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^{13}C and ^{1}H Nuclear Magnetic Resonance Relaxation Measurements of the Lipids of Sarcoplasmic Reticulum Membranes†

J. D. Robinson,‡ N. J. M. Birdsall, A. G. Lee,§ and J. C. Metcalfe*

ABSTRACT: ^{13}C nuclear magnetic resonance spectra of sarcoplasmic reticular membranes yield several well-defined resonances from the membrane lipids, identified as the terminal methyl, $(CH_2)_n$, and olefinic carbons of the fatty acid chains and the N^+Me_3 of the choline head group of lecithins. The spectra correspond in intensity to 60–90% of the membrane lipids which is substantially more than is observed in the sharp components of the ^{1}H spectrum. The proton spectra are very similar to those described by Davis and Inesi (Davis, D. G., and Inesi, G. (1971), *Biochim. Biophys. Acta 241*, 1) in which, unlike the ^{13}C spectra, the N^+Me_3 resonance is very weak at 30°. The spin–lattice (T_1) relaxation times have been measured for the assigned resonances in both the ^{13}C and ^{1}H spectra and are compared with the relaxation times of sonicated vesicles of the lipids extracted from the membrane. The ^{13}C chain resonances have very similar T_1 values in both the membrane and vesicle preparations, but the N^+Me_3 T_1 value is significantly shorter in the membrane. This is tentatively attributed to interaction with membrane protein. The relaxation of the $(CH_2)_n$ proton resonance is not characterized by a single T_1 relaxation time in the membrane, and the relaxation of the $(CH_2)_n$, CH_3, and N^+Me_3 groups are all complex in the extracted lipid preparation, unlike the relaxation observed in vesicles of homogeneous lecithins. The results are used to define the problems of relating relaxation measurements to the organization of the lipids in the membrane. An independent estimate of the extent of the bilayer in the membranes from measurement of the binding of the spin-label 2,2,6,6-tetramethylpiperidine-1-oxyl is consistent with the ^{13}C intensity and relaxation data.

The proton nuclear magnetic resonance (nmr) spectrum of the sarcoplasmic reticulum membrane recently described by Davis and Inesi (1971) is the first high-resolution spectrum from a functionally intact membrane. These spectra contain sharp well-defined resonances corresponding in intensity to about 20% of the protons in the fatty acid chains of the phospholipids ($(CH_2)_n$ and CH_3), while the remaining fatty acid protons appear to be included in a much broader peak under these sharp resonances. In addition, although about 75% of the membrane phospholipids are lecithins (Fiehn and Hasselbach, 1970; Meissner and Fleischer, 1971), the intensity of the choline N^+Me_3 resonance was very weak, and at higher temperatures where the resonance became more fully developed there was a simultaneous decline in the ability of the vesicles to accumulate calcium (Davis and Inesi, 1971).

These spectral features raise several issues regarding the organization of the lipids within the membrane. The appearance of only 20% of the fatty acid chain protons in the sharp components of the membrane spectra might correspond to that fraction of the lipids in a simple bilayer structure unperturbed by the presence of membrane protein, with the major fraction of the lipid resonances broadened either as a result of some more tightly packed structural organization, or by direct intermolecular interaction with membrane proteins. Alternatively, the sharp resonances might represent a "superfluid" fraction of the lipids with motional characteristics that reflect the functional environment of the calcium-transport system, which involves a major portion of the membrane protein (Ikemoto *et al.*, 1971). Clearly the possibility of heterogeneous regions of lipid composition or organization is of considerable interest. Similarly the diminished N^+Me_3 resonance, apparently related to functional integrity, suggests intimate intermolecular interactions with other membrane components, since in sonicated lipid vesicles of synthetic lecithins and natural lecithin mixtures, the N^+Me_3 proton resonance is fully developed (Sheard, 1969; Birdsall *et al.*, 1971; Lee *et al.*, 1972).

† From the Medical Research Council, Molecular Pharmacology Unit, Medical School, Hills Road, Cambridge. *Received March 21, 1972.*
‡ National Science Foundation senior post doctoral fellow; present address: Department of Pharmacology, State University of New York, Upstate Medical Center, Syracuse, N. Y.
§ Research Fellow of Kings College, Cambridge.

Recently we have shown that the relaxation times of ^1H and ^{13}C nuclei of phospholipids depend on both the structural organization of the lipid molecules and on the chemical structure of the lipids themselves (Levine *et al.*, 1972a). It therefore seems possible that the membrane proteins will impose a characteristic pattern of molecular motion on the lipids with which they interact, which will result in a T_1 value distinct from those of the separated lipids organized in a bilayer structure. In this paper, the spin–lattice relaxation time (T_1) of both the proton and ^{13}C nuclei of the lipids in the membrane and in vesicles of the extracted lipids are compared to determine whether the T_1 values are consistent with a simple bilayer organization of the lipids in the membrane. The ^{13}C spectra have the advantage over the proton spectra that a much larger fraction of the lipid chains is observed in the membrane, and the N^+Me_3 resonance is also well defined under conditions where the corresponding proton resonance is not observed.

