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EFFECT OF CHEMICAL MODIFIERS OF PASSIVE PERMEABILITY ON THE CONFORMATION OF SPIN-LABELED ERYTHROCYTE MEMBRANES

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SUMMARY

Red blood cell membranes were labeled with stearic acid spin labels and with two protein spin labels (4-maleimido-2,2,6,6-tetramethylpiperidinooxyl and 4,2-iodoacetamido-2,2,6,6-tetramethylpiperidinooxyl) covalently bound to the membrane sulfhydryl groups.

Blocking the sulfhydryl groups with *p*-chloromercuribenzoate, *p*-chloromercuribenzosulfonate, *N*-ethylmaleimide and iodoacetamide provides information about the binding selectivity and the accessibility to their sites of the protein spin labels.

The ESR spectra of labeled red blood cell membranes were studied in various experimental conditions.

The ESR spectra of fatty acid spin-labeled intact erythrocytes are not modified by large variations of pH and osmolarity, indicating a stability of the phospholipid region.

Alterations of the ESR spectra of the protein spin labels were produced upon exposure of the ghosts to chemical reagents that bind to amino groups (dinitrofluorobenzene, 2-methoxy-5-nitrotropone, trinitrobenzene sulfonic acid), indicating changes in the local environment surrounding the probes.

The ESR spectra of the same protein spin labels are altered by pH variations of the external medium. These changes are different from those observed when the labeled ghosts are exposed to amino reagents. These data, observed by ESR, may be related to the well known effect of the NH_2 reagents on the ionic permeability of the red blood cell.

INTRODUCTION

Several chemical agents are known to modify passive permeability of red blood cells [1]. These substances may be characterized either by their permeability action (on ionic selectivity) or by their ligand specificity (binding with sulfhydryl or amino protein groups). The amino reactive reagents 1-fluoro-2,4-dinitrobenzene (DNFB), 2-methoxy-5-nitrotropone and 2,3,4-trinitrobenzene sulfonic acid (TNBS) decrease the anion permeability but also cause an increase in the cation permeability [2]. In

contrast, sulfhydryl reagents such as *p*-chloromercuribenzoate (PCMB) and *p*-chloromercuribenzosulfonate (PCMBS) decrease the cation permeability without any effect on the anion permeability of human red blood cells. However, it is usually difficult to establish a meaningful relationship between binding and effect.

The spin-labeling technique is a sensitive method of detecting changes in membranes [3]. One can either use lipid labels or protein labels and the technique has been recently applied to the investigation of conformational changes in a variety of systems [4, 5].

In the present work on red blood cell membranes, attempts were made to study the possible conformational changes induced either by chemical modifiers of passive permeability or by physicochemical variations (pH, osmotic strength). Two groups of labels were used: (1) spin-labeled stearic acids were dissolved in membrane lipids; (2) spin-labeled analogs of maleimide and iodoacetamide were covalently bound to membrane protein SH groups. Moreover, a study of the competitive reactions between the chemical reagents and the protein spin labels, bound to the membrane proteins, provides information about the binding selectivity of these labels.

MATERIALS AND METHODS

Biological

Human red cells were isolated from freshly drawn heparinized blood, washed in isotonic phosphate buffer several times, and the buffy coat removed by aspiration.

Membranes were prepared by the method of Dodge et al. [6] and used within three days. This method produces intact ghosts ($\approx$ 3 mg of membrane protein/ml) with no visible evidence of residual hemoglobin.

Chemicals

Fatty acid spin labeling. The spin labels used, I (*m,n*)-stearic acid analogs, were purchased from Synvar Associates. Probe I [12, 3] [2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl] has the nitroxide ring located at the polar end whereas Probe II [1, 14] [2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxyl] has the ring near the hydrophobic end.

Red cells were spin labeled by exchange from bovine serum albumin as described by Landsberger et al. [7]. After labeling, the samples were washed (4–5-times) until a constant EPR spectrum was obtained.

