Membrane Fluidity Change in Erythrocytes Induced by Complement System†

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ABSTRACT: The structural change in erythrocyte membranes induced by antibody and complement was studied using phospholipid spin-labels. Sheep erythrocytes were labeled with phosphatidylcholine spin-label and various intermediate cells (erythrocyte-antibody complex (EA), EA bound with complement components from C1 to C7 (EAC1-7), EAC1-8, and EAC1-9) were prepared. Electron spin resonance spectra of EA, EAC1-7, and EAC1-8 were very similar to that of the erythrocytes, while that of EAC1-9 was markedly different. The overall splitting value for the lysed EAC1-9 (53 G) was much smaller than that for the erythrocytes (57 G), indicating a marked fluidization around the phosphatidylcholine label. The unlysed EAC1-9 membranes contained a limited fraction of the fluidized area. When EA was reacted with complement in the presence of 36% bovine serum albumin, the membranes were fluidized similarly to the lysed EAC1-9, although the hemolysis was largely blocked. The membranes of unlysed EAC1-9 prepared in isotonic (ethylenedinitrilo)tetraacetic acid were also fluidized, but to somewhat smaller extent. The role of C9 in the modification of erythrocyte membranes was also demonstrated using Mg^{2+} ghosts, which were prepared by hypotonic hemolysis in the presence of Mg^{2+}. The membranes of Mg^{2+} ghost of EAC1-7 were markedly fluidized when bound with C8 and C9, but not affected by binding of C8 only. The component C8 was found to give a latent effect on the membranes that caused irreversible fluidization upon osmotic shock. The terminal component thus creates a fluidized area in the erythrocyte membranes through which small ions and molecules may diffuse more easily and the resulting osmotic unbalance may finally cause hemolysis.

Complement-mediated hemolysis is the most investigated system among various immunological reactions by which men and other animals defend themselves against microscopic invaders. Effective hemolysis is caused by the action of antibody and complement components from C1 to C9[1] (Mayer, 1973; Müller-Eberhard, 1975). Preparation of the intermediate cells using sheep erythrocytes, hemolysin, and purified complement components has been established and opened up a way for detailed investigations of successive mechanism of immune hemolysis (Borsos et al., 1961; Nelson et al., 1961; Rommel and Mayer, 1973; Kolb and Müller-Eberhard, 1974; Kitamura and Inai, 1974). Immunolysis involves at least two steps: modification of the cell membranes by antibody and complement, and increased permeation of ions and molecules through the membranes. Morphological studies have shown the presence of circular structures of about 10-nm diameter in erythrocyte membranes (for example, see Iles et al., 1973). A model for the membrane lesions, called the doughnut model, has been proposed that assumed creation of an annular structure composed of terminal complement components inserted into phospholipid bilayer (Mayer, 1972, 1973; Hammer et al., 1975).

In order to obtain deeper insight into the molecular mechanism, we observed the membrane structural changes in various intermediate cells using phospholipid spin-labels as a probe. The EAC1-9 cell membranes were studied in the early stage after binding of C9 to EAC1-8, in isotonic EDTA medium, and also in the presence of 36% bovine serum albumin. The results have clearly demonstrated a marked fluidity change in erythrocyte membranes induced at the stage of C9 binding.

The present work is based upon our previous spin-label study of erythrocyte membranes (Tanaka and Ohnishi, 1975). Intact erythrocyte membranes had a heterogeneity in the fluidity, more rigid by phosphatidylcholine spin-label, and more fluid by phosphatidylserine and phosphatidylethanolamine spin-labels. The heterogeneity disappeared upon osmotic hemolysis. Mg^{2+} inhibited the homogenization, while Ca^{2+} acted antagonistically. The preservation of heterogeneity by Mg^{2+} was nullified when *N*-ethylmaleimide, trypsin, or heavy meromyosin coexisted in the hemolysis buffer. It was suggested that the heterogeneity arose from interactions between membrane proteins (lipids) and the inner fibrous proteins, presumably spectrin and actin, and disruption of the interaction leads to the homogenization. The present immunological results suggested that the terminal component C9 modified the interaction between the membrane constituents and the inner proteins, and caused local fluidization of the membranes, thus producing leaky patches.

Materials and Methods

Buffers. Isotonic veronal buffer contains 142 mM NaCl and 5 mM sodium veronal, pH 7.4. VB^{2+} is isotonic veronal saline containing 0.15 mM $CaCl_2$ and 1.0 mM $MgCl_2$. $GGVB^{2+}$ represents isotonic veronal saline containing 3% glucose, 0.1% gelatin, 0.15 mM $CaCl_2$, and 1.0 mM $MgCl_2$, pH 7.4. Isotonic EDTA consists of 90 mM EDTA and pH was adjusted with NaOH to 7.4.

