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SPIN LABEL STUDIES ON THE HUMAN ERYTHROCYTE MEMBRANE

TWO SITES AND TWO PHASES FOR FATTY ACID SPIN LABELS

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Summary

Human erythrocytes, untreated and glutaraldehyde-treated, were spin labeled with three kinds of fatty acid labels, and their electron spin resonance (ESR) spectra were studied in detail at various temperatures.

1. The better spectral resolution could be obtained by packing the erythrocytes in a hematocrit capillary tube, because of the preferential parallel orientation of the cylindrical axes of erythrocyte-disc to the centrifugal axis.

2. It was demonstrated by the incorporation and the release of the labels that the membrane possessed two kinds of the fatty acid "sites": the tightly and weakly binding "sites" at the approximate molar ratio of 1 : 1. The rough estimates of the binding constants were obtained, which reproducibly varied with the blood donors over a period of a year.

3. The temperature dependency of the ESR spectra revealed the presence of two distinct phases, perhaps the solid and fluid phases. With lowering of the temperature, the fluid phase became more solid but the solid phase unchanged. The pretreatment of the erythrocytes with glutaraldehyde increased the amount of the frozen phase, corresponding to the decrease of the membrane flexibility.

Introduction

The spin label technique has been widely used to investigate the molecular phenomena in the biological membrane [1]. Concerning the erythrocyte membrane, various studies have been made, e.g., the effect of certain hormones and drugs on the fluidity of the membrane [2—4], the application to the diagnosis of the myotonic disease [5,6] and the virus-induced fusion of the erythrocytes [7]. However, Bieri et al. [8] showed that an excess incorporation of the fatty

acid spin labels caused the deformation of the erythrocytes. Also, the heterogeneity of the membrane consituents must be taken into account for the analyses of the electron spin resonance (ESR) spectra, because the "sites" available for the label molecules will not be always uniform in their very local environments. Actually, the erythrocyte membrane possesses two kinds of sites for laurate anion, as demonstrated by its partition between albumin and membrane [9].

We originally intended to find the relation between the motion of the label molecule in the membrane and the rheological properties of the erythrocytes. For this purpose, the fatty acid spin labels have the following advantages: (a) they can sense the different parts (depth) of the membrane, (b) they are incorporated into the membrane within a relatively short time, namely the possible changes of the metabolic states or the deformation of the cell will be less and (c) they are commercially available. However, we faced difficulties, i.e., the heterogeneity of the "binding sites" contributed to the ambiguity in explaining the observed spectra and the imperfection of the technical procedures limited the spectral resolution.

The present paper, thus, describes the "sites" for the fatty acid spin labels and the phase of the "sites" of the normal and artificially hardened erythrocytes.

Materials and Methods

Materials. The spin labels, 2-(14-carboxytetradecyl)-2-ethyl-, 2-(10-carboxydecyl)-2-hexyl- and 2-(3-carboxypropyl)-2-tridecyl-4,4-dimethyl-3-oxazolidinyloxyl (abbreviated as I(1,14), I(5,10) and I(12,3), respectively) were purchased from Syva Co. and used without further purification. Human serum albumin was obtained from NBC Co. (Human, 4x cryst., Lot No. 9624). Glutaraldehyde was purchased from Wako Chem. Co. Other chemicals were analytical grade.

Preparation of the spin labeled erythrocytes. The blood was collected in a heparinized tube from the antecubital vein of two healthy adult donors. After removing the plasma by centrifugation, the erythrocytes were washed 3 times with 10 volumes of 0.9% NaCl solution. It was noted that the number of washings affected the total amount of the "sites" for the spin label molecules, i.e., the extensive washing increased the number of "sites".

The spin labels were dissolved in ether and dried in a test tube in vacuo. The albumin solution (50 mg/ml) was added to the test tube and stood for 24 h at 4°C. The spin labeled albumin was added to the erythrocyte suspension [10] to give the final hematocrit value of about 50%, and the mixture was incubated for 30 min at 0°C. The phosphate buffered saline (0.9% NaCl/0.160 M phosphate buffer (1 : 1, v/v), pH 7.4) was used as solvent. The shape of the erythrocytes was frequently monitored with a microscope.

In order to determine the "binding" constants of the spin labels with the membrane, two types of experiments were carried out. (a) In the titration experiment, the quantity of the spin labeled albumin to the erythrocytes was varied, and the amount of the incorporated labels was measured after incubation. (b) In the dilution experiment, the spin labeled erythrocyte suspension (hematocrit = 50%) was immersed in the solvent of the desired volume for

30 min at 4°C. The erythrocytes were packed in the sample tube, and the amount of labels remaining in the erythrocytes was measured.

