J. Membrane Biol. 84, 249–258 (1985)

The Journal of
Membrane Biology
© Springer-Verlag 1985

Cyanine Dye Fluorescence Used to Measure Membrane Potential Changes due to the Assembly of Complement Proteins C5b-9

Therese Wiedmer and Peter J. Sims
Immunohematology Laboratory, Department of Pathology,
University of Virginia School of Medicine, Charlottesville, Virginia 22908

Summary. The fluorescent potentiometric indicator diS-C_3-(5) has been used to investigate changes in membrane potential due to assembly of the C5b-9 membrane attack complex of the complement system. EAC1-7 human red blood cells and resealed erythrocyte ghosts—bearing membrane-assembled C5b67 complexes—were generated by immune activation in C8-deficient human serum. Studies performed with these cellular intermediates revealed that the membrane potential of EAC1-7 red cells and ghosts is unchanged from control red cells (−7 mV) and ghosts (0 mV), respectively. Addition of complement proteins C8 and C9 to EAC1-7 red cells results in a dose-dependent depolarization of membrane potential which precedes hemolysis. This prelytic depolarization of membrane potential—and the consequent onset of hemolysis—is accelerated by raising external [K^+], suggesting that the diffusional equilibration of transmembrane cation gradients is rate limiting to the cytolytic event. In the case of EAC1-7 resealed ghosts suspended at either high external [K^+] or [Na^+], no change in membrane potential (from 0 mV) could be detected after C8/C9 additions. When the membrane potential of the EAC1-7 ghost was displaced from 0 mV by selectively increasing the K^+ conductance with valinomycin, a dose-dependent depolarization of the membrane was observed upon addition of C8 and C9. In these experiments, lytic breakdown of the ghost membranes was <5%. Conclusions derived from this study include: (i) measured prelytic depolarization of the red cell Donnan potential directly confirms the colloid-osmotic theory of immune cytolysis. (ii) The diffusional transmembrane equilibration of Na^+ and K^+ through the C5b-9 pore results in a dose-dependent depolarization of the membrane potential (E_m) which appears to be rate-limiting to cytolytic rupture of the target erythrocyte. (iii) Enhanced immune hemolysis observed in high K^+ media cannot be attributed to cation-selective conductance across the C5b-9 pore, and is probably related to the near-equilibrium condition of potassium-containing red cells when suspended at high external K^+. These experiments demonstrate that carbocyanine dye fluorescent indicators can be used to monitor electrochemical changes arising from immune damage to the plasma membrane under both cytolytic and noncytolytic conditions. Potential application of this method to the detection of sublytic pathophysiological changes in the plasma membrane of complement-damaged cells are discussed.

Key Words complement · membrane potential · membrane pore · erythrocyte · carbocyanine dye · fluorescence

Introduction

The cytolytic activity of human serum resides in the C5b-9 membrane attack complex composed of complement[1] proteins C5b, C6, C7, C8 and C9 (reviewed by Esser, 1981; Bhakdi & Tranum-Jensen, 1983; Muller-Eberhard, 1984). The assembly of this complex from its five component glycoproteins initiates hydrophilic-to-hydrophobic conformational transitions within the proteins, which results in their insertion into membrane lipid with a concomitant increase in membrane permeability to aqueous solute. By undermining the solute barrier function of the plasma membrane, the C5b-9 proteins initiate the collapse of ionic gradients which can ultimately lead to cell death (Green & Goldberg, 1960; Mayer, 1972).

The detection and analysis of functional membrane damage due to the insertion of the C5b-9 proteins has for the most part derived from measurements made on the release from the intracellular space of normally impermeant aqueous marker solute. Most commonly, these measurements have been performed with red blood cell target membranes, relying upon the detection of hemoglobin release as a quantitative indicator of membrane damage due to C5b-9 binding (Mayer, 1961). In the case of target cells other than erythrocytes (including liposomal and vesicle membranes) a variety of other impermeant membrane-encapsulated radioactive, fluorescent, spin-labeled, or chromogenic marker solutes have been employed for these measurements (e.g., Vistnes, 1984, and references therein).

[1] C, Complement proteins are named in accordance with recommendations of *Bull. World Health Org.* **39**:935 (1968). EAC1-7 refers to antibody-sensitized erythrocyte membranes treated with complement proteins C1 to C7.

Although the efflux of intracellularly trapped marker solute has generally been interpreted to indicate the functional state of the C5b-9 membrane pore *per se,* accumulated evidence pertaining to both the membranolytic and pore-forming properties of these proteins suggest that these measurements may fail to detect functional changes in the target membrane that are directly related to the insertion of the C5b-9 proteins, and that can have direct consequence for normal cellular homeostasis. For example, it is now widely recognized that the membrane 'pores' formed by these proteins are functionally heterogeneous, with apparent exclusion radii ranging from <4 Å to >40 Å, depending not only upon the multiplicity of C5b-9 binding and the molecular stoichiometry of these protein complexes, but also upon the composition and physical properties of the target membrane (Mayer, 1981; Sims, 1981). Furthermore, there is now increasing evidence to suggest that initial changes in the electrochemical state of the cell upon C5b-9 binding, including changes in the transmembrane electrochemical potential and the levels of intracellular Ca^{++} and ATP, may significantly determine the ultimate extent of plasma membrane damage and its consequence for normal cellular function and survival (Esser, 1980; Campbell et al., 1981; Hallet & Campbell, 1984; Tirosh & Berke, 1984). In this context, changes in plasma membrane integrity arising secondarily to the colloid-osmotic influx of water (Lauf, 1975; 1978), or due to the release of free fatty acids by activated endogenous phospholipases (Imagawa et al., 1983) are each likely to contribute to the apparent 'pore properties' of the C5b-9 membrane complex as detected solely by the efflux of trapped marker solute.

These considerations suggest that the detection and analysis of pathophysiological changes in the immune-damaged plasma membrane requires the application of methods that can dynamically resolve sublytic changes in normal plasma membrane function which may arise directly due to the membrane-bound C5b-9 proteins. Accordingly, we have employed the fluorescent potentiometric indicator diS-C_3-(5)[2] (Hoffman & Laris, 1974; Sims et al., 1974) to monitor changes in electrochemical gradients maintained across erythrocyte membranes during assembly of the C5b-9 proteins under both lytic and noncytolytic conditions. In addition to providing insight into the electrochemical changes that arise due to the effects of these proteins on normal membrane ion conductance, our results suggest that this simple and sensitive method may have wide application to the detection of sublytic functional changes in the plasma membrane of a variety of cells exposed to these activated serum immunoproteins.

