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STUDIES OF THE MOBILITY OF MALEIMIDE SPIN LABELS WITHIN THE ERYTHROCYTE MEMBRANE

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We have confirmed a method yielding reproducible and reliable spectrometric parameters derived from spin-labeled erythrocyte ghosts using nitroxide derivatives of maleimide compounds. The disorder parameter, W/S, was shown to vary with changes in the structure of the label, the conditions utilized for labeling such as ionic strength and erythrocyte age and the presence of drugs such as alcohol and acetaminophen. The nitroxide spectrum was also found to change with increasing and decreasing temperature in an irreversible manner. These findings should permit increased reliance to be placed on the spin-labeling technique when used to monitor changes in membrane lipid or protein assembly.

Introduction

Spin-label methodologies have been extensively applied to monitor changes in the motional and structural behavior of membrane proteins and phospholipids [1]. Non-covalently bound probes such as 2,2,6,6,-tetramethylpiperidinoxyl (TEMPO), which are soluble in polar and non-polar phases and consequently partition between aqueous and membrane regions [2] when added to erythrocyte preparations [3], in addition to covalently bound nitroxide labels such as 3-[(2-(2-(2-iodoacetamido)ethoxy)ethyl)-carbamoyl]-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl [4] attached to either unspecified protein or lipid components, have been utilized.

A number of procedures have been developed to specifically bind maleimide-analog sulfhydryl spin labels to proteins incorporated within erythrocyte membranes [5–8]. While all exhibit extensive similarities, small perturbations of the labeling conditions may produce significant changes in the nitroxide spectrum used to monitor the physical state of these proteins. The most common spectral parameter for protein-bound labels is defined as the W/S ratio. This ratio reflects signal amplitudes of the two dominant classes of the probe, the weakly (W) bound or more mobile species and the strongly (S) bound or more immobilized spin label (Fig. 1). This ratio has been shown to be extremely sensitive to a number of factors, including preparation and labeling conditions of the membranes and also changes in membrane structure.

Confirmation of a method that provides reproducible and reliable W/S ratios from human erythrocyte ghosts is an important prerequisite for further membrane investigations. In addition, elucidation of such factors as the effects of label structure, temperature, aging and ionic strength on this parameter must be determined such that these conditions can be controlled during investigations of possible changes in membrane organization.

Materials and Methods

Human erythrocyte ghosts were prepared as described by Butterfield and Markesbery [5] using blood from healthy adult volunteers. Erythrocytes were sedimented by centrifugation at $1000 \times g$ for 10 min at 20°C. The plasma and buffy coat were removed by aspiration and the cells washed three times in 5 mM sodium phosphate buffer, containing 150 mM NaCl, pH 8.0 at 4°C. Hemoglobin was removed by osmotic lysis using 5 mM sodium phosphate buffer, pH 8.0, followed by repeated washing at $25\,000 \times g$ for 10 min until the supernatant exhibited no discernable hemoglobin contamination. Maleimide spin labels of varying structure, shown in Fig. 2. (Syva, Palo Alto, CA) were added to the erythrocyte ghosts in a ratio of 1 mg of label per 25 mg of membrane protein and the mixture incubated at 4°C for 16 h in the dark following which the membranes were sedimented and washed four times to remove unreacted spin label. Protein was determined by the method of Bradford [9].

Reduction of the spin-labeled membranes was achieved by incubating the labeled erythrocyte ghosts (3–4 mg/ml protein in 5 mM sodium phosphate buffer, pH 8.00) at 20°C with varying concentrations of ascorbate (0.1–1 mM), adjusted to pH 8 prior to addition. With 1 mM ascorbate, loss of the nitroxide EPR spectrum was complete within less than 1 min. For hydrogen reduction, labeled membranes (3 mg/ml protein) were incubated with Pt and hydrogen (1 atm) for 40 min at 4°C following which the Pt was removed by centrifugation at $500 \times g$ for 5 min. Control studies were performed using identical conditions on the free nitroxides.

