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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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a great extent by specific enrichment with ^2H and ^{13}C, this remains an often difficult and expensive proposition. In addition, the low sensitivity of these nuclei necessitates a high level of enrichment in order to obtain a usable NMR signal within a reasonable time period. Thus, in order to study the individual methylene segments of a variety of fatty-acyl groups, a complete series of specifically enriched probes must be synthesized and incorporated at high levels into the subject membrane for each fatty acid of interest.

Each of these disadvantages of ^1H, ^2H, or ^{13}C NMR spectroscopy can be overcome by utilizing ^{19}F NMR, particularly by the use of monofluorinated fatty acid probes. These probes, as well as being relatively nonperturbing, avoid the complication of multiple resonance lines since they themselves are the sole source of the fluorine signal. In addition, the sensitivity of the fluorine nucleus in the NMR experiment alleviates the necessity of high levels of probe incorporation. Thus the same series of specifically monofluorinated probes can be used to report upon the properties of a wide variety of different fatty-acyl structures.

We report here studies of the orientational order of a series of fatty acids incorporated into membranes of the microorganism *Acholeplasma laidlawii* B. Orientational order parameters were acquired after line-shape analysis of ^{19}F NMR spectra obtained from membranes containing small quantities of one of a series of positional isomers of monofluoropalmitic acid in addition to the particular fatty acid of interest.

It is possible to produce, in *A. laidlawii* B, membranes that are virtually homogeneous with respect to a particular fatty acid (Silvius & McElhaney, 1978a). Consequently, this organism provides the opportunity to manipulate, in a biological situation, the membrane fatty acid composition and, hence, the membrane lipid physical properties within wide limits. The total absence of any cell wall structure, as well as the presence of only a single membraneous structure, the plasma membrane, considerably simplifies the acquisition of "pure" membrane preparations (Razin, 1975). When considered together, these various points indicate that *A. laidlawii* B represents a nearly ideal organism in which to study membrane fatty acid phenomena.

The present studies have elucidated the effects, at a variety of temperatures, of the iso-branch, anteiso-branch, and trans double bond fatty acyl chain substituents upon the overall membrane order, as well as the membrane order profile, in *A. laidlawii* B. We conclude that, while changes in the overall membrane order in the presence of a particular substituent at a given temperature can be related to the proximity of the membrane lipids to their gel to liquid-crystalline phase transition, when compared at a constant temperature relative to the main transition, these substituents have an overall ordering effect relative to straight-chain saturated fatty acids. In addition, differential effects of these groups upon the membrane order profile itself have been observed. These particular effects can be interpreted, when considered within the framework of a rotational isomeric model, as having been mediated through alterations in the probability of the occurrence of trans–gauche isomerization at particular methylene segments.

Theory

The dominant interactions in the NMR experiment for a spin $^1/_2$ nucleus such as fluorine or phosphorus are the Zeeman coupling and dipolar interactions (Wennerström & Lindblöm, 1977).

The Zeeman coupling is influenced by the chemical shift tensor σ, which contains both orientation-dependent and orientation-independent terms (Niederberger & Seelig, 1976).

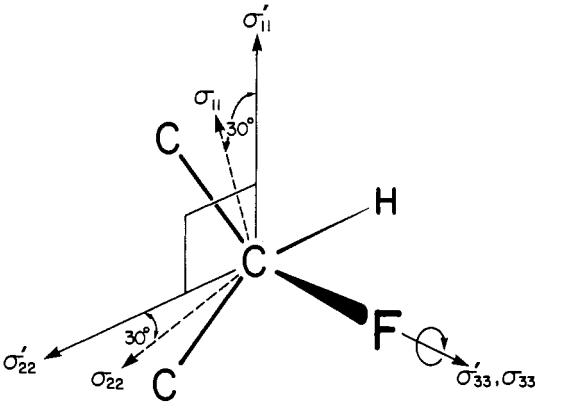

FIGURE 1: Geometry of H–C–F group and coordinate system of the fluorine chemical shift tensor.

One can write the observable chemical shift for any given orientation of the normal to the bilayer relative to the applied magnetic field as

$$\sigma = \sigma'_i + (2/3)\sigma'_a\left(\frac{3\cos^2\theta - 1}{2}\right)$$

where θ denotes the angle between the bilayer normal and the magnetic field and

$$\sigma'_i = (1/3)(\sigma'_{11} + \sigma'_{22} + \sigma'_{33})$$

$$\sigma'_a = \sigma'_{11}S_{11} + \sigma'_{22}S_{22} + \sigma'_{33}S_{33}$$

The axis system of the fluorine chemical shift tensor (in a molecule-fixed reference frame rotated from the principal axis system) is illustrated in Figure 1 and is defined as follows: σ'_{33} lies along the C–F bond direction, σ'_{22} is perpendicular to σ'_{33} in the H–C–F plane, and σ'_{11} is parallel to the normal to that plane. S_{kk} is the order parameter of the kth principal axis as introduced by Saupe (1964).

Motional averaging due to rotation about the fatty acyl chain long axis yields a pseudoaxial symmetry of the chemical shift tensor and allows the system to be defined by one independent order parameter. Consequently, the observable chemical shifts can be written as

$$\sigma'_i = (1/3)(2\sigma_\perp + \sigma_\parallel)$$

$$\sigma'_a = S_{11}(\sigma_\parallel - \sigma_\perp)$$

$$\sigma_\parallel = \sigma'_{11}$$

$$\sigma_\perp = (1/2)(\sigma'_{22} + \sigma'_{33})$$

In the absence of dipolar line broadening, the sum of the resonances of all possible orientations θ results in a "powder-type" spectrum that can be described by a probability function $p(\nu)$ (Niederberger & Seelig, 1976) where

$$p(\nu) \propto 1 - \left(\frac{2(\nu - \nu_i)}{\nu_a}\right)^{-1/2}$$

and

$$\nu_a = (2/3)\nu_0\sigma'_a$$

The contribution of dipolar interactions to the ^{19}F NMR line shape can be separated into orientation-independent (predominantly interchain contributions, Δ_0) and orientation-dependent (intrachain contributions, Δ_1) terms (Niederberger & Seelig, 1976; Bloom et al., 1975). The $3\cos^2\theta - 1$ dependence of Δ_1 (Bloom et al., 1975; McLaughlin et al.,

