid bilayer. However, in two studies (Davis et al., 1980; Koblin & Wang, 1981), much higher levels of cholesterol incorporation were reported, 39 and 40 mol %, respectively.

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[1] Abbreviations: DSC, differential scanning calorimetry; ESR, electron spin resonance spectroscopy; FID, free induction decay; T_1, longitudinal relaxation time; T_2, transverse relaxation time; T_R repetition time.

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These values seem high for *A. laidlawii* B, particularly since in both of these studies the membrane lipids were enriched in saturated fatty acids, which usually results in lower levels of cholesterol incorporation (typically 12–15 mol %) than with lipids enriched in primarily unsaturated fatty acids (deKruijff et al., 1972, 1973; Wieslander & Selstram, 1987; Bhakoo & McElhaney, 1988; Rilfors et al., 1987).

Using ^{2}H NMR dePakeing methods previously developed (Sternin et al., 1988; Lafleur et al., 1989), we have recently found that membrane hydrocarbon order falls within a defined range if good rates and extents of *A. laidlawii* growth are to be observed. Thus, changing the membrane composition to achieve a significant increase in order would be expected to influence the growth characteristics of the microorganism. In light of the effects of cholesterol on the plasma membranes of *A. laidlawii* B listed above, it is of interest to observe the effects of cholesterol on the ^{2}H NMR order profile derived from *A. laidlawii* membranes. We note that several different physical techniques have been used to study the membrane of the organism *Acholeplasma laidlawii* B [see McElhaney (1984, 1989)]; thus, it has been the most well studied of the mycoplasma membranes to date.

In the present study, we investigate the effect of addition of cholesterol on the lipid thermotropic phase behavior and on the orientational (hydrocarbon) order in intact *A. laidlawii* membranes and derived liposomes. The organism was grown in the presence of perdeuteriated palmitic acid and elaidic, oleic, or linoleic acid (and cholesterol). ^{2}H NMR order parameters, DSC studies, and chemical methods were used to determine the relative locations and quantities of cholesterol associated with the membrane. Additionally, we have used deuteriated cholesterol ([2,2,3,4,6-^{2}H]cholesterol) to further examine the phase state of cholesterol associated with the *A. laidlawii* membrane. Our results indicate that two pools of cholesterol exist, both tightly associated with the intact *A. laidlawii* membrane. Some of the cholesterol is in direct contact with the lipid fatty acyl chains while the bulk of the exogenously supplied sterol is present in a solid form in close association with the membrane.

MATERIALS AND METHODS

The fatty acids used in this study were obtained from Nuchek Prep Inc. (Elysian, MN). Palmitic acid was perdeuteriated using the methodology of Hsiao et al. (1974), purified by column chromatography, and recrystallized from ethanol. The [2,2,3,4,6-^{2}H]cholesterol was a generous gift from Dr. Harold Jarrell, National Research Council of Canada, Ottawa, Canada.

Isolation of A. laidlawii Membranes. The microorganism *A. laidlawii* B was cultured in the presence of the desired exogenously supplied fatty acids and cholesterol (where applicable) under conditions where endogenous fatty acid biosynthesis and elongation have been inhibited by the inclusion of avidin in the growth medium [see Silvius and McElhaney (1978)]. For all cultures in which either fatty acids only or cholesterol and fatty acids were added to the growth medium, they were presented in the form of a mixed micelle in a small amount of ethanol. The membranes were isolated by differential centrifugation after cell lysis by osmotic shock (cited above).

Determination of Glycerolipid Content. The glycerolipid content of *A. laidlawii* membranes was determined by gas chromatographic analysis of the fatty acid methyl esters formed by transesterification of dried membrane samples with acidic methanol. A known amount of an appropriate phosphatidylcholine was added to the dried membrane samples as an internal standard.

Determination of Cholesterol. The cholesterol content of *A. laidlawii* membranes was determined by the colorimetric method of Watson (1960). Briefly, a known volume of membrane sample was placed in a clean glass test tube and then heated at 110 °C until all of the water had evaporated. Next, 200 μL of each H_2O and glacial acetic acid was added followed by 5 mL of a chromogenic solution. The chromogenic solution contained a mixture of 1 volume of 0.25 M 2,5-dimethylbenzenesulfonic acid in glacial acetic acid, 3 volumes of acetic anhydride, and 1 volume of glacial acetic acid. After the samples were allowed to cool (approximately 15–20 min), 0.6 mL of concentrated H_2SO_4 was added to each of the above solutions which were then mixed thoroughly, and allowed to stand for 20 min to enable completion of color development. Afterward, the absorbance was read at 620 nm.

Differential Scanning Calorimetry. DSC thermograms were recorded with a Microcal MC2 high-sensitivity scanning calorimeter operating at heating rates near 11.5 °C/h.

