IMMUNOLOGICAL AND PHYSIOLOGICAL CHARACTERISTICS OF THE RAPID IMMUNE HEMOLYSIS OF NEURAMINIDASE-TREATED SHEEP RED CELLS PRODUCED BY FRESH GUINEA PIG SERUM*

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Since the studies of Yachnin et al. (1), Arquilla et al. (2), and Dalmasso and Müller-Eberhard (3) it has been known that neuraminidase (VCN)[1]-treated red blood cells hemolyze in fresh, complement-containing sera. While working on sheep red blood cell (SRBC) antigens associated with the cation transport polymorphism in these cells (4), hemolysis of neuraminidase-treated N-SRBC by fresh guinea pig serum was recognized as an attractive and simple immunological model system for an exploratory physiological study on the nature of immune hemolysis.

This paper reports (a) complement hemolysis of neuraminidase-treated (N)-SRBC is preceded by binding of a naturally occurring IgM antibody protein, and (b) the ensuing hemolysis appears to be colloid osmotic with a striking temperature independency above a transition temperature. It is reasoned that the membrane lesion produced in these cells cannot be one or more large holes, because sucrose permeation at a rate slower than free diffusion becomes the rate-limiting factor in cell swelling before hemolysis. Preliminary accounts of this work have been given elsewhere (5).

Materials and Methods

Red Cells and Neuraminidase Treatment.   Sheep blood drawn into heparin from healthy, only genetically high potassium (HK) sheep (4) was stored on ice and used within a few hours. The cells were washed in physiological saline buffered with 1 mM Tris/HCl, pH 7.4 (TBS).

For removal of neuraminic acid (NA), about 10^{10} SRBC/ml TBS were incubated for 1 h at 37°C with 0–33 U of protease-free VCN from Vibrio Cholerae (mucopolysaccharide *N*-acetylneuraminoyl hydrolase, E.C. 3.2.1.1., 500 U/ml, Behringwerke AG Marburg, West Germany). Sheep red cells contain both *N*-acetyl- and *N*-glycolylneuraminic acid, and it is known that VCN cleaves both sugar moieties at the α-glucosidic linkage (6). VCN-treated (N-SRBC) and untreated control (C-SRBC) cells were washed five times each with 30 vol of TBS. The amounts of NA liberated during exposure of SRBC to VCN was determined in the cell supernates by the thiobarbituric acid methods (7) using pure commercial *N*-acetylneuraminic acid as standard.

[1] *Abbreviations used in this paper:* GPS, guinea pig serum; HK, high potassium; NA, neuraminic acid; N-SRBC, neuraminidase-treated sheep red blood cells; TBS, 1 mM Tris/HCl, pH 7.4; VBS, veronal-buffered saline; VCN, neuraminidase from Vibrio Cholerae.

Standardization of Red Cell Suspensions and Sampling of Hemolytic Supernates. Unless stated otherwise, for most experiments SRBC were suspended in veronal-buffered saline pH 7.4 (VBS), containing 5 mM KCl (K^+-VBS). Cell suspensions were always adjusted spectrophotometrically at 414 nm to 10^8 cells/ml by standard procedures (8).

In the experiments shown in Figs. 1–4 (see Results) 1.0 ml of 10^8 N- or C-SRBC was incubated with 0.5 ml guinea pig serum (GPS) or buffer as control for 1 h at 37° C, followed by placing the tubes on ice, adding 6 ml ice cold K^+-VBS, and centrifuging for 5 min at 4° C. The degree of hemolysis was determined spectrophotometrically in the supernates and corrected for serum color and cell buffer blanks. In the kinetic experiments of Figs. 5–9 (see Results) a rapid sampling technique (9, 10) was adopted using Millipore AP 2502500 prefilters and RAWP 02500 filters connected to 10-ml syringes in order to separate unlysed SRBC from their suspending medium, which subsequently was analyzed spectrophotometrically for hemolysis. In the experiments on the thermal dependence of hemolysis, cell suspensions were kept in a waterbath with constant agitation at the indicated temperatures ($\pm 0.05°$ C).

Cell Water and Chloride Analysis; Adjustment of Chloride Ratio. Cell water was determined by drying aliquots of packed SRBC to constant weights. The apparent water fraction was corrected assuming 2% extracellular water and expressed as kg H_2O/kg cell solids. The determination of the chloride ions concentration ratio, $(Cl)_i/(Cl)_o$, was carried out by $AgNO_3$ titration of the perchloric acid (7%) extract of the extracellular medium and the packed SRBC.

To obtain SRBC with $(Cl)_i/(Cl)_o$ near unity, about 30% SRBC suspensions in 140 mM KCl and 10 mM NaCl containing none, 27, or 54 mM sucrose were titrated with CO_2 gas at 37° C to a final pH of 6.6 using a Radiometer titrator. This pH was reached after several intermitting washes in the medium during which HCO_3^- ions released from the cells in exchange for extracellular Cl^- ions are removed. As the pH approaches 6.6, hemoglobin within the cells becomes the primary buffer, and $(Cl)_i/(Cl)_o$ approaches unity.