Table I: Fatty Acid Composition of Membranes of *A. laidlawii* B Enriched with 8F-16:0 plus One of the Particular Fatty Acids of Interest

fatty acid supplement	fatty acid composition (mol %)										
	12:0	14:0	15:0	16:0	F-16:0	16:0i	16:0ai	16:1tΔ9	18:0	18:1	nd[a]
90% 16:0 + 10% 8F-16:0	2.1	11.4		66.7	9.6				2.0	1.0	7.2
90% 15:0 + 10% 8F-16:0	0.2		87.0	1.6	8.7				0.9	0.7	0.9
90% 16:0i + 10% 8F-16:0					10.2	87.9			1.6	0.3	
90% 16:0ai + 10% 8F-16:0				0.7	10.0		89.0		0.3		
90% 16:1tΔ9 + 10% 8F-16:0					10.0			88.0	1.8		0.2

[a] Not determined. In this case, no single fatty acid represented more than 1% of the total fatty acid present.

1975) is assumed to be further modulated by the acyl-chain order parameter (Gent & Ho, 1978) so that

$$\Delta = \Delta_0 + \Delta_1\left(\frac{3\cos^2\theta - 1}{2}\right)S_{11}$$

where $(\delta\nu)_{1/2} = 2.36\Delta$ is the Gaussian line width at half-height.

The ^{19}F NMR line shape is considered to consist of individual resonances centered at frequency ν^* corresponding to particular orientations θ of the H–C–F segments with respect to the magnetic field H_0. The intensity of an individual resonance line centered at frequency ν^* as a function of the observed frequency ν can be approximated by a normalized Gaussian of the form $I(\nu - \nu^*)$ described by Niederberger & Seelig (1976). The total absorption intensity $S(\nu)$ at a frequency ν is then the sum over all overlapping resonances ν^* weighted with their corresponding probabilities $p(\nu^*)$

$$S(\nu) = \int_{-\infty}^{\infty} I(\nu - \nu^*)p(\nu^*)\,d\nu^*$$

The shape of the spectrum is therefore governed by the chemical shift anisotropy, by the line-width parameters Δ_0 and Δ_1, and by the order parameter S_{11}.

Materials and Methods

Materials. The synthesis of the various monofluoropalmitic acids has been described in the accompanying paper (McDonough et al., 1983). All other fatty acids used were products of either NuCheck Prep Co. (Elysian, MN) or Analab Co. (North Haven, CT). All chemicals used were of reagent grade or better.

Cell Culture, Membrane Isolation, and Lipid Analysis. The growth medium and conditions used for culturing *A. laidlawii* B with fatty acids and avidin have been described previously (Silvius & McElhaney, 1978a). Membranes were prepared from late log phase cultures of this organism by osmotic lysis essentially as previously described (Silvius et al., 1977). The extraction and purification of total membrane lipids, preparation of methyl esters, and analysis by gas–liquid chromatography have been described elsewhere (Saito & McElhaney, 1977).

NMR. Membrane samples for NMR analysis were suspended in buffer (0.154 M NaCl, 0.05 M Tris-HCl, 20 mM β-mercaptoethanol, pH 7.4) diluted 20-fold with 95% deuterium oxide. ^{19}F NMR spectra were collected at 254.025 MHz on a Bruker HXS-270 NMR spectrometer equipped with a ^{2}H lock, operating in the Fourier-transform mode, by using quadrature detection, at a spectral width of ±50 000 Hz. Bessel filters with a filter width of ±100 000 Hz were used. The ^{19}F probe was constructed entirely of fluorine-free materials and was free of any ^{19}F background signal. The temperature of the probe was maintained at the specified temperature to within ±1 °C. Samples were equilibrated at a particular temperature for 30 min prior to data acquisition. Samples were subjected to a 15-μs (~45°) pulse followed by a 20-μs receiver blanking delay and a 200-ms acquisition time. Computer simulations indicate that the use of a 20-μs receiver blanking pulse has little effect upon the spectral line shape and results in only a small (~10%) reduction in the extracted order parameters. Typically, 25K scans were accumulated for samples in which the membrane lipids were entirely in the liquid-crystalline phase state. The signal was enhanced with an exponential multiplication of 50 Hz, and the FID was Fourier transformed to 4K data points in the real domain. Spectra collected at 188.217 and at 376.333 MHz were obtained under similar experimental conditions.

Results

Fatty Acid Composition of A. laidlawii B Membranes. The results of the analysis of the fatty acid composition of the various membrane preparations investigated are presented, for the typical case of supplementation with 8F-16:0, in Table I. The fatty acid compositions were virtually identical when other fluoroprobes replaced 8F-16:0. Of those fatty acids under investigation, that is, pentadecanoic acid (15:0), hexadecanoic acid (16:0), 14-methylpentadecanoic acid (16:0i), 13-methylpentadecanoic acid (16:0ai), and *trans*-9-hexadecanoic acid (16:1tΔ9), cells cultured in the presence of one of the latter three together with avidin and the pertinent fluorinated probe yielded membranes that were 96.7–99.6% homogeneous with respect to the particular fatty acids supplied exogenously. In all cases the mole ratio of primary supplement to fluorinated probe extant in the membranes (about 90:10) closely approximated that originally provided exogenously in the supplement.

When 15:0 was the primary fatty acid supplement, the background level of endogenous fatty acid incorporation increased, and the resulting membranes were 93.3–96.0% homogeneous with respect to the exogenous fatty acid supplement. Silvius & McElhaney (1978a,b) have demonstrated that 15:0 represents the upper chain-length limit of the straight-chain, saturated fatty acids capable of supporting the growth of *A. laidlawii* B at 37 °C in the presence of avidin. The increased incorporation of endogenous fatty acids in this case may then represent an attempt by the organism to compensate for somewhat suboptimal growth conditions.

