Fluorine-19 Nuclear Magnetic Resonance Studies of Lipid Fatty Acyl Chain Order and Dynamics in *Acholeplasma laidlawii* B Membranes. A Physical, Biochemical, and Biological Evaluation of Monofluoropalmitic Acids as Membrane Probes†

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ABSTRACT: Fluorine-19 nuclear magnetic resonance spectroscopy offers a number of unique advantages for studies of lipid fatty acyl chain order and dynamics in model and biological membranes. However, the geminal difluoromethylene fatty acids commonly employed as ^{19}F membrane probes appear to appreciably perturb the organization of model membranes and biomembranes. We have thus synthesized a series of specifically labeled monofluoropalmitic acids and evaluated these as suitable membrane probes. Differential scanning calorimetric studies of aqueous dispersions of several bis(monofluoropalmitoyl)phosphatidylcholines reveal that a fluorine substitution near the carbonyl group of palmitic acid has only a modest effect on the thermotropic phase behavior of these model membranes and that substitutions in the center or toward the methyl terminus are relatively nonperturbing. Moreover, all bis(monofluoropalmitoyl)phosphatidylcholines tested exhibit nearly ideal mixing in all proportions with dipalmitoylphosphatidylcholine. The thermotropic phase behavior of membranes of the simple, cell-wall-less prokaryote *Acholeplasma laidlawii* B is also not detectably altered by the presence of appreciable amounts of biosynthetically incorporated monofluoropalmitic acid. We also find that the biosynthetic incorporation of even large amounts of monofluoropalmitic acids into the membrane lipids of *A. laidlawii* B has no effect upon the growth and survival of this organism. The presence of exogenous monofluoropalmitic acids in the growth medium does not alter the polar head group composition or lipid/protein ratio of the *A. laidlawii* B membrane. In addition, all monofluoropalmitic acids tested are biosynthetically incorporated as well as palmitic acid itself and distribute relatively evenly between the various membrane glyco- and phospholipids. Since in most respects these monofluorinated palmitic acids are considerably less perturbing than the geminal difluoro fatty acids thus far studied, they appear to be the ^{19}F fatty acid probes of choice for nuclear magnetic resonance studies of membranes.

The technique of ^{19}F NMR[1] spectroscopy offers a number of advantages for studies of the order and dynamics of lipid fatty acyl chains in model and biological membranes. The ^{19}F nucleus is unique among those nuclei (^{1}H, ^{2}H, ^{13}C, and ^{19}F) commonly employed in NMR studies of membrane lipid hydrocarbon chains in possessing both a high sensitivity and a natural abundance of zero. Since the fluorine atom may be selectively incorporated into specific positions along the fatty acyl chain by established chemical procedures, the orientation and motional rates of individual segments of this chain can also be measured. In addition, the relatively similar sizes, geometries, and physical properties of fluorine and hydrogen atoms and the low chemical reactivity of the carbon–fluorine bond [see Sturtevant et al. (1979)] suggest that the substitution of a fluorine for a hydrogen atom should not greatly perturb the organization of hydrocarbon chains in lipid bilayers. Thus ^{19}F NMR spectroscopy of membrane lipids, which contain relatively small amounts of a series of specifically fluorinated fatty acid probes, appears to be a sensitive and specific technique for studying hydrocarbon chain order and dynamics in model and biological membranes.

In recent years, a number of ^{19}F NMR studies of model (Birdsall et al., 1971; Gent et al., 1976; Longmuir & Dahlquist, 1976; Longmuir et al., 1977; Post et al., 1981) and biological (Gent & Ho, 1978; Gent et al., 1978, 1981; Esfahani et al., 1981) membranes have indeed been published. In all of these investigations except two (Birdsall et al., 1971; Esfahani et al., 1981), fatty acid probes containing two fluorine atoms substituted on a single carbon atom have been utilized. There appear, however, to be several potential problems associated with the use of these difluoro fatty acid probes. Recent DSC (Longmuir et al., 1977; Sturtevant et al., 1979) and ^{2}H NMR (Oldfield et al., 1980) work has shown that the presence of the geminal difluoromethylene group can substantially alter the thermotropic phase behavior and fatty acyl group order of synthetic phosphatidylcholine model membranes. Moreover, the biosynthetic incorporation of relatively small amounts of difluoromyristic acid into the membrane lipids of several unsaturated fatty acid auxotrophs of *Escherichia coli* can result in a substantial inhibition of cell growth and membrane transport activity (Gent et al., 1981). Thus the suitability of

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[1] Abbreviations: NMR, nuclear magnetic resonance; DSC, differential scanning calorimetry; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; DFPPC, bis(monofluoropalmitoyl)phosphatidylcholine; DF_2MPC, bis(difluoromyristoyl)phosphatidylcholine; DF_2SPC, bis(difluorostearoyl)phosphatidylcholine; PA, palmitic acid; FPA, monofluoropalmitic acid; F_2PA, difluoropalmitic acid; F_2MA, difluoromyristic acid; MA, myristic acid; MGDG, monoglucosyl diglyceride; DGDG, diglucosyl diglyceride; GPDGDG, glycerylphosphoryldiglucosyl diglyceride; PG, phosphatidylglycerol; OAPG, O amino acid ester of phosphatidylglycerol; GLC, gas–liquid chromatography; TLC, thin-layer chromatography. Though the common symbols DFPPC, DF_2MPC, and DF_2SPC have been retained throughout this paper, the editorial office has modified the full names to more systematic variations, using "bis" rather than "di" where appropriate.

