Proc. Natl. Acad. Sci. USA
Vol. 75, No. 10, pp. 4930–4934, October 1978
Biophysics

Electron spin resonance studies on interaction of complement proteins with erythrocyte membranes

(protein–lipid interaction/decreased fluidity)

CHARLES E. DAHL* AND R. P. LEVINE†

The Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138

Communicated by Konrad Bloch, July 17, 1978

ABSTRACT **Sheep erythrocytes have been spin labeled with 5-, 12-, and 16-nitroxystearic acid in order to investigate complement-induced changes in the physical state of the lipid bilayer. Formation of osmotic lesions in the membrane causes an increase in the fluidity of the membrane which overcomes the decrease in membrane fluidity caused by the interaction of the complement proteins. A decrease in membrane fluidity is observed only when complement-lysed membranes are resealed or when complement proteins react with isosmolar ghosts that do not undergo osmotic lysis. The decrease in bulk fluidity of the membrane is first observed when C8 binds to the membranes bearing C5b67 and is enhanced upon the subsequent binding of C9. The decrease in membrane fluidity shown by the electron spin resonance spectra of spin-labeled fatty acids suggests that certain of the complement proteins penetrate the membrane and interact with hydrophobic regions of the lipid bilayer.**

The complement-mediated killing of bacteria and other cells by the serum complement proteins is among the most intensely investigated immunological reactions. The proteins circulate as inactive precursors in the serum until they are activated sequentially by highly specific biochemical reactions. This activation process changes water-soluble inactive proteins into membrane-bound proteins that destroy the osmotic integrity of the cell membrane. We are particularly interested in the assembly of the terminal complex of complement proteins (C5b–9), which binds to cell membranes and initiates the lytic process (1). The C5b67 complement proteins form a complex that binds to the cell membranes and provides a receptor site for C8 (2). C8 is directly responsible for producing membrane damage (3, 4), and C9 acts to enhance the lytic activity of the terminal complex (5). Recent evidence indicates that membrane damage by complement requires the insertion of hydrophobic portions of the C5b–9 complex into the hydrophobic regions of the cell membrane (6, 7).

The exact sequence of events or the molecular mechanism by which this terminal complex damages the membrane has not been rigorously defined. Two recent studies in which electron spin resonance (ESR) spectroscopy has been used to investigate changes in the physical state of the erythrocyte membrane brought on by the interaction of the erythrocyte with complement (8, 9) have produced conflicting results. This confusion stems from the fact that the end result of complement interaction with erythrocytes is osmotic lysis of the cells, and osmotic lysis itself produces a change in the spin-label spectrum which is superimposed on the change caused by the interaction of the complement proteins with the lipid bilayer. Accordingly, we have used ESR spectroscopy to monitor the changes in the physical state of erythrocyte membranes brought on by the action of the complement proteins under conditions that eliminate any effect due to osmotic lysis. This was accomplished by either resealing erythrocyte ghosts bearing the complement proteins or by using isosmolar ghosts, which do not undergo osmotic lysis upon interaction with the complement proteins.

This communication describes these experiments and reports the following observations: (*i*) the terminal complement proteins (C5b–9) bind to the cell membrane and reduce the fluidity of the membrane around the spin-label site; and (*ii*) this reduction in fluidity is first noticed when C8 binds to the membrane and is enhanced by the subsequent binding of C9. A preliminary account of the work has been published (10).

MATERIALS AND METHODS

Fatty Acid Spin Labels. The experiments described here used 5-, 12-, and 16-nitroxystearic acid purchased from Syva Associates, Palo Alto, CA.