Measurement of ESR spectra. A Varian E-3 spectrometer equipped with a variable temperature accessory was used. The temperature was controlled by the N_2 gas stream. The spectra were recorded with the 100 kHz modulation amplitude of 1 gauss, at the incident microwave power of 5 mW. A hematocrit capillary (Corning Co., 1.1 mm inner diameter, non-heparinized) was filled with the spin labeled erythrocyte suspension and centrifuged at 12 000 rev./min for 5 min at 4°C. After centrifugation, the portion of the packed erythrocytes was placed in the variable temperature dewar.

The spin concentrations were determined by comparing the doubly integrated areas of the spectra for the samples and the solution of Fremy's salt. The concentration of Fremy's salt in the solution was calculated by using the molar extinction coefficient of 20.8 at 540 nm [11].

Modification of the membrane state. In order to modify the state of the erythrocyte membrane, the erythrocytes were immersed in the glutaraldehyde solution of the desired concentrations, incubated for 30 min at 0°C, and then washed three times with the solvent. After glutaraldehyde treatment, the spin labeled albumin was added to the erythrocyte suspension.

Results

ESR spectra of the packed, spin labeled erythrocytes

The representative spectra are shown in Fig. 1. The top spectra (line a in Fig. 1), which were measured for the labeled erythrocyte suspension in a Varian flat

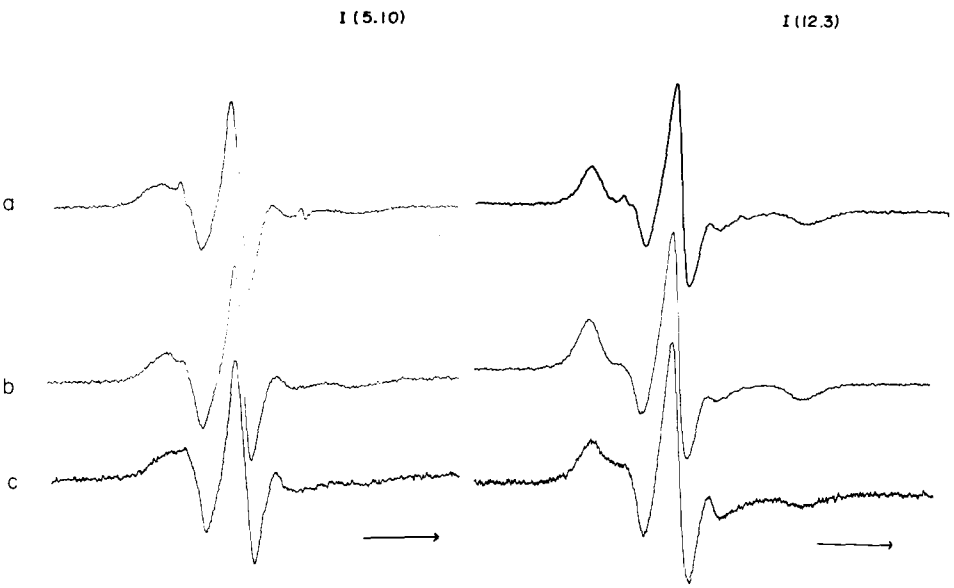

Fig. 1. ESR spectra of (a) randomly oriented erythrocyte suspension in the flat cell, (b) packed erythrocytes, the capillary axis perpendicular to the magnetic field, and (c) packed erythrocytes, the capillary axis parallel to the magnetic field. (left) I(5,10)-labeled; (right) I(12,3)-labeled. The arrow indicates 20 gauss. Measured at 20°C.

cell, consisted of two kinds of signals: one was from labels bound to the membrane and other from those free in the solution. The middle and the bottom spectra (lines b and c in Fig. 1) were recorded for the packed erythrocytes, in which the longitudinal axis of the capillary was perpendicular and parallel to the applied magnetic field, respectively. It was evident that the contribution of the free labels on the spectra was very little. Moreover, it was noticed that the spectral width of I(12,3) labeled erythrocytes depended on the orientation of the capillary to the magnetic filed. Such spectral difference was also observed for the flowing erythrocytes in the flat cell by McConnell et al. [2,13]. They showed that the cylindrical axes of the flowing erythrocytes in the flat cell perpendicularly oriented to the flat plane of the cell.