Phospholipid Spin-Labels. Phosphatidylcholine spin-label

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[1] Abbreviations used are: C, C1, C2, C3, etc.—C refers to complement, and the numbers indicate the component of the complement system. E, EA, EAC1-7, EAC1-8, and EAC1-9 are shorthand designations for erythrocytes, sensitized erythrocytes, erythrocytes carrying antibody and complements from C1 to C7, from C1 to C8, and from C1 to C9, respectively. Nomenclature of *Bull. W.H.O. 39*, 935 (1968). EAC represents EA reacted with whole complement. CH50 is the quantity of complement required for 50% hemolysis and SFU the site-forming units which correspond to effective molecules (see Mayer, 1961); ESR, electron spin resonance; EDTA, (ethylenedinitrilo)tetraacetic acid.

with 12-nitroxide stearic acid attached at the 2-position was prepared by the reaction of lysolecithin, obtained from egg lecithin, and anhydride of the stearic acid label (Hubbell and McConnell, 1971). 12-Nitroxide stearic acid was synthesized according to Waggoner et al. (1969) with slight modifications (Ito et al., 1975). Analogous spin-label for phosphatidylserine was synthesized by the method of Ito et al. (1975).

Spin-Labeling of Erythrocytes. Phosphatidylcholine spin-label was suspended in isotonic veronal buffer at a concentration of 1 mg/ml, sonicated for a few minutes in an ice bath, and centrifuged. Washed sheep erythrocytes (packed volume 2 ml) were mixed with the supernatant (4 ml) and incubated at 37 °C for 3 h. The spin-labeled erythrocytes were washed three times with isotonic saline to remove unincorporated spin-labels. Labeling with phosphatidylserine spin-label was carried out in a similar way. Human erythrocytes were also used for comparison. In these cases, the amount of spin-label was halved and the incubation time was 2 h.

Complement Components. The reagent containing C4, C2, C3, C5, C6, and C7 (7 cell reagent) was obtained from fresh human serum by the method of Manni and Müller-Eberhard (1969) with a slight modification. The eighth component of guinea pig complement was purified according to Inoue et al. (1967). The ninth component of human complement was purchased from Cordis Laboratories, Miami, Florida. Guinea pig serum was obtained from Toshiba and used as whole complement.

Preparation of Intermediate Cells, Lysed EAC and Lysed EAC1–9. Sheep erythrocytes (E), rabbit anti-sheep erythrocyte serum (hemolysin, A), sensitized erythrocytes (EA), and EA with various complement components were prepared as described previously (Kitamura and Inai, 1974), except for the use of spin-labeled erythrocytes. Briefly, EAC1–7 cells were prepared by incubating EAC1 with 7 cell reagent at 30 °C for 25 min. EAC1–8 cells were prepared by incubating EAC1–7 with 200 site-forming units (SFU)/cell of C8 at a final cell concentration of 7.5×10^7 cells/ml in $GGVB^{2+}$ at 30 °C for 20 min.

Unlysed EAC1–9 cells were prepared in three different conditions. In the first preparation, EAC1–8 was incubated at 37 °C with a limited amount of C9 (2.5 or 10 SFU/cell) in $GGVB^{2+}$ and the reaction was stopped after a short time (15 or 30 min). In the second preparation, EAC1–8 was incubated with 20 SFU/cell of C9 in isotonic EDTA at 30 °C for 20 min. For the third preparation, EA (2×10^9 cells) was incubated with guinea pig serum (20 CH50 units) in VB^{2+} containing 36% bovine serum albumin at 37 °C for 30 min. The reaction mixtures were chilled after incubation and centrifuged for 10 min at 700*g*. The unlysed erythrocytes were pelleted, while the lysed cells remained in the supernatant. ESR spectra of the pellets were measured. When a portion of the pellet was taken and resuspended in $GGVB^{2+}$, complete hemolysis was observed immediately for the third preparation, and in a few hours for the first and second preparations.

Lysed EAC was prepared by incubating EA with guinea pig serum at 37 °C for 30 min in VB^{2+}, followed by centrifugation at 20 000*g* for 40 min. Lysed EAC1–9 was obtained as pellet by centrifugation at 20 000*g* after reaction of EAC1–8 with C9 at 37 °C for sufficient time for complete hemolysis.