The osmolarity of the suspending media was measured with an osmometer (Osmette, Precision Instrument Co., Santa Clara, Calif.). For computation of volume changes and permeability coefficients see Results.

Preparation and Processing of Guinea Pig Serum. Large guinea pigs were bled by heart puncture. The blood was permitted to clot for 3–5 h at 4° C and for 1 h at room temperature, after which the serum was collected by centrifugation at 0° C. The cell-free serum was then absorbed three times for 10 min at 0° C, each with one-tenth volume of washed packed HK SRBC from sheep whose blood was used in the later experiments. GPS prepared in this manner never exhibited any significant hemolytic effect on normal C-SRBC even with incubation periods of several hours. The "CH50 titer" (usually between 200–220) was measured with Forssman antibody and C-SRBC according to standard methods (8). The sera were stored at −90° C before use.

Whole gamma (γ) globulin was prepared by ammonium sulfate precipitation. Pure γ-macroglobulins (IgM) were isolated from GPS by three major steps: the high molecular weight serum proteins were collected by gel filtration on Sephadex G-200 and separated into macroglobulins and lipoproteins by ultracentrifugation at 0° C for 16 h in a buffered high density ($\rho = 1.08$) KBr/NaCl solution (11). The macroglobulins were further separated by preparative starch block electrophoresis into IgM and α_2-M proteins and verified by immunodiffusion analysis. The dilutions of GPS added to the cell suspensions are expressed in terms of relative GPS concentrations (i.e. 1 = undiluted, 0.5 = 1:1 diluted etc.) or in milligram protein/ml which, as in the case of whole γ-globulin or IgM protein, was determined by the Lowry method (12).

Results

Correlation between Neuraminidase Treatment and Susceptibility to Hemolysis. Sheep red cells pretreated for 1 h at 37° C with increasing units of VCN become correspondingly more susceptible to hemolysis in fresh GPS. Fig. 1 shows that pretreatment with 16.6 U VCN produces maximum hemolysis by fresh GPS. Control SRBC hemolyzed only negligibly at 0.25 relative GPS concentration.

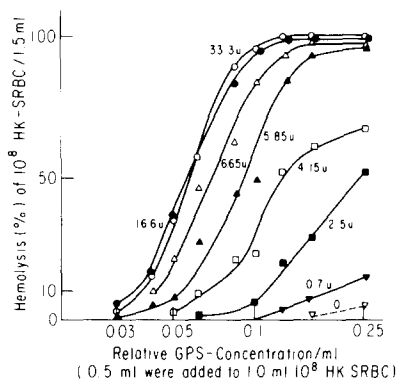The degree of hemolysis in fresh GPS is related to the amount of NA enzymati-

FIG. 1.   The effect of pretreatment of SRBC with increasing units of VCN on the degree of hemolysis (1 h, 37° C) in fresh GPS. U = units of VCN/10^{10} SRBC.
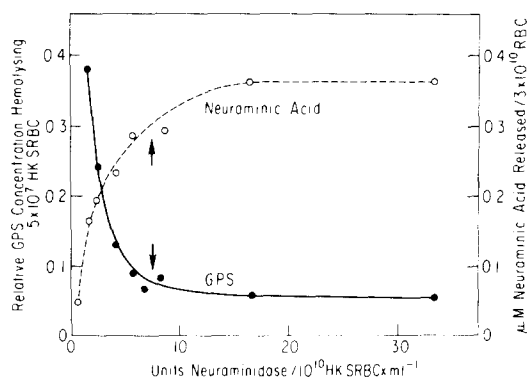

FIG. 2.   Relation between amounts of neuraminic acid released (right abscissa) and the relative GPS concentration required for 50% hemolysis of 10^8 SRBC (left abscissa) as function of units VCN/10^{10} cells used during the 1 h pretreatment.

cally cleaved from the cells. Fig. 2 shows an almost symmetric inverse relationship between the relative hemolytic GPS concentration required (left ordinate) and the amounts of NA released (right ordinate) as the VCN units are increased during the 1 h pretreatment of the cells (abscissa). When approximately 80% (i.e. 0.3 μM/3 × 10^{10} SRBC) of the total NA present on SRBC (6) is hydrolyzed by about 8–10 enzyme units, maximum hemolysis occurs (arrows), i.e. a relative concentration of less than 0.1 GPS is required to hemolyze 50% N-SRBC. In a time study, no further release of NA occurred beyond 1 h incubation time.