^{2}H NMR Measurements. ^{2}H NMR measurements were performed at 46.175 MHz on a home-built spectrometer (Sternin, 1985). The quadrupolar echo pulse sequence (Davis, 1983) was used with a 90° pulse length of 4 μs, a 300-ms recycle delay time (T_R), a 50-μs interpulse spacing, and a 30.5-μs ring-down delay. The spectrum shown in Figure 6 was measured using a 20-s T_R. All FID's were acquired with an 8-step phase cycle sequence. Spectral widths for all spectra shown were either 200 or 500 kHz.

T_1 Measurements. A saturation recovery method was used to measure the T_1 relaxation time of the solid cholesterol. The quadrupolar echo pulse sequence (Davis, 1983) was used with a variable T_R, a 50-μs interpulse spacing, and a 30.5-μs ring-down delay. All FID's were acquired with an 8-step phase cycle sequence. The spectral width was 500 kHz. The echo height was fit to an exponential function of the form $S(T_R) = S_\infty(1 - e^{-T_R/T_1})$ where $S(T_R)$ is the signal intensity at time T_R, T_1 is the longitudinal relaxation time, and S_∞ is the signal intensity for $T_R > 5T_1$.

Derivation of Order Profiles. Orientational order profiles were derived as previously described (Monck et al., 1992). Briefly, since the lipid system employed gives rise to an axially symmetric spectrum, it is possible to apply a method of spectral simplification involving dePakeing and integration of the dePaked spectrum (Sternin et al., 1988; Lafleur et al., 1989) to derive the general shape of the order profile. The dePaked spectrum represents the continuous probability distribution of order for the deuteriated acyl chain. Assuming that CD_2 groups contribute equal intensity to the dePaked spectrum and that there is a monotonic decrease of order from the interface toward the middle of the bilayer, an average value of the quadrupolar splitting, $\Delta\nu_q$, was assigned to each methylene group denoted by its acyl chain position, $n = 2, 3, \ldots, 16$. The order parameters, $|S(n)|$, were then calculated using eq 1 where e^2qQ/h = 167 kHz is the quadrupolar

$$\Delta\nu_q = \frac{3}{4}\frac{e^2qQ}{h}|S(n)| \qquad (1)$$

coupling constant (Davis, 1983). Average order values, $\langle|S|\rangle$, mentioned in the text, were determined by calculating an arithmetic mean for $|S(n)|$, for $2 \le n \le 16$. Corrections were made for $|S(16)|$ by a linear extrapolation of $|S(14)|$ and $|S(15)|$ (Lafluer et al., 1989).

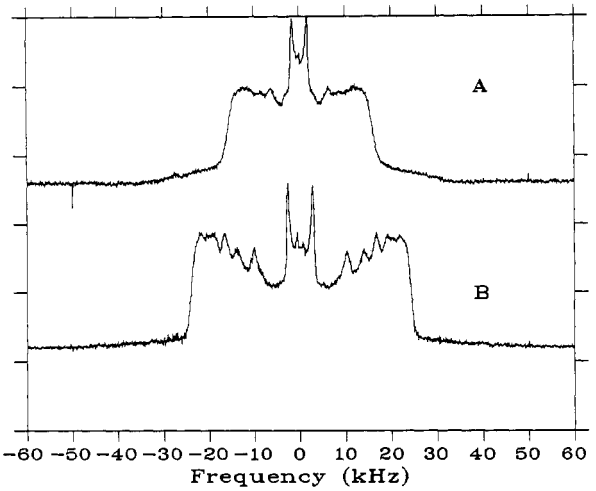

FIGURE 1: Deuterium NMR spectra of (A) intact *A. laidlawii* B membranes and (B) derived liposomes containing equimolar mixtures of 16:0_{d31} and 18:1tΔ9. Both spectra were measured at 37 °C and were the result of 60 000 scans.

Table I: Cholesterol Content (Mole Percent Total Lipid) in *A. laidlawii* Membrane Preparations of Given Fatty Acid Composition

16:0_{d31}/18:2cΔ9,Δ12	16:0_{d31}/18:1cΔ9	16:0_{d31}/18:1tΔ9
29	37	41

RESULTS

In a recent paper (Monck et al., 1992), we have demonstrated that there is a fairly narrow range of hydrocarbon order that is compatible with good growth of the microorganism *A. laidlawii* B. Cholesterol has a (well-known) large ordering effect in the hydrocarbon region of model and biological membranes (Huang et al., 1991; Rance et al., 1982; Davis et al., 1980; Gally et al., 1979; Brown & Seelig, 1978; Stockton & Smith, 1976). Thus, we were interested in examining the effect of cholesterol on the membrane hydrocarbon order and growth characteristics of *A. laidlawii* B. For this experiment, equimolar mixtures of cholesterol, perdeuteriated palmitic acid (16:0_{d31}), and elaidic acid (18:1tΔ9) were presented to the growth medium as a mixed micelle in a small volume (≥500 μL/L of culture).[2] Progressive growth of the microorganism was observed at a rate (and to an extent) similar to that previously observed (approximately 24 h at 37 °C). The ^{2}H NMR spectra obtained for the intact membranes and extracted lipids are presented in Figure 1. Hydrocarbon order profiles and average order parameters, $\langle|S|\rangle$, were derived from ^{2}H NMR spectra using the dePakeing and integration methods described under Materials and Methods. The average order parameter obtained from the intact membrane preparation, $\langle|S|\rangle$ = 0.20, is slightly greater than that found for a similar (16:0_{d31}/18:1tΔ9) intact membrane preparation lacking cholesterol, and corresponds to a relatively small (≈13%) increase in order over $\langle|S|\rangle$ = 0.176, the average order parameter for the sample lacking cholesterol. This is surprisingly low given the high levels of cholesterol assayed in the membranes used for this study (41 mol %; see Table I). We note that the $\langle|S|\rangle$ value obtained is close to the upper limit of the range 0.176 > $\langle|S|\rangle$ > 0.140 compatible with good growth characteristics for *A. laidlawii* B found recently (Monck et al., 1992).

