^{13}C Fourier Transform Nuclear Magnetic Resonance Studies of Fractionated *Candida utilis* Membranes†

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ABSTRACT: ^{13}C Fourier transform nuclear magnetic resonance has been used to study the lipid structure and dynamics of fractionated *Candida utilis* cell membranes. Measurements of the spin-lattice relaxation times indicate the existence of mobility gradients in the direction of increased mobility from the glycerol backbone toward the terminal methyl group of the fatty acid and toward the choline methyls. The temperature dependence of the relaxation times gives activation energies of ~4–6 kcal/mol for the rotations about the various carbon–carbon bonds which determine the relaxation rates. In general, comparison with data which have been reported for artificial membrane systems indicates that the contributions of protein–lipid interactions to the T_1 gradient are of negligible importance in the yeast membrane system. A dynamical model for the motion about bonds near unsaturated bonds which determines the relaxation of the unsaturated carbons is also proposed. Measurements of chemical shifts with temperature also exhibit a correlation with chain position. On the basis of these data a correlation of ΔE, the energy difference between gauche and anti conformations for γ carbons, with chain position is inferred. In addition, an estimate of 1.2 kcal/mol can be obtained for ΔE for carbons near the end of the fatty acid chain. This value indicates that intermolecular interactions contribute substantially to ΔE since a value of ~0.5 kcal/mol can be ascribed to intramolecular interactions.

Carbon-13 nuclear magnetic resonance (NMR) spectroscopy has been recognized as a valuable tool for the study of both synthetic liposome membranes (Oldfield and Chapman, 1971; Levine et al., 1972a,b; Birdsall et al., 1972; Williams et al., 1973; Batchelor et al., 1972; Godici and Landsberger, 1974; Gent and Prestegard, 1974) and, in several cases, naturally occurring membrane systems (Metcalfe et al., 1971, 1972; Metcalfe, 1972; Robinson et al., 1972; Lee et al., 1973; Keough et al., 1973). Line width, spin-lattice relaxation time, and chemical shift data provide detailed information about the structure and dynamics of membrane lipids without introducing into the system under study a perturbation such as a spin-label (Seelig and Niederberger, 1974). Recent investigations of naturally occurring membranes have, however, been somewhat less successful than studies of synthetic liposomes due to the low ^{13}C concentrations and the complexity of the former. The technique of specific enrichment of growing cells with a ^{13}C enriched membrane component has recently been exploited as one method for dealing with these problems (Metcalf et al., 1972). In the present study, nonspecific enrichment of yeast (*Candida utilis*) cells by growth on 20 atom % ^{13}C doubly enriched acetate was used to obtain adequate signal to noise levels for the cell membrane spectra, the 20% enrichment level avoiding the complication of ^{13}C–^{13}C multiplets which become significant at higher ^{13}C levels (London et al., 1975). A substantial simplification of the ^{13}C NMR spectrum was then obtained by removing water-soluble constituents by a combination of freeze–thaw cycling and osmotic shock; the remaining sample consists of lipid–protein complex, as well as the peptidoglycan cell wall, the latter being physically separated from the former according to microscopic examination. The resulting cell wall–membrane system is amenable to NMR study, the resolution of the fatty acid resonances being superior to that of any previously reported membrane ^{13}C spectrum, synthetic or natural. The separation techniques are also sufficiently gentle so that most of the proteins associated with the lipid are probably not denatured significantly, a conclusion which is supported by the NMR spectrum. Studies of these systems are important, we believe, for determining how closely the synthetic or "model" membrane systems which have been extensively studied resemble the more "natural" systems in which protein–lipid interactions may be significant. The high resolution character of the ^{13}C spectra of this system, for the fatty acid resonances particularly, has permitted precise measurements of the spin-lattice relaxation times, T_1, and the chemical shifts as a function of temperature. Based on these data, we conclude that this "fractionated" natural system exhibits mobility gradients for the fatty acid chains similar to those determined in synthetic and natural liposome systems (Godici and Landsberger, 1974, and references therein). A chemical shift gradient has also been measured and if such shifts arise from a steric interaction between γ carbons as postulated previously (Batchelor et al., 1972), the data reflect a gradient in ΔE, the energy difference between gauche and anti conformations, along the fatty acid chain. In addition, it is possible to estimate the effect of intermolecular lipid interactions on the magnitude of ΔE.

Materials and Methods

Yeast Growth. Agar slants of *Candida utilis*, strain CU-1, were obtained from Dr. James Mattoon, Department of Physiological Chemistry, The Johns Hopkins University School of Medicine. The cells were grown in a minimal medium described by Hernandez and Johnson (1967) with the trace element mixture of Button and Garver (1966) added. The carbon source for the isotopically enriched cells was acetic acid prepared in this laboratory by V. Kerr via the catalytic reduction of [^{13}C]carbon monoxide or [^{13}C]car-

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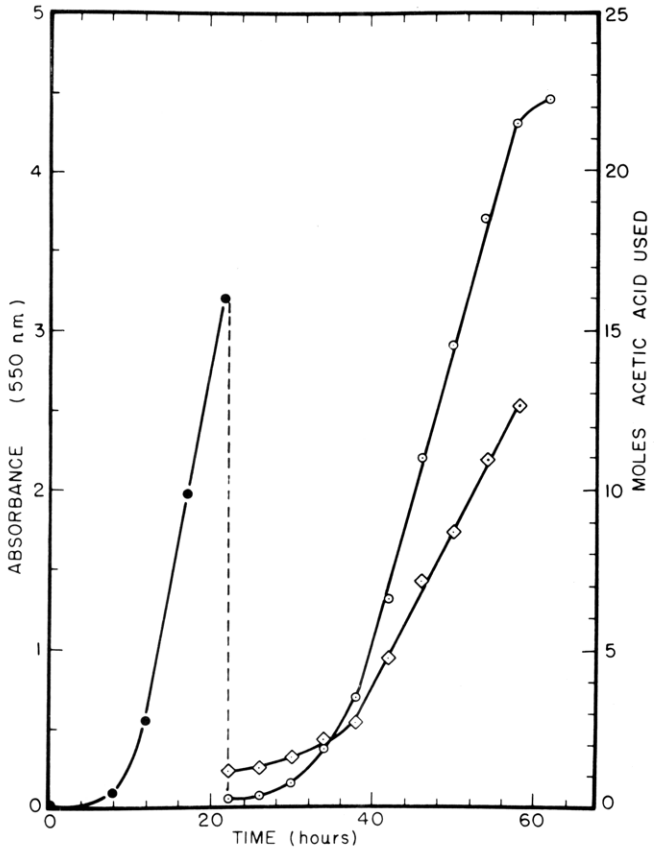

FIGURE 1: Growth of *C. utilis* on natural abundance and ^{13}C enriched acetic acid as measured by absorbance at 550 nm. A 1-l. portion of the natural abundance yeast grown in a 14-l. fermentor (●) was used to inoculate ~64 l. of medium in the 250-l. fermentor (⊙). Moles of carbon-13 acetic acid consumed as a function of time is also shown (◇).

bon dioxide to [^{13}C]methanol followed by catalytic carbonylation with [^{13}C]carbon monoxide to acetic acid. The acetic acid was randomly enriched for these studies to the 20 atom % level and its concentration in the growth medium was initially ~20 m*M*.