Properties of Hemolytic Serum Activity.   The hemolytic effect of GPS depends on the presence of (a) an antibody-like specificity against N-SRBC and (b) hemolytically active serum complement. Fig. 3 shows an experiment in which N-SRBC were preincubated in the cold for 1 h with increasing amounts of GPS, IgM protein, or whole γ-globulin. At this temperature no decrease of the CH50 titer was observed. After three washes in K⁺-VBS the N-SRBC were incubated for 1 h at 37° C with fresh GPS, absorbed at 0° C with N-SRBC, thus containing only the hemolytically active complement and not the sensitizing IgM fraction. There was a pronounced hemolysis of N-SRBC pre-exposed to IgM protein or
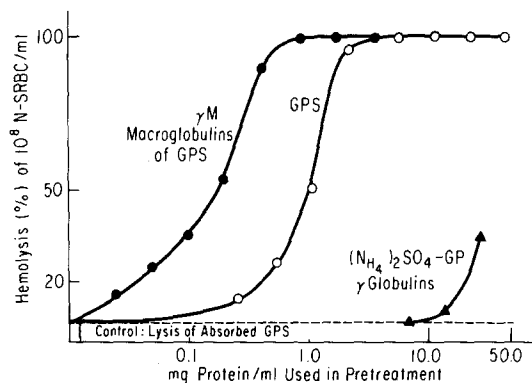
FIG. 3. Hemolysis at 37° C (1 h) of N-SRBC preexposed in the cold to γM protein, GPS or whole γ-globulin. Complement containing GPS in falling concentrations, absorbed in the cold with N-SRBC. The dotted line indicates the background lysis of unsensitized N-SRBC by the absorbed GPS.

GPS containing this protein while sensitization with whole γ-globulin produced rather little hemolysis. That whole γ-globulin failed to sensitize N-SRBC can be ascribed to the fact that IgG proteins appear not to have the antibody specificity found in the IgM fraction, and that most of the latter precipitated during dialysis of the ammonium sulfate precipitate being subsequently removed by centrifugation. Apparently, the shift of the lysis curves to the left produced with IgM reflects the relative purification of the fraction of sensitizing IgM protein. As control, nonsensitized N-SRBC remained virtually unaffected by the absorbed GPS (Fig. 3; control: lysis of absorbed GPS).

Like to intact N-SRBC, the antibody activity of fresh GPS can also be adsorbed to hemoglobin-free ghosts prepared by osmotic lysis from N-SRBC. Fig. 4 shows that 50% of the hemolytic activity of finally $1/10$ diluted GPS was removed with about 12 μg membranes from N-SRBC, while incubation with membranes from C-SRBC did not affect the hemolytic activity of GPS at all. Exposure to ghosts from either source did not lower the CH50 titer of GPS (not shown).

The hemolytic potency of fresh GPS is not reduced by heating at 50° C for 1 h, but it is abolished by incubation around 52° C, as would be expected since serum complement is inactivated at this temperature. Nevertheless, sensitization of N-SRBC with the noncomplement component was still feasible, even when the serum was heated for 1 h at 60° C. Thus, upon binding of an IgM serum protein with an antibody specificity against an antigenic surface determinant on N-SRBC, activation of the hemolytic complement cascade occurs.

Thermal Kinetic of Hemolysis. Hemolysis by GPS of N-SRBC nearly completely depleted of their surface NA is extremely rapid. The rate of hemolysis at 37° C dramatically increases with higher relative GPS concentrations. For example, at a GPS concentration of 0.5, hemolysis of all cells is completed within 200 s.

The thermal kinetics are shown in Fig. 5—hemolysis changes from a rather shallow curve at 23° C to a very steep but sigmoidal curve at 28° C. The rate
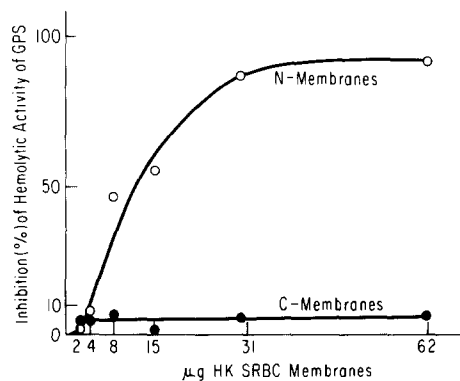
FIG. 4. Effect of absorption of GPS with ghosts from N- or C-SRBC on its hemolytic activity toward N-SRBC. 1.0-ml ghosts were incubated with 1.0 ml ¹/₅ diluted GPS for 1 h at 0° C. After incubation the membranes were pelleted by centrifugation and the supernates assayed for hemolytic activity toward N-SRBC.
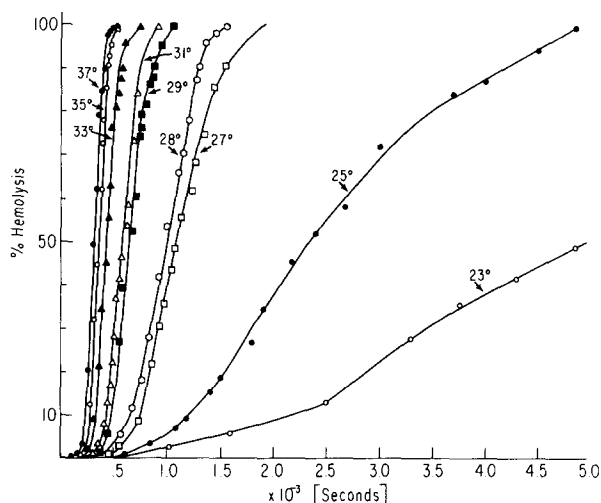