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difluoro fatty acids as relatively nonperturbing probes of membrane structure can be questioned.

It is unclear from previous studies whether the physical and biological perturbations associated with the incorporation of difluoro fatty acid probes are due simply to the two fluorine-for-hydrogen substitutions or whether the special properties of the geminal difluoromethylene group itself are primarily responsible for the observed effects. In order to address this question, we have synthesized a series of monofluorinated palmitic acids that, at any rate, would be expected to be less perturbing than their difluorinated analogues. In this paper, we assess the physical, biochemical, and biological suitability of several of these monofluorinated fatty acids as probes of biological membrane structure.

Materials and Methods

Monofluorinated fatty acids of the form $CH_3(CH_2)_m$-CHF-$(CH_2)_n$-$COOH$, where $m + n = 13$, were synthesized from the corresponding keto acid methyl esters following a modified procedure of Birdsall et al. (1971). The keto acid methyl ester was selectively reduced to the hydroxy acid methyl ester with $NaBH_4$. The product (yield 90%) was purified by silicic acid column chromatography and concentrated in vacuo. The hydroxy derivative was mesylated with methanesulfonyl chloride in the presence of triethylamine, and the mesylated fatty acid methyl ester was extracted with CH_2Cl_2, concentrated in vacuo, and used without further purification (yield 98%). This product was dissolved in dry acetonitrile in the presence of 5 equiv of dry tetrabutylammonium fluoride and reacted for 3 days at room temperature under anhydrous conditions. This reaction yields 30–40% of a saturated monofluorinated fatty acid methyl ester. The unsaturated byproduct was oxidized following the procedure of Von Rudloff (Christie, 1973), and the monofluorinated fatty acid methyl ester was isolated by acid–base extraction, concentrated in vacuo, and crystallized twice, once from acetone and once from hexane, at −20 °C. The purity was estimated to be greater than 99.5% by GLC and analytical TLC. The methyl ester was hydrolyzed to the free fatty acid in distilled methanol in the presence of 0.1 N KOH.

The synthesis of the keto acid methyl esters followed exactly the procedure of Hubbell & McConnell (1971) for the 5-, 8-, 10-, and 12-fluorinated species. The precursors required for the synthesis of the 14-keto acid methyl ester via this pathway were not soluble in the reaction solvents, so an alternate method was used for this analogue. The lithio anion of the ethylethynylcarbinol dissolved in liquid ammonia was coupled to 11-bromoundecanol in which the primary alcohol was protected with dihydropyran in the presence of a mild acid catalyst (Ames & Covell, 1963). The product was hydrogenated at 50 psi of hydrogen gas in the presence of platinum oxide as a catalyst, deprotected in methanol in the presence of an acid catalyst, and oxidized to 14-ketopalmitic acid with 3 equiv of $K_2Cr_2O_7$ dissolved in acetone at 0 °C.

The bis(monofluoropalmitoyl)phosphatidylcholines and DPPC were synthesized according to the procedure of Patel et al. (1979). The fatty acid was reacted with dicyclohexylcarbodiimide in dry carbon tetrachloride overnight at room temperature to produce the fatty acid anhydride. This was reacted with the cadmium chloride adduct of glycerophosphocholine in an equal volume solution of dry benzene and dimethyl sulfoxide in the presence of 4-pyrrolidinylpyridine, which acts as a catalyst. The products were purified by silicic acid column chromatography to remove free fatty acid and preparative TLC with silica gel G and the solvent system 50:50:4 methanol:chloroform:ammonium hydroxide to remove the lysophosphatidylcholine present. The isolated phosphatidylcholine was greater than 99% pure as determined by GLC and analytical TLC.

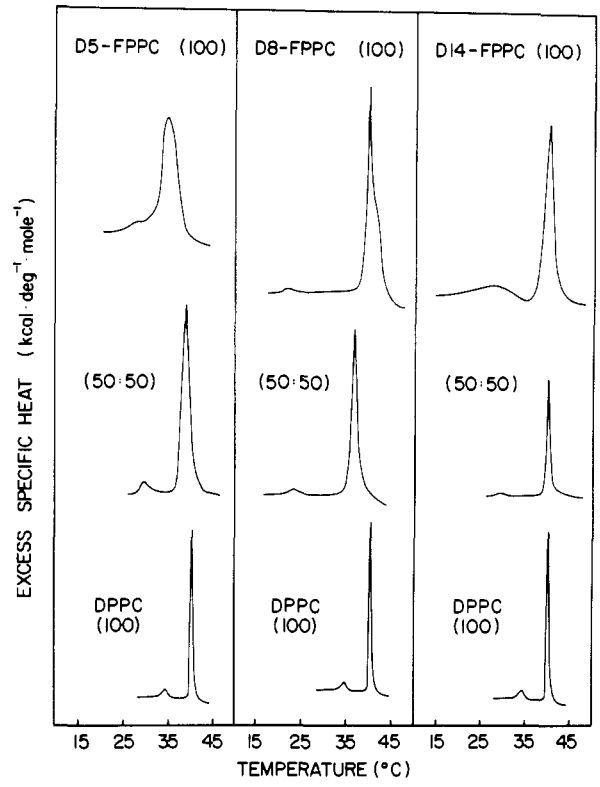

FIGURE 1: DSC traces of aqueous, multilamellar dispersions of pure D5FPPC, D8FPPC, D14FPPC, and DPPC and of equimolar mixtures of each DFPPC with DPPC. The peak areas for the different phosphatidylcholines are not drawn to scale.

