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ACTION OF SURFACE-ACTIVE SUBSTANCES ON BIOLOGICAL MEMBRANES

IV. HEMOLYTIC AND MEMBRANE-PERTURBING ACTION OF HOMOLOGOUS SERIES OF β-D-GLUCOPYRANOSYL-1-ALKYLPHOSPHATES

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Summary

The hemolytic action of a homologous series of β-D-glucopyranosyl-1-alkylphosphates on human erythrocytes has been examined. The agent's affinity for the red cell membrane and the mean number of the agent's molecules which, upon interaction with an erythrocyte, make it undergo hemolysis have been measured. The contribution of the head group and that of a CH_2 group of the surfactants to the free energy of the agents' binding to the cell membrane have been estimated. The effect of the surfactants on the red cell volume and the lytic concentrations of the agents have been measured. The contribution of a CH_2 group to the free energy of the interaction of the amphiphiles embedded in the membrane bilayer with their environment has been evaluated and is proposed to be used as a measure of the membrane matrix stability.

Introduction

A wide variety of physical and chemical techniques used to probe the architecture of biological membranes [1] include the study of the molecular processes involved in surfactant-induced cell membrane lysis [2—8].

The red blood cell membrane is most popular as a model membrane system

due to both the availability and the large body of background information [9]. This appears to be the reason for the extensive studies made on the hemolytic action of different surfactants [2—8]. A great number of various approaches was developed for the study of the mechanisms involved in surfactant's hemolytic effect [5—8]. It seems to us that the easiest way to determine the main characteristics of the surfactant-induced hemolysis, namely, the affinity of the agent for the cell membrane and the mean number of the agent's molecules which, upon interaction with an erythrocyte, make it undergo hemolysis, is to study the agent's concentrations providing different hemolysis degrees at different concentrations of erythrocytes.

This approach was developed in the present study and was used to examine the hemolytic action of sodium salts of β-D-glucopyranosyl-1-alkylphosphates on human red cells. The dependence of the hemolytic effect of the agents on the alkyl chain length was studied and compared with the literature data on amphiphile-membrane interactions. The effect of the surfactants on the red cell volume was studied and is discussed in relation to the agent's action on the cell membrane.

Materials and Methods

The red cell suspension was prepared from outdated human bank blood as described earlier [4]. The determination of the degree of hemolysis under surfactant treatment was made by estimating spectrophotometrically the amount of hemoglobin released from the cells as in Ref. 4. The treatment of the erythrocyte suspension ($8 \cdot 10^7$—$2 \cdot 10^9$ cells/ml) by surfactants was carried out for 10 min. Each experiment was repeated 2—3 times with essentially identical results.

Cell volume frequency distribution histograms on all erythrocyte samples were made with the help of a model ZBIC Coulter counter supplemented with a Channelyzer model C-1000. The red cells suspended in isotonic sodium phosphate buffered saline (pH 7.2) at a concentration of approximately $6.8 \cdot 10^6$ cells/ml were incubated for 10 min with the surfactants the final concentrations of which are shown below. After that the cell suspensions were diluted with particle-free isotonic saline immediately before measuring to give the final concentrations of the order of $3—4 \cdot 10^4$ cells/ml. A 100 μm orifice tube was used in the measurements. The size range distribution data were analyzed in terms of the mean cell volume using the mode or peak of the distribution curve [10]. The counter was standardized against fresh human red blood cells and particles of known mean corpuscular volume. Mean volumes of the cells were determined as the corresponding model volumes. 3—6 mean cell volume readings were recorded for each sample with the accuracy and reproducibility of results within 2—3%. The experiments were repeated 2—3 times with at least two variously freshly drawn blood samples producing essentially identical results.

Sodium salts of β-D-glucopyranosyl-1-alkylphosphates with different alkyl chain lengths (C_nH_{2n+1}, n = 10, 13, 15, 16) were obtained by the method described in Ref. 11.

The critical micelle concentration values of the surfactants in sodium

phosphate-buffered isotonic saline (pH 7.2) were determined by the eosin solubilization technique as in Ref. 12.

Results

Typical curves showing the degree of human red cell hemolysis versus the agent's concentration at various concentrations of erythrocytes are presented in Fig. 1. The hemolytic activity of surfactants was determined as the agent's concentration C^x required to bring about an X% hemolysis degree (X = 10, 25, 50 and 75%) during 10 min treatment.