[2] In all *A. laidlawii* preparations used, except where specified otherwise, the fatty acids and cholesterol were presented to the growth medium as a mixed micelle in ethanol to achieve solubility of the lipids in the growth medium.

The derived liposome dispersion gave rise to the spectrum shown in Figure 1B from which an $\langle|S|\rangle$ = 0.3 was derived. This contrasts strongly with the value of $\langle|S|\rangle$ = 0.2 obtained for the intact membranes. The concentration of cholesterol in this membrane preparation constituted 41 mol % of the total membrane lipid content as determined by chemical methods (see Materials and Methods). Because of this large difference between $\langle|S|\rangle$ values in the intact membrane and the extracted lipid preparations, it appeared that either less of the endogenous cholesterol was interacting with the fatty acyl chains of the intact membrane than with those of the lipid dispersions or the influence of cholesterol was reduced in the intact membranes, possibly due to the presence of proteins.

In order to characterize the generality of this effect, we investigated the amount of cholesterol associated with intact *A. laidlawii* membranes with different fatty acyl chain compositions. Cholesterol and equimolar mixtures of perdeuteriated palmitic (16:0_{d31}) and either elaidic (18:1tΔ9), oleic (18:1cΔ9), or linoleic (18:2cΔ9,Δ12) acids were exogenously supplied to the growth medium. ^{2}H NMR spectra were obtained for each of the cholesterol-containing intact membrane and derived liposome samples. Hydrocarbon order profiles were derived using the dePakeing and integration methods (as above) and were compared with corresponding samples lacking cholesterol. The results are presented in Figure 2. Approximately the same fractional increase in order was observed for each of the above systems.

As shown above, a large increase in acyl chain orientational order is obtained upon lipid extraction and redispersion of the lipid mixture at temperatures above the gel/liquid-crystalline main phase transition. It was found that heating the intact membrane also gave this result. Normally, an increase in temperature results in increased motional freedom of the acyl chains in a bilayer which, in a ^{2}H NMR experiment, is measured as a reduction in the quadrupolar splitting or a decrease in the hydrocarbon order. Thus, one would expect to see a decrease in hydrocarbon order in *A. laidlawii* membranes as the temperature is increased. We examined the temperature dependence of order profiles measured for *A. laidlawii* membrane preparations containing cholesterol and equimolar mixtures of 16:0_{d31} and 18:1tΔ9. The results are shown in Figure 3 and Table II. As can be seen in the table, from 37 to 47 °C a decrease in $\langle|S|\rangle$ is observed as would be expected. However, at higher temperatures, 57–77 °C, $\langle|S|\rangle$ increases monotonically and then decreases again at 87 °C. A second measurement at 37 °C after cooling showed $\langle|S|\rangle$ to be approximately 50% higher than in the initial measurement. This is the type of result that would be obtained in model membranes if cholesterol was incorporated into the membrane bilayer upon heating.

DSC studies of cholesterol-containing and cholesterol-free *A. laidlawii* membranes were performed to further characterize the effects of heating on the amount of cholesterol interacting with the membrane hydrocarbon chains. DSC thermograms typically found for cholesterol-free and cholesterol-containing *A. laidlawii* B membranes are presented in Figure 4. With both sets of membranes, the membrane lipid chain-melting phase transition occurs as a reversible lower temperature thermal event, whereas the endothermic transitions attributable to the thermal denaturation of the membrane proteins are the broad (and irreversible) higher temperature events. With the cholesterol-free membrane samples, the area under the peak attributable to the gel/liquid-crystalline phase transition of the membrane lipids is