The DSC studies were performed on a MicroCal MC-1 differential scanning microcalorimeter operating at a heating scan rate of 0.5 $K \cdot min^{-1}$.

Acholeplasma laidlawii B was cultured at 37 °C in a lipid-extracted growth medium, and the cells were harvested, the plasma membranes isolated, and the membrane lipids extracted, separated, and analyzed all as previously described (Silvius & McElhaney, 1978; Silvius et al., 1978).

Results

Thermotropic Phase Behavior of Pure Bis(monofluoropalmitoyl)phosphatidylcholines. Monofluoropalmitic acids containing a fluorine atom at either the 5-, 8-, or 14-position were used to synthesize the corresponding bis(monofluoropalmitoyl)phosphatidylcholines, and aqueous multilamellar dispersions of these phospholipids were analyzed by DSC. The transition curves for these three phosphatidylcholines, as well as that for DPPC, are presented in Figure 1. One can see that all three bis(monofluoropalmitoyl)phosphatidylcholines exhibit both a lower temperature, lower energy pretransition and a higher temperature, higher energy main transition, just as does the parent phospholipid, DPPC. Also, the main transition of the three bis(monofluoropalmitoyl)phosphatidylcholines is clearly broader than that of the DPPC dispersion. The main transition of D8FPPC, in fact, appears itself to consist of two components, a relatively sharp lower temperature transition and a broader, higher temperature peak. The physical basis for this complex thermotropic behavior is presently unknown. The positions and enthalpies of the pretransitions and of the main transitions are summarized in Table I.

Table I: Thermotropic Phase Transition Properties of Pure Bis(monofluoropalmitoyl)phosphatidylcholines and Dipalmitoylphosphatidylcholine As Studied by DSC

	pretransition			main transition		
phosphatidylcholine	T_{m1} (°C)	ΔH_{cal} (kcal/mol)	CU[a] (molecules)	T_{m2} (°C)	ΔH_{cal} (kcal/mol)	CU (molecules)
D5FPPC	30.0	<0.5		35.8	5.8 ± 0.3	30
D8FPPC	23.0	<0.5		40.5	6.5	60
D14FPPC	30.0	~1.0		41.0	8.8	35
DPPC	35.9	1.0	110	41.2	8.3	110

[a] The CU was calculated from the relationship CU = $\Delta H_{vH}/\Delta H_{cal}$ where $\Delta H_{vH} \approx 6.9(T_m^2/\Delta T_{1/2})$, as defined by Mabrey & Sturtevant (1978).

The main transition temperature corresponds to the gel to liquid-crystalline chain-melting temperature of these phosphatidylcholine bilayers. In each case, this transition temperature is reduced in the bis(monofluoropalmitoyl)phosphatidylcholine in comparison to that of DPPC by 5.4 °C for D5FPPC, 0.7 °C for D8FPPC, and 0.2 °C for D14FPPC, respectively. The enthalpies of the main transitions are also substantially reduced for the D5FP- and D8FPPC's, by some 2.5 and 1.8 kcal/mol, respectively, in comparison to that of DPPC, while the transition enthalpy of D14FPPC is unaffected or slightly elevated. Finally, the main transition cooperative unit sizes of the bis(monofluoropalmitoyl)phosphatidylcholines all appear to be significantly reduced in comparison to that of DPPC, with the reduction being greatest for the 5-fluoropalmitoyl and smallest for the 14-fluoropalmitoyl derivatives; however, the somewhat greater purity of the palmitoyl fatty acyl groups (>99.9%) as compared to the monofluoropalmitoyl groups (>99.5%) may have contributed to the smaller cooperative unit sizes measured for the bis(monofluoropalmitoyl) phospholipids. It thus appears that the presence of a fluorine atom near the carbonyl group of the palmitoyl chain does significantly perturb the organization of phosphatidylcholine bilayers but that this perturbation becomes increasing less serious as the position of the fluorine substitution approaches the methyl terminus of the hydrocarbon chain.

