Deuterium nuclear magnetic resonance spectra of *Acholeplasma laidlawii* (PG9) membranes and lipid extracts enriched biosynthetically in the presence of avidin, with either [1-14]H]tetradecan-1-oic acid, [16-1H]hexadecan-1-oic acid, [4-1H]1, [6-1H]1, or [8-1H]1 tetradecan-1-oic acids, have been recorded at a variety of temperatures. The results indicate that at their growth temperature (37°C) the *A. laidlawii* membrane lipids are ~90% in a rigid gel-like state. Plasma membranes which had been lyophilized, then rehydrated, behaved in the 1H-NMR experiment as did fresh plasma membranes. The 2H-NMR quadrupole splittings (Δχq) were very similar for all of the fluid phase spectra recorded. These results indicate that protein has little effect on lipid order in the *A. laidlawii* B membrane system. The 2H-quadrupole splittings observed for the 4, 6, 8, and 14-labeled tetradecanoic acid-enriched membranes were within experimental error the same as those observed previously for bilayers of pure 1,2-myristoyl-sn-glycero-3-phosphocholine (DMPC) (Oldfield, E., Meadows, M., Rice, D., and Jacobs, R. (1978) Biochemistry 17, 2727-2740) when examined immediately above the end of the solid-to-fluid phase transition temperature range. Relatively small decreases in order in the DMPC molecule were seen using cytochrome oxidase as a model membrane protein at high protein to lipid ratio, the effects being largest near the chain terminus (C12-C14).

By contrast, 3H-NMR spectra of the [6-2H]1 or [10-2H]1-hexadecan-1-oic acid-enriched *Escherichia coli* L48-2 cell membranes showed extreme line broadening compared to spectra of their lipid extracts, and Δχq values were slightly decreased. Results with intact *E. coli* cell membranes show essentially the same NMR line shapes as those seen previously with the DMPC-gramicidin A' system (Rice, D., and Oldfield, E. (1979) Biochemistry 18, 3272-3279) including collapsed terminal methyl group quadrupole splittings and large (4 to 6 kHz) line widths of methylene segment chain resonances.

The plasma membrane of *Acholeplasma laidlawii* and the cell membranes of various *Escherichia coli* strains are some of the natural biological membranes studied most frequently using physical techniques. The cell wall less *Acholeplasma* is particularly attractive since the plasma membranes can be readily isolated as a pure preparation (1) while *E. coli* is attractive because of its ease of culture, its well understood genetics, and the ready availability of a variety of mutants.

Some of the earliest physical studies of these systems involved the use of differential scanning calorimetric methods (2-4) and for *A. laidlawii* B it was concluded (5) that 90 ± 10% of the lipids were in an extended bilayer configuration organized in a Danielli-Davson sandwich structure (6). The assumptions used in arriving at this conclusion were later questioned (7). The early differential scanning calorimetric studies were followed shortly by x-ray diffraction investigations (8, 9) which attempted to delineate the gel to liquid crystal phase transition undergone by these systems, although it proved to be difficult to monitor the low temperature end of the transition, which corresponds to a loss of a broad 4.6 Å-1 reflection. At about the same time, the first 3H- and 13C-NMR studies of membrane structure, using 2H- or 13C-labeled species, were reported (10,11). However, these early NMR studies, together with essentially all of those reported to date, were limited, allowing only incomplete comparisons of the intact biological membrane (with protein) and its lipid extract. For example, Metcalfe et al. (10) compared spectra of intact 13CO-palmitate-labeled *A. laidlawii* B (PG9) with sonicated lipid extracts, which are known to have narrower line widths than unsonicated dispersions, while Oldfield et al. (11) did not make any comparisons with *Acholeplasma* lipid extracts. Smith et al. (12) later also observed the 3H-NMR spectra of 1H-labeled *A. laidlawii*; however, only freeze-dried membranes were studied, and spectra were poorly resolved. Subsequently, with new instrumentation, Stockton et al. (13) reported improved 3H-spectra of lyophilized membranes 5°C above their growth temperature, together with a profile of chain ordering and a spectrum showing the effect of cholesterol incorporation on lipid ordering, although again a comparison with the membrane's lipid extract was not made. Most recently these authors have repeated their earlier work at additional temperatures (14) and by analogy with the work of others concluded that phospholipid, and presumably glycolipid, molecules exchange rapidly between sites in the membrane and that the average perturbation of the local orientational order of the acyl chain of phospholipid (and glycolipid) molecules by proteins must be small at 45°C. However, no measurements on lipid extracts or purified lipid fractions were reported so although these observations were consistent with more detailed results in model systems (15-17) no direct independent...
1156 results were reported on the effects of lyophilization on membranes, but no detailed interpretations of the results for intact membranes. Birdsall et al. (19) incorporated [1-13C]acetate labeling resonances from all of the membrane components. Membranes were possible since the spectra consisted of overlapping resonances from all of the membrane components. Experiments by Urbina and Waugh (20, 21) used selective enrichment of a fatty acid auxotroph (K-12 30E) with 9,10-octadecadienoic acid and a cross-polarization NMR method to investigate the solid-fluid membrane phase transition in this system, but again no membrane-lipid extract comparisons were made to assess the nature of protein-lipid interaction.