Preparation of Cells Lysed Osmotically in the Presence of Mg^{2+} (Mg^{2+} Ghost). E, EA, and various intermediate cells were lysed in a hypotonic veronal buffer containing $MgCl_2$ as described previously (Tanaka and Ohnishi, 1976). Washed intermediate cells were mixed at 0 °C with 20 volumes of 5 ml of veronal buffer containing 1.0 mM $MgCl_2$ (pH 7.5) and kept at the temperature for 30 min. After restoration of isotonicity with NaCl, the ghost cells were incubated at 37 °C for 30 min for resealing and centrifuged down at 20 000*g* for 40 min. EAC1–7 ghosts were further reacted with C8 and C9 or with C8 only at 37 °C for 30 min at concentration ratios comparable to the respective intermediate cell preparations.

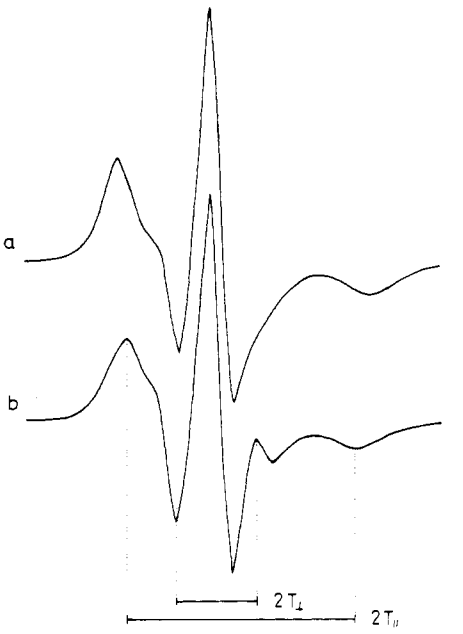

FIGURE 1: The ESR spectrum of sheep erythrocytes labeled with phosphatidylcholine spin-label (a) and phosphatidylserine spin-label (b). The spin-labeled erythrocytes were prepared as described under Materials and Methods and the ESR spectrum was measured at 22 °C.

Preparation of Liposomes. Total lipid was extracted from sheep erythrocyte ghost by the method described by Haxby et al. (1968). Phosphatidylcholine spin-label was added to the lipid extract by an amount of 2%, suspended in VB^{2+} at a concentration of 4 mg/ml, and dispersed by agitation with a Vortex mixer. Hemolysin (10 μl) and guinea pig serum (50 μl containing 12 CH50 units) were added to 0.2 ml of liposomes and incubated at room temperature for 10 min or at 37 °C for 1 h.

Measurement of ESR Spectra. Spin-labeled erythrocytes and intermediate cells were centrifuged down at 700*g*. The corresponding lysed cells were collected by centrifugation at 20 000*g*. The cell pellet was taken into a quartz capillary tube (i.d. ~0.8 mm) and the ESR spectrum was measured at 22 °C routinely using an X-band spectrometer (JEOLCO Model ME-X). Typically, 2×10^9 cells were reacted in 24 ml or larger volumes and centrifuged for one ESR measurement. All the spectra were recorded together with the signal of Mn^{2+} in MgO for the field-scan calibration. The accuracy for the overall splitting values given in the text are within 0.5 G.

Results

ESR Spectrum of Spin-Labeled Erythrocytes. Figure 1 shows ESR spectra of sheep erythrocytes labeled with phosphatidylcholine and phosphatidylserine spin-labels. The spectral characteristics indicate axially symmetric motion of the lipid alkyl chains and suggest incorporation of the spin-labels into the lipid bilayer portion of the membranes. The anisotropic principal values of the hyperfine tensor, $T_\parallel$ and $T_\perp$, can be obtained from the spectra. The overall splitting represents $2T_\parallel$ and the inner splitting equals $2T_\perp$ (see Figure 1b). An order parameter defined by $S = (3 \cos^2 \theta - 1)/2$ can be

