The authors recognize that the dilution analog computer has limitations with respect to ease of manipulation and applicability to very complex models. It does have the advantages of simplicity, low cost, and availability.

REFERENCES

(1) J. D. Taylor and R. G. Wiegland, *Clin. Pharmacol. Therap.*, **3**, 464(1962).

(2) J. G. Wagner, "Use of Computers in Pharmacokinetics," Brook Lodge Symposium on Computers in Biomedical Research, November 1965.

(3) E. Krüger-Thiemer, W. Diller, L. Dettli, P. Bünger, and J. Seydal, *Antibiot. Chemotherapia*, **12**, 171(1964).

(4) O. W. Wendell, *Phys. Med. Biol.* **5**, 411(1961).

(5) J. G. Wagner, unpublished data.

ACKNOWLEDGMENTS AND ADDRESSES

Received June 4, 1969 from the *Research Laboratories, The Upjohn Company, Kalamazoo, MI 49001*

Accepted for publication July 25, 1969.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.

Mechanism of Hemolysis by Cationic Surface-Active Agents

TAMOTSU KONDO and MICHIKO TOMIZAWA

Abstract □ A study was made of the mechanism of hemolysis by cationic surface-active agents. Phospholipids were found to be released from the cell membrane prior to lysis by the cationic agents under the conditions of physiological pH and ionic strength, indicating an important role of electrostatic interaction between the surface-active cations and the phospholipid anions in hemolysis. The adsorption experiments showed that the binding of surface-active cations to the lipoprotein layer of cell membrane is a prerequisite for the hemolysis to take place. The temperature dependence of adsorption suggested a hydrophobic nature of the binding. It was proposed that the adsorbed surface-active cations make the channels through which the phospholipid molecules are removed out of the cell membrane and that the removal of phospholipids from the lipoprotein layer causes the alteration of protein conformation, thereby allowing the release of hemoglobin into the surrounding medium.

Keyphrases □ Hemolysis mechanism—cationic surfactants □ Cell membrane phospholipids—surfactant effect □ Phospholipid release—cell lysis □ Temperature dependence—membrane-surfactant binding □ TLC—separation, identification

Previous reports (1–3) from this laboratory have revealed that the surface-active cations with alkyl chain length from C_1 to C_{12} are able to cause hemolysis of dog red cells under the conditions of physiological pH and ionic strength, liberating phospholipids in considerable amount from the cell membrane prior to lysis. This has led us to a hypothesis that the surface-active cations strongly interact with the phospholipids in the cell membrane to give rise to hemolysis.

In this paper an attempt was made to examine the validity of the hypothesis in the case of red cells from other animal species than dog and to construct a common mechanism of hemolysis by cationic surface-active agents responsible for all kinds of red cells based on the experimental results obtained.

MATERIALS AND METHODS

Materials—Alkyl pyridinium iodides were synthesized and purified as previously described (4). Alkylamine hydrochlorides and polymethylenediamine dihydrochlorides were prepared by passing dried hydrogen chloride through the benzene solutions of the corresponding amines and diamines. The precipitated salts were recrystallized from ethanol.

Table I—Hemolytic Concentrations of Cationic Surface-Active Agents (m*M*/l.)

Compd.	Species: Dog	Rabbit	Sheep
$C_{12}H_{25}NH_2HCl$	3.41×10^{-1}	2.75×10^{-1}	5.23×10^{-1}
$C_{10}H_{21}NH_2HCl$	2.97	1.88	3.85
$C_8H_{17}NH_2HCl$	3.43×10	2.23×10	4.50×10
$C_6H_{13}NH_2HCl$	4.50×10^2	3.65×10^2	4.58×10^2
$C_{12}H_{25}PyI$	6.20×10^{-1}	4.72×10^{-1}	1.13
$C_{10}H_{21}PyI$	5.40	5.89	7.88
$C_8H_{17}PyI$	3.35×10	3.08×10	3.45×10

The red cell suspension for the experiments was prepared as follows. Citrated blood from normal, healthy animals (dog, rabbit, and sheep) was centrifuged and the cells were washed three times with the phosphate-buffered isotonic saline (pH 7.4) or nonbuffered isotonic saline. The former was used for the hemolysis experiments by alkyl pyridinium iodides, whereas the latter for the ones by alkylamine hydrochlorides and polymethylenediamine dihydrochlorides. The washed, packed cells were then suspended in the same medium as that used in washing to give a 2.5% v/v suspension. The number of cells in unit volume of the suspension was counted on a hemocytometer.