[2] *Abbreviations:* DiS-C_3-(5), 3,3'-dipropylthiodicarbocyanine iodide; MOPS, 3-[N-morpholino] propanesulfonic acid; DIDS, 4,4'-Diisothiocyano-2,2'disulfonic acid stilbene; C8D, human serum deficient in complement protein C8; ghosts, human erythrocyte resealed ghost membranes.

Materials and Methods

Materials

DiS-C_3-(5) was obtained from Molecular Probes (Junction City, Ore.). Valinomycin and albumin (bovine, fatty-acid-free) were from Sigma (St. Louis, Mo.). $Na^{125}I$ was obtained in dilute NaOH solution from Amersham (Chicago, Ill.). All chemicals were of analytical grade, and solvents of spectroscopic grade.

Solutions

All solutions were freshly prepared using H_2O obtained by reverse osmosis and ultrafiltration (Millipore, Boston, Mass.). Unless indicated otherwise, all solutions contained 0.02% (wt/vol) sodium azide. KCl-MOPS: 153 mM KCl, 10 mM MOPS, pH 7.4. NaCl-MOPS: 153 mM NaCl, 10 mM MOPS, pH 7.4. Wash Buffer A: NaCl-MOPS made 0.25% (wt/vol) in albumin. Wash Buffer B: 102 mM KCl, 51 mM NaCl, 10 mM MOPS, pH 7.4 made 0.25% (wt/vol) in albumin.

Complement Proteins

Human complement proteins C8 and C9 were purified and assayed for functional (hemolytic) activity according to published methods (Sims, 1983; 1984). To ensure removal of trace contaminating amounts of IgG or C9, purified C8 was subjected to immunoabsorption against monospecific anti-C9 coupled to Affigel 10 (*see below*) and staph protein A-sepharose-CL-6B (Pharmacia, Piscataway, N.J.). Likewise, purified C9 was subject to absorption against solid phase anti-C8 and staph protein A.

Immunochemical Procedures

Monospecific antisera against human complement proteins C8 and C9 were raised in goats as previously described (Sims, 1984). The IgG fractions were prepared by successive precipitation with octanoic acid and ammonium sulfate (Steinbuch & Audran, 1969). Freshly drawn human serum was immunochemically depleted of complement protein C8 by multiple absorption against anti-C8 (above) covalently coupled to Affigel 10 (Bio Rad). The C8 deficient serum (C8D) was concentrated to its original volume and repleted with Clq (40 μg/ml), $CaCl_2$ (0.15 mM), and $MgCl_2$ (0.5 mM). When tested against antibody-sensitized sheep red blood cells, this serum was devoid of hemolytic activity. For use in preparing EAC1-7 erythrocytes or resealed ghosts (*see below*) the serum was dialyzed against either NaCl-MOPS (erythrocytes) or Wash Buffer B (ghosts) supplemented with 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$.

Preparation of Normal Human Red Blood Cells

Blood was drawn into heparinized vacutainers from healthy adult volunteers, the red cells washed five times with 0.9% (wt/vol) NaCl and suspended at 2×10^9 cells/ml. This red cell suspension was immediately used to prepare resealed ghost membranes and EAC1-7 cells (below), for experiments performed the same day.

Resealed Erythrocyte Ghosts

Resealed ghost membranes of human erythrocytes (ghosts) were prepared by reversible hypotonic lysis at 0°C using a modification of published procedures (Sims & Lauf, 1978; Sims & Wiedmer, 1984). Lysis of 1 vol red cell suspension (2×10^9/ml) was performed at pH 6.0 to 6.1 in 10 vol 4 mM $MgSO_4$, 2 mM ATP. Isotonicity was restored to 102 mM KCl, 51 mM NaCl, 10 mM MOPS, pH 7.4, by addition of 1 vol of the concentrated salts. Following resealing (60 min at 37°C) the ghosts were washed into Wash Buffer B and suspended to 2×10^9 cells/ml. For certain experiments, trace quantities of ^{125}I-albumin were incorporated into the ghost water prior to resealing, according to the methods of Funder and Wieth (1976).

EAC1-7 Erythrocytes and Resealed Ghosts

The C5b67 complex was assembled on human erythrocytes and resealed ghosts by immune activation in C8D. One ml of erythrocytes or resealed ghosts (2×10^9 cells) was mixed with 5 ml of C8D to which was added rabbit antibody against human red blood cell stroma (IgG fraction, Cappel Laboratories, West Chester, Pa.). The final dilution of the antibody was 1/80. After successive incubations of 5 min at 4°C and 30 min at 37°C, the resulting EAC1-7 erythrocytes (EAC1-7 cells) or resealed ghosts (EAC1-7 ghosts) were collected by centrifugation. After washing and suspension to 1×10^9/ml in ice-cold Wash Buffer A or Wash Buffer B, respectively, the EAC1-7 cells and EAC1-7 ghosts were kept on ice for use the same day.

Radiolabeling

Bovine serum albumin was radiolabeled with Na^{125}I using Iodo-Gen (Pierce Chemical Co., Rockford, Ill.) One hundred μg of albumin were incubated with 0.5 mCi of Na^{125}I in a test tube coated with 10 μg of Iodo-Gen for 15 min at 4°C. Unreacted radiolabel was removed by gel filtration on Sephadex G-25 (PD-10 column, Pharmacia) and subsequent dialysis. A specific activity of 3.7×10^6 cpm/μg protein was achieved.

Fluorescence Spectroscopy

All fluorescence measurements were made in 1 cm quartz cuvettes using an SLM 4800S spectrofluorometer equipped for sample stirring and temperature control as described fully in Sims (1984). Excitation was always at 622 nm (4-nm slits) and emission at 670 nm (8-nm slits). To minimize the contribution of light scattering, polarizers were configured vertically (excitation) and horizontally (emission). To reduce photobleaching, shutters were closed except during data acquisition. Provision was made for additions to the stirred sample by injection (Hamilton syringe) through a light-tight port above the sample compartment.

Estimation of Membrane Potential

The membrane potential of EAC1-7 cells was calibrated to diS-C_3-(5) fluorescence by the methods of Hoffman and Laris (1974). Calculations were performed for 30°C on the basis of cell $[K^+]$ = 126 mM, cell $[Cl^-]$ = 117 mM, and a permeability ratio of P_K/P_{Cl} = 18 in the presence of 1.25 μM valinomycin (estimated from data of Hunter, 1977; Knauf et al., 1977).

Assays

Hemoglobin concentrations were determined spectrophotometrically at 415 or 542 nm. Cell concentrations were determined by resistive counting (Coulter ZBI) or from measured hemoglobin, utilizing a mean corpuscular volume of 94.1 fl and cell water of 969 kg/kg ghosts (Funder & Weith, 1976). Gamma counting was performed in a Beckman Gamma 4000.