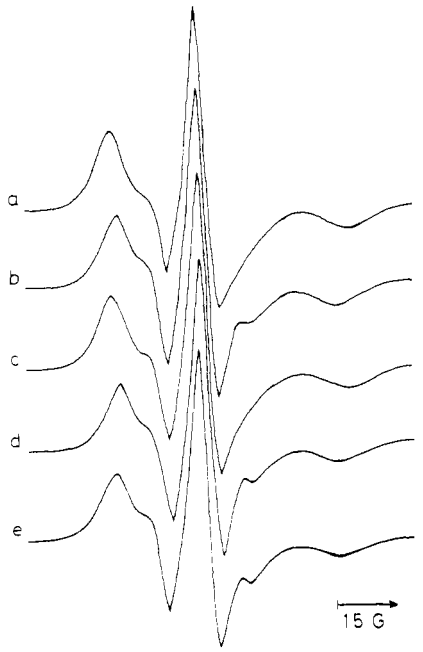

FIGURE 2: The ESR spectrum of spin-labeled sheep erythrocytes (a), the cells lysed in a hypotonic solution (b), the cells lysed in the hypotonic solution containing Mg^{2+} (c), the cells lysed by incubation with antibody and complement (d), and the cells incubated with antibody and complement in the presence of 36% bovine serum albumin (hemolysis 19%) (e). Erythrocytes labeled with phosphatidylcholine spin-label were lysed at 37 °C in 5 mM veronal buffer (b) and lysed at 0 °C in the veronal buffer containing 1.0 mM $MgCl_2$ followed by incubation at 37 °C after restoration of isotonicity (c). Incubation with antibody and complement was done as described under Materials and Methods.

calculated using a relation $S = (T_{\parallel} - T_{\perp})/27$. Here, θ is the angle between one of the principal axes (z) of the hyperfine tensor and the normal to bilayer membranes (Hubbell and McConnell, 1971). As the anisotropic motion becomes more vigorous, the mean angular deviation of the z axis becomes larger, the order parameter becomes smaller, or, simply, the overall splitting smaller. These spectral parameters are conveniently used as a measure of the fluidity of the membranes (Ohnishi, 1975).

A predominant feature in Figure 1 is that the spectrum for phosphatidylcholine spin-label was markedly different from that for phosphatidylserine spin-label. The overall splitting for the former (57 G) was much larger than that for the latter (52 G). The order parameter for phosphatidylserine spin-label was calculated as 0.62, the mean angular deviation θ being 30°. For phosphatidylcholine spin-label, the motion of alkyl chains was much more frozen so that the inner splitting was not resolved. The results clearly demonstrate a heterogeneity in the membrane fluidity of sheep erythrocytes, more rigid surroundings of phosphatidylcholine spin label, and more fluid environments around phosphatidylserine spin label.

The ESR spectrum of spin-labeled erythrocytes was greatly modified upon hypotonic hemolysis (see Figure 2a,b). The overall splitting for phosphatidylcholine spin-label markedly decreased to 53 G, indicating a fluidization of the membranes around the label. The fluidization was prevented and the heterogeneity was preserved when Mg^{2+} was present in the hemolysis buffer and the hemolysis was carried out at lower temperatures (e.g., 0 °C). The spectrum for phosphatidylcholine spin-label was essentially unchanged (Figure 2c), the overall splitting remaining to be 57 G. The ESR spectrum for phosphatidylserine spin-label was not largely modified on the hypotonic hemolysis, even in the absence of Mg^{2+}. The overall splitting value became 52.2 G, which was almost the same as that for intact erythrocytes (51.8 G) within the experimental error.

TABLE I: Overall Splitting Value at 22 °C of the ESR Spectrum of Spin-Labeled Sheep Erythrocytes (E) and Their Various Complexes with Antibody (A) and Guinea Pig Serum (C).[a]

	Splitting Value (G)
E	56.7
EA	56.8
E + C	56.8
EAC lysed	52.9
EAC in 36% albumin[b]	53.0
$EAC_{inactivated}$ in 36% albumin[b]	56.9
E lysed osmotically	52.8
Total lipid liposomes	49.3
Total lipid liposomes + A + C	49.8

[a] Erythrocytes were labeled with phosphatidylcholine spin-label and the complexes were prepared as described under Materials and Methods. [b] The sensitized erythrocytes (EA) were incubated with guinea pig serum (C) or with heat-inactivated guinea pig serum ($C_{inactivated}$) in the presence of 36% bovine serum albumin. Hemolysis was 19% for EAC and 0% for $EAC_{inactivated}$.

Effect of Antibody and Complement. Binding of hemolysin to spin-labeled erythrocytes did not affect the ESR spectrum at all. When guinea pig serum was added to the sensitized erythrocytes and incubated at 37 °C for 30 min, the cells were completely hemolyzed and the ESR spectrum for phosphatidylcholine spin-label changed markedly (see Figure 2d). The overall splitting decreased to 53 G, indicating fluidization of the membranes around the label. However, the spectrum for phosphatidylserine spin-label was almost unchanged in the immune hemolysis.