The ^{13}C experiments on membranes at the natural abundance of ^{13}C of 1.1% are at the limits imposed by instrumental sensitivity, and it is difficult to quantitate precisely the fraction of lipids observed in the membrane spectrum. The binding of the spin-label Tempo[1] depends critically on the fluidity of the lipid structure to which it binds (Hubbell and McConnell, 1968, 1971; Hubbell *et al.*, 1970) and can be used as an independent technique for probing the organization of the lipids in the membrane (Metcalfe *et al.*, 1972). For this purpose the binding of Tempo to the microsomal membranes has been compared with the binding to an equivalent concentration of extracted lipid.

Materials and Methods

Membranes. Sarcoplasmic reticulum from the white leg muscles of the rabbit was prepared by a slight modification of the method of Davis and Inesi (1971). The muscle was minced in a meat grinder and then homogenized in a blender in three volumes of 0.3 M sucrose and 0.02 M histidine (pH 7.3). The homogenate was centrifuged at 10,000*g* for 15 min, and the supernatant material was decanted; the pellet was rehomogenized and again centrifuged as before. The combined supernatant material was then centrifuged at 54,000*g* for 1 hr. The pellets were suspended in 0.6 M KCl, 0.3 M sucrose, and 0.01 M histidine (pH 7.3) with a TenBroek homogenizer, and allowed to stand at 0–4° for 30–60 min. The mixture was then centrifuged at 10,000*g* for 15 min, and the resultant supernatant material at 54,000*g* for 1 hr. Finally, the pellet was washed by resuspension and centrifugation at 54,000*g*; twice in 5 mM phosphate buffer (pH 7.0) in 90% D_2O, and twice in 99.8% D_2O. For nmr studies the pellet was usually transferred directly to 12-mm tubes without resuspension; for studies of ATPase activity and calcium accumulation the pellet was resuspended in the original homogenizing medium with a TenBroek homogenizer.

Lipids were extracted from the sedimented sarcoplasmic reticulum by homogenizing in 20 volumes of chloroform–methanol (2:1, v/v), following the procedure of Folch *et al.* (1957). After filtration, the extract was washed with one-fifth volume of 1 mM $MgCl_2$, and the lower phase evaporated to dryness under N_2 in a rotary evaporator. The lipid was suspended in D_2O by vigorous shaking, and dispersed to optical clarity by sonication (Lee *et al.*, 1972). Microsomal protein was prepared in a soluble form by adaptation of the butanol extraction procedure described by Maddy (1964) for erythrocyte membranes.

ATPase activity was measured in terms of the production of inorganic phosphate after incubation for 5–10 min at 30° in media containing 30 mM histidine (pH 7.0), 3 mM ATP, 3 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 100 mM KCl. Activity in the absence of added $CaCl_2$ and the presence of 0.1 mM EGTA was measured concurrently. The reaction was stopped by the addition of trichloroacetic acid, the mixture was centrifuged, and inorganic phosphate was measured by the method of Lowry and Lopez (1946).

Calcium accumulation was measured in terms of the removal of ^{45}Ca from the medium following incubation for 3–12 min at 30° in 30 mM histidine (pH 7.0), 3 mM ATP, 3 mM $MgCl_2$, 0.1 mM $CaCl_2$ containing tracer amounts of ^{45}Ca, 100 mM KCl, and 5 mM sodium oxalate. Accumulation in the absence of ATP was measured concurrently. The reaction was terminated by filtration through Millipore filters (pore size 0.45 μ), and the radioactivity in a portion of the filtrate was measured in Bray's solution with a liquid scintillation counter.

Protein content was measured by the biuret method, using bovine serum albumin as a standard. Lipid phosphorus was measured using the method of Fiske and Subbarow (1925).

Nuclear Magnetic Resonance Measurements. ^{13}C and ^1H spectra were obtained by the Fourier transform technique on a Varian XL-100 spectrometer, locked on to deuterium in the D_2O solvent, and accumulated in a Varian 620i computer. The T_1 relaxation times were measured by the inversion recovery method described by Freeman and Hill (1971), where a $\pi - t - \pi/2 - t_\infty - \pi/2 - t_\infty$ pulse sequence gives alternate measurements of the magnetization S_t at time t (seconds) after the π pulse, and the thermal equilibrium magnetization, S_∞. The value of t_∞ between the $\pi/2$ pulses was at least five times the longest T_1 value in the sample. The deviation from equilibrium ($S_\infty - S_t$) was then displayed directly as the Fourier transform of the free induction decay accumulated from a series of such pulse sequences. It is generally assumed that the decay of the intensity of the resonance ($S_\infty - S_t$) *vs.* t is exponential for molecules in solution at room temperature, and that the slope of a plot of log ($S_\infty - S_t$) *vs.* t is determined by the relaxation time T_1. Any nonlinearity in the decay of the signal intensity then implies that the nuceli contributing to the resonance have more than one relaxation time T_1. Previous work has shown that substantial proportions of the nuclei giving rise to the observed resonance must have significantly different relaxation rates to be readily distinguishable from a single relaxation process by this technique, since the errors in measuring resonance intensities of low signal to noise at long t values usually imply that accurate data can only be obtained for a limited range of t values ($t < 5T_1$).