Results

The interaction of complement proteins C8 and C9 with membrane-bound C5b67 is known to initiate a rapid increase in transmembrane ion flux which can ultimately result in the cytolytic rupture of the plasma membrane (Green & Goldberg, 1960; Hingson et al., 1969; Hallett & Campbell, 1984). In order to determine how this rapidly evolving prelytic change in ion permeability alters the electrochemical steady state of the target erythrocyte, we used the fluorescent lipophilic cation diS-C_3-(5) to continuously monitor relative changes in membrane potential that occur upon the binding of C8 and C9 to the membrane C5b67 complex. As first described by Sims et al. (1974), the fluorescence emission of this membrane-permeant carbocyanine dye changes with its electrochemical field-dependent distribution across the membrane, allowing real-time optical monitoring of the membrane potential of a variety of cells and organelles maintained in suspension. In order to facilitate the detection of prelytic changes in the membrane potential which occur directly upon the membrane insertion of C8 and C9, and to permit fluorescence measurement in a serum-free system, C5b67 complexes were preassembled on target red cells by immune activation in C8-deficient serum, and the cytolytic C5b-9 membrane lesion then generated by the addition of purified C8 and C9 to the washed EAC1-7 cells equilibrated with dye in a measuring cuvette.

Characterization of the Electrochemical Steady State of the EAC1-7 Erythrocyte

In Fig. 1 are shown typical results obtained for EAC1-7 erythrocytes (in the absence of added C8/C9) equilibrated with diS-C_3-(5) at 30°C. In these

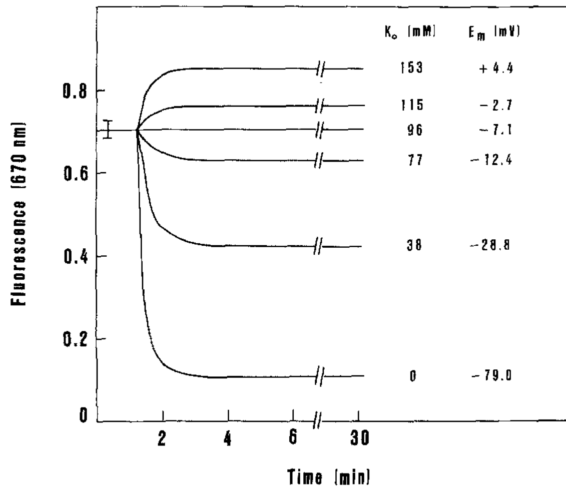

Fig. 1. Electrochemical steady state of EAC1-7 cells. EAC1-7 cells (2.5×10^7) were suspended with diS-C_3-(5) (4.7×10^{-7}M) in a total volume of 2 ml KCl-MOPS, NaCl-MOPS or mixtures thereof to achieve external [K_o^+] indicated. After 5-min equilibration in the dark at 30°C, fluorescence trace was started. Error bar shows range of fluorescence intensity measured after equilibration of dye. At t = 80 sec, valinomycin (val; 0.33 mg/ml ETOH) was added to a final concentration of 1.25 μM. Membrane potential (E_m) was estimated for indicated [K_o^+] as described in Materials and Methods. On the basis of [K_o^+] at the apparent reversal potential (K_o^+ = 96 mM) the membrane potential of the EAC1-7 cells was determined to be −7 mV

experiments, the potassium-selective ionophore valinomycin was employed to shift the membrane potential towards the potassium equilibrium potential (E_K) for suspension of the cells at various external [KCl]. As shown by the figure, in the absence of added valinomycin, EAC1-7 cells equilibrated to the same level of diS-C_3-(5) fluorescence, irrespective of the external [KCl]. Upon addition of the ionophore, the external [KCl]-dependent dye response of these cells was similar to that obtained for control erythrocytes suspended under equivalent conditions, with the level of fluorescence attained in response to valinomycin remaining constant during 30-min measurement (*cf.* Fig. 1, Sims et al., 1974). The concentration of external KCl at the equilibrium reversal potential for the EAC1-7 cells was 96 mM (range 90 to 97 mM). These results demonstrate that EAC1-7 cells maintain a near-normal distribution of ions across the membrane, with the membrane potential governed (in the absence of ionophore) by the Donnan distribution of chloride (E_m = −7 mV). Using an assumed ratio of the valinomycin-induced potassium to chloride permeabilities of P_K/P_{Cl} = 18 the level of dye fluorescence measured for EAC1-7 cells in the presence of ionophore and various external [KCl] was calibrated to the calculated membrane potential as described in Materials and Methods. It should be emphasized, however, that in the absence of measured ion permeabilities for these cells in the presence of valinomycin, the absolute values we assign to the membrane potential at various external [KCl] are only approximate.

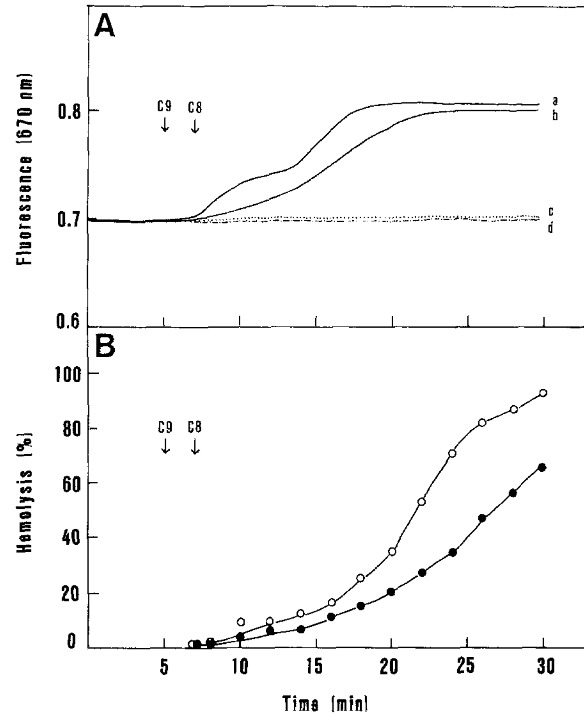

Fig. 2. Effect of C5b-9 assembly on the membrane potential of red blood cells. Panel (*A*): EAC1-7 cells (2.5×10^7) were suspended with diS-C_3-(5) (4.7×10^{-7}M) in a total volume of 2 ml KCl-MOPS (traces *a,c*) or NaCl-MOPS (traces *b,d*). After 5-min equilibration in the dark at 30°C, fluorescence trace was started. At times indicated by arrows, C9 (265 ng) and C8 (53 ng) were delivered to the cuvette. No additions were made to controls (traces *c,d*). Note expanded ordinate scale. Panel (*B*): Reaction mixtures identical to those in (*A*) were incubated at 30°C. At the times indicated, aliquots were removed, layered onto a 200 μl phthalate cushion (1 vol bis(2-ethylhexyl): 2 vol dibutyl-) and spun immediately (Eppendorf Microfuge Model 5414). Cell hemolysis was derived on the basis of supernatant hemoglobin (●–●) NaCl-MOPS; (○–○) KCl-MOPS