EPR spectra were recorded using a Varian Associates spectrometer model E-9 interfaced to a Hewlett-Packard HP9845B computer. For the majority of measurements, a standard flat cell was utilized. However, to examine the effect of temperature on label mobility, samples were placed in a micro flat cell (Wilmad Glass, Buano, NJ) situated inside a Varian variable temperature accessory.

Samples were prepared in triplicate and the average of three scans used for analysis. Double integration of the spectra was performed according to the method of Wyard [10] using dilute solutions of the free spin label in phosphate buffer as standards. Separation of the complex spectrum into its two components was achieved using the following method. EPR spectra were obtained and integrated for samples of labeled membranes maintained at temperatures in the range -50°C to 50°C. At either temperature extreme, the spectrum was considered to be representative of the individual nitroxide components. For any given spectrum, recorded at 20°C, the relative amounts of the weakly and strongly immobilized labels could be determined from the normalized peak heights (W and S of Fig. 1) of the g_1 features.

Results

EPR spectra typical of both the free spin label and that attached to proteins associated with human erythrocyte ghosts are shown in Fig. 1. In aqueous solution, low concentrations of the free spin label gave rise to a narrow, three line spectrum centered around $g = 2$ with coupling constants of 1.68 mT and an isotropic 0.2 mT linewidth. The nitroxide moiety bound to the ghost membranes exhibited a markedly altered spectrum, comprising of two components. The first, a

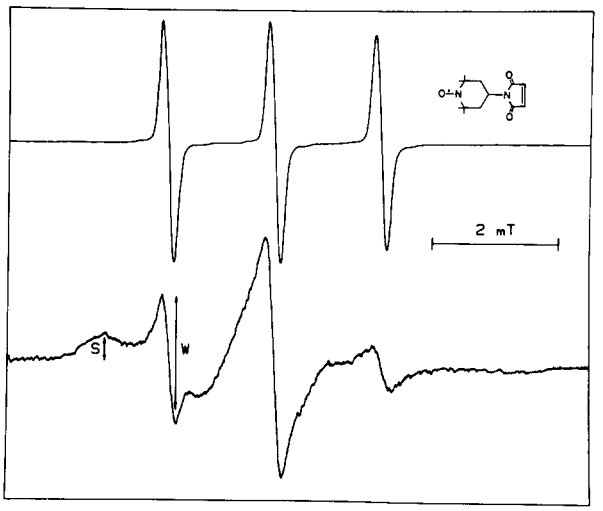


Fig. 1. Electron paramagnetic resonance spectra of free and membrane-bound 4-maleimido-2,2,6,6-tetramethyl-piperidino-oxyl. Spectrum A, free spin label (0.1 mM) in 5 mM phosphate buffer, pH 8. Spectrum B, spin-labeled erythrocyte ghosts (4 mg/ml) in 5 mM sodium phosphate buffer, pH 8. Spectra were recorded at 20°C using 14 mW microwave power and a modulation amplitude of 0.032 mT.

broad anisotropic spectrum arose from labels bound in a strongly immobilized environment and the second, a narrower spectrum due to nitroxide molecules exhibiting features characteristic of a less rotationally constrained environment. Analysis of this type of spectrum has been previously published [11].

The ratio of the signal amplitudes of the low-field transitions for both the weakly (W) bound and the strongly (S) bound spin labels, referred to as the *W/S* ratio, has been proposed to be an extremely sensitive indicator of nitroxide mobility and hence the ratio measures variations in protein conformation and/or environment within the membrane. Applying the procedures outlined by Butterfield and Markesbery [5], we have confirmed that labeling normal erythrocyte ghosts under carefully controlled conditions yields a highly reproducible value of 5.4 ± 0.2 for the *W/S* ratio using 4-maleimido-2,2,6,6-tetramethylpiperidinooxyl (Fig. 1) in 5 mM phosphate buffer, pH 8.0. Double integration of the composite spectrum indicated that the spin populations of the two components were significantly different, with the strongly immobilized species accounting for approx. 90% of the spectrum. In addition to yielding a highly reproducible value for the *W/S* ratio, the overall extent of label incorporation, determined by integration of various spectra was effectively constant, when corrected for differences in protein concentration.

