SPIN-LABEL STUDIES OF THE LIPID AND PROTEIN COMPONENTS OF ERYTHROCYTE MEMBRANES

A Comparison of Electron Paramagnetic Resonance and Saturation Transfer Electron Paramagnetic Resonance Methods

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ABSTRACT We have used both a protein spin label and a lipid spin probe to study some of the slow motions of proteins and of lipids, respectively, in intact erythrocyte membranes. Three electron paramagnetic resonance (EPR) methods, conventional (V_1) EPR, second harmonic out-of-phase absorption saturation transfer (ST) EPR (V_2'), and first harmonic out-of-phase dispersion ST EPR (U_1') were used to compare the experimental methods and spectral sensitivities with different kinds of molecular motions in human erythrocyte membranes under different experimental conditions. The results show that the V_2' display is relatively more sensitive to the protein motion, while the U_1' display appears more sensitive to the lipid motions, and the V_2' display is substantially more convenient to obtain than the U_1' display.

INTRODUCTION

The dynamic properties of the components of biological membranes are generally believed to be essential for their functions. Extensive literature describing the motional behavior of the lipid hydrocarbon chains is available (1). However, we have relatively little information about the motions of the membrane proteins and of the lipid molecules in the head group region. Partly due to their large sizes, viscous environments, and/or their association with other molecules, many membrane components exhibit slow motion, in a time range to which many physical methods no longer show sensitivity. For example, spin label electron paramagnetic resonance (EPR) studies of spin labeled human erythrocyte membranes have given qualitative descriptions of some of the motions of proteins as being weakly, and some strongly, immobilized (2,3). Unfortunately, with conventional EPR, the strongly immobilized component, which is the major spectral component in the system, shows little or no change in spectral shape as correlation times slow down beyond $\sim 10^{-7}$ s. The recent development of rapid passage saturation transfer (ST) EPR techniques (4–6) has shown the potential of using such techniques for studying molecules undergoing very slow rotational motion. Model system studies of hemoglobin in glycerol-water solution initially demonstrated the usefulness of the ST EPR techniques to simple slow, isotropic motions (4). Recently, several more complicated membrane systems have also been studied by the second harmonic out-of-phase absorption ST EPR technique (7–14). Theoretical analysis indicates that several detection methods, including second harmonic out-of-phase absorption and first harmonic out-of-phase dispersion, can

be used to obtain both qualitative and quantitative dynamic information about molecules which exhibit motions in the microsecond to millisecond region (6). Experimentally, the methods appear to be rather simple and the instrumental requirements, which are commercially available, are fairly modest. It has been suggested that the second harmonic absorption method may be more sensitive and convenient to use, but that the dispersion method is potentially more powerful for most biological systems (4,6). We have used a nitroxide protein spin label and a nitroxide fatty acid spin probe to monitor the motional behavior of the protein and lipid components of intact erythrocyte membrane ghosts at different temperatures and pH values over a wide range of motions and to compare the experimental methods and spectral sensitivity of the three EPR displays: conventional (first harmonic in-phase absorption) EPR, second harmonic out-of-phase absorption ST EPR, and first harmonic out-of-phase dispersion ST EPR.

Our data show the sensitivity of ST EPR over conventional EPR to the motions we have studied. Between the ST EPR methods, the second harmonic out-of-phase absorption is more practical, at least at this time, than is the first harmonic out-of-phase dispersion, due to signal-to-noise limitations. Furthermore, our data indicate that, at least for this system, the protein motions occur on a time scale for which only the second harmonic absorption display yields useful information; in the dispersion display the proteins appear to be essentially immobilized under all conditions. However, the dispersion ST EPR spectra of the lipid spin probe exhibit small changes under different conditions which are not detected by either the conventional EPR or the second harmonic absorption ST EPR displays. These results show that the first harmonic dispersion and second harmonic absorption ST EPR methods have different time scales of sensitivity. The dispersion method appears to be uniquely sensitive to motions for rotational correlation times around 10^{-7} s and the absorption method around 10^{-7} to 10^{-3} s.