Available sites for spin labeled fatty acids

(1) The labels in the membrane were liberated into the solvent by dilution of the labeled erythrocytes. The degree of the liberation depended on the structures of the label molecules and on the dilution factors (Fig. 2a). No change in the spectral shape was observed. The results clearly showed the presence of two kinds of sites: one was the weakly binding site in which the label molecules were easily liberated from the membrane, and the other the tightly binding site. I(12,3) in the tightly binding sites was scarecely liberated but I(5,10) and I(1,14) were easily expelled upon dilution.

A rough measure of the intrinsic binding constant for the tightly binding sites may be estimated by the following calculation. The total number of the tightly binding sites, N_{max}, will be the sum of the number of the membrane bound labels, N_{init}, and the number of unbound sites, N_o, at the initial stage before dilution. The dissociation constant of the spin labels for the sites may be defined as K = [free label] · [free sites]/[occupied sites]. If the occupied sites decrease to N_x, upon dilution to the total volume of V_x, $(N_{init} - N_x)$ labels are

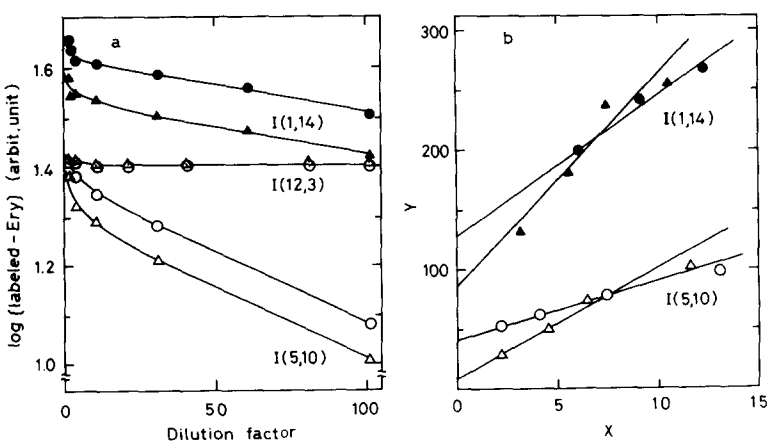

Fig. 2. Liberation of labels from the erythrocyte (Ery) membrane by dilution. (a) The total label concentration (in arbitrary unit) in the membrane vs. the dilution factors, and (b) the plot of Y vs. X. (see text). ●, ▲: I(1,14); ○, △: I(5,10); and ⊙, ⋔: I(12,3); circles for donor A and triangles for donor B. Measured at 20°C.

TABLE I

THE BINDING CONSTANTS AND THE MAXIMAL INCORPORATION

K_{ass} obtained by the dilution experiments are for the tightly binding sites only. K_{ass} obtained by the titration experiments represent actually the partition between the membrane and albumin. N_{max} is an extrapolated value (mol/l of the packed erythrocytes).

Expt.	Label	Donor A		Donor B	
		K_{ass} (M^{-1})	(N_{max}) (M)	K_{ass} (M^{-1})	(N_{max}) (M)
Dilution					
	I(5,10)	$5.4 \cdot 10^3$	$(3.4 \cdot 10^{-4})$	$8.0 \cdot 10^3$	$(2.6 \cdot 10^{-4})$
	I(1,14)	$1.1 \cdot 10^5$	$(5.7 \cdot 10^{-4})$	$1.8 \cdot 10^5$	$(4.2 \cdot 10^{-4})$
Titration					
	I(5,10)				
	[tightly]	$7.7 \cdot 10^3$		$14.1 \cdot 10^3$	
	[weakly]	$3.0 \cdot 10^3$		$3.0 \cdot 10^3$	
	[total]		$(5.0 \cdot 10^{-4})$		$(4.4 \cdot 10^{-4})$

liberated. Therefore, [free sites] = $(N_o + N_{init} - N_x)/V_x$. Neglecting the free labels present at the initial stage, [free labels] = $(N_{init} - N_x)/V_x$.

Finally the following equation is obtained: $V_x \cdot N_x/(N_{init} - N_x) = (N_{init} - N_x)/K + N_o/K$. In Fig. 2b, the plot of $(N_{init} - N_x)$ [$= X$] vs. $V_x \cdot N_x/(N_{init} - N_x)$ [$= Y$] was shown. At lower dilution factors, however, a non-linear relation was obtained, since (i) the weakly binding site was ignored in the process of deriving the equation, (ii) the volume of the intercellular space was not corrected and (iii) the presence of a subtle amount of albumin might be contributing factor. K's were summarized in Table I. I(12,3) bound too tightly to determine the constant.

