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ELECTRON SPIN RESONANCE STUDIES OF CHANGES IN MEMBRANE FLUIDITY OF CHINESE HAMSTER OVARY CELLS DURING THE CELL CYCLE

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Summary

Electron spin resonance (ESR) spin-label methods were used with 5-doxyl-stearic acid as a probe to investigate membrane fluidity of Chinese hamster ovary (CHO) cells during the cell cycle. A 35 GHz ESR technique was developed to study membrane fluidity of intact cells. This technique requires only about 1/6 the amount of cells compared to the conventional spin-label techniques. With this technique we observed a cyclic change of membrane fluidity during the cell cycle of CHO cells: cells in mitosis had the highest membrane fluidity, whereas cells in G_1 and early S phases had the lowest membrane fluidity.

Introduction

Studies of the cell surface of mammalian cells in culture indicate that changes occurring in the cell surface are related to growth and to the cell cycle [1,2]. For example, the cell surface of Chinese hamster ovary (CHO) cells undergoes morphological changes characteristic of each stage in the cell cycle [3].

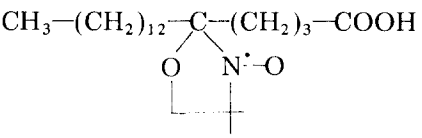
Biological membranes exist as a fluid mosaic of protein and lipid which possess various degrees of motion [4]. Many fundamental properties of membranes are affected by molecular motions of the membrane components. For example, transport processes, electrical potential, enzyme activities and osmotic properties, all of which have been implicated in the cell cycle events,

Abbreviation: doxyl, the *N*-oxyl-4′, 4′-dimethyloxazolidine derivatives of keto fatty acids.

have been related to membrane fluidity [5,6].

There are at least four reports [7—10] on the fluidity of the membrane lipids during the cell cycle by using fluorescence polarization techniques with 1,6-diphenyl-1,3,5-hexatriene as a probe. DeLaat et al. [7] reported that the microviscosity of mitotic neuroblastoma cells was higher than that of interphase cells, suggesting that mitotic cells had a more rigid membrane compared to interphase cells. In their studies, however, the mitotic cells were incubated with the fluorescent probe at 37°C for 30 min. According to Nagasawa and Dewey [11], under those experimental conditions, there is a great chance for mitotic cells to progress into the G_1 phase. Conversely, Collard et al. [8] found that mitotic lymphocytes, fibroblasts, and regenerating liver cells were more fluid than interphase cells. Also, Beiderman et al. [9] reported recently that mitotic hepatoma cells are more fluid than interphase cells. On the other hand, Obrenovitch et al. [10] showed that there was no significant change in membrane fluidity of mouse leukemic L1210 cells. In their system, unfortunately, the mitotic index was only 35—40%. Measurements made with fluorescence polarization techniques have therefore produced conflicting results [7—10], perhaps because of different experimental conditions and/or uncertainties and variations in the location of labels [12—15].

In this study, we have used electron spin resonance (ESR) spin-label methods to study membrane fluidity of CHO cells during the cell cycle with 5-doxylstearic acid as a probe.



It is now generally accepted that fatty acid spin labels reside in the phospholipid portion of the cell surface membrane [16,17] and therefore can provide unambiguous results. The rotational diffusion about the long axis of these labels (this axis is normal to the surface of the membrane) is rapid whereas the rotational diffusion about the other axes is rather restricted. This has been interpreted to be due to the ordering of the lipids that make up the lipid bilayer portion of the membrane [18]. In this study, membrane fluidity (i.e., the flexibility of lipid chains) was determined by measuring the motion of 5-doxylstearic acid in the cell surface membrane of CHO cells.

Conventional ESR spin-label methods generally require large numbers of cells for each measurement, making it difficult to study mitotically synchronized cells. We have used high-frequency (35 GHz) ESR techniques to study this problem. The high frequency significantly reduced the number of cells required for each point. Recent studies also make it possible to obtain additional information on the motion using 35 GHz studies in combination with 9 GHz studies [19,20].

Methods

Cell line. The CHO cell line was obtained from Dr. William C. Dewey (Colorado State University) and cultured as monolayers in α minimal essential

medium supplemented with 10% fetal calf serum and the antibiotics, penicillin, streptomycin, polymixin 'B' sulfate and neomycin sulfate. Cells were subcultured three times per week using a solution of 0.25% trypsin in McCoy's medium to strip the monolayers from the surface of tissue culture flasks. Fresh cell stocks were obtained from mycoplasma-free liquid nitrogen stocks every 3—4 months.