When the sensitized erythrocytes were incubated with guinea pig serum in the presence of 36% bovine serum albumin, the hemolysis was largely blocked (Frank et al., 1965), but the ESR spectrum for phosphatidylcholine spin-label changed in nearly the same way as that for complete hemolysis (Figure 2e). A control experiment using heat-inactivated guinea pig serum showed no change in the ESR spectrum and no hemolysis. The overall splitting values for various cell preparations are summarized in Table I.

Essentially, the same change was observed for human erythrocytes labeled with phosphatidylcholine spin-label. The splitting value for human E, EA, lysed EAC, and EAC prepared in 36% bovine serum albumin (6% hemolysis) was 53, 53, 50, and 51 G, respectively.

Effect of antibody and complement on the lipid part of erythrocyte membranes was investigated using liposomes made of total lipid extract from sheep erythrocytes. Phosphatidylcholine spin-label was included in the liposomes. The ESR spectrum of the liposomes was not affected by the action of antibody and complement (Figure 3), the overall splitting value being nearly the same (Table I). The amount of antibody and complement used was large enough to cause extensive leakage of a marker glucose molecule out of the liposomes, according to the data by Haxby et al. (1968).

No effect of antibody and complement was observed on erythrocyte ghosts prepared by hypotonic hemolysis. The overall splitting for phosphatidylcholine spin-label remained to be 53 G.

Membrane Fluidity Change by C9 Binding. In order to in-

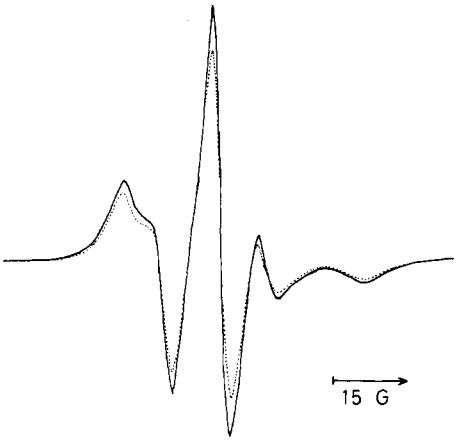

FIGURE 3: The ESR spectrum of spin-labeled liposomes (—) and effect of antibody and complement (• • •). The liposomes were made of total lipid extract from sheep erythrocytes and labeled with phosphatidylcholine spin-label.

TABLE II: Overall Splitting Value at 22 °C of the ESR Spectrum of Spin-Labeled Intermediate Cells.[a]

	Splitting Value (G)
E	56.7
EA	56.8
EAC1-7	56.8
EAC1-8[b]	56.4
EAC1-9[c]	
2.5 SFU/cell, 15 min, 8% hemolysis	55.8
10 SFU/cell, 15 min, 30% hemolysis	54.8
10 SFU/cell, 60 min, 65% hemolysis	55.1
in isotonic EDTA	54.4
EAC1-9 lysed	52.9

[a] Sheep erythrocytes were labeled with phosphatidylcholine spin-label and intermediate cells were prepared using the spin-labeled erythrocytes. The splitting values are averages of four to six measurements, in most cases. [b] EAC1-8 suffering from partial spontaneous hemolysis gave the splitting value of 56.0 G. [c] Prepared by incubating EAC1-8 with different concentrations of C9 for different intervals, as described under Materials and Methods. The resulting hemolysis is indicated. The splitting values are for the unlysed EAC1-9.

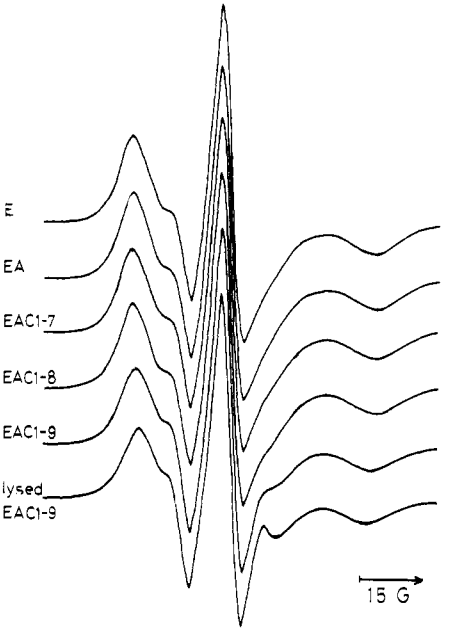

FIGURE 4: The ESR spectrum of spin-labeled intermediate cells. The intermediate cells, EA, EAC1-7, EAC1-8, and EAC1-9, were prepared using sheep erythrocytes labeled with phosphatidylcholine spin-label. All the spectra were measured at 22 °C with the unlysed intermediate cells, except for one designated as lysed EAC1-9. The unlysed EAC1-9 was collected by centrifugation at 700*g* after incubating EAC1-8 with 10 SFU/cell of C9 at 37 °C for 15 min.