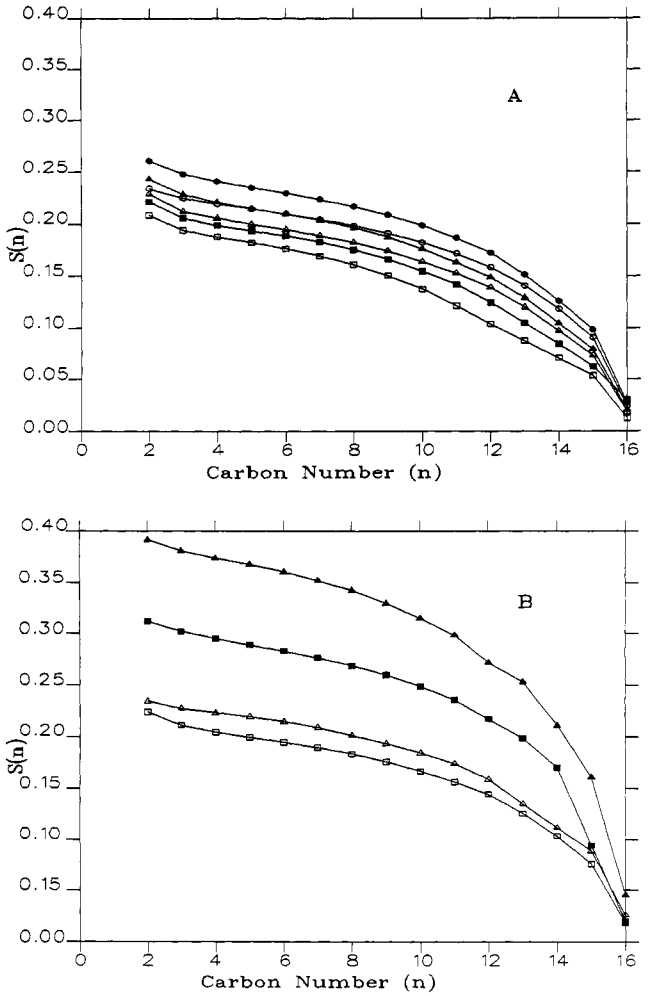

FIGURE 2: Order profiles obtained (A) from intact *A. laidlawii* B membranes containing 16:0$_{d31}$ and 18:2cΔ9,Δ12 (rectangles), 18:1cΔ9 (triangles), and 18:1tΔ9 (circles) and (B) from lipid dispersions of extracted (total) *A. laidlawii* lipids containing 16:0$_{d31}$ and 18:1cΔ9 (rectangles) and 18:1tΔ9 (triangles). The filled symbols represent order parameters from membrane preparations in which cholesterol was added to the growth medium. The open symbols represent order parameters from membrane preparations completely lacking cholesterol.

usually 10–15% lower in the first heating scan, when compared with subsequent heating scans (see Figure 4, left panel). The smaller change in enthalpy in the first scan probably reflects the amount of lipid whose phase behavior is perturbed prior to the thermal unfolding of the membrane protein. However, in the case of the cholesterol-containing membranes, the thermograms shown in Figure 4 (right panel) clearly indicate that the enthalpy of the chain-melting phase transition of the membrane lipids is some 50–55% higher in the first heating scan (A) than in subsequent heating scans (B). A decrease in the enthalpy of the lipid chain-melting phase transition is precisely what would be expected if the high-temperature incubation had resulted in an increase in the amount of cholesterol interacting with the acyl chains of the membrane lipids. It is well-known that the incorporation of cholesterol into lipid bilayer model membranes reduces both the enthalpy and the cooperativity of the gel/liquid-crystalline phase transition (McElhaney, 1982; Keough, 1984).

Previous ^{2}H NMR studies of *A. laidlawii* showed that cholesterol causes a large increase in hydrocarbon order. However, these studies involved sample lyophilization (Rance et al., 1982; Davis et al., 1980) in contrast to the intact wet membrane samples used here. An intact *A. laidlawii*

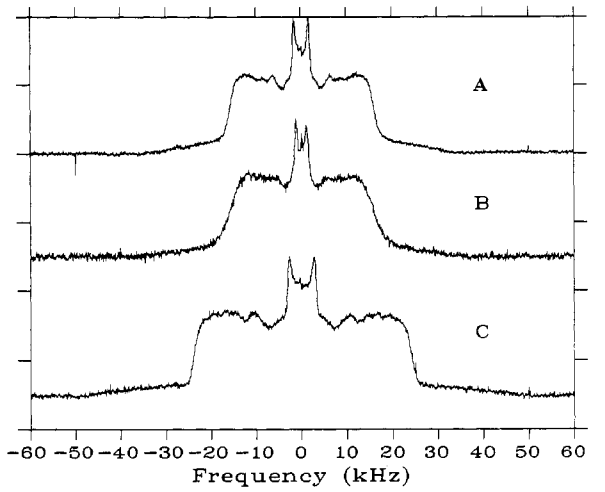

FIGURE 3: $\langle|S|\rangle$ obtained for an *A. laidlawii* intact membrane as a function of temperature. For this experiment, the microorganism was grown on 16:0$_{d31}$, 18:1tΔ9, and 20 mM cholesterol, and spectra were measured at (A) 37 °C (initial spectrum), (B) 77 °C, and (3) 37 °C (after cooling from 87 °C).