Synchronization of CHO cells. Mitotic cells were obtained by selective detachment [21]. T150 plastic flasks containing approx. $1 \cdot 10^7$ cells were placed in a reciprocating shaker at 37°C for 10 s at 10-min intervals. The detached cells were collected and held for a maximum of 3—4 h at 4°C to prevent entry into G_1. Storage of mitotic cells at 4°C for at least 4 h did not affect membrane fluidity as measured by ESR spin-label methods.

After collection of sufficient numbers of cells, they were concentrated by centrifugation and diluted to the desired concentration for immediate ESR measurement and for culture in a spinner flask at 37°C in an atmosphere of 5% CO_2/95% air with slow stirring (2 rev./s).

Entry of cells into the S phase was determined by pulse labeling with [^{3}H]-thymidine (5 μCi/ml) for 20 min at 37°C at various times after mitotic collection. Labeling was stopped by cold centrifugation and dilution. The cells were fixed with methanol/acetic acid (3 : 1, v/v) and air-dried on a glass slide. The nuclear track emulsion (Kodak NTB2) was applied and exposed 1 week before developing. The percentage of cells labeled was obtained from 200 cells counted per slide. The mitotic index determined by the hypotonic squash method [22] was greater than 95% in all experiments.

Spin labeling of CHO cells. $3 \cdot 10^6$ cells were centrifuged at 4°C at 300 × *g* for 5 min, washed once with cold phosphate-buffered saline (OXOID Dulbecco 'A' solution without Mg^{2+} and Ca^{2+}), resuspended in 2 ml of phosphate-buffered saline, and added to the spin-label solution. We prepared the spin-label solution by drying an ethanolic solution of 5-doxylstearic acid (from Molecular Probes) under N_2 in a 10 ml beaker and then adding 2 ml of phosphate-buffered saline and vigorously stirring for 5 min at 23°C. 2 ml of cell suspension were gently transferred to the spin-label solution and incubated at 23°C for 10 min with slow stirring (1 rev./s). The concentration of spin label used in these studies was 12—30 μM. The spin-labeled CHO cells were washed twice and the thick cell suspension was transferred to a 2 μl disposable pipette for studies at 35 GHz. For 9.5 GHz measurements, $20 \cdot 10^6$ cells were placed in a 50 μl quartz flat cell. The spin-labeling procedure did not affect the cell viability as measured by plating efficiency (70—80%) or the mitotic index of mitotic cells as measured by the hypotonic squash method.

The concentration of 5-doxylstearic acid was determined by double integration of ESR spectra with a digital signal analyzer (Tracor Northern NS-570A). The Tempone (4-oxo-2,2,6,6-tetramethylpiperidinooxyl) spin label with an extinction coefficient of 2550 $M^{-1} \cdot cm^{-1}$ at 236 nm was used as a standard.

ESR measurements. All ESR spectra were recorded at 23 ± 0.2°C with a Varian Century-line 9 GHz spectrometer and a Varian E-9 35 GHz spectrometer. Each spectrometer was equipped with a variable temperature controller and a digital thermometer (Fluke 2100A model). The incident microwave powers were 10 mW (9 GHz) and 2 mW (35 GHz). The field sweep was 100 G

and the modulation amplitude was 1.0 G.

Spectral parameters, $2T_{\parallel}$ and $2T_{\perp}$ (in G) at 9 GHz are shown in Fig. 1. These correspond to the separation of the outer and inner spectra extrema, respectively, and reflect rotational diffusion about the molecular axes of fatty acid spin labels in membranes. These were used to calculate order parameters [18]. The order parameter reflects the mean angular deviation of a spin-labeled fatty acid long chain from its average orientation in the lipid bilayer. High values of order parameters indicate relatively low mobility of the lipids. At 9 GHz, A_z is equal to 1/2 of $2T_{\parallel}$.

Mason and Freed [23] have developed an alternative method of determining membrane fluidity at 9 GHz by measuring the width parameter, Δ_L (see Fig. 1), the outer half-width at half-height of the low-field extremum. This method has been suggested to be a more sensitive assay for detecting changes in membrane fluidity [24,25].

The analyses of the 35 GHz spectra can be based on A_z (see Fig. 2), a reflection of the wobbling motion about the long axis of the spin label and the width parameter, ΔW_L which is a reflection of how well A_x and, to a lesser extent A_y, are averaged by motion about the z-axis; i.e.. the width is a reflection of the rate of rotation about the long axis of the spin label [26].