Figure 1 illustrates the results of a typical experiment in which a yeast inoculum was grown on natural abundance acetate in a 14-l. fermentor from which an aliquot was taken and transferred to a 250-l. fermentor containing about 65 l. of medium. Growth was continued on carbon-13 enriched acetic acid for the time period shown, during which the acetic acid was added automatically to maintain the pH at 5.7. The cultures were aerobically grown at 35°C using carbon dioxide free air until the labeled substrate was consumed; generally, this occurred when the culture density was about 3.5 g/l.

The yeast were passed from the fermentor into a 200-l. harvest cart and the temperature of the cell suspension was very rapidly changed from 35 to 5°C. The cells were removed from the medium by centrifugation through a Sharples A-16 centrifuge, resuspended, washed with cold normal saline and cold distilled water, then lyophylized, and stored in the freezer. Cells to be used fresh were washed in cold saline. *C. utilis* has been grown extensively (>2 kg dry cell wt) in this laboratory, using carbon-13 acetic acid (20–90 atom %) (Eakin et al., 1972). No significant differences in the growth rate between yeast grown on natural abundance acetic acid and enriched acetic acid were observed (Figure 1) (Edwards et al. 1970).

Cell Preparation. Lyophilized yeast cells randomly enriched to the 20 atom % level were suspended in distilled water and osmotically shocked by repeated freeze–thaw procedures. The osmotically shocked cells were removed from the supernate by centrifugation, and then were resuspended in water and placed in an NMR tube for spectral analysis.

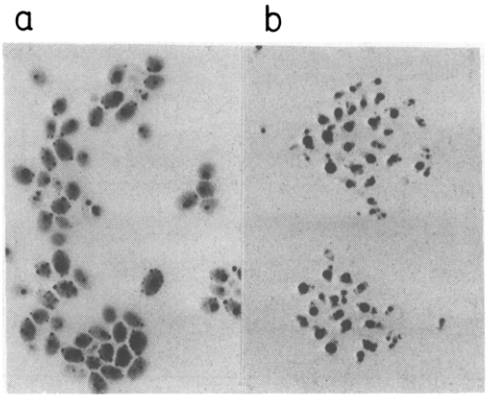

FIGURE 2: Microscope pictures of yeast cells prior to osmotic shock (a) and subsequent to osmotic shock (b). Cells were stained with the lipid specific stain, Oil Red O.

^{13}C NMR Measurements. Proton decoupled ^{13}C NMR Fourier transform spectra were obtained at 25.2 MHz using a Varian XL-100-15 spectrometer interfaced to a Data Genreal Supernova computer in the manner described previously (Eakin et al., 1972), a capillary containing D_2O being used for the lock. For most of the experiments reported, 4096 data points in the time domain were used. Spin–lattice relaxation (T_1) measurements were based on the method of partially relaxed Fourier transform spectra (Vold et al., 1968), requiring a $180° - \tau - 90°$ T pulse sequence, where T is four times the maximum T_1 being measured to allow >96% recovery of the equilibrium magnetization between pulse sequences.

Results and Discussion

Characterization of the Samples under Study. The lipid composition of a number of different strains of *Candida utilis* has been investigated (Dawson and Craig, 1966; Babij et al., 1969; Matile et al., 1969; Hunter and Rose, 1969; Farrell and Rose, 1971; Johnson et al., 1972). In general, it has been found that the bulk of the lipids present are localized in the cellular membranes, specifically the plasmalemma, internal endoplasmic reticulum, and the vacuolar, nuclear, and mitochondrial membranes. However, the existence of lipid vesicles has also been reported (Johnson et al., 1972; Matile et al., 1969). In particular, the size and fatty acid content of the vesicles, which probably contain primarily triglycerides, depend on the medium and on the growth phase of the organisms at the time of harvest. In the minimal acetate medium in which the cells were grown, and in the logarithmic growth phase (Figure 1) during which they were harvested, the amount of stored lipid present in nonmembranous vesicles is probably minimal (Johnson et al., 1972; Matile et al., 1969).

Microscopic examination of normal yeast cells prior to osmotic shock (Figure 2a) reveals a number of small areas which are darkly stained with the lipid specific stain Oil Red O, many of which appear to be localized near the cell wall interface. These areas are tentatively identified with lipid granules, i.e., small membrane bound lipid vesicles (Matile et al., 1969). In addition, most cells appear to con-

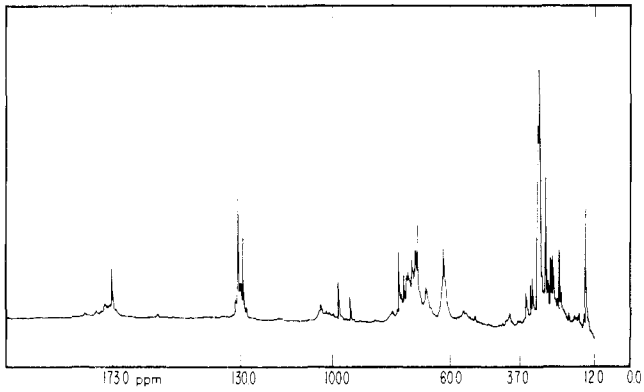

FIGURE 3: ^{13}C FT NMR spectrum of osmotically shocked *C. utilis* cells. The spectrum represents 3320 transients with 4096 data points in the time domain. Sample temperature was 26°C. A spectrum of *C. utilis* cells prior to osmotic shock is given in Eakin et al. (1972).

tain one larger vacuole with a significant lipid content. Osmotically shocked cells which were similarly stained with Oil Red O show the lipid material to be concentrated onto one large mass having an average diameter of about 1.4 μm (Figure 2b). Only a few undisturbed smaller vesicles are detectable. In addition, Figure 2b shows a significant number of cells to be ruptured and either totally or partially devoid of Oil Red O staining material. Similar results were obtained using a Sudan Black stain which has a high affinity for phospholipids (Pearse, 1968). The mean cell size for normal *C. utilis* cells is 4–5 μm (Figure 2a). Osmotic shocking results in the contraction of the Oil Red O staining material into large masses, as well as the expansion of the cell wall to a diameter larger than observed for normal cells (Figure 2b).

The major lipids of *C. utilis* include neutral lipids (mostly triglycerides and diglycerides), phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, and cardiolipin (Johnson et al., 1972). Additional information about the fatty acid composition can be obtained from the ^{13}C NMR spectrum of the osmotically shocked "ghost" cells (Figure 3), a preliminary report of which has appeared recently (Eakin et al., 1972). The spectrum is dominated by fatty acid residues of the lipids and the glucose and mannose residues of the carbohydrates in the cell wall peptidoglycans. The latter resonances occur in the 60–105-ppm region of the spectrum (relative to external Me_4Si), whereas the former occur in the regions 15–35 ppm for the saturated carbons, 128–131 ppm for the unsaturated carbons, and at 174.5 ppm for the carbonyl resonance. Choline and glycerol resonances can also be identified, although there is some overlap with the carbohydrate peaks. Comparison of the yeast spectrum with the spectra obtained with sonicated egg yolk lecithin vesicles (Godici and Landsberger, 1974; Batchelor et al., 1972) indicates that the choline resonance is significantly broadened in the former system, perhaps as a result of the interaction of the choline residues with protein or the polar head groups of the other phospholipids present. The integrated area of that resonance is substantial, corresponding to ~¼ the area of the terminal methyl groups of the fatty acid chains. If it is assumed that roughly similar proportions of all of the phospholipids listed above are present (Johnson et al., 1972), then only a relatively small fraction of the fatty acid resonances need be ascribed to neutral lipids, some of which may be in lipid vesicles.

