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ACTION OF SURFACE-ACTIVE SUBSTANCES ON BIOLOGICAL MEMBRANES

II. HEMOLYTIC ACTIVITY OF NONIONIC SURFACTANTS

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Summary

The hemolytic action of commercially available nonionic surfactants and synthesized polyoxyethylene fatty acids and mercaptans on human erythrocytes was measured. It is shown that the hemolytic power of the detergents depends on the mutual effect of the hydrophobic and hydrophylic fragments of the agent molecule and does not depend on the hydrophile-lipophile balance of the compounds. A graphical image of the structure-activity relationship obtained in the study is similar to the one found in the literature when studying the analgesic effect of imidazoline derivatives on rats in vivo. This fact is discussed on the basis of assumption that the mechanism of both processes in vivo and in vitro is related to influence of the agents on cellular membranes. It is suggested that the role of the polyoxyethylene chain is its effect on the dipole moment and the relative lipophilicity of the compound or in the participation of the fragment in the interaction with the surface components when the agent is sorbed on the membrane. It is concluded that when the correlation between the hydrophile-lipophile balance values and a membrane effect the capacity of the surfactants this indicates that the effect is caused not by destruction of the membrane but by some rearrangement of the membrane structure accompanying the surfactant adsorption.

Introduction

Extensive studies have been made recently on the action of nonionic surfactants on natural biological membranes and model lipid bilayers [1—11]. The results obtained are as varied as the objects used in the studies. One of the most widely used characteristics of nonionic detergents is the quantity called the

hydrophile-lipophile balance. This quantity is a general measure for the balance of the opposing hydrophilic and hydrophobic groups in nonionic surfactant and many authors attempt to correlate the detergents' hydrophile-lipophile balance values to their biological activity [1—3,9,11]. Some authors [1,2] conclude that the active hydrophile-lipophile balance range of surfactants is between 12.5 and 14.5, others [9] obtained data indicating the active hydrophile-lipophile balance range to be between 16 and 17. At present, however, it is unclear whether the hydrophile-lipophile balance value of the nonionic surfactant is really one of the primary parameters determining particularly the detergents' membrane-solubilizing capacity as it is argued in the literature [3] or whether the structure of the agent molecule also determines its biological activity. When nonionic detergents with the polyoxyethylene ester structure were used in a number of studies there was found a decrease of the membrane disaggregating power with an increase of the polyoxyethylene group length [7,8,10]. There is not a great deal known about the dependence of the membrane-solubilizing capacity on the hydrophobic moiety of nonionic detergents [5,8]. Thus, experiments have been undertaken in this study to examine the effects of hydrophobic as well as hydrophilic moieties on lytic activity of nonionic surfactants towards human erythrocytes.

Materials and Methods

The erythrocyte suspension was prepared from human blood as described earlier [12]. 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. 12. As in the case of sodium alkyl sulfates [12] the treatment of the erythrocyte suspension ($3 \cdot 10^8$ cells/ml) by detergents was carried out for 10 min. The experiments were performed 3—4 times and the found deviation from the given average C^{50} value did not exceed 5—7% for all the agents under study.

The commercially available nonionic surfactants used in the study were Tweens 20, 40, 60 and 80, Triton X-305, polyethylene glycol-600-monolaurate and polyethylene glycol-300-monolaurate, supplied from Ferak (Berlin), Triton X-100, X-405 and Brij 35 purchased from Serva Fine Biochemicals (Germany), Triton N-101 from Koch-Light (England), and Nonidet P-40 from BDH Chemicals (U.K.).

The polyoxyethylene esters of fatty acids represented by the formula $R_nCOO(EO)_mH$ ($n = 6$, $m = 5.6$ and 7.0; $n = 9$, $m = 4.0$, 5.2, 8.0; $n = 10$, $m = 5.5$, 7.15, 8.8 and 11.0 $n = 11$, $m = 25.0$; $n = 12$, $m = 12.0$, 25.0 and 40.0; $n = 15$, $m = 8.0$ and 10.4) were prepared by esterification with ethylene oxide as described in ref. 13.

The polyoxyethylene mercaptans represented by the formula $R_nS(EO)_mH$ ($n = 7$, $m = 3.5$, 4.55, 5.6 and 7.0; $n = 8$, $m = 4.0$, 5.2, 6.4 and 8.0; $n = 10$, $m = 5.0$, 6.5, 8.0 and 10.0; $n = 12$, $m = 6.0$, 7.8, 9.6 and 12.0) were obtained by the method similar to the one described in ref. 14.

In the above formulae R_n represents an alkyl chain of n length C_nH_{2n+1}, (EO) represents the oxyethylene unit (CH_2CH_2O) and m, the degree of EO units polymerization.

The mean value of EO groups number per molecule for each of the prepared compounds was determined by the technique described in ref. 15. The above substances were also characterized by the hydrophile-lipophile balance values experimentally determined by the method described in ref. 16.