The pretransition of certain saturated phospholipids is currently thought to be associated with a transformation from a planar lamellar phase, in which the hydrocarbon chains are closely packed, fully extended, and tilted with respect to the bilayer plane, to a rippled lamellar phase in which the hydrocarbons are more loosely packed, perhaps less than fully extended, and perpendicular to the local bilayer normal [see Silvius et al. (1979)]. The pretransition is very sensitive to impurities in the phosphatidylcholine bilayer, being abolished by the addition of more than a few mole percent of free fatty acid or cholesterol, for example [for a review, see McElhaney (1982)]. The pretransition temperatures of D5FPPC and D14FPPC both exhibit reductions of about 6 °C in comparison with DPPC, while D8FPPC shows a more marked reduction of 12.9 °C. However, relative to their reduced main transition temperatures, the most marked *relative* reduction of the pretransition temperature occurs with D8FPPC and the smallest reduction with D5FPPC. Although it proved difficult to obtain acurate and reproducible enthalpy values for the pretransitions of D5FP- and D8FPPC's, those obtained were always considerably lower than those exhibited by the other two phospholipids. It appears from these results that the presence of the fluorine atom can alter the properties of the pretransition somewhat, particularly when present near the center of the hydrocarbon chain. However, the persistence of the pretransition in all three bis(monofluoropalmitoyl)-phosphatidylcholines indicate that these monofluoropalmitoyl residues mimic fairly closely the behavior of the palmitoyl chains in phospholipid bilayers.

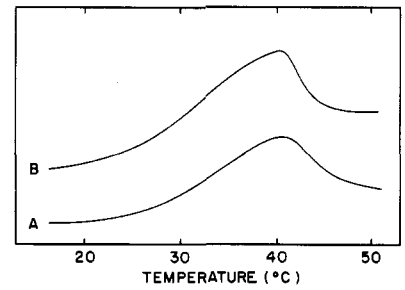

FIGURE 2: DSC traces of aqueous dispersions of *A. laidlawii* B membranes from cells enriched to about 80 mol % in PA (curve A) or to about 40 mol % each in PA and FPA (curve B).

Thermotropic Phase Behavior of Mixtures of Bis(monofluoropalmitoyl)phosphatidylcholine and Dipalmitoylphosphatidylcholine. An examination of the thermotropic behavior of binary mixtures of various bis(monofluoropalmitoyl)phosphatidylcholines with DPPC by DSC provides a good means to estimate the extent to which the fluorine substitution perturbs the lipid bilayer structure. If the perturbation is minimal, nearly ideal mixtures should be formed that exhibit complete miscibility of both components in the gel and liquid-crystalline states. Figure 1 shows the transition curves for equimolar mixtures of each of the three bis(monofluoropalmitoyl)phosphatidylcholines with DPPC. It is apparent from these curves that each of these binary mixtures exhibit nearly ideal behavior, since the temperatures, enthalpies, and degrees of cooperativity of the main transitions fall on or just slightly below the average of the values for the individual phosphatidylcholine components (data not presented). The only exception to this behavior was the D8FPPC–DPPC equimolar mixture, where the transition temperature fell below that of either of the pure components. Similar behavior was observed when the proportions of the bis(monofluoropalmitoyl) and dipalmitoyl components were varied. These results indicate that these bis(monofluoropalmitoyl)phosphatidylcholines mix nearly ideally with their nonfluorinated parent phospholipid.

Thermotropic Phase Behavior of A. laidlawii B Membrane Enriched in Monofluoropalmitic Acids. Phase transition curves of *A. laidlawii* B membranes enriched in PA (A), or with both PA and 8FPA (B), are presented in Figure 2. The membranes giving curve A were derived from cells grown in 120 μM/L of exogenous PA without avidin, and about 80% of the total esterified fatty acid was palmitate; the membranes giving curve B were from cells grown in 60 μM/L of exogenous PA plus 60 μM/L of exogenous 8FPA, and each of these exogenous acids made up about 40 mol % of the total esterified fatty acid. In both cases, DSC reveals a single, broad, asymmetric transition extending from just below 20 °C to just over 45 °C, with the peak temperature lying near 40 °C and the

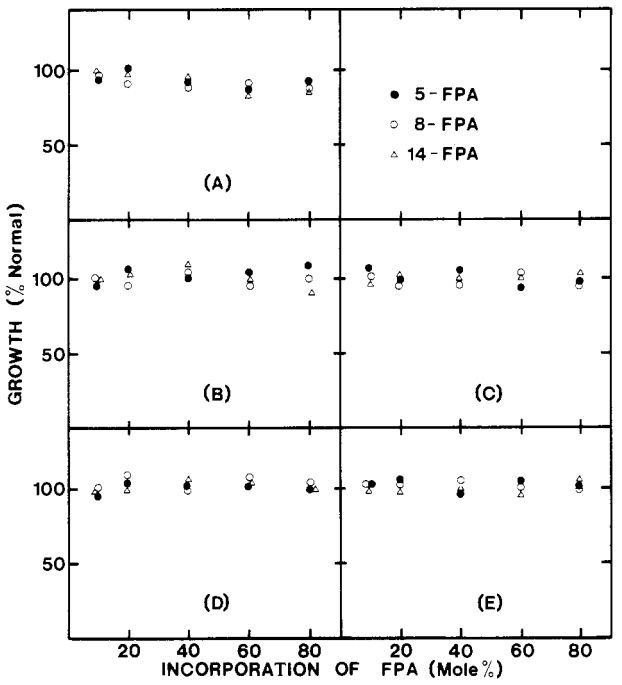

FIGURE 3: Effect of biosynthetic incorporation of increasing quantities of FPA on growth of *A. laidlawii* B. Cell growth was determined by measuring the A_{450nm}, which is a valid measure of cell mass during the logarithmic phase of growth of this organism. In panel A, the results for cells grown in various proportions of exogenous FPA and PA, without avidin, are presented. In panels B–E, the results for cells grown in various proportions of FPA and pentadecanoic acid (B), palmitelaidic acid (C), methylisopalmitic acid (D), or methylanteisopalmitic acid (E) in the presence of avidin, are presented.

actual phase transition midpoint temperature (estimated from the peak areas) lying near the growth temperature of 37 °C). Uncertainties in assigning the correct base lines made an accurate determination of the transition enthalpies impossible, but in general both membranes give roughly comparable values of approximately 3.5 kcal/g of membrane lipid. It is clear from these results that the biosynthetic incorporation of appreciable amounts of FPA into the glyco- and phospholipids of the *A. laidlawii* B membrane has a very little effect on the membrane lipid gel to liquid-crystalline phase transition.