MATERIALS AND METHODS

Membrane Samples

Hemoglobin-free white membrane ghosts were prepared from normal adult human erythrocytes according to the methods of Dodge et al. (15) and Steck and Kant (16). Membrane samples (usually ~4 mg/ml in protein concentration) were incubated with the protein spin label *N*-(1-oxyl-2, 2, 6, 6-tetramethyl 4-piperidinyl) maleimide (Mal-6) (from Syva Research Chemicals, Palo Alto, Calif.) at a concentration of 30–50 μg Mal-6 per milligram of protein in the dark at 4°C for 1 h. Excess spin label was removed by washing with phosphate buffer until the samples gave constant EPR signals. A portion of this labeled membrane sample was set aside as the Mal-6 EPR sample at pH 8. The remaining membranes were then dialyzed at pH 4.5. A trace amount of sodium azide was added to all samples as a preservative. The membrane samples were concentrated by centrifugation to obtain a protein concentration of ~15 mg/ml. The Mal-6 spin label concentration is ~25 nmol of labeled sulfhydryl per milligram proteins. Fatty acid spin probe 2-(3-carboxy-propyl)-4, 4-dimethyl-2-tridecyl-3-oxazolidinyloxyl (5-doxyl stearate) was also used for a separate series of samples. The membrane sample was incubated with 5-doxyl stearate at a concentration of 100 μg/mg protein for 30 min at room temperature. The labeled membrane samples of different pH were prepared similarly to Mal-6 labeled membrane samples.

EPR Experiments

The samples were transferred to 50-μl glass capillaries (nonheparinized microhematocrit tubes from Dade Div., American Hospital Supply Corp., Miami, Fla.). One end of the capillary was then sealed

with flame and a table top centrifuge was used to pack samples to the bottom of the capillary tubes. For each EPR measurement, the capillary was placed in a 3-mm EPR quartz tube filled with a small amount of silicon fluid to give thermal stability. A Varian E109E EPR spectrometer (Varian Associates, Palo Alto, Calif.) equipped with a TM cavity and a variable temperature control set-up was used for all measurements. The sample temperature was monitored by a digital read-out device connected to a copper constantan thermocouple. A Nicolet 535 time averager (Nicolet Instrument Corp., Madison, Wis.) was interfaced with the EPR spectrometer to improve signal-to-noise ratios. The microwave power was calibrated such that the ST spectra of hemoglobin in water-glycerol were similar to those published (3); this was found to occur at a bridge attenuation of 7 dB. The modulation amplitude was calibrated according to the procedures given in the Varian instruction manual. Since the ST EPR spectra are quite sensitive to microwave power and modulation amplitude, these calibrations were exercised with care. We have found that to have a stable (nondrifting) phase setting, the console of the EPR spectrometer needed to be on for at least 4 h before running.

Spectrometer parameters used in first harmonic in-phase absorption, (V_1) (terminologies and abbreviations of reference 4 have been followed), second harmonic out-of-phase absorption, (V_2') and first harmonic out-of-phase dispersion, (U_1') experiments are presented in Table I.

RESULTS AND DISCUSSION

The maleimide-analogue Mal-6 spin labels alkylate primarily the sulfhydryl (SH) groups of the protein molecules. Under our labeling conditions, we have found that we label ~25 nmol of SH groups per milligram protein, which are ~20% of the total SH groups in membrane. It has been reported that membranes in SDS consist of 130 nmol per milligram membrane proteins (17). Furthermore, ~80% of the spin label intensity arises from label sites at the inner membrane surface, with most of the spin labels attached to the peripheral proteins, the spectrin-actin complex (18), which covers the entire inner membrane surface. Thus, the Mal-6 spectra are primarily monitoring the molecular mobility (flexing and wobbling) of this protein complex.