Spin Labeling of Erythrocytes. Antibody-sensitized sheep erythrocytes (EA) prepared according to Nelson *et al.* (11), were centrifuged in the cold at 1570 × *g* for 5 min, washed three times with 2.5 mM Veronal buffer (pH 7.4)/75 mM NaCl/2.5% dextrose/0.05% gelatin/0.15 mM Ca^{2+}/0.5 mM Mg^{2+} ($DGVB^{2+}$), and then resuspended in $DGVB^{2+}$ at 1.25×10^{10} cells per ml. In order to incorporate the spin label into membranes of intact cells, we measured 0.5 ml of a 1.0 mM solution of the appropriate spin label in ethanol into an aluminum foil-shielded test tube. The ethanol was evaporated by a stream of N_2 gas, leaving a thin film of the spin label. One milliliter of the washed cells was added to the test tube and incubated for 30 min at 37°. The cells were washed once with $DGVB^{2+}$ to remove any unincorporated spin label and used immediately. The 30-min incubation period was found to be optimum for incorporation of spin label.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: Terminology for complement components is that recommended by the World Health Organization Committee on Complement Nomenclature (1968); C1, C2, C3, C4, C5, C6, C7, C8, and C9, nine complement components in the classical pathway. E, sheep erythrocytes; EA, antibody-sensitized erythrocytes; EAC1, EAC14, EAC142, and EAC1423, cell–complement intermediates with the designated complement proteins attached to the cell; EAC, EA that have been lysed with guinea pig serum. $DGVB^{2+}$, 2.5 mM Veronal buffer (pH 7.4)/75 mM NaCl/2.5% dextrose/0.05% gelatin/0.15 mM Ca^{2+}/0.5 mM Mg^{2+}; GVB^{2-}, 5 mM Veronal buffer (pH 7.4)/147 mM NaCl/0.1% gelatin. 10 mM EDTA, prepared by mixing 57.5 ml of 86 mM EDTA (pH 7.4) and 442.5 ml of GVB^{2-}; C-EDTA, normal guinea pig serum diluted with 10 mM EDTA; GA, antibody-sensitized isosmolar ghosts; GAC, GA that have been reacted with normal guinea pig serum. ESR, electron spin resonance. 5-NSA, 12-NSA, and 16-NSA, 1-oxyl-2,2-dimethyloxazolidine derivatives of 5-, 12-, and 16-ketostearic acid, respectively.

* Present address: James Bryant Conant Laboratories, Department of Chemistry, Harvard University, Cambridge, MA 02138.
† Present address: Department of Genetics, School of Medicine, Washington University, St. Louis, MO 63110.

Table 1. Complement protein interaction with cell membranes: Classical pathway with guinea pig serum*

	5-NSA†	12-NSA†	16-NSA†
EA	60.0	53.0	36.5
EA (ghost)	57.0	51.5	35.5
EA (resealed ghost)	60.0	53.3	36.8
EAC (heat-inactivated)	60.0	53.0	36.5
EAC (ghost)	57.3	51.0	35.3
EAC (resealed ghost)	61.0	54.5	37.4

Erythrocytes were labeled with stearic acid spin labels, and the intermediate cells were prepared as described. Hemolysis was 100% for EAC (ghosts) and 0% for EAC (heat-inactivated).

* Hyperfine interaction ($2T_{\parallel}$) in gauss at 22°.

† 1-Oxyl-2,2-dimethyloxazolidine derivatives of 5-, 12-, and 16-ketostearic acid, respectively.

Cells, Antisera, and Complement Components. Sheep blood drawn into Alsever's solution was obtained from a single sheep. Rabbit anti-sheep hemolysin was purchased from Microbiological Associates. Guinea pig serum obtained from Pel Freez Biologicals (Rogers, AR) was used as whole complement. C8 and C9 were purchased from Cordis Laboratories (Miami, FL). C5b6 was isolated from human serum by the procedure of Baker *et al.* (12), and C7 according to Podack *et al.* (13). The titer of both C5b6 and C7 was determined by reactive lysis (14). A 25-μl suspension of sheep erythrocytes (1×10^8 cells per ml in $DGVB^{2+}$) was mixed in microtiter plates with 50 μl of an appropriate dilution of C7 or C5b6 and incubated with shaking at 30° for 20 min. Then 25 μl of 1:200 dilution of normal guinea pig serum in 10 mM EDTA/GVB^{2+} (C-EDTA) was added and incubation was continued for 40 min at 37°. C5b6 contained 500 units/ml and C7 100,000 units/ml, where 1 unit is defined as the highest dilution giving total lysis of sheep erythrocytes.