More recently, Davis et al. (22) have reported the results of incorporating perdeuterated palmitic acid into E. coli L51. They found that most of the phospholipid molecules partici-pated in the phase transition and that the 1H-NMR spectra of intact membranes were similar to those of their total lipid extracts, although profiles of molecular ordering were not obtained. In another study using E. coli Kang et al. (23) observed the 1H-NMR spectra of biosynthetically incorporated 16-d-jabeled palmitic acid and found for it that protein had the effect of disordering the hydrocarbon chain organization, although the 1H-NMR spectra of other labeled positions were not investigated.

In this publication we report results of a detailed comparison between the 2H-NMR spectra of intact A. laidlawii B (PG9) plasma membranes and their lipid extracts and of E. coli L-48 cell membranes and their lipid extracts, into which we have biosynthetically incorporated specifically chain-deuterated fatty acids. In this way we investigate the nature of protein-lipid interaction in these systems. We also investigate the effects of lyophilization on A. laidlawii membrane structure and assess the necessity of having fluid liquid-crystalline regions present in the A. laidlawii membrane in order to achieve good cell growth. Our results are compared with others recently obtained in these laboratories (15, 16), and models of protein-lipid interaction are proposed that involve characteristic of lipids in a disordered, liquid-crystalline state, as we have done previously for E. coli labeled with [16-2H]palmitic acid (23). We then obtain the results shown in Table I which give the percentage of fluid phase lipids in freshly lyophilized plasma membranes, and a lipid extract of the plasma membranes of Acholeplasma laidlawii B (PG9) grown in the presence of [14-2H]tetradecanoic acid and avidin.

### EXPERIMENTAL PROCEDURES

#### Nuclear Magnetic Resonance Spectroscopy

**Materials and Methods—Deuterium NMR spectra were obtained at 34.1 and 55.3 MHz (corresponding to magnetic field strengths of 5.2 and 8.5 Tesla) using the quadrupole-echo Fourier transform technique (24). Spectra were proton coupled. The low-field spectra were obtained as outlined in the accompanying publication (25). The high-field spectra were obtained on another "home built" spectrometer, which consisted of an 8.45 Tesla 3½-inch bore Oxford Instrument Co. high resolution superconducting solenoid (Oxford Instrument Co., Osney Mead, Oxford, U.K.), together with assorted digital and radiofrequency components.**

**We used a Nicolet NIF-808 data system (Nicolet Instrument Corporation, Madison, WI) to acquire and process most 1H-spectra, using a 100 kHz effective spectral width (25). For some spectra we used a home built 400 kHz data system, consisting of an LSI-11 microcomputer and dual floppy discs, to achieve increased spectral widths. The 90° pulse at 34.1 MHz was 6 to 7 µs and at 55.3 MHz ~7 µs.**

**Spectral Simulations—Deuterium spectral simulations were carried out on the University of Illinois Digital Computer Laboratory’s Control Data Corporation Cyber-175 computer as described (25).**

Single-component spectra were fitted to a theoretical lineshape function \(g(\delta, \Delta) = \frac{1}{2} [1/(\text{full width at half-height})]^2 \times (3.03 - 4\delta^2)\), \(\delta\) is the first-order quadrupole coupling constant, \(\Delta\) is the quadrupole splitting. Two component spectra were fitted using linear combinations of such theoretical powder patterns.

