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Carbon-13 Nuclear Magnetic Resonance Spectroscopy of Suspensions of Chinese Hamster Ovary Cells Specifically Enriched with [methyl-^{13}C]Choline†

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ABSTRACT: The mammalian Chinese hamster ovary cell line (CHO) was labeled with [methyl-^{13}C]choline. Under the conditions used, ~42% of the cellular choline was found to be associated with cellular lipids and the remaining 58% was present in the water-soluble fraction of the cell, primarily as phosphorylcholine. The spin–lattice relaxation time (T_1) of this pool of phosphorylcholine indicates an intracellular viscosity ~1.2 times that of H_2O. Cells fixed with formaldehyde and washed several times did not exhibit ^{13}C resonances corresponding to free choline or phosphorylcholine and were sufficiently stable to be studied up to 43 °C. The T_1 and line width behavior of the methyl resonance was studied as function of temperature in the fixed cells. The T_1 data exhibited an Arrhenius dependence with an activation energy of 4.3 kcal/mol, similar to that observed for free choline. The line width does not conform to an Arrhenius law and the values obtained for the CHO cells are similar to those previously reported for sonicated lecithin vesicles and to the value obtained in sonicated lecithin vesicles prepared from the extracted CHO cell lipids. The values are significantly smaller than the values reported for unsonicated lecithin dispersions. The data are discussed in terms of a theoretical model involving multiple internal rotations. A measurement of the ^{13}C–^{1}H nuclear Overhauser enhancement for the choline methyl carbons in vesicles prepared from the extracted CHO cell lipids gave a value of 3.0 ± 0.3.

Carbon-13 nuclear magnetic resonance spectroscopy has been used extensively to study the architecture and dynamics of the lipid bilayers in aqueous suspensions of liposomes and synthetic single-walled vesicles (Oldfield and Chapman, 1971; Birdsall et al., 1972; Levine et al., 1972a,b; Williams et al., 1973; Assmann et al., 1974; Gent and Prestegard, 1974; Godici and Landsberger, 1974, 1975; Sears et al., 1974; Stoffel et al., 1974). In contrast to the situation generally encountered in proton magnetic resonance spectroscopy (for a review, see Horwitz, 1972), the ^{13}C NMR[1] spectra of these lipid bilayers exhibit a remarkably high degree of resolution, frequently allowing the extraction of line width, spin–lattice relaxation (T_1), and chemical-shift data for individual lipid carbon atoms. These systems have been offered as models for the state of the lipids in natural membranes, but whether they are appropriate ones is questionable, given the high protein content of natural membranes and the important contributions that lipoprotein interactions probably make to membrane structure. In addition, Chan et al. (1973) have pointed out that the small liposomes and vesicles (250–500 Å) have a much shorter radius of curvature than cells (20 000 Å or more) and this could lead to packing disorders in the former which would strongly affect lipid mobilities.

Recently, ^{13}C NMR studies of natural membrane systems have been reported (Metcalfe et al., 1971; Metcalf, 1972; Robinson et al., 1972; Lee et al., 1973; Keough et al., 1973; Williams et al., 1973; Brown et al., 1975; Nicolau et al., 1975), but, due to many overlapping resonances and the low natural abundance and low magnetogyric ratio of ^{13}C, the generally poor quality of the spectra recorded has prevented a proper appreciation of the great potential of ^{13}C NMR spectroscopy for investigating the structure of the components of suspensions of intact cells, such as those considered here. These problems of low sensitivity and large natural background can be overcome to a large extent by employing systems uniformly or specifically labeled with ^{13}C. Thus, in an early study, Metcalfe

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[1] Abbreviations used are: CHO, Chinese hamster ovary; NMR, nuclear magnetic resonance; EPR, electron paramagnetic resonance; NOE, nuclear Overhauser effect; FT, Fourier transform.

et al. (1972) were able to study, by high-resolution ^{13}C NMR spectroscopy, the mobility of ^{13}C-enriched phospholipids in acholeplasma membranes above the lipid-phase transition. In another early study (Matwiyoff and Needham, 1972), the ^{13}C NMR spectra of erythrocyte suspensions treated with $^{13}CO_2$ revealed narrow resonances not only for the expected $^{13}CO_2$ and $^{13}CO_3H^-$ constituents but also for the elusive carbamino moiety. More recently, Stoffel and Bister (1975) have been able to record promising high-resolution ^{13}C NMR spectra of suspensions of specifically enriched intact vesicular Stomatitis Virus derived from host BHK 21 cells into which [11-^{13}C]oleic acid and [methyl-^{13}C]choline had been incorporated. The high-resolution ^{13}C NMR spectra of intact erythrocyte suspensions containing hemoglobin labeled with [2-^{13}C]histidine have also been reported (London et al., 1975a) in a study of the effect of intracellular viscosity on the rotational mobility of the protein. Finally, London et al. (1975b) have investigated the high-resolution ^{13}C NMR spectra of fractionated yeast membranes enriched with carbon-13 and have been able to determine the gradients in the mobility of the lipid carbon atoms.