Table I: Spin–Lattice Relaxation Times for Fatty Acid Resonances of Yeast Cell Membranes.

Peak	Chemical Shift[a] (ppm)	Assignment	T_1 (sec) 7°C	26°C	65°C
1	15.00	CH_3CH_2	1.02	1.70	4.25
2	23.70	CH_3CH_2	0.35	0.65	2.34
3	25.83	$-CO_2CH_2CH_2-$	0.11[c]	0.25[c]	0.51[c]
4	26.56	$-CH=CHCH_2CH=CH-$	0.16	0.39	1.05
5	28.18	$-CH=CHCH_2CH_2-$	0.13	0.41	0.90
6	30.37	$-(CH_2)_n-$	0.15	0.30	0.51
7	32.56	$CH_3CH_2CH_2$ (linoleic)[b]	0.35	0.62	1.63
8	33.04	$CH_3CH_2CH_2$	0.29	0.51	1.45
9	34.67	$-CO_2CH_2-$	0.05[c]	0.11[c]	0.30
10	128.77	$-CH=CHCH_2CH=CH-$	0.31	0.59	1.25
11	130.48	$-CH=CHCH_2CH_2-$	0.26	0.41	1.48
12	55.38	Choline-CH_3	0.19	0.46	1.05

[a] Chemical shifts relative to external Me_4Si. [b] C(16) of linoleic acid, assignment based on Hamilton et al. (1973). [c] Short time constant of a nonexponential decay after graphical separation into two exponentials.

The assignments of the ^{13}C resonances of the fatty acids summarized in Table I are based on those reported for similar fatty acid derivatives (Stoffel et al., 1972; Hamilton et al., 1973; Dorman et al., 1971). The excellent resolution evident in the spectrum reproduced in Figure 3 is consistent with the relatively high fluidity characteristic of the fatty acid residues in membranes rich in polyunsaturates, the presence of the latter confirmed by the peaks at 128.8 ppm corresponding to two unsaturated carbons located β to each other, the peak at 26.6 ppm corresponding to saturated carbons α to two unsaturated carbons, and by the peak at 32.6 ppm corresponding to the C-16 of linoleic acid (Hamilton et al., 1973). Based on the relative heights of the two C-16 peaks, ca. half of the lipid is linoleic acid. The degree of unsaturation appears to be similar to other strains of *C. utilis*. For example, Farrell and Rose (1971) reported that *C. utilis* strain NCYC grown at 30°C, pH 4.5 on a glucose medium exhibits a lipid composition consisting primarily of palmitic (16%), oleic (28.2%), linoleic (30.2%), and linolenic (17.8%) acids. Nevertheless, the lipid composition is strongly dependent on growth conditions and, in some systems, has been found to be considerably more saturated (Dawson and Craig, 1966).

The osmotically shocked preparation contains significant amounts of protein, indicated, for example, by the prominent narrow resonance at 40.0 ppm which corresponds to the ε carbons of lysine and by the broad resonances in the ~175-ppm region which correspond to amide carbonyl carbons (Stothers, 1972). Since the osmotic shock and freeze–thaw procedures are relatively mild, the proteins associated with the membrane may not be appreciably denatured. The absence of any additional sharp amino acid resonances characteristic of denatured protein is consistent with this conclusion.

The simple fractionation procedure which was used to obtain a high resolution spectrum of the lipids in the yeast membranes is not universally applicable. In particular, whole cells of the blue green alga *Anacystis nidulans* grown on a 20% ^{13}C enriched medium (details to be published elsewhere) yield a much more poorly resolved ^{13}C NMR spectrum (Figure 4A). The freeze–thaw and osmotic shock procedures applied to the yeast system, as well as sonication

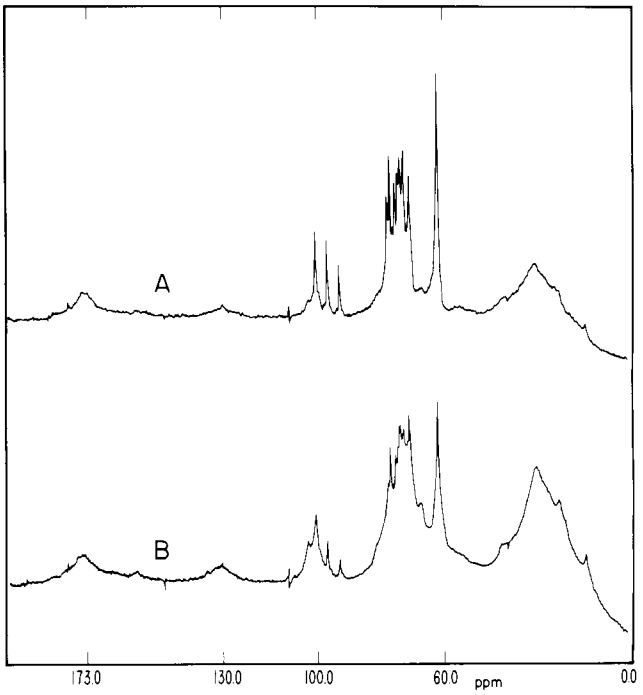

FIGURE 4: ^{13}C FT NMR spectra of whole *Anacystis nidulans* cells (A) and of the same cells after repeated osmotic shock treatments and 6 min of sonication (B). Sample temperature was 23°C. Spectrum A represents 43729 transients and spectrum B represents 182582 transients. Some diminution in the relative intensity of the water soluble carbohydrate resonances (primarily corresponding to galactose) is evident in spectrum B.

treatments, failed to appreciably increase the resolution (Figure 4B). Differences in the spectra may reflect a number of structural factors as well as differences in membrane composition. In particular, *A. nidulans* contains no polyunsaturated fatty acids, the membranes consisting primarily of palmitic acid (55%), palmitoleic acid (35%), and oleic acid (6%) as determined by gas chromatography. Similar results have been reported by Hirayama (1967). In the context of theories for the relaxation rates of molecules whose motion is characterized by two correlation times, one reflecting overall rotational mobility and one reflecting more rapid internal motion, the slower overall motion will only affect the line width if the faster internal motion is anisotropic (Finer, 1974; Gutowsky and Pake, 1950). The spectra obtained here with the yeast and algal cells suggest that the line widths and the "apparent" isotropy of the fatty acid motion depend strongly on the presence of polyunsaturated fatty acids in the membrane lipids.