An approximately linear relation was found by several authors [2,8,13,14] to exist between the agent concentration C^x needed for the X% hemolysis and the number of erythrocytes present. The relation is expressed as

$$C^x = a_x + b_x \cdot N \quad (1)$$

where N is the cell count per unit volume, a_x and b_x are constants.

The physical meaning of a_x and b_x was discussed earlier [8] and it was concluded that the parameter a_x provides a measure of the agent's affinity for the membrane according to the equation $\Delta G_{aff} = RT\ln a$, and the parameter b_x can be used as a measure of the agent's hemolytic power.

Both parameters a_x and b_x appear to depend on the hemolysis degree X% [2,8]. Therefore to get a general description of the hemolytic process it is necessary to determine the a_0 and b_0 values independent of hemolysis. In order to find these parameters the following approach was used. The agent's concentrations C^x needed for various hemolysis degrees versus cell concentration curves were plotted as shown in Fig. 2. The a_x and b_x values for various X values were determined. The relationship between the parameter a_x and the degree of hemolysis X% was plotted as indicated in Fig. 3, and the a_0 value was obtained by graphical extrapolation to zero hemolysis degree. The b_0 value was determined in the same way.

The a_0 and b_0 values are listed in Table I together with the affinity for the human red cell membrane, ΔG_{aff}, values (calculated as above from the a_0

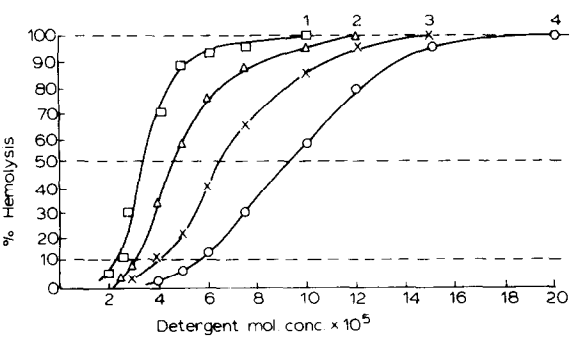
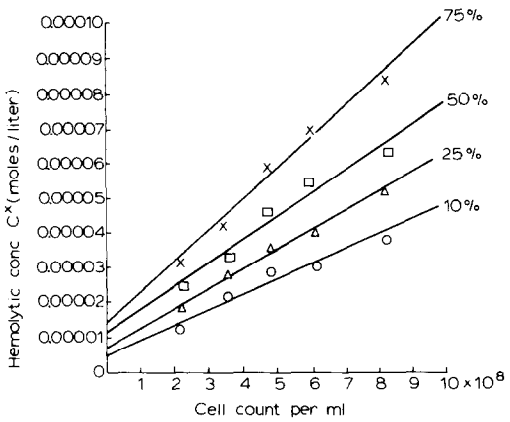


Fig. 1. Hemolysis of human erythrocytes by β-D-glucopyranosyl-1-pentadecylphosphate (sodium salt) in phosphate-buffered isotonic saline (pH 7.2) at various cell concentrations. 1, $3.5 \cdot 10^8$ cell/ml; 2, $6.1 \cdot 10^8$ cell/ml; 3, $8.2 \cdot 10^8$ cell/ml; 4, $10.7 \cdot 10^8$ cell/ml.



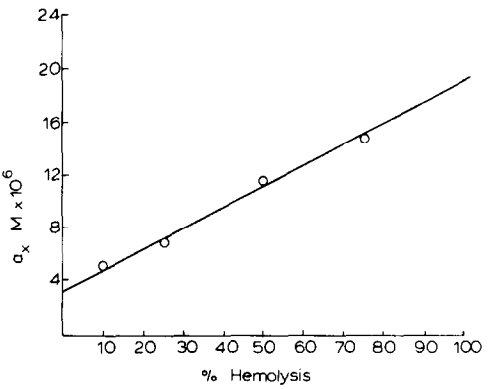


Fig. 2. Concentrations C^x of β-D-glucopyranosyl-1-pentadecylphosphate required for X% hemolysis (X = 10, 25, 50, 75%) as functions of human red cell concentrations.