Table II: Average Order Parameter vs Temperature for Intact *A. laidlawii* B Membrane Preparation with Cholesterol[a]

temp (°C)	$\langle\|S\|\rangle$
37	0.201
47	0.154
57	0.178
67	0.185
77	0.195
87	0.177
37[b]	0.294

[a] Fatty acid composition was an equimolar mixture of 16:0$_{d31}$ and 18:1tΔ9. [b] Obtained after cooling from 87 °C for 1 h.

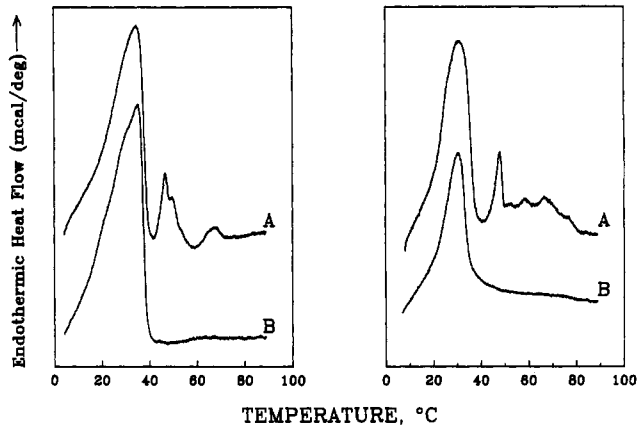

FIGURE 4: Differential scanning calorimetric tracings of an intact *A. laidlawii* membrane preparation containing 16:0$_{d31}$ and 18:1tΔ9 without cholesterol (left panel) and with cholesterol (right panel). (A) Initial heating scan (scan rate of 10 °C/h). (B) Final heating scan after cooling for several hours [same rate as in (A)].

membrane (16:0$_{d31}$/18:1tΔ9/cholesterol preparation was lyophilized and redispersed in deuterium-depleted buffer (see Materials and Methods) in order to compare the effect of variation of sample preparation on the values of $\langle|S|\rangle$ obtained from ^{2}H NMR dePakeing methods. Values of $\langle|S|\rangle \approx 0.3$ were observed for such samples compared to $\langle|S|\rangle = 0.20$ for nonlyophilized intact membrane samples prepared from the same *A. laidlawii* cell culture. These results indicate that lyophilization and redispersion of intact cells containing large amounts of cholesterol give rise to the same type of irreversible changes in acyl chain order as the heating and/or

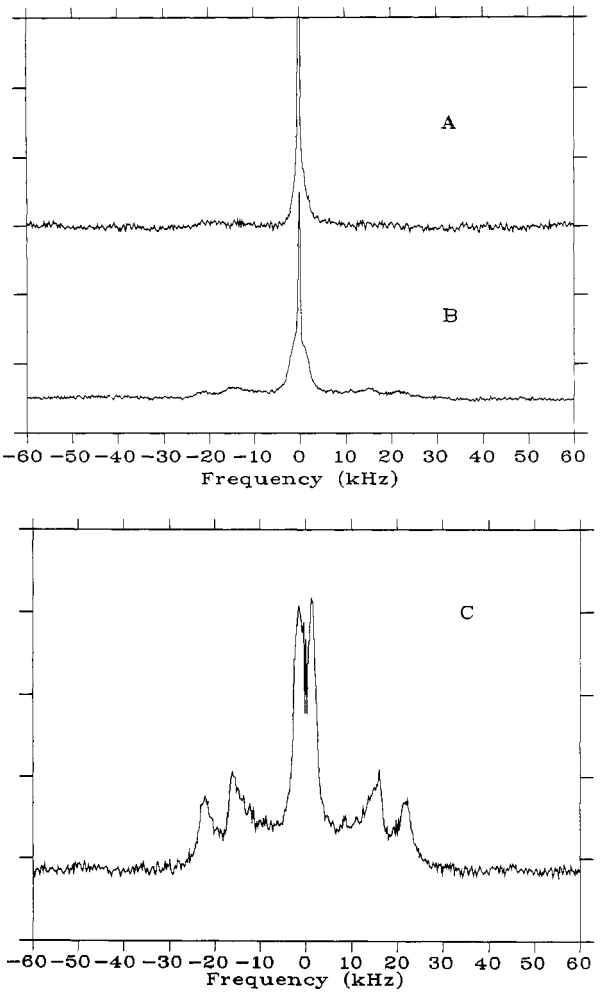

FIGURE 5: Deuterium NMR spectra of (A and B) intact *A. laidlawii* membranes and (C) derived liposomes containing 16:0/18:1tΔ9 (50:50 mol %) and [2,2,3,4,6-^{3}H]cholesterol. Spectra were taken at (A) 37 °C, (B) 37 °C (acquired after cooling for 1 h from 70 °C), and (C) 37 °C. The intensity of the central peak (due to residual HDO, membrane fragments, and small vesicles) was reduced in order to show the details of the broadline spectrum. Quadrupolar splittings in (C) were measured from the dePaked spectrum (not shown) and are as indicated in the text.

solubilization procedures described earlier.