**Production of H-Labeled Membranes—A. laidlawii B (PG9) were obtained from the National Institute of Allergy and Infectious Diseases Catalog of Research Reagents. E. coli L-48 was the kind gift of Professor David F. Silbert, Washington University, St. Louis, MO. The A. laidlawii were grown basically as described previously (11) except that avidin (grade II, Sigma Chemical Company, St. Louis, MO) was incorporated into the growth medium at a level of 25 units liter\(^{-1}\) (26, 27). Specifically deuterated fatty acids from the batches whose syntheses have been described previously (15, 28) were added at a level of 50 µg ml\(^{-1}\). A. laidlawii plasma membranes were isolated using a hypotonic lysis method (11, 29). E. coli were grown and membranes isolated as described previously (22). Lipids were extracted from both A. laidlawii and E. coli membranes using a chloroform-methanol procedure (29). For 1H-NMR spectroscopy, intact membranes were exchanged with a 50 mM pH 7.4 phosphate buffer made using 2H-depleted H2O (Aldrich Chemical Company, Milwaukee, WI) to reduce the intensity of the natural abundance HOD signal. The dried chloroform-methanol lipid extracts were dispersed at ~40°C in the same buffer on a Vortex mixer.**

**Protein Isolation and Reconstitution Method—The cytochrome oxidase samples were prepared using methods described previously (15, 16). We used a cholate dilution method with lipid-depleted cytochrome oxidase, removing excess cholate by extensive dialysis (30) over Amberlite XAD-2 resin (British Drug Houses, Poole, Dorset, U.K.). Enzyme activities were typically 15 to 20 µmol of cytochrome c oxidized min\(^{-1}\) mg\(^{-1}\).**

### RESULTS AND DISCUSSION

#### The Acholeplasma Phase Transition

We show in Fig. 1 the results of a series of 1H Fourier transform NMR experiments at 34.1 MHz on 14-d, myristate-enriched A. laidlawii B (PG9) membranes (Fig. 1A), lyophilized membranes which have been reuspended in 50 mM pH 7.4 phosphate buffer (Fig. 1B), and chloroform-methanol (2:1, v/v) extracted lipids hand dispersed in excess water (Fig. 1C), as a function of temperature.

It is a straightforward matter to analyze quantitatively the results of Fig. 1 if we assume that the broad (~10 kHz) components of the spectra are characteristic of gel state lipid while the narrow (~4 kHz) quadrupole-split doublet is characteristic of lipids in a disordered, liquid-crystalline state, as we have done previously for E. coli labeled with [16-2H]palmitic acid (23). We then obtain the results shown in Table I which give the percentage of fluid phase lipids in freshly

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isolated plasma membranes, lyophilized and rehydrated plasma membranes, and a hydrated lipid extract of the plasma membranes, as a function of temperature, and a comparison with results obtained on lyophilized membranes, and their lipid extracts

| Temperature (°C) | Plasma membrane | Lyophilized membrane | Lipid extract |
|----------------|-----------------|----------------------|---------------|
| 50             | 100             | 100                  | 100           |
| 46             | 45              | 49                   | 25            |
| 42             | 20              | 20                   | 10            |
| 37             | 10              | 5                    | 5             |
| 31             | 5               | NM                   | 5             |

* The accuracy of the temperature is ±1°C over the whole sample.
* Obtained from computer simulations of the data shown in Fig. 1.
* NM, not measured.

The conclusion that lyophilization causes no change in membrane structure detectable by $^1$H-NMR is also true for membranes obtained at the high temperature end of the phase transition.

Profiles of Chain Ordering—The above results and conclusions are based on observation of only one chain label, a terminal methyl group, and we have shown previously that the effect of protein is largest at this position (16). It is, therefore, of some interest to investigate the effect of protein on lipid order as a function of the position of the $^1$H-label in the fatty acid hydrocarbon chain. Our previous results (16, 30, 31) would suggest that there should be essentially no change in $\Delta q$, but perhaps a change in line width parameter ($\delta$, HWHH).