(2) The incorporation of the spin labels into the erythrocyte membrane was examined by the titration with the spin labeled albumin. The incorporated amount of the label increased hyperbolically with the increase of the amount

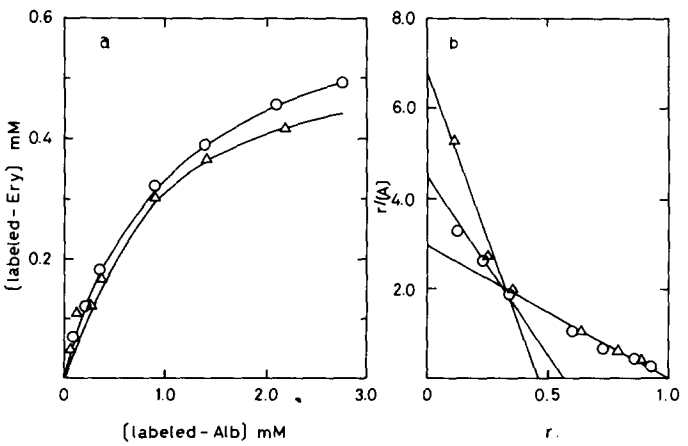

Fig. 3. Incorporation of I(5,10) from the labeled albumin. (a) The amount of the label incorporation vs. the concentration of the labeled albumin added, and (b) Scatchard plot: r vs. $r/[\text{labeled albumin}]_{free}$. ○, donor A and △, donor B. Measured at 27° C.

of the spin labeled albumin (Fig. 3a), though the fraction of the echinocytes gradually increased as pointed out by Bieri et al. [8]. The spectral shapes were the same and the cluster formation of the labels was not observed, as shown by Verma and Wallach [13].

A Scatchard plot of the data (Fig. 3b) showed the presence of, at least, two kinds of the binding sites with the different intrinsic binding constants: apparently one was about 2—5 times greater than the other, as summerized in Table I. The constants obtained by the titration experiment, however, were influenced by the partition of the labels between albumin and membrane, as suggested by Lovrien et al. [9].

The ambiguity of the dilution and the titration experiments for obtaining the exact binding constants will be discussed later.