Palmitic acid cannot support the growth of *A. laidlawii* B in the presence of avidin (Silvius & McElhaney, 1978a), and the exclusion of avidin necessarily permits the occurrence of significant de novo fatty acid biosynthesis. Thus, membranes prepared from cells grown in the presence of 16:0 plus a particular fluoroprobe, and in the absence of avidin, were only 76.1–78.7% homogeneous with respect to the exogenously supplied fatty acids. However, this situation is, in fact, desirable for the purpose of comparing our ^{19}F NMR results with the ^{2}H NMR studies of Stockton et al. (1977) in which, under comparable growth conditions, a very similar fatty acid composition was obtained.

Despite these complications, it seems most probable, even in the case of 16:0 enrichment, that the order parameters obtained by analysis of the ^{19}F NMR line shapes represent

Table II: Values of ^{19}F NMR Line-Shape Parameters Providing the Best Fit of Simulated Spectra to Experimental Spectra at Various Field Strengths

	field strength								
	188.217 MHz			254.025 MHz			376.333 MHz		
T (°C)	Δ_0	Δ_1	S_{11}	Δ_0	Δ_1	S_{11}	Δ_0	Δ_1	S_{11}
16:1tΔ9-Enriched Membranes Containing 14F-16:0									
37	0	20 000	0.09	0	21 000	0.09	50	22 000	0.11
20	0	22 000	0.19	0	20 000	0.18	50	23 000	0.18
16:0-Enriched Membranes Containing 5F-16:0									
45	0	20 000	0.24	0	20 000	0.25			
37	0	20 000	0.19	0	20 000	0.31			

predominantly the effects of the major component of the membrane fatty acids upon the orientational order of the fluorinated probe.

Evaluation of ^{19}F NMR Line-Shape Parameters. As outlined under Theory, the ^{19}F NMR line shape is considered to be influenced by four parameters: $\sigma_\parallel - \sigma_\perp$, Δ_0, Δ_1, and S_{11}.

(*i*) *Estimation of $\sigma_\parallel - \sigma_\perp$.* Although the chemical shift tensor elements for the H–C–F group have not been determined, they can be estimated from model compounds. Three distinct elements are found in Teflon: $\sigma_{11} = -80$ ppm, $\sigma_{22} = 21$ ppm, $\sigma_{33} = 59$ ppm, where σ_{33} is along the C–F bond direction and σ_{11} is perpendicular to the 33 axis in the C–C–F plane (Mehring et al., 1971). Rotation of this Cartesian coordinate system by 30° about the 33 axis reorients the principal axis system of the fluorine chemical shift tensors into a coordinate system with an axis parallel to the bilayer normal. This transformation is accomplished by means of the orthogonal transformation matrix $\mathbf{M} = \mathbf{M}_{ik}$ (Seelig, 1977; Van et al., 1974) such that

$$\sigma' = \mathbf{M}\cdot\sigma\cdot\mathbf{M}^{-1}$$

Here, $\mathbf{M}\cdot\mathbf{M}^{-1} = \mathbf{M}^{-1}\cdot\mathbf{M} = 1$, since the Hamiltonian must be invariant to orthogonal coordinate transformation. After transformation into the new coordinate system, the chemical shift tensor elements correspond to $\sigma'_{11} = -54.75$ ppm, $\sigma'_{22} = -4.25$ ppm, and $\sigma'_{33} = 59$ ppm, where σ'_{11} is normal to the H–C–F plane and σ'_{33} is along the C–F bond direction. The results of this transformation and the new axis system of the chemical shift anisotropy tensor relative to the geometry of the H–C–F group are illustrated in Figure 1. Note that this molecule-fixed coordinate system corresponds to that defined under Theory to describe the orientation dependence of the chemical shift anisotropy. Invoking motional averaging due to rotation about the 11 axis yields the values

$$\sigma_\parallel = \sigma'_{11} = -54.75 \text{ ppm}$$

$$\sigma_\perp = (1/2)(\sigma'_{22} + \sigma'_{33}) = 27.375 \text{ ppm}$$

$$\sigma_\parallel - \sigma_\perp = -82.2 \text{ ppm}$$

(*ii*) *Angular-Independent Dipolar Contributions.* Δ_0 represents the contribution of angular-independent dipole–dipole interactions to the observed line shape. At higher temperatures and, in particular, when membrane lipids are in the liquid-crystalline phase state, rapid translational diffusion of the lipid molecules tends to severely reduce the efficacy of these interactions, and their contribution to the observed ^{19}F NMR line shape approaches a minimal value. When the rate of such motions decreases with, for example, decreasing temperature or the onset of the gel to liquid-crystalline phase transition, it is necessary to introduce increasingly large positive values of Δ_0, measured in hertz, in order to simulate the observed ^{19}F NMR line shapes.

(*iii*) *Angular-Dependent Dipolar Contributions.* The contributions to the observed line shape of the angular-dependent dipole–dipole interactions, consisting primarily of intrachain interactions, are considered in the term Δ_1. The value of Δ_1 would be modulated by θ (the angle between $\vec{\eta}$, the dipole–dipole interaction vector, and the magnetic field $\vec{H}_0$), as well as by S_{11}. Since only the chemical shift anisotropy contribution to the ^{19}F NMR line shape should vary with the magnetic field strength, the value of Δ_1 is approximated from a comparison of the ^{19}F NMR spectra of identical membrane samples collected at various magnetic field strengths.