Effect of Biosynthetic Incorporation of Various Monofluoropalmitic Acids on Growth of A. laidlawii B. When *A. laidlawii* B is grown in lipid-depleted medium supplemented with an excess of free PA, it grows normally when cultured at 37 °C (McElhaney, 1974a,b) and biosynthetically incorporates substantial quantities of exogenous PA into its membrane glyco- and phospholipids (see the following section). In order to investigate the effect of FPA incorporation on cell growth, *A. laidlawii* B was cultured on media containing various proportions of exogenous FPA and PA but with the total amount of fluorinated and nonfluorinated fatty acid being held constant. Since FPA and PA were found to be incorporated almost equally well by this organism (see below), the proportion of fluorinated to nonfluorinated fatty acid in the total membrane lipid could thus be varied widely. In Figure 3A, the effect of the biosynthetic incorporation of increasing amounts of three different FPA's on cell growth is illustrated. At all levels of incorporation up to 80 mol % of the total esterified fatty acid, none of the FPA's tested inhibited cell growth, measured as either growth rate or growth yield.

Exogenous PA alone cannot support the growth of *A. laidlawii* B in the presence of avidin, a potent inhibitor of de novo fatty acid biosynthesis and chain elongation (Silvius & McElhaney, 1978), since at 37 °C the phase transition temperature of the membrane lipids begins to exceed the growth temperature as progressively higher levels of PA are biosynthetically incorporated (Silvius et al., 1978). However, several shorter chain saturated fatty acids and a number of branched-chain, monounsaturated, and *trans*-cyclopropane fatty acids will support good growth in the presence of avidin, resulting in the production of plasma membranes whose lipids contain essentially only a single fatty acyl group (Silvius & McElhaney, 1978). In order to test whether the biosynthetic incorporation of FPA's would inhibit cell growth in avidin-supplemented cells, *A. laidlawii* B was grown in media supplemented with various levels of fluorinated or nonfluorinated PA, plus a second exogenous fatty acid capable of supporting good growth when added alone. The results obtained for several different fatty acid combinations show that the biosynthetic incorporation of up to 80 mol % of the various FPA's into the membrane lipids had no significant effect on maximum cell growth (Figure 3B–E). These experiments demonstrate that the presence of substantial quantities of monofluoropalmitoyl fatty acyl groups in the membrane lipids of this organism has little or no detectable effect on membrane function.

Table II: Effect of Biosynthetic Incorporation of Palmitic Acid and Several Monofluorinated Fatty Acids on Membrane Lipid Polar Head Group Composition of *A. laidlawii* B[a]

membrane lipid species	fatty acid supplement			
	PA (control)	5-FPA	8-FPA	14-FPA
MGDG	51.5	41.8	44.0	43.8
DGDG	11.6	10.6	9.1	14.6
GPDGDG	8.4	9.1	7.5	6.4
PG	20.8	30.2	32.7	28.7
OAPG	7.8	8.3	6.4	6.5

[a] The membrane lipid composition is expressed in percent by weight of the total membrane polar lipids. Cells were grown in the absence of avidin with either 120 μM palmitic acid (control) or a combination of 100 μM palmitic acid plus 20 μM monofluoropalmitic acid.

Similar experiments were performed with the corresponding geminal F_2PA's (data not presented). Again, no growth inhibition was noted with these fatty acids either, at least at low-to-moderate levels of incorporation. Since the F_2PA's tested were biosynthetically incorporated only about half as well as the corresponding FPA's and most other exogenous fatty acids by this organism, the effect on these fatty acids on growth at levels of incorporation of greater than 35–40 mol % could not be tested.

Effect of Biosynthetic Incorporation of Various Monofluoropalmitic Acids on Membrane Lipid Composition and Metabolism in A. laidlawii B. A desirable property of a fatty acid membrane probe is that it does not significantly alter the lipid polar head group composition or the lipid/protein ratio of the membrane into which it is incorporated. To test whether or not the incorporation of FPA probes cause such alterations in the *A. laidlawii* B membrane, cells were grown with either 120 μM PA or 100 μM PA plus 20 μM 5-, 8-, or 14FPA, in the absence of avidin, and the composition of the membrane glyco- and phospholipids was determined. The results of these experiments are presented in Table II. When cells were enriched with PA, MGDG was the major lipid component, accounting for just over half of the total polar lipid fraction, with DGDG accounting for a much smaller proportion; together, these two neutral glycolipids made up over 60% (by weight) of the membrane polar lipids. The anionic phosphatide PG was the next largest membrane component, accounting