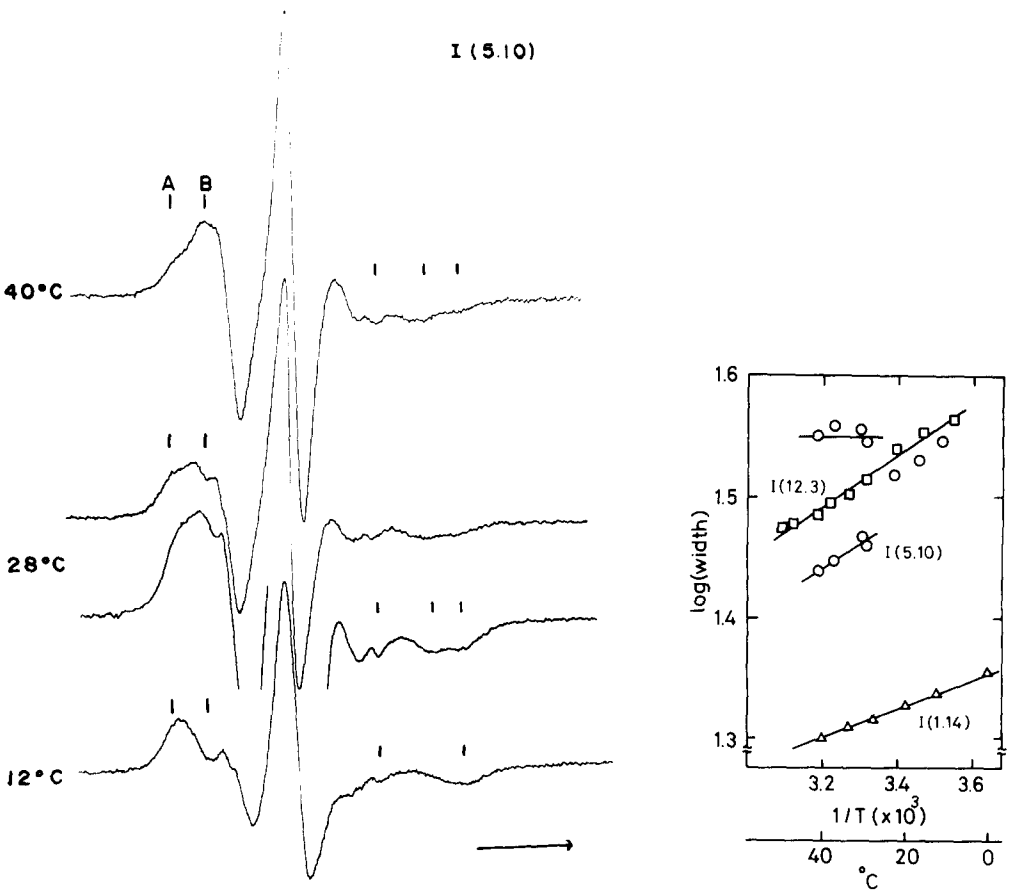

Fig. 4. ESR spectra of the I(5,10)-labeled erythrocytes. (Top) measured at 40°C, (middle) at 28°C, accompanied with an amplified trace (×2.5) and (bottom) at 12°C. The arrow indicates 20 gauss.

Fig. 5. Temperature dependency of the ESR spectra of I(5,10)-labeled erythrocytes. (a) The width between the spectral center and the high field extrema (in gauss). △, I(1,14); ○, I(5,10) and □, I(12,3).

Temperature dependence of ESR spectra

The motion parameter, S, which reflects the molecular motion of the spin labels, has been frequently used [1,14—16]. Also, the ratio of the representative peak heights [17,18] or the width of the hyperfine extrema [19] of the first derivative spectrum has been used. In the present case, the S values were 0.75 for I(12,3), 0.65 for I(5,10) and 0.29 for I(1,14) at 20°C.

In Fig. 4, the ESR spectra of the packed erythrocytes labeled with I(5,10) at various temperatures were shown. It was recognized that the high field extreme began to split into two peaks at higher temperatures. The phenomena could barely be observed only in certain samples labeled with a suitable amount of I(5,10), but not observed in the different label concentrations and for other labels. The separation between the spectral center and the high field extreme was plotted against the reciprocal of temperature (Fig. 5). It was obvious that the spectrum consisted of two overlapped signals which originated from the strongly immobilized and the weakly immobilized labels, presumably the label molecule sensed two phases corresponding to the solid and fluid phases. Further, the spectral width of the strongly immobilized signal was independent of temperature, but that of the weakly immobilized signal was temperature-

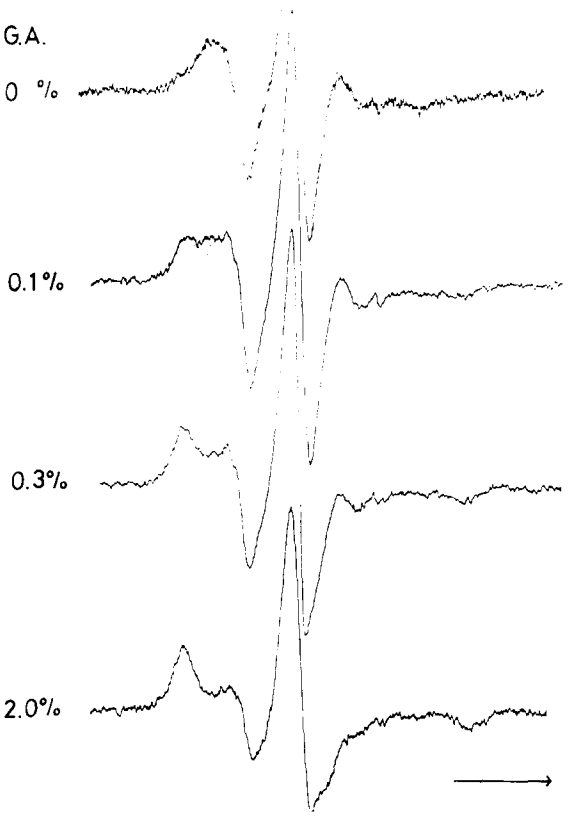

Fig. 6. ESR spectra of the glutaraldehyde treated erythrocytes, labeled with I(5,10), at 40°C. Glutaraldehyde (G.A.) concentrations 0, 0.1, 0.3 and 2% as indicated. The arrow indicates 20 gauss.