We show in Fig. 2 spectra of A. laidlawii plasma membranes and of their lipid extracts, obtained from cells grown in the presence of avidin, and myristic acid labeled as CD$_3$ at one of positions 4, 6, or 8. Visual inspection of the results of Fig. 2 indicates clearly that within our experimental error ($\sim 2\%$) the quadrupole splitting ($\Delta q$) of each plasma membrane spectrum is the same as that of its lipid extract. These observations are confirmed by more accurate spectral simulations (data not shown) which give the following $\Delta q_0$ parameter: 4-label, $\Delta q_0 = 31 \pm 0.6$ kHz; 6-label, $\Delta q_0 = 33 \pm 0.7$ kHz; 8-label, $\Delta q_0 = 29 \pm 0.6$ kHz. These results are within experimental error the same as those seen in a 1,2-dimyristoyl-sn-glycero-3-phosphocholine bilayer at $25 \pm 2°C$ (27). There are no large line broadening effects seen in the intact plasma membrane spectra of Fig. 2, such as we have observed in model systems (16, 30), although as explained previously the relatively low protein-lipid ratio in the Achetseplasma membranes compared to the model systems investigated would make any differences difficult to detect. Similar results were obtained at the high temperature end of the phase transition (50°C, data not shown). A similar lack of any significant line broadening at 1:1 protein-lipid ratios but large broadening at 4:1 ratios is seen in the $^3$P-NMR spectra of DMPC$^-$/cytochrome oxidase complexes shown in the accompanying publication (25). Such line broadening effects, if they exist in intact biomembranes, may only be easy to demonstrate in systems containing high protein to lipid ratios.

The results at high temperature strongly suggest that fast exchange occurs between the various fluid lipid classes. Since the overall transition width in the case of myristate-labeled membranes is 15 to 20°C, we may assume that the difference in transition temperatures between the main lipid components is ± 0.1 kHz, essentially within our experimental error. This might at first be thought to be in contradiction to our previous observation that protein causes a disordering of hydrocarbon chain organization, especially toward the methyl terminus of a chain (15, 16, 31). However, it must be remembered that the Achetseplasma have very low protein-lipid ratios for biological membranes (7). At the 1:1 protein-lipid ratio present in the plasma membranes, assuming quadrupole splittings similar to those found previously for a free and protein-associated lipid (16) then only about a 0.1 kHz decrease in $\Delta q_0$ should be observed at 50 weight% protein (16).

Incorporation of [16$^-$$^3$H]palmitic acid into the A. laidlawii B (PG9) plasma membrane, in the presence of avidin, again results in a highly enriched labeled membrane preparation, with some 92 ± 2% of the fatty acid chains being palmitate. The phase transition temperature of the membrane lipids increases considerably due to the increased increase (2), and cell growth is slow with only fair yields (27). At their growth temperature of 37°C there is no evidence for liquid phase (from spectral simulations).
lipid extracts then some lipid components should be close to their central narrow features are due primarily to the spectral conditions described in Fig. 1. Sample temperature was at least this large. At 50°C, when chain melting is complete using basically the spectrometer conditions described in Fig. 1. We find that the effects of protein on $\Delta\nu_q$ are relatively small for all positions except those near the methyl terminus. At ~70 weight% protein the quadrupole splitting decreases in the order C-14 (30%), C-12 (15%), C-10 (2%), C-6 (0%), and C-2 (–5%) with an error of ~±5% for each decrease. These results support the idea that in many biological membranes having relatively low protein to lipid ratios (1:1 to 2:1 protein to lipid weight ratio), there will be only minor effects of protein on the average lipid hydrocarbon chain organization. By contrast, the sterol cholesterol has a dramatic effect, even at lower weight ratios (28), increasing $\Delta\nu_q$ by about a factor of 2 at 30 weight%.