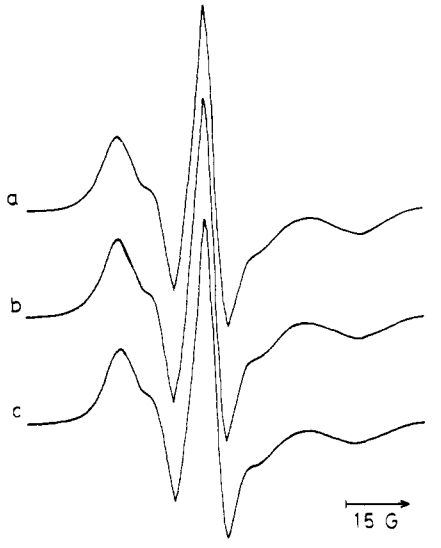

FIGURE 5: The ESR spectrum of spin-labeled EAC1-9 prepared by different concentrations of C9. EAC1-8 was incubated with 2.5 SFU/cell of C9 for 15 min (a), with 10 SFU/cell of C9 for 15 min (b), and with 10 SFU/cell of C9 for 60 min (c). The unlysed EAC1-9 was collected by centrifugation at 700*g* after the incubations. Hemolysis was 8, 30, and 65% for a, b, and c, respectively.

vestigate how and at which stage the fluidity change occurred, various intermediate cells were prepared using sheep erythrocytes labeled with phosphatidylcholine spin-label. As shown in Figure 4, a drastic change in the ESR spectrum occurred at the stage of C9 binding, whereas the spectra of the preceding intermediate cells were quite similar to that of erythrocytes. The membrane fluidity was not largely modified by binding of complement components up to C8, as far as the phosphatidylcholine label was concerned.

The spectrum of unlysed EAC1-9 was different from those of erythrocytes and other intermediate cells in its shape, as well as in the overall splitting (Table II). The difference in the spectral shape can be most easily recognized in the high-field side of the central peak. The spectrum of unlysed EAC1-9 was also different from that of lysed EAC1-9 and lay somewhere in between those of erythrocytes and lysed cells. The spectrum was interpreted as superposition of these two spectra at a certain ratio. This indicates that the unlysed EAC1-9 contained some fraction of the fluidized area in the membranes.

The membrane fluidity of EAC1-9 was investigated as a function of the amount of C9 used for the preparation. Figure 5 shows the ESR spectrum of EAC1-9 prepared by incubating EAC1-8 with various dilutions of C9 for various intervals. The spectra appeared rather similar to each other in spite of the large difference in the degree of hemolysis reactions. The apparent overall splitting values were also not very different (Table II). The spectrum of unlysed EAC1-9 taken from preparations with more extensive hemolysis approached slightly more that of lysed cells.

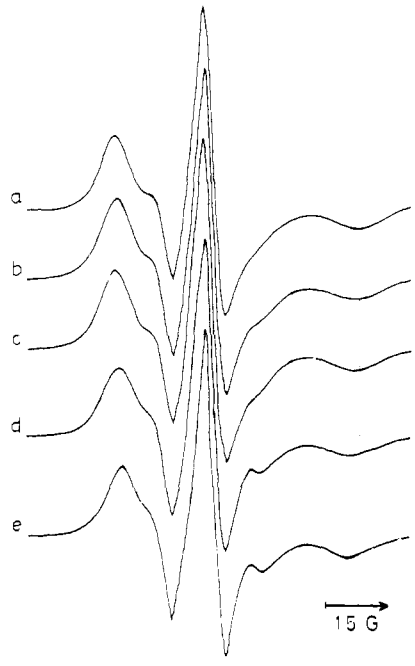

FIGURE 6: The ESR spectrum of Mg^{2+} ghost of E (a), EA (b), EAC1-7 (c), and EAC1-8 (d). (e) The spectrum after incubation of Mg^{2+} ghost of EAC1-7 with C8 + C9. The various intermediate cells were prepared using sheep erythrocytes labeled with phosphatidylcholine spin-label and lysed at 0 °C in 5 mM veronal buffer containing 1.0 mM $MgCl_2$. The ESR spectrum was measured at 22 °C.

Unlysed EAC1-9 was also prepared in isotonic EDTA, where hemolysis was largely blocked (Frank et al., 1965). The ESR spectrum was again different from those of the preceding intermediate cells. The overall splitting was smaller (54.4 G), indicating a fluidization of the membranes around the label. The fluidization was not as large as that for unlysed EAC prepared in the presence of albumin, where the splitting value was 53.0 G (see Table II).