Fig. 3. Parameter a_x as a function of X% hemolysis degree of human red cells hemolyzed by β-D-glucopyranosyl-1-pentadecylphosphate.

TABLE I

The affinity for the human red cell membrane (expressed as a_0 and $\Delta G_{aff} = RT\ln a_0$), hemolytic activity (expressed as b_0), the critical micelle concentration and the free energy of micellization (ΔG_{mic}) values of sodium salts of β-D-glucopyranosyl-1-alkylphosphates.

Alkyl chain length of surfactant	a_0 (M)	$-\Delta G_{aff}$ (kcal/mol)	b_0 (mol/cell)	Critical micelle concn. (M)	ΔG_{mic} (kcal/mol)
10	$2.0 \cdot 10^{-3}$	3.68	$1.0 \cdot 10^{-11}$	$8.0 \cdot 10^{-3}$	2.86
13	$7.5 \cdot 10^{-5}$	5.63	$5.0 \cdot 10^{-14}$	$6.2 \cdot 10^{-4}$	4.38
15	$3.0 \cdot 10^{-6}$	7.54	$4.0 \cdot 10^{-15}$	$8.8 \cdot 10^{-5}$	5.54
16	$4.9 \cdot 10^{-7}$	8.62	$2.5 \cdot 10^{-16}$	$3.75 \cdot 10^{-6}$	6.04

TABLE II

ACTION OF SODIUM SALTS OF β-D-GLUCOPYRANOSYL-1-ALKYLPHOSPHATES ON THE HUMAN RED CELL VOLUME

Alkyl chain length of surfactants	V_{max} μm^3	V_{min} μm^3	C_{lys} mol/l	C^*_{lys} mol/cell
10	122	110	$2.3 \cdot 10^{-2}$	$3.09 \cdot 10^{-12}$
13	116	99	$4.4 \cdot 10^{-4}$	$5.37 \cdot 10^{-14}$
15	108	84	$2.6 \cdot 10^{-5}$	$3.44 \cdot 10^{-15}$
16	96	76	$1.8 \cdot 10^{-5}$	$2.58 \cdot 10^{-15}$

V_{max} is the maximum volume achieved by the red cells under agent treatment; V_{min} is the minimum volume achieved by the red cells under agent treatment; C_{lys} is the agent concentration at which the complete membrane break-down occurs; $C^*_{lys} = (C_{lys} - a_0)/N$, where N is the cell concentration amounting to $6.8 \cdot 10^6$ cell/ml.

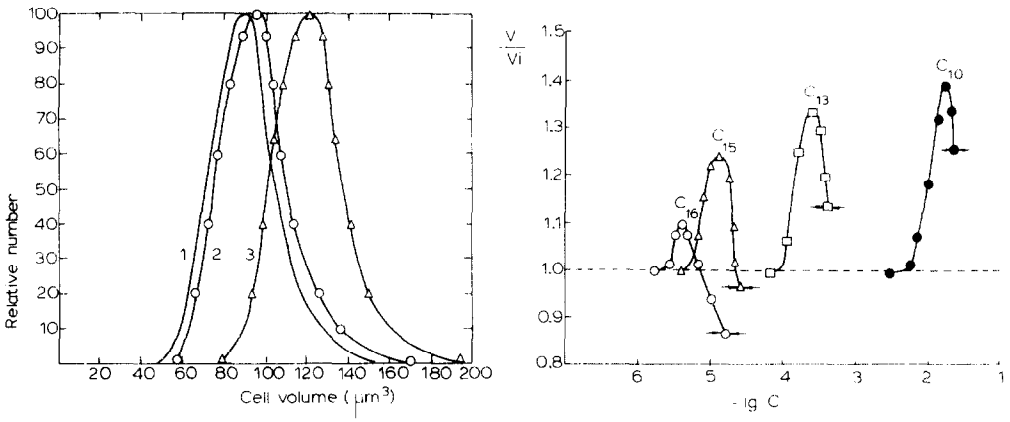


Fig. 4. Histograms of typical human red cell volume frequency distribution in phosphate-buffered isotonic saline at pH 7.2: 1, intact human erythrocytes; 2, maximum effect achieved under the C_{16} surfactant treatment; 3, maximum effect provided by the C_{10} surfactant treatment.