Our results with cytochrome oxidase also show that, even in a model protein-lipid complex, the nonequivalence in $^2$H-quadrupole splittings of the 2-chain 2-position is preserved (as in the case of the interaction with cholesterol, Ref. 34) and that the inner and outer resonances of the 2-chain $\alpha$-methylene doublet signal (28) retain their 1:1 intensity ratios. Similar results have been reported for A. laidlawii (13) suggesting that the two signals arise from the nonequivalent deuterons at the 2-position and that this nonequivalence is maintained even in protein-lipid complexes.

**Escherichia coli L48-2**—We present in Fig. 3 our $^2$H-NMR spectra obtained with *E. coli* L48-2 membranes (and lipid extracts) containing biosynthetically incorporated [6-$^2$H$_2$]- and [10-$^2$H$_2$]palmitic acids. Although the lipid extract and intact cell membrane spectra have approximately the same quadrupole splittings (or order parameters) as judged from spectral simulation (results not shown), there are clearly very long-lived (>10$^{-4}$ s) lipid clusters.

Below the high temperature end of the solid-fluid phase transition both gel and liquid-crystalline regions co-exist and when using 333 kHz spectral widths, an additional broad component may be discerned in spectra of intact lipids labeled at other than the terminal methyl$^3$ (see also Ref. 14). The observation of both broad and narrow components requires that at these temperatures the average lifetime of a given lipid molecule in either fluid or solid state must be longer than ~10$^{-4}$ s. However, since the nature of the diffusion between lipid domains, the domain sizes, etc., are unknown, it is possible that diffusion is the rate-determining step for exchange between the fluid and solid phases, rather than the actual exchange of individual molecules between these states (14).

**The Cytochrome Oxidase-DMPC System**—We reported previously (15, 16) spectra of DMPC labeled at either the 14 or 6 positions, in the presence and absence of the membrane protein cytochrome $c$ oxidase (EC 1.9.3.1). We have now carried out additional experiments with DMPC's labeled on the 2-chain at one of positions 2, 10, or 12 (data not shown).

FIG. 2. Deuterium NMR spectra of plasma membranes and lipid extracts of *A. laidlawii B* (PG9) grown in the presence of one of [4-$^2$H$_4$]-, [6-$^2$H$_6$]-, or [8-$^2$H$_8$]tetradecanoic acids and avidin. Sample temperature was 46°C. Spectra were recorded at 55 MHz using basically the spectrometer conditions described in Fig. 1. The central narrow features are due primarily to residual HOH.
considerable differences in line shapes between the intact cell membrane and lipid extract spectra, the cell membrane spectra closely resembling those obtained previously (35) with the model system gramicidin A'-DMPC.

The spectra of Fig. 3 do show a very small decrease in \( \Delta \nu \) on going from lipid extract to intact cell membrane. \( \Delta \nu \) (simulated) decreasing from 27 to 25.8 kHz (Fig. 3, A and B) for the C-6 label, and from 17.5 to 16.5 kHz (Fig. 3, C and D) for the C-10 label. In principle, the decrease in order could be apparent rather than real. Such broad lines could arise from fast isotropic rigid body rotation of entire lipid molecules, in essence to obtain reliable collapsed to isotropic line shapes with fast exchange between "normal" bilayer regions and regions containing isotropic "lipidic particles" (36). Moreover, both the observation that while the terminal methyl (\( \Delta \nu \sim 3 \text{ kHz in pure bilayer} \)) and \( ^{31} \text{P} \) resonances (\( \Delta \nu \sim 48 \text{ ppm or } 3 \text{ kHz at the fields employed} \)) were collapsed to isotropic line shapes with \( W \approx 100 \text{ Hz} \), the line shapes for the other \( ^{1} \text{H} \) resonances (\( \Delta \nu \sim 25 \text{ kHz in the pure bilayer} \)) were very broad and appeared to originate in some type of isotropic methylene segment reorientation (35). Moreover, these authors have also very recently reported that \textit{E. coli} membranes and lipid extracts may also show the presence of considerable fractions of phospholipids undergoing almost isotropic motion (37), so it seems likely that such exchange could occur in the case of intact \textit{E. coli} cell membranes, causing the collapsed terminal \( \Delta \nu \) splittings (23) and broad line shapes (Fig. 3, A and C).

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