The effect of spin-label structure, or more specifically the number of carbon atoms in the nitroxide ring was found to alter the hyperfine coupling constants of the free spin-label spectrum. Changing the ring structure from a pyrrolidinyloxyl (a five-membered ring) to a piperidinooxyl (a six-membered ring) increased the coupling constant from 1.59 mT to 1.68 mT [1]. Increasing the number of atoms between the nitroxide moiety and the maleimide grouping did not change the nitrogen hyperfine coupling constants of the resulting EPR spectrum. In contrast, protein-bound labels incorporated within the membranes exhibited marked variations in the *W/S* ratio, given in Fig. 2. Typical spectra are shown in Fig. 3. A plot of the *W/S* ratio versus spin label length (previously published by Zeidan et al. [12]) is shown in Fig. 4. Inclusion of a methyl or ethylcarbamoyl group between the maleimide and pyrrolidine ring resulted in an increase in the *W/S* ratio from 10.6 to 16.6 and 24, respectively. Incorporation of an additional methylene or methoxy group drastically increased the *W/S* ratio to 38.3 and 67, respectively.

The effect of varying temperature on the mobility of the strongly and weakly immobilized piperidinooxyl spin labels are shown in Fig. 5. Samples of the labeled membranes were incubated at the indicated temperatures for 10 min prior to recording the spectrum. Control spectra were initially obtained at 20°C following which the tem-

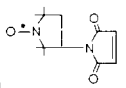
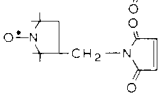
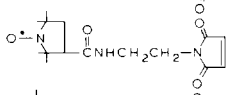
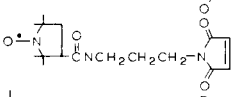
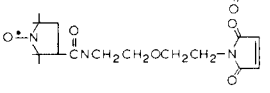
Code	Structure	Systematic Name	Length Å	W/S Ratio
A		3-Maleimido-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl	4.4	10.6
B		3-(Maleimidomethyl)-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl	5.7	16.6
C		3-[(2-Maleimidoethyl)carbamoyl]-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl	9.3	24.0
D		3-[(2-Maleimidopropyl)carbamoyl]-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl	10.5	38.3
E		3-[2-(2-Maleimidoethoxy)ethyl]carbamoyl]-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl	12.9	67.0

Fig 2. Chemical structures of pyrrolidinyloxyl maleimide spin labels. Spin-label dimensions were taken from Zeidan et al. [12]. The *W/S* ratio were determined as described in Methods.

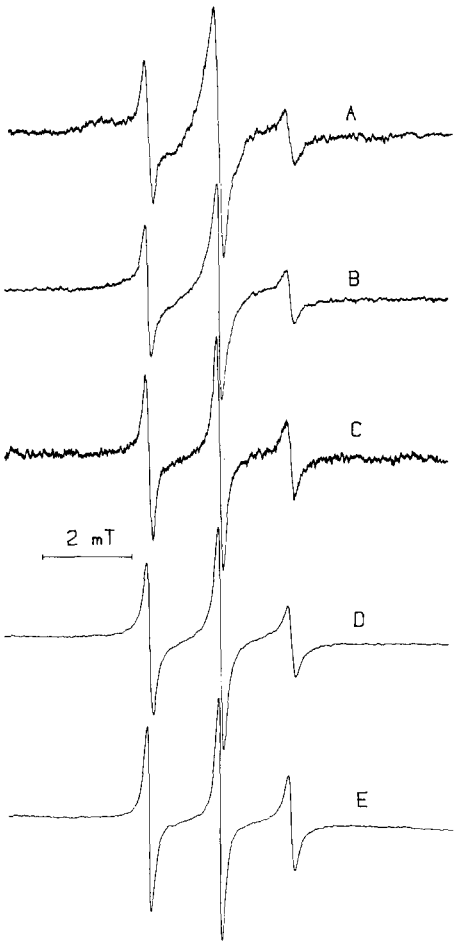


Fig. 3. Electron paramagnetic resonance spectra of pyrrolidinyloxyl spin labels bound to erythrocyte ghosts. Erythrocyte ghosts in 5 mM sodium phosphate buffer, pH 8, were reacted for 16 h with the nitroxides (designated A through E) shown in Fig. 2, as described in Methods. The corresponding EPR spectra, A through E, were then recorded using 14 mW microwave power and 0.032 mT modulation amplitude.