Fig. 5. Relative human red cell volume (expressed as the ratio of the observed volume to the initial volume value) as a function of the surfactant concentration: 1, C_{10} surfactant; 2, C_{13}-surfactant; 3, C_{15}-surfactant; 4, C_{16}-surfactant. The sign →○← indicates the point corresponding to a concentration C_{lys} at which the complete membrane break-down occurs. Initial volume of the untreated cells is 87 μm^3.

values) and the data on the critical micelle concentration and the free energy of micellization, ΔG_{mic}, values.

To estimate the membrane-perturbing effects of the surfactants more closely we have studied the action of the agents on the red cell volume. Typical cell volume distribution curves are shown in Fig. 4. It should be noted here that the changes of the width and the asymmetry of the peak appear to be independent of the agent's concentration in contrast to the model volume position.

The cell volume changes under surfactant treatment are shown in Fig. 5 as the relationships between the relative erythrocyte volumes and the agents' concentrations. The maximum cell volumes, V_{max}, and the minimum cell volumes, V_{min}, achieved by erythrocytes under the surfactants' treatment are present in Table II together with the agents' concentrations at which the complete breakdown of the cell membrane occurs.

Discussion

From the data given in Table I it appears that the affinity for the erythrocyte membrane is a strictly linear function of the agent's alkyl chain length, n. It allows one to use the conventional approach [15] considering the contributions of the hydrophilic and hydrophobic portions of an amphiphile to the free energy of micellization or to the free energy of binding as nearly independent. It consists in dissecting the free energy of micellization into contributions from the hydrophilic head group, ΔG_{mic}^{head}, and from the methylene group, $\Delta G_{mic}^{CH_2}$, by investigating the alkyl chain length dependence of the critical micell concentration for a given homologous series of surfactants [15]. Contributions of the groups to the free energy of micellization estimated as above amount to ΔG_{mic}^{head} = 2.44 kcal/mol and $\Delta G_{mic}^{CH_2}$ = −0.53 kcal/mol. A similar approach

applied to evaluate the contributions from the head group and from a CH_2 group to the free energy of the surfactants' binding to the red cell membrane gives: $\Delta G_{aff}^{head} = 4.04$ kcal/mol and $\Delta G_{aff}^{CH_2} = -0.77$ kcal/mol.

It should be noted that Seeman et al. [16] in studying the relation between the binding of the alcohol anesthetics to the human erythrocyte membrane and the chain length of the alcohols have been shown that the mean free energy of alcohol adsorption was characterized by -0.695 ± 0.081 kcal/mol of methylene groups. Relative partition coefficients of saturated fatty acids between the phosphate buffered saline at pH 7.4 and human erythrocyte ghost membranes have been determined by Salleen [17] from the linear relationship isotope content of sedimented membranes and aqueous concentrations of the agents. Incremental free energy for methylene group addition was found to be -0.883 kcal/mol per methylene group. The above data obtained by direct determinations of amphiphiles binding to the red cell membranes [16,17] appear to be in good agreement with our results obtained by the approach suggested here. This fact seems to support the validity of the approach.

The b_0 values listed in Table I indicate that the hemolytic capacity of the agents under study increases with the increasing alkyl chain length. The quantity of the C_{16}-surfactant needed to bring about hemolysis of single red cell amounts to $15 \cdot 10^7$ molecules per cell. If the hemolysis is caused just by the agent's binding to the cell membrane the surface of the cell covered by the agent molecule can be calculated. Taking a value of $163 \cdot 10^8$ Å^2 for the cell surface [7], the number of the C_{16}-surfactant's molecules adsorbed per single cell is equivalent to one molecule per 108 Å^2 red cell area. This value appears to be similar to those found in the literature [7,13]. When the other agents under study are considered in this manner, however, the improbable values such as one molecule per $2.7 \cdot 10^{-3}$ Å^2 cell area for the C_{10}-derivative or one molecule per 6.8 Å^2 cell are in the case of the C_{15} agent are obtained. The fact that the same approach gives reasonable value for the C_{16}-agent and the improbable one for the C_{15} analog seems to be accounted by the incorrect treatment of the hemolytic capacity of surfactants expressed as b_0 values. It is known that the release of some membrane constituents occurs at the initial stage of the hemolytic process caused by surfactants [2,18]. The b_0-value represents the total quantity of an agent required to bring about hemolysis, hence it must include the number of the agent's molecules involved in the solubilization of the membrane constituents and the number of the molecules adsorbed per cell. Solubilizing capacity of amphiphiles is known to increase with the increasing alkyl chain length [19]. It seems possible that the dependence of the surfactant's solubilizing capacity on the alkyl chain length may account for the observed dependence of the b_0 values on the agents' alkyl chain length.