^{19}F NMR spectra of *A. laidlawii* B membranes enriched with 16:0 plus 5F-16:0 were acquired at both 37 and 45 °C at field strengths of 188.217 and 254.025 MHz, while ^{19}F NMR spectra of *A. laidlawii* B membranes enriched with 16:1tΔ9 plus 14F-16:0 were acquired at both 20 and 37 °C at field strengths of 188.217, 254.025, and 376.333 MHz. Theoretical spectra were generated by computer with the outlined line-shape theory, and various values of the line-shape parameters Δ_0, Δ_1, and S_{11} were tested for their fit to the observed spectra. The line-shape parameters giving the best fit to the observed spectra are listed in Table II. Δ_0 was set close to or at zero in these cases since the angular-independent dipole–dipole interactions become significant only at temperatures well below the membrane lipid phase transition (P. M. Macdonald, B. D. Sykes, and R. N. McElhaney, unpublished results). The values of S_{11} providing the best fit in the case of 16:0 enrichment were somewhat lower than those obtained in a similar situation by ^2H NMR (Stockton et al., 1977). The final value of 20 000 Hz chosen for Δ_1 is biased somewhat toward the theoretical fits obtained at the two lower field strengths, primarily because of considerations of spectral quality and the consequent reliability of the theoretical fit and, hence, the extracted line-shape parameters. This value may vary with both temperature and lipid phase state and was adjusted as required.

(*iv*) *Order Parameter.* In the absence of dipolar line broadening, the experimentally accessible parameter $(\sigma_\parallel - \sigma_\perp)/(\sigma_\parallel - \sigma_\perp)_{MAX}$ would be related to the order parameter, S_{11}, of the hydrocarbon long axis. The ^{19}F NMR spectral line shape contains contributions from both chemical shift anisotropic and dipolar interactions, and as such, no one spectral parameter is readily interpretable in terms of an order parameter.

We have, therefore, resorted to computer simulation of the experimentally observed ^{19}F NMR spectra utilizing the theoretical description outlined previously and the input variables Δ_0, Δ_1, $\sigma_\parallel - \sigma_\perp$, and S_{11}. Examples of typical experimentally obtained ^{19}F NMR spectra and the corresponding computer-generated "best fit" simulated spectra are illustrated in Figure 2 for the case of the 16:0i-enriched membranes. In practice, the simulated spectra, having been generated on the

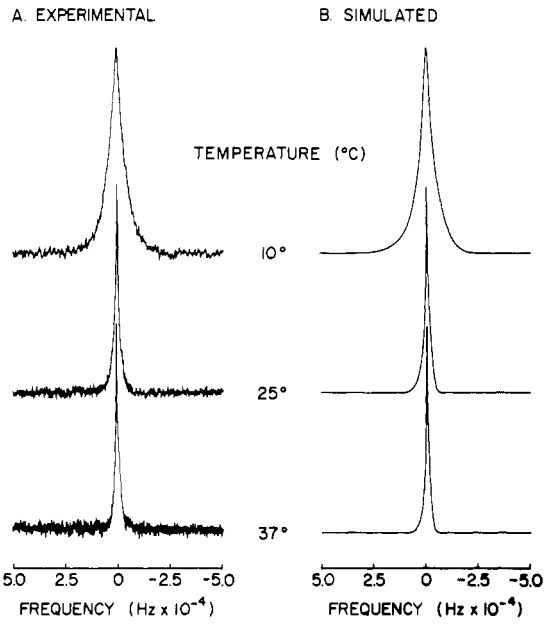

FIGURE 2: Experimental (obtained at 254.025 MHz) and simulated ^{19}F NMR spectra of membranes of *A. laidlawii* B enriched with 10% 10F-16:0 plus 90% 16:0i. (A) (Experimental spectra) 10 °C, 150 000 transients, line broadening 250 Hz; 25 °C, 25 000 transients, line broadening 100 Hz; 37 °C, 25 000 transients, line broadening 50 Hz. (B) (Simulated spectra) 10 °C, Δ_0 = 700 Hz, Δ_1 = 35 000 Hz, S_{11} = 0.60; 25 °C, Δ_0 = 100 Hz, Δ_1 = 20 000 Hz, S_{11} = 0.30; 37 °C, Δ_0 = 50 Hz, Δ_1 = 20 000 Hz, S_{11} = 0.26.

basis of the aforementioned input variables, were superimposed upon the experimental spectra in order to judge the quality of the fit. In general, only differences greater than ±5% in the value of the order parameter S_{11} could be distinguished via this visual comparison.

Effect of Fatty Acid Structure and Temperature on Order Parameter Profile of A. laidlawii B Membranes. The orientational order profile of membranes of *A. laidlawii* B enriched with palmitic acid, as determined by analysis of ^{19}F NMR spectra, obtained at 254.025 MHz, has been compared with that determined from ^{2}H NMR analysis of the same system enriched with specifically deuterated palmitic acids (Stockton et al., 1977) in Figure 3. The two order profiles were similar in that the order parameters remained constant out to approximately carbon atom number ten and subsequently decreased rapidly toward the methyl terminus of the acyl chain. The absolute value of the ^{19}F NMR order parameters were in most cases approximately 50% lower than those determined by ^{2}H NMR. The largest discrepancies existed in the plateau region of the profile, while beyond the 10-position the values of the order parameters obtained by the two techniques tended to converge. We have observed previously that any perturbing effect of the fluorine nucleus in monofluorinated palmitic acids becomes more pronounced with increasing proximity to the carbonyl head group of the fatty acid (McDonough et al., 1983). Studies in which difluorinated myristic acids were deuterium-labeled at positions next to the *gem*-difluoro group indicated that values of S_{MOL} determined by ^{2}H NMR were approximately 30% lower in the presence of difluoro substitution than in its absence (Oldfield et al., 1980). Thus, some underestimation of the degree of order may be due to a perturbation of the acyl-chain conformation by the fluorine substituent. It seems most likely, however, that the discrepancies between the absolute values of the order parameters obtained by ^{19}F NMR and ^{2}H NMR can be attributed to the estimated quantity $\sigma_\parallel - \sigma_\perp$. Examination of the equations relating chemical shift anisotropy and observed