FIG. 5. Thermal dependence of GPS-induced hemolysis of N-SRBC. 1 vol of standard N-SRBC suspension pre-equilibrated to the experimental temperature was mixed at time zero with half volume of similarly equilibrated, five fold diluted GPS and incubated in a shaker bath at the indicated temperatures. Samples were rapidly withdrawn at given time intervals.

increment of hemolysis is less dramatic between 28° and 37° C; at the latter temperature hemolysis is completed at 200–400 s.

From the Arrhenius Eq. 1 a plot of log rate constant, k,

$$k = s \cdot e - \frac{E_a^H}{R \cdot T},$$

(1)

vs. $1/T$, the reciprocal value of the absolute temperature T, may provide information about E_a^H, the apparent energy of activation of the hemolytic process. In Fig. 6 k was expressed as $1/t$, where t defines the time in seconds to
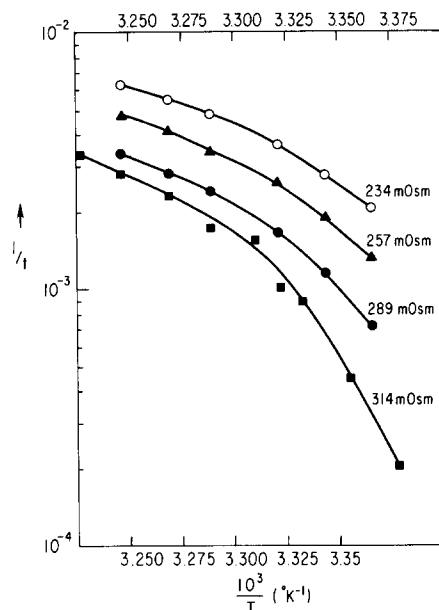
FIG. 6.   Arrhenius plot of hemolysis of N-SRBC by GPS at different osmolarities. Data replotted from Fig. 5 are in the 314 mOsm curve.

hemolyze 50% of N-SRBC. There is a curvilinear relationship between $1/t$ and $1/T$ which is not significantly altered when, before hemolysis by GPS, the N-SRBC were osmotically swollen (in 234 and 254 mOsm K^+-VBS) or shrunken (in 314 mOsm K^+-VBS) as compared to nominal isotonic conditions (289 mOsm). The transition temperature (T_c) observed at 28°–29° C apparently is independent of volume and, therefore, water content of the N-SRBC. For the more linear portions of the curves above T_c, E_a^H values of about 19 kcal·mol^{-1} were computed. Likewise, VCN-treated human red cells also hemolyze in fresh GPS. The Arrhenius diagram, not shown, revealed a quite linear relationship between log $1/t$ and $1/T$ above and below a well-defined T_c of 28° C. The E_a^H values above T_c were 14–16 kcal·mol^{-1} and below T_c 28–30 kcal·mol^{-1}.

Although the information to be gained from an Arrhenius plot of the multistage immune lysis is rather limited, the data indicate that hemolysis of VCN-treated sheep or human red cells by GPS above T_c is less temperature sensitive. The observation of a T_c in both sheep and, more pronounced, in human red cells with much higher activation energies below T_c cannot be explained at present but may be related either to membrane events or to activation of complement introducing a new rate-limiting factor for hemolysis.

Physiological Mechanism of Hemolysis.   The almost parallel upward shift of the portion of the Arrhenius curves linear above T_c (Fig. 6) indicated that hemolysis proceeded faster when the cells are swollen in hypotonic media before addition of GPS. This assumption was confirmed in the experiments shown in Fig. 7, in which the hemolysis kinetics were measured in N-SRBC suspended in salt (Fig. 7 A and B) or in salt (130 mM) plus sucrose (Fig. 7 C and D) solutions of various osmolarities below and above nominal isotonicity (Fig. 7, A–D, dashed

lines). The rate of hemolysis dramatically increases below and diminishes considerably above nominal isotonicity (292–299 mOsm). Note that higher osmolarities achieved with sucrose are less effective in delaying hemolysis than those with KCl or NaCl. However, ultimately all cells suspended in hypertonic media will be hemolyzed. In Fig. 8 the degree of hemolysis at time intervals selected from 300–2,000 s was replotted from Fig. 7 A and B as a function of the measured osmolarities of NaCl and KCl. The almost parallel displacement of the curves suggests that the higher the osmolarity of the medium, the longer will be the time required for 50% hemolysis ($t_{50\%}$) of all cells.