For the ^{13}C T_1 measurements of the microsomal membranes and lipid vesicles, 2000 ($\pi - t - \pi/2 - t_\infty - \pi/2 - t_\infty$) pulse sequences were generally accumulated over 3 hr for each t value, whereas for the ^1H measurements, 50 pulse sequences were accumulated over 10 min for each t value. The membrane samples were approximately 15% w/w for the ^{13}C experiments, and 5% for the ^1H measurements; the experiments on lipid vesicles were performed on equivalent concentrations of lipids to those in the membranes. Proton line width measurements were made using the XL-100 spectrometer in the continuous wave mode.

Tempo binding measurements were made using a Varian E3 electron spin resonance (esr) spectrometer at Tempo con-

[1] Abbreviations used are: T_1, spin–lattice relaxation time; T_2, spin–spin relaxation time; Tempo, 2,2,6,6-tetramethylpiperidine-1-oxyl.

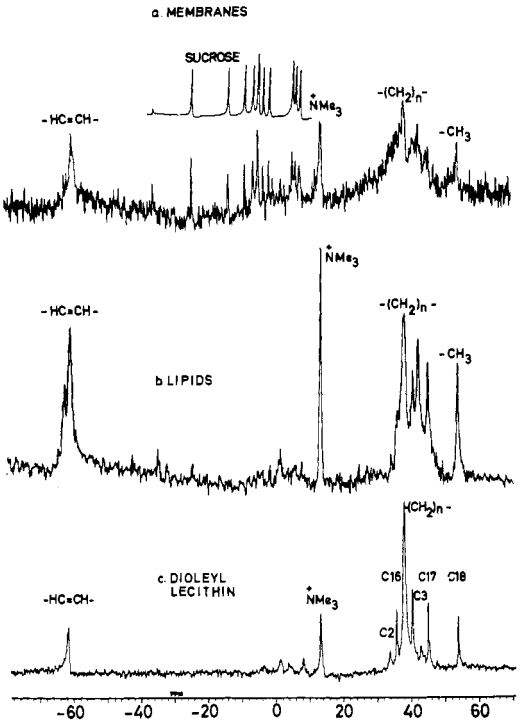

FIGURE 1: ^{13}C nmr spectra of (a) microsomal membranes (15% w/w) at 31° (the inset sucrose spectrum indicates the resonances which arise from sucrose trapped in the vesicles during preparation); (b) sonicated microsomal lipids (20% w/w) at 31°; (c) sonicated dioleyllecithin (20% w/w) at 52°. All samples were in D_2O; details of the assignment of the resonances are given elsewhere (Levine *et al.*, 1972a; Birdsall *et al.*, 1972).

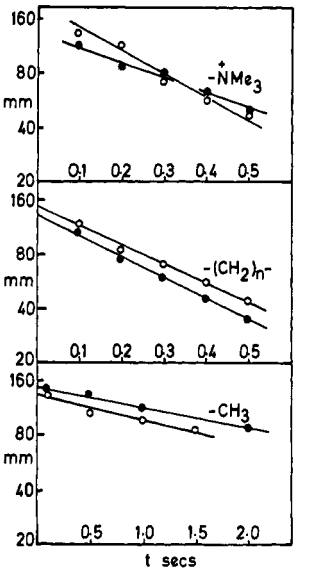

FIGURE 2: Amplitudes (mm) of ^{13}C nmr resonances at 31° as a function of t of microsomal membranes (○) and sonicated microsomal lipids (●).

centrations of 1×10^{-4} to 5×10^{-4} M. The proportion of Tempo bound to the membranes and lipid vesicles was estimated from the difference in intensity of the high-field resonance from Tempo free in the aqueous medium and of the corresponding resonance of a standard Tempo solution of the same total concentration in the absence of the membranes (Metcalfe *et al.*, 1972). Equilibrium dialysis experiments to estimate Tempo binding to the membrane protein were performed as described elsewhere (Colley *et al.*, 1971); the depleted Tempo concentrations were estimated from the intensities of the esr spectra.