In this work, we have specifically labeled Chinese hamster ovary (CHO) cells grown in a medium supplemented with [methyl-^{13}C]choline. We report here the ^{13}C NMR spectra of aqueous suspensions of the whole cells and of the phospholipid vesicles derived therefrom and discuss the results in terms of several models for the internal motions of the choline moiety.

Materials and Methods

Preparation of [methyl-$^{13}C_3$]Choline. [$^{13}C_3$]Hydroxyethyltrimethylammonium chloride ([$^{13}C_3$]choline chloride) was prepared using a procedure similar to that described for deuterium-labeled choline chloride (duVigneaud et al., 1941). Ethanolamine, 8.8 g (0.14 mol), was cooled in a dry ice–acetone bath and 17.3 g (0.122 mol) of [^{13}C]iodomethane (91.9 mol % ^{13}C) was added dropwise with stirring. The solution was stirred for 5 days at room temperature. The conversion to the Reineckate salt and chloride was carried out as described (duVigneaud et al., 1941; Kapfhammer and Bischoff, 1930). Purification of the ^{13}C-labeled choline chloride was affected by removing water by azeotropic distillation with ethanol and crystallization from absolute ethanol–diethyl ether. White needles, 2.46 g, were obtained by suction filtration in a drybox. A second crop yielded 1.51 g (70% yield). ^{1}H NMR (D_2O): δ 3.2 ($^{13}CH_3$), $^1J_{CH}$ = 146 Hz, $^3J_{CNCH}$ = 3.5 Hz; δ 3.55 (CH_2N) multiplet, δ 4.05 (CH_2OH) multiplet, δ 4.70 (OH).

Growth of [methyl-$^{13}C_3$]Choline-Labeled CHO Cells. Chinese hamster cells (line CHO) were cultured in suspension free from contamination by mycoplasma in F-10 medium (Gibco) supplemented with 15% bovine Kadet serum (Biocell Laboratories) and antibiotics. F-10 medium contains 5.0 μM choline chloride. [methyl-^{13}C]Choline in sterile aqueous solution was added to the growth medium to 74 μM final concentration. As shown in previous studies (Hildebrand and Tobey, 1973) and confirmed during the experiments reported here, the methyl groups of choline are not redistributed to other cellular components (e.g., protein or nucleic acids) and are recovered from cells either as phosphatidylcholine or as water-soluble precursors of phosphatidylcholine. These studies were facilitated by addition of [methyl-^{14}C]choline (New England Nuclear Corp.) (0.1 μCi/ml final radioisotope concentration) and following the distribution of radioisotope.

Cells were harvested from culture by centrifugation (500g for 5 min) and washed by resuspension and centrifugation in cold (0–4 °C) [^{13}C]choline-free growth medium or cold buffer (0.01 M Tris-Cl, pH 7.3, 0.150 M NaCl, 0.0015 M $MgCl_2$). Washing of cells in these solutions resulted in removal of only 10% of the "free" choline (water soluble) from the cell.

Cell fixation was effected by incubation of cells at 4 °C for 18 h in 4% formaldehyde in a buffered solution (6.0 g of glucose, 6.4 g of NaCl, 0.32 g of KCl, 0.31 g of $Na_2HPO{\cdot}12H_2O$, 0.12 g of KH_2PO_4, 0.123 g of $MgSO_4{\cdot}7H_2O$, 0.13 g of $CaCl_2{\cdot}2H_2O$, 0.96 mg of phenol red/l. of solution adjusted to pH 7.0).

Cell viability was determined by the trypan blue dye exclusion method described elsewhere (Phillips, 1973).

In order to characterize the cellular incorporation of [^{13}C]choline, cells were cultured in [^{14}C]choline-containing medium along with [^{13}C]choline as described above. Cells were harvested and washed, as described, and resuspended in 0.01 M Tris-Cl, pH 7.3, at 4 °C. Homogenization was performed with an Omnimixer microcup, the cell homogenate was extracted with 1 volume of $CHCl_3$–MeOH (2:1), and the aqueous and organic phases were reextracted several times with $CHCl_3$–MeOH (2:1) and water, respectively. Thin-layer (Skipski et al., 1964) and paper chromatography systems (Schneider et al., 1966) were employed to determine the subcellular distribution of the [^{13}C]choline. In the organic phase, the labeled choline (42% of the total cellular label) was found exclusively in phosphatidylcholine. The aqueous phase of the cell homogenate contained 58% of the total intracellular choline, which was present as phosphorylcholine (82%) and "free" choline (18%).

[^{13}C]NMR Spectroscopy. Proton-decoupled ^{13}C Fourier transform NMR spectra were obtained at 25.2 MHz with a Varian XL-100-15 spectrometer interfaced to a Nova 1210 computer. A D_2O capillary was used for the lock. Typical resolution was 0.5 Hz and data acquisition times were 2–3 s. Spin–lattice relaxation times (T_1) were determined using a 180°–τ–90°–T pulse sequence with $T \geq 5\ T_1$ (Vold et al., 1968; Freeman and Hill, 1971). The nuclear Overhauser enhancement was determined by comparing the intensities of fully decoupled and coupled spectra. Peak intensities were determined by Xerox copying of the spectra, followed by cutting and weighing of the peak. This method has proved most reliable for obtaining intensities where the signal to noise ratio is low (London et al., 1975c).

