Hemolysis by Nonionic Surface-Active Agents

By TAMOTSU KONDO and MICHIKO TOMIZAWA

The hemolytic actions of polyoxyethylene glycol monododecyl ethers, $C_{12}H_{25}(C_2H_4O)_nOH$, have been studied. The amount required of these agents to produce lysis was found to be linearly related to the red cell concentration within experimental error. This was interpreted as showing the significant role of the adsorption of nonionic agent molecules onto the red cells prior to lysis. The average amount adsorbed of the agents necessary to cause lysis was estimated.

HEMOLYTIC ACTIONS of nonionic surface-active agents have been described by several authors (1–3). The mechanisms of the hemolysis, however, have not yet been well understood.

Pethica and Schulman stressed the importance of the surface effects of nonionics (4). Thus, they claimed that the nonionics used in their work lysed on attaining surface pressures of 34 dynes/cm. or above. On the other hand, the present authors suggested a significant role of the adsorption of nonionics onto the red cell membranes, because they found a proportionality between the hemolytic concentration of polyoxyethylene carboxylates and the cell count (5). The estimation of the amount adsorbed of the nonionics, however, was not possible owing to the heterogeneity of the materials.

Recently, polyoxyethylene glycol monoalkyl ethers have become available, which are homogeneous with respect to alkyl and polyoxyethylene chain length. This has now made it possible to determine the amount of nonionics adsorbed. The aim of this study has been to confirm the adsorption mechanism and to find a relation between the hemolytic activity and molecular structure of polyoxyethylene glycol monoalkyl ethers.

EXPERIMENTAL

Materials—The nonionic surface-active agents used were polyoxyethylene glycol monododecyl ethers having the general formula, $C_{12}H_{25}(C_2H_4O)_nOH$. These agents were synthesized by a successive condensation reaction between carefully fractionated dodecyl alcohol and diethylene glycol and/or triethylene glycol (6), and were generously supplied by Nihon Surfactants, Co., Ltd., Tokyo. The homogeneity of these nonionics were checked by gas or thin-layer chromatography. In Table I are listed the critical micelle concentrations and cloud points of the nonionics, which are referred to as $C_{12}E_n$ for brevity.

The red cell suspension used was prepared from dog blood as follows. Citrated blood was centrifuged and the cells were washed three times with the phosphate-buffered isotonic saline (pH 7.4). The washed, packed cells were then suspended in the above medium to yield a suspension of a desired concentration. A concentration of 1% v/v of the suspension was found in a hemocytometer to correspond to a cell count of 170–180 $\times$ 10^6/ml.

Hemolysis Techniques—The determination of the degree of hemolysis was done in the following way. Two milliliters of the cell suspension were pipeted into test tubes, into which an equal volume of various concentrations of the nonionics was added quickly by a syringe to prevent any local lysis. The mixtures were then allowed to react for an hour in a water bath controlled at 27° or 37 ± 0.1° with shaking so that the adsorption of nonionic molecules attained an equilibrium as quickly as possible. At the end of this period, the mixtures were immediately centrifuged to remove the unhemolyzed cells. The degree of hemolysis was estimated by determining spectrophotometrically the amount of hemoglobin released in the supernatant liquid.

Received March 12, 1968, from the Faculty of Pharmaceutical Sciences, Science University of Tokyo, Shinjuku, Tokyo, Japan.

Accepted for publication April 18, 1968.

The authors are indebted to Misses Naoko Fukuhara and Tomoko Yoshida for their assistance in this work.

RESULTS AND DISCUSSION

Hemolytic Activity—Effect of Polyoxyethylene Chain Length—Figure 1 shows the relation between the final percent hemolysis and the concentration of the nonionics studied at a cell concentation of 5% v/v.

All the hemolysis curves are of the similar shape and they shift toward the higher concentration region as the chain length of polyoxyethylene becomes longer. This means that the hemolytic activity of $C_{12}E_n$ decreases as the value of n increases. The same tendency was observed at all the cell concentrations studied.

Effect of Cell Concentration—In general, as the cell

TABLE I—CRITICAL MICELLE CONCENTRATIONS AND CLOUD POINTS OF NONIONIC SURFACE-ACTIVE AGENTS

Material	Cloud Point, °C.	C.M.C.	Temp., °C.
$C_{12}E_6$	55	$8.0 \times 10^{-5} M$	30
$C_{12}E_7$	70	$8.8 \times 10^{-5} M$	30
$C_{12}E_8$	82	$1.4 \times 10^{-4} M$	30

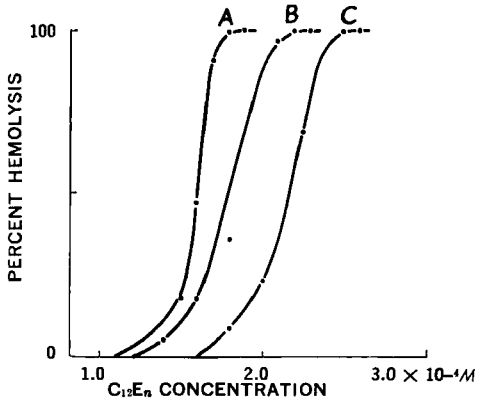

Fig. 1—Percent hemolysis versus *$C_{12}E_n$ concentration curves for 5% red cell suspension at 37°. Key:* A, n = *6;* B, n = *7;* C, n = *8.*