The data are unequivocal evidence that N-SRBC undergo a colloid-osmotic swelling before hemolysis (see also Discussion). The cells, however, were not at equilibrium with respect to cations and membrane potential (about -10 mV, see ref. 13, 14) which is a function of the chloride ion distribution ratio [$(Cl)_i/(Cl)_o =$ 0.7]. Thus an experiment was designed to test how sucrose added on top of an isotonic high potassium (K^+)-low sodium (Na^+) medium may affect the rate of hemolysis of HK N-SRBC with $(Cl)_i/(Cl)_o$ near unity. The latter was achieved by use of the Hamburger shift and titration of hemoglobin (see Methods). The concentrations of sucrose (27 and 54 mM) used to protect human and sheep red cells treated with the antibiotic nystatin (which nonselectively enhances cation permeability) against colloid osmotic hemolysis (15).

Fig. 9 shows that presence of either sucrose concentration delayed the onset of hemolysis, shifted the sigmoidal hemolysis time curves to the right, but did not protect the cells against the hemolytic attack. In the absence of sucrose, there is a chemical driving force for K^+ ions (plus Cl and water) to enter the cells through the GPS damaged membrane since, due to the presence of hemoglobin,
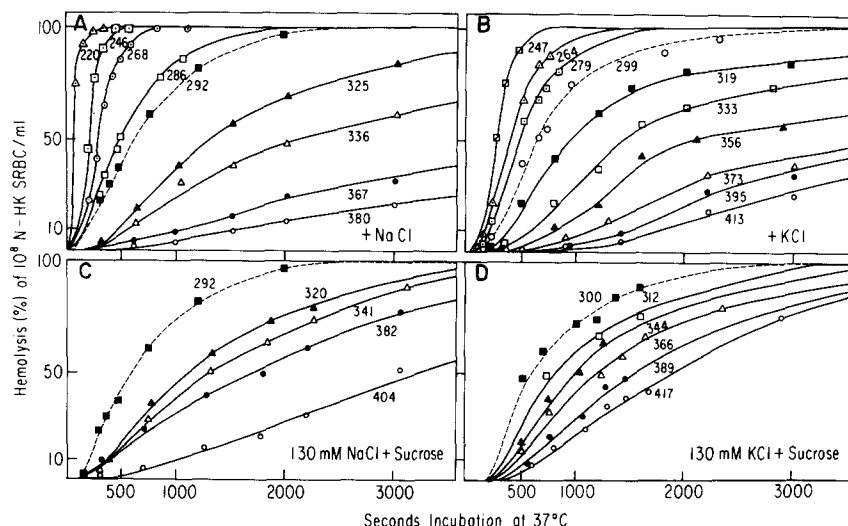

Fig. 7. Kinetics of hemolysis of N-SRBC by GPS in veronal-buffered salt solutions (pH 7.4, 37° C) of various osmolarities. (A), NaCl; (B), KCl; (C), 130 mM NaCl plus sucrose; (D), 130 mM KCl plus sucrose. Dashed lines indicate nominal isotonicity; solid lines plus numbers indicate hypo- and hypertonic osmolarities.
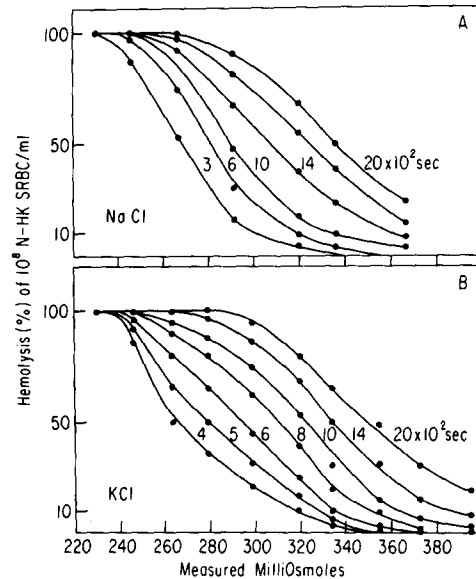
FIG. 8.   Replot of Fig. 7 A and B. Hemolysis of N-SRBC by GPS at selected time intervals as function of measured osmolarity.
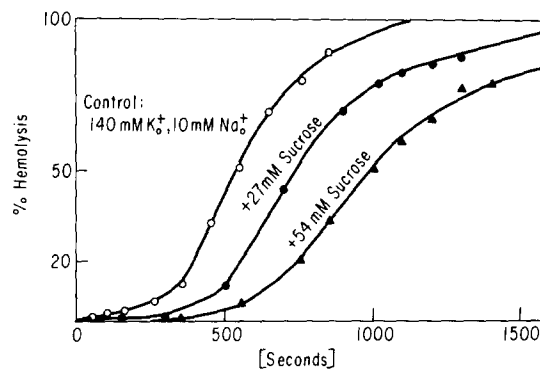