Results

^{13}C Nuclear Magnetic Resonance Spectra. Although the natural abundance of ^{13}C is only 1.1%, a useful spectrum of the sarcoplasmic reticulum membrane can be obtained, and this is compared to the spectrum of a sonicated suspension of the extracted membrane lipids in D_2O (Figure 1a,b). The N^+Me_3 and chain resonances in the membrane and lipid spectra are assigned by comparison with the chemical shifts of dioleyllecithin (Figure 1c), since these shifts have been found to be very similar in lecithins of different chain structure (Levine *et al.*, 1972a). Comparison of the N^+Me_3 intensities in the membrane and the lipid spectra after comparable spectral accumulation indicated that 75 ± 15% of the lipids are observed in the membrane spectrum. Although a more precise estimate is not possible at the signal to noise level obtainable, it is clear that a much larger fraction of the membrane lipid chain nuclei are observed in the ^{13}C spectrum than in the sharp components of the ^{1}H spectra described below. The N^+Me_3 resonance is also well defined in the ^{13}C spectrum, in contrast to the very weak proton N^+Me_3 resonance obtained from the same membrane sample at 30°.

Despite the signal to noise problem in the membrane spectra, it was possible to measure the T_1 relaxation times for three of the membrane lipid resonances (N^+Me_3, $(CH_2)_n$, and CH_3) in Table I. Plots of peak amplitude *vs.* t were consistent with a single T_1 relaxation time for each resonance (Figure 2), although the limited number of t values compatible with the accumulation times required does not rule out the possibility of a small fraction of the resonance intensity decaying at a faster rate (*e.g.*, at very short t values <0.1 sec) as found in the ^{1}H measurements described below. Comparison of the T_1 values from the membrane and the lipids shows no significant difference for the $(CH_2)_n$ and the CH_3 resonances, but the T_1 value of the N^+Me_3 resonance is about 35% shorter in the membrane than in the extracted lipid bilayer. There were no major differences in the line widths of the N^+Me_3 resonances in the two preparations, but the heavy smoothing function applied to the Fourier-transformed spectra to improve the signal to noise ratio will tend to obscure any small differences in the line widths. The membrane samples are much more glutinous at 10% (w/w) than the corresponding sonicated vesicle suspensions, but this does not appear to be an important factor in determining the ^{13}C nmr line widths of membrane lipid resonances (Metcalfe *et al.*, 1972).

TABLE I

	T_1 (sec)[a]		
	$N^+(CH_3)_3$	$(CH_2)_n$	CH_3
Membrane	0.36 ± 0.03	0.42 ± 0.03	3.1 ± 0.6
Lipid vesicles	0.55 ± 0.06	0.37 ± 0.01	3.7 ± 0.3

[a] T_1 relaxation times of ^{13}C lipid nuclei in microsomal membranes and extracted lipid vesicles at 31 ± 1°.

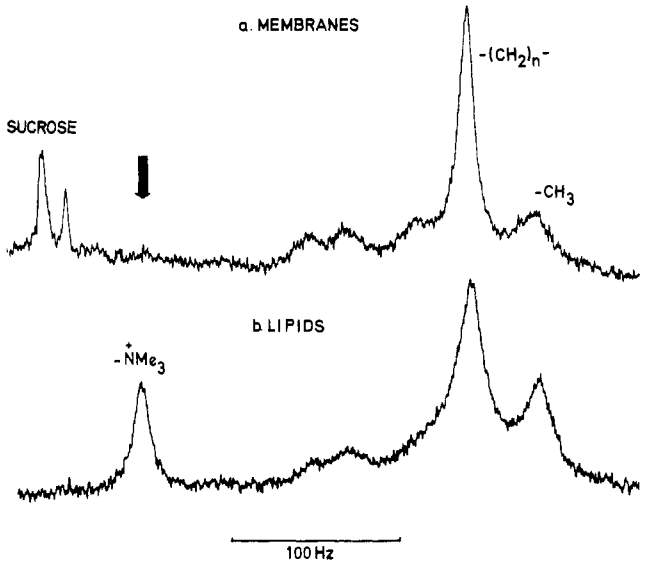

FIGURE 3: ^1H nmr spectra at 32° of (a) microsomal membranes (5% w/w) and (b) sonicated microsomal lipids (5% w/w) in D_2O.

^1H Nuclear Magnetic Resonance Spectra. The ^1H nmr spectrum of the membrane preparation (Figure 3a) is in general agreement with that of Davis and Inesi (1971); at 30° the N^+Me_3 resonance is barely detectable, and a large broad peak appears to lie beneath the sharp chain resonances. In contrast, the ^1H spectrum of the lipids extracted from the sarcoplasmic reticulum showed a well-developed N^+Me_3 resonance (Figure 3b). Comparison of the area beneath the sharp resonances in the membrane spectrum and the corresponding resonances of lipid vesicles at an equivalent lipid concentration, indicated that only 24 ± 5% of the total membrane lipid signal appeared in the sharp components of the membrane spectrum. The addition of 0.1 M KCl did not significantly alter the appearance of the membrane spectrum, so that the sharp resonances do not merely represent an effect of low ionic strength on the membrane structure.