Lipid Composition. Membrane lipids were extracted with chloroform–methanol (2:1, v/v) after homogenization in methanol. The filtrates were subsequently washed with 0.88% potassium chloride in water. Neutral lipids and phospholipid fractions were separated on a silicic acid column, transmethylated, and the lipid composition was determined by gas chromatography (Christie, 1973). The liquid phase was Silar 9CP (Applied Science Labs). The neutral lipids were found to be considerably more saturated than the phospholipids, containing primarily the fatty acids: 14:0 (6.7%); 14:1 (5.8%); 16:0 (21.5%); 16:1 (7.3%); 18:0 (21.6%); 18:1 (23.7%); and 18:2 (13.3%), as well as traces of higher molecular weight species. The phospholipid composition included 14:0 (1.2%); 14:1 (2.0%); 16:0 (16.0%); 16:1 (4.6%); 18:0 (23.9%); 18:1 (15.2%); 18:2 (13.5%); 20:4 (16.6%). In addition, several unidentified higher molecular weight fatty acids were present at relatively low concentrations. The arachidonic acid of the phospholipids can be seen directly in the natural abundance spectrum of the CHO cells (Walker et al., in preparation). Thin-layer chromatography indicates that the large majority of the phospholipids are phosphatidylcholine (~80%) with

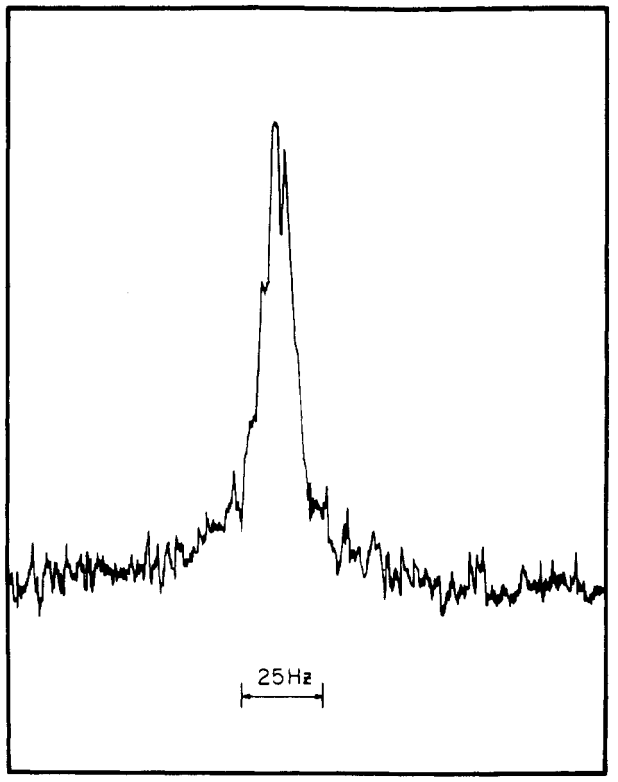

FIGURE 1: Proton-decoupled ^{13}C FT NMR spectrum of the methyl resonances of the [methyl-$^{13}C_3$]choline-labeled CHO cells *not* fixed with formaldehyde.

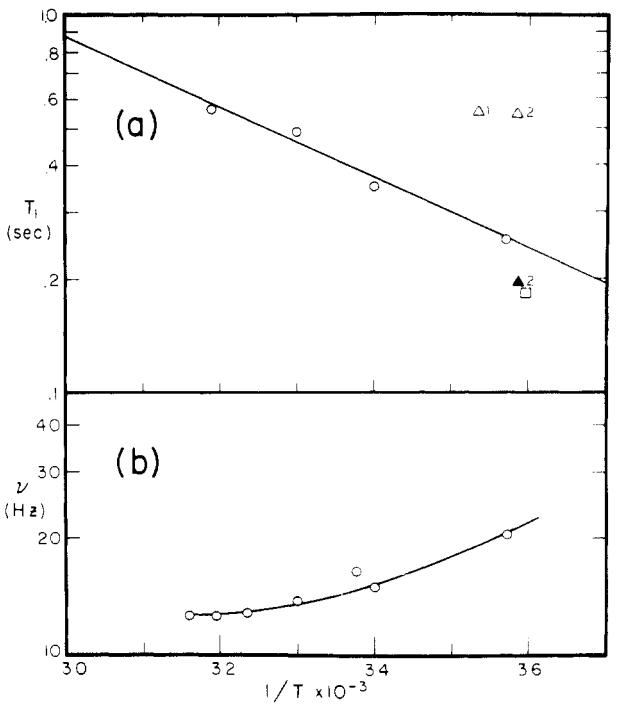

FIGURE 2: (a) A plot of the T_1 data obtained vs. inverse temperature for several different preparations of [methyl-$^{13}C_3$]choline-labeled CHO cells and lipid vesicles: O cells fixed in formaldehyde, washed five times with distilled water, and resuspended, Δ1, Δ2. Two runs of cells suspended in F10 medium immediately after harvest and kept at 7 °C through the initial runs, ▲2. The Δ2 cells after being allowed to die at elevated temperature (see text) and subsequently fixed with formaldehyde and resuspended; (□) sonicated vesicles prepared from [methyl-$^{13}C_3$]choline-labeled lipids extracted from the labeled CHO cells. (b) Line width data obtained with the formaldehyde-fixed CHO cells plotted as a function of inverse temperature.

sphingomyelin, phosphatidylethanolamine, and phosphatidylserine present in relatively small quantities. These results reflect the lipid composition under growth conditions which include a large quantity of additional choline (74 μM) and may differ from the lipid composition which exists for CHO cells grown in F10 medium plus calf serum only.