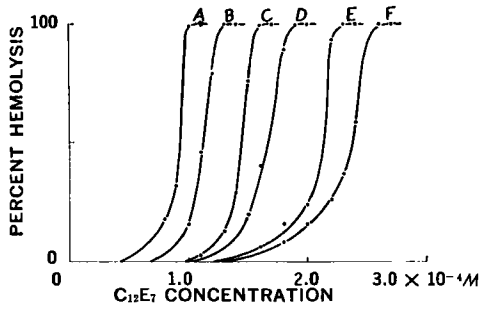

*Fig. 2—Shift of $C_{12}E_7$ hemolysis curves with change in cell concentration at 27°. Key: cell concentration % v/v—*A, *0.5;* B, *1;* C, *2;* D, *3;* E, *4;* F, *5.*

TABLE II—$C_{12}E_7$ CONCENTRATIONS REQUIRED TO PRODUCE 100% HEMOLYSIS FOR 5% v/v RED CELL SUSPENSION

Run	27°C.	37°C.
1	$1.2 \times 10^{-4}M$	$0.96 \times 10^{-4}M$
2	$1.4 \times 10^{-4}M$	$1.2 \times 10^{-4}M$
3	$1.3 \times 10^{-4}M$	$1.1 \times 10^{-4}M$

concentration was raised, the hemolytic curves were shifted to higher concentrations of the nonionics without change in shape. A typical example for $C_{12}E_7$ is given in Fig. 2. This result may rule out the possibility of the surface pressure mechanism, because the hemolytic concentrations are independent of surface tension of solutions.

Effect of Temperature—The rise in temperature appeared to make the red cells more sensitive to the lyzing agents. Thus, the hemolytic concentrations of the nonionics at 37° were lower than those at 27°. Table II gives the $C_{12}E_7$ concentrations to cause 100% hemolysis at both temperatures.

In connection with these findings, it is worth noting that the release of lipids from the red cell membranes was detected after shaking the cell suspension without the presence of surface-active agent in water bath for an hour. Moreover, the amount released of the lipids was observed to increase as the temperature rose. It may be inferred, therefore, that the lipid release from the red cell membranes correlates with the adsorption of the nonionics in the hemolysis, especially at elevated temperatures.

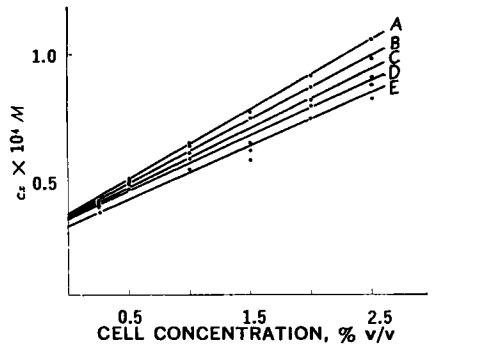

Fig. 3—$C_{12}E_7$ concentration required for various degrees of hemolysis, c_x, versus *cell concentration at 37°. Key: hemolysis percent—*A, *100;* B, *90;* C, *60;* D, *40;* E, *20.*

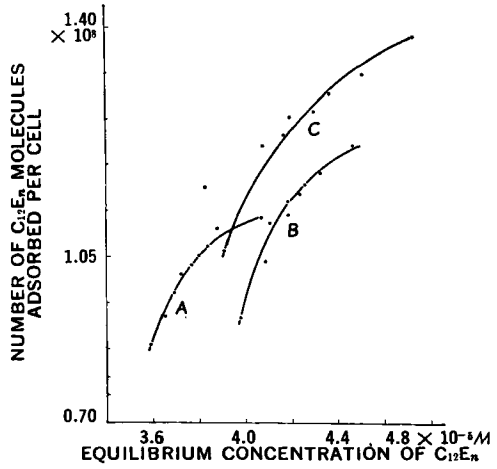

Fig. 4—Adsorption isotherms for $C_{12}E_n$ at 27°. Key: A, n = *6;* B, n = *7;* C, n = *8.*

Adsorption—The amount adsorbed of the nonionics on the red cells was estimated by an indirect method proposed by Thron (8), because several attempts have failed to determine it directly due presumably to the presence of surface-active materials released from the red cells. This method is based on the assumption that an individual cell undergoes lysis if and only if it adsorbs nonionic agent molecules in excess of a certain amount (not necessarily the same amount for different individual cells). If each cell comes into an adsorption equilibrium with its surroundings, then its uptake of the nonionic agent will be a function of the concentration of unadsorbed nonionic agent.