Membrane Potential Changes Initiated by the Binding of C8 and C9 to EAC1-7 Erythrocytes

In Fig. 2 are shown the fluorescence traces obtained for EAC1-7 cells exposed to C8 and C9 during suspension at 30°C. The cells were first equilibrated with dye and excess C9, and the C5b-9 lesion initiated by rapid injection into the cuvette of 53 ng C8. The addition of C8 resulted in an increase in fluores-

cence to a level approximately corresponding to the 0 mV potential, as derived for EAC1-7 by methods described above (*see* Fig. 1). This apparent C8/C9-dependent depolarization of the EAC1-7 membrane potential preceded the hemolysis of the cells, as would be anticipated for a cytolytic colloid osmotic expansion of cell water under Donnan equilibrium conditions. No change in diS-C_3-(5) fluorescence was observed when control erythrocytes were exposed to C8 and C9 under identical conditions, confirming the specificity of the fluorescence change observed with EAC1-7 for the assembly of the membrane-bound C5b-9 complex *per se*.

When exposed to identical concentrations of C8 and C9, the onset of the fluorescence response of EAC1-7, which presumably signals depolarization of the membrane potential to the 0 mV level, occurred substantially faster when the cells were suspended in KCl-MOPS versus NaCl-MOPS, coinciding with the increased complement-mediated hemolysis that is also observed for these high potassium-containing erythrocytes when suspended in KCl versus NaCl (Fig. 2, and Dalmasso et al., 1975; Sims & Wiedmer, 1984). A close inspection of the traces of Fig. 2 suggest that the increase in dye fluorescence measured after C8/C9 binding follows a multiphasic time-course, which is most evident for experiments performed in KCl-MOPS (*see also* Fig. 3, below). These data suggest that the membrane potential of these cells after C8/C9 binding may be directly affected by E_K or E_{Na} (as well as the Donnan chloride potential), which rapidly depolarize to 0 mV. Alternatively, this apparent multiphasic dye response may be due to changes in the emission of the fluorophore that arise secondarily, for example, as a consequence of the concomitant increase in cell volume or the lytic release of cell hemoglobin (*see below* and Discussion).

Since the onset of the terminal membrane-depolarizing (and cytolytic) stage of C5b-9 lysis was observed to be influenced by the initial cation gradient maintained across the target membrane, it was of particular interest to monitor the effect of the C5b-9 proteins on the membrane potential of these cells in the circumstance that the pre-existing membrane potential is dominated by a cation (rather than the chloride) conductance. Accordingly, EAC1-7 cells were suspended in 153 mM [K^+] or [Na^+] and treated with valinomycin, prior to the addition of C8 and C9 (Fig. 3). As shown by the data of the figure, the addition of C8 to the valinomycin-treated EAC1-7 cells (equilibrated with C9) initiates a decrease in fluorescence towards the level of the untreated EAC1-7 cell, suggesting an initial collapse of the K^+-electrochemical potential (*cf.* Fig. 1). Consequent to this apparent collapse of the K^+ gradient, a secondary increase in fluorescence can be discerned, analogous to the response observed for these cells in the absence of valinomycin (*cf.* trace *a*, Fig. 2). These results suggest that the initial change in the membrane potential of these cells occurs by the collapse of the transmembrane gradient of potassium (which in the presence of a valinomycin-induced conductance dominates the membrane potential). Consequent to this collapse of the cation gradient, a terminal depolarizing (and cytolytic) collapse of the chloride (Donnan) potential occurs.

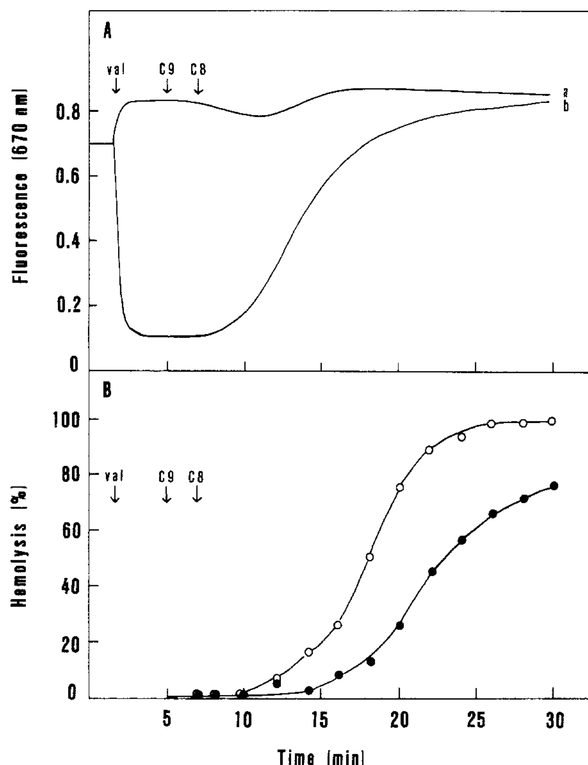

Fig. 3. Effect of C5b-9 assembly on the membrane potential of red blood cells in the presence of valinomycin. Panel (*A*): EAC1-7 cells (2.5 × 10^7) were suspended with diS-C_3-(5) (4.7 × 10^{-7}M) in a total volume of 2 ml KCl-MOPS (trace *a*) or NaCl-MOPS (trace *b*). After 5-min equilibration in the dark at 30°C, fluorescence trace was started. At times indicated by arrows, valinomycin (1.25 × 10^{-6}M), C9 (265 ng) and C8 (53 ng) were injected. Panel (*B*): Reaction mixtures identical to those in (*A*) were incubated at 30°C. At times indicated, aliquots were removed and cell hemolysis was determined as described for Fig. 2. (●–●) NaCl-MOPS; (○–○) KCl-MOPS

As shown by the data of Fig. 4, the rate of change in dye fluorescence was directly proportional to the amount of C8 (in the presence of excess C9) added to the EAC1-7 cells. These results demonstrate that under the range of conditions employed in these experiments, the time-course of the fluorescence response we observe after C8/C9 addition reflects the rate of change of the membrane