In some measurements, a 5 G modulation amplitude was employed to measure $2T_{\parallel}$ at 9 GHz (Fig. 1) and A_z at 35 GHz (Fig. 2). The increase in modulation amplitude did not change the values for $2T_{\parallel}$ (9 GHz) and A_z (35 GHz). The accuracy of measurements was ±0.2 G.

Results

Membrane fluidity of CHO cells was determined at both 9 and 35 GHz by the incorporation of 5-doxylstearic acid spin label. Fig. 1 shows the ESR spectrum of asynchronous CHO cells grown in spinner culture and labeled with a 5-doxylstearic acid spin label. It is typical of anisotropically immobilized spectra obtained from a 5-doxylstearic acid spin label in either lipid bilayer vesicles or from biological membranes [18]. The 35 GHz ESR spectrum of asynchronous CHO cells grown in spinner culture and labeled with a 5-doxylstearic acid is shown in Fig. 2. This spectrum is also consistent with the label being confined in a phospholipid-like bilayer [19].

At a fixed cell number, as shown in Fig. 3, the width parameter Δ_L (9 GHz) or ΔW_L (35 GHz) increased as the amount of spin label increased, suggesting that spin-spin interactions contributed to the broadening of the lines. Attempts to avoid spin-spin interactions by decreasing the concentration of spin label in these studies were not successful because the ESR signal became too weak to be detected. We also found that both Δ_L and ΔW_L narrowed as a function of time, presumably due to the reduction of spin label in cell membranes, which decreased the concentration of spin label and thereby diminished spin-spin interactions. However, the spectral parameters, $2T_{\parallel}$ and $2T_{\perp}$ (9 GHz), and A_z (35 GHz) did not change appreciably over the range of the concentration of spin label used. We therefore performed all our data analyses by measurements of $2T_{\parallel}$ and $2T_{\perp}$ (9 GHz) and A_z (35 GHz).

The results in Fig. 4 show that the order parameter, S, or spectral parameter,

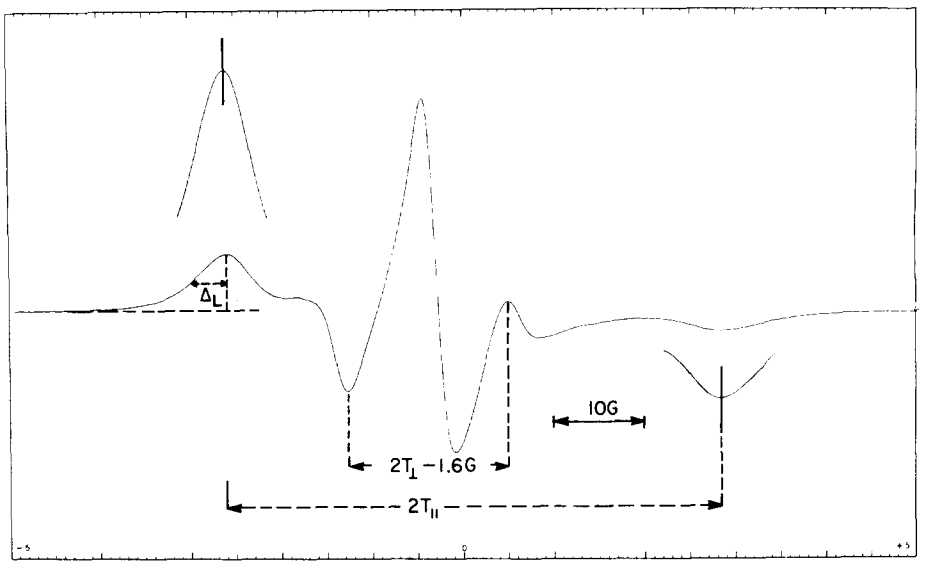


Fig. 1. 9 GHz ESR spectrum of asynchronous CHO cells grown in spinner culture labeled with 5-doxylstearic acid. $2T_{\parallel}$, $2T_{\perp}$ and Δ_L were measured as indicated. The complete spectrum was scanned with 1 G modulation amplitude while the partially resolved two outer extrema were scanned with 5 G modulation amplitude.