The fatty acid spin probe intercalates amongst the lipid molecules, with the nitroxide moiety of 5-doxyl stearate located near the carboxyl group, and has been used to monitor the behavior of the lipid hydrocarbon chains in the region near the polar head group (19).

We have used these two spin label molecules to follow different kinds of motions associated with the peripheral proteins and the lipids in intact erythrocyte membranes at pH 8 and 20°C. Decreasing the temperature to 5°C results in motional changes in membrane components and therefore allows us to compare the sensitivity of the three EPR detection methods. It has been well-documented from morphological studies that, upon decreasing pH, the erythrocyte membranes shrink and eventually form aggregates at pH 4.5, the isoelectric point of spectrin-actin (20). Purified spectrin-actin also form similar, large aggregates at pH 4.5 (21).

TABLE I
SPECTROMETER PARAMETERS FOR EPR AND ST EPR EXPERIMENTS

Spectral display	V_1	V_2'	U_1'
Microwave power attenuation (*decibels*)	20	7	7
Modulation frequency (*kilohertz*)	100	50	100
Modulation amplitude (*Gauss*)	1	5	5
Time constant (*seconds*)	0.128	0.128	0.128
Scan time (*minutes*)	2	2	1
Number of scans	4	16	128

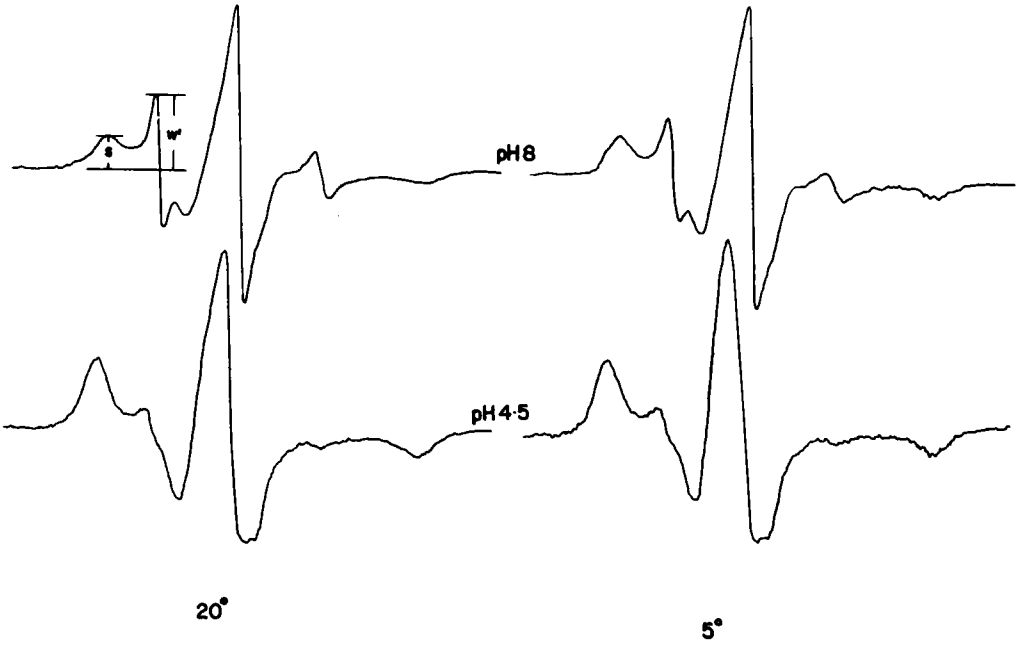

FIGURE 1 Conventional V_1 EPR spectra of Mal-6 labeled membrane samples at pH 8 and pH 4.5 at 20 and 5°C.

At this pH value, we therefore expect the mobility of the labeled proteins to differ markedly from that at pH 8 (22). Thus, membranes at pH 4.5 provide us with another system to test the motional sensitivity of the EPR methods.