The T_1 relaxation of the sharp $(CH_2)_n$ component in the membrane spectrum did not follow a simple exponential decay, whereas the decay of the terminal CH_3 was indistinguishable from a single relaxation process (Figure 4a). The proportion of the $(CH_2)_n$ signal amplitude in the more rapidly relaxing component (t = <0.1 sec) was about 30%, which is a very small proportion of the total lipid chains in the membrane.

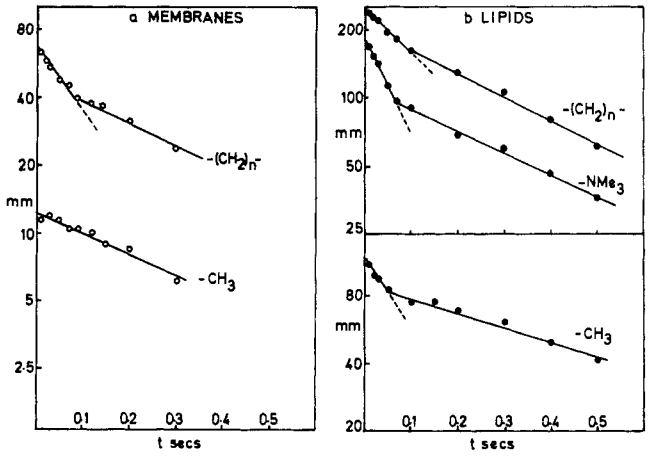

FIGURE 4: Amplitudes (mm) of ^1H nmr resonances at 32° as a function of t of (a) microsomal membranes and (b) sonicated microsomal lipids. All the resonances show complex relaxation processes except for the terminal CH_3 of the membranes.

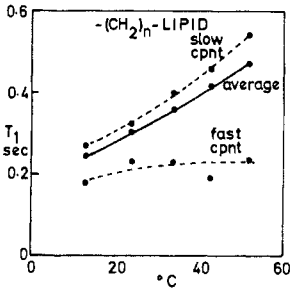

FIGURE 5: The proton T_1 relaxation times of the $(CH_2)_n$ resonance of the microsomal membranes as a function of temperature. The dashed curves are the approximate relaxation times of the fast and slow components in the resonance decay curves (see Figure 4). The average values (full line) were calculated using all points on the resonance decay curves.

For all three major resonances in the spectra of the extracted lipids ($(CH_2)_n$, CH_3, and N^+Me_3) the time course of relaxation did not follow a simple exponential decay (Figure 4b). Approximate T_1 values for the fast and slow components could be estimated graphically, and are compared to an average T_1 value calculated by fitting all the points by the method of least squares to a single exponential process. It can be seen in Figure 5 that for the $(CH_2)_n$ resonance from the lipid vesicles, the major component with the longer relaxation time dominates the average T_1 and they have a very similar temperature dependence. However the shorter T_1 values are inherently sensitive to errors in the graphical analysis of complex exponential curves, and for comparison of the T_1 values of the membrane and the extracted lipid the average T_1 values calculated over all t values have been used, as shown in Figure 6a,b. Although the temperature dependence of T_1 for the terminal methyl is apparently smaller in the membrane than the lipid vesicles, the average T_1 values for the two structures are generally simillar. More detailed comparisons are not justified by the treatment of the data described above. The relaxation data are also compared to data for sonicated suspensions of egg lecithin (Figure 6c), which show similar relaxation times to the membrane lipid vesicles; in the egg lecithin preparation only the $(CH_2)_n$ resonance does not relax with a single exponential time course (Lee *et al.*, 1972).

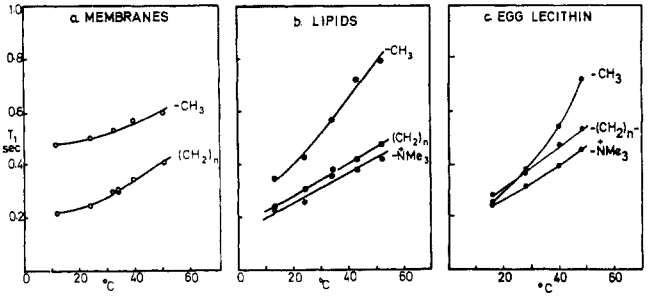

FIGURE 6: Proton T_1 relaxation times as a function of temperature for (a) microsomal membranes, (b) sonicated microsomal lipids, and (c) sonicated egg lecithin (from Lee *et al.*, 1972).

The line widths at half-height ($\Delta\nu_{1/2}$) of the sharp $(CH_2)_n$ resonance from the membrane decreases from 14.6 Hz at 8° to 8.1 Hz at 50°, which is approximately half the line width of the $(CH_2)_n$ resonance from the sonicated lipid vesicles of 21.5 ± 2.0 Hz over the same temperature range. The terminal methyl line widths are similar in both preparations and are only slightly affected by temperature in both preparations (20–25 Hz between 17 and 50°). Accurate measurements of the terminal methyl are not possible because of overlap with the $(CH_2)_n$ peak. The line widths indicate that $\Delta\nu_{1/2} \gg 1/\pi T_1$ and that T_2 values estimated from the line widths are much shorter than T_1 values, as described previously for egg lecithin and dipalmitoyllecithin preparations (Lee *et al.*, 1972).