Experiments involving ^{2}H-labeled cholesterol should provide more information on the physical environment of the cholesterol "associated" with the *A. laidlawii* membranes that exhibit these irreversible effects. Cholesterol, deuterium-labeled in the 2, 2, 3, 4, and 6 ring positions, was used in the growth media in place of deuteriated palmitate for the following experiments in which palmitate and elaidate were supplied exogenously. All other conditions concerning the growth of the organism remained the same as in the experiments described earlier.

The ^{2}H NMR spectra obtained at 37 °C for the intact *A. laidlawii* wet membranes containing deuteriated cholesterol and for their derived liposomes are given in Figure 5 A,C. Following a heating and cooling cycle over the course of 48 h, during which spectra were taken at 50 and 70 °C (not shown), the intact membrane sample was remeasured at 37 °C. This spectrum is shown in Figure 5B. The quadrupolar splittings of the individual resonances for the 2-, 2-, 3-, 4-, and 6-positions were determined from the dePaked spectrum of the derived liposomes (Figure 5C) and were 4, 33, and 44 kHz for the deuterons on the 6, 2, and 4 equatorial, the 2 axial, and the 3-positions on the deuteriated cholesterol, respectively. The splittings from the intact wet membrane spectrum (Figure 5B) are not as easily obtained by this method due to the poor signal-to-noise ratio although some of the splittings are evident and can be estimated from the spectrum. In both cases, the splittings correlate well with previously determined splittings of [2,2,3,4,4,6-^{2}H]cholesterol in membranes measured at 35 °C and are typical of cholesterol interacting with hydrocarbon chains [see Dufourc (1983), Kelusky et al. (1983), Dufourc and Smith (1986), and Bonmatin et al. (1990)].

The integrated area under a ^{2}H NMR spectrum reflects the number of contributing deuterons in the sample of interest. The ratio of the relative integrated areas of the two spectra before and after heating (Figure 5A,B), where the spectra were normalized to the number of scans, was 1:2.2, indicating that differences existed in the environments of some of the cholesterol before and after heating. This suggests that a large fraction of cholesterol does not contribute to the type of ^{2}H NMR signal arising from a fluid lipid bilayer, consistent with the calorimetric and ^{2}H NMR results above. A possible organization for the pool of cholesterol not associated with the acyl chains is a solid or crystalline form. In this regard, lipids in the solid phase can have long T_1 relaxation times which are on the order of seconds (Valic et al., 1979). If the time, T_R, between signal acquisition is short compared with $5T_1$, a loss in signal intensity will occur. A T_1 of 4.3 s was measured for solid deuteriated cholesterol in a separate experiment [see Valic et al. (1979) for comparison]. A spectrum of solid [2,2,3,4,6-^{2}H]cholesterol is shown in Figure 6A and of [2,2,3,4,6-^{1}H]cholesterol in an intact *A. laidlawii* membrane preparation in Figure 6B. A T_R = 20 s was used in both cases. Although the signal-to-noise ratio is poor, the broad spectrum (Figure 6B) with a $\Delta\nu_q$ = 127 kHz is clearly characteristic of a spectrum of solid [2,2,3,4,4,6-H]cholesterol.

The free induction decay of the spectrum in Figure 6B is shown in Figure 6C. Two major components can be identified in the figure. The magnitudes of the fast and slower decaying components are denoted by I_1 and I_2, respectively, as shown in the Figure.[3] A distortion in the spectrum, derived from I_2, has been identified and is due to finite radio-frequency pulse width effects as described previously (Bloom et al., 1980). As determined from Bloom et al. (1980), the expected magnitude for the I_2 component, given by I_{2corr}, should be $I_{2corr} = I_2/0.8$. The ratio of the heights, I_1/I_{2corr}, measured at the peak of the echo gives the relative proportions of membrane-associated cholesterol to solid cholesterol in the sample. We found a ratio of I_1/I_{2corr} = 0.30 in this case which suggests further that the bulk of the cholesterol is in the solid phase.

DISCUSSION

The motivation for this study was largely due to the recent identification of a range of hydrocarbon order that is consistent with good growth characteristics of the organism *A. laidlawii* B (Monck et al., 1992). It is well-known that cholesterol increases orientational order in the hydrocarbon region of model membranes (Brown & Seelig, 1978; Stockton & Smith, 1976; Lafleur et al., 1990). Cholesterol has also been shown to be effective in this regard in *A. laidlawii* membranes (Davis et al., 1980; Rance et al., 1982), and this has been thought to be true in general for *A. laidlawii* membranes (Bloom et al., 1991). However, if the observed range in order, 0.176 >

[3] The measurement of I_1 does not originate from zero intensity due to the presence of a slowly decaying component in the FID which contributes to the isotropic peak in the spectrum. This component defines a base line from which I_1 is measured.