Osmotic Hemolysis of Intermediate Cells in the Presence of Mg^{2+}. E, EA, EAC1-7, and EAC1-8 were subjected to hypotonic hemolysis in the presence of Mg^{2+} and the ESR spectrum of the lysed cells (Mg^{2+} ghost) was measured. It is shown in Figure 6 that a marked change occurred in the ESR spectrum of EAC1-8. The overall splitting decreased to 54.1 G (see Table III), indicating considerable fluidization of the cell membranes on the hemolysis. The ESR spectrum of EAC1-7 was also somewhat modified but the modification was slight. Practically no change was observed for EA.

Another interesting result with the Mg^{2+} ghost was that the ESR spectrum for EAC1-7 was markedly modified when bound with both C8 and C9 (Figure 6e), while no effect was observed on binding of C8 only. The overall splitting decreased remarkedly to 52.0 G (Table III). This conforms to the role of C9 in the membrane fluidity change obtained with unlysed intermediate cells and mimics the spectral changes, marked change on C9 binding but no change up to C8 binding. The degree of fluidization by C9 was also nearly the same for intact cells and Mg^{2+} ghosts.

An interesting difference is noted on the action of C8 to EAC1-7. The component affected the erythrocyte membranes when bound before hemolysis, but gave no effect when bound after the hemolysis in Mg^{2+} (Table III).

TABLE III: Overall Splitting Value at 22 °C of the ESR Spectrum of Spin-Labeled Intermediate Cells Lysed Osmotically in the Presence of Magnesium Ion.[a]

Mg^{2+} ghost of	Incubated with	Splitting Value (G)
E		56.8
	C9	56.8
EA		56.3
EAC1-7		55.5
	C8	55.6
	C8 and C9	52.0
EAC1-8		54.1

[a] Intermediate cells were prepared using sheep erythrocytes labeled with phosphatidylcholine spin-label and lysed at 0 °C in 5 mM veronal buffer containing 1.0 mM $MgCl_2$.

Discussion

The present spin-label study has demonstrated that a definite fluidity change was induced in sheep erythrocyte membranes at the C9 binding stage of immune hemolysis. That is, although the ESR spectra of EA, EAC1-7, and EAC1-8 were nearly the same as that of the erythrocytes, the spectrum of EAC1-9 was markedly different from other intermediate cells. The overall splitting value for phosphatidylcholine spin-label in the lysed EAC1-9 (53 G) was much smaller than that for the other intermediate cells (57 G), becoming closer to that for the membranes consisting only of the erythrocyte lipids (49 G). The fluidization therefore suggests formation of lipid bilayer area that is less influenced by the membrane proteins. If we take the results obtained for osmotic hemolysis (Tanaka and Ohnishi, 1976) into account, the complement-induced fluidization appears to arise from disruption of the interactions between membrane constituents and the inner fibrous proteins by C9.

The unlysed EAC1-9 prepared by smaller amounts of C9 contained some limited fraction of the fluidized area. The membranes of EAC prepared in the presence of 36% bovine serum albumin had extensive fluidized area. In this case, the membranes seemed to be ready for lysis, as evidenced by immediate complete hemolysis after removal of the protein from the medium. The presence of high-molecular-weight protein outside may have prevented proceeding of hemolysis. The unlysed EAC1-9 prepared in isotonic EDTA also contained fluidized membranes, but the degree of fluidization was somewhat smaller than that of EAC in bovine albumin or that of lysed EAC1-9. The effect of EDTA may be interpreted similarly to the case of bovine serum albumin, although the molecular action of EDTA is not clear. Remarkable fluidity change in Mg^{2+} ghosts of EAC1-7 induced by binding of both C8 and C9 confirmed the role of C9 in the membrane structural change (Table III). This result also indicates that the membrane fluidity change was independent of the osmotic pressure.

The membrane fluidity of erythrocytes was not much modified by binding of complement components up to C8. A pronounced difference emerged, however, when the intermediate cells were lysed in the presence of Mg^{2+}. The membrane fluidity of EAC1-8 was markedly increased, indicating that C8 must have given some latent effect on the erythrocyte membranes. This latent effect leads to the irreversible membrane damage (fluidization) when the cells were subjected to osmotic shock. In the absence of C8, the membrane heterogeneity was preserved, probably by restoration by Mg^{2+} of the membrane damage once formed during osmotic shock. Interaction of C8 with erythrocyte proteins may have inhibited

the restoration. Some agglutinins gave a similar latent effect on the erythrocyte membranes; a marked fluidization was observed only after hemolysis in the presence of Mg^{2+} (unpublished results).