$2T_{\parallel}$ (9 GHz) obtained from asynchronous CHO cells grown in spinner culture labeled with 5-doxylstearic acid changed linearly as a function of temperature. Over the temperature range 20—30°C, a change of the order parameter by 0.025 or a change of $2T_{\parallel}$ by 1 G is equivalent to a change in the fluidity of

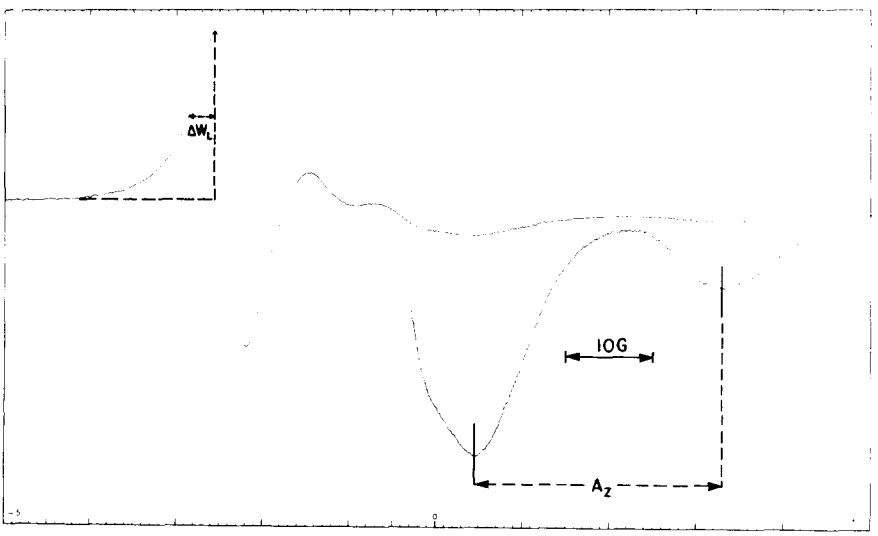
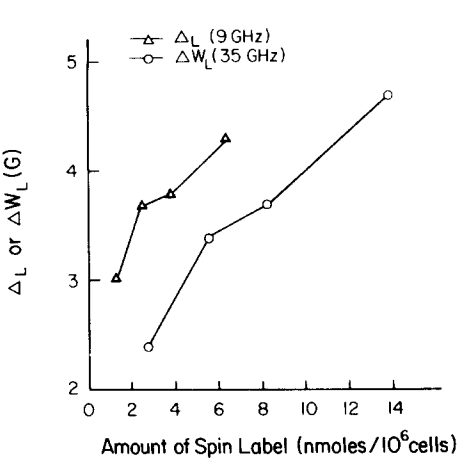


Fig. 2. 35 GHz ESR spectrum of asynchronous CHO cells grown in spinner culture labeled with 5-doxylstearic acid. A_z and ΔW_L were measured as indicated. The complete spectrum was scanned with 1 G modulation amplitude while the partially resolved A_z was scanned with 5 G modulation amplitude.



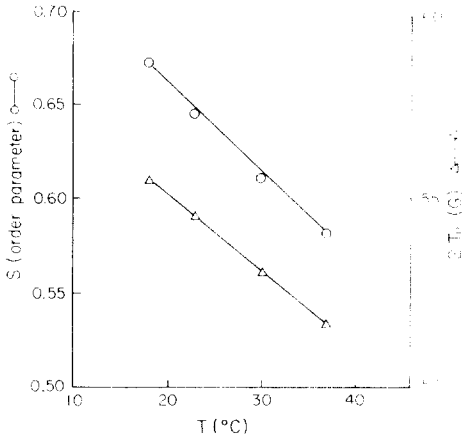


Fig. 3. Effects of the amount of 5-doxylstearic acid spin label on Δ_L (9 GHz) and ΔW_L (35 GHz). Width parameters were measured as indicated in Figs. 1 and 2, respectively. The samples were equilibrated at 23°C for 10 min prior to the ESR measurement.

Fig. 4. Changes in order parameter, S, or spectral parameter, $2T_\parallel$, as a function of temperature. The measurements were carried out at 9 GHz. The samples were equilibrated at each temperature for at least 10 min prior to the ESR measurement. Order parameter was calculated by using the method of Hubbell and McConnell [18].

cell membranes produced by a temperature change of about 5°C. The sensitivity to temperature of A_z at 35 GHz was found to be similar to that of $2T_\parallel$ at 9 GHz.

The differences in the spectral parameters and order parameter between CHO cells in asynchronous population and CHO cells in mitosis are shown in Table I. These results suggest that mitotic CHO cells have a more fluid membrane than asynchronous CHO cells grown in spinner culture.