The data on the effects of the surfactants under study on the red cell volume appear to indicate that these effects reflect some differences in the agents' membrane-perturbing action. It is known that the permeation of normally impermeant solutes leads to the swelling and eventual hemolysis and lysis of the red cells. This type of hemolysis is known as colloid osmotic hemolysis [20]. Since surfactants are known to change the membrane permeability [2,3, 21] it seemed possible that the agents under study could produce this type of hemolysis. The colloid osmotic hemolysis, however, would be accompanied by

the same erythrocyte swelling independent of the agent used to damage the cell membrane. The data in Table II indicate that the maximum volume achieved by the cells under a given agent treatment depends on the agent's alkyl chain length. This fact seems to rule out the possibility regarding the colloid osmotic hemolysis induced by the surfactants through the changes of the cell membrane permeability.

The observed increase of the cell volume can be explained also by the bilayer couple hypothesis of Singer et al. [22]. It was proposed by Singer et al. [22] that anionic amphiphiles intercalate mainly into the lipids in the exterior half of the membrane bilayer, expand that layer relative to the cytoplasmic half, and thereby induce the cell to crenate. It is possible that the agents differing in the alkyl chain length may induce various expanding of the membrane, and hence cause different increase of the cell volume.

It seems reasonable to assume that once the maximum volume is achieved hemolysis occurs. After that a progressive shrinkage of the ghosts formed is observed as the concentration of the agent is increased. This fact is in agreement with the observations of Coleman et al. [23] studied the residues remaining after extensive treatment of human erythrocyte ghosts with different surfactants by the phase contrast microscopy. Hence, it seems possible to conclude that the observed ghosts shrinkage is due to the solubilization of the membrane constituents by the surfactants. The observed dependence of the minimum ghost volume on the alkyl chain length can be attributed to the different degree of solubilization of the membrane constituents providing by various agents.

The results presented in Table II show that the C_{lys} value decreases as the chain length of the surfactant increases. According to the model proposed by Haydon and Taylor [24] the point at which the lipid bilayer breaks up should, to a first approximation, be dependent only on a number of adsorbed amphiphile molecules. According to the model [24] this number is related to the free energy of the interaction of the adsorbed agent with its membrane environment, ΔG_{lys}, and this relation can be expressed as:

$$\Delta G_{lys} = -RT\ln(C^*_{lys}) \tag{2}$$

where $C^*_{lys} = (C_{lys} - a_0)/N$, C_{lys} is the agent's concentration at which the complete break-down of the cell membrane occurs, N is the cell concentration, a_0 is as defined above.

This equation permits one to estimate the free energy of the membrane break-up under a given compound treatment. Since the free energy of the lytic interaction of the agents bounded to the red cell membrane with their environment appears to be a linear function of the agent's alkyl chain length, it is possible to use the conventional approach to estimate the contribution from a CH_2 group and from the head group of the compounds to the free energy of the lytic interaction. These contributions amount to $\Delta G^{CH_2}_{lys} = 0.80$ kcal/mol and $\Delta G^{head}_{lys} = 7.72$ kcal/mol.

The fact that both contributions are positive indicates that the lytic interactions of both parts of the amphiphile molecule partially embedded in the membrane bilayer with their environment reduce the membrane stability. The above $\Delta G^{CH_2}_{lys}$ value appears to provide a measure of a given membrane matrix stability.

The data obtained in this work do not allow one to conclude which mechanism of the surfactants' hemolytic action is the most probable one. For a more comprehensive understanding of the membrane-perturbing, hemolytic and lytic effects of surfactants the study of the action of amphiphiles with the same hydrophobic portion size but with different polar head groups is called for and it is in progress at present.

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