Erythrocyte–Complement Intermediates. Erythrocyte–complement intermediates EAC1 and EAC14 were prepared from spin-labeled erythrocytes by the method of Borsos and Rapp (14). EAC142 and EAC1423 were prepared by the procedure of Fearon *et al* (15). EAs were treated with complement by incubating 5.0 ml of spin-labeled cells (1×10^9 cells per ml) in $DGVB^{2+}$ with 0.5 ml of normal guinea pig serum for 60 min at 37°. The ghosts, produced by complement lysis, were concentrated by centrifugation at 27,000 × *g* for 15 min. They were washed once and resuspended in 1.0 ml of $DGVB^{2+}$, and the suspension was divided into two equal parts; one part was used for an ESR experiment without further modification and the second part was resuspended in 100 vol of resealing buffer (4.0 mM sodium phosphate/0.9% NaCl/4 mM $MgCl_2$, pH 7.0)

Table 2. Complement protein interaction with cell membranes: Isosmolar ghosts with guinea pig serum*

	5-NSA	12-NSA	16-NSA
GA	60.0	53.2	36.3
GAC (heat-inactivated)	60.0	53.1	36.4
GAC	60.8	55.0	37.5
GAC (resealed)	61.0	55.0	37.5

Isosmolar ghosts (G) were labeled with stearic acid spin labels, and the intermediates were prepared with the spin-labeled isosmolar ghosts.

* Hyperfine interaction ($2T_{\parallel}$) in gauss at 22°.

and incubated at 37° for 45 min. The resealed complement-lysed ghosts were then centrifuged at 100,000 × *g* for 1 hr on 12% Ficoll in isotonic NaCl (0.9%), a process that sediments cells retaining complement lesions (16, 17). After centrifugation, the membrane pellet was resuspended in $DGVB^{2+}$ for the ESR experiments.

Hypotonic Hemolysis. Hypotonically lysed cells (spin-labeled E or EA) were prepared by mixing 1.0 ml of packed cells (2.50×10^{10} cells per ml) with at least 50 vol of ice-cold lysing buffer (5.0 mM sodium phosphate/5.0 mM $MgCl_2$, pH 7.0) for 10 min at 0°. The spin-labeled ghosts were sedimented for 15 min at 27,000 × *g* and washed twice with the same buffer, and ESR spectra were recorded. Resealed ghosts were obtained by the procedure described above.

Complement Lysis of Isosmolar Ghosts. Spin-labeled EA (1.0 ml of 1.25×10^{10} cells per ml) were suspended in at least 50 vol of ice-cold lysing buffer for 10 min at 0°. Isotonicity was restored by adding one part of a 4.5% NaCl solution to four parts of cell suspension (10 ml to 40 ml) and incubating at 37° for 45 min. The isosmolar ghosts (GA) were centrifuged at 27,000 × *g* for 15 min, resuspended in 3.0 ml of $DGVB^{2+}$, and then mixed with 0.5 ml of guinea pig serum. This mixture was then incubated for 1 hr at 37°. After incubation, the ghosts (GAC) were centrifuged at 27,000 × *g* for 15 min, washed once with isotonic NaCl, and resuspended in 1.0 ml of isotonic NaCl. The suspension was divided into two equal parts (0.5 ml each). After centrifugation at 27,000 × *g*, one part was resuspended in resealing buffer and incubated for 45 min at 37°. This incubation was followed by ultracentrifugation on 12% Ficoll to pellet isosmolar ghosts having complement lesions. The pellet was resuspended in isotonic NaCl and the ESR experiment was performed. The second part (0.5 ml) was centrifuged at 100,000 × *g* in the same manner as above without incubation in resealing buffer, and the ESR spectra were recorded. Antibody-sensitized spin-labeled ghosts reacted with heat-inactivated