perature of the flat cell was either raised or lowered to the desired value. The results indicate that between 10 and 25°C, the *W/S* ratio remained nearly independent of temperature. Outside this narrow range, the *W/S* ratio decreased or increased as the applied temperature was lowered or raised. These changes were judged to be irreversible from the observation that incubating membranes at 37°C for 10 min followed by measurement of the *W/S* ratio at 20°C failed to restore the initial value of 5.4. Examination of the effect

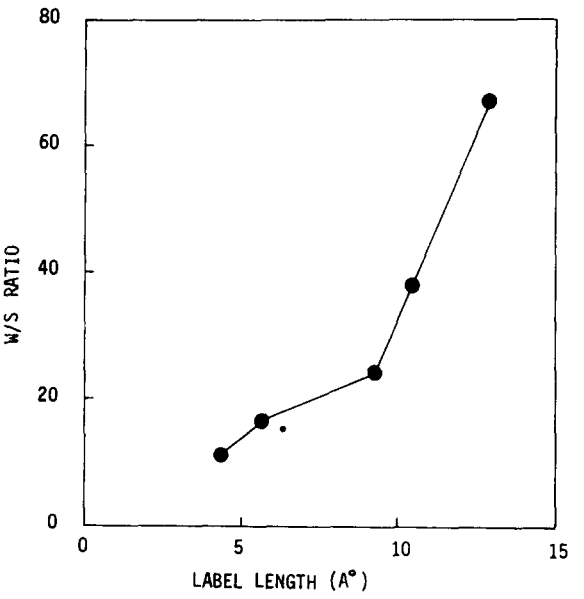


Fig. 4. Variation of *W/S* ratio of pyrrolidinyloxyl maleimide labeled ghosts as a function of chain length. The *W/S* ratios were determined as described in Methods and the chain length was taken from Zeidan et al. [12].

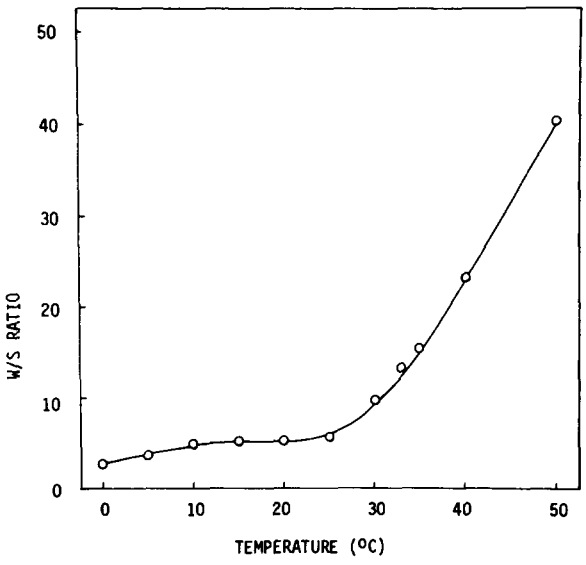


Fig. 5. Changes in the *W/S* ratio for the 4-maleimido-2,2,6,6-tetramethylpiperidinooxyl spin label bound to erythrocyte membranes with increasing temperature. The temperature varied from 0°C to 50°C. Samples were prepared as described in Methods and spectra recorded after incubation at the indicated temperature for 10 min.

of freezing the membranes lowered the ratio to 0.80 at $-10°C$, indicating substantial changes in membrane or protein organization. Samples of frozen membranes maintained at $-100°C$ yielded signals considered to be representative of powder spectrum, exhibiting a single class of randomly orientated, immobilized, spin labels. Thawing of the frozen samples resulted in a W/S ratio of 6.0, again indicating that irreversible changes in nitroxide mobility had occurred. To determine if the freezing process resulted in extrusion of labeled protein into the aqueous phase, which would be expected to increase the W/S ratio, thawed membranes were sedimented and the supernatant examined for the presence of free nitroxide, and protein. Neither spin label nor protein was detected.