The fluidity of liposome membranes made of total lipid extract was not modified by antibody and complement. This is in line with the above conclusion that proteins are involved in the C9-induced fluidity change in erythrocyte membranes. This result may suggest that immune lysis of erythrocytes and liposomes occurs through different mechanisms.

A speculation can be made on the mechanism(s) of the immune hemolysis from the present results. The terminal complement component C9 creates a fluidized spot in the erythrocyte membranes, which may develop into larger area with time. The fluidized area may be leaky so that smaller ions and molecules may penetrate more easily and the penetration may eventually cause hemolysis as a result of the Donnan-type effect. The rate of hemolysis will be larger as the fluidized area becomes larger. This model is not new but one of the oldest concepts (see, for example, Mayer, 1972), and may not be directly compatible to the doughnut model (Mayer, 1972, 1973; Hammer et al., 1975). A big difference between the old and the revived leaky-patch model is that, while the old model assumed patches produced by lytic agents, the present model assumes, with some experimental evidence, patches created by interaction of C9 with the membrane constituents and the inner fibrous proteins. The erythrocyte lipids are in a rigid state as a result of interactions with the membrane and inner proteins. The modification by C9 causes weakening of the lipid–protein interactions, thus producing a pure lipid bilayer-like area in the membranes. The fluidized area may be mechanically weak and deformed or even broken more easily. It is now being generally accepted that the inner proteins, spectrin and actin, form fibrous structures and support the lipid bilayer in erythrocyte membranes (see Steck, 1974).

Manipulation of local membrane fluidity through modification of interactions between the membrane constituents and the inner fibrous proteins may be a more general control mechanism for various membrane activities. In the osmotic and immune hemolysis, the trigger for the modification was osmotic shock and binding of a protein (C9) to the membranes, respectively. Our recent study has shown that human erythrocyte membranes were markedly fluidized in Sendai virus-induced hemolysis and fusion. The trigger was binding of a viral protein, F glycoprotein, to the membranes (Maeda et al., in preparation). Various physiological reagents, hormones, and divalent cations, such as Ca^{2+}, may also act as trigger.

Acknowledgments

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References

Borsos, T., Rapp, H. J., and Mayer, M. M. (1961), *J. Immunol. 87,* 310.

Frank, M. M., Rapp, H. J., and Borsos, T. (1965), *J. Immunol. 94,* 295.

Hammer, C. H., Nicholson, A., and Mayer, M. M. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 5076.

Haxby, J. A., Kinsky, C. B., and Kinsky, S. C. (1968), *Proc. Natl. Acad. Sci. U.S.A. 61,* 300.

Hubbell, W. L., and Connell, H. M. (1971), *J. Am. Chem. Soc. 93,* 314.

Iles, G. H., S ıan, P., Naylor, D., and Cinader, B. (1973), *J. Cell Bic 6,* 528.

Inoue, K., M , T., and Yonematsu, K. (1967), *Biken J. 10,* 143.

Ito, T., Obnishi, S., Ishinaga, M., and Kito, M. (1975), *Biochemistry 14,* 3064.

Kitamura, H., and Inai, S. (1974), *J. Immunol. 113,* 1992.

Kolb, W. P., and Müller-Eberhard, H. J. (1974), *J. Immunol. 113,* 479.

Manni, J. A., and Müller-Eberhard, H. J. (1969), *J. Exp. Med. 130,* 1145.

Mayer, M. M. (1961), in Experimental Immunochemistry, Kabat, E. A., and Mayer, M. M., Ed., Springfield, Ill., Charles C. Thomas, Chapter 4.

Mayer, M. M. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 2954.

Mayer, M. M. (1973), *Sci. Am. 229,* 54.

Müller-Eberhard, H. J. (1975), *Annu. Rev. Biochem. 44,* 697.

Nelson, R. A. Jr., Jensen, J., Gigli, I., and Tamura, N. (1961), *Immunochemistry 3,* 111.

Ohnishi, S. (1975), *Adv. Biophys. 8,* 35.

Rommel, F. A., and Mayer, M. M. (1973), *J. Immunol. 110,* 637.

Steck, T. L. (1974), *J. Cell Biol. 62,* 1.

Tanaka, K., and Ohnishi, S. (1975), *Biochim. Biophys. Acta 426,* 218.

Waggoner, A. S., Kingzett, T. J., Rottschaeffer, S., and Griffith, O. H. (1969), *Chem. Phys. Lipids 3,* 245.