Relaxation Time Measurements. The spin-lattice relaxation times for the fatty acid resonances determined at three temperatures are summarized in Table I. Nonexponential recovery of the equilibrium magnetization was noted for the resonances assigned to C-2 and C-3, for which the *initial* relaxation rates were estimated and included in the table. The apparent nonexponential recovery of magnetization can result from several factors, including a heterogeneity in the environments of these carbon atoms or from the fact that the low intensity resonances reside on the tail of an intense multiresonance methylene peak (exhibiting a different relaxation time) so that the latter may make a variable contribution to the measured value of the delay time, τ. Whereas the latter effect may obtain for some of the other resonances studied, the very short T_1 values for

Table II: Activation Energies for Fatty Acid Carbons Determined from T_1 Data.

Carbon	E_a (kcal/mol)
CH_3CH_2	4.7
$CH_3CH_2CH_2$	6.1
$CH_3CH_2CH_2CH_2$ (downfield peak)	5.3
$CH_3CH_2CH_2CH_2$ (upfield peak, linoleic acid)	5.1
$-CH=CHCH_2CH_2-$	5.3
$-CH=CHCH_2CH=CH-$	5.0
$-CH=CHCH_2CH_2-$	6.3
$-CH=CHCH_2CH=CH-$	6.2
$-CO_2CH_2CH_2-$	5.7
$-CO_2CH_2CH_2CH_2-$	5.1
$-(CH_2)_n-$	4.3

the C-2 and C-3 nuclei would make the apparent effect more pronounced. In the cases of the other nuclei, however, the relaxation did appear to be exponential.

The ^{13}C relaxation rates, at least for those atoms containing directly bonded H atoms in molecules of moderate to high molecular weights, have been shown to be dominated by intramolecular dipolar interactions with the directly bonded protons (Kuhlman et al., 1970; Levine et al., 1972b). The fact that in all cases T_1 increases with increasing temperature is consistent with a short correlation time for the motion so that $T_1 \propto 1/\tau_c \propto$ temperature, where τ_c is the correlation time for the motion. This condition, in turn, leads to the conclusion that $\omega_0\tau_c \ll 1$, or $\tau_c < 6.3 \times 10^{-9}$ sec. Arrhenius plots of the spin-lattice relaxation times vs. reciprocal temperature indicate that, to the accuracy of the T_1 measurements the T_1 (or $1/\tau_c$) values for the carbons near the end of the chain, which are denoted as C_N, C_{N-1}, and C_{N-2} for an N carbon fatty acid chain, as well as for both unsaturated carbon resonances follow an Arrhenius type law. The calculated apparent activation energies (E_a) for the T_1 values of the latter are listed in Table II. In general they are slightly larger than those reported by Gent and Prestegard (1974) for ^{13}C-labeled lecithin vesicles and multilayers, a result which may reflect differences in the compositions of the two systems. In this context, it should be noted that somewhat higher activation energies than the ones we report here have been measured by Stoffel et al. (1974) for lipid vesicles composed of phosphatidylcholine and sphingomyelins. The deviation of the relaxation times of the remaining nuclei from a simple exponential dependence on the reciprocal temperature appeared to be somewhat greater than the experimental error; thus activation energies listed in Table II for the latter are only rough approximations which may be considerably in error. Since measurements were made at only three temperatures, a more complete analysis seemed unjustified. It is interesting to note, however, that if the 65°C–26°C temperature range and the 26°C–7°C temperature range are considered separately, the activation energies are invariably higher in the lower temperature range, a parameter which could reflect the proximity to a low temperature phase transition such as occurs in lecithin vesicles (Ladbrooke and Chapman, 1969). It should be noted also that although the lower temperature phase may be characterized by larger values of E_a, the condition $\omega_0\tau_c \ll 1$ still holds, since if it did not the Arrhenius plot would bend in the opposite direction as T_1 becomes proportional to τ_c and thus begins to increase with reciprocal temperature.

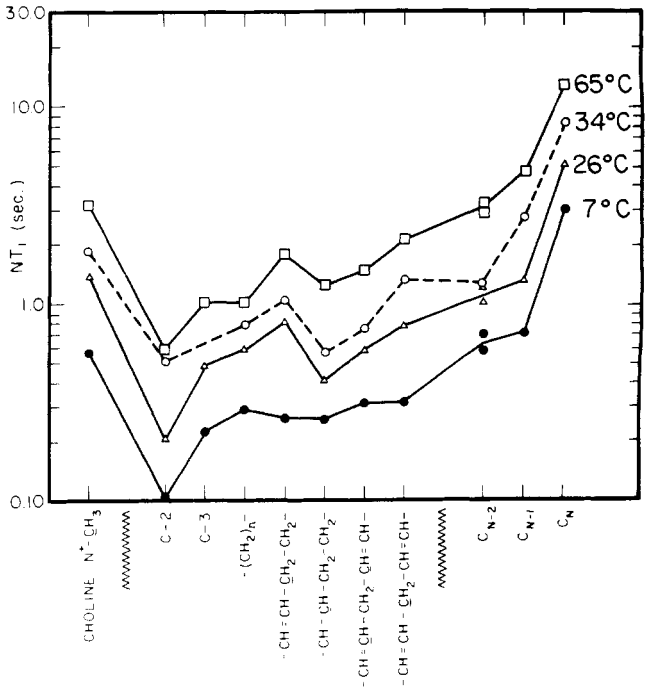

FIGURE 5: Plots of NT_1 vs. carbon position where N is the number of directly bonded protons corresponding to the carbon atom whose T_1 is measured. The data were taken at 7°C (●), 26°C (△), and 65°C (□). For each temperature, two values are given for C_{N-2}. The longer value corresponds to the upfield linoleic acid C-16 peak and the shorter value corresponds to the downfield peak which includes all of the methylene carbons two bonds from the terminal carbon of the remaining fatty acids. Also shown are data from Godici and Landsberger (1974) for sonicated egg yolk lecithin measured at 34°C, (○).

The T_1 data obtained are consistent with the existence of a mobility gradient along the fatty acid chain remarkably similar to the one described by Godici and Landsberger (1974) for sonicated egg yolk lecithin. Plots of NT_1 vs. carbon position for the yeast data (where N is the number of protons directly bonded to the carbon whose relaxation time is being measured) as well as the egg yolk lecithin ^{13}C relaxation data obtained by Godici and Landsberger are shown in Figure 5. In addition to the overall gradient in T_1 values with carbon position, the deviations of many of the individual carbon relaxation times from the values for the methylene envelope are similar in the two systems. In particular, the T_1 value for the upfield unsaturated resonance, representing only the unsaturated carbon atoms of polyunsaturated fatty acids, is longer than the T_1 value for the downfield peak which represents both poly- and monounsaturated fatty acids. This observation is again consistent with a shorter correlation time or greater "fluidity" characterizing the polyunsaturated fatty acids, even though there is no evidence that these acids are in any way segregated in the membrane. A similar conclusion follows from the fact that the linoleic acid C-16 peak has, at all temperatures, a longer T_1 than the downfield C_{N-2} peak which corresponds to the equivalent carbon of the other fatty acids present, most of which are more highly saturated. Also, there is a sharp increase in the terminal methyl T_1 which can be ascribed to a rapid rotation about the terminal C–C bond, a rotation which is expected to be less restricted than that about any other C–C bond because no additional volume is swept when it takes place.