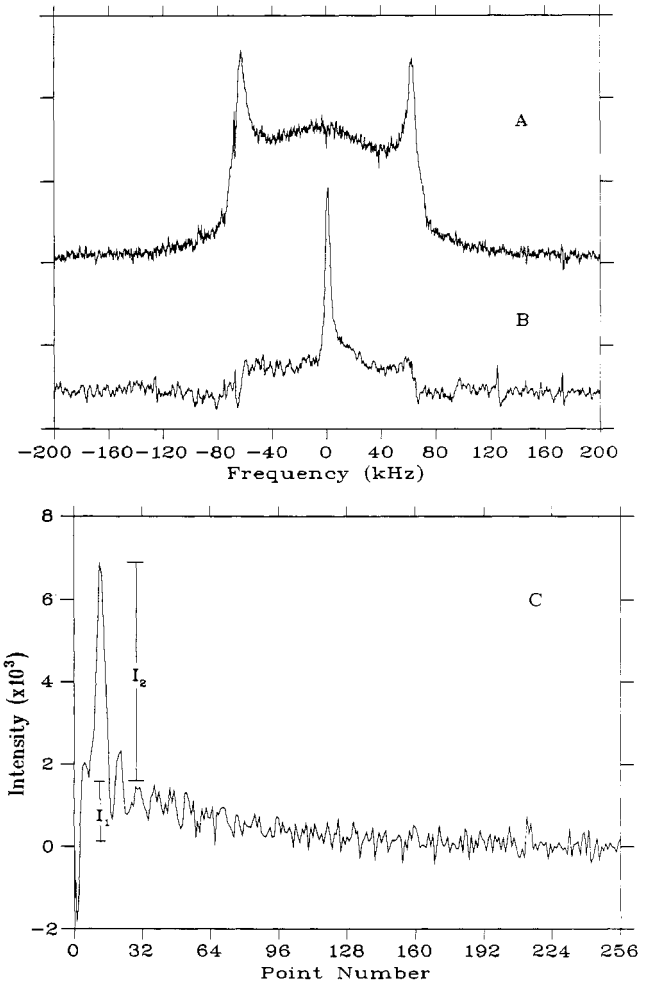

FIGURE 6: Deuterium NMR spectra of (A) solid [2,2,3,4,6-^{2}H]cholesterol, (B) an intact *A. laidlawii* preparation containing 16:0/18:1tΔ9 (50:50 mol %) and [2,2,3,4,6-^{2}H]cholesterol, and (C) an FID of (B) with solubilized cholesterol (I_1) and solid cholesterol (I_2) components identified. Both spectra A and B were measured at 37 °C with T_R = 20 s. The maximum splitting shown is 127 kHz. We note that in (B) a line-broadening function was applied to the FID prior to Fourier transformation. The applied function involved an exponential decay with T_2 = 0.6 s.

$\langle|S|\rangle > 0.140$ (Monck et al., 1992), is necessary for growth of the organism at 37 °C, the optimal growth temperature, one would expect that the incorporation of significant amounts of cholesterol, giving rise to $\langle|S|\rangle > \gg 0.176$, would result in diminished *A. laidlawii* growth rates. As outlined under Results, we found that the presence of cholesterol in the culture medium did not decrease the rate or extent of cell growth compared to cultures lacking cholesterol. In addition, the range of order found in these membranes corresponded to $\langle|S|\rangle$ = 0.20 for 16:0$_{d31}$/18:1tΔ9 down to $\langle|S|\rangle$ = 0.149 for 16:0$_{d31}$/18:2cΔ9,Δ12, an increase of no more than 13% over that of membranes lacking cholesterol in any case. In the previous study (see above), it was difficult to determine an upper boundary for hydrocarbon order due to poor growth of the organism on long-chain saturated fatty acids at 37 °C. It was presumed that the presence of more than 50% gel-state lipid, under these conditions, inhibited *A. laidlawii* growth. Given that cholesterol fluidizes a gel-state membrane bilayer, it is possible that the value of $\langle|S|\rangle$ = 0.20 better approximates the upper bound in the range of order than does $\langle|S|\rangle$ = 0.176.

A striking feature of these cholesterol-containing membrane systems is the remarkably large $\langle|S|\rangle$ values observed for the derived liposomes as compared to those observed for the intact parent membranes. Such a difference is well illustrated by the spectra in Figure 1 and the order profiles of Figure 2. From these data, it is obvious that cholesterol, in large amounts (see Table I), is not having the same effect on the membrane hydrocarbon order in *A. laidlawii* B as was shown previously (Davis et al., 1980; Rance et al., 1982). A logical explanation is that a large fraction of the cholesterol is initially excluded from the intact *A. laidlawii* membrane lipid bilayer and may become incorporated through perturbation of the membranes, for example, by extraction and resuspension of the total *A. laidlawii* lipids.

Large increases in hydrocarbon order were also observed after incubation at elevated temperatures and/or after lyophilization and resuspension of the intact membranes, provided that resuspension was performed at temperatures well above the gel/liquid-crystalline phase transition temperature of the intact membrane. In addition, the DSC studies of the lipid thermotropic phase behavior showed a decrease in the enthalpy of transition typical of that due to cholesterol in a membrane, only after the first scan to 70 °C (see Figure 4). It is possible that protein denaturation influences the increases in hydrocarbon order and decrease in transition enthalpy seen in the ^{2}H NMR and DSC measurements, respectively. We cannot rule out this possibility. However, a more reasonable explanation is that a large fraction of the cholesterol "associated" with the lipid bilayer but not interacting with the lipid acyl chains is incorporated into the membranes during heating, lyophilization, and resuspension of the membranes or during lipid extraction and that this is responsible for the large increase in hydrocarbon order or decrease in transition enthalpy. This is discussed more fully below.