Hemolysis Techniques—The determination of the degree of hemolysis was made in the following way. Various dilutions of cationic surface-active agents in isotonic buffer or saline were made and brought to the required temperature (30°). Two milliliters of each dilution at the same temperature was pipeted into small test tubes, and then an equal volume of the cell suspension was added. The mixtures were allowed to react for 15 min. in the water bath with shaking. At the end of this period, the mixtures were immediately centrifuged to remove the unhemolyzed cells. The degree of hemolysis was evaluated by determining spectrophotometrically the amount of hemoglobin released in the supernatant liquid with a Bausch & Lomb spectrophotometer. The standard for 100% hemolysis was assumed to be given by an optical density value for complete hemolysis. Finally, the hemolysis curve was set up by plotting percent hemolysis against hemolytic agent concentration.

Surface Tension Measurements of Hemolytic Agents Solutions—The surface tension measurements were carried out using a du Nouy tensiometer.

Adsorption of Surface-Active Cations on Red Cells—The amount adsorbed of surface-active cations on dog red cells was estimated

Table II—Critical Micelle Concentrations of Cationic Surface-Active Agents (M/l)

Compd.	CMC
$C_{12}H_{25}NH_2HCl$	14×10^{-3}
$C_{10}H_{21}NH_2HCl$	48×10^{-3}
$C_8H_{17}NH_2HCl$	17.5×10^{-2}
$C_6H_{13}NH_2HCl$	—
$C_{12}H_{25}PyI$	4×10^{-3}
$C_{10}H_{21}PyI$	16×10^{-3}
$C_8H_{17}PyI$	64×10^{-3}

by the method adopted in a previous work (5). Thus, the total amount of surface active cations, c_x, needed to produce x% hemolysis after 1 hr. reaction time was determined as a function of cell count, N, in unit volume of the system:

$$c_x = a_x N + b_x$$

The values of a_x and b_x give the amounts adsorbed and unadsorbed of surface-active cations per cell, respectively, and were determined by the least-squares method at various degrees of hemolysis. The adsorption isotherms were obtained by plotting a_x against b_x.

Determination of Amount of Phospholipids Released—The determination of the amount of phospholipids released from the red cell membrane by surface-active cations was made by the method described in the previous paper (2). Forty milliliters of 10% v/v red cell suspension in the buffered or nonbuffered isotonic saline were pipeted into a 100-ml. glass stoppered flask and was added an equal volume of surface-active agent solution of a concentration far less than the hemolytic concentration. The mixture was then permitted to react for 15 min. at 30° in a thermostat with continuous shaking. At the end of the period, it was centrifuged without delay to separate the cells. The supernatant was withdrawn by a pipet and placed in a 200-ml. glass-stoppered flask, to which was added 40 ml. of a chloroform–methanol (2:1, v/v) mixture. Phospholipids were extracted by shaking for 6 hr. at 40°. The liquid was centrifuged, followed by separation in a separator. The upper layer was discarded, and lower chloroform-rich layer was concentrated under reduced pressure in nitrogen atmosphere at a temperature not higher than 40° to yield 0.25 ml. of the concentrated extract containing phospholipids.

Aliquots of the extract (usually 100 μl.) were spotted on thin-layer plates (0.25 mm. thick) using silica gel with 5% binder (Wakogel B5, Wako Pure Chemical Industries, Ltd., Osaka) as the adsorbent, and chloroform–methanol–water (65:25:4, v/v) mixture as the developing solvent. The plates had been air-dried for 20 min. and baked in an oven at 100° for 1 hr. before use. After developing, the separated phospholipid spots were colored by spraying with 4% ammonium molybdate solution in 12% perchloric acid containing 1.6% hydrochloric acid, followed by heating at 105° for 10 min.

The amounts of phospholipids on the chromatoplates were determined by an Atago Ozumar 8 recording densitometer (Atago Optical Works Co., Ltd., Tokyo).