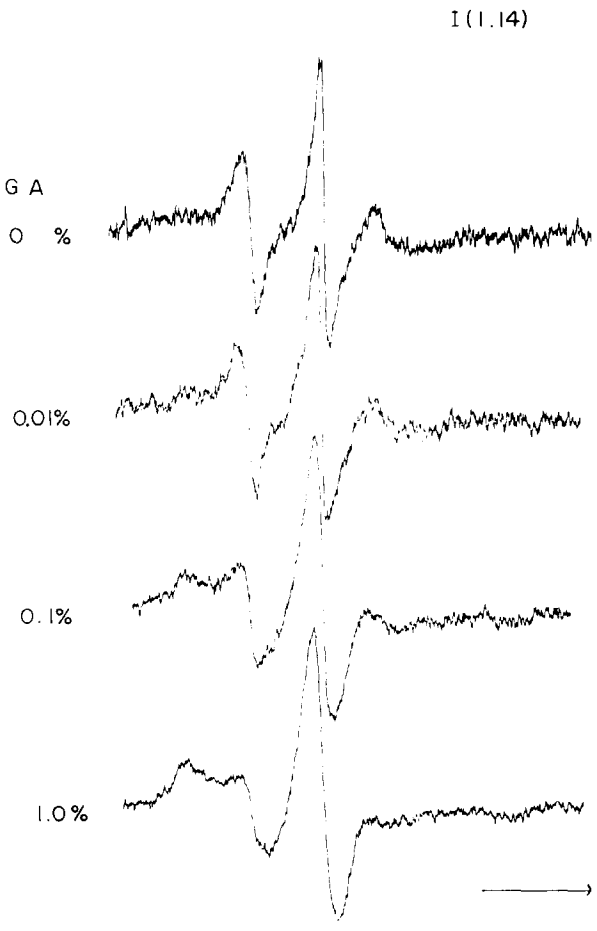

Fig. 7. ESR spectra of the glutaraldehyde (G.A.) treated erythrocytes, labeled with I(1,14), at 20°C. Glutaraldehyde concentrations 0, 0.01, 0.1 and 1% as indicated. The arrow indicates 20 gauss.

dependent. Below certain temperature, two kinds of signals fused into one. If two distinct signals could be estimated separately, either experimentally or by a computer simulation [16,20—23] at various temperatures, it would be possible to measure the "concentration" of two kinds of phases. However, it was too hard to deduce two separated signals from the observed spectra.

Effect of glutaraldehyde

Glutaraldehyde is commonly used not only as a fixation reagent for the electron microscopy but also as a modifier of the flexibility of the erythrocytes.

The representative spectra of the normal and hardened erythrocytes were shown in Figs. 6 and 7. As increasing the glutaraldehyde concentrations, the strongly immobilized signal developed and the incorporation of the labels into the membrane decreased. The spectral changes became apparent at the glutaraldehyde concentration above 0.05%.

Discussion

Orientation of the packed erythrocytes

Judging from the spectral shape, the erythrocytes are preferentially packed with their cylindrical axes parallel to the centrifugal direction, since the spectra obtained with the packed capillary placed perpendicular to the applied magnetic field, were similar to the spectrum reported for the flowing erythrocytes. For these erythrocytes the cylindrical axes were oriented perpendicular to the magnetic field [2,12] and vice versa. Although such orientational preference obtained by the packing method was less effective than that obtained by the flowing method, the packing method much increased the spectral resolution.

The method has the following advantages, compared with a conventional measurement using a flat cell or a capillary filled with the erythrocyte suspension: (i) the erythrocytes are fairly well oriented, so that a better spectral resolution can be obtained. (ii) The contribution of the unbound labels (or of the albumin bound labels) to the spectrum is minimal, since the intercellular liquid space is minimal. (iii) The quantity of the erythrocytes (thus, of the labels) is minimal, i.e., 0.1 ml of the erythrocyte suspension with 50% in hematocrit. (iv) No attention is necessary for the sedimentation of erythrocytes in the sample tube during the measurement. (v) The operational procedures, e.g., handling, temperature control, storage, disposal, etc., are easy.

Quantitative aspects

The present study demonstrates the presence of two kinds of fatty acid (spin label) sites in the erythrocyte membrane: one is the tightly binding site and the other is the weakly binding site, independently of the molecular structure of the labels. A Scatchard plot, obtained from the titration experiment with the spin labeled albumin (Fig. 3b and Table I), shows that the ratio of the amount of two sites is about 1 : 1 and that the ratio of the intrinsic binding constants for two sites is about 1 : 2—5. Concerning the tightly binding sites, the following information was obtained: (i) the personal difference in the binding capacity and in the binding constant was reproducibly detected, (ii) the ratio of the binding constants (donor B vs. donor A) coincided in the dilution experiment and the titration experiment, and (iii) the ratio also coincided for two kinds of spin labels, I(5,10) and I(1,14), as measured by the dilution experiment.