Results

Although initial experiments were undertaken on formaldehyde-fixed cells due to the need for maintaining the integrity of the sample over long periods of time and over a temperature range sufficient to obtain an activation energy for the spin–lattice relaxation process, it was found that by working at sufficiently low temperatures studies on intact, viable cells could be carried out. Cellular viability is determined at various times during such experiments by examining the ability of the cells to maintain impermeability to trypan blue dye, as described under Materials and Methods. The [^{13}C]choline *N*-trimethyl resonances exhibited by aqueous suspensions of the nonfixed cells are reproduced in Figure 1. Compared to the two previous studies in which cells (Stoffel and Bister, 1975) and vesicles (Sears et al., 1974) were labeled with ^{13}C-enriched choline containing only 1 labeled methyl group, the increase in sensitivity of a factor of 3 can lead to a reduction in the required signal averaging time by a factor of 9. In experiments, such as these, where sensitivity can be marginal at best, the latter procedure is to be recommended highly.

Formaldehyde-Fixed Cells. In all cases, T_1 measurements were found to be exponential within experimental error. A plot of T_1 vs. inverse temperature is consistent with Arrhenius-type behavior with an activation energy of 4.3 kcal/mol (Figure 2a). An Arrhenius plot of line width vs. inverse temperature was not linear, the line widths (Figure 2b) tending to level off at higher temperatures. As has been reported in other model membrane systems (Sears et al., 1974), the value of T_2 derived from the line width assuming a Lorentzian line is considerably shorter than T_1 despite the fact that both appear to increase with increasing temperature.

Nonfixed CHO Cells. Two striking differences were observed between the results obtained with the fixed and nonfixed cells. In the latter, the T_1 values for the *N*-methyl groups are considerably longer and the resonance is not a single Lorentzian line but appears to possess some resolvable structure (Figure 1).

T_1 measurements were made on viable cells at 5 °C, the total loss of viability during the measurement amounting to ~20%. As illustrated in Figure 2a, the T_1 for these cells of 546 ms is significantly longer than the value for the fixed cells at corresponding temperatures. In a second series of experiments, a similarly long T_1 value was obtained. The cells were subsequently examined at higher temperatures (from 6 to 33 °C). Heating of the cells was accompanied by a rapid loss of viability and cellular integrity indicated in photomicrographs by massive disruption of the cells and a considerable amount of dissociated cytoplasm and bare nuclei. The dissociated cytoplasm was decanted (fraction I) and the remaining cellular material was fixed with formaldehyde (fraction II). The T_1 observed for fraction II (194 ms at ~6 °C) corresponded closely to the values obtained for the fixed cells. Although the relaxation behavior for the unfixed cells appeared to be approximately exponential, an examination of the partially relaxed spectra indicated clearly that the wings of the resonance relax faster than the center peaks, suggesting that the com-

position of the sample is inhomogeneous. Further studies of the incorporation of the radioactively labeled choline with CHO cells indicate that, *under the growth conditions corresponding to high levels of added choline,* there are significant pools of free phosphorylcholine in the cells (~50%), accounting for the relatively long T_1 exhibited by the unfixed cells. In the fixed cells, the cell membranes are sufficiently leaky to allow the free choline to be washed out of the preparation so that the resonance observed for them corresponds only to membrane-bound choline, which, of course, exhibits a shorter T_1. These observations suggest also that the apparent structure in the choline methyl resonance of the unfixed cells is due to free phosphorylcholine, which has a short rotational correlation time and gives rise to a sharper resonance, which may, in fact, be a triplet due to the ^{14}N coupling. This interpretation is also consistent with the observation that the decanted cytoplasm fraction I described above, which should contain the free choline as well as some phosphatidylcholine, exhibited the same structure of the choline resonance.

Sonicated Lipids Extracted from CHO Cells. The CHO lipids were extracted with a 3:1 chloroform–methanol solution, dried, resuspended in D_2O containing 0.04 M NaCl and sonicated to optical clarity. The resulting line width and T_1 values obtained for the *N*-methyl group were similar to the values obtained with the fixed cells (Figure 2). The nuclear Overhauser enhancement was obtained by comparing the intensities of proton coupled and decoupled spectra (Figure 3) and a value of 3.0 ± 0.2 was obtained. It is evident from the figure that the ^{13}C lines are broader in the proton-coupled spectra. This probably reflects the long range C–H coupling between the methyl carbon and the protons in the other methyl and methylene carbons. This coupling produces a nonette for each of the four peaks in Figure 3b which is easily observed with free choline. The coupling constants are ~4 Hz.