The W/S ratio was found to be susceptible to storage or 'aging' of the erythrocyte ghosts. The order parameter was observed to be effectively constant for labeled membranes maintained at 4°C for up to 2 days. However, increasing the storage time interval resulted in a progressive elevation of the W/S ratio, reaching values of 6.7 after 7 days and 25 after 14 days.

Butterfield and Markesbery [5] have shown that the W/S ratio of maleimide-labeled ghosts is extremely susceptible to alterations in the ionic strength of the medium following the addition of choline chloride. We have confirmed these results using sodium chloride (Fig. 6). Increasing the sodium chloride concentration from 0 to 170 mM was found to decrease the W/S ratio to a limiting value of approx. 3. Thus, accurate control of the conditions used to generate a spectrum are required for meaningful evaluation of the results.

Addition of ethanol to isolated spin labeled ghosts was found to perturb the W/S ratio as shown in Fig. 7. Low concentrations were observed to decrease the ratio significantly. However, at concentrations up to 10% v/v, these effects were readily reversible both by dilution or washing of the ghosts with buffer. Thus, in vitro, relatively high concentrations of ethanol were found not to elicit irreversible membrane damage although changes in nitroxide mobility were apparent. These alterations are a result of an overall decrease in the amplitude of the weakly immobilized component.

We have also examined the W/S ratio of labeled

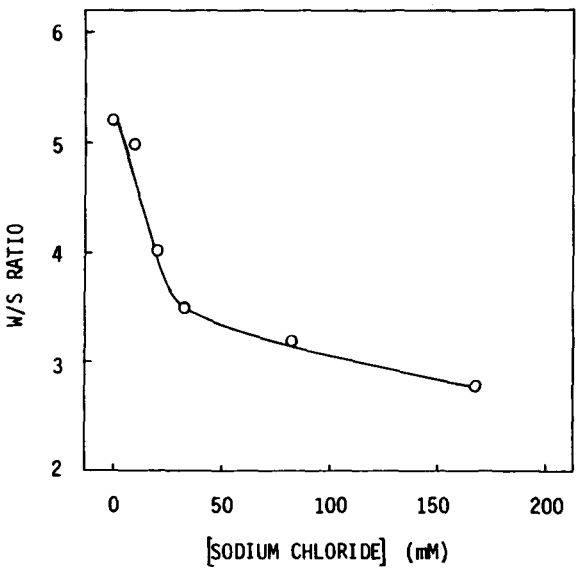


Fig. 6. Changes in the W/S ratio for the 4-maleimido-2,2,6,6-tetramethylpiperidinooxyl spin label with increasing ionic strength. The final concentration of NaCl ranged from 0 to 170 mM. Spectra were recorded as described in Fig. 1.

ghosts from a sample of blood from an adult volunteer whose blood concentration of alcohol was 0.3%. The value of the W/S ratio obtained, 5.3, showed no irreversible changes in the membrane fluidity had occurred in vivo.

Contrary to our findings with ethanol, acetaminophen increased the W/S ratio from 5.4 ± 0.2 for control to 5.9 ± 0.2 at 10 μM. However, this increase in ratio rapidly reached its maximum at

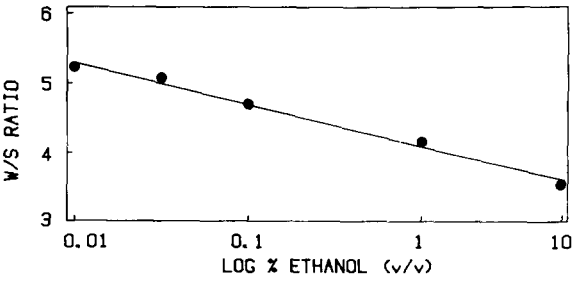


Fig. 7. Changes in the W/S ratio of 4-maleimido-2,2,6,6-tetramethylpiperidinooxyl bound to erythrocyte membranes in the presence of ethanol. The final concentration of ethanol varied from 0% to 10% v/v. Spectra were recorded using conditions given in Fig 1.

concentrations of acetaminophen above 0.1 mM. As in the case of ethanol, this alteration in the *W/S* ratio was reversible upon washing with 5 mM phosphate buffer at pH 8.0.