SPECTRAL BEHAVIOR OF MAL-6 LABELED PROTEINS

The conventional EPR (V_1) spectra in Fig. 1 show that the Mal-6 labeled membrane samples at pH 8 exhibit relatively broad spectral lines and "powder" patterns characteristic of strongly immobilized motions. Both the hyperfine separation and the line widths of the hyperfine extremal lines have been used to monitor motional behavior in the correlation time domain of 10^{-8} to 10^{-6} s (23–25); these parameters were measured from the spectra and compared with those obtained from spectra of membranes at pH 4.5. We found that these parameters exhibit little or no significant change upon changing the pH from 8 to 4.5 at either 20 or 5°C, suggesting that the proteins at both pH values are essentially "immobilized" (strongly immobilized) on the conventional EPR time scale ($\tau > 10^{-7}$ s).

In addition to the broad, slow-motion spectral components, the V_1 spectra of the Mal-6 labeled membranes at pH 8 and 20°C (Fig. 1) also show that a minor portion, ~10%, of the signal exhibits the comparatively narrow line widths and derivative shapes which are considered to be from species with weakly immobilized motions (2–3). The amplitude ratio of the weakly and strongly immobilized signals (W'/S) of the Mal-6 samples has often been used to monitor the relative amount of weakly and strongly immobilized label motions in the protein molecules in membranes under different conditions. Values of this ratio for membranes at pH 8 are 2.2 ± 0.2 and 1.2 ± 0.2 for 20 and 5°C, respectively. The values at pH

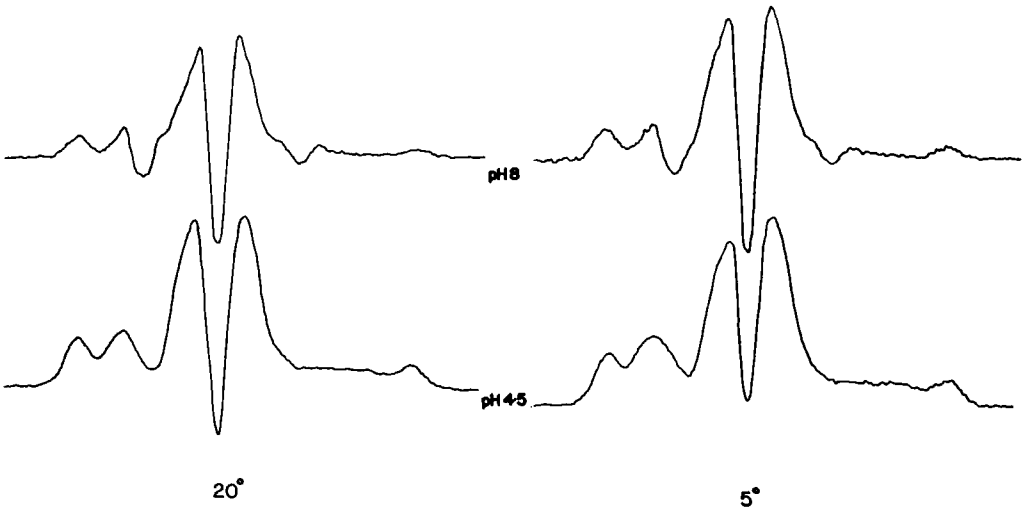

FIGURE 2 Second harmonic V_2' ST EPR spectra of Mal-6 labeled membrane samples at pH 8 and pH 4.5 at 20 and 5°C.

4.5 are 0.51 ± 0.03 at 20°C and 0.34 ± 0.02 at 5°C. From these values, it can be seen that lowering the pH to 4.5 virtually eliminates the weakly immobilized component, presumably by conversion to the strongly immobilized component.