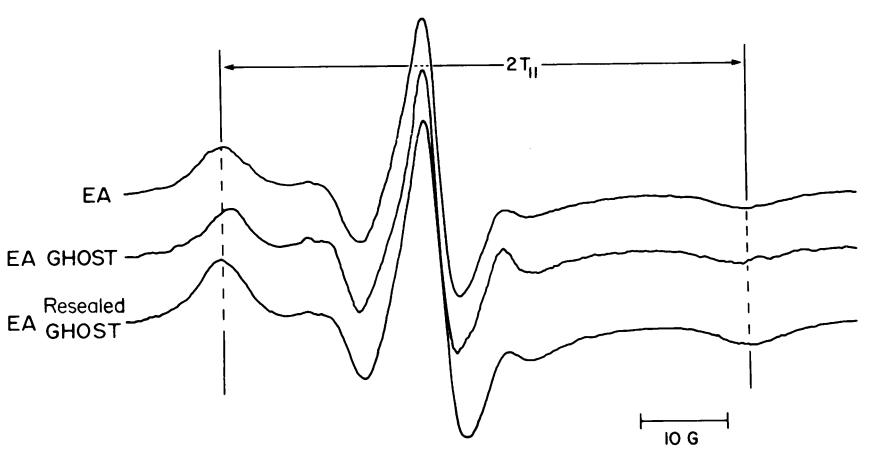

FIG. 1. ESR spectra of sheep erythrocytes spin labeled with 5-nitroxystearic acid. All spectra were measured at 22°.

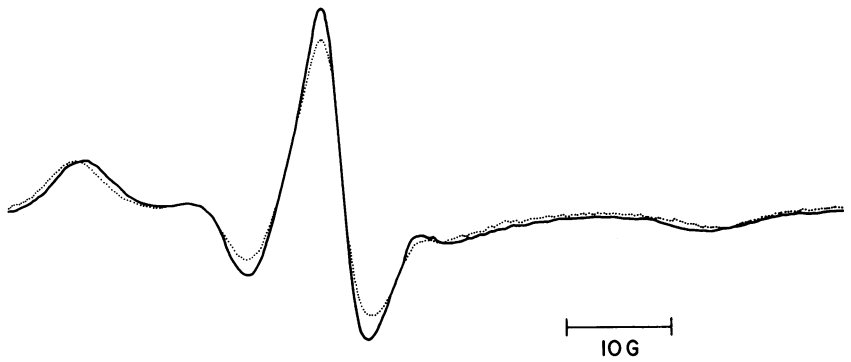

FIG. 2. ESR spectra of 5-nitroxystearic acid in EA resealed (solid curve) and EAC resealed (dotted curve). Spectra were recorded at 22°.

guinea pig serum (56°, 30 min) served as controls.

Reactive Lysis. Spin-labeled erythrocytes were washed once with $DGVB^{2+}$ and resuspended in 0.5 ml of $DGVB^{2+}$ at 1×10^9 cells per ml. The cells were incubated with 10 μl of C5b6 (500 units/ml) and 10 μl of C7 (100,000 units/ml) in a total volume of 0.5 ml for 20 min at 30°. The cells were washed once with 0.5 ml of $DGVB^{2+}$; then 1.0 ml of C-EDTA (1:200) as a source of C8 and C9 was added and the incubation was continued for 1 hr at 37°. The clear red solution was then centrifuged at 1570 × *g* to remove any unlysed cells. The supernatant was transferred to another centrifuge tube and the spin-labeled ghosts containing the complement proteins were pelleted by centrifugation at 27,000 × *g* for 15 min. Spin-labeled isosmolar ghosts, prepared in the manner described above, were incubated with C5b6 and C7 for 20 min at 30°. These ghosts, with C5b67 bound to their membranes, were incubated with either 300 μl of C8 (1000 units/ml) or the same amount of C8 and 700 μl of C9 (1000 units/ml) for 1 hr at 37°. After incubation, the membranes were spun down at 27,000 × *g* and ESR spectra were recorded.