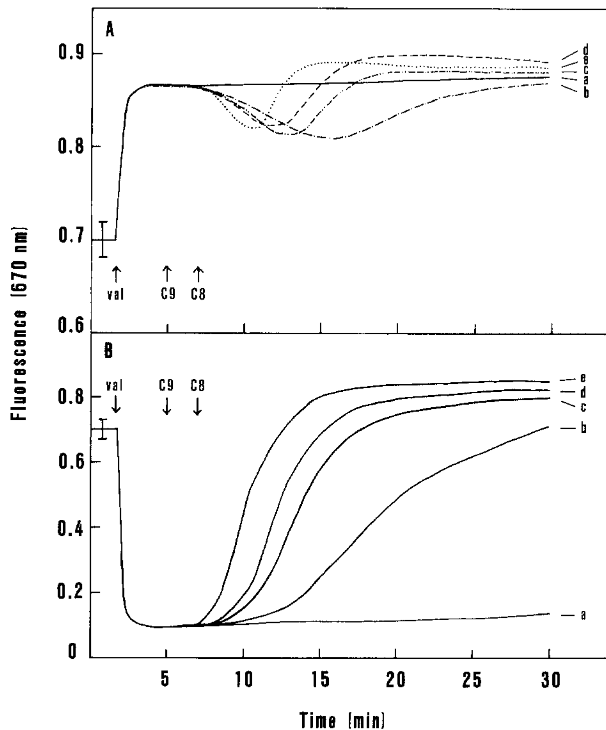

Fig. 4. Dose-dependence of the membrane potential change of C5b-9-treated red blood cells. EAC1-7 cells (2.5×10^7) were suspended with diS-C_3-(5) (4.7×10^{-7}M) in a total volume of 2 ml KCl-MOPS (*A*) or NaCl-MOPS (*B*). After 5-min equilibration in the dark at 30°C, fluorescence trace was started. Error bar shows range of fluorescence intensity after equilibration of dye. At times indicated by arrows, valinomycin (1.25×10^{-6}M), C9 (265 ng) and the following amounts of C8 were injected: 0 ng (trace *a*), 5.3 ng (trace *b*), 53 ng (trace *c*), 106 ng (trace *d*) and 212 ng (trace *e*). Note expanded ordinate scale in upper panel

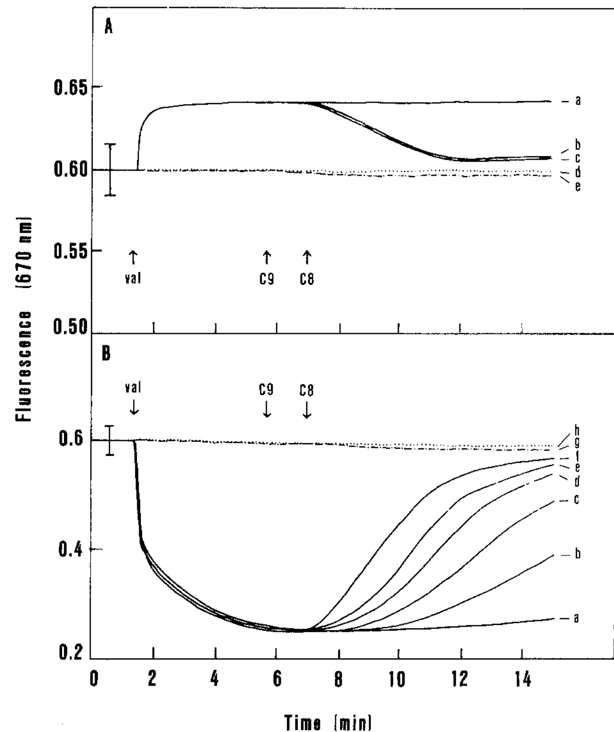

Fig. 5. Effect of C5b-9 assembly on the membrane potential of resealed erythrocyte ghosts. EAC1-7 ghosts (5×10^7) were suspended with diS-C_3-(5) (2.3×10^{-7}M) in a total volume of 2 ml KCl-MOPS (*A*) or NaCl-MOPS (*B*). After 5-min equilibration in the dark at 30°C, fluorescence trace was started. Error bar shows range of fluorescence intensities measured after equilibration of dye. Panel (*A*): At times indicated by arrows, valinomycin (1.25×10^{-6}M, to solid traces *a-c* only), C9 (265 ng) and the following amounts of C8 were injected: 0 ng (traces *a,d*), 106 ng (traces *b,e*) and 212 ng (trace *c*). Note expanded ordinate scale. Panel (*B*): At times indicated by arrows, valinomycin (1.25×10^{-6}M, solid traces *a-f* only), C9 (265 ng) and following amounts of C8 were injected: 0 ng (traces *a,h*), 5.3 ng (trace *b*), 26.5 ng (trace *c*), 53 ng (trace *d*) 106 ng (traces *e,g*) and 212 ng (trace *f*)

potential of the target cells *per se*, and is not limited by the response time of the fluorophore. These data therefore suggest that the depolarization of the membrane potential occurs through the dissipation of ion gradients after assembly of the C5b-9 pore, rather than from a *selective* change in the ion conductance of the membrane (an electrogenic change in membrane conductance expected to be immediately reflected by E_m; *see below*). Furthermore, this collapse of the electrochemical potential requires minutes to reach equilibrium and would appear to be rate-limiting to the ultimate lysis of the target cell (*see* Figs. 2 and 3). In this context it is interesting to note that on the basis of apparent osmotic permeabilities measured for complement-treated red cells, Lauf (1978) also concluded that the net flux of ions across the C5b-9 membrane pore must be limiting to the rate of immune hemolysis.

Effect of C8/C9 Binding on Membrane Potentials Maintained Across EAC1-7 Resealed Ghosts

Although results obtained with diS-C_3-(5) stained EAC1-7 erythrocytes suggest that the binding of complement proteins C8 and C9 initiates the collapse of transmembrane electrochemical gradients resulting in a net depolarization of the membrane potential prior to cell lysis, the short lag between the fluorescence response and measured hemolysis (Figs. 2 and 3) led us to question whether the dye response we measured for these cells corresponds directly to their changing membrane potential (as considered above), or rather, is due to the effects of

cell lysis on the emission of the fluorophore *per se*. This was of particular concern in light of the concentration-dependent quenching mechanism that underlies the potentiometric response of this fluorescent probe, as well as the well-known potential for cell hemoglobin to affect its fluorescence intensity (Sims et al., 1974; Tsien & Hladky, 1978; Guillet & Kimmich, 1981). Accordingly, we undertook to monitor changes in diS-C_3–(5) fluorescence initiated by the binding of the C5b-9 proteins to resealed erythrocyte ghost membranes, during suspension under conditions that imposed various electrochemical potentials across the ghost membrane.