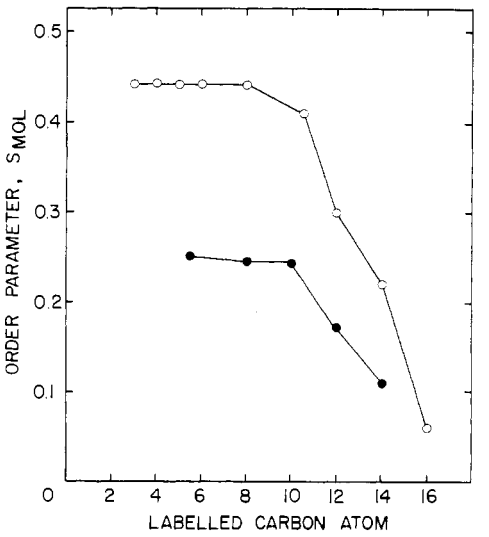

FIGURE 3: Comparison of order parameter profiles of palmitic acid enriched membranes of *A. laidlawii* B obtained with specifically deuterated palmitic acids (○), taken from Stockton et al. (1977), and monofluorinated palmitic acids with the value of $\sigma_\parallel - \sigma_\perp$ estimated from Teflon (●).

line shape reveals the direct inverse relationship between the value of the maximum chemical shift anisotropy $\sigma_\parallel - \sigma_\perp$ and the hydrocarbon-chain order parameter S_{11}. An overestimation of $\sigma_\parallel - \sigma_\perp$ will result in a correspondingly undervalued S_{11}. We have estimated the value of $\sigma_\parallel - \sigma_\perp$ for the monofluoropalmitate probe to be 82.2 ppm from a consideration of the chemical shift tensor elements of the model compound Teflon. Recently, Engelsberg et al. (1982) have estimated the maximum chemical shift anisotropy of the *gem*-difluoro group from studies of the orientation dependence of the resonance line position in macroscopically oriented bilayers. 1-Myristoyl-2-(8,8-difluoro[2,2,7,7,9,9-$^2H_6$]myristoyl)-*sn*-glycero-3-phosphocholine and its nondeuterated analogue 1-myristoyl-2-(8,8-difluoromyristoyl)-*sn*-glycero-3-phosphocholine yielded values of 58 and 64 ppm, respectively, for the maximum chemical shift anisotropy. These results indicate that the value of $\sigma_\parallel - \sigma_\perp$ that we have calculated from Teflon and have used throughout this study may have been overestimated. Our ^{19}F NMR order parameters approach to within 20% of the order parameters obtained by ^{2}H NMR when the values obtained by Engelsberg et al. (1982) are used. This remaining discrepancy between the results obtained by ^{2}H NMR and by ^{19}F NMR may, in part, be attributed to the somewhat higher temperature we have employed and the consequent reduction in the degree of order. Until a more accurate estimate of the value of the maximum chemical shift anisotropy of the monofluoro probe is obtained by, for example, studies of the orientation dependence of the resonance line position in oriented bilayers, the quantitative correspondence between the order parameters obtained by ^{19}F NMR and those obtained by ^{2}H NMR cannot be definitively evaluated. To put this point in perspective, it can be stated tht the two techniques provide the same qualitative picture of segmental order through the bilayer, and as pointed out by Griffith & Jost (1976), the main point is that the data provide an operationally defined parameter S and that it is the relative change in this parameter at some depth in the bilayer and not the absolute value that provides a handle on the experimental variable.

The ^{19}F NMR orientational order profiles obtained at 254.025 MHz and 37 °C for 15:0-, 16:0i-, 16:0ai-, and 16:1tΔ9-enriched membranes isolated from cells cultured in the presence of avidin are compared in Figure 4. At 37 °C,

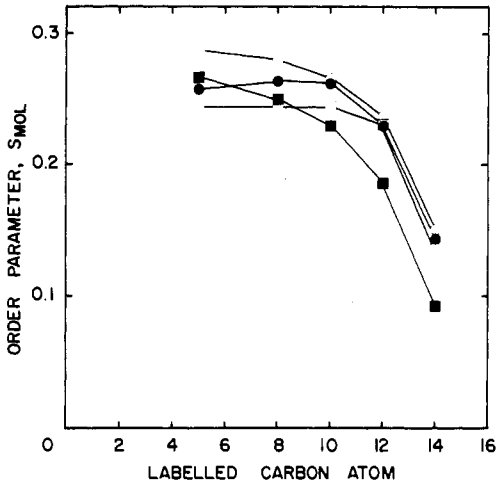

FIGURE 4: ^{19}F NMR order parameter profiles at 37 °C of membranes of *A. laidlawii* B enriched with 15:0 (○), 16:0i (●), 16:0ai (□), and 16:1tΔ9 (■).

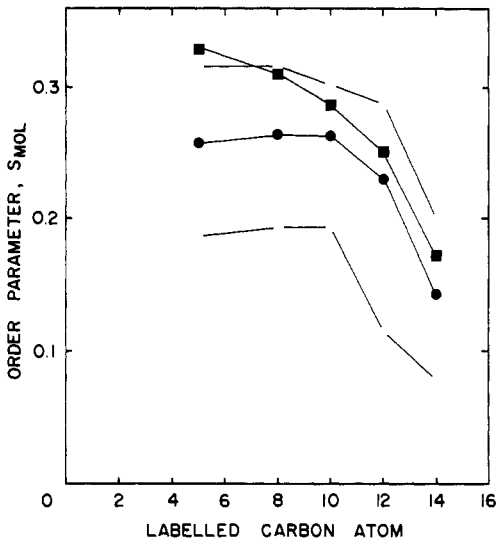

FIGURE 5: ^{19}F NMR order parameter profiles at T_m + 15 °C of membranes of *A. laidlawii* B enriched with 15:0 (○), 16:0i (●), 16:0ai (□), and 16:1tΔ9 (■).

the overall orientation order through the bilayer decreased in the progression 15:0 > 16:0i > 16:0ai > 16:1tΔ9. The thermal transition midpoints (T_m) of the membranes enriched with these fatty acids decrease in the order 15:0 > 16:0i > 16:1tΔ9 > 16:0ai (Silvius & McElhaney, 1978b). Thus, the overall membrane order, at a constant temperature, varied with the position of the gel to liquid-crystalline phase transition temperature. Order parameters generally increased with increasing proximity to the gel to liquid-crystalline phase transition. Therefore, the decrease in overall membrane order resulting from the addition of a methyl-branch substituent proximal to the methyl terminus of the fatty acid, or from the addition of a trans double bond, can be correlated with changes in the thermotropic phase transition temperature of the particular membrane. Clearly, the veracity of this relationship may not necessarily hold when two species of fatty acids having very similar thermotropic properties but markedly dissimilar order profiles are compared. This point is relevant to the case of 16:0ai and 16:1tΔ9 where the T_m's are similar [T_m = 4.1 and 6.7 °C, respectively (Silvius & McElhaney, 1978b)], and the dissimilarity of the two order profiles masks the small contribution, in this case, of relative proximity to the phase transition.