FIG. 9.   Hemolysis kinetics of N-HK SRBC with $(Cl)_i/(Cl)_o$ near unity by GPS in high K^+-media containing: 140 mM KCl, 10 mM NaCl $\pm$ 27 or 54 mM sucrose. At time zero, one-half portion of $^1/_5$ diluted GPS was added previously dialyzed against K^+-medium containing Ca^{++} and Mg^{++} ions so that the concentration of these ions in the final suspensions were 0.15 and 0.5 mM, respectively (8).

the K^+-concentration is lower inside the cell. In the presence of sucrose, the cells are shrunken, thus increasing the intracellular K^+-concentration and thereby reducing the chemical driving force for salt. Thus, the fact that delayed hemolysis occurs in presence of sucrose must be explained by the entrance of sucrose plus water (plus salt), accompanied by a reswelling of the cells from their shrunken state to a critical hemolytic volume before bursting, which is probably identical to that in absence of sucrose. The time required to reach the critical hemolytic volume for a shrunken cell can be estimated from the shift of the $t_{50\%}$ points in presence of either sucrose concentration. Then the water lost (Δkg

H_2O) due to the hypertonic sucrose containing K^+-medium will be reaccumulated in the given time difference ($\Delta t_{50\%}$) after the GPS-induced membrane damage permits sucrose (plus water and salt) to enter the cell.

This influx of sucrose (M_{app}^{suc}) is only an apparent influx since sucrose is not at equilibrium across the membrane before lysis and is a function of the extracellular sucrose concentration $(C_{suc})_0$ and a rate constant (k) according to Eq. 2:

$$M_{app}^{suc} = (C_{suc})_o \cdot k \qquad (2)$$

Table I shows that (M_{app}^{suc}) increases at higher $(C_{suc})_o$ while k remains rather constant. Thus, the more the cells are shrunken in sucrose before lysis, the more driving force $(C_{suc})_o$ is required for reswelling.

Provided sucrose is indeed the permeating species when the membrane lesion is set, its apparent permeability coefficient may be computed from Eq. 3:

$$P_{app}^{suc} = M \cdot A^{-1} \cdot C^{-1}; \qquad (3)$$

where P_{app}^{suc} = apparent permeability coefficient for sucrose in $cm \cdot s^{-1}$; M = influx of sucrose in millimoles·(kg cell solids)$^{-1} \cdot s^{-1}$; C = millimoles sucrose·cm^{-3}; A = surface area in square centimeters·(kg cell solids)$^{-1}$. Using Ponder's (16) formula $A = 12/5\pi \cdot r^2$ and a radius for sheep red cells of $r = 2.6$ μm, the surface area was computed as $51 \cdot 10^{-8}$ cm^2/cell or $5.141 \cdot 10^7$ $cm^2 \cdot$(kg cell solids)$^{-1}$. Table I shows that P_{app}^{suc} is about of 10^{-8} $cm \cdot s^{-1}$ and practically independent of M and $\Delta t_{50\%}$. Extrapolation to zero concentration of sucrose yielded a P_{exp}^{suc} of $3 \cdot 10^{-8}$ $cm \cdot s^{-1}$ indicating only a very small concentration dependence.

The experiments are indirect proof that GPS caused in N-SRBC penetration of sucrose and that, before hemolysis, a colloid osmotic swelling occurs with a rate determined by the membrane permeability for sucrose.

Discussion

The immunological and physiological observations may be aligned to the following order of events: (a) Removal of more than 80% of surface-bound NA by VCN renders SRBC highly susceptible to complement hemolysis triggered by membrane binding of an IgM antibody protein; (b) The lytic release of hemoglo-

TABLE I*

Osmotically Determined Permeability Constants of Sucrose for Neuraminidase-Treated HK-SRBC Immune Hemolyzed by GPS

Sucrose in pH 6.6 medium	$(Cl)_i/(Cl)_o$	kg cell H_2O/kg cell solids	Δ kgH_2O	Δt_{50}	M_{app}^{suc}	k	P_{app}^{suc}
mM				S	$mmol \cdot s^{-1}/kg$ cell solids	h^{-1}	$cm \cdot s^{-1}$
—	0.99	1.979	—	—	—	—	—
27	1.02	1.856	0.123	190	0.01748	0.76	$1.26 \cdot 10^{-8}$
54	1.13	1.721	0.258	450	0.03108	0.68	$1.12 \cdot 10^{-8}$

* Computed from Fig. 9; for explanation see Results.

bin is less temperature-dependent above a transition temperature (T_c) of about 28° C, while below this temperature the rate of hemolysis decreases much faster. The computed energies of activation above and below T_c differ by a factor of two; (c) Before hemolysis by GPS complement, N-SRBC undergo a colloid osmotic swelling which was not prevented by sucrose (27 or 54 mM) under conditions where no electrochemical driving force for salt movement existed.