RESULTS

Hemolytic Concentrations—In Table I are presented the minimum concentrations of cationic surface-active agents required to cause complete hemolysis for 2.5% v/v red cell suspension. These values were graphically evaluated on the corresponding hemolysis curves.

Table III—Relative Amounts of Phospholipids Released from Red Cell Membrane by Alkylamine Hydrochlorides[a]

	Species								
	Dog[b]			Rabbit[c]			Sheep[c]		
Phospholipid	C_{12}	C_8	C_4	C_{12}	C_8	C_4	C_{12}	C_8	C_4
Cephalin	1.8	1.6	1.5	2.23	1.32	1.18	1.43	1.12	1.08
Lecithin	2.4	2.4	1.6	3.34	1.62	1.29	3.72	2.92	1.72
Sphingomyelin							3.67	2.23	1.67

[a] Data are averages obtained from duplicate determinations. [b] Taken from *Reference* 2. [c] Concentration used of alkylamine hydrochlorides was 0.01 mM.

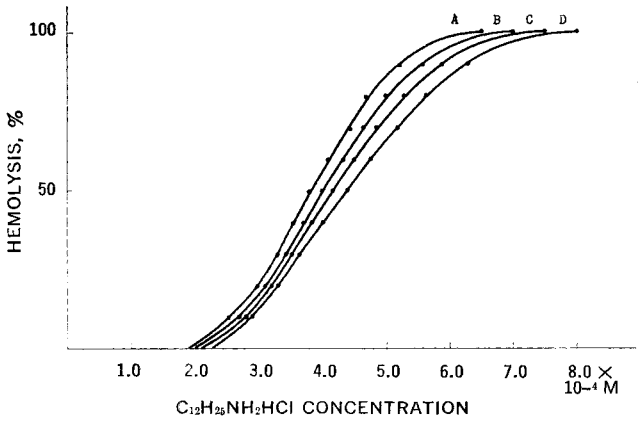

Figure 1—*Percent hemolysis* versus $C_{12}H_{25}NH_2HCl$ *concentration curves for dog red cell suspensions at 30°. Key: cell concentration % v/v:* A, *1;* B, *2;* C, *3;* D, *4*

An inspection of Table I clearly indicates the following facts. First, the longer the alkyl chain length of surface-active cations is, the lower is the hemolytic concentration, irrespective of animal species. This results from the increase in surface activity of each homologous series with increasing chain length. Secondly, the alkylamine hydrochlorides are, in general, more hemolytically active than the corresponding alkyl pyridinium salts. However, there seems to be no direct correlation between the surface activity and the hemolytic activity of these two homologous series since the critical micelle concentrations (CMC) as determined by surface tension measurements are not in parallel with the hemolytic concentrations. Thus, for example, the CMC values of dodecylamine hydrochloride and dodecyl pyridinium iodide are 14.0 and 4.0 mM, respectively, while the hemolytic concentrations of the former are smaller than those of the latter as shown in Table II. Thirdly, the sensitivity of red cells to the surface-active cations is in the order, rabbit > dog > sheep, which may reflect the difference in membrane composition.

The polymethylenediamine dihydrochlorides studied in this work were not hemolytically active enough to produce complete lysis. Thus, only dodeca- and decamethylenediamine dihydrochlorides could cause 80–90% and 20–30% hemolysis at 30 and 37°, re-