The character of the order profile itself was altered by the addition of either methyl-branch substituents or by the addition of a trans double bond. Membranes enriched with straight-chain saturated fatty acids yielded order profiles very similar to those obtained by ^2H NMR (Stockton et al., 1977) with an initial order plateau region extending out to approximately carbon number eight or ten of the acyl chain and a subsequent rapid decrease toward the methyl terminus. This situation can be seen to pertain to the case of 16:0-enriched membranes in Figure 3, as well as to the case of 15:0-enriched membranes in Figure 4.

The presence of a methyl-branch substituent extended the plateau region of the order profile to the 12-position of the fatty acyl chain in the case of the anteiso-branched fatty acid (16:0ai) while the rate of decrease of the order parameters in the postplateau region of the order profile was markedly reduced in the case of the iso-branched fatty acid (16:0i). It can be concluded then that the presence of a methyl-branch substituent tends to increase the orientational order of neighboring fatty acids in the immediate vicinity of that substituent. The rather more clear-cut effect of the anteiso-methyl branch upon the order profile, when compared to the iso-methyl branch, can be related to the different depths of penetration of these two groups into the hydrocarbon milieu of the membrane. The ability of the anteiso methyl branch substituent to cause a more pronounced disruption is evident as well when the temperatures of the major phase transitions of membranes enriched with 15:0, 16:0i, or 16:0ai are compared [T_m = 36.7, 21.8, 4.1 °C, respectively (Silvius & McElhaney, 1978b)].

The effect of the trans double bond upon the character of the orientational order profile was to decrease the length of the order plateau relative to that observed with straight chain saturated with fatty acids. In particular, the values of the order parameters at positions 8 and 10 showed a significant decrease. Thus, our results indicate that the trans double bond affects a local decrease in the order of the probe molecule.

In order to eliminate, or to at least minimize, the contribution to the absolute overall order of the relative proximity to the gel to liquid-crystalline phase transition, the order parameter profiles of the above membranes were acquired at a constant temperature of 15 °C above their respective phase transition temperatures. The resulting order parameter profiles are illustrated in Figure 5. Under these conditions, the effects of methyl-branch and trans double bond structural substituents upon the character of the order profile that were observed at 37 °C were again evident. The relative ordering effect of a methyl-branch substituent of either an iso or anteiso configuration or the relative disordering effect of a trans double bond appears then to be independent of the acquisition temperature provided the phase state of the membrane lipids remains constant. This observation is consistent with the findings of other investigators, who have noted that the character of the order profile is not altered as the temperature is changed but rather the values of the order parameters increase or decrease in a similar fashion across the width of the bilayer with decreasing or increasing temperature [see, for example, Seelig & Seelig (1977)].

The situation with regard to the relative overall orientational order through the bilayer of the various enriched membranes, when compared at 15 °C above each particular T_m, was found to be almost completely reversed from that observed at 37 °C. Under conditions in which the various membrane fatty acids could be considered to be in a constant thermodynamic state relative to the thermotropic phase transition, the overall orientational order decreased in the progression: 16:0ai >

16:1tΔ9 > 16:0i > 15:0. Therefore, in addition to the local ordering effect of the iso or anteiso methyl branch substituent or the local disordering effect of the trans double bond, these structural substituents appear to mediate as well as overall increase in orientational order throughout the width of the bilayer.

Discussion

The ^{19}F NMR line shape can be interpreted in terms of the orientational order parameter S_{MOL} of the hydrocarbon long molecular axis. S_{MOL} represents the time-average angular excursions of particular methylene segments away from the bilayer normal. Tilting of the whole molecule with respect to the bilayer normal, as well as trans–gauche isomerization at individual methylene segments, can contribute to the deviation of S_{MOL} from a value of 1.0, indicative of acyl chains assuming a fully extended all-trans configuration and packing parallel to the bilayer normal, toward a value of zero at which individual chain segments would experience essentially free isotropic motion. S_{MOL} is found to be (Seelig, 1977)

$$S_{MOL} = (1/2)(3\langle\cos^2\theta\rangle - 1)$$

Here θ is the angle between the segment direction and the bilayer normal where the segment direction is defined as the normal to the plane formed by the H–C–H atoms of the methylene segment. The broken brackets represent an average over time.

Schindler & Seelig (1975) have demonstrated that the theory for chain ordering in lipid bilayers presented by Marcelja (1974) can be used successfully to reproduce, in the case of a bilayer of dipalmitoylphosphatidylcholine, orientational order parameters obtained by ^{2}H NMR, as well as to predict with precision the associated transition temperature, thermodynamic properties, and physical characteristics. Marcelja's mean-field model of chain ordering considers all physical forces contributing to bilayer stability. Meraldi & Schlitter (1981a,b), utilizing a model that contained many elements of Marcelja's mean-field theory, were able to further refine the correlation of theoretical prediction with experimental results. In particular, these investigators, having scaled their theoretically obtained deuterium order parameter profile via a suitable adjustment of various phenomenological parameters, were subsequently able to demonstrate the manner in which the orientational order profile could be altered when a particular phenomenological parameter was varied. These scaling parameters included values intended to fit repulsive (hard-core steric) and attractive (van der Waals) energies of acyl-chain interaction. The forces responsible for the interactions between head groups and between head groups and water were scaled via an effective lateral-pressure parameter. The values of the repulsive and attractive phenomenological parameters when varied had no large effect upon the shape of the simulated order profile. In contrast, alteration of the lateral-pressure parameter drastically altered the shape of the simulated order profile, as well as the overall order present throughout the bilayer. Increasing the value of the lateral-pressure parameter broadened the plateau region of the order profile, as well as increasing the absolute overall order. Additionally, Meraldi and Schlitter demonstrated that, by restricting the distribution of possible chain conformations near the methyl terminus of the acyl chain, the length of the plateau region of the simulated order profile was increased, as were the simulated order parameters themselves toward the methyl terminus. Do the relative values of these phenomenological parameters, which one would predict from the established physical properties of the fatty acids we have investigated, alter the order profiles in a manner that correlates with the experimental observations?