Recent studies demonstrated that measurements of A_z at both 35 GHz and 9 GHz ESR spectra permit the direct calculation of rotational correlation times between 10^{-6} and 10^{-8} s [20]. Table II shows the A_z values of 35 GHz and 9 GHz ESR spectra of CHO cells in asynchronous populations and in mitosis labeled with 5-doxylstearic acid. At different frequencies, namely 35 and 9 GHz, the results in Table II also indicate that mitotic CHO cells have

TABLE I

SPECTRAL PARAMETERS AND S VALUES OF 9 GHz ESR SPECTRA OF SPIN-LABELED CHO CELLS IN MITOSIS AND IN ASYNCHRONOUS POPULATION

Mitotic cells obtained by the mitotic shake-off method and asynchronous cells grown exponentially in spinner culture were labeled with 5-doxylstearic acid as described in Methods. The results are given as mean ± S.E. (number of independent measurements in parentheses). S (order parameter) was calculated by using the method of Hubbell and McConnell [18].

CHO cells	$2T_\parallel$ (G)	$2T_\perp$ (G)	S
Asynchronous	54.3 ± 0.2 (14)	19.1 ± 0.1 (14)	0.645 ± 0.003
Mitotic	53.5 ± 0.2 (4)	19.3 ± 0.1 (4)	0.630 ± 0.002

TABLE II

THE A_Z VALUES OF 35 AND 9 GHz ESR SPECTRA OF SPIN-LABELED CHO CELLS IN MITOSIS AND IN ASYNCHRONOUS POPULATION

Mitotic cells obtained by the mitotic shake-off method and asynchronous cells grown exponentially in spinner culture were labeled with 5-doxylstearic acid as described in Methods. The results are given as mean ± S.E. (number of independent measurements in parentheses). At 9 GHz A_Z is equal to 1/2 of $2T_{\|}$.

CHO cells	A_Z (G)		ΔA_Z (G) (A_Z(Q)–A_Z(X))
	35 GHz	9 GHZ	
Asynchronous	28.6 ± 0.2 (16)	27.2 ± 0.1 (14)	1.4
Mitotic	27.9 ± 0.2 (6)	26.6 ± 0.1 (4)	1.2

a more fluid membrane than the asynchronous CHO cells. The ΔA_z value (in G) is defined as the difference in A_z values between 35 GHz (Q-band) and 9 GHz (X-band). The ΔA_z (A_z(Q) – A_z(X)) values of 1.2–1.4 G corresponds to an effective rotational correlation time of about $1.2 \cdot 10^{-8}$ s [20]. According to Hyde and Rao [20], ΔA_z increases as the rate of motion increases. If mitotic cells are more fluid than asynchronous cells, one should expect that the ΔA_z value of mitotic cells is greater than that of asynchronous cells. However, the A_z value between mitotic and asynchronous cells is not significantly different in this experiment (the ΔA_z differences of 1.2–1.5 G are within our experi-

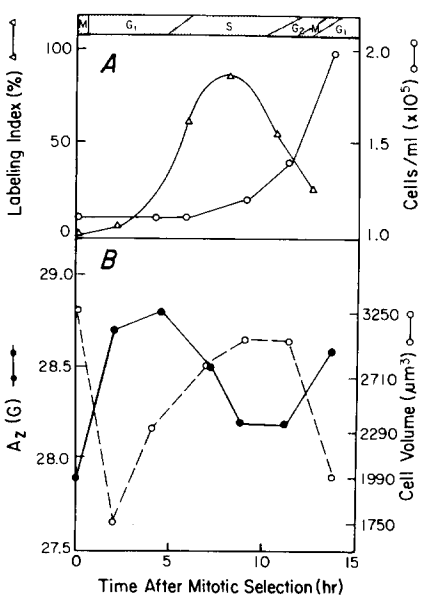


Fig. 5. The relationship between membrane fluidity and cell cycle. (A) Synchronization of CHO cells: ○———○, number of cells; △———△, ^{3}[H]thymidine labeling index. (B) A_Z and cell volume during the cell cycle: ●———●, the A_Z values of 35 GHz ESR spectra of synchronous CHO cells labeled with 5-doxylstearic acid. ○- - - - -○, cell volume measured by using Coulter cell-counter set for log scale expansion. Each data point indicates an average of two measurements giving essentially the same value. All measurements were made on cells grown in suspension cultures after selection by the mitotic method.

mental error). One possible explanation is that the rotational diffusion of the fatty acid spin label along the long axes in membrane is rather fast ($1.2 \cdot 10^{-8}$ s) and is close to the limit of the method of Hyde and Rao [20].