ESR Measurements. All ESR spectra were recorded at 22° with 100 G field scans on a Varian E-Line 9.5 GHz ESR spectrometer with a variable temperature accessory. The changes in ESR parameters reported here do not reflect changes resulting from variations in temperature, power saturation, or magnetic field modulation. Intact cells and suspensions of membrane ghosts were placed in a 1.0-μm quartz capillary which was then placed in a standard Varian 9.5 GHz quartz tube, and the ESR spectra were recorded. The accuracy for the overall hyperfine splitting values given in the *Results* is about 0.5 G.

RESULTS

ESR Spectra. All the ESR spectra of sheep erythrocytes labeled with stearic acid spin labels show spectral characteristics indicating axially symmetric motion of the lipid alkyl chains. The anisotropic principal values of the hyperfine tensor $T_\parallel$ and $T_\perp$ can be obtained from the spectra. Nuclear hyperfine interactions generate the spectral parameters $2T_\parallel$ and $2T_\perp$, which may be used as a convenient measure of the fluidity of membranes (18, 19). The binding of antibody, C1, $C\overline{42}$, or C3 to E or EA membranes showed no change in $2T_\parallel$ as compared to E or EA.

Classical Pathway Lysis. Table 1 summarizes the results of using guinea pig serum as a complement source for the lysis of EA. The $2T_\parallel$ values for spin-labeled EA decrease as the covalently bound nitroxide free radical moves toward the center of the bilayer. This means that the amphipathic spin probe is oriented in the membrane with the polar carboxyl group at the lipid–water interface of the membrane surface and the hydrophobic alkyl chain extended parallel to the fatty acyl chains of the membrane phospholipids. When osmotic lesions are generated in the membrane by lysis of EA in hypotonic buffer (EA ghost), there is a decrease in the $2T_\parallel$ values at all positions down the alkyl chain bearing a paramagnetic center. This indicates an increase in the fluidity of the bilayer (Fig. 1). When the osmotic lesion in the hypotonically lysed EA ghost is resealed (EA resealed ghost) by incubating the membranes in resealing buffer, $2T_\parallel$ returns to the values characteristic of the EA membrane. A control experiment with heat-inactivated guinea pig serum showed no change in the ESR spectrum and no hemolysis. When EA were lysed by incubating with normal guinea pig serum (EAC ghost), the fluidity of the EAC ghost,

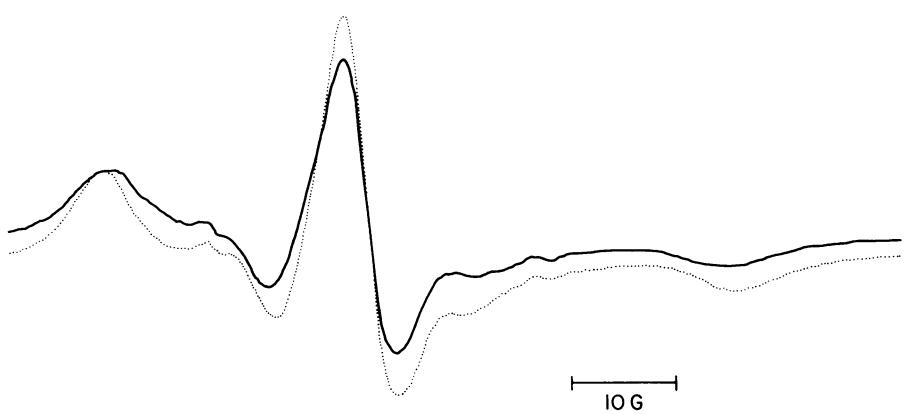

FIG. 3. ESR spectra of 5-nitroxystearic acid in GA (solid line) and GA treated with complement (dotted line). Spectra were recorded at 22°.