Table III: Incorporation of Palmitic Acid and Monofluoropalmitic Acids into Individual Membrane Polar Lipid Species of *A. laidlawii* B[a]

membrane lipid species	fatty acid supplement			
	PA (control)	5-FPA	8-FPA	14-FPA
total membrane lipids	82.4 (1.00)	9.1 (1.00)	11.1 (1.00)	12.2 (1.00)
MGDG	74.6 (0.91)	8.4 (0.92)	10.5 (0.95)	12.2 (1.00)
DGDG	74.2 (0.90)	9.0 (0.99)	5.7 (0.51)	9.6 (0.79)
GPDGDG	65.9 (0.80)	6.1 (0.67)	10.8 (0.97)	9.8 (0.80)
PG	82.7 (1.00)	10.4 (1.14)	11.4 (1.03)	13.6 (1.12)
OPAG	65.0 (0.79)	8.6 (0.95)	8.4 (0.76)	4.6 (0.38)

[a] The incorporation is expressed first absolutely as the mole fraction of FPA present in the individual polar lipid species relative to that present in the total membrane lipid fraction and second (in parentheses) relatively as the ratio of the mole fraction present in the individual polar lipid species relative to the mole fraction in the total membrane lipids, the latter being arbitrarily set at 1.00. Note that the total membrane lipid fraction contains small amounts (5–10 wt %) of nonpolar lipids, as well as the five major polar lipid species indicated above. Cells were grown in the absence of avidin with either 120 μM palmitic acid (control) or with a combination of 100 μM palmitic acid and 20 μM monofluoropalmitic acid.

for about 21% of the total polar lipid, with its zwitterionic derivative OAPG being much less abundant. The anionic phosphoglycolipid GPDGDG was also a minor component. When cells were grown in the presence of one of the FPA's, there were minor shifts in the distribution of the glyco- and phospholipid head groups. The only consistent alterations produced by the biosynthetic incorporation of these FPA's were a decrease in MGDG levels and a nearly corresponding increase in the levels of PG. It thus appears that the presence of monofluoropalmitic acids perturbs the normal pathways of membrane glyco- and phospholipid biosynthesis only moderately. Moreover, due to compensatory changes in the levels of the other more minor components, only modest changes in the ratio of neutral to anionic lipids occurred. We have previously demonstrated that considerably larger alterations in glyco- and phospholipid composition, which can be produced by growing *A. laidlawii* in different exogenous fatty acids in the presence of avidin, do not adversely affect cell growth (Silvius & McElhaney, 1978; Silvius et al., 1978) or ATPase activity (Silvius & McElhaney, 1980). It thus seems unlikely that the relatively modest alterations in polar head group distribution produced by the biosynthetic incorporation of FPA will result in plasma membranes whose structure or function are appreciably different from normal.

The effect of the biosynthetic incorporation of FPA on the lipid/protein ratio of the *A. laidlawii* B membrane was also investigated, by employment of an experimental approach similar to that used to study the effect of FPA incorporation of cell growth (see previous section). We found that the incorporation of the three FPA's to levels up to 50 mol % did not alter the lipid/protein ratio of the plasma membrane, in comparison to similar experiments in which nonfluorinated PA was used (data not presented).

Other desirable features of a fatty acid probe are that it distributes among the various membrane lipid components relatively evenly and that it mimics the distribution characteristic of its natural analogue. The distributions of PA and of 5-, 8-, and 14FPA between the five major membrane polar lipids of *A. laidlawii* B are illustrated in Table III. In these experiments, *A. laidlawii* B was grown in the presence of 120 μM PA, or in the presence of 20 μM FPA plus 100 μM PA, in the absence of avidin. The relative levels of PA (85% of total exogenous fatty acid) and of FPA (15% of total) were chosen so as to give a FPA incorporation of greater than the 5–10 mol % required for a typical NMR experiment (see accompanying paper, Macdonald et al., 1983). Since the level of incorporation of fluorinated and nonfluorinated fatty acid into the total membrane lipid was typically 75–80 mol %, one would expect the FPA acids to account for 15% of the total exogenous palmitoyl groups incorporated or about 11–12 mol % of the total esterified fatty acid in the total lipid fraction. Since the actual incorporation into the *A. laidlawii* B total lipid fraction is near the expected value, this result again shows that the fluorinated and nonfluorinated fatty acids are incorporated about equally well by this organism. Moreover, the relative distribution of the FPA's between the various membrane glyco- and phospholipids parallels fairly closely the distribution of PA. Although the FPA's, like PA itself, tend to be enriched in the PG fraction, appreciable fatty acid probe is found in each of the five membrane polar lipids. Thus, these FPA probes, after their biosynthetic incorporation into the membrane lipids of *A. laidlawii* B, should report on the behavior of each of the glyco- and phospholipid constituents, although the relative contributions of the various fractions may not be precisely the same as their relative abundances in the membrane (see Table II).