The use of deuteriated cholesterol ([2,2,3,4,6-^{2}H]cholesterol) proved fruitful in determining some physical characteristics of the cholesterol associated with the intact *A. laidlawii* membrane bilayer. The initial spectrum obtained at 37 °C for *A. laidlawii* membranes grown on 16:0/18:1tΔ9 and [2,2,3,4,6-^{2}H]cholesterol contained a sharp isotropic resonance which, on the basis of further experiment, probably arose from a combination of some small membrane fragments, residual HDO, and small vesicles in the sample. The ratio of 1:2.2 observed for the integrated ^{2}H NMR signal intensities arising from deuteriated cholesterol before and after heating the *A. laidlawii* membranes to 37 °C is clearly consistent with the presence of two pools of cholesterol. It is estimated that with a T_1 = 4.3 s, a solid cholesterol signal would contribute approximately 7% of the observable ^{2}H NMR signal obtained under the experimental conditions used here (T_R = 300 ms). The solid cholesterol (see Figure 6), although absent from the membrane bilayer, must be tightly associated with the *A. laidlawii* membrane.

As discussed in the introduction, in most of the early studies of the effect of cholesterol on the structure and function of the *A. laidlawii* B membrane, it appeared that most of the exogenous cholesterol present resided in the lipid bilayer. Thus, although exogenous cholesterol was presented to cultures of this organism in the same manner as in the present study, the existence of two types of cholesterol in these *A. laidlawii* membranes is not obvious. In the ^{2}H NMR studies of Davis et al. (1980) and the ESR studies of Koblin and Wang (1981), where quite high levels of cholesterol were reported, it is possible that these high values arose, at least in part, because of the presence of two pools of cholesterol, one solubilized in the bilayer and one absent from it. Although the values of hydrocarbon order observed by Davis and co-workers for palmitate-enriched *A. laidlawii* membranes were compatible

with their reported cholesterol levels of 39 mol %, we note that the membranes utilized in those ^2H NMR studies were lyophilized and rehydrated at an elevated temperature. Thus, the phenomena described in this study could provide a rationale both for the unusually high levels of cholesterol incorporation observed in the study of Davis et al. (1980) and for their experimental results.

Although the location of the solidlike pool of cholesterol is not clarified by the results presented here, in principle the two pools appearing in our spectra could be related to some previously published data on the rates of cholesterol exchange between *A. laidlawii* cells or isolated membranes and egg phosphatidylcholine/cholesterol vesicles. Davis et al., (1984) reported that in intact cells, about half of the cholesterol associated with the cell exchanges relatively rapidly while the other half exchanges much more slowly. These workers initially discussed the possibility that the cholesterol present in the outer monolayer of the membrane bilayer could exchange rapidly while that present in the inner monolayer first had to undergo transverse diffusion (flip-flop) to the outer monolayer before exchange could occur. However, since cholesterol transbilayer movement is quite rapid in model membranes and in most biological membranes (Philips et al., 1987), this was not a completely convincing explanation for these results. Further experiments by the same workers using unsealed isolated membranes also gave two exchange rates for cholesterol, indicating that the observed differences are not due to a particular transbilayer distribution of cholesterol. A second suggestion was that the slowly exchanging cholesterol is due to some sort of preferential interaction of cholesterol with certain classes of lipids or with certain integral membrane proteins in the *A. laidlawii* membrane. If the slowly exchanging cholesterol is identified with our solidlike pool, then the interaction presumably gives rise to an immobilization of the cholesterol molecules. It is clear that additional experimental work will be required to firmly establish any of these hypotheses.

Perhaps the simplest mechanism for the production of a solid pool of cholesterol is crystallization of the cholesterol and its subsequent association with the mycoplasma membrane surfaces. Behavior analogous to this has been observed for some macrophages [see Johnson et al., (1991) for a review]. Such a crystalline state should give rise to Bragg peaks in X-ray diffraction that are characteristic of crystalline cholesterol. In an attempt to observe such peaks, we detected none. However, this result does not completely rule out the presence of crystalline cholesterol.

In summary, ^2H NMR and DSC measurements of the effect of cholesterol on *A. laidlawii* membranes have given two major results. First, two pools of cholesterol are associated with the membrane bilayer. One is dissolved in the membrane while the other is solidlike, associated with the membrane, and can be incorporated in the membrane by various solubilization procedures. Finally, the level of cholesterol residing in the bilayer results in a maximum $\langle|S|\rangle = 0.20$ which is compatible with good growth characteristics of the organism *A. laidlawii* B.

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