Tempo Binding. The resolution of the high-field resonance of Tempo into a free (F) component in the aqueous medium, and a bound (B) component in a fluid hydrophobic region of the membrane is shown in Figure 7. The percentage of Tempo bound to the membranes compared with an equivalent concentration of extracted lipid vesicles at 22° is 73 ± 8% (average of experiments at three Tempo concentrations: 1×10^{-4}, 3×10^{-4}, and 5×10^{-4} M). Less than 5% of the Tempo bound by the membrane was bound by an equivalent concentration of separated membrane protein, estimated by equilibrium dialysis.

(Mg^{2+} + Ca^{2+})-ATPase and Ca^{2+} Accumulating Activity. To assess the functional capacity of the sarcoplasmic reticulum membranes used in the nmr experiments, a preparation that was washed with D_2O was resuspended in 0.3 M sucrose and compared with an unwashed control preparation in the same buffer. The calcium-dependent ATPase activity for the D_2O-washed preparations and unwashed control was 1.2 and 1.3 μmoles of P_i liberated per min per mg of protein; the ATP-dependent calcium accumulating capacity was 1.2 μg-atoms of Ca^{2+} accumulated in 10 min/mg of protein for both preparations. Both sets of data for activity are within the ranges reported for other sarcoplasmic reticulum preparations (Sreter *et al.*, 1970; Martonosi and Feretos, 1964; Inesi and Asai, 1968). These experiments indicate that the sharp resonances observed in the membrane ^{1}H nmr spectra are not associated with irreversible disruption of the membrane at low ionic strength in D_2O.

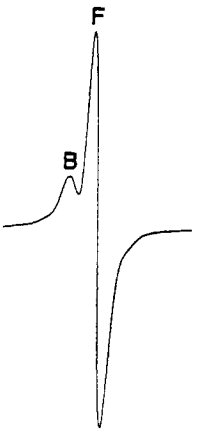

FIGURE 7: The high-field resonance of 5×10^{-4} M Tempo in the presence of 3.0% w/w microsomal membranes at 25°.

Discussion

The ability to compare the relaxation times of lipids in a membrane with those of the same lipids organized in a simple bilayer structure in vesicles provides a technique for examining the effect of membrane proteins on the organization of the lipids. It is very probable that constraints will be imposed on the molecular motion and organization of at least some of the lipids in the membrane compared to the unperturbed bilayer, since strong intermolecular interactions between lipid and protein are required to maintain the structure. These intermolecular interactions may affect only those lipids in direct contact with the proteins, or the effects may extend throughout the structure and affect the overall packing of bilayer regions in the membrane.

If the proteins also require specific lipids for maintenance of the membrane structure or their functions, as suggested, for example, by the activation of several membrane-bound enzymes by specific phospholipids (*e.g.*, Levey, 1971) this may impose some segregation of lipids in different regions of the membrane, distinct from that in the simple bilayer structure of the vesicle. Even in the vesicles the local lipid composition is known to be nonrandom in mixtures of lipids of sufficiently different chain structure (Phillips *et al.*, 1970). It is also possible that in addition to modifying the basic bilayer structure of the membrane lipids, some other distinctive structural arrangement may be imposed on some membrane lipids, although as yet there is no experimental evidence to support this, and in general physical studies of membranes have been consistent with a basic bilayer organization of the membrane lipids. However, it remains difficult to establish with precision the proportion of lipids which can be shown to be in an unmodified bilayer, or to define the effect of protein interactions as outlined above.

Comparison of the relative intensities of the well-defined lipid resonances in the ^{13}C nmr spectra from the membranes and the extracted lipids indicates that about three-fourths of the lipids is represented in the membrane spectra. The relaxation times of the lipid chain resonances in the two preparations were similar, the only significant difference being for the N^+Me_3 resonance, where the value was shorter in the membrane by 35%. The similarity between the chain relaxation times in the membrane and the simple bilayer is consistent with the interaction of the spin-label Tempo with the membrane; the membrane binds 73% of the amount of Tempo bound by vesicles containing an equivalent concentration of extracted lipid. Although these Tempo measurements can only be regarded as preliminary estimates, the observation of a resolved resonance from a fluid hydrophobic region of the membrane is certainly characteristic of a bilayer structure, since no resolved resonance is observed from Tempo in micelles of sodium dodecyl sulfate (J. R. S. Hoult and J. C. Metcalfe, unpublished observations). It should be noted that in comparing Tempo binding to the membrane and to the lipid vesicles it is essential to use a representative sample of the membrane lipids. For example, dioleyllecithin vesicles bind more Tempo than dipalmitoyllecithin below the thermal transition, while cholesterol displaces less Tempo from dioleyllecithin than from dipalmitoyllecithin (J. R. S. Hoult and J. C. Metcalfe, unpublished observations). Modification of the bilayer regions of the membrane by the protein, for example, by affecting the packing of the lipids, or by causing separation into bilayer regions of differing lipid composition could affect the binding of Tempo compared to the simple bilayer structure. The very low level of Tempo binding to the separated microsomal membrane protein is taken as strong evidence that protein binding in the intact membrane is also unimportant, since separated membrane