Discussion

The preceeding results confirm the disorder parameter, referred to as the *W/S* ratio, for nitroxide moieties incorporated into erythrocyte ghosts to be significantly influenced by a number of factors. However, with adequate controls, this ratio may be used to monitor changes in the organization of proteins associated with the cell membrane since the spin-label technique is a convenient method of investigating reporter group sensitivity to the mobility and chemical environment of its host molecule. The precise location of the label binding sites have not been determined. However, the maleimide spin probe presumably alkylates primarily the sulfhydryl groups of membrane proteins, although some amino acid binding may occur. No EPR signals have been observed from lipid samples extracted from the labeled ghosts [6], but Spectrin, a high molecular weight protein complex extracted from the membrane has been shown to represent approx. 25% of the membrane protein and has been proposed to account for binding of approx. 30 % of the nitroxide label by analogy with *N*-[^{3}H]ethylmaleimide binding studies while Peak III (30%) binds significantly less label (11%). Overall, the maleimide spin probe has been proposed as having a greater affinity for high molecular weight membrane proteins under labeling conditions similar to those utilized in this study [6]. Our results indicate that for the piperidinoxyl probe, attached to unsealed ghosts, all the label binding sites are accessible to added ascorbate (at concentrations above 1 mM) suggesting that the majority of nitroxide free radicals are situated very close to the membrane surface. This hypothesis is supported by fact that the magnitude of the nitrogen hyperfine splitting constants are very similar to those obtained for the free spin label in a polar environment. This finding is in contrast to the results of Butterfield et al. [6] who proposed that a significant proportion of the maleimide binding sites were inaccessible to ascorbate and must consequently be situated within the ascorbate-impermeable membrane. However, our findings do confirm those of Butterfield and Markesbery [5] that there are two dominant classes of binding sites and that the EPR parameters suggest that both are labeled to comparable extents to those otained with previous studies using similar conditions.

Comparison of the results obtained using spin probes of differing chain length indicate that the piperidinoxyl species is the most suitable for mobility studies of membrane proteins. The observation that changing the ring structure from the piperidinoxyl to the pyrrolidinoxyl increases the *W/S* ratio suggests that some of the strongly-bound label sites may be located within clefts in the protein structure that are of sufficient dimensions to afford the latter nitroxide increased mobility. In addition, increasing the chain length would be expected to position the nitroxide portion of the probe at a greater distance from the membrane surface facilitating near complete rotational mobility. The analysis of the variation in the disorder parameter with label length may indicate that some binding sites could be located at a depth of approx. 8 Å within the membrane. In attempt to more completely define the surface location of these bound nitroxide probes, reduction experiments using a combination of Pt and hydrogen were performed. Samples of membranes labeled with the compounds shown in Fig. 1 revealed near total stability of the spin probes towards Pt-catalyzed, hydrogen reduction, while the nitroxides in 5 mM sodium phosphate buffer, pH 8.0 in the absence of membranes are rapidly reduced to their corresponding hydroxylamines. These findings indicate that the nitroxide-portion of the probes are not readily accessible in solution but are possibly localized in hydrophobic pockets within the overall membrane organization.

The modifications of probe fluidity introduced by temperature variations may be a significat source of error. Our findings indicate that within a narrow temperature range, reproducible *W/S* ratios may be obtained. Significant deviations from the 4–20°C interval may generate irreversible changes in membrane organization that are not the result of extrusion of labeled proteins into the aqueous medium. In addition, we may propose that nitroxide-labeled proteins could be utilized to

monitor phase transitions of biological membranes from both mammalian and bacterial sources. Recent work by Fung [13] support this hypothesis.

Alterations in the W/S ratio of isolated erythrocyte ghosts have been applied to investigate phenomena ranging from interaction between the membrane and hemoglobin [8] to clinical disorders, such as myotonic muscular dystrophy [6], which are thought to effect changes in membrane structure. We have extended these type of studies to alcohol and the commonly used analgesic acetaminophen. While the effects of both drugs are reversible, at low concentrations, both have been shown to exert significant perturbations in the local environment of spin-labeled, membrane-bound proteins.

Acknowledgment

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