Protein spin labeling. 4-Maleimido-2,2,6,6-tetramethyl piperidinooxyl (maleimide spin label: Probe III) and 4-(2-iodoacetamido)-2,2,6,6-tetramethyl piperidinooxyl (iodoacetamide spin label: Probe IV) were purchased from Synvar Associates. For labeling, 1 mg of the spin label was added to three ml of packed ghosts ($\approx$ 9 mg of membrane protein) resuspended in phosphate buffer (pH = 7.4). The mixture was incubated for 3 h at room temperature (25 °C) or overnight at 4 °C. After the incubation period, the excess label was removed by washing 3–4-times in an appropriate buffer and recentrifuged (40 000 $\times$ *g* for 10 min).

Chemical reagents. The sulfhydryl-group specific reagents *N*-ethylmaleimide, iodoacetamide, *p*-chloromercuribenzoate (PCMB), *p*-chloromercuribenzosulfonate (PCMBS) and the amino group specific reagents were incubated, before or after labeling with the erythrocyte ghosts at a concentration of 10^{-3} M in phosphate

buffer (pH = 7.4) for variable times at room temperature (25 °C). The excess of reagent was removed by two washes in buffer (pH = 7.4).

Spectral measurements

EPR spectra were recorded at 25 °C on a Varian E-9 spectrometer equipped with the Varian variable temperature accessory.

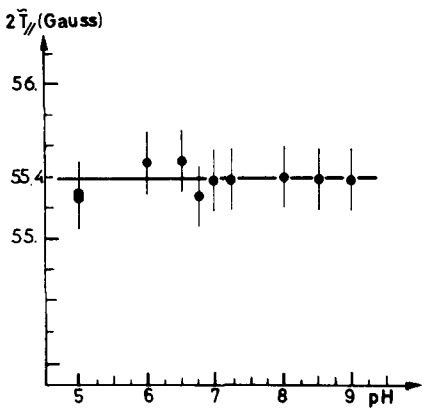
RESULTS

Fatty acid spin labels

The ESR spectra of such labels have been thoroughly investigated in model systems [3, 8] and biological membranes [7, 10]. As shown by McConnell [11] the hyperfine splitting $2\tilde{T}_{//}$ can be related to the freedom of motion of the nitroxide radical and the order parameter of the probe, greater freedom being associated with smaller values of $2\tilde{T}_{//}$.

The ESR spectra obtained in the present study on intact erythrocytes appear to be similar, both qualitatively and quantitatively, to those previously described [7, 12]. They confirm the pronounced dependence of splitting values on the position of the nitroxide radical on the aliphatic chain.

The effects of pH and osmolarity have been studied using Probe I [12, 3]. Between pH 4 and pH 9 (Fig. 1) no significant changes of the spectra were detected. The spectra appear equally insensitive to the variations of NaCl concentration. As shown in Fig. 2, no modifications were observed when the red cell was swollen in hypotonic medium (150 mosM) or shrunk in hypertonic solution (600 mosM).



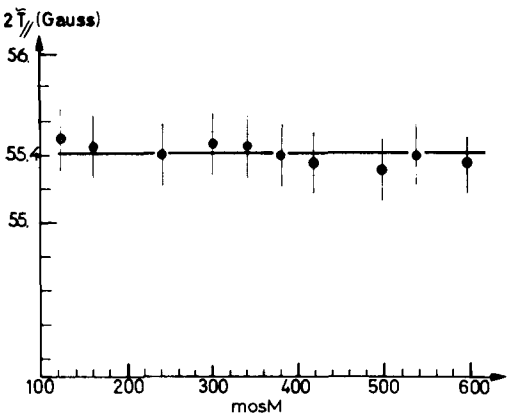


Fig. 1. Hyperfine splitting 2 $\tilde{T}_{//}$ (±0.15 G) of Probe I [12, 3] in red blood cells as a function of pH. After labeling, the red cells were resuspended in phosphate buffers to yield suspensions having final pH values between 5 and 9.