as measured by $2T_{\parallel}$, is comparable to that of the hypotonically lysed EA. When these EAC ghosts are incubated in resealing buffer, there is a significant decrease in membrane fluidity around the spin-label site, for $2T_{\parallel}$ increases to values greater than those obtained for resealed ghosts of EA by approximately 1.0 G at all positions down the fatty acid chain (Fig. 2). These experiments were repeated with 5-nitroxylmethylstearate with similar results. This indicates that localization of spin-labeled fatty acids in the membrane after interaction of the complement proteins does not occur due to charge effects of the carboxyl group.

Isosmolar Ghosts with Guinea Pig Serum. Isosmolar ghosts were used to study complement-induced changes in $2T_{\parallel}$ in the absence of osmotic lesions since these ghosts do not undergo colloid osmotic lysis. Table 2 shows that $2T_{\parallel}$ values for antibody-sensitized isosmolar ghosts (GA) are comparable to those for spin-labeled EA. In a control experiment, GA treated with heat-inactivated guinea pig serum showed no change in $2T_{\parallel}$ values from GA. When normal guinea pig serum is incubated with GA for 1 hr at 37°, there is a significant increase in $2T_{\parallel}$ of the GAC, especially at the 12-carbon atom of the fatty acid chain (Fig. 3). After GAC was incubated in resealing buffer (GAC-resealed), there was no change in the $2T_{\parallel}$ values as compared to GAC. Thus, Mg^{2+} was not interacting with the membrane to change the fluidity of the lipid bilayer.

Reactive Lysis. Table 3 summarizes the results of experiments using reactive lysis in which only the C5b–9 proteins are bound to the E membrane. The $2T_{\parallel}$ values obtained for E bearing C5b67 are comparable to those found for EA or GA. When EC5b67 were lysed by incubating with C-EDTA and then resealed in resealing buffer, there was an increase in the $2T_{\parallel}$ values at all positions down the fatty acid chains. This increase was particularly dramatic at the 12-carbon atom showing an increase of 4.2 G. When C8 and C9 are added to EC5b67 and the membranes resealed, there is an increase in $2T_{\parallel}$ values comparable to those found in the experiment with whole complement. Addition of C8 alone to C5b67 causes a partial increase in $2T_{\parallel}$ values that reaches its maximum when C9 is added.

DISCUSSION

We conclude from the data presented here that the binding of the terminal complex of complement proteins has a pronounced effect on the ESR spectra of fatty acid spin labels intercalated into sheep erythrocyte membranes. The resulting line shapes consistently show larger $2T_{\parallel}$ values, indicating that complement protein–lipid interaction decreases the fluidity of the membranes. This effect is first observed when C8 binds to membranes bearing C5b67, and it is enhanced upon the subsequent binding of C9.

The binding of the terminal complex of complement proteins damages the cell membrane, and colloid osmotic lysis ensues.

Table 3. Complement protein interaction with cell membranes: Reactive lysis*

	5-NSA	12-NSA	16-NSA
EC5b67	59.3	53.3	36.4
EC5b67+ C-EDTA (resealed)	60.9	57.5	38.1
EC5b67+ C8,9-EDTA (resealed)	61.0	57.6	37.9
GC5b67 + C8-EDTA	59.7	54.4	36.9
GC5b67 + C8,C9-EDTA	60.9	57.5	37.7

Sheep erythrocytes (E) and isosmolar ghosts (G) were labeled with stearic acid spin labels, and the intermediates were prepared.
* Hyperfine interaction ($2T_{\parallel}$) in gauss at 22°.