The presence of an iso- or anteiso-methyl branch must necessarily increase the energy required for the particular chain segment containing that methyl branch to undergo rotational isomerization. This would effectively restrict the distribution of conformational possibilities available to that acyl chain near the methyl terminus, resulting in a local ordering effect. The order profiles of iso- and anteiso-branched fatty acids, when compared with those of straight chain saturated fatty acids, as in Figure 4, show such an effect, and the comparison is qualitatively similar to that made by Meraldi & Schlitter (1981a) of restricted and unrestricted conformational possibilities near the methyl terminus.

The importance of the lateral-pressure parameter, π, to the scaling of the orientational order profiles simulated by Meraldi & Schlitter (1981a,b) has been recognized as well by Dill & Flory (1980) in their analogous term σ, representing the relative surface density of the acyl chains. As defined by Dill & Flory (1980), the relative surface density, σ, is obtained as the ratio a_0/a, where a_0 is the cross-sectional area of the all-trans chain and a is the cross-sectional area in the liquid-crystalline phase. When σ was increased, the simulated order profile was altered in a manner very similar to that observed by Meraldi & Schlitter (1981a) when π was increased; that is, the plateau region of the order profile was lengthened and the absolute overall order was increased.

Several lines of evidence indicate that branched-chain fatty acids, in the gel state, occupy a larger area in the bilayer than do linear saturated fatty acids. Wide-angle X-ray diffraction experiments indicate that the sharp 4.2-Å reflection associated with reflections from closely packed hydrocarbon chains in gel-state lipids is replaced by a broader reflection with a spacing of 4.3–4.4 Å in biological membranes naturally or artificially enriched with branched-chain fatty acids (Haest et al., 1974; Bouvier et al., 1981). Pig pancreatic phospholipase A_2, which cannot hydrolyze gel-state phosphatidylglycerol in *A. laidlawii* membranes enriched with linear, saturated, or unsaturated fatty acids, can attack this phospholipid in membranes enriched with branched-chain fatty acids, even at temperatures below the phase transition lower boundary temperature (Bouvier et al., 1981). Monolayer studies of di-(16:0)-, di-(17:0i)-, and di-(17:0ai)PC's have indicated that, in the gel-state, di-(16:0)PC molecules occupy a much smaller area (50 Å^2) than do molecules of di-(17:0i)PC (57 Å^2) or di-(17:0ai)PC (60 Å^2) (Karl Poralla, personal communication). Little difference was discernible in the liquid-crystalline state between the cross-sectional areas occupied by these three species.

These various points lead to the prediction that the term σ, as defined by Dill & Flory (1980), should increase in the order:

$$\sigma(\text{straight chain}) < \sigma(\text{iso branched}) < \sigma(\text{anteiso branched})$$

In turn, the absolute overall order at comparable thermodynamic conditions should increase in the sequence: straight chain < iso branched < anteiso branched. Indeed, this prediction corresponds to our observations via ^{19}F NMR.

The trans double bond imposes upon an acyl chain a stiffening that is the result of the exclusion of the possibility of trans–gauche isomerization about that particular carbon–carbon bond. This occurs in the absence of any tilting of the acyl chain posterior to the site of trans unsaturation, as would occur in the presence of a cis double bond. The energy of a single gauche conformer, relative to a trans, would not nec-

essarily be altered by the presence of a trans double bond, but rather, certain sequences or combinations of trans–gauche isomers would be forbidden.

^{2}H NMR studies by Seelig & Seelig (1977) concerning the effects of a cis double bond upon the deuterium order profile of 1-(16:0),2-(18:1cΔ9)PC indicated that, when corrected for geometric considerations, the order profile of the unsaturated chain closely resembled that of DPPC, while the order profile of the neighboring saturated chain manifested a local increase in orientational order proximal to the adjacent site of cis unsaturation. This effect was attributed to the rigidity of the cis double bond and a consequent restriction to the rotational freedom that reduced the number of isomeric states available to the neighboring saturated acyl chain. A later study by Seelig & Waespe-Sarčević (1978) demonstrated that the ^{2}H NMR order profile of PC's containing trans-unsaturated acyl chains again resembled that of DPPC after correction for geometric considerations. The effects of the trans double bond upon the order profile of a neighboring saturated acyl chain were not reported. The results presented herein would suggest that the trans double bond allows an increase in rotational freedom and, therefore, an increase in the number of isomeric states available to an adjacent saturated acyl chain. In the case of the cis double bond, the stiffening effect, manifested as a local increase in order upon the neighboring chain, was presumed to supercede the effect of increased rotational freedom about single bonds connected to the double bond. The reverse apparently occurs in the case of the trans double bond and is manifest as a local decrease in order upon a neighboring acyl chain. Clearly, these differences can be attributed to the differing geometries of the cis and trans double bond.