Fig. 2. Hyperfine splitting 2 $\tilde{T}_{//}$ (±0.15 G) of Probe I [12, 3] in red blood cells as a function of ionic strength. After labeling, the red cells were resuspended in phosphate buffers of increasing ionic strength.

The study of spectra as a function of temperature yields a gradual decrease of the splitting values with increasing temperature between 5 °C and 45 °C. A classical

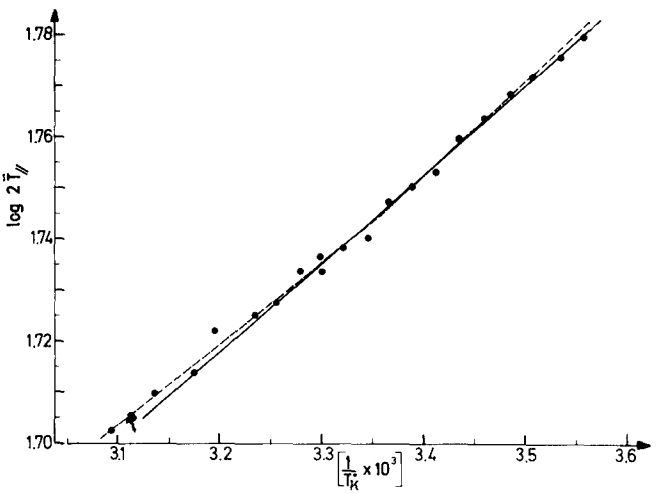


Fig. 3. Logarithmic plot of the hyperfine splitting $2\,\tilde{T}_{//}$ against $1/T$ for red cells labeled with Probe I [12, 3].

plot of the logarithm of the splitting against $1/T°$ K is shown in Fig. 3. A slight inflexion might occur near 30 °C. However, this slope change is too small to be considered significant. Moreover, upon exposure to chemical reagents (PCMB, PCMBS, DNFB) the spectra of the fatty acid spin labels incorporated into intact erythrocytes remain unaffected.

Fatty acid spin labels are known to provide information about the physical state of membrane lipids. Our data indicate that large variations of pH and osmolarity do not modify the spectra of such labels incorporated in the membrane of intact red blood cells. The chemical agents, either specific of the SH groups (PCMB-PCMBS) or of the NH_2 groups (DNFB) do not affect the spectra. This indicates that the general molecular organization of the lipidic leaflet of the membrane, as seen through fatty acid spin labels, is not affected by large variations of the physicochemical conditions known to affect the protein components of the membranes. Such a conclusion is consistent with the observation [12] that the spectra obtained from the same labels on intact cells, their ghosts and their lipid extracts are qualitatively similar.

On the other hand, from a physiological standpoint, the apparent stability of lipid organization over a large range of physicochemical conditions is worth noticing: the deformation of the red blood cell membrane under large osmotic gradients seems not to imply any modification in the state of the lipids. The important permeability changes induced either by alterations of physicochemical conditions or by chemical modifiers appear to be due to membrane modification at the protein level (including, probably, the thin local lipidic environment).

Consequently, in the following experiments with protein spin labels, any change in the spectra, which occurs upon variation of the physicochemical conditions or exposure to chemical reagents, may be attributed exclusively to changes in the protein region.

Protein spin labels

This study was carried out on red blood cell ghosts. Preliminary experiments on

intact erythrocytes show that the labeling intensity by the maleimide spin label is too low to permit an accurate analysis of the spectra. On the other hand, the iodoacetamide spin label penetrates inside the cell and the membrane signal is blurred by a large inner free signal.

However, the characteristics of the spectra of both ghosts and intact cells are similar, only the intensity of labeling is increased by the ghost preparation [13].