It should be mentioned, however, that these values were rough estimates, because the following factors contributed to the ambiguity for determining the binding constants.

(a) It has been established that albumin possesses multiple binding sites for fatty acids. They can be grouped into three: two strongly, 4—5 intermediate and one weakly, 20—30 binding sites [24]. In the present experiments, one molecule of albumin carried originally 12—15 molecules of the label, since the molecular sieving was not used for the final preparation of the spin labeled albumin, and, after incorporation of the labels into the membrane, more than 2—3 labels (perhaps attached to the tightly binding sites of albumin) always remained on albumin. Therefore, the constants measured by the titration experiments were actually the "partition" constants between the membrane and the intermediate binding sites of albumin.

(b) It is known that albumin itself interacts with the erythrocyte membrane. A recent report suggested the formation of a monolayer on the membrane surface [25]. If this could be true for the spin labeled albumin, certain amounts of labels would attach to the membrane-adsorbed albumin. However, the typical ESR signal of the spin labeled albumin of which the spectral width would be wider than that of the membrane bound labels was not observed in the present study. The result agreed with that of Landsberger et al. [26]. Supporting evidence was that the same spectra were obtained without albumin (by evaporating the solution of the spin label in a test tube, followed by introducing the erythrocytes and incubating).

(c) The disadvantage of the dilution experiment is the assumption of one kind of site instead of two. In Fig. 2b, I(5,10) gave a straight line but I(1,14) did not, probably because two binding constants for I(5,10) were close together.

In spite of the above disadvantages, the qualitative conclusions summarized earlier would be of importance for the application of the spin label technique on the erythrocytes.

On the personal difference of the binding constant, many factors may be considered. The hematological and chemical characteristics of blood from two normal, healthy donors are summarized in Table II. The fact, that the erythrocytes of the donor A always accessed more labels and bound weaker than those of the other, will be probably related to the mean corpuscular volume, the cholesterol content, the cholesterol/phospholipid molar ratio, the compositions of the phospholipids, etc. Although such quantitative data were fairly constant

TABLE II

COMPARISON OF THE ERYTHROCYTES FROM TWO DONORS

Donors	A	B
Mean corpuscular volume (μm^3)	93	83
Fragility [a] (in NaCl vol. %)	0.41	0.43
Total phospholipids [b] (μmol/10^{11} cells)	38	39
Total cholesterol [c] (μmol/10^{11} cells)	34	29
Cholesterol/phospholipids (molar ratio)	0.90	0.74
Relative ratio of K_{ass} (tightly binding sites) [d]	1	~1.5
Maximum label incorporation (tightly binding sites) [e]	1	~0.7
(total binding sites) [f]	1	~0.9

[a] Expressed by NaCl vol.%, at 50% hemolysis [33].

[b] Lipids were extracted by the method of Hurter et al. [34], the amounts of phosphate were determined by the method of Bartlett [35].

[c] The amounts of cholesterol in the extracted lipids were determined by the method of Zak [36], and converted to μmol/10^{11} cells [37].

[d] From Table I, the dilution experiments for I(5,10) and for I(1,14).

[e] From Table I, the dilution experiments for I(5,10).

[f] From Table I, the titration experiments for I(5,10)-labeled albumin.

over a year without controlling the diet, further extensive and statistical analyses on the number of blood (donors) will be necessary to draw a conclusion.

Qualitative aspects

The motion parameter, S [15], of the incorporated spin labels is frequently used as a measure of the membrane lipid phase and consequently employed as an index of the membrane flexibility [2—4]. In cases of the homogeneous membrane, such as the phospholipid vesicles, a simple parameter could represent the overall phase. In the heterogeneous membrane, such as erythrocyte membrane, the local environments around the spin label sites would differ from each other. Therefore, the phases of the membrane would be multiple and the parameter S should be understood as an averaged value.

In the present study, I(5,10) apparently sensed the distinct two phases at higher temperatures, which could be roughly classified into the strongly immobilized, solid phase and the weakly immobilized, fluid phase. The width between the spectral center and the high field extreme was independent of temperature for the spin labels in the solid phase, but not for those in the fluid phase. Below 25°C, two kinds of signals fused into one. That these changes of the spectral shape were reversible in the range of 5—40°C suggested the gradual phase transition between fluid and solid.