To undertake these experiments, erythrocyte ghosts resealed to 102 mM internal [KCl] were treated with antibody and C8D to assemble the membrane-bound C5b67 complex, under conditions similar to those employed for red cells (*see* Materials and Methods). These EAC1-7 ghosts were then washed and equilibrated with diS-C_3-(5) during isotonic suspension in 0 or 153 mM [KCl]. As shown by the data of Fig. 5, EAC1-7 ghosts in the absence of valinomycin equilibrate to virtually the same level of diS-C_3-(5) fluorescence irrespective of the external [KCl]. The addition of valinomycin initiates a change in fluorescence to levels consistent with a shift of the membrane potential towards E_K (i.e., increased fluorescence corresponding to positive potentials for external $K^+ >$ cell K^+; decreased fluorescence corresponding to negative potentials for external $K^+ <$ cell K^+; *cf.* Fig. 1). These results confirm that within the time-frame of these experiments, the EAC1-7 ghosts maintain a transmembrane cation gradient, and that the membrane potential of these cells (in the absence of valinomycin) is insensitive to the equilibrium potentials of Na^+ or K^+ (i.e., is dominated by the chloride ratio). As shown by the data of Fig. 5, in the absence of valinomycin the addition of C8 and C9 to these membranes has no measureable effect upon diS-C_3-(5) fluorescence, suggesting that within the level of resolution of this indicator, the membrane potential of the target EAC1-7 ghost remains constant at its initial level of 0 mV (based on a Donnan ratio of unity as determined by Funder and Wieth, 1976). Under these conditions, potassium completely equilibrates across the ghost membrane, as evidenced by a complete loss of dye response to valinomycin when the ionophore was added 10 min after C8 and C9 additions (*not shown*). These results indicate that the transmembrane equilibration of Na^+ and K^+ across the C5b-9 lesion does not directly alter the net membrane potential of the target ghost, suggesting that any selective component of the cation conductance through the C5b-9 pore must be negligibly small as compared to the net chloride permeability of these membranes.

When the membrane potential of the EAC1-7 ghost was shifted towards E_K by selectively increasing membrane permeability to potassium (with valinomycin), the capacity of the C5b-9 proteins to rapidly depolarize that pre-existing membrane potential was again evident (Fig. 5). Note that in the case of EAC1-7 ghosts, the addition of C8 and C9 invariably collapses dye fluorescence to the initial (0 mV) level (*cf.* results for EAC1-7 red cells, Fig. 3) underscoring the stable Donnan equilibrium condition of these target membranes irrespective of C5b-9 assembly. In this context, it is also to be noted that under the conditions of these experiments, the lytic breakdown of the target ghost membranes never exceeded 5%, as determined by the measured release of either residual cell hemoglobin or ^{125}I-albumin incorporated in the cell water at the time of resealing.

Discussion

The results of this study provide direct experimental evidence that the binding of the C5b-9 proteins to the plasma membrane initiates a prelytic depolarization of membrane potential, thereby confirming the basic assumptions implicit to the colloid-osmotic theory of immune lysis. This membrane depolarization in the red cell appears to occur by the net equilibration of ion gradients (caused by net transmembrane ion flow) and not by selective changes in membrane conductance *per se*. By demonstrating that this change in membrane potential can also occur independent of any subsequent cytolytic event, these results underscore the potential of the complement proteins to induce *sublytic* changes in the electrochemical steady state of the target cell, of possible significance to the immune etiology of various disorders of cellular function. Finally, based on our evidence that diS-C_3-(5) can be employed to monitor changes in membrane potential induced by C5b-9 binding to the red blood cell, it can be suggested that this simple fluorescence assay can also be used to investigate early pathophysiological changes which occur in a variety of other cells consequent to immunoinjury and that might otherwise go undetected by conventional methods used to assess plasma membrane integrity or cell viability.

One of the particular goals of this study was to determine whether ion-selective conductance across the C5b-9 membrane pore contributes to

early prelytic changes in the membrane potential of the target cell. The possibility that this putative transmembrane channel might selectively conduct potassium (versus sodium) was suggested by: (i) the enhanced lysis observed when C5b-9-treated erythrocytes are suspended in isotonic KCl versus NaCl, which we have recently shown reflects an effect on the target cell which is exerted *after* the binding of the C5b-9 proteins (Dalmasso et al., 1975; Sims & Wiedmer, 1984); (ii) the differential effect of anisoosmotic solutions of KCl versus NaCl on the kinetics of complement-mediated hemolysis (Lauf, 1975; 1978); (iii) data pertaining to the molecular exclusion size of the C5b-9 pore, which suggest a minimum pore radius of <4 Å (Sims & Lauf, 1978; 1980); (iv) recent analysis of the single-channel conductance associated with binding of the C9b fragment of complement protein C9 to lipid bilayer membranes, which indicates a K/Na selectivity ratio of 3.5 : 1 (Esser et al., 1984).

In the present study, we were unable to demonstrate ion-selective conductance through the C5b-9 membrane pore. In the case of EAC1-7 red cells (suspended in the absence of valinomycin) the binding of C8/C9 depolarizes the red cell Donnan potential (Fig. 2), which is observed irrespective of the initial polarities of E_K and E_{Na} (both of which reverse upon substitution of external Na^+ by K^+). In the case of EAC1-7 ghost membranes suspended under these conditions (E_m = 0), no measureable change in diS-C_3-(5) fluorescence was detected upon C8 and C9 addition (Fig. 5), suggesting that the diffusional equilibration of Na^+ and K^+ across the C5b-9 pore is effectively "electrically silent" in these membranes. In the context of these data, several points warrant particular consideration. First, it is to be noted that by comparison to lipid bilayer membranes—and many cell plasma membranes—the erythrocyte membrane exhibits a relatively low electrical resistance, due principally to its high intrinsic permeability to Cl^- (the conductive permeability of the red cell membrane to Cl^- exceeding that of Na^+ or K^+ by two orders of magnitude; Knauf et al., 1977). Consequently, electrogenic conductances arising from selective permeability of the C5b-9 pore to either Na^+ or K^+ may go undetected in the erythrocyte membrane, due to "shunting" through the chloride conductance pathway (*see* for example, Hoffman et al., 1979). Finally, it is important to note that the effective response time of the potentiometric dye indicator used in these studies is relatively slow (the field-dependent redistribution of diS-C_3-(5) requiring several seconds to reach equilibrium). Accordingly, we would not have been able to resolve fluctuations in membrane potential upon C8/C9 binding that do not persist for at least this duration. Direct electrical measurements on single cells will be required to identify fast transients in membrane potential that might arise due to the binding of these proteins.