proteins generally have a much higher binding capacity for small molecules than the intact membrane (Colley *et al.*, 1971; Metcalfe *et al.*, 1971).

In contrast to these estimates of the bilayer organization based on Tempo binding and on ^{13}C nmr resonance intensities and relaxation times, the ^{1}H nmr intensities of the sharp well-defined lipid chain resonances in the membrane spectra were only one-fourth of that of the corresponding resonances in the extracted lipid bilayer. Davis and Inesi (1971) commented that the binding of Tempo to the microsomal membranes is consistent with the presence of a low viscosity region in the membrane, but the present Tempo binding experiments indicate that this region is much more extensive than the proportion of lipid chain protons observed in the sharp resonances of the ^{1}H nmr membrane spectrum. However, the lipids giving rise to the sharp resonances are certainly derived from a fluid hydrophobic region of the membrane, since preliminary experiments have shown that the T_1 values of the resonances are reduced by the presence of 10^{-4} M Tempo.

The sensitivity of the proton line widths to intermolecular interactions is probably responsible for the small proportion of the lipid chain protons in the sharp resonances, compared to the proportion of the corresponding ^{13}C nuclei observed in the ^{13}C spectrum. The ^{13}C spectrum must contain resonances from many of the chains for which the proton resonances are severely broadened, and since the T_1 values of the ^{13}C chain resonances are consistent with a bilayer structure, the proton resonance broadening in the membrane is most likely to be due to the membrane protein causing intermolecular line broadening. This effect will be much greater for the proton nuclei in the lipid chains than for the carbon nuclei, since intermolecular proton–proton distances will be smaller than intermolecular carbon–proton distances and since the gyromagnetic ratio for protons is greater than that of carbon by a factor of 4. However, the reduced temperature dependence of the proton T_1 value of the sharp terminal methyl resonance in the membrane compared to the vesicle could either be due to the effect of the protein, or to heterogeneity of lipid regions in the membrane in which the sharp resonances are derived from a selected lipid population. For example, the single T_1 relaxation process observed for the terminal methyl in the membrane compared to the more complex relaxation process in the lipid vesicles may indicate that only a selected class of lipids are observed in the membrane. Approximately 65% of the fatty acid chains of the microsomal lipids are unsaturated (Fiehn and Hasselbach, 1970) so that at least 30% of the lipids must have two unsaturated chains; these may be likely candidates for the chain resonances observed in the sharp component of the membrane ^{1}H spectrum. Davis and Inesi (1971) have pointed out that the relatively large signals from the $CH_2CH{=}$ and $CH{=}C$ in unsaturated chains suggest that a high proportion of the protons in the sharp resonances are derived from unsaturated lipids. It would also be of interest to know whether the lipids giving rise to the sharp chain resonances are lecithins of which the N^+Me_3 head groups are not observed, or whether all the lecithin chain protons are contained in the broad component of the membrane spectrum.

The absence of the N^+Me_3 resonance in the ^{1}H spectra cannot be accounted for by the relatively small difference in the T_1 values of the ^{13}C N^+Me_3 resonance in the membrane and the vesicles, and it is clear that the proton line width is increased by a much larger factor than the change in T_1. The most probable explanation is that the proton broadening is due to interaction of the N^+Me_3 protons with membrane protein, causing intermolecular dipolar broadening as described above. The reduction in the ^{13}C T_1 value for the N^+Me_3 is larger than any effect which has been observed on addition of polyvalent cations to lecithin vesicles (Levine *et al.*, 1972b) and probably cannot be attributed directly to ionic interaction of the head group with simple solvated ions.

In vesicles of single defined lipids (*e.g.*, dipalmitoyllecithin and dioleyllecithin) the relaxation processes of all three major proton resonances are indistinguishable from a single T_1 process, while in egg lecithin only the $(CH_2)_n$ resonance decays nonexponentially. This was attributed to the heterogeneity of the chain structure (Lee *et al.*, 1972) which was insufficient to cause any detectable nonexponential relaxation of the N^+Me_3 or terminal methyl resonances. The heterogeneity of the relaxation processes has to be quite pronounced to be readily detectable by this method of measuring relaxation times, and the complex relaxation processes of all three major resonances in the microsomal lipid vesicles could simply be a feature of extreme heterogeneity of the lipid composition, or it may imply that lipids with different chemical structures are grouped in mosaic regions of the bilayer, in which lateral diffusion between the regions is too slow to average the relaxation time of the different lipids. It is concluded that analysis of the behavior of model systems by the techniques described should allow the organization of the lipids in the membrane to be defined in more detail.

