

and a number of theories^{16-20,27)} proposed on the mode of action of sulfonamides, it may be followed that there are two binding sites essential to the action located apart about 6.5 to 7 Å each other on the enzyme receptor: a site for specific binding of N⁴-nitrogen and a cationic site for non-specific and electrostatic binding of the acidic group of PABA or sulfonamides. Besides these major bindings, there may possibly be other non-specific and non-essential bindings somewhat contributing to the increasing action such as hydrophobic binding, hydrogen binding, charge transfer forces, and π -hydrogen binding between the substituents on N¹ of sulfonamides and the receptor protein. The strength of the specific binding of N⁴-nitrogen to the receptor may be approximately same with PABA and many sulfonamides, while the strength may be diverse in case of non-specific bindings. It was reported that the ability of non-specific binding of sulfonamides to a number of different proteins and macromolecular substances increased approximately in the same sequence as to serum albumin.¹⁵⁾ Accordingly the explanation on the relationship between bacteriostatic activities and ability of protein bindings of sulfonamides proposed in the previous paper¹⁾ may be reasonable.

From the general point of view, however, the activity of sulfonamides seems to be related to many other properties such as solubility, permeability to biological membranes, and metabolism rate. Most of these properties may be more or less dependent on the electronic structure of the molecules. Consequently, it may be a matter of course that the Q -value, one of the indices for the electronic structure, is approximately related to the bacteriostatic activities as well as the protein-binding properties of sulfonamides. A better correlation is probably expected by a more complete calculation procedure.

Anyway, it is of great interest to note that some biological properties can be predicted by the calculation of an electronic index, and furthermore that the index may play the part of a pilot for creation of new potent sulfonamides.

Acknowledgement The authors are very grateful to Professors Z. Tamura and M. Tsuboi of the University of Tokyo for valued criticisms and advices, and to Prof. Emeritus T. Akiba of the University of Tokyo and Director, and Dr. H. Sano, Chief Member of these Laboratories for continuing encouragement.

27) M. Tsuruoka, *Yakugaku Zasshi*, **71**, 336, 347, 350, 354 (1951).

[Chem. Pharm. Bull.]
[16(4) 738-740 (1968)]

UDC 612.397.8-08

Release of Lipids from Red Cell Membrane by Surface-active Agents

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(Received June 30, 1967)

In due course of studies on the hemolytic action of homologous surface-active electrolytes, a marked difference in the action between the surface-active cations and anions has been found²⁾. Thus, it has been demonstrated that the cations with shorter alkyl chain than octyl radical still keep the hemolytic activity, while the corresponding members of the anions are not

1) Location: 12 Funakawara-machi, Ichigaya, Shinjuku-ku, Tokyo.

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

active at all. This leads us to an idea that the site and/or mode of action of the cations may differ from that of the anions, though both types of the ions may interact with lipids. In fact, a larger amount released of phospholipids was detected for the cations than the anions by means of thin-layer chromatography²⁾. On the other hand, a similar study on the hemolytic action of nonionic surface-active agents has shown no appreciable release of phospholipids prior to lysis.³⁾

These findings have prompted us to carry out a more quantitative study on the release of lipids from red cell membrane at the initial stage of the hemolytic process caused by surface active agents. This note describes some of the results obtained in the experiments and their possible implications.

Experimental

Red Cell Suspension—The red cell suspension used in this work was prepared as follows. Citrated dog blood was centrifuged and the packed cells were washed three times on the centrifuge with the phosphate-buffered isotonic saline (pH=7.1). The washed cells were then suspended in the above medium to yield a 10% v/v suspension.

Surface-active Agents—Sodium alkyl sulfates and alkylamine hydrochlorides (C₄, C₈, and C₁₂ compounds) were the same lots as those used in a previous work.²⁾ Octaoxyethylene laurate, a nonionic agent, was provided by Dr. N. Ohba of Nikko Chemicals Co., Tokyo.

All solutions of the surface-active agents were made up with the buffered isotonic saline.

Lipid Extraction—Forty milliliters of the red cell suspension were pipetted into a 100 ml glass stoppered flask and was added an equal volume of surface-active agent solution. The concentrations used⁴⁾ of solutions of the cationic, anionic, and nonionic agents were 0.001, 0.01, and 0.05 mM, respectively, all of which were far less than the hemolytic concentrations of these agents. Thus, the hemolytic concentrations of the agents with C₁₂ chain were 2.0, 1.0, and 2.0 mM, respectively.⁵⁾

The mixture was then allowed to react for 15 min at 30° in a thermostat with continuous shaking. At the end of this period, it was centrifuged without delay to remove unhemolyzed cells.