Spectra analysis. The ESR spectra of ghosts labeled with maleimide spin label (Probe III-) and iodoacetamide spin label (Probe IV) are shown on fig 4. The spectra

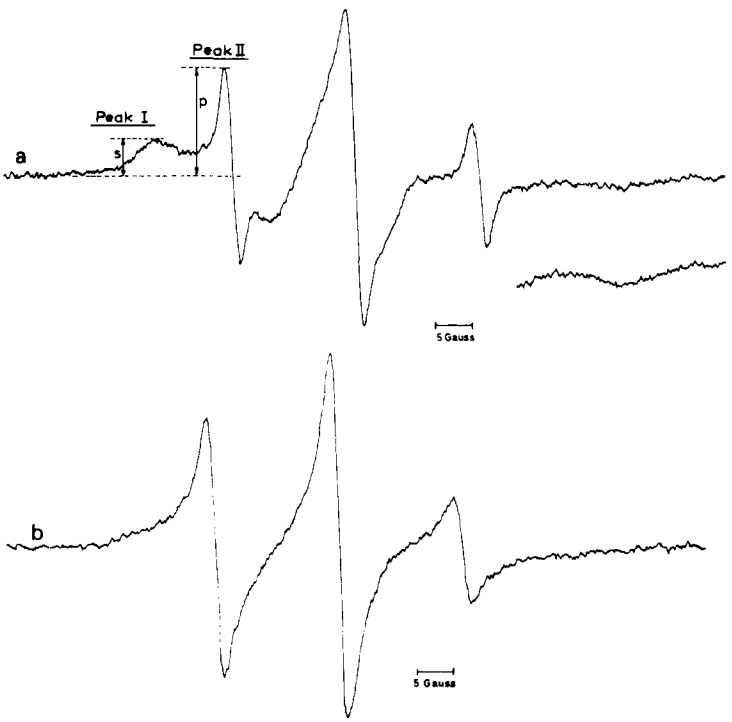


Fig. 4. ESR spectra of spin-labeled erythrocyte ghosts. (a) With the maleimide spin label (Probe III), (b) with the iodoacetamide spin label (Probe IV).

of the two labels are different as noticed by several authors [4, 13]. At first sight, the iodoacetamide spin label spectrum would seem to be due to one tumbling spectrum and the maleimide spin label to two spectra: a rapidly tumbling one and a so-called "immobilized" one. In fact, this means that the two spectra may be synthesized with only one set (or two sets for Probe III) of parameters: hyperfine tensors and widths, these sets being interpreted as the "mean" parameters of the labeled sites.

(1) In the case of the iodoacetamide spin label, apart from the total intensity, several parameters are interesting.

The isotropic hyperfine splitting a, is known to be related to the polarity of the environment of the label. For the iodoacetamide spin-labeled ghosts the measured value of 17 G is very close to the value of 17.1 G of the label dissolved in water.

The widths and relative heights of the peaks depend on the tumbling rate of the

label. Assuming that the motion is isotropic, a mean rotation time, τ, of about $2 \cdot 10^{-9}$ s for the iodoacetamide spin-labeled ghosts is obtained, which can be compared to a correlation time of $7 \cdot 10^{-11}$ s for the label dissolved in water at room temperature.

(2) By contrast, there are mainly two components in the spectrum of the maleimide spin label (Probe III). The intensities of these components will be measured by the heights of, respectively, Peak I for the so-called "immobilized" one and Peak II for the mobile one as shown on Fig. 4. The ratio of the height of Peak II to Peak I (called R) represents a rough estimate of the overall mobility of all the spin label sites. This method of interpretation was used previously by various authors [18, 9]. The hyperfine splitting of the mobile component indicates a polar aqueous environment for the label. The "immobilized" component (Peak I), so called because it resembles a rigid powder spectrum provides information about the slow motions of the nitroxide. Two types of empirical measurements were proposed.

The first one, used by McConnell [15], considers, the separation of the outer hyperfine extremes, which decreases when the rotational motion increases. Graphs of the variation of this separation versus the rotational correlation time were published [15].

The second method has been proposed by Hyde [16] who considered the changes in shape of the out-of-phase dispersion spectra and is valid only for correlation times in the range from $2 \cdot 10^{-7}$ s to $2 \cdot 10^{-5}$ s.