Osmotic lesions form in the membrane, where their presence appears to increase the fluidity of the membrane around the spin-label site. These results are comparable to other studies which demonstrate fluidization of the lipid bilayer by complement (8). This fluidization suggests that there are areas of the membrane in which normal, cooperative protein–lipid and lipid–lipid interactions have been altered to give a highly disordered state in the bilayer. Significantly, the decrease in fluidity is observed only when the membranes are resealed or when complement proteins react with isosmolar ghosts, which do not undergo osmotic lysis.

Although no definitive statement can be made at this time, it is intriguing to speculate as to the cause of the decrease in membrane fluidity brought about by the presence of proteins of the terminal complex. The ESR spectra of the membrane-bound fatty acid spin labels suggest that the proteins of the terminal complex penetrate the membrane and interact with hydrophobic regions of the lipid bilayer. This complement protein–lipid interaction causes a reduction in the flexibility of lipid alkyl chains in close proximity to the proteins. The fact that we observe a motionally restricted homogeneous spectrum implies that the complement proteins alter the cooperative lipid–lipid interactions over a distance greater than that occupied by lipids that are in close association with the complement proteins (20). Preliminary studies in which the concentration of complement proteins has been varied support this hypothesis, for we have observed a reduction in the bulk fluidity of the membrane at concentrations of complement where there are only a few complement lesions per cell. Interestingly, it has been shown that the binding to human erythrocytes of as few as one to three molecules of prostaglandin E_2 per cell increases the order parameter of 5-nitroxypalmitate by as much as 1% (21). Of further interest are recent spin-label studies showing that many membrane proteins that penetrate deeply into or completely through the bilayer are solvated by an immobilized layer of lipid (22–27). In other pertinent studies it has been observed that melittin, an oligopeptide toxin from bee venom, interacts with liposomes to reduce the fluidity of the bilayer and to increase ion permeability (28, 29). It has also been reported that gramicidin A interacts with black lipid membranes to form a polar channel spanning the liquid hydrocarbon interior of the membrane as a conducting dimer in equilibrium with nonconducting monomers (30).

The loss of membrane phospholipids into the fluid phase after membrane damage by complement proteins (31–33) is another potential mechanism by which complement could cause a decrease in the fluidity of the membrane since a loss of phospholipid would result in an increase in the cholesterol-to-phospholipid ratio. It is well documented that increasing the cholesterol concentration in a phospholipid bilayer can reduce the flexibility of the lipid alkyl chains (34).

The precise mechanism by which the terminal complex of complement proteins brings about a decrease in the fluidity of sheep erythrocyte membranes remains unknown, but the results presented here clearly demonstrate the presence of a population of lipids whose motion becomes more restricted when the proteins bind to the cell membrane.

We thank Sam Wright and Alex Law for protein purification and K. N. Conan Li for helpful discussions. C.E.D. thanks the Department of Chemistry, Harvard University, for use of a Varian ESR spectrometer purchased on a grant from the National Science Foundation. This research was supported by grants from the Camille and Henry Dreyfus Foundation and the National Science Foundation Program in Human Cell Biology (PCB-77-10206).