The supernatant was withdrawn by a pipette and placed in a 200 ml glass stoppered flask, to which was added 40 ml of a chloroform-methanol (2:1, v/v) mixture. Lipids were extracted by shaking for 6 hours at 40°. The liquid was centrifuged, followed by the separation in a separatory funnel. The upper layer was discarded. The solvent was distilled out of the lower chloroform-rich layer containing lipids to leave 0.25 ml of the concentrated extract in a nitrogen atmosphere under a reduced pressure at 40°.

The same procedures were applied to the mixture of red cell suspension and the buffered saline to detect the release of lipids in the absence of surface-active agent.

In addition, the total amounts of lipids in red cell membrane were determined after osmotically hemolyzing red cells by distilled water.

Chromatography—Aliquots of the extract (usually 100 μl) were spotted on thin-layer plates (0.25 mm thick) using Wakogel B-5 (silica gel with 5% binder, a product of Wako Pure Chemical Industries, Ltd., Osaka) as an absorbent, and petroleum ether-ether-acetic acid (60:40:1, v/v) and chloroform-methanol-water (65:25:4, v/v) mixtures as developing solvents for cholesterol and phospholipids, respectively. The plates had been air-dried for 20 min and baked in an oven at 100° for an hour prior to use. After developing, the separated spots of lipids were colorated by spraying with the reagents described below, followed by heating at 105° for 10 min.

The spraying reagents were 33% antimony trichloride solution in chloroform for cholesterol and 4% ammonium molybdate solution in 12% perchloric acid for phospholipids.

Quantitative Determination of Lipids—The amounts of lipids on the chromatoplates were determined by an Atago Ozumar 8 recording densitometer (Atago Optical Works Co., Ltd., Tokyo) which permits simultaneous integration of the area under absorption curve on a densitogram.

Results and Discussion

Lecithin and cephalin were selected as the phospholipids to be determined, because they are major phospholipid components in dog red cell membrane.⁶⁾

3) T. Kondo and M. Tomizawa, *J. Pharm. Soc. Japan*, **86**, 251 (1966).

4) These were chosen to give comparable release of lipids for all surface-active agents.

5) No change in shape and size of red cells was detected microscopically under these conditions.

6) E. Condrea, Z. Mammon, S. Aloof, and A. DeVries, *Biochem. Biophys. Acta*, **84**, 365 (1964).

The results obtained are summarized in Table I. The figures in this table are the ratios of the released amounts of the lipids by the surface-active agents to those by the buffered saline alone.

TABLE I. Relative Amounts Released of Lipids by Surface-Active Agents

Surface-active agents	Lecithin	Cephalin	Cholesterol
None	1.0	1.0	1.0
$C_{11}H_{23}COO(C_2H_4O)_8H$	1.3	1.2	1.4
$C_{12}H_{25}NH_2HCl$	2.4	1.8	0.9
$C_8H_{17}NH_2HCl$	2.4	1.6	
$C_4H_9NH_2HCl$	1.8	1.5	
$C_{12}H_{25}OSO_3Na$	2.1	1.7	1.0
$C_8H_{17}OSO_3Na$	2.0	1.5	
$C_4H_9OSO_3Na$	1.7	1.4	

The effect of ionic head of the surface-active agents on the extraction of the phospholipids seems to be stronger than that of nonionic one, indicating ionic nature of the interaction of the phospholipids with the surface-active agents.

A considerable difference in phospholipid extracting power between the cationic and anionic agents is also noted. Thus, the cationic agents at 0.001 mM have almost as strong an extracting power as do the anionic agents at 0.01 mM.

As to the effect of chain length of hydrophobic tail of the ionic agents, we may say the phospholipid extracting power increases with alkyl chain length for both of the cationic and anionic agents.

In all cases mentioned so far, approximately 1% of total phospholipids present in red cell membrane was found to be released by surface-active agents.

The nonionic agent is likely to be slightly more effective in cholesterol extracting power than the ionic agents. In this case, the amount released of cholesterol is about 1–2% of the total amount of this lipid in red cell membrane.

These results will be very suggestive in further study of the mechanism of hemolysis by surface-active agents.

Acknowledgement The authors are indebted to Dr. H. Moriya for supplying fresh dog blood throughout this work. The gift of nonionic surface-active agent from Dr. N. Ohba of Nikko Chemicals Co., is also gratefully acknowledged.