In our case, the first method gives a mean rotation time of the order of 10^{-8} s. At this point it is interesting to note that independently of the site of fixation one can expect a higher mobility for the iodoacetamide spin label relative to the maleimide spin label both because of the longer chain between the site and the nitroxide in the iodoacetamide spin label and of the presence of the ring in the maleimide spin label (see Fig. 5).

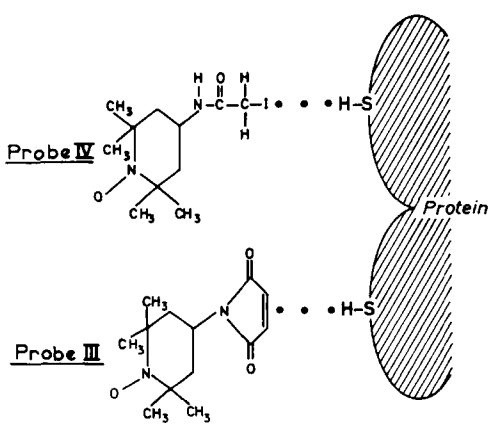


Fig. 5. The molecular formula of the protein spin labels, 4-maleimido-2,2,6,6 tetramethyl piperidinooxyl (Probe III), 4-(2-iodoacetamido)-2,2,6,6-tetramethyl piperidinooxyl (Probe IV), showing the greater independence of Probe IV movements from its protein binding site.

Study of the spin label sites of fixation. The selectivity of spin labels for SH groups was checked by studying the amount of label fixation on ghosts after exposure

to SH specific reagents: PCMB,PCMBS, the unlabeled *N*-ethylmaleimide and iodoacetamide.

TABLE I

Ghosts exposed different times to sulfhydryl reagents prior to labeling by Probe III. The intensities of the spectra are expressed as a percentage of the control intensity. The percent error was within ±2.5 %.

Reagent (10^{-3} M)	20 min of exposure		2 h exposure		16 h exposure	
	Intensity of Peak I (%)	Intensity of Peak II (%)	Intensity of Peak I (%)	Intensity of Peak II (%)	Intensity of Peak I (%)	Intensity of Peak II (%)
Control	100	100	100	100	100	100
N-Ethylmaleimide	70	100	30	100	0	50
PCMB	0	100	0	50	0	40
PCMBS	0	100	0	25	0	30
Iodoacetamide	70	94	90	100	70	100

In Table I are given the intensities of two components of the maleimide spin label signal (Peak I and Peak II as defined in Fig. 4) after different times of exposure to the reagents. Table II gives the equivalent data for the iodoacetamide spin label (Probe IV).

TABLE II

Ghosts exposed for different times to sulfhydryl reagents prior to labeling by Probe IV. The intensity of the spectrum is expressed as a percent of the control intensity. The percent error was within ±2.5 %.

Reagent (10^{-3} M)	Intensity of signal (%)		
Exposure time:	5 min	20 min	2 h
Control	100	100	100
N-Ethylmaleimide	26	10	0
PCMB	10	0	0
PCMBS	15	9	0
Iodoacetamide	90	70	40

PCMB and PCMBS exposures inhibit completely the iodoacetamide spin label fixation. The SH specificity of the two organic mercurial compounds being well established [2], it may be concluded that the iodoacetamide spin label only binds to SH groups.

In the case of the maleimide spin label (Probe III), PCMB and PCMBS inhibit completely the label fixation on the sites giving rise to Peak I but only partially the fixation on the sites giving rise to Peak II. This incomplete inhibition, obtained on ghosts exposed to PCMB or PCMBS concentrations able to react with virtually all the SH binding sites available, suggests that part of the Peak II sites corresponds to fixation on other functional groups, probably NH_2 groups as already suggested by Chapman [17].

In order to further differentiate the fixation sites of the two protein spin labels, the effect of exposure to *N*-ethylmaleimide on iodoacetamide spin labeling and exposure to iodoacetamide prior to maleimide spin labeling was studied. 20 min of exposure of the ghosts to *N*-ethylmaleimide results in a complete inhibition of Probe IV fixation (absence of spectrum). On the contrary, even after 15 h of exposure to iodoacetamide, only a small decrease of Probe III fixation is obtained. By comparing these two sets of results, it can be concluded that the maleimide spin label has few common sites of fixation with the iodoacetamide spin label, and can label a lot of additional sites not accessible to the other label.