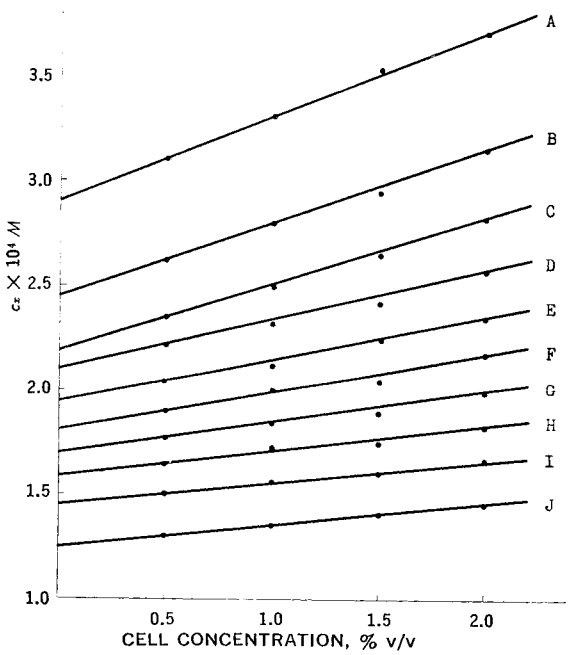

Figure 2—$C_{12}H_{25}NH_2HCl$ *concentration needed for various degrees of hemolysis,* c_x, versus *cell concentration at 30°. Key: hemolysis percent:* A, *100;* B, *90;* C, *80;* D, *70;* E, *60;* F, *50;* G, *40;* H, *30;* I, *20;* J, *10.*

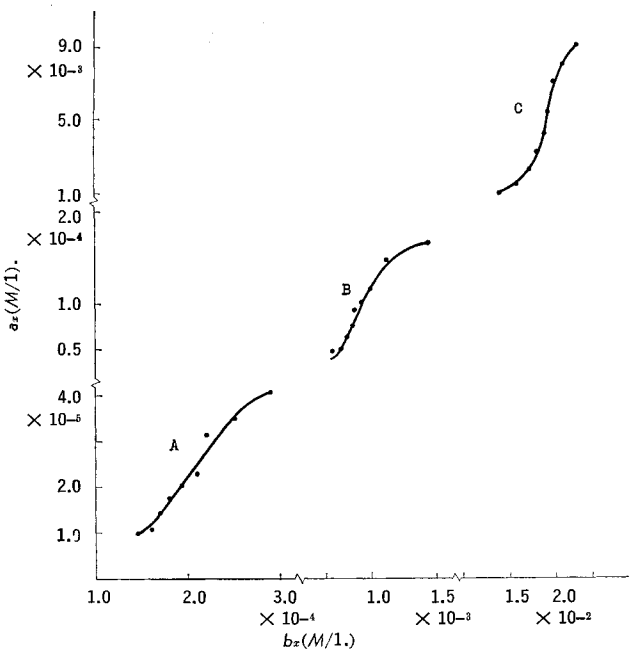

Figure 3—*Adsorption isotherms for alkylamine hydrochlorides, RNH_2HCl, at 30°. Key: R:* A, $C_{12}H_{25}$; B, $C_{10}H_{21}$; C, C_8H_{17}.

spectively, whereas the lower members were not active at all within the limit of their solubility. These agents did not lower appreciably the surface tension of water (at most down to 55 dynes/cm.) even when hemolysis was observed.

Amounts of Phospholipids Released—Table III gives the amounts of cephalin, lecithin, and sphingomyelin released from red cell membrane by alkylamine hydrochlorides.

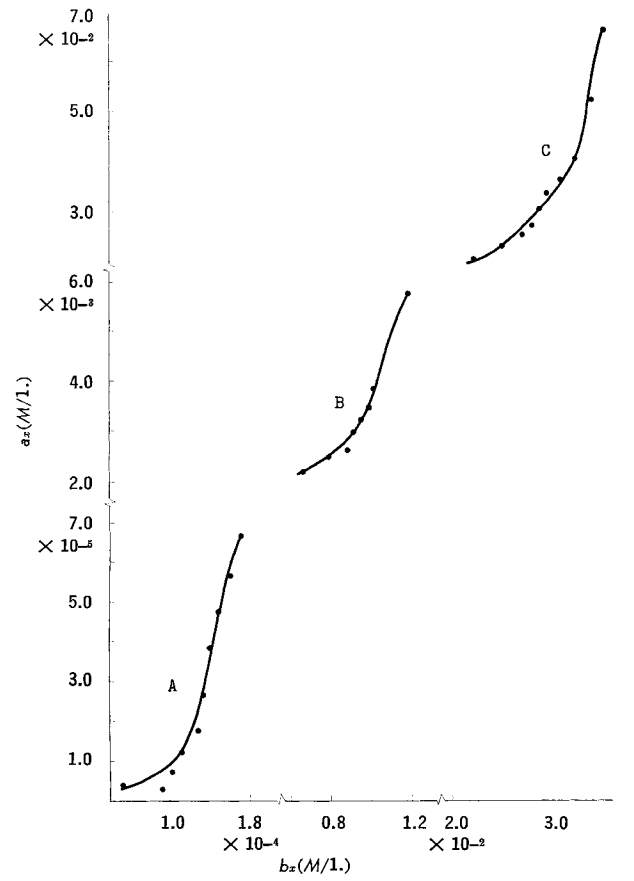

Figure 4—*Adsorption isotherms for alkyl pyridinium iodides, RPyI, at 37°. Key: R:* A, $C_{12}H_{25}$; B, C_8H_{17}; C, C_4H_9.