Some relation may exist between the two kinds of sites, classified by the binding constant, and the two kinds of phase, barely separated to the strongly and the weakly immobilized signals at higher temperatures. A plausible hypothesis would be as follows, the labels incorporated into the tightly binding sites will show the signal of the strongly immobilized, solid phase and vice versa. To test the idea, the spectra obtained from two samples, of which the amounts of the label differed considerably, were compared in detail. For example, ratios of the heights at points A and B in Fig. 4 were compared, because such ratio was sensitive for analyzing the overlapped signals [27—31]. However, no clearcut relation could not be proved quantitatively for the following experimental difficulties; (i) the difference in the intrinsic binding constants between two sites was so small that two sites were simultaneously occupied and (ii) the difference in the signal shapes was too small to be separated.

Several explanations could account the phenomenon. (a) The different phases might correspond to the outer and inner layer of the membrane as suggested for the spin labeled phospholipids [32]. However, this possibility could be denied by the ascorbate experiment: when the labeled erythrocytes were immersed into the ascorbate solution, 85—90% of the labels were immediately disappeared, i.e., most of labels were in the outer layer.

(b) The localization of the labels in the flat surface or in the rim of the erythrocyte-disc might give the different signals. If this is the case, the temperature-independence of the strongly immobilized signal cannot be understood. In addition, the increase of the strongly immobilized signal by glutaraldehyde treatment cannot be explained.

(c) According to a surface pressure study on the monomolecular film [38], the label I(5,10) can adopt two orientations: one with the nitroxide group in the interior of the film and other the group at the lipid-water interface. This

possibility is again less plausible for explaining the temperature dependent and independent changes of the signals. Of course, this mechanism may be operative, if the nitroxide orientations are affected by the phase changes.

(d) The membrane states might depend on the ages of the individual erythrocytes, but a preliminary experiment on the younger and the older erythrocytes (separated by the method of Murphy, ref. 39) did not support the idea.

(e) Our preliminary experiment has suggested the dependence of the overall phase on the cholesterol contents or on the cholesterol/phospholipid molar ratio of the membrane, using an androstane spin label. Furthermore, the composition of the fatty acid chains of the phospholipids might influence the properties of the label sites, consistently with the model experiments [40—42].

(f) The phase distinction may be affected by the protein-lipid interaction in the membrane (vide infra), as in cases of the model experiments [43—46]. It might be assumed that the strongly immobilized labels localized in the proximity of certain membrane proteins, as in the case of the spin labels bound to the intact cytochrome b_5 [47].

(g) The influence of the morphological changes induced by the label could be neglected, because (i) the spectral shape was not altered by the contaminant, deformed erythrocytes in the present technique and (ii) the amount of the incorporated labels was less than the safety value (label/lipid molar ratio of 1 : 10) suggested by Butterfield et al. [48].

The most possible interpretation would be the mechanisms (e) and (f).

Effect of glutaraldehyde

In cases of the glutaraldehyde treated erythrocytes, the spectral changes were drastic, i.e., the weakly immobilized signal diminished and the strongly immobilized signal augmented. The observed spectral width of I(5,10) coincided with that of the signal just before freezing point (about 4°C). Further, the appearance of the strongly immobilized signal of I(1,14) showed that the effect of glutaraldehyde was not only superficial but penetrated into the middle portion of the lipid bilayer. These spectral changes clearly demonstrated the alteration of the membrane state ("freezing") induced by glutaraldehyde treatment.

Corresponding to the spectral changes, the viscosity of the 2% glutaraldehyde treated erythrocyte suspension (hematocrit = 45%) at 25°C increased about 20% compared with the untreated erythrocyte suspension. The "freezing" effect could be detected at a relatively low concentration of glutaraldehyde (above 0.05%; incubated for 30 min at 0°C). The effect of glutaraldehyde treatment on the erythrocyte membrane has been extensively studied [49—52], e.g., the modification of the cell shape and of the ionic equilibrium, the penetration of glutaraldehyde molecule, the protein cross-linking, the rheological behavior and so on. However, the information on the lipid portion of the membrane was poor. In contrast to the present study, Jost et al. [53] found no effect of 2% glutaraldehyde on the fatty acid spin label in lobster nerves nor in the phospholipid multilayer. Such conflicting results may be due to the differences of the lipid and/or protein composition and of the protein-lipid interaction in the membrane.

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