Moreover, the inhibition kinetics by sulfhydryl reagents, as studied on the spectra, indicate a different accessibility of the various sites. These results lead us to the following conclusions: the iodoacetamide spin label has easy access to its binding sites either in the intact erythrocytes or in the ghosts, in contrast to the maleimide spin label.

These results have to be compared to the analysis by gel electrophoresis of ghosts labeled with radioactive iodoacetamide or *N*-ethylmaleimide made by Carraway et al [19]: the authors showed that iodoacetamide specifically binds to a single protein (Component VIII), which is easily extractable and may be localized at one of the membrane surfaces. On the contrary, *N*-ethylmaleimide does not exhibit such a specificity binding to many proteins, although mainly to the high molecular weight protein (spectrin) which is considered as a major structural component of the membrane.

TABLE III

Ghosts labeled by Probe III and then exposed for 4 h to sulfhydryl and amino reagents. The intensities of the spectra are expressed as a percentage of the control intensity. The percent error was within $\pm 2.5\,\%$.

Reagent (10^{-3} M)	Intensity of Peak I (%)	Intensity of Peak II (%)	$\frac{\text{Intensity of Peak II}}{\text{Intensity of Peak I}} = R$
Control	100	100	1
N-Ethylmaleimide	100	100	1
PCMB	100	100	1
PCMBS	100	100	1
Iodoacetamide	100	100	1
DNFB	112	80	0.71
2-Methoxy-5-nitrotropone	70	40	0.57
TNBS	126	73	0.57

Finally, Tables III and IV show that there is no action of the sulfhydryl reagents after labeling by Probe III or Probe IV. There are no changes either in the amplitude or in the characteristics of the different spectra. It cannot be concluded that no protein conformational changes are produced by SH reagents since the label fixation by itself might induce comparable changes. On quantitative grounds, the concentration of the maleimide spin label binding sites was evaluated to be $9.8 \cdot 10^{-18}$ mole/cell [13]. This concentration may be compared to the $38.2 \cdot 10^{-18}$ mole/ cell for the PCMBS sites of fixation [2].

TABLE IV

Ghosts labelled by Probe IV and then exposed for 4 h to sulfhydryl and amino reagents. The intensity of the spectrum is expressed as a percentage of the control intensity. The percent error was within ±2.5 %.

Reagent (10^{-3} M)	$\tau \times 10^{10}$ (s)	Intensity of signal (%)
Control	19.6	100
DNFB	21.15	86
2-Methoxy-5-nitrotropone	21.15	57
TNBS	20.90	60
N-Ethylmaleimide	19.60	100
Iodoacetamide	19.50	100

Action of amino specific reagents. The effects of amino specific reagents (DNFB, MNT and TNBS) were studied before and after labeling the ghosts with each protein spin label.

In the case of the maleimide spin label, exposure to NH_2 reagents before labeling (Table V) strongly inhibits the label fixation.

TABLE V

Ghosts exposed for 3 h to amino reagents prior to labeling by Probe III. The intensities of the spectra are expressed as a percentage of the control intensity. The percent error was within ±2.5 %.

Reagent (10^{-3} M)	Intensity of Peak I (%)	Intensity of Peak II (%)	$\frac{\text{Intensity of Peak II}}{\text{Intensity of Peak I}} = R$
Control	100	100	1
DNFB	40	30	0.75
2-Methoxy-5-nitrotropone	70	50	0.72
TNBS	65	45	0.70