Even though 9 GHz studies indicate the differences in membrane fluidity between mitotic and asynchronous cells, it is not possible to follow multiple time points during the cell cycle with this conventional ESR method. We then used a 35 GHz ESR method to study the changes in membrane fluidity during the complete cell cycle of CHO cells.

Fig. 5 summarizes the results of a representative experiment in which ESR and biological measurements were made during the cell cycle. Fig. 5a shows the degree of synchronization as determined by measuring the [^{3}H]thymidine labeling index and cell number as a function of time after mitotic shake-off. The cell numbers doubled within 12—13 h, which is similar to the normal doubling time for CHO cells grown in either spinner culture or tissue culture flasks. The duration of the G_1, S and G_2 phases were about 5, 6 and 1.5 h, respectively. The dashed line in Fig. 5b shows the change of cell volume during the cell cycle. The change of membrane fluidity during the cell cycle as measured by changes in A_z values of 35 GHz ESR spectra is shown in Fig. 5b (solid line). Cells in mitosis had the greatest membrane fluidity whereas cells in G_1 and early S phases had the lowest membrane fluidity. Partial loss of synchrony during the second wave of division at 12—14 h makes the comparison of A_z values invalid for the second generation.

Discussion

These data clearly indicate that the mobility of the spin label changes during the cell cycle, with greatest mobility during mitosis and the least during G_1. Although the absolute magnitude of the differences between the experimental data points is small (e.g., A_z for mitotic cells = 27.9 G and A_z for G_1 cells = 28.6 G), such differences are well within the accuracy of our measurements (less than 0.2 G). Differences of A_z of this magnitude correspond to relatively large differences in molecular motions and membrane structure, being equivalent to differences observed in membranes on changing temperature by approx. 3—4°C or altering the percent of cholesterol by about 7% [27].

An important consideration in the interpretation of these results is the location of the spin label: i.e., whether the spin label is confined to the plasma membrane. Hatten et al. [28] reported that the order parameters of purified plasma membranes of 3T3 cells closely resemble those of intact cells. Kaplan et al. [29] have demonstrated that $K_3Fe(CN)_6$, which is impermeable to cell membranes, can preferentially oxidize the fatty acid spin labels which are being reduced in the cell surface membrane. We added $K_3Fe(CN)_6$ to the spin-labeled cells in which the ESR signal had decayed and observed an ESR signal which was identical to the original one in order parameters. The intensity of the revived ESR signal was about 20% stronger than that of the original one. It is possibly due to the revival of the spin label which was reduced during the initial labeling and washing periods. This result here suggests that the vast majority of the probes are in the plasma membrane during the time course of our experiment.

The basis for the change of mobility of the spin label during the cell cycle is not established by these experiments. The changes could be reasonably ascribed to a number of factors including changes in the amount and type of proteins or changes in the amount of cholesterol in the cell surface membrane. Recent publications indicate that all of these factors may change during the cell cycle [5,30].

These experiments have also indicated the usefulness of using both 9 and 35 GHz spectra to obtain an independent measurement of the rotational correlation time of the spin label in biological membranes. It should be noted, however, that the technique developed by Hyde and Rao [20] is based on isotropic motion while the spin label in these experiments is clearly undergoing anisotropic motion, rotating freely only about the long molecular axis. The theory probably applies reasonably well to such motion but the calculations to prove this remain to be performed.

Furthermore, our data suggest the difficulty of using linewidth techniques such as those advocated by Mason and Freed [23] to measure motion of spin labels in membranes of viable cells. The reduction of the spin labels during the measuring period changes the linewidth significantly, presumably by reducing the frequency of spin-spin interactions. We could not obtain an adequate signal-to-noise ratio at levels of spin labels at which spin-spin interactions become negligible.

These experiments have also demonstrated the feasibility and usefulness of high-frequency ESR techniques to study membranes of viable cells. Some of the advantages of such studies have been suggested by other authors and data have been obtained at 35 GHz on model membrane systems [19,26,31]. We have now extended this approach to viable cells and also correlated our ESR measurements with biological data on aliquots of the same samples. With the reduced number of cells required by this technique (about 1/6 of the cell number required by the conventional ESR) it may now be more feasible to study the effects of other physiological, pharmacological, and pathological variables on cell membranes by ESR techniques.

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