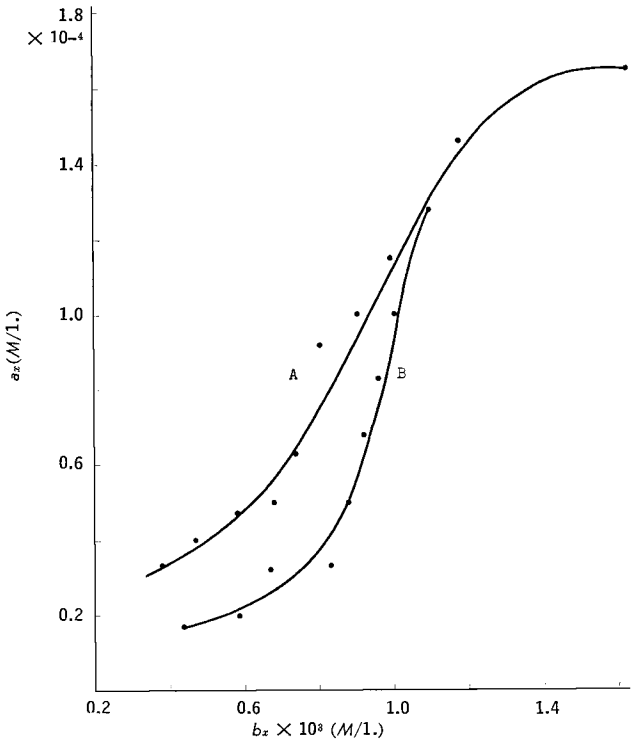

Figure 5—*Effect of temperature on the adsorption isotherm for $C_8H_{17}NH_2HCl$. Key: temperature:* A, *30°*; B, *37°*.

The phospholipid composition of red cell membrane varies from one species to another (6, 7). Thus, dog and rabbit red cells are abundant in cepahlin and lecithin and not in sphingomyelin, whereas sheep red cells contain sphingomyelin as a major component. This situation appears to be reflected in the amounts of phospholipids released as shown in the table. The alkyl chain length affects the amounts released to a certain extent.

All members of the polymethylenediamine dihydrochlorides were also capable of liberating phospholipids from red cells.

Adsorption of Surface-Active Cations—Figures 1 and 2 show the hemolysis curve and hemolytic concentration *versus* cell concentration curve for dodecylamine hydrochloride at 30°. Similar results were obtained with other cationic agents employed.

Adsorption isotherms for alkylamine hydrochlorides and alkyl pyridinium iodides are shown in Figs. 3 and 4, respectively. All of the isotherms are sigmoid in shape, suggesting a complex nature of the adsorption of these agents. The effect of increasing alkyl chain length at a fixed temperature is to decrease in stepwise the amount adsorbed of the agents necessary to produce lysis.

The temperature dependence of the adsorption is indicated in Fig. 5 for octylamine hydrochloride as a typical example. A rise in temperature brings about an increase in hemolytic activity of the cationic agents. The same trend was true of all other agents.

In Table IV are summarized the values of a_{100} and b_{100} at 30 and 37° for aklylamine hydrochlorides and alkyl pyridinium iodides. Both a_{100} and b_{100} decrease with increasing alkyl chain length. An increase in temperature results in a decrease in a_{100} and b_{100}.

DISCUSSION

The experimental results reported herein seem to confirm the hypothesis that a strong interaction of surface-active cations with phospholipids in the red cell membrane causes hemolysis of mammalian red cells. This is most evidenced by the release of phospholipids in large amount from the cell membrane prior to lysis as indicated in Table II. In order to set up a detailed mechanism of hemolysis, however, it is necessary to consider the adsorption of surface-active cations on red cells.