The qualitative correlation between the amount of NA removed and the degree of hemolysis by GPS observed by others (1, 2) was quantitatively confirmed in these studies. Apparently, NA masks "hidden" membrane structures which are capable of interacting with antibodies present in the IgM serum proteins, thereby triggering the complement cascade. Such an increased sensitivity to cytolytic serum action is not restricted to red cells alone: VCN-treated mouse spleen cells are sensitive to *H-2* antisera (17) and CH3 mouse sera contain natural cytotoxicity for VCN-treated but not control TA3 mouse adeno-carcinoma cells (18, 19); anti-HL-A allo-antisera are cytotoxic for VCN-treated human lymphocytes (20) and complement lysis of autologous lymphoid cells is mediated by a circulating IGM antibody (21). Between 61–92% of all NA had to be removed to produce the effect (22), a number range only coincidentally correlating with that found for sheep red cells in this study (Fig. 2).

Although the data clearly establish the requirement of binding of an IgM serum protein before the activation of complement, no final conclusion can yet be reached about the nature of the antigen-antibody reaction involved. A priori, one would suspect the binding of "panagglutinins" or "T-agglutinins" occurring in all vertebrate sera to galactosyl moieties of "T-transformed" N-SRBC (23, 24). In support of this hypothesis is the hemagglutination of N-SRBC by the isolated IgM protein observed here.

It is possible that there are "natural" antibodies against membrane surface-adsorbed VCN (25). Normal mammalian sera contain IgM antibodies reacting with free VCN as well as with VCN bound to lymphocytes (26). Furthermore, anti-VCN antibodies agglutinate VCN-treated human red cells (27).

Once the membrane of N-SRBC is sensitized with the IgM antibody protein, hemolysis by complement ensues. It is unknown at present which of the two main pathways of complement activation prevails, but the fact that high GPS concentrations are required to bring about hemolysis suggests participation of the "alternate pathway" (28).

Irrespective of the mode of complement activation, hemolysis of N-SRBC by GPS is less dependent of temperature above T_c of about 28° C. The magnitude of the activation energies computed above and below T_c is in the range of those found for other membrane processes such as the passive diffusion of cations across human red cell membranes (14–16 kcal·mol^{-1}, see ref. 29) and the fast anion exchange (28–32 kcal·mol^{-1}, see ref. 30). Assuming that the hemolysis of N-SRBC by GPS constitutes a series of consecutive irreversible first order reactions, it was computed from the temperature dependence data that at least eight different steps may be involved in the lytic process (unpublished data in collaboration with Dr. F. H. Schmidt, Dept. of Statistics and Biometry, Emory University, Atlanta, Ga.). In light of this multitude of reactions, an interpretation of the values of the activation energies found awaits further dissection of

the lytic process at the membrane molecular level. Similar arguments apply to the observation of a T_c around 28°–29° C. If the T_c value found relates to the "fluidity" of the membranes, it probably is not related to the predominant phospholipids which are quite different in human (lecithin) and sheep (sphingomyelin) membranes. Alternatively, the existence of a T_c value may reflect differences in activation and binding of complement at low and higher temperatures.

In several reports (31–35) the colloid osmotic nature of immune cytolysis has been inferred from the observation that large nonpenetrating solutes or polymers counterbalance the colloid-osmotic pressure of the intracellular protein and thus prevent lysis. Wilbrandt (36) stated that, in the strict sense, colloid osmotic hemolysis commences when the red cell membrane is made nonselectively permeable to cations, but that colloid osmotic swelling is not restricted to the equilibration of small cations across the membrane. Immune lysis of sheep red cells by amboceptor and complement occurred without a preceding swelling phase and thus was distinguished from colloid osmotic hemolysis (36). However, Valet and Opferkuch[2] recently showed that, before immune lysis, sheep red cells exposed to antibody plus complement components C1-8 (EAC1-8 cells) and upon final addition of C9 underwent an irreversible swelling which persisted into the ghosting stage of the EAC1-9 cells[2] indicating membrane structural damage. In contradiction to Wilbrandt's observation, the present study clearly implies a colloid osmotic aspect of immune hemolysis. The parallel displacement of the lysis curves (Fig. 8) is characteristic of a colloid osmotic swelling as it was shown by Wilbrandt for certain types of nonimmune hemolysis, but is opposed to his immune lysis (36).

The rate of swelling appears to be related to the type of solute in the external media. At present, it is difficult to explain why the smaller K^+ and Na^+ ions are also effective in delaying hemolysis and to some extent more than sucrose (see Fig. 7). It seems improbable that the differences in swelling before lysis of N-SRBC can be attributed alone to a differential effect of the various solutes used on the reaction mechanism of the complement cascade. It is, nevertheless, possible that ionic and nonionic solutes differ in their interaction with the membrane as they pass through the immune "lesion". In a discussion of a hydrophilic pore within a cylindrical protein barrier floating in the lipid bilayer (doughnut theory of Mayer, see ref. 39), one must also consider solute-water interactions. In osmotic lysis studies Good (37, 38) has shown that sucrose is more effective than K^+ ions in breaking water structure.