Second harmonic ST EPR (V_2') spectra for these systems are shown in Fig. 2. It can be seen that these spectra are much more sensitive to temperature and pH changes than are the V_1 spectra. The spectral parameters L''/L, C'/C, and H''/H, introduced by Thomas et al. (4), are given in Table IIA. For an isotropic, single motional component system, the rotational correlation time, τ, can be obtained from either L''/L, C'/C, or H''/H (4). For simple systems with anisotropic motions, one would expect that τ_L and τ_H would yield roughly similar results, but that τ_C might differ markedly, depending on the orientation and extent of motional anisotropy (6,13). However, our system is a heterogeneous system with probably several different types of anisotropic motion. The τ_L, and τ_C, and τ_H values obtained from calibration curves of isotropic motion (4) all differ (data not shown). These τ values may have rather complicated physical meaning and the interpretation of these values is not straightforward.

TABLE IIA
SPECTRAL AMPLITUDE PARAMETERS OBTAINED FROM FIG. 2

System	Temperature	L''/L	C/C	H''/H
	(°C)			
pH 8	20	*	−0.90‡	0.35
	5	*	−0.66	0.39
pH 4.5	20	1.30	−0.16	0.81
	5	1.44	0.16	0.90

*No values were obtained due to overlapping signals.
‡For simplicity in presentation, the standard deviations were omitted. All spectral ratio values have standard deviations $< \pm 0.2$.

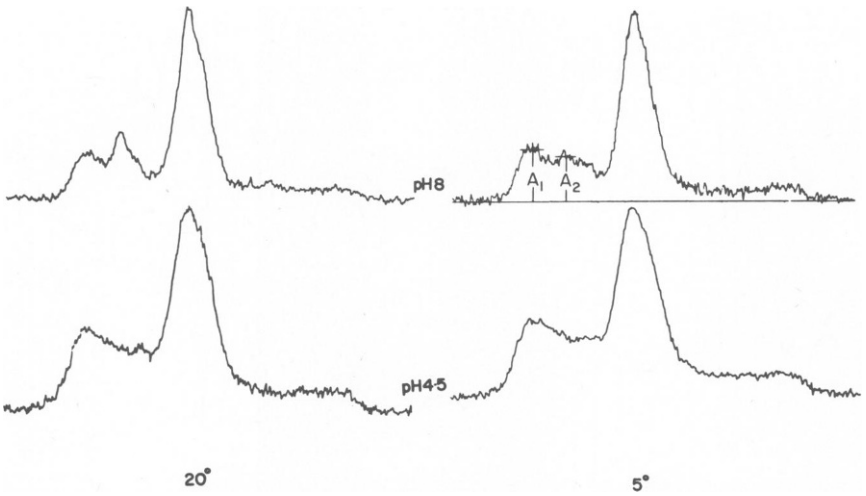

FIGURE 3 First harmonic U_1' ST EPR spectra of Mal-6 labeled membrane samples at pH 8 and pH 4.5 at 20 and 5°C.

Thus, for our purpose of comparing the sensitivity of techniques, we will simply use the spectral parameter L''/L, C'/C, and H''/H without interpretation in terms of apparent τ values. As indicated in Table IIA, the magnitudes of these ratios change significantly upon decreasing the temperature from 20 to 5°C for both pH 8 and pH 4.5 samples.

In contrast to the second harmonic ST EPR (V_2') display, the dispersion ST EPR (U_1') spectra shown in Fig. 3 exhibit little sensitivity to various sample conditions. The general spectral shapes are quite similar to those shown for fully immobilized systems (4,6). Little information on the quantitative analysis of U_1' spectra is available in the literature as yet. However, a qualitative interpretation may be obtained by analogy with that developed for the V_2' display. Two amplitude parameters, A_1 and A_2, and the amplitude ratio A_2/A_1, are shown in Table IIB. These values show little change for the sample at pH 4.5 with temperature changes from 20 to 5°C. The values for the sample at pH 8 show some difference at the two temperatures, presumably reflecting a small amount of the weakly immobilized component overlapping the major component at 20°C, but not at 5°C. It has been noted that the U_1' spectra exhibit maximum motional sensitivity over the τ range of $\sim 10^{-7}$ s to 10^{-5} s, reaching an asymptotic behavior at a τ of $\sim 10^{-5}$ s at 100 kHz modulation (6). Thus, it would appear that the protein motions in these systems are too slow for significant information to be obtained from the U_1' display.