1. Müller-Eberhard, H. J. (1975) *Annu. Rev. Biochem.* **44,** 697–724.
2. Kolb, W. P., Haxby, J. A., Arroyave, C. M. & Müller-Eberhard, H. J. (1972) *J. Exp. Med.* **135,** 549–566.
3. Stolfi, R. L. (1968) *J. Immunol.* **100,** 46–54.
4. Tamura, N., Shimada, A. & Chang, S. (1972) *Immunology* **22,** 131–140.
5. Hadding, U. & Müller-Eberhard, H. J. (1969) *Immunology* **16,** 719–735.
6. Hammer, C. H., Nicholson, A. & Mayer, M. M. (1975) *Proc. Natl. Acad. Sci. USA* **73,** 5076–5080.
7. Michaels, D. W., Abramovitz, A. S., Hammer, C. H. & Mayer, M. M. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 2852–2856.
8. Nakamura, M., Ohnishi, S., Kitamura, H. & Inai, S. (1976) *Biochemistry* **15,** 4838–4843.
9. Mason, R. P., Giavedoni, E. B. & Dalmasso, A. P. (1977) *Biochemistry* **16,** 1196–1201.
10. Dahl, C. E. & Levine, R. P. (1978) *J. Immunol.* **120,** 1770.
11. Nelson, R. A., Jensen, J., Gigli, I. & Tamura, N. (1966) *Immunochemistry* **3,** 111–135.
12. Baker, P. J., Rubin, L. G., Lint, T. F., McLeod, B. C. & Gewurz, H. (1975) *Clin. Exp. Immunol.* **20,** 113–121.
13. Podack, E. R., Kolb, W. P. & Müller-Eberhard, H. J. (1976) *J. Immunol.* **116,** 263–269.
14. Borsos, T. & Rapp, H. L. (1967) *J. Immunol.* **99,** 263–268.
15. Fearon, D. T., Austin, K. F. & Ruddy, S. (1973) *J. Exp. Med.* **138,** 1305.
16. Li, C. K. N. (1978) Dissertation (Harvard University, Cambridge, MA).
17. Steck, T. (1974) in *Methods in Membrane Biology,* ed. Korn, E. D. (Plenum, New York), Vol. 2, pp. 245–281.
18. Hubbel, W. L. & McConnell, H. (1971) *J. Am. Chem. Soc.* **93,** 314–326.
19. Ohnishi, S. (1975) *Adv. Biophys.* **8,** 35–82.
20. Longmuir, K. S., Capaldi, R. & Dahlquist, F. (1977) *Biochemistry* **16,** 5746–5755.
21. Kury, P. G., Ramwell, P. W. & McConnell, H. M. (1973) *Biochem. Biophys. Res. Commun.* **56,** 478–483.
22. Jost, P. C., Griffith, O. H., Capaldi, R. A. & Vanderkooi, G. (1973) *Proc. Natl. Acad. Sci. USA* **70,** 480–484.
23. Warren, G. B., Toon, P. A., Birdsall, N. J. M., Lee, A. G. & Metcalfe, J. C. (1974) *Proc. Natl. Acad. Sci. USA* **71,** 622–626.
24. Stier, A. & Sackmann, E. (1973) *Biochim. Biophys. Acta* **311,** 400–408.
25. Trauble, H. & Overath, P. (1973) *Biochim. Biophys. Acta* **308,** 491–512.
26. Nakamura, M. & Ohnishi, S. (1975) *J. Biochem. (Tokyo)* **78,** 1039–1945.
27. Brisson, E. G., Scandella, C. J., Bienvenue, A., Devaux, P. F., Cohen, J. B. & Changeux, J. P. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 1087–1091.
28. Verma, S. P., Wallach, D. F. H. & Smith, I. C. P. (1974) *Biochim. Biophys. Acta* **345,** 129–140.
29. Williams, J. C. & Bell, R. M. (1972) *Biochim. Biophys. Acta* **288,** 255–262.
30. Veatch, W. R., Mathies, R., Eisenberg, M. & Styer, L. (1975) *Biophys. J.* **15,** 305a.
31. Giavedoni, E. B. & Dalmasso, A. P. (1976) *J. Immunol.* **116,** 1163–1169.
32. Inoue, K., Kinoshita, T., Akiyama, Y., Okada, M. & Amano, T. (1976) *J. Immunol.* **116,** 1737.
33. Shin, M. L., Paznekas, W. A., Abramovitz, A. S. & Mayer, M. M. (1977) *J. Immunol.* **119,** 1358–1364.
34. Oldfield, E. & Chapman, D. (1972) *FEBS Lett.* **23,** 285–297.