Recent works on the red cell surface (8–10) have established that the negative surface charges arise from the carboxyl groups of *N*-acylated neuraminic acids. In view of this fact, the adsorption sites of surface-active cations at the outer surface of plasma mem-

Table IV—Values of a_{100} and b_{100} at 30 and 37° (ions/cell)

Compd.	30°		37°	
	a_{100}	b_{100}	a_{100}	b_{100}
$C_{12}H_{25}NH_2HCl$	1.40×10^8	1.00×10^9	1.25×10^8	7.72×10^8
$C_{10}H_{21}NH_2HCl$	5.71×10^8	5.64×10^9	4.43×10^8	3.74×10^9
$C_8H_{17}NH_2HCl$	3.11×10^{10}	7.77×10^{10}	6.92×10^9	6.92×10^{10}
$C_6H_{13}NH_2HCl$	3.51×10^{11}	8.79×10^{11}	1.25×10^{11}	8.40×10^{11}
$C_{12}H_{25}PyI$	2.39×10^8	1.08×10^9	2.32×10^8	5.77×10^8
$C_8H_{17}PyI$	2.47×10^{10}	5.29×10^{10}	1.99×10^{10}	4.02×10^{10}
C_4H_9PyI	3.18×10^{11}	1.28×10^{12}	2.28×10^{11}	1.20×10^{12}

brane are most likely to be the carboxyl groups. The surface-active cations thus adsorbed will neutralize the negative charges, thereby concomitantly making the outer cell surface lipophilic by their alkyl chains. This will facilitate the surface-active cations in solution to concentrate at the cell surface and make them easier to penetrate into the inner part of the membrane, even though their surface activity is not strong enough to lower the surface tension of water to a large extent. This may also cause the aggregation of red cells. Actually, in some cases, the formation of aggregates prior to lysis was observed on the addition of surface-active cations to red cell suspension (3). However, this adsorption does not necessarily produce lysis (3) and the total number of surface-active cations adsorbed per cell to cause complete hemolysis is more than ten times the number of carboxyl groups per cell, which ranges from 10^6 to 10^7 for all red cells of various animal species thus far obtained (11, 12). More than 90% of surface-active cations required to cause complete lysis will penetrate, therefore, into the inner part of plasma membrane where they may be adsorbed in a somewhat different manner from that at the outer surface.

A significant role of hydrophobic interaction should be emphasized in the binding of surface active cations to the inner part of plasma membrane. This view will be supported by the following facts. First, the ratio of the amount adsorbed per cell, a, to the amount remaining in the surrounding medium, b, of surface-active cations increases as the temperature rises if the chain length is sufficient. Thus, for example, the values of ratio a_{100}/b_{100} for dodecyl pyridinium iodide and dodecylamine hydrochloride increase from 0.22 and 0.14 at 30° to 0.40 and 0.16 at 37°, respectively, indicating the increased affinity of these surface-active cations to the membrane at higher temperature. This trend is more favored by hydrophobic than ionic bond since the former becomes stronger as the temperature increases at low temperatures (13, 14) while the latter is expected to be weakened by increase in thermal motion of the molecules involved. The stronger affinity to the membrane and larger temperature effect of the pyridinium salt will be ascribable to the presence of an aromatic ring in the molecule. However, in order to correlate the hemolytic activity of cationic surface-active agents with their chemical structure, much further study will be needed using a wide variety of cationic agents. The tendency of surface-active cations to remain in the aqueous phase will increase as the chain length decreases. This may lead to the inversion of the temperature effect. Secondly, the amount adsorbed of polymethylenediamine dihydrochlorides is not large enough to give rise to lysis when the number of methylene groups is less than 8. Moreover, the ratio, a/b, for the higher members increases with temperature. For instance, the values of a_{80}/b_{80} for dodecamethylenediamine dihydrochloride increases from 0.06 at 30° to 0.12 at 37°, suggesting again an important role of hydrophobic bond.