Despite the similarities, there are several differences between the yeast and egg yolk lecithin data. In particular, the relaxation time of the C-16 carbons of the egg yolk lecithin does not exhibit the increased NT_1 which the C-16 peaks of the yeast sample do. This could reflect a difference in the mobility gradient; however, examination of the egg yolk spectrum (Godici and Landsberger, 1974) indicates that the C-16 peak is not well resolved from the methylene envelope so that a shorter T_1 would be measured. Significant differences in the mobility gradient may exist, however, near the glycerol end of the fatty acid chain since nonexponential decays were not reported for egg yolk lecithin data. The short relaxation times determined for the C-2 carbon seem to indicate a steeper gradient in the yeast cells.

As reflected in the T_1 relaxation time, the choline N^+CH_3 carbon atoms also exhibit an increased mobility relative to the fatty acid carbon atoms near the glycerol backbone, similar to the situation which obtains for sonicated vesicles (Levine et al., 1972b; Metcalfe et al., 1971). As with the fatty acid resonances, T_1 increases with increasing temperature so that it can be concluded that $\tau_c < 1/\omega$, $\sim 6.3 \times 10^{-9}$ sec. However, the description of the motion of the choline methyl group in terms of a single rotational correlation time must be crude in view of the anisotropic nature of the motion. In particular, the large N^+CH_3 line width may reflect the slower rotation of the entire membrane system. Recent ^{13}C NMR studies indicate that the apparent T_2 as obtained from the choline line width for both lecithin multilayers and sonicated vesicles is considerably shorter than T_1 (Sears et al., 1974). Thus, the large line width of the choline methyls cannot be attributed to immobilization of the NCH_3 groups. Alternatively, the broadening may reflect quadrupolar interaction with ^{14}N as well as some unresolved triplet structure due to interaction with ^{14}N, or a spread in chemical shifts due to inequivalent chemical environments. (We defer a more detailed discussion of the T_1 gradients observed in these systems until a more rigorous mathematical analysis of the motions in bilayer structures is available.)

Chemical Shift Measurements. The excellent resolution obtained for the fatty acid resonances indicates that the yeast membrane system should be useful for studying chemical shift effects. Small, temperature dependent chemical shifts of the fatty acid resonances of phospholipid bilayers have been reported by Batchelor et al. (1972). Without the use of an internal standard, such shifts are difficult to determine absolutely. Comparison of the measured shifts with the shifts measured for an external aqueous tetramethylammonium bromide sample (or for sodium 2,2-dimethyl-2-silapentane-5-sulfonate) at each temperature results in positive shifts for all resonances, the maximum shift being ~0.8 ppm (Figure 6). These shifts may reflect, in part, differences in bulk susceptibility between the two samples. Despite the difficulty of measuring absolute shifts, relative shifts can be determined with greater accuracy and significant differences do exist for the various carbon atoms. A plot of the total shift observed for each carbon between 65 and 7°C vs. carbon position along the chain is shown in Figure 7. The C-2 shift has been arbitrarily set to 0 and the remaining shifts are, thus, given relative to C-2. There are several possible explanations for these shifts, including: solvent effects arising from the penetration of water into the bilayer as noted for the fluorine resonances in fluorohydrocarbon micelles (Muller and Johnson, 1969; Muller and Simsohn, 1971; Muller et al., 1972, and references therein); interactions with dissolved proteins; or electron delocalization effects resulting from interactions between γ carbons

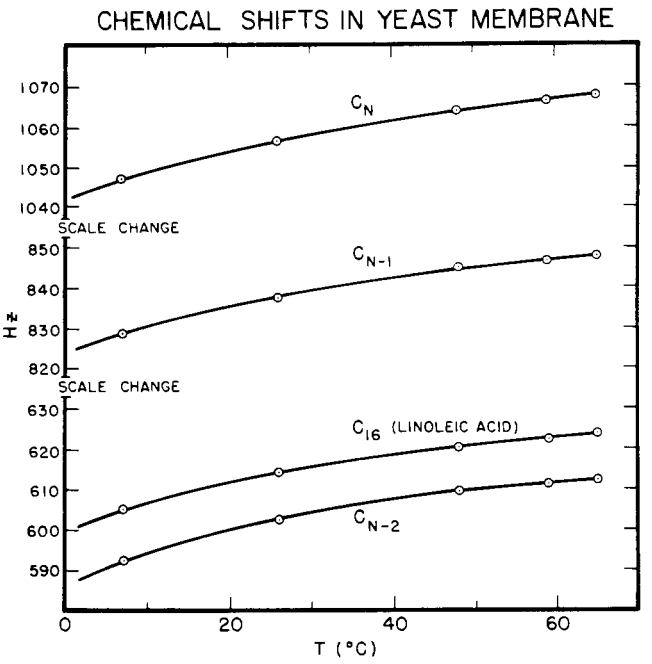

FIGURE 6: ^{13}C chemical shifts as a function of temperature for the fatty acid carbon resonances of osmotically shocked *C. utilis* cells. The shifts are given relative to a tetramethylammonium bromide reference sample measured at the same temperatures.

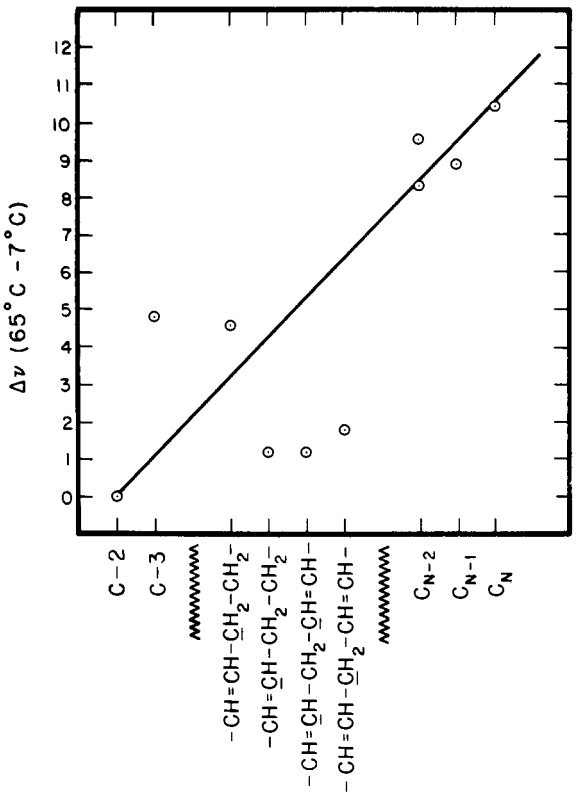

FIGURE 7: ^{13}C chemical shifts vs. carbon position for the fatty acid resonances. The shifts plotted represent the total shift measured between 65 and 7°C. The straight line in the figure illustrates the correlation of shift with carbon position but has no quantitative significance. Two values are given for the C_{N-2} shift, the smaller values corresponding to the linoleic acid C-16 peak and the larger to the C_{N-2} carbons of the other fatty acids present.

as suggested by Batchelor et al. (1971) to account for the shifts observed in lecithin micelles. In developing a rationalization for these data we note first that there is an apparent correlation between the shifts and the carbon position along the chain, in the direction of increasing shifts toward the terminal methyl group (Figure 7). A gradation in chemical shift has also been observed by Muller and Simpsohn (1971) for the fluorine atoms of aqueous micelles of sodium pentafluoroctanoate. It was found that the fluorine atoms nearer the acid head group exhibit chemical shifts closer to the "aqueous solvent value" and the fluorine atoms closer to the methyl terminal exhibit shifts closer to the "fluorocarbon solvent" value. If the penetration of water molecules into the micelle is temperature dependent, and if the water causes a downfield shift for the carbon resonances, the observed chemical shift correlation with chain position might reflect a temperature dependent gradient in the amount of dissolved water in the bilayer. However, Batchelor et al. (1972) conclude, on the basis of studies with model compounds, that such solvent effects should be negligible for ^{13}C in lecithin vesicles. In addition, with this mechanism we cannot account for the relatively lower shifts for the unsaturated carbon atoms and for C-11 of linoleic acid. Of the remaining explanations, the effect of dissolved proteins is the most difficult to predict.