Moreover, in the resulting spectra, the ratio R of the amplitude of Peak II to the amplitude of Peak I decreases. This fact can be explained in two ways. First there could be a slightly greater inhibition of spin label fixation on the sites giving rise to Peak II, presumably because a larger proportion of the mobile signal (Peak II) originates in the NH_2 fixation of Probe III. Secondly, the more immobile population could increase at the expense of the mobile sites. In this case modification of the ratio R would suggest a protein structural change. The second hypothesis seems to be correct, since exposure to the same amino specific reagents, after labeling by the maleimide spin label (Table III) results in a decrease of the ratio R without any change in the integrated intensity of the spectra. The conversion of the rapidly tumbling spectrum (Peak II) to an immobilized spectrum as shown in Fig. 6 and the increase of the distance between the outer hyperfine extremes would indicate that a change has occurred in the environment of the spin label, resulting in a reduction of the label's rotational freedom. In contrast, in the case of the iodoacetamide spin label, exposure to the NH_2 reagents, before or after labeling, strongly inhibits the spectrum intensity

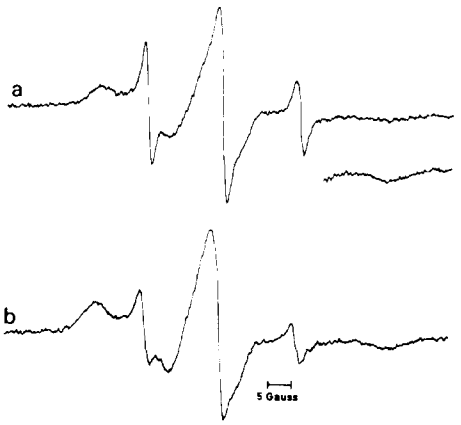


Fig. 6. ESR spectra of ghosts spin labeled with Probe III: (a) Control, (b) Ghosts exposed to DNFB after labeling.

(Tables IV and VI). Moreover, the residual spectrum indicates a reduced mobility of the remaining labeled proteins, as seen by the increase of a factor 2 of the mean rotational time calculated according to ref. 14. All these data clearly indicate a membrane protein structural change under the exposure of ghosts to DNFB, 2-methoxy-5-nitrotropone, and TNBS.

TABLE VI

Ghosts exposed for 4 h to amino reagents prior to labeling by Probe IV. The intensity of the spectrum is expressed as a percentage of the control intensity. The percent error was within $\pm 2.5\,\%$.

Reagent (10^{-3} M)	$\tau \times 10^{10}$ (s)	Intensity of signal (%)
Control	19.6	100
DNFB	not measurable	5
TNBS	21	40
2-Methoxy-5-nitrotropone	32.5	40

Following these results, we can differentiate between two effects of the NH_2 reagents whether the ghosts are labeled with Probe III or Probe IV. In the case of iodoacetamide spin labeling the decrease in spectra intensity obtained by exposure to NH_2 agents, prior to or after labeling, may be interpreted as an extraction of some of the ghost proteins. A conformational change is induced by these agents as indicated by both the lower accessibility of maleimide sites before labeling and the changes of the spectra of Probe III (without changes of intensity) upon exposure to the agents after labeling.

The results obtained with the NH_2 specific reagents are of interest with respect to the ionic permeability properties of the red blood cell membranes. From a physiological standpoint, there is evidence [20] that positively charged amino groups are directly involved in the control of ion permeation through the red cell and the effects

of amino reagents have been related to a reduction of the fixed charges within the ion permeable pathways. Reactions of a number of NH_2 reagents, as well as high pH values which discharge amino groups, lead to an increase in cation fluxes together with a decrease in anion fluxes. According to this hypothesis, the change in the number of fixed charges of proteins by the amino reagents might be responsible for the modification of the spectra observed, and comparable modification should be caused by the pH variation of the external medium.

Effect of pH. The effects of pH (pH 2 to 9) on the maleimide spin-labeled ghosts are shown on Fig. 7. The ratio R shows a minimum in mobility over the range of pH 4.5 to 5.5. On either side of this minimum, there is an increase of the amplitude of the rapid tumbling component in the spectrum.