Discussion

Thermotropic Phase Behavior of Synthetic Phosphatidylcholines Containing Monofluoropalmitic Acids. Sturtevant et al. (1979) have studied the thermotropic phase behavior of three DF_2MPC's where the geminal difluoro group was present at carbons 4, 8, or 12 by DSC. It is clear from a comparison of these results with ours that both qualitative and quantitative differences exist in the phase behavior of synthetic phosphatidylcholines containing mono- or difluorinated fatty acids. The transition temperatures of the DFPPC's are always *reduced* in comparison to that of DPPC, and the magnitude of the reduction ranges from about 5 °C when the fluorine atom is near the carbonyl function to only a few tenths of a degree when the fluorine substitution is near the methyl terminus of the palmitoyl chain. In contrast, the transition temperature of D4F_2MPC is *elevated* by 5 °C, although D8F_2MPC and D12F_2MPC exhibit transition temperatures that are *reduced* by 7.4 and 3.8 °C, respectively, as compared to that of DMPC. Longmuir et al. (1977) also reported that the transition temperatures of D7F_2SPC and D12F_2SPC were reduced by about 10 and 6 °C, respectively, in comparison to that of DSPC. Similarly, the transition enthalpies of D5FPPC and D8FPPC are *reduced* by about 30 and 22%, respectively, in comparison to that of DPPC, while that of D14FPPC is unchanged or slightly elevated. In contrast, the transition enthalpies of DF_2MPC's are *increased* by about 100% in comparison to that of DMPC. However, in both studies the cooperativity of the main transition was found to be substantially reduced when fluorinated fatty acyl chains were present. It thus appears that not only is the magnitude of the perturbation produced by the difluoro substitution considerably greater than that produced by a monofluoro substitution but also the nature of the perturbation is different. This implies that the interactions of the

mono- and difluoromethylene groups with each other in the lipid bilayer are fundamentally different. This difference is also manifested in the melting behavior of mixtures of phosphatidylcholines containing fluorinated and nonfluorinated fatty acyl chains. Thus, the three DFPPC's investigated in the present study exhibit nearly ideal mixing with DPPC in all proportions, whereas the DF_2MPC's studied by Sturtevant et al. (1979) exhibited markedly nonideal mixing with their nonfluorinated analogue, DMPC. We have also shown that glyco- and phospholipids containing FPA groups appear to mix almost ideally with those containing palmitoyl groups in the plasma membrane of *A. laidlawii* B. One might note that the perturbation of phosphatidylcholine bilayer structure produced by the most perturbing FPA probe is comparable to that of a fully perdeuterated palmitoyl group [see Silvius (1982)] and that the perturbation introduced by probes containing the fluorine atom near the chain terminus is negligible. The perturbation introduced by a single fluorine atom would be expected to be considerably less than that of the considerably bulkier electron spin and fluorescence fatty acid probes currently in use [see Chen & Gaffney (1978) and Chen et al. (1982)].

Effect of Biosynthetic Incorporation of Monofluoropalmitic Acids on Growth of A. laidlawii B. Gent et al. (1978, 1981) have shown that $4F_2MA$ is unable to support the growth of *E. coli* unsaturated fatty acid auxotrophs grown in the absence of exogenous oleate but that $8F_2MA$ and $13F_2MA$ can support some cell growth. However, after one generation, when the levels of incorporation of F_2MA reach 20–30%, growth becomes progressively inhibited, and the cells become morphologically aberrant. In addition, the transport of methyl β-D-thiogalactopyranoside is markedly inhibited after one generation of growth in the presence of $8F_2MA$. When the levels of $8F_2MA$ and $13F_2MA$ incorporation reach 50 and 37%, respectively, growth ceases. It is difficult to discern whether the inhibition of cell growth and membrane transport observed in these *E. coli* unsaturated fatty acid auxotrophs is due primarily to the accumulation of F_2MA groups in the membrane lipids of this organism or to the depletion of monounsaturated fatty acids.

In contrast, *A. laidlawii* B can be grown for many generations in the presence of 5-, 8-, or 14FPA, and the biosynthetic incorporation of up to 80 mol % of the FPA's tested did not reduce growth rates or yields. Moreover, these high levels of FPA incorporation did not alter cellular morphology. It would thus appear that the FPA's tested here are physiologically much less perturbing than are the F_2MA's studied by Gent et al. (1978, 1981). However, *A. laidlawii* B may be more tolerant of changes in its fatty acid composition than is *E. coli* [see McElhaney (1983)]. Also, the F_2MA's may not support the prolonged growth of *E. coli* auxotrophs because they cannot fully mimic the behavior of monounsaturated fatty acids and not because they are inherently perturbing. Support for this latter view is provided by the observation that the biosynthetic incorporation of up to 35–40 mol % of several geminal F_2PA's into the *A. laidlawii* B membrane lipids did not result in growth inhibition.