Alternatively, most of the chemical shift results can be explained by the carbon-γ carbon interaction mechanism if the appropriate values of ΔE, the energy difference between gauche and anti-conformational states, are selected. Assuming that the population differences between gauche and anti states can be described by a Boltzmann factor, and taking into account the two possible gauche conformations for each anti conformation, the probability of obtaining a gauche conformation, P_G, will be given by:

$$P_G = 1 \Big/ \left(1 + \frac{1}{2} e^{\Delta E/kT}\right) \qquad (1)$$

The chemical shift difference between two temperatures, T_a and T_b, will then be given by:

$$\Delta\nu_{T_a-T_b} = \left(\frac{1}{1+\frac{1}{2}e^{\Delta E/kT_a}} - \frac{1}{1+\frac{1}{2}e^{\Delta E/kT_b}}\right)\Delta\nu_0 \qquad (2)$$

where $\Delta\nu_0$ is the total chemical shift between pure gauche and pure anti conformations taken as 4.8 ppm (Cheney and Grant, 1967). Equation 1 defines a family of curves whose solutions for P_G change from 0 to 2/3 as T varies from 0 to ∞, most of the change occurring in the region $\Delta E \sim kT$. Considering $\Delta\nu_{T_a-T_b}$ as a function of ΔE, $\Delta\nu_{T_a-T_b}$ will equal 0 at $\Delta E = 0$, pass through a maximum whose position depends on the temperatures T_a and T_b, and again approach 0 as $\Delta E \rightarrow \infty$ (Figure 8).

A qualitative explanation for the observed chemical shift gradient can then be made as follows. For carbons near the end of the chain ΔE would be approximately the energy difference between a gauche and an anti conformation. A value of 0.5 kcal/mol was suggested by Batchelor et al. (1972) based on a calculation by Flory (1969). For saturated carbon atoms of the fatty acids closer to the glycerol backbone, the gauche state may be very much less favorable since a large bend must be introduced into the fatty acid chain. The β coupled isomerism model (Horwitz et al., 1973) predicts that pairs of gauche conformations may be more likely to occur, so that in this case ΔE would be approximately twice the value for carbons near the end of the chain, $\Delta E \sim 1.0$ kcal/mol. For the unsaturated carbon

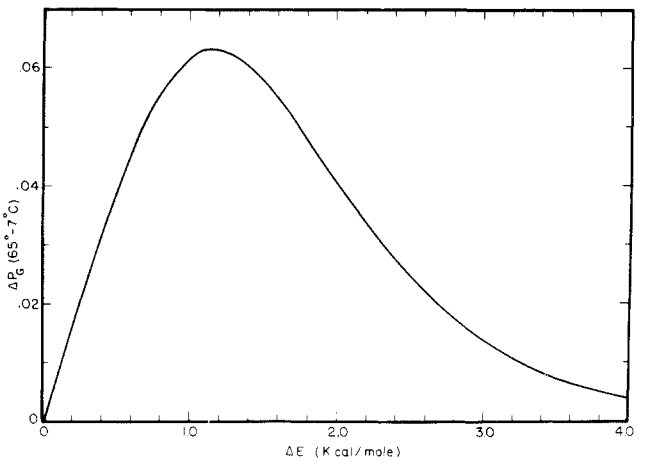

FIGURE 8: The theoretical dependence of ΔP_G (65°C-7°C), or, equivalently, $\Delta\nu/\Delta\nu_0$ (65°C-7°C) on ΔE. $\Delta\nu$ passes through a maximum at ΔE = 1.15 kcal/mol. Thus, the shifts do not uniquely determine ΔE.

atoms, calculations indicate that $\Delta E \sim 0$ so that, as observed, the chemical shift will also be ~0 (Batchelor et al., 1972; Flory, 1969). However, using Figure 8 to obtain the predicted gauche probabilities (or shifts) it is clear that the above values of ΔE for the saturated carbon atoms are inconsistent with the observed data, since in the 65°C-7°C range a larger shift will result for the carbons with $\Delta E \sim$ 1.0 kcal/mol than for carbons with $\Delta E \sim 0.5$ kcal/mol. The value of 0.5 kcal/mol was based on a calculation which includes only intramolecular interactions. However, interchain interactions would be expected to destabilize the energy of gauche states which lead to nonlinear fatty acid conformations that do not pack well. Thus, such interactions would be expected to increase the values for the ΔE's. In order to explain the magnitude of the effects observed, it is necessary to take $\Delta E \sim 1.15$ kcal/mol for rotations about the C_{N-2}-C_{N-1} (the value corresponding to the maximum in Figure 8), and to assume that ΔE will increase further proceeding toward the glycerol backbone. The conclusion that intermolecular effects increase ΔE has also been deduced by Seelig and Niederberger (1974) in a deuterium magnetic resonance study of sodium decanoate bilayers, and by Marsh (1974) as a result of an electron paramagnetic resonance (EPR) study of stearic acid spin-labels in oriented egg lecithin multibilayers. Seelig and Niederberger (1974) calculated that the energy for a gauche-trans-gauche conformation is ~0.5 + 0.5 + 2.6 kcal/mol, where the first two values reflect the intramolecular contribution and the third value reflects the intermolecular contribution due to the extra volume occupied by the gauche-trans-gauche kink. The latter authors noted, however, that the actual value could be smaller due to cooperative kink formation among the chains. We have not calculated values for ΔE from the chemical shift data since such values would depend on the value of $\Delta\nu_0$, and would assume all other effects to be negligible, which may not be the case. However, the Seelig and Niederberger calculation is consistent with the chemical shift data. Marsh (1974) determined a value of 6.4 kcal/mol for the energy of a single gauche conformation relative to a trans conformation. This value is, however, much too large to explain the chemical shifts obtained in the yeast membrane system and may reflect differences in the systems under study or perturbation effects of the spin-label.

Support for the interpretation that the γ-carbon interaction is responsible for the observed chemical shifts is also obtained from the chemical shifts of the unsaturated carbon atoms. Batchelor et al. (1972) reported a downfield shift for the unsaturated carbon atoms which was attributed to the proximity to a phase transition at lower temperatures for lecithin vesicle. Since in the present experiment absolute values for the shifts could not be obtained, we are not able to verify this result for the yeast system. However, it can be noted that the resonance corresponding to C-11 was shifted by approximately the same amount as those of the unsaturated carbon atoms. Since the C-11 is held fixed in a cis orientation relative to the two carbon atoms γ to it, C-8 and C-14, a zero chemical shift would be predicted on the basis of the Cheney and Grant (1967) mechanism since the relative orientations of these carbon atoms cannot change with temperature. Thus, if the mechanism responsible for the upfield shifts is the γ-carbon interaction, it can be concluded that the shift of the unsaturated resonances is small and positive in the present system. This result is consistent with the fact that the energy difference between gauche and anti conformations for bonds β to cis unsaturates is approximately zero (Batchelor et al., 1972).

An additional interesting feature of the observed spectrum is the chemical shift difference between the resonances of the C-16 of linoleic acid and those of the remaining fatty acids. The C-16 atom of linoleic acid is γ to the unsaturated carbon C-13; the greater likelihood of a gauche orientation for bonds β to cis unsaturates (Batchelor et al., 1972) might account for the upfield shift. However, it must be pointed out that the interaction between the C-16 protons and the C-13 atom and C-14 proton is fundamentally different from the interaction between the protons of two γ carbons which are saturated. Thus, the total chemical shift difference between pure gauche and pure trans isomers, $\Delta\nu_0$, may be larger than the value of 4.8 ppm which describes the interaction of saturated carbons. One way of sorting out the contribution due to differences in $\Delta\nu_0$ and ΔE is by consideration of the temperature dependence of the observed shift. It appears that the linoleic acid C-16 peak is shifted upfield with temperature almost as much as the other C-16 resonance (Figure 7). If *bonds* β to cis unsaturates have little preference for either gauche or trans conformations so that $\Delta E \sim 0$, there should be very little temperature dependence for the linoleic acid C-16 chemical shift. The fact that so large a shift is observed suggests, contrary to the prediction of Batchelor et al. (1972), that the gauche conformation has a significantly higher energy than the trans. In fact, for linoleic acid a nearly linear conformation can be constructed in which the two bends introduced by the two double bonds nearly compensate for each other. In this case, no additional gauche conformations need be present to straighten out the fatty acid chains and intermolecular constraints may even tend to favor a trans orientation for C-13 and C-16.

The Dynamics of Fatty Acid Chains Near Double Bonds: δ-Coupled Isomerism. Recent models for the dynamical motion of fatty acid chains have had considerable success in explaining the nmr relaxation and chemical shift data of lipid bilayers and vesicles (Horwitz et al., 1972, 1973; Horwitz, 1972). In particular, the concept of a localized disturbance of β-coupled isomerism, whose probability increases along the chain, is consistent with the observed mobility gradient as measured, for example, by the T_1 data given here. It has been suggested that unsaturated carbons can

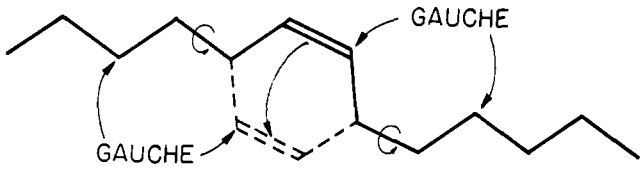

FIGURE 9: A model for a Δ-coupled isomerism about an unsaturated bond in the fatty acid chain. The transition to an isomerized state is indicated by the dotted lines.

undergo motions analogous to β-coupled isomerisms, but only half as many as those allowed for methylene carbons (Gent and Prestegard, 1974). Then taking into account the number of directly bonded protons, T_1 for the unsaturated carbon atoms should be approximately the same as T_1 for the methylene carbon atoms. As is clear from Table I, however, this prediction deviates considerably from the observed relaxation data at low temperatures. The fact that the unsaturated carbons obey a simple Arrhenius relationship suggests that a simple mechanism may be operative throughout the temperature range studied. A particularly simple model can be constructed by assuming the existence, on the average, of a single gauche conformation which is distributed between the two bonds β to the unsaturated bond. The relaxation data then reflects the interconversion of the gauche conformation between two bonds which are δ to each other (Figure 9):

$$t\,t\,e\,t\,g \rightleftarrows g\,t\,e\,t\,t \qquad (3)$$

where e represents the eclipsed conformation which is fixed by the presence of the double bond. This mechanism would then be consistent with the greater stability of gauche conformation β to a cis-unsaturated bond. The fact that interconversions of bonds δ to each other may be coupled if there is an intervening unsaturated bond reflects the basic fact that the rigidity of the double bond makes possible an information transfer over a larger distance. Experiments with space filling models indicate that the interconversion envisioned in eq 3 can occur with minimal perturbation of the remaining fatty acid chain. Finally, we point out that even if, as postulated by Batchelor et al. (1972), a gauche conformation is frozen in at lower temperatures, the δ coupled isomerism could still be operative since the position, but not the existence of the gauche conformation, is allow to vary.

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References

Babij, T., Moss, F. J., and Ralph, B. J. (1969), *Biotech. Bioeng. 11,* 593.

Batchelor, J. G., Prestegard, J. H., Cushley, R. J., and Lipsky, S. R. (1972), *Biochem. Biophys. Res. Commun. 48,* 70.

Birdsall, N. J. M., Feeney, J., Lee, A. G., Levine, Y. K., and Metcalfe, J. C. (1972), *J. Chem. Soc., Perkin Trans. 2,* 1441.

Button, D. K., and Garver, J. C. (1966) *J. Gen. Microbiol. 45,* 195.

Cheney, V. B., and Grant, D. M. (1967), *J. Am. Chem. Soc. 89,* 5319.

Dawson, P. S. S., and Craig, B. M. (1966), *Can. J. Microbiol. 12,* 775.

Dorman, D. E., Jautelet, M., and Roberts, J. D. (1971), *J. Org. Chem. 36,* 2757.

Eakin, R. T., Morgan, L. O., Gregg, C. T., and Matwiyoff, N. A. (1972), *FEBS Lett. 28,* 259.

Edwards, V. H., Gottschalk, M. J., Noojin, A. Y. III, Tuthill, L. B., and Tannahill, A. L. (1970), *Biotech. Bioeng. 12,* 975.

Farrell, J., and Rose, A. H. (1971), *Arch. Mikrobiol. 79,* 122.

Finer, E. (1974), *J. Magn. Reson. 13,* 76.

Flory, P. J. (1969), *Statistical Mechanics of Chain Molecules,* New York, N.Y., Wiley, Chapter 3.

Gent, M. P. N., and Prestegard, J. H. (1974), *Biochem. Biophys. Res. Commun. 58,* 549.

Godici, P. E., and Landsberger, F. R. (1974), *Biochemistry 13,* 362.

Gutowsky, H. S., and Pake, G. E. (1950), *J. Chem. Phys. 18,* 162.

Hamilton, J. A., Talkowski, C., Williams, E., Avila, E. A., Allerhand, A., Cordes, E. H., and Camejo, G. (1973), *Science 180,* 193.

Hernandez, E., and Johnson, M. J. (1967), *J. Bacteriol. 94,* 996.

Hirayama, O. (1967), *J. Biochem. 61,* 179.

Horwitz, A. (1972), in Membrane Molecular Biology, Fox, C. F., and Keith, A. D., Ed., Stamford, Conn., Sinauer Associates, pp 164–191.

Horwitz, A. F., Horseley, W. J., and Klein, M. P. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 590.

Horwitz, A. F., Michaelson, D., and Klein, M. P. (1973), *Biochim. Biophys. Acta 298,* 1.

Hunter, K., and Rose, A. H. (1969) in The Yeasts, Vol. II (Physiology and Biochemistry of Yeasts), Rose, A. H., and Harrison, J. S., Ed., New York, N.Y., Academic Press, p 211.

Johnson, B., Nelson, S. J., and Brown, C. M. (1972), *Antonie van Leeuwenhoek 38,* 129.

Keough, K. M., Oldfield, E., Chapman, D., and Beynon, P. (1973), *Chem. Phys. Lipids 10,* 37.

Kuhlman, K. F., Grant, D. M., and Harris, R. K. (1970), *J. Chem. Phys. 52,* 3439.

Ladbrooke, B. D., and Chapman, D. (1969), *Chem. Phys. Lipids 3,* 304.

Lee, A. G., Birdsall, N. J. M., and Metcalfe, J. C. (1973), *Chem. Br. 9,* 116.

Levine, Y. K., Birdsall, N. J. M., Lee, A. G., and Metcalfe, J. C. (1972b), *Biochemistry 11,* 1416.

Levine, Y. K., Partington, P., Roberts, G. C. K., Birdsall, N. J. M., Lee, A. G., and Metcalfe, J. C. (1972a), *FEBS Lett. 23,* 203.

London, R. E., Kollman, V. H., and Matwiyoff, N. A. (1975), *J. Am. Chem. Soc. 97,* 3565.

Marsh, D. (1974), *Can. J. Biochem. 52,* 631.

Marshall, A. G., Schmidt, P. G., and Sykes, B. D. (1972), *Biochemistry 11,* 3875.

Matile, Ph., Moor, H., and Robinow, C. F. (1969), in The Yeasts, Vol. I (Biology of Yeasts), Rose, A. H., and Harrison, J. S., Ed., New York, N.Y., Academic Press, Chapter 6, p 219.

Metcalfe, J. C. (1972), *Chem. Phys. Lipids 8,* 333.

Metcalfe, J. C., Birdsall, N. J. M., Feeney, J., Lee, A. G., Levine, Y. K., and Partington, P. (1971), *Nature (London) 233,* 199.

Metcalfe, J. C., Birdsall, N. J. M., and Lee, A. G. (1972),

FEBS Lett. 21, 335.
Muller, N., and Johnson, T. W. (1969), *J. Phys. Chem. 73,* 2042.
Muller, N., Pellerin, J. H., and Chen, W. W. (1972), *J. Phys. Chem. 76,* 3012.
Muller, N., and Simsohn, H. (1971), *J. Phys. Chem. 75,* 942.
Oldfield, E., and Chapman, D. (1971), *Biochem. Biophys. Res. Commun. 43,* 949.
Pearse, A. G. E. (1968), Histochemistry, Vol. I, 3rd ed, Boston, Mass., Little, Brown and Co., p 415.
Robinson, J. D., Birdsall, N. J. M., Lee, A. G., and Metcalfe, J. C. (1972), *Biochemistry 11,* 2903.
Sears, B., Hutton, W. C., and Thompson, T. E. (1974), *Biochem. Biophys. Res. Commun. 60,* 1141.
Seelig, J., and Niederberger, W. (1974), *Biochemistry 13,* 1585.
Stoffel, W., Tungal, B. D., Zierenberg, O., Schreiber, E., and Binczek, E. (1974), *Hoppe-Seyler's Z. Physiol. Chem.* 355, 1367.
Stoffel, W., Zierenburg, O., and Tungal, B. D. (1972), *Hoppe-Seyler's Z. Physiol. Chem. 353,* 1962.
Stothers, J. B. (1972), Carbon-13 NMR Spectroscopy, New York, N.Y., Academic Press.
Vold, R. L., Waugh, J. S., Klein, M. P., and Phelps, D. E. (1968), *J. Chem. Phys. 48,* 3831.
Williams, E., Sears, B., Allerhand, A., and Cordes, E. H. (1973), *J. Am. Chem. Soc. 95,* 4871.

Constitution and Properties of Axonal Membranes of Crustacean Nerves†

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ABSTRACT: The purification of axonal membranes of crustaceans was followed by measuring enrichment in [^{3}H]tetrodotoxin binding capacity and in Na^+,K^+-ATPase activity. A characteristic of these membranes is their high content of lipids and their low content of protein as compared to other types of plasmatic membranes. The axonal membrane contains myosin-like, actin-like, tropomyosin-like, and tubulin-like proteins. It also contains Na^+,K^+-ATPase and acetylcholinesterase. The molecular weights of these two enzymes after solubilization are 280000 and 270000, respectively. The molecular weights of the catalytic subunits are 96000 for ATPase and 71000 for acetylcholinesterase. We confirmed the presence of a nicotine binding component in the axonal membrane of the lobster but we have been unable to find [^{3}H]nicotine binding to crab axonal membranes. The binding to axonal membranes of two neurotoxic compounds, tetrodotoxin and veratridine, which affect the functioning of the sodium channel, has been studied in detail. The dissociation constant for the binding of [^{3}H]tetrodotoxin to the axonal membrane receptor is 2.9 n*M* at pH 7.4. The concentration of the tetrodotoxin receptor in crustacean membranes is about 10 pmol/mg of membrane protein, 7 times less than the acetylcholinesterase, 30 times less than the Na^+,K^+-ATPase, and 30 times less than the nicotine binding component in the lobster membrane. A reasonable estimate indicates that approximately only one peptide chain in 1000 constitutes the tetrodotoxin binding part of the sodium channel in the axonal membrane. Veratridine, which acts selectively on the resting sodium permeability, binds to the phospholipid part of the axonal membrane. [^{3}H]Veratridine binding to membranes parallels the electrophysiological effect. Veratridine and tetrodotoxin have different receptor sites. Although tetrodotoxin can repolarize the excitable membrane of a giant axon depolarized by veratridine, veratridine does not affect the binding of [^{3}H]tetrodotoxin to purified axonal membranes. Similarily, tetrodotoxin does not affect the binding of [^{3}H]veratridine to axonal membranes. Scorpion neurotoxin I, a presynaptic toxin which affects both the Na^+ and the K^+ channels, does not interfere with the binding of [^{3}H]tetrodotoxin or [^{3}H]veratridine to axonal membranes. Tetrodotoxin, veratridine, and scorpion neurotoxin I, which have in common the perturbation of the normal functioning of the sodium channel, act upon three different types of receptor sites.

Understanding of the molecular aspects of nerve conduction necessitates both a biochemical and an electrophysiological approach. Axons from crustacean nerves constitute a biological system that permits both approaches: (1) they are unmyelinated, (2) walking-leg nerves can be easily dissected out and obtained in large quantities, (3) a number of crustaceans like crayfishes or lobsters have giant axons which permit detailed electrophysiological studies.

Fisher et al. (1970) and Camejo et al. (1969) have chosen to study the molecular organization of nerve membranes from squid axons, which are also unmyelinated and which have been extensively used in electrophysiological experiments. The garfish olfactory nerve which is probably a very good system for biochemists (Chacko et al., 1972; Gre-

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