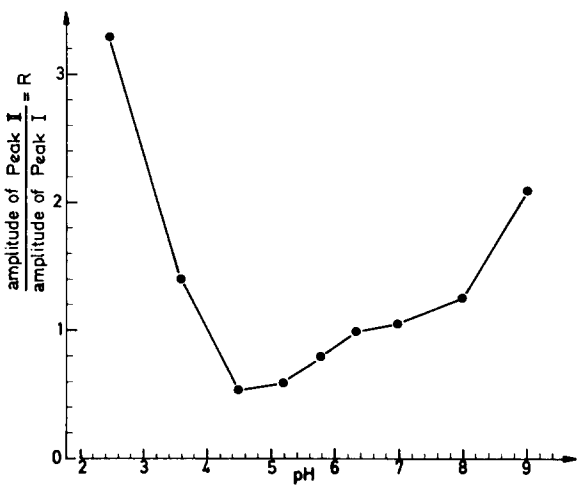


Fig. 7. Ratio of the intensities of Peak I and Peak II as a function of pH in ghosts labeled with Probe III. The same experimental conditions exist as in Fig. 1.

The results are similar to the observations reported by Chapman [17]. However, a different evolution of the rotational freedom with pH was obtained on iodoacetamide spin-labeled ghosts (Fig. 8) the mobility gradually decreases from pH 2 to 4.5 and then remains constant from pH 5 to 9. Clearly, the effects of pH and of the NH_2 reagents are different: alkalinisation of the medium increases the mobility of the maleimide spin label and has no action on the iodoacetamide spin label. Instead, a decrease, in mobility for both labels was observed under exposure of the ghosts to NH_2 reagents.

The present data indicate that the mode of action of pH and NH_2 reagents is different and suggest that the protein structural change obtained with NH_2 reagents cannot simply be ascribed to a direct modification of the membrane electric charge.

Many features of the red blood cell membranes selective permeability to ions (different responses of Na^+ and K^+ fluxes to pH, Ca^{2+} concentration and NH_2 reagent action) cannot be easily accounted for by the fixed-charge hypothesis alone. Moreover the very fast halide exchange phenomena in red blood cells has been interpreted rather in terms of a carrier-mediated transport [21]. From this point of

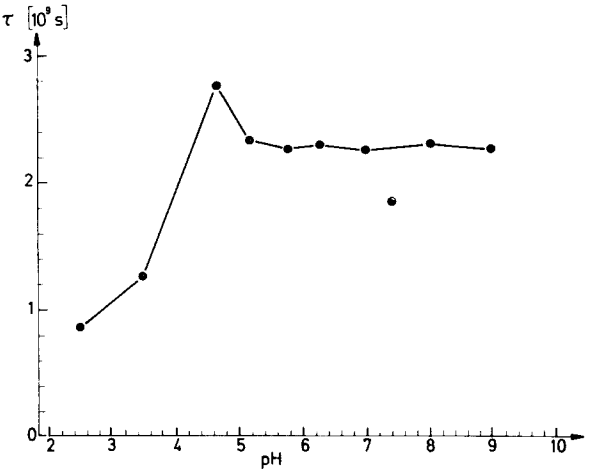


Fig. 8. Mean rotation time (τ) of Probe IV in ghosts as a function of pH. The same experimental conditions exist as in Fig. 1.

view, beside the general physicochemical effect of the reduction of the positive fixed charge by NH_2 reagent, the protein conformational change observed by the present spin labeling experiment could reflect a more specific action on the proteins responsible for the ionic permeability.

CONCLUSION

It seems clear that the phospholipid matrix of the red blood cell membrane remains stable in a large range of pH and osmolarity of the medium and is unaffected by the changes occurring at the protein level.

The present investigation indicates that important differences in the spectra of protein spin labels can be induced by the exposure of red blood cell ghosts to amino-specific reagents. These spectral modifications are probably the results of protein conformational changes and have to be related to the well known effects of these NH_2 reagents on the permeability of the red blood cell.

Due to the complex protein composition of red blood cell membranes and the rather non specific character of the protein labeling, no more precise correlation can be made at the present time. However, the isolation of the proteins labeled under various conditions which are currently being carried out in our laboratory will undoubtedly help to make more accurate relationships.

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