Results and Discussion

Typical curves showing the degree of cell hemolysis versus agent concentration are given in Fig. 1. The hemolytic activity of nonionic detergents was determined as the agent concentration C^{50} required to bring about 50% hemolysis during 10 min treatment.

As is known from the literature [5,7,8,10] the membrane action of polyoxyethylene compounds may be effected by the size of the hydrophobic molecule fragment as well as by the polyoxyethylene chain length. To establish the mutual influence of both the opposing parts of the surfactant molecule on its disaggregating action toward the erythrocyte membrane, the effect of the molecule structure on the hemolytic activity (C^{50}) of the nonionic detergents is presented in Fig. 2 in a three-dimensional coordinate system. The structure-activity surface in Fig. 2 is obtained by plotting the C^{50} values against the alkyl chain length (n) and the polyoxyethylene chain length (m). The dependence of hemolytic activity of sodium alkyl sulfates (expressed in C^{50} terms) on the alkyl chain size (n) [12] is presented in Fig. 2 near the surface for comparing the lytic power of ionic and nonionic agents. It should be pointed out that the surface given in Fig. 2 is produced by the extrapolation of the data obtained with the compounds noted above and it presents just the total trend observed in the experiments but is not absolutely precise. To obtain a more closely comprehensive relationship it would be desirable to study the hemolytic activity of a greater number of compounds, however, they were not available for the present investigation.

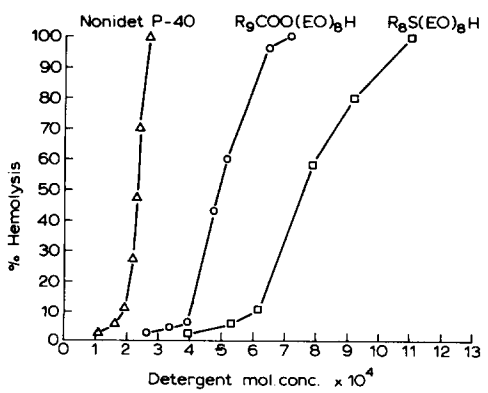

Fig. 1. Hemolysis of human erythrocytes by nonionic surfactants in the phosphate-buffered isotonic saline (pH 7.2) at a cell concentration of $3 \cdot 10^8$ cells/ml.

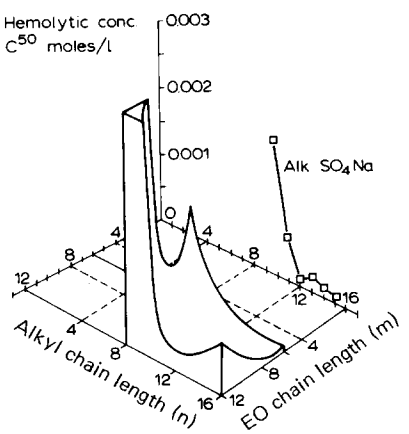

Fig. 2. Structure-hemolytic activity relationship for polyoxyethylene compounds of varying alkyl chain length (n) and polyoxyethylene chain length (m).

The general trend that can be seen from the relationship in Fig. 2 is characterized by the following points. First of all, in each homologous series of the compounds used in the study independently of the hydrophobic group size the hemolytic capacity decreases with the increasing polyoxyethylene chain length. When the number m of EO groups does not exceed 7.0 the elongation of the alkyl chain above R_{12}—R_{14} does not effect the hemolytic power of the detergents. When the polyoxyethylene chain length m is in the range 7.0—12.0 the compounds with the hydrophobic moiety R_{12} show the highest hemolytic activity. It should be noted that the polyoxyethylene mercaptans and the polyoxyethylene esters of fatty acids with the same number (m) of EO units show exactly the same lytic capacity in the case of equal numbers of C atoms in the alkyl and the acyl moieties respectively, i.e. $R_nS(EO)_mH$ has the same hemolytic activity as $R_{n-1}COO(EO)_mH$. Since the compounds are polydisperse it seems reasonable that the additive hydrophilicity provided in the polyoxyethylene esters of fatty acids by the COO-group cannot be displayed in the experimental conditions used.

It was found possible to evaluate the conventional size of the hydrophobic moiety of polyoxyethylene alkylphenol derivatives from the relationship in Fig. 2 when approximating the moiety by the straight alkyl chain. The C^{50} value corresponding to the lytic activity of Nonidet P-40 (polyoxyethylene derivative of nonylphenol) was plotted on the surface of the relationship in Fig. 2 in accordance with the degree of polymerization of the substance (m = 9.0) and it was found that the nonylphenol fragment in the conditions used conforms to R_{11} straight alkyl chain. It means that the phenyl moiety, when being estimated by its contribution into lipophility of the total hydrophobic fragment of this agent molecule, resembles 2 CH_2 groups. It agrees reasonably with the contribution evaluated earlier by the technique of detergent partition in aqueous polymeric biphasic system [17]. The diisobutyl moiety of the Triton-series of nonionic detergents when similarly estimated was found to comply with a nonyl chain, probably due to the greater volume of this group which compensates for the lack of its length when the agent displays its hemolytic activity.