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α-Methylaminomalonate-Dependent Reactions of 5-Deoxypyridoxal†

John W. Thanassi

ABSTRACT: The reactions between α-methylaminomalonic acid and the B_6 analog, 5-deoxypyridoxal, have been studied at pH 5.2 (30°). The initial and required decarboxylation step results in a carbanionic Schiff base which partitions into the formation of four ultraviolet-absorbing products, three of which have been identified by their nuclear magentic resonance and mass spectra. The reaction pathways leading to these products involve a transaminative decarboxylation and at least two carbanion condensations, one of which leads to a dimer (structure I) incorporating one molecule of 5-deoxypyridoxal and one molecule of 5-deoxypyridoxamine. In addition to these reactions, there is buffer catalysis of the 5-deoxypyridoxal-catalyzed decarboxylation of α-methylaminomalonate; this catalysis is suggested to be general acid in nature. The results of these experiments are discussed in terms of the mechanism of action of B_6-dependent enzymes, with particular reference to Dunathan's suggestions concerning the reaction specificity of such enzymes.

Vitamin B_6 dependent enzymes catalyze a wide variety of extremely important reactions in the intermediary metabolism of amino acids. For example, decarboxylation of histidine and 5-hydroxytryptophan by B_6-requiring decarboxylases give rise to the pharmacologically active substances, histamine and serotonin, respectively. All of the amino acid transaminases also require vitamin B_6. These enzymes fulfill a key role in overall nitrogen balance and serve to connect amino acid and carbohydrate metabolism. Many other types of reactions are catalyzed by pyridoxal phosphate requiring enzymes, and there are a number of general reviews available which discuss these in detail (Braunstein, 1960; Snell, 1958).

In an attempt to elucidate the role of this vitamin in the molecular mechanisms of an apparently large variety of enzyme-catalyzed reactions, model systems have been extensively examined (Metzler *et al.*, 1954; Auld and Bruice, 1967; Bruice and Benkovic, 1966). Although most of the enzymatic reactions have been reproduced in such studies, they generally require elevated temperatures and/or relatively long reaction times. In contrast, as shown in a previous communication (Thanassi, 1970), the reactions between aminomalonic acid, $NH_2CH(COOH)_2$, and 5-deoxypyridoxal, a B_6 analog, occur very rapidly at 30°. Thus, aminomalonate derivatives appear to be uniquely sensitive to pyridoxal-catalyzed reactions. The experiments reported herein deal with the reactions occurring between 5-deoxypyridoxal and α-methylaminomalonic acid, $NH_2C(CH_3)(COOH)_2$.

Experimental Section

Materials. 5-Deoxypyridoxal was synthesized by the method of Mühlradt and Snell (1967), as described previously (Thanassi, 1970). The preparation of the monoammonium salt of α-methylaminomalonic acid is described elsewhere (Thanassi, 1971). All other chemicals were reagent grade. Water employed in these experiments was house-distilled water, redistilled from all-glass apparatus. D_2O (99.8%) and AG 50W ion-exchange resin were obtained from Bio-Rad.

Reaction between 5-Deoxypyridoxal and α-Methylaminomalonic Acid; Isolation of Fractions I–IV. 5-Deoxypyridoxal (608 mg, 4.0 mmoles) was dissolved in 400 ml of 0.04 N ammonium acetate buffer at pH 5.2. To this solution was added 2.42 g (16 mmoles) of the monoammonium salt of α-methylaminomalonic acid. The reaction was allowed to proceed for 1 hr in the dark in a nitrogen atmosphere at room temperature. After acidification from pH 5.3 to pH 1 with concentrated HCl, the reaction solution was applied to a 1.5 × 100 cm column of AG 50W × 8 (200–400 mesh) in the hydrogen form. The column was washed with 2.0 N HCl and then eluted in a stepwise fashion with increasing concentrations of HCl at a flow rate of 30–40 ml per hour (Figure 1). Fractions were collected at 30-min intervals, and the absorbancies at 295 nm were measured. The tubes containing the individual fractions designated I through IV in Figure 1 were combined, and the separate pooled fractions were concentrated to dryness on a rotary evaporator at a bath temperature not exceeding 40°. Approximately 80% of the absorbancy applied to the column was recovered in the four fractions. The individual fractions were then decolorized with charcoal. Fraction IV was recrystallized from 2-propanol–ether. Fractions I–III were recrystallized from water–2-

† From the Department of Biochemistry, University of Vermont College of Medicine, Burlington, Vermont 05401. *Received August 16, 1971.* These studies were supported by U. S. Public Health Service Grant No. 5-R01-AM 12436.