Chapman et al. (1966) have investigated the monolayer properties of phosphatidylethanolamines containing cis-unsaturated or trans-unsaturated fatty acids esterified to both positions of the glycerol backbone. These workers concluded that, at comparable surface pressures, di-(18:1cΔ9)PE occupied a cross-sectional area significantly larger than that occupied by di-(18:0)PE, while di-(18:1tΔ9)PE was intermediate to the relatively expanded cis species and the relatively condensed straight chain saturated species. The same trend was evident when 1-(18:0),2-(18:1tΔ9)PE was compared with 1-(18:0),2-(18:1cΔ9)PE. The differences in the relative cross-sectional areas were much more pronounced in the liquid-compressed than in the liquid-expanded state. These studies tend to indicate that the surface density parameter, σ, of Dill & Flory (1980), or, by analogy, the lateral-pressure parameter, π, of Meraldi & Schlitter (1981a) should be greater in the case of the trans-unsaturated fatty acid relative to a straight chain saturated fatty acid. As in the case of the branched-chain fatty acids, this prediction would explain the i.e., a plane taken through the bilayer can be approximated by a latticed network, then it is necessary to invoke a greater lateral pressure in order to pack an acyl chain of larger cross-sectional area into that lattice. If the lateral pressure is constant, the σ term of Dill & Flory (1980) must necessarily be altered to accommodate differences in acyl-chain cross-sectional area.

The potential now exists to rapidly elucidate, via ^{19}F NMR, the effects of the many variables encountered in fatty acyl chain chemistry upon membrane orientational order. Such questions as the effects of chain length, the effects of chain mismatch, the role of the many other structural substituents yet to be investigated, the relationship between the position along the acyl chain, and the effects of a specific structural substituent all promise to be amenable to investigation through the versatility of the monofluoropalmitic acid probes.

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Registry No. 15:0, 1002-84-2; 16:0, 57-10-3; 16:0i, 4669-02-7; 16:0ai, 20121-96-4; 16:1tΔ9, 10030-73-6.

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Inhibition of Nitrogenase-Catalyzed NH_3 Formation by H_2[†]

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ABSTRACT: We have investigated the inhibition by H_2 (D_2) of NH_3 formation by nitrogenase from *Klebsiella pneumoniae* and have confirmed that the inhibition is competitive vs. N_2. D_2 inhibits NH_3 formation by diverting nitrogenase from production of NH_3 to production of HD (one electron per HD). By careful exclusion of N_2 from the reaction mixture, we have been able to place an upper limit on N_2-independent HD formation by nitrogenase, under 1 atm of D_2, at 1% of the total electron flux. Formation of NH_3 and formation of HD were inhibited identically by CO. We observed that as the ratio of dinitrogenase to dinitrogenase reductase is increased, the ratio of HD formed to NH_3 formed rises, and D_2 becomes a stronger inhibitor of N_2 reduction. This may be caused in part by an accompanying increase that is observed in the K_m of nitrogenase for N_2. We propose a model for D_2 inhibition of NH_3 formation in which D_2 and N_2 compete for the same form of nitrogenase. According to our proposal, when N_2 reacts with nitrogenase, either N_2 reduction proceeds to completion if H_2 (D_2) is absent or, if D_2 already is bound to nitrogenase, N_2 reduction is aborted and two molecules of HD are produced at the net expense of one electron per HD. Key consequences of the model are that it predicts that H_2 (D_2) is a competitive inhibitor of NH_3 formation and that the apparent $K_m(N_2)$ for formation of HD and NH_3 may differ.

Nitrogenase participates in several reactions involving H_2. In the absence of other substrates, nitrogenase catalyzes ATP- and reductant-dependent H_2 evolution from protons (Bulen et al., 1965). H_2 evolution in vitro cannot be completely suppressed by N_2 (Hadfield & Bulen, 1969), though the substrates C_2H_2 and HCN (Rivera-Ortiz & Burris, 1975) can completely suppress H_2 evolution in the extrapolated limit of infinite substrate concentration.

H_2 not only is a product of nitrogenase but also is an inhibitor of N_2 reduction by nitrogenase (Wilson & Umbreit, 1937). Hoch et al. (1960) observed the production of HD when N_2 fixation by soybean nodules was inhibited by D_2 (rather than H_2) and proposed that HD is formed when D_2 reacts with an enzyme-bound intermediate in N_2 reduction. Bulen (1976) combined these observations by proposing that inhibition of NH_3 formation by H_2 (D_2) and N_2-dependent HD formation from D_2 and H_2O are different manifestations of the same molecular process.

There is convincing experimental support for Bulen's proposal. First, H_2 inhibits N_2 reduction specifically, and HD formation is supported specifically by N_2. Thus, H_2 (D_2) inhibits reduction of N_2, but not of H^+ (Burns & Bulen, 1965), nitrous oxide (Hoch et al., 1960), azide, acetylene, cyanide, methylisonitrile (Hwang et al., 1972), or hydrazine (Burgess et al., 1981), all of which are substrates of nitrogenase. Correspondingly, HD formation from D_2 and H_2O is greatly enhanced by N_2 (Hoch et al., 1960) but not by N_2O (Hoch et al., 1960), azide, acetylene, cyanide, methylisonitrile (Jackson et al., 1968), or hydrazine (Newton et al., 1977). [It has been proposed (Wherland et al., 1981) and challenged (Li & Burris, 1983) that there is a low rate of HD formation during reduction of protons.] Second, several studies have led to the conclusion that HD formation is an electron-utilizing reaction, requiring one electron per HD formed. Under N_2, H_2 does not inhibit ATP hydrolysis or total product formation but redirects nitrogenase from production of NH_3 to increased production of H_2 (Hadfield & Bulen, 1969). Burgess et al. (1981) directly established that H_2 does not alter the total electron flux under N_2 by following the rate of dithionite oxidation in the presence and absence of H_2. These same electron allocation effects hold in the presence of D_2 only if the production of HD is allotted one electron per HD (Bulen, 1976; Newton et al., 1977). In addition, HD formation occurs almost entirely at the expense of NH_3 formation, and very few electrons, if any, are diverted by D_2 from H_2 evolution into HD production (Newton et al., 1977). Similarly, it is thought that H_2 inhibits NH_3 formation by causing production of H_2 in an N_2-dependent reaction without significantly affecting the other form of H_2 evolution, which occurs maximally in the absence of N_2.

Burgess et al. (1981) further ruled out the involvement of an exchange mechanism for HD production by demonstrating that the rate of incorporation of tritium from T_2 into the

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