Although we were unable to detect transient diffusion potentials arising directly from the equilibration of Na^+ and K^+ across the C5b-9 pore *per se*, it is interesting to note that the onset of membrane depolarization after C8/C9 addition to EAC1-7 cells occurs measureably faster when the red cells are suspended in isotonic KCl versus NaCl (Fig. 2). The differential effect of the suspending cation on the C5-b-induced collapse of the Donnan potential coincides with the enhanced lytic effect observed for these proteins in K^+-containing media (Fig. 2, and Dalmasso et al., 1975). As would be predicted for a colloid-osmotic lytic process, and as directly demonstrated by the data of Figs. 2 and 3, the collapse of the Donnan ratio of these cells (and their concomitant hemolysis) is directly related to the C5b-9-mediated equilibration of transmembrane cation gradients, which is detected in Fig. 3 by the collapse of E_K (measured in the presence of valinomycin). Accordingly, it is likely that the enhanced C5b-9 hemolyis observed in high-K^+ media, and the accelerated membrane depolarization under these conditions, is a direct consequence of the near-equilibrium state of E_K when these high-K^+ erythrocytes are suspended in the presence of isotonic KCl.

It is of interest to note that the addition of C8 and C9 to EAC1-7 red cells results in the same maximal depolarization of the membrane potential in K^+ versus Na^+ medium, even though the measured hemolysis (at submaximal C8 inputs) in Na^+ medium was always significantly less than observed in K^+ medium (Figs. 2 and 3) even after prolonged (2-hr) incubation (*data not shown*; *see also* Dalmasso et al., 1975; Sims & Wiedmer, 1984). These results suggest that although depolarization of E_m after C5b-9 binding is a necessary antecedent to cell lysis, this electrochemical change is not invariably sufficient to achieve cytolytic rupture of the plasma membrane. As previously reported by Esser (1980), C5b-8 in the absence of C9 can dissipate membrane potential without consequent cytolytic changes. Although in our experiments C9 was always present in excess (simulating conditions in whole serum) the possibility remains that the sublytic depolarization of E_m we observe after C8/C9 addition reflects target cells that have either failed to bind C9, or that escape cytolytic rupture due to dissociation or inactivation of bound C9. The possibility for plasma membrane repair after C5b-9 binding to nucleated target cells has previously been considered (Ramm et al., 1983).

Our selection of diS-C_3-(5) to monitor changes in membrane potential subsequent to C5b-9 assembly was dictated by : (i) the non-first-order kinetics displayed by the prelytic ion flux, indicative of time-variant changes in membrane permeability, which precludes a direct analysis of individual rate constants measured for transmembrane ion equilibration after C8/C9 binding (P.J. Sims, *unpublished observations*), (ii) the need to resolve relatively small changes in the membrane potential of the EAC1-7 target cell, arising from the collapse of the Donnan chloride distribution (Δ7 mV); (iii) the requirement that the potentiometric indicator be relatively insensitive to changes in the physical properties of the membrane *per se,* including those arising from the direct effects of protein binding and membrane insertion. In light of the above considerations, diS-C_3-(5) was particularly well-suited for use in this study. Nevertheless, it is important to note that the analysis of results obtained with diS-C_3-(5) in the circumstance of a hemolytic process (such as our measurements using target EAC1-7 red cells) is potentially limited by changes in fluorescence which may arise due to the interaction of this fluorophore with hemoglobin (Tsien & Hladky, 1978; Guillet & Kimmich, 1981). Although we cannot exclude the possibility that the increased fluorescence we observe upon C8/C9 binding to the EAC1-7 red cell is due to the lytic release of cell hemoglobin rather than the collapse of the membrane potential *per se* (Figs. 2 and 3), a close inspection of these data suggests that contributions of dye-hemoglobin interactions to the C8/C9-dependent change in fluorescence are of secondary significance as compared to the total transmembrane field-dependent response of the fluorophore: First, it is to be noted that the increase in fluorescence observed after C8 addition invariably precedes cell hemolysis by 1 to 3 min, suggesting that the increase in dye fluorescence cannot be accounted for strictly on the basis of a release of cell hemoglobin. For example, when EAC1-7 cells are suspended in KCl-MOPS (plus C9), the dye response to the addition of C8 is virtually complete at $t = 16$ min, a time at which only 16% hemolysis has occurred. A comparable lag between the dye response and hemoglobin release can also be discerned for experiments performed in NaCl-MOPS (Figs. 2 and 3). Furthermore, results obtained with EAC1-7 ghosts (Fig. 5) demonstrate that changes in dye fluorescence which are consistent with its well-documented field-dependent response do occur upon C8/C9 binding in the absence of lytic membrane breakdown (or hemoglobin release), provided that the membrane potential of the target cell is initially displaced from 0 mV.

It is interesting to note that the fluorophore used in this study fundamentally differs from the various radioactive, chromogenic, fluorescent, or spin-labeled indicators commonly employed to assess membrane damage by the complement proteins: By contrast to conventional immunoinjury markers, which are normally membrane impermeant, and which signal membrane damage by their entry or release from the target cell, diS-C_3-(5) is *freely permeable* across the undamaged plasma membrane, its transmembrane distribution (and, in turn, fluorescence) governed by the electrochemical potential (Sims et al., 1974; Hladky & Rink, 1976). Consequently, the fluorescence response measured for this indicator is completely independent of a change in membrane permeability to the dye itself (the lipophilic chromophore always freely equilibrating), but instead responds to changes in the electrochemical state of the target cell which might arise due to changes in membrane permeability to one or more cellular ions. Thus it can be suggested that this indicator should prove particularly useful for assessing *sublytic* changes in the electrochemical steady state of those cell membranes that normally fail to show cytolytic changes after C5b-9 binding.

Cohn Fraction III used in the purification of C8 was a generous gift from the State Biological Laboratories, Massachusetts Department of Health. Superb technical assistance in purifying the complement proteins was provided by Ellen Boswell. The assistance of Cathy Murphy in preparing the manuscript is gratefully acknowledged. This work was supported by a Grant-In-Aid from the American Heart Association with funds contributed in part by the AHA Virginia Affiliate; and by a grant from the Thomas F. Jeffress Memorial Trust. Peter Jay Sims is a John A. Hartford Fellow.