TABLE IIB
SPECTRAL AMPLITUDE PARAMETERS OBTAINED FROM FIG. 3

System	Temperature	A_2/A_1
	(°C)	
pH 8	20	1.47
	5	0.83
pH 4.5	20	0.74
	5	0.74

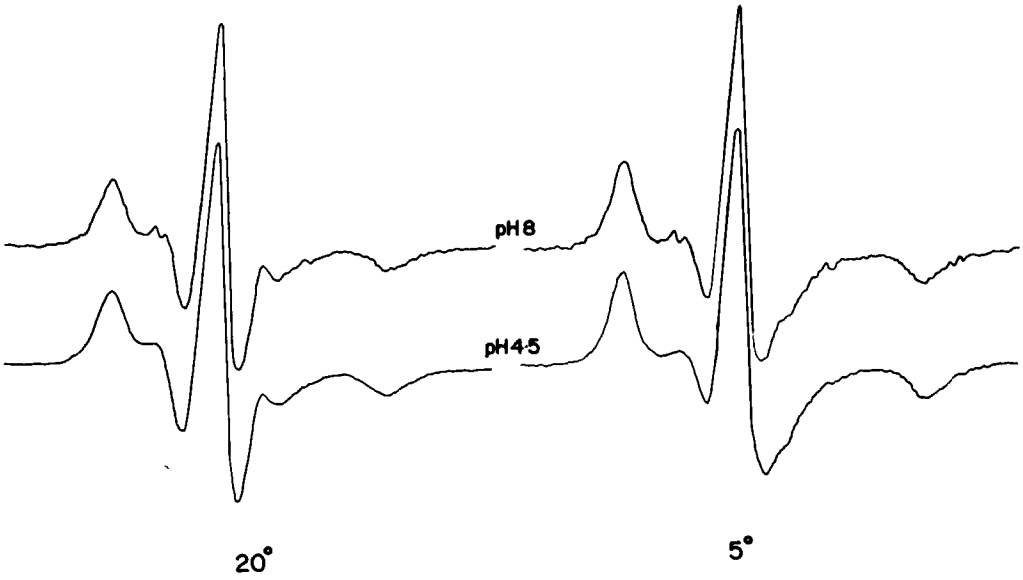

FIGURE 4 Conventional V_1 EPR spectra of 5-doxyl stearate labeled membrane samples at pH 8 and pH 4.5 at 20 and 5°C.

SPECTRAL BEHAVIOR OF THE LIPID SPIN PROBE

The conventional V_1 spectra of membranes with 5-doxyl stearate incorporated are shown in Fig. 4. In going from 20°C down to 5°C, the hyperfine splitting at pH 8 increases from 58.8 ± 0.6 to 63.1 ± 0.2 G, the hyperfine extremal line widths decrease slightly, and the high field