Effect of Biosynthetic Incorporation of Various Monofluoropalmitic Acids on Membrane Lipid Composition and Metabolism in A. laidlawii B. The results of the present study may be compared with those of Gent et al. (1978, 1981), who studied the effect of the incorporation 4-, 8-, and $13F_2MA$ on membrane lipid composition and metabolism in several unsaturated auxotrophs of *E. coli*. In *E. coli* auxotrophs, the extent of biosynthetic incorporation varies markedly with the position of the geminal difluoro group. Under optimal conditions, $8F_2MA$ was incorporated to a maximum level of 50% of the total membrane lipid and $13F_2MA$ to a maximum level of 37%, while $4F_2MA$ was not biosynthetically incorporated at all. Although relatively high levels of incorporation of $8F_2MA$ and $13F_2MA$ could be achieved, these fatty acid probes competed poorly for incorporation with exogenous oleic acid in unsaturated fatty acid auxotrophs, and in wild-type *E. coli* $8F_2MA$ incorporation was less than 10%. The incorporation of only 25–30% of $8F_2MA$ is sufficient to produce significant alterations in the lipid-to-protein ratio of the *E. coli* cytoplasmic membrane. The effect of F_2MA incorporation on the membrane phospholipid polar head group composition was not determined in these studies nor was the distribution of these fatty acid probes among the phospholipid classes. However, it was reported that the $8F_2MA$ incorporated was esterified almost exclusively at the 2-position of the *E. coli* glycerophospholipids, unlike MA, which is found primarily at the 1-position. Gent et al. (1978, 1981) concluded in fact that the $8F_2MA$ and $13F_2MA$ act to some extent as unsaturated, rather than as saturated, fatty acids, and this conclusion has recently been supported by ^2H NMR studies of specifically deuterated difluoromyristic acids (Oldfield et al., 1980).

The biochemical behavior of the FPA's observed in the present study is quite different. All of the FPA's tested including 5FPA are biosynthetically incorporated to high levels by *A. laidlawii* B. In fact, the maximum extent of incorporation (nearly 80 mol %) is as great or almost as great as can be obtained with exogenous PA. Moreover, the FPA's compete well for incorporation with other exogenous fatty acids, as does PA. In addition, preliminary experiments indicate that these FPA's are located primarily at the 1-position of the membrane glyco- and phospholipids of this organism, as is PA. It thus appears that the FPA probes much more closely resemble PA in their biochemical properties than do the F_2MA probes resemble MA. However, it is possible that some of the differences observed in the biochemical behavior of these mono- and difluoro fatty acids could be due to the differences in the biochemical machinery of the organisms in which they were studied. However, we found in the present study that geminal F_2PA's were biosynthetically incorporated by *A. laidlawii* only about 40–50% as well as their FPA analogues. Whatever, the lack of any effect of the biosynthetic incorporation of FPA's on the lipid polar head group composition or the lipid/protein ratio of the *A. laidlawii* membrane and the relatively even incorporation of these fatty acids into all the major membrane glyco- and phospholipids make these fatty acids excellent probes, at least for this organism.

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Registry No. D5FPPC, 86569-17-7; D8FPPC, 86569-18-8; D14FPPC, 86569-19-9; 5-FPA, 86569-20-2; 8-FPA, 86569-21-3; 14-FPA, 86569-22-4.

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Fluorine-19 Nuclear Magnetic Resonance Studies of Lipid Fatty Acyl Chain Order and Dynamics in *Acholeplasma laidlawii* B Membranes. Effects of Methyl-Branch Substitution and of Trans Unsaturation upon Membrane Acyl-Chain Orientational Order†

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ABSTRACT: The hydrocarbon-chain orientational order parameters of membranes of *Acholeplasma laidlawii* B enriched with straight-chain saturated, methyl iso-branched, methyl anteiso-branched, or trans-unsaturated fatty acids have been determined via fluorine-19 nuclear magnetic resonance spectroscopy (^{19}F NMR). A theoretical description of the ^{19}F NMR spectral line shape is presented that permits the determination of the orientational order parameters associated with specifically monofluorinated palmitic acid probes biosynthetically incorporated into membrane glycerolipids. Membrane orientational order profiles determined by ^{19}F NMR in the case of straight-chain saturated fatty acid enrichment were qualitatively similar to profiles obtained by ^{2}H NMR. The methyl iso-branch and methyl anteiso-branch structural substituents induced a local ordering while the trans double bond substituent induced a local disordering evident from alterations to the character of the orientational order profile. These various effects could be understood in terms of an altered probability of the occurrence of rotational isomerization in the presence of particular substituents. At 37 °C the overall orientational order decreased in the progression η-acyl > iso branched > anteiso branched ≥ trans double bonded. The relative overall order was then a direct function of the relative proximity of the membrane lipids to their respective gel to liquid-crystalline phase transitions. When observed at T_m + 15 °C, where the different species of fatty acids could be considered to be in a comparable thermodynamic state, the overall order decreased in the progression anteiso branch > trans double bond > iso branch > η-acyl. The overall ordering effect of these substituents, observed upon elimination of the effect of relative proximity to the T_m, could be interpreted in terms of their effects upon membrane acyl-chain packing.

The hydrocarbon milieu of biological and model membranes has been the subject of intensive investigation via nuclear magnetic resonance (NMR)[1] techniques [for reviews, see Seelig & Seelig (1980) and Jacobs & Oldfield (1981)]. The variety of nuclei that have been studied each possesses inherent advantages and disadvantages. Studies focusing upon ^{1}H or ^{13}C nuclei are complicated by the multiplicity and breadth of the resonance lines present in the NMR spectrum of lipid membranes. While these disadvantages can be overcome to

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[1] Abbreviations: NMR, nuclear magnetic resonance; Tris, tris(hydroxymethyl)aminomethane; FID, free induction decay; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

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