References

Bhakdi, S., Tranum-Jensen, J. 1983. Membrane damage by complement. *Biochim. Biophys. Acta* **737**:343–372

Campbell, A.K., Daw, R.A., Hallett, M.D., Luzio, J.P. 1981. Direct measurement of the increase in intracellular free calcium ion concentration in response to the action of complement. *Biochem. J.* **194**:551–560

Dalmasso, A.P., Lelchuk, R., Giavedoni, E.G., Isola, E.D. de 1975. The modification of the terminal stages of the complement reaction by alkali metal cations. *J. Immunol.* **115**:63–68

Esser, A.F. 1980. Bactericidal action of complement. *Fed. Proc.* **39**:1755

Esser, A.F. 1981. Interactions between complement proteins and biological and model membranes. *In:* Biological Membranes. D. Chapman, editor. pp. 277–322. Academic, New York

Esser, A.F., Shiver, J.W., Dankert, J.R., Donovan, J.D. 1984. The ninth component of complement: Functional activity of the B-domain. *Fed. Proc.* **43**:1491

Funder, J., Wieth, J.O. 1976. Chloride transport in human erythrocytes and ghosts: A quantitative comparison. *J. Physiol. (London)* **262**:679–698

Green, H., Goldberg, B. 1960. The action of antibody and complement. *Ann. N.Y. Acad. Sci.* **87**:352–362

Guillet, E.G., Kimmich, G.A. 1981. DiO-C_3-(5) and DiS-C_3-(5): Interactions with RBC, ghosts and phospholipid vesicles. *J. Membrane Biol.* **59**:1–11

Hallet, M.B., Campbell, A.K. 1984. Is intracellular Ca^{2+} the trigger for oxygen radical production by polymorphonuclear lymphocytes? *Cell Calcium* **5**:1–19

Hingson, D.J., Massengill, R.K., Mayer, M.M. 1969. The kinetics of release of ^{86}rubidium and hemoglobin from erythrocytes damaged by antibody and complement. *Immunochemistry* **6**:295–307

Hladky, S.B., Rink, T.J. 1976. Potential difference and distribution of ions across the human red blood cell membrane: A study of the mechanism by which the fluorescent cation, diS-C_3-(5) reports membrane potential. *J. Physiol. (London)* **263**:287–319

Hoffman, J.F., Kaplan, J.H., Callahan, T. 1979. The Na:K pump in red cells is electrogenic. *Fed. Proc.* **38**:2440–2441

Hoffman, J.F., Laris, P.C. 1974. Determination of membrane potentials in human and *Amphiuma* red blood cells by means of a fluorescent probe. *J. Physiol. (London)* **239**:519–552

Hunter, M.J. 1977. Human erythrocyte anion permeabilities measured under conditions of net charge transfer. *J. Physiol. (London)* **268**:35–49

Imagawa, D.K., Osifchin, N.E., Paznekas, W.A., Shin, M.L., Mayer, M.M. 1983. Consequences of cell membrane attack by complement: Release of arachidonate and formation of inflammatory derivatives. *Proc. Natl. Acad. Sci. USA* **80**:6647–6651

Knauf, P.A., Fuhrmann, G.I., Rothstein, S., Rothstein, A. 1977. The relationship between anion exchange and net anion flow across the human red blood cell membrane. *J. Gen. Physiol.* **69**:363–386

Lauf, P.K. 1975. Immunological and physiological characteristics of the rapid immune hemolysis of neuraminidase-treated sheep red cells produced by fresh guinea pig serum. *J. Exp. Med.* **142**:974–988

Lauf, P.K. 1978. Membrane immunology and permeability functions. *In*: Physiology of Membrane Disorders. T.E. Andreoli, J.F. Hoffman and D.D. Fanestil, editors. pp. 369–398. Plenum, New York

Mayer, M.M. 1961. Complement and complement fixation. *In*: Kabat and Mayer's Experimental Immunochemistry, 2nd Edition. E.A. Kabat and M.M. Mayer, editors, pp. 133–240. Thomas, Springfield, Illinois

Mayer, M.M. 1972. Mechanism of cytolysis by complement. *Proc. Natl. Acad. Sci. USA* **69**:2954–2958

Mayer, M.M. 1981. Membrane damage by complement. *Johns Hopkins Med. J.* **148**:243

Muller-Eberhard, H.J. 1984. The membrane attack complex. *Springer Semin. Immunopathol* **7**:93–141

Ramm, L.E., Whitlow, M.B., Koski, C.L., Shin, M.L., Mayer, M.M. 1983. Elimination of complement channels from the plasma membranes of U937, a nucleated mammalian cell line: Temperature dependence of the elimination rate. *J. Immunol.* **134**:1411–1417

Sims, P.J. 1981. Permeability characteristics of complement damaged membranes: Evaluation of the membrane leak generated by the complement proteins C5b-9. *Proc. Natl. Acad. Sci. USA* **78**:1838–1842

Sims, P.J. 1983. Complement pores in erythrocyte membranes. Analysis of C8/C9 binding required for functional membrane damage. *Biochim. Biophys. Acta* **732**:541–552

Sims, P.J. 1984. Complement protein C9 labeled with fluorescein isothiocyanate can be used to monitor C9 polymerization and formation of the cytolytic membrane lesion. *Biochemistry* **23**:3248–3260

Sims, P.J., Lauf, P.K. 1978. Steady-state analysis of tracer exchange across the C5b-9 complement lesion in a biological membrane. *Proc. Natl. Acad. Sci. USA* **75**:5669–5673

Sims, P.J., Lauf, P.K. 1980. Analysis of solute diffusion across the C5b-9 membrane lesion of complement: Evidence that individual C5b-9 complexes do not function as discrete, uniform pores. *J. Immunol.* **125**:2617–2625

Sims, P.J., Waggoner, A.S., Wang, C.-H., Hoffman, J.F. 1974. Studies on the mechanism by which cyanine dyes measure membrane potential in red blood cells and phosphatidylcholine vesicles. *Biochemistry* **13**:3315–3330

Sims, P.J., Wiedmer, T. 1984. The influence of electrochemical gradients of Na^+ and K^+ upon the membrane binding and pore-forming activity of the terminal complement proteins. *J. Membrane Biol.* **78**:169–176

Steinbuch, M., Audran, R. 1969. The isolation of IgG from mammalian sera with the aid of caprylic acid. *Arch. Biochem. Biophys.* **134**:279–284

Tirosh, R., Berke, G. 1984. Mechanism of C-mediated lysis of nucleated cells: Prelytic depletion of high energy phosphates detected by ^{31}P-NMR. *Fed. Proc.* **43**:1764

Tsien, R.Y., Hladky, S.B. 1978. A quantitative resolution of the spectra of a membrane potential indicator, diS-C_3-(5) bound to cell components and to red blood cells. *J. Membrane Biol.* **38**:73–97

Vistnes, A.I. 1984. A new method of evaluating complement mediated lysis of liposomes. *J. Immunol. Methods* **68**:251–261

Received 6 September 1984; revised 19 November 1984