Discussion

Qualitative Features of the Spin–Lattice Relaxation Times. Unfixed Cells. As noted under Results, studies with [methyl-^{14}C]choline carried out under typical growth conditions for these experiments indicated the presence of significant quantities of the label in the water-soluble fraction of the unfixed cells. Most of this label was found to correspond to phosphorylcholine. This is consistent with the work of Plagemann (1968, 1971) and Plagemann and Roth (1969) who found that most of the choline in the acid-soluble fraction of Novikoff hepatoma cells is in the form of phosphorylcholine. Since the recovery of the magnetization of the $N(^{13}CH_3)_3$ resonance for these cells is approximately exponential and corresponds to a T_1 value (546 ms, 6 °C) which is significantly longer than that (280 ms, 6 °C) for the fixed cells, it can be assumed that the T_1 measured for the unfixed cells is dominated by the narrow free phosphorylcholine resonance. This results partially from the fact that the phosphorylcholine resonance is considerably sharper than the phospholidylcholine resonance. In fact, examination of the partially relaxed spectra appear to indicate that the wings of the resonance are relaxing more rapidly than the center. Use can be made of the T_1 data to obtain an estimate of the intracellular viscosity of the CHO cells. We have measured the T_1 for the calcium salt of phosphorylcholine dissolved in H_2O (0.386 M, pH 7.0, 7 °C) and obtained a value of 660 ms. Using the extreme narrowing approximation and treating the phosphorylcholine molecule as an isotropic molecule that reorients rigidly, the ratio of the viscosity of the CHO cells to H_2O can be obtained using the Stokes–Einstein equation and the value obtained is $\eta^{CHO}/\eta^{H_2O} \sim 1.20$.

This value is of interest because different probes of cellular viscosity appear to give different results. The estimate given above is identical to the value obtained for mouse erythrocytes by London et al. (1975a) who obtained a viscosity ratio, erythrocyte–H_2O, of 1.2 from the relative rotational correlation times of [2-^{13}C]histidine-enriched hemoglobin in the two systems. The value is, however, considerably *less* than that (~500 cp) obtained by Keith and Snipes (1974) for chlamydomonas on the basis of an EPR spin-label study. The validity of the latter result has been questioned by Finch and Harmon (1974) who suggested that the correlation time of the spin-label probe could be a weighted average of its predominant occupation of a "mobile" site and a small fractional occupation of a highly immobilized one.

In the case of the results reported here, it should be emphasized that the relative values of the viscosities are only approximate because we have taken into account neither chemical exchange phenomena nor the effects of possible differences in the rate of internal methyl group rotation. Thus, it is possible that a small fraction of the choline occupies a highly restricted environment with a relatively short T_1. The T_1 measured ($T_{1,obsd}$) will then obey the equation

$$\frac{1}{T_{1,obsd}} = \frac{1}{T_{1,free}} + \frac{f}{\tau + T_{1,B}}$$

where $T_{1,free}$ corresponds to the bulk of the choline and $T_{1,B}$ corresponds to the fraction, f, of the "immobilized" choline occupying the restricted site with a mean lifetime τ. Accordingly, T_1 for the bulk site could be longer than measured and the viscosity ratio derived could be an *upper* limit. Changes in the rate of internal rotation of the methyl groups may also affect the apparent viscosity ratio.[2]

Fixed Cells. There are several interesting features to the relaxation times of the fixed cells. First, the value of the apparent activation energy, 4.3 ± 0.5 kcal/mol, associated with the temperature dependence of T_1 is identical, within the error limits, to that (4.0 ± 0.5 kcal/mol) obtained by Behr and Lehn (1972) for free acetylcholine. The value is typical for a neopentyl barrier (Pitzer and Kilpatrick, 1946), suggesting that relaxation is dominated by internal motion of the methyl groups about the *N*-methyl axis (Figure 4). There should be little hindrance to this motion from adjacent groups on the membrane, since a rotating methyl group requires the same volume as a stationary one. Despite the similarity in the activation energy, it is apparent from the line width and from the values obtained for T_1 that the $N(CH_3)_3$ resonance of the fixed cells does not arise from free choline. In addition, we note that ^{13}C T_1 measurements have been made in a variety of other

[2] Taking into account the possibility of internal rotation of the methyl groups, we can write $1/T_1 \propto \tau_R \chi(\rho,\theta)$, where τ_R is the overall rotational correlation time and $\chi(\rho,\theta)$ is a function of $\rho = \tau_R/\tau_G + 1$, where τ_G is the rotational correlation time for the internal rotation of the methyl protons and $\theta = 109.5°$ is the angle between the axis of internal rotation and the C–H vector. $\chi(\rho,\theta)$ has the form (Doddrell et al., 1972):

$$\chi(\rho,\theta) = 1 + \frac{3(1-\rho)}{5+\rho}\sin^2\theta\left[1 + \frac{3(1-\rho)}{2+4\rho}\sin^2\theta\right]$$

Assuming that $\eta \propto \tau_r$, and using the extreme narrowing approximation we obtain $\eta^{CHO}/\eta^{free} = (T_1^{free}/T_1^{CHO})(\chi^{free}/\chi_{CHO})$. Now if there is either no internal rotation ($\rho = 1$) or very rapid internal rotation ($\rho \gg 1$) the factor $(\chi^{free}/\chi_{CHO}) = 1$ and the result stated above is correct. Alternatively, in the intermediate case this is not necessarily true. Assuming that τ_G is not affected by solution viscosity, ρ will increase as τ_R increases and, thus, we will have $(\chi^{free}/\chi_{CHO}) > 1$. Therefore, the result stated above represents a minimum estimate of the viscosity difference between the CHO cells and an H_2O solution of choline. It is unlikely, however, that this effect will be significant.