When the free nonionic agent concentration is b_x, where x is a given % hemolysis, the average amount adsorbed per cell has a corresponding value a_x, and the total amount adsorbed by the cells is a_xN, N being the cell count per unit volume of the system. This quantity and the free nonionic agent concentration, b_x, make up the total amount of nonionics, c_x, required to produce x% hemolysis:

$$c_x = a_xN + b_x$$

This equation means that a linear relation should hold between c_x and N. As shown in Fig. 3 the linear relation was obtained within experimental error.

Using the values of a_x and b_x, determined by the least-square method at various degrees of hemolysis, an adsorption isotherm can be set up. The results for the nonionics used at 27° are shown in Fig. 4.

As the polyoxyethylene chain length is decreased, the amount adsorbed necessary to cause lysis is also decreased. This will reflect an enhanced effectiveness in hemolysis of the nonionics with shorter polyoxyethylene chain. Thus, for example, the $C_{12}E_6$ molecules are more easily adsorbed onto the red cells from the solution and more strongly incorporated into the interior of cell membrane than are the $C_{12}E_7$ or $C_{12}E_8$ molecules. In other words, $C_{12}E_6$ causes the complete lysis at lower concentrations than does $C_{12}E_7$ or $C_{12}E_8$.

In Table III are given the amounts adsorbed of $C_{12}E_7$ per cell at 100% hemolysis. A decrease in the

TABLE III—NUMBER OF $C_{12}E_7$ MOLECULES ADSORBED PER CELL AT 100% HEMOLYSIS

Run	27°C.	37°C.
1	1.3×10^8	1.1×10^8
2	1.2×10^8	1.0×10^8
3	1.0×10^8	0.86×10^8

amount adsorbed is clearly seen with a rise in temperature.

As the slope of the adsorption isotherms at 37° was generally steeper than that at 27°, the rise in temperature would result in an increased tendency of the adsorption of nonionic agent molecules onto the red cell surface from aqueous solution owing to the temperature-dependent hydration of the molecules, thereby promoting the hemolysis. A similar adsorption behavior of the polyoxyethylene glycol monoalkyl ethers has been reported (9). The increased release of lipids from the red cells mentioned earlier may also contribute to a certain extent to the enhancement of the hemolysis at elevated temperatures.

As to the attacking site of the polyoxyethylated nonionics molecules, Mima and co-workers have concluded, based on the monolayer experiments, that the cholesterol portion in the cell membrane would be predominant (10). There still remains some doubt on this conclusion. In view of the limited scope of this study, however, any conclusion on the attacking site of the nonionics cannot be drawn.

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Keyphrases

Hemolysis—nonionic surfactants
Polyoxyethylene glycol monododecyl ethers—hemolysis
Temperature effect—hemolysis
Erythrocytes—surfactant adsorption
Spectrophotometry—analysis

Simple Method for Resuscitating Rats from Ether Overdosage

By L. F. SANCILIO, P. KRAUS, C. MYERS, and L. WAGNER

A simple method using compressed air for resuscitating rats from ether overdosage is described. Exposure to ether vapors for 55 and 90 sec., respectively, was lethal to 70 and 100 percent of the animals. The compressed air technique afforded protection to animals exposed to the anesthetic for 55, 90, 120, and 150 sec., while the conventional hand technique was ineffective against the 55- and 90-sec. exposure.

DURING THE PERFORMANCE of various experimental techniques in rats, *e.g.*, intrapleural injection (1), cotton pellet implantation (2), and adrenalectomy (3), using ether as the anesthetic agent, marked respiratory depression leading to death sometimes occurs unless the animal is immediately hyperventilated. This study describes a simple and effective technique used in our laboratory for reviving rats manifesting respiratory arrest following exposure to this anesthetic agent.

METHOD

Compressed air is required in performing this technique. In our laboratory, the air is pressurized at 60 p.s.i., and the tapered outlet (labcock) contains a valve to regulate its flow. The depressed animal is placed in a prone position in the palm of the hand with the head held gently but firmly between the thumb and index finger. The valve is partially opened (approximately 25%), and the air directed toward the rat's nostrils. The animal is moved back and forth rapidly across the stream of air to allow for inflation and deflation of the lungs. Spontaneous breathing is usually restored within 30 sec. An excess of pressurized air must be avoided as this will inflate the gastrointestinal tract.

The following study was undertaken to demonstrate the superiority of the compressed air to the manual technique. In the latter method, intermittent pressure was applied to hyperventilate the animal by placing the thumb on one side and the index and ring fingers on the other side of the rib cage.

Charles River CD rats (100–150 Gm.) of either sex were etherized in the following manner. One hundred milliliters of ether was poured into a 41-oz. glass dressing jar (Aloe Medical), the base of which was immersed in a water bath maintained at 39°. Ether was added periodically to replace that which had evaporated. The animal was placed on a wire mesh platform 7.5 cm. (3 in.) from the floor of the jar. After covering the jar, the exposure time to the ether vapors was determined with a Universal timer.

Received September 11, 1967, from the Therapeutics Research Division, Miles Laboratories, Inc., Elkhart, IN 46514
Accepted for publication February 14, 1968.