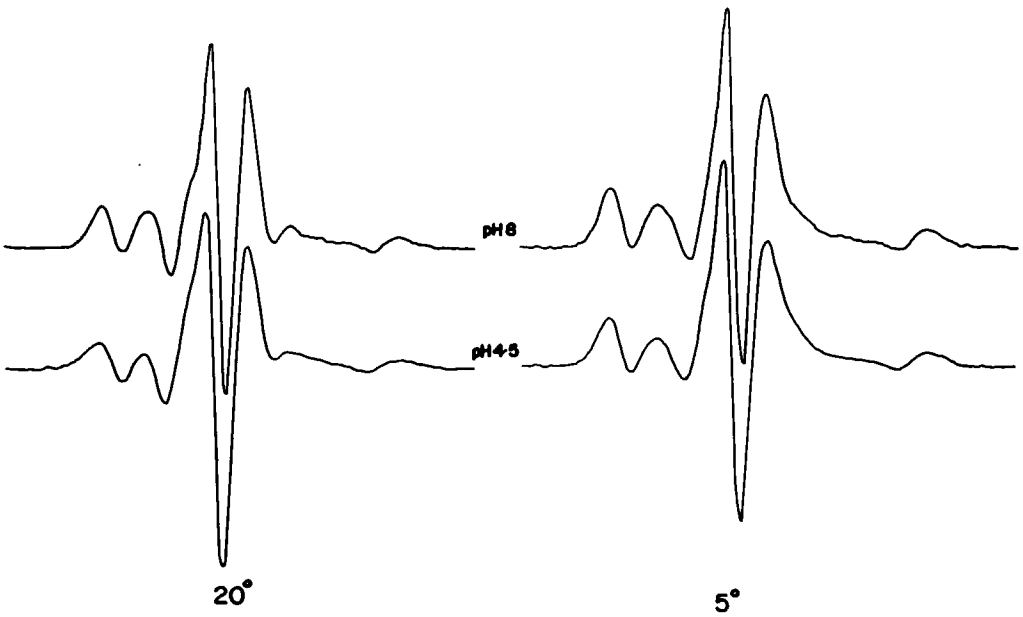

FIGURE 5 Second harmonic V_2' ST EPR spectra of 5-doxyl stearate labeled membrane samples at pH 8 and pH 4.5 at 20 and 5°C.

TABLE IIIA
SPECTRAL AMPLITUDE PARAMETERS OBTAINED FROM FIG. 5

System	Temperature	L''/L	C/C	H''/H
	(°C)			
pH 8	20	0.70 ± 0.03	−1.32 ± 0.01	0.40 ± 0.06
	5	0.61 ± 0.01	−0.84 ± 0.01	0.38 ± 0.03
pH 4.5	20	0.52 ± 0.05	−1.35 ± 0.05	0.43 ± 0.01
	5	0.56 ± 0.03	−0.84 ± 0.01	0.38 ± 0.03

side of the center line changes shape, with all changes indicating decreased motion, as would be expected. However, the membrane perturbant of reducing pH appears to produce little or no effect upon the spectral behavior at each of the two temperatures.

Likewise, the V_2' spectra shown in Fig. 5 also exhibit little or no change in going from 20 to 5°C for samples at both pH 8 and pH 4.5. The values of L''/L, C'/C, and H''/H are given in Table IIIA.

The dispersion ST EPR spectra for these systems are shown in Fig. 6. In contrast to the V_1 and V_2' displays, these spectra appear to show differences under the various conditions. The amplitude ratios of A_2/A_1 of membranes at pH 8 and 4.5 are 0.90 and 0.50, respectively, at 20°C and decrease to 0.50 and 0.32, respectively, at 5°C. In comparing the U_1' spectra of membranes at pH 4.5 with those at pH 8 at both 5 and 20°C, the central regions appear essentially identical. However, the spectral amplitude ratio (A_2/A_1) comparable to the L''/L of the V_2' display appears to be somewhat lower at pH 4.5 than at pH 8, suggesting that lowering the pH may allow a slightly increased motion in the region of the molecule being