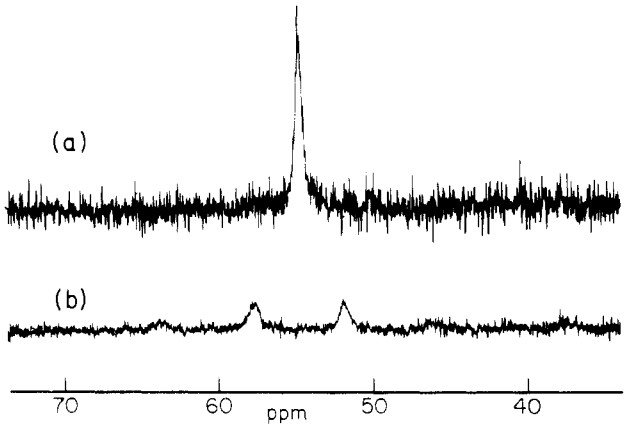

FIGURE 3: Proton decoupled (a) and coupled (b) ^{13}C FT NMR spectra of the choline resonance of sonicated vesicles prepared from extracted CHO cell lipids. Spectrum a represents 8200 pulses and spectrum b 100 850. Sample temperature was 11 °C and a 2-s delay between pulses was used for the accumulation time.

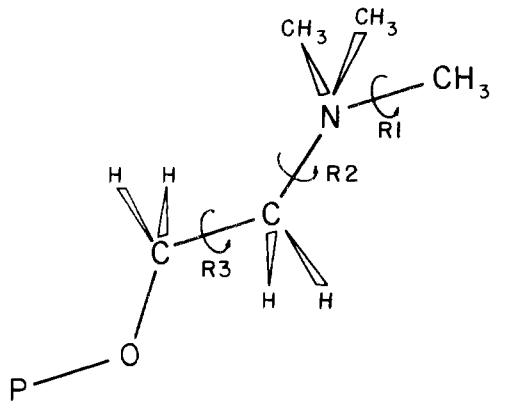

FIGURE 4: Illustration of the possible motions contributing to the relaxation of the choline methyl groups. As discussed in the text, rotations R1 and R2 require very little additional volume and should, therefore, be relatively insensitive to intermolecular interactions. In addition, these rotations do not change the direction of the field gradient at the nitrogen and should, therefore, not contribute to the quadrupolar relaxation of ^{14}N.

lipid systems and the T_1 values obtained for sonicated or unsonicated dispersions are similar, again suggesting that relaxation is dominated by segmental motion (Sears et al., 1974).

The activation energy for the choline relaxation is close to the value of 3.7 kcal/mol obtained for Gally et al. (1975) for the choline methyl groups in dipalmitoyllecithin bilayers using deuterium relaxation, and to proton data obtained previously (Lee et al., 1972; Horwitz et al., 1972), but somewhat shorter than the value of 5.7 kcal/mol obtained by Stoffel et al. (1974) for a system of mixed lipid vesicles. The significantly longer value obtained for that case suggests that lipid–lipid interactions may hinder the mobility of the choline head groups to a larger extent in the vesicle than in the fixed CHO cells. It must be noted, however, that the data given by Stoffel et al. appear to indicate the opposite temperature dependence of that reported here and in the other references. That is, in Figure 6 of Stoffel et al. (1974) the rotational correlation time ($\alpha(1/T_1)$) decreases with increasing temperature. Rotation about the $N\text{-}CH_2$ bond should also be relatively insensitive to interlipid interactions, since it also requires very little additional volume relative to the nonrotating conformation (Figure 4). Thus, the rather long T_1 values which have been obtained for the choline methyl carbons can be explained, in part, by the fact that rotations R_1 and R_2 illustrated in Figure 4 require little additional volume and could even be consistent with relatively rigid conformations. This point is further illustrated by the fact that below the lipid phase transition in dipalmitoyllecithin vesicles only the choline methyl peak remains sufficiently narrow to be observed (Levine et al., 1972a).