In the absence of an electrochemical driving force for electrolytes (Fig. 9), sucrose did not balance the osmotic pressure of intracellular hemoglobin. Thus, once the lesion is produced, sucrose enters the cell together with extracellular water (and salt) causing cell swelling and finally rupture of the cell with loss of hemoglobin (not necessarily through the lesions, see ref. 35, 40). If, on the basis of Mayer's one hit theory (41), one assumes that GPS complement produces one fixed pore (i.e. which does not expand with time) per N-SRBC with an effective

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[2] Valet, G., and W. Opferkuch. 1975. Mechanism of complement induced cell lysis. Demonstration of a three step mechanism of EAC1-8 cell lysis by C9 and of a nonosmotic swelling of erythrocytes. J. Immunol. In press.

diameter of d = ca. 60 Å or greater as proposed by Humphrey and Dourmashkin (42) and a length (l) equivalent to an average membrane thickness of 75–90 Å (39), the theoretical permeability coefficient for sucrose in such a cylindrical pore filled with water can be computed from Eq. 4:

$$P_{theor}^{suc} = \frac{\pi r^2 \cdot D}{A \cdot l};$$ (4)

where P_{theor}^{suc} = theoretical permeability coefficient in cm·s⁻¹; r = $\frac{1}{2} d$ = $3 \cdot 10^{-7}$ cm; A = $51 \cdot 10^{-8}$ cm²·cell⁻¹ (see Eq. 3); l = $7.5 \cdot 10^{-7}$ cm; D = diffusion coefficient for sucrose in water = $0.5223 \cdot 10^{-5}$ cm²·s⁻¹ (ref. 43). According to Eq. 4, P_{theor}^{suc} was computed to be about $4 \cdot 10^{-6}$ cm·s⁻¹ and thus about two orders of magnitude greater than the experimentally obtained value (P_{exp}^{suc} = $3 \cdot 10^{-8}$ cm·s⁻¹). This finding indicates that GPS complement produces in N-SRBC sensitized with IgM antibody a lesion which restricts the free passage of sucrose across the membrane, thus excluding the possibility of large, water-filled holes. From Eq. 4 it can also be easily seen that generating 100 holes per cell would make P_{theor}^{suc} even higher (i.e. $4 \cdot 10^{-4}$ cm·s⁻ms¹). Alternatively, assuming a hole with a radius of 4.4 Å, which is identical to the molecular radius of a single sucrose molecule (44), P_{theor}^{suc} would be about $8 \cdot 10^{-8}$ cm·s⁻¹. Although quite close to P_{exp}^{suc}, this value is incompatible with the view of free and unrestricted diffusion of sucrose for which a rather small ratio (ca. 0.1) of the radius of the molecule to that of the pore would be required (44).

The observation that sucrose permeates the complement-damaged membrane at a rate slower than free diffusion is consistent with the reports of others that the lesions seen on the surface of membranes after immune hemolysis may not penetrate its entire thickness (40, 42, 35). Preliminary electron microscopic screening of immune hemolyzed N-SRBC are affirmative (unpublished data).

SUMMARY

The rapid hemolysis by fresh guinea pig serum known to occur with neuraminidase-treated sheep red cells has been investigated with respect to the immunological and physiological properties of the lytic process. The following observations were made: (a) The susceptibility to hemolysis is directly proportional to the amounts of neuraminic acid enzymatically released from the cell surface. Complement lysis is mediated through binding of an IgM antibody protein to membranes of neuraminidase-treated cells. (b) Hemolysis is relatively temperature-independent above about 28° C but below which a decrease in the hemolysis rate occurs. Arrhenius activation energies above and below the transition temperature were therefore found to be different. (c) Colloid osmotic swelling of neuraminidase-treated high potassium sheep red cells with a chloride ion concentration ratio near unity suspended in high potassium medium could not be prevented by sucrose. Hence, colloid osmotic swelling before lysis must be due to the entrance of sucrose and water since sucrose was the only external solute not at equilibrium. (d) From the rate of swelling and the apparent flux of sucrose under nonsteady state conditions an experimental permeability coefficient (P) for sucrose of $3 \cdot 10^{-8}$ cm·s⁻¹ was computed. Comparison with a theoretical P of $4 \cdot 10^{-6}$ cm·s⁻¹ for sucrose freely permeating through a single, hypothetical membrane lesion per cell of 60 Å effective diameter indicates a membrane lesion which permits the passage of solutes larger than cations, but clearly constrains the free diffusion of sucrose.

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