The surface-active cations adsorbed on the inner part of plasma membrane by hydrophobic bond will interact electrostatically with the phospholipid molecules in the innermost lipoprotein layer of the membrane, which are prevalently in the anionic form under the condition of physiological pH. These surface-active cations will also turn out to make the channels through which the interacting phospholipid molecules move out of the cell membrane. The removal of phospholipid molecules to a certain extent from the lipoprotein layer may result in the alteration of protein conformation to allow the release of hemoglobin into the surrounding medium, thereby producing hemolysis.

Recently, a large body of evidence has been accumulated, showing the predominant binding mode between lipids and protein is hydrophobic rather than electrostatic (15–20). The hydrophobic bonding established so far implies deep penetration of the hydrophobic region of protein by the hydrocarbon residues of phospholipid molecules. The surface-active cations adsorbed will exert the electrostatic force on the phospholipid molecules to leave from the protein chains with a net decrease in free energy of the system. Thus, the decrease in enthalpy brought about by the electrostatic interaction between the phospholipid molecules and the surface-active cations will more than make up for the entropy decrease caused by the destruction of hydrophobic bonds, resulting in the net negative free energy change. The entropy change due to the alteration of protein conformation may compensate in part the decrease in entropy mentioned above.

A plausible explanation for the effect of temperature on hemolysis may be given by the consideration of entropy change involved. An entropy increase will arise from an increase in the number of configurations of protein due to temperature rise. The increase in entropy will favorably contribute to the net negative free energy change in the hemolytic process caused by surface active cations. This may in turn lead to enhanced hemolysis at elevated temperatures.

REFERENCES

(1) T. Kondo and M. Tomizawa, *J. Colloid Interface Sci.*, **21,** 224(1966).

(2) T. Kondo and M. Tomizawa, *Chem. Pharm. Bull. (Tokyo)*, **16,** 738(1968).

(3) T. Kondo and M. Tomizawa, *J. Pharm. Sci.*, **58,** 255(1969).

(4) K. Mrguro and T. Kondo, *J. Chem. Soc. Japan, Pure Chem. Sect.*, **80,** 818(1959).

(5) T. Kondo and M. Tomizawa, *J. Pharm. Sci.*, **57,** 1246(1968).

(6) P. F. Elbers, in "Recent Progress in Surface Science," Vol. 2, J. F. Danielli, K. G. A. Pankhurst, and A. C. Riddiford, Eds., Academic, New York, N. Y., 1964, p. 467.

(7) G. J. Nelson, *Biochim. Biophys. Acta*, **144,** 221(1967).

(8) E. Klenk and G. Uhlenbruck, *Z. Physiol. Chem.*, **311,** 227 (1958).

(9) G. M. Cook, D. H. Heard, and G. V. F. Seaman, *Nature*, **191,** 44(1961).

(10) R. M. Glaser and H. C. Mel, *Biochim. Biophys. Acta*, **79,** 604(1964).

(11) E. H. Eylar, M. A. Madoff, O. V. Brody, and J. L. Oncley, *J. Biol. Chem.*, **237,** 1992(1962).

(12) G. V. F. Seaman and G. Uhlenbruck, *Arch. Biochem. Biophys.*, **100,** 493(1963).

(13) G. Nemethy and H. A. Scheraga, *J. Chem Phys.*, **36,** 3382 (1962).

(14) G. Nemethy, *Angew. Chem.*, **6,** 195(1967).

(15) D. E. Green and S. Fleisher, *Biochim. Biophys. Acta*, **70,** 554(1963).

(16) S. Richardson, H. O. Hultin, and S. Fleisher, *Arch. Biochem. Biophys.*, **105,** 254(1964).

(17) A. D. Brown, *J. Mol. Biol.*, **12,** 491(1965).

(18) A. A. Benson, *Ann. Rev. Plant Physiol.*, **15,** 1(1964).

(19) A. A. Benson, *J. Am. Oil Chemists' Soc.*, **43,** 265(1966).

(20) D. E. Green and J. F. Perdue, *Proc. Natl. Acad. Sci.*, **55,** 1295(1966).

ACKNOWLEDGMENTS AND ADDRESSES

Received May 27, 1969 from the *Faculty of Pharmaceutical Sciences, Science University of Tokyo, Shinjuku, Tokyo, Japan.*

Accepted for publication August 5, 1969.

The authors wish to express their thanks for the experimental aids of S. Akutsu, S. Ito, K. Obi, M. Saito, and A. Usami.