Although the spin–lattice relaxation times in the systems considered here appear to be dominated by motions described by rotation R_1 and R_2 illustrated in Figure 3, theoretical calculations (vide infra) suggest that rotation R_3, which sweeps out additional volume, can have an important effect. That the effect does occur, in fact, is suggested by the dependence of the T_1 values of a variety of lipid systems on the degree of unsaturation of the fatty acids. Assuming all other conditions equal, T_1 increases with increasing unsaturation of the lipids. Thus, Levine et al. (1972a) have reported that at 52 °C for sonicated vesicles made from dipalmitoyllecithin or dioleyllecithin the T_1 values are 700 and 1060 ms, respectively. Similarly, Stoffel and Bister (1975) have attributed the much greater T_1 values observed in sonicated vesicles compared with membranes of vesicular stomatitis virions in part to the greater unsaturation of the former system. The physical basis for the dependence of the choline methyl T_1 on fatty acid saturation may be the looser packing or greater average distance between unsaturated lipid molecules which has been demonstrated for lipid bilayers (Seelig et al., 1973). In particular, x-ray diffraction studies indicate that the area occupied by the polar groups in an oleate bilayer is 35 $Å^2$ compared with 25 $Å^2$ in a bilayer with saturated hydrocarbon chains (Ekwall et al., 1969). The longer T_1 could then reflect a greater average volume in which the choline moiety can execute segmental motion in the more unsaturated lipid systems. Interesting in this regard is the fact that the choline T_1 of the fixed cells is somewhat larger than that of the vesicles obtained from them, suggesting that the packing of the lipids in the former is somewhat *looser* than in the vesicles. Alternatively, this difference may reflect oxidation of the polyunsaturated fatty acids which may occur to some extent despite the precautions taken during the extraction and sonication procedures.

The T_1 data obtained with the fixed CHO cells is consistent with the fairly high degree of unsaturation found for the phospholipids. Thus, at equivalent temperatures, the T_1 values for the fixed cells and the vesicles are similar to values obtained with sonicated egg yolk lecithin (Godici and Landsberger, 1974) and with lipid vesicles containing dilinoleyllecithin (Stoffel et al., 1974), but significantly longer than the values measured for 10-dimethylstearoyllecithin (Sears et al., 1974) or for the vesicular stomatitis virions with a lipid composition of 25% palmitic, 20% stearic, and 34% oleic acids (Stoffel and Bister, 1975). One exception to this trend is the data obtained at 52 °C for dipalmitoyllecithin by Levine et al. (1972a) which is close to that extrapolated for the fixed CHO cells at this temperature.

We next consider the width of the choline $N(CH_3)_3$ peak. If it is treated as a single Lorentzian resonance and a value of T_2 is extracted from the line width, we find that $T_2 \ll T_1$ in both the fixed cells and the vesicles. Similar results have been obtained previously for unsonicated lecithin dispersions and for sonicated lecithin vesicles (Sears et al., 1974). The line width in the fixed CHO cells at 21 °C of 14.8 Hz is considerably closer to the value reported for the sonicated lecithin vesicles (11.8 Hz) than to the value obtained with unsonicated multilayers (28.9 Hz) (Sears et al., 1974) consisting of 10-

dimethylstearoylphosphatidylcholine. Levine et al. (1972a) have also reported a [methyl-^{13}C]choline line width of 25 Hz for an unsonicated dispersion of dipalmitoyllecithin. A comparison among these systems may not be particularly meaningful due to the differences in the lipid compositions. It is interesting to note, however, that the line width in the fixed cells is close to the value of 10.8 Hz obtained at 24 °C for sonicated vesicles prepared from the extracted lipids. This result is somewhat surprising, since it has been suggested that the small radius of curvature in sonicated vesicles makes them a poorer model for cellular membranes than unsonicated multilayers (Feigenson and Chan, 1974).

Theoretical Interpretation of Choline Relaxation Data. Line Width. There are three possible sources contributing to the observed line width of the choline methyl carbons.

(a) In a heterogeneous system, such as has been examined here, significant contributions may reflect chemical-shift heterogeneity of different types of membrane-bound choline throughout the cell. Even in sonicated single-walled lecithin vesicles, chemical-shift differences can be observed between the [^{1}H]choline resonances corresponding to the inner and outer leaflets of the bilayer wall (Kostelnik and Castellano, 1973). Although no shift inequivalence has been resolved for the [^{13}C]choline resonances, such differences probably exist but are $\lesssim$ a few Hz. It appears particularly significant that the observed line width is quite similar to that reported for the sonicated lipid vesicles as discussed above. This result suggests that chemical shift inequivalence of the different cellular cholines cannot be significant for the bulk of the choline molecules contributing to the observed resonance.

(b) A second possible contribution to the observed line width can arise from interactions with the directly bonded ^{14}N nucleus. Two types of interaction are possible: scalar broadening due to the modulated ^{14}N spin, which relaxes by a quadrupolar mechanism and unresolved scalar coupling. The ^{14}N-^{13}C dipolar interaction, which may be significant for nonprotonated carbons is of negligible importance for the choline methyl carbons (Oldfield et al., 1975). Using the quadrupolar coupling constant determined by Behr and Lehn (1972): e^2qQ/h = 0.14 MHz and the coupling constant J_{CN} = 4.1 Hz, the effects of the ^{14}N nucleus can be calculated if the relevent rotational correlation time is known. The rotational correlation time, which can be obtained from the choline methyl carbon relaxation, is not relevant to this problem, since internal rotation of the methyl group, which dominates the choline methyl T_1, does not lead to ^{14}N relaxation. Behr and Lehn have used the rotational correlation time for the choline C-2 carbon to obtain the ^{14}N quadrupolar coupling constant so that the T_1 for carbon C-2 would be useful. This value has recently been obtained by studying the relaxation of CHO cells grown on [$^{13}C_5$]choline (Walker et al., in preparation). A calculation using the rotational correlation time estimated from the choline C-2 relaxation data suggests that the ^{14}N triplet is incompletely collapsed and, therefore, may contribute somewhat to the observed line width. In order to evaluate this possibility, we have recently undertaken a double frequency decoupling experiment in which both the ^{1}H and ^{14}N frequencies are irradiated. The ^{13}C [^{14}N,^{1}H] NMR spectra thus obtained indicated a ^{14}N contribution of several hertz. Additional work is in progress (Walker et al., in preparation).