It should be noted that in the study [18] of the analgesic potencies of a series of substituted imidazolines on rats, the structure-activity relationship identical to the one in Fig. 2 was obtained. The structure-activity surface in ref. 18 is shown in a three-dimensional coordinate system on the axes of which the following parameters are presented: ED_{50}, the dose of the agent that will prevent 50% of the animals from exhibiting the characteristic HCl-induced writhing response; the parameter π introduced by Hansch [19] defined by $\pi = \log P_s - \log P_0$, where P_s is the partition coefficient of a substituted compound in water-octanol biphasic system and P_0 that of the parent compound; and the energy of the highest occupied molecular orbital E_{HOMO} of the compounds calculated with the aid of molecular orbital technique (this index is a relative measure of the ability of an electron to be transferred from the agent molecule to an acceptor). The identity of the relationships obtained in ref. 18 and in our experiments seems to indicate that the mechanisms of the agents actions have something in common with each other in spite of all the evident differences. The only common part seems to be just that both processes occur on the

cellular membranes (when an analgesic effect is displayed by the imidazolines on rats in vivo and when the lytic effect is manifested by nonionic detergents toward human red cells in vitro). Thus it seems reasonable to suggest that the dose ED_{50} in the work [18] is similar to the C^{50}, both corresponding to the agent concentration providing the given response of a biological system (because of the cellular membrane being the site of the agent action in both cases). The parameter π in ref. 18 as it is known [19] is a measure of the relative lipophilicity of the agent in a homologous series of compounds, i.e. it is close to parameter n (the size of the hydrophobic fragment of the agent molecule) used by us. The ability of the agent molecule to give an electron to an acceptor (E_{HOMO}) may effect the molecule's amphiphilicity and this probable role of the agent property must not be overlooked. It is known particularly from the physicochemical studies of detergents [20] and for example from the data concerning the influence of pH on the partition behaviour of fatty acids in a water-heptane biphasic system [21]. Such an oversimplified approach to the parameter E_{HOMO} is incorrect as it ignores the agent ability to form a charge · transfer complex in the biological system but it has some advantages, particularly in allowing one to understand the identities of both relationships in ref. 18 and in Fig. 2. It also allows the suggestion that the hemolytic capacity of the polyoxyethylene compounds may depend on the polyoxyethylene chain length because of its effect on the dipole moment of the nonionic surfactant [22], on the hydrophile-lipophile balance value or due to the participation of this molecule fragment in the interaction with the membrane surface components.

Experimentally found hydrophile-lipophile balance values of some of the substances used in the study and those from the literature [23] for some of the commercially available detergents are given in Table I together with the relative values of these compounds' hemolytic capacities.

It follows from the data in Table I that the hemolytic activity does not correlate with the hydrophile-lipophile balance values of the compounds used in the study. It is known from the literature however that the hydrophile-lipophile balance, widely used as a general characteristic of nonionic surfactants [3], correlates with the physicochemical properties of the detergents, e.g. with critical micellar concentration values [24], emulsifying abilities [25] and particularly with the effectiveness in the membrane action [1—3,9,11]. The seeming contradiction arising between our results and the literature data however is overcome when the disaggregating interaction of the detergent with the membrane is considered. The disaggregating process can be regarded as including two main stages: the detergent adsorption stage and the lytic stage. The lytic stage is a complicated process including a lot of different steps [26] and in spite of the great number of papers that deal with this subject (e.g. see ref. 3) the mechanisms of the process remain almost totally unclear. The adsorption stage has to be dependent on the affinity of the agent to the membrane, to the bulk water phase and on its critical micellar concentration value in the given conditions. If the specific affinity is not taken into account, as it remains still to be elucidated, it is evident that the affinity of the surfactant to the membrane is effected by its relative lipophility or hydrophile-lipophile balance value. This does not contradict the lack of correlation between the

TABLE I

Hydrophile-lipophile balance (HLB) values of some of the nonionic surfactants used in the study and the relative values of the hemolytic activities (calculated as C^{50}/C^{*50}, where C^{*50} is the activity of $R_{12}S(EO)_{7.8}H$).

Formula or chemical name (commercial name)	n	m	HLB value	C^{50}/C^{*50}
$R_nCOO(EO)_mH$	9	5.2	9.1	12.1
	10	7.15	10.3	5.7
$R_nS(EO)_mH$	12	7.8	11.2	1.0
$R_nCOO(EO)_mH$	9	8.0	11.4	7.1
	10	8.8	11.4	3.8
$R_nS(EO)_mH$	10	6.5	11.4	64.3
	10	8.0	11.6	6.6
$R_nCOO(EO)_mH$	10	11.0	12.0	3.6
$R_nS(EO)_mH$	8	6.4	12.0	16.7
	12	9.6	12.1	1.5
$R_nCOO(EO)_mH$	6	7.0	12.3	49.3
$R_nS(EO)_mH$	10	10.0	12.6	30.0
	8	8.0	12.6	10.7
	12	12.0	12.6	2.9
Polyoxethylene nonylphenol (Nonidet P-40)		9