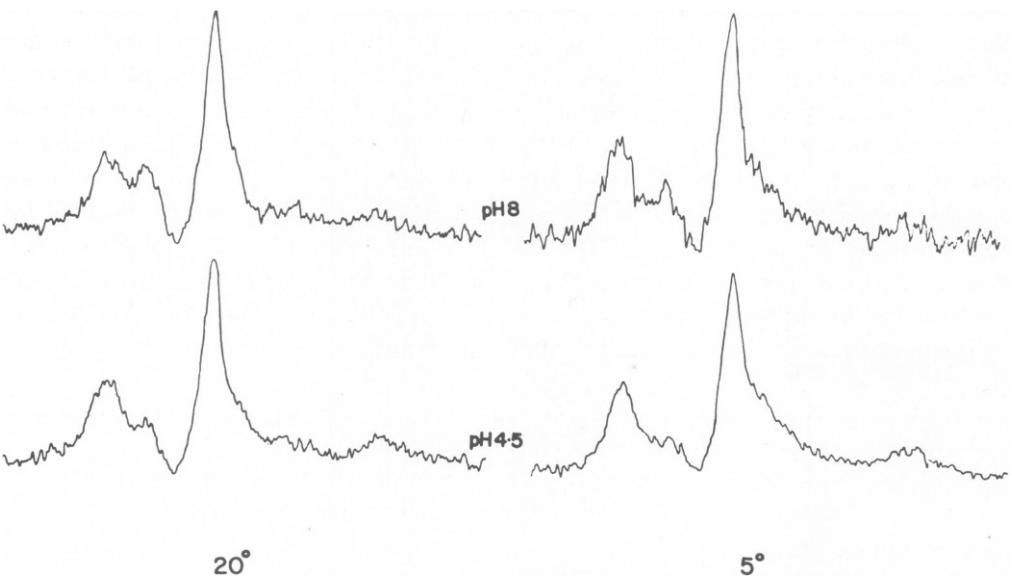

FIGURE 6 First harmonic U_1' ST EPR spectra of 5-doxyl stearate labeled membrane samples at pH 8 and pH 4.5 at 20 and 5°C.

TABLE IIIB
SPECTRAL AMPLITUDE PARAMETERS OBTAINED FROM FIG. 6

System	Temperature	A_2/A_1
	(°C)	
pH 8	20	0.90
	5	0.50
pH 4.5	20	0.50
	5	0.32

monitored. Thus, the U'_1 spectra appear to be relatively more sensitive to the motions of the lipid molecules in erythrocyte membranes than either the V_1 or V'_2 spectra.

In summary, we have used different membrane systems to generate a series of dynamic states for molecules in erythrocyte membrane ghosts, and systematically tested the experimental convenience and motional sensitivity of V_1, V'_2, and U'_1 spectral displays in the study of human erythrocyte membranes. The first harmonic conventional in-phase absorption (V_1) spectra show that the 5-doxyl stearate lipid labels in erythrocyte membranes at pH 8 undergo motions of $\sim 10^{-7}$ s or slower. For the protein motion a small fraction, ~10% of the signals, shows a much faster motion (about or faster than 10^{-9} s). The majority of the signals show slow motion. At pH 4.5, the fast motion is converted to the slower motion. However, no additional information about these slow motions in membranes at either pH 8 or pH 4.5 can be obtained from the V_1 spectra due to their low sensitivity to slow motion. Likewise, the first harmonic out-of-phase dispersion (U'_1) spectra of the Mal-6 labeled membranes indicate only that protein motions are too slow for any changes to be detected. However, the second harmonic out-of-phase absorption (V'_2) ST EPR spectra show that the protein molecules undergo marked motional changes upon decreasing temperature or pH. The lipid molecules show little spectral change in the V_1 and V'_2 display, but the U'_1 spectra appear to show changes in going from 20 to 5°C and also small changes in going from pH 8 to pH 4.5 at each temperature measurement. Experimentally, the V'_2 spectra can generally be obtained within half an hour with good signal-to-noise ratios, whereas each U'_1 spectrum takes ~2 h to obtain and the signal-to-noise ratios are still rather poor. Since membrane samples are rather unstable and are not amenable to further concentration, both the long time required for data acquisition and the poor signal-to-noise ratios make the U'_1 detection undesirable for routine runs at the present time. The U'_1 display does, however, appear to be more sensitive than either the V_1 or the V'_2 display to motional changes for correlation times around 10^{-7} s. Thus, for some applications in this time range, its use may be important.

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