(c) The third possible contribution to the observed line width is ^{13}C-^{1}H dipolar broadening. This effect is considered in connection with the T_1 data discussed below.

Spin-Lattice Relaxation. There can be little doubt that the relaxation of the [^{13}C]choline methyl groups is dominated by the internal motion of the choline methyl carbons. As noted above, the value of 4.3 kcal/mol obtained for the activation energy of the motion leading to the relaxation is similar to that expected for a neopentyl-type barrier. The fact that the relaxation time increases with increasing temperature is also consistent with a motion whose rate lies in the extreme narrowing limit. Dominance of the spin-lattice relaxation by rapid internal motion is also consistent with a maximal nuclear Overhauser enhancement (NOE) (Doddrell et al., 1972). Although no measurement was made on the CHO cells, a maximum NOE value of 3.0 was obtained for the extracted sonicated lipids which exhibit T_1 and line width values similar to the fixed CHO cells.

Given the dominance of the spin-lattice relaxation by the ^{13}C-^{1}H dipolar interaction, it is reasonable to attempt to calculate the ^{13}C-^{1}H dipolar contribution to the line width. If the approximation of isotropic motion is made, then, since T_1 is a double-valued function, two possible values of the rotational correlation time can correspond to the observed T_1. Using the fact that T_1 increases with increasing temperature, it is necessary to take the shorter value of τ, in which case $T_1 = T_2$. Therefore, the isotropic motion assumption is incompatible with the observed data if the line width is assumed to represent primarily ^{13}C-^{1}H dipolar broadening.

Several models exist for predicting the effects of anisotropic motion on T_1 and T_2. In particular, Levine et al. (1973) have modified a model of Wallach (1967) for multiple internal rotations to describe the relaxation of lipid systems (Levine et al., 1972b; Lee, 1974). This model is capable of predicting $T_1 > T_2$, as well as the observed temperature dependences of T_1 and T_2. This results from the fact that the T_1 process is dominated by the internal motion and the T_2 process is dominated by the overall motion, at least for the first several bonds from the portion of the molecule which is fixed in the membrane. According to the Levine model, a particular carbon, C_0, is chosen fixed in the membrane so that its motion is highly restricted. Free, uncorrelated internal rotations are allowed to occur about the bonds between C_0 and the carbon whose relaxation times are being calculated. One possible identification of C_0 arises from the work of Gally et al. (1975) who showed that for dipalmitoyllecithin above the lipid phase transition the motion of the choline C-1 is substantially restricted, while motion about the C-1-C-2 axis, the C-2-N axis, and the N-(CH_3) axes occurs to a significant extent. Making the extrapolation from this system to the CHO cells so that the choline C-1 is identified with C_0, we find that it is possible to predict reasonable values for both the T_1 and line width data of the choline methyls. In particular, choosing overall and internal diffusion coefficients as $D_0 = 7.2 \times 10^4\ s^{-1}$ ($\tau_0 = 2.3 \times 10^{-6}$ s) and $D_i = 5.4 \times 10^9\ s^{-1}$ ($\tau_{int} = 3.1 \times 10^{-11}$ s) we obtain values of T_1 = 452 ms and ν = 13.6 Hz, compared with measured values at 30 °C of T_1 = 460 ms and ν = 13.6 Hz. In the above calculation, we have assumed that each internal motion has the same correlation time, although it is more probable that the terminal methyls rotate faster than the other internal rotations (Lee, 1974). Although the assumption of negligible motion for the choline C-1 is a fairly severe approximation, recent data obtained with CHO cells grown on choline enriched in all carbons is reasonably consistent with this approximation (Walker et al., in preparation). We conclude, therefore, that a model qualitatively similar to that used by Levine and co-workers appears to be the most likely explanation for the choline T_1 and line width data obtained for the formaldehyde-fixed CHO cells. The dominance of the T_1 by the internal motion using this model is again illustrated by

the fact that, if isotropic motion of the choline methyls is assumed, the calculated rotational correlation time $\tau_{iso} = 3.4 \times 10^{-11}$ s, a value extremely close to the internal rotational correlation time τ_{int} obtained above. The slower correlation time is similar to that suggested for sonicated liposomes (Levine et al., 1972b). Although the CHO cells are considerably larger (cell diameter ~10 μm), the maximum correlation time for rotation of each lipid molecule may be determined by lateral diffusion. Since most membranes are reported to be highly invaginated structures, this diffusion will lead to reorientation of the lipid molecule on a considerably shorter time scale than that required for overall cellular rotation. Comparison of the data with results calculated using a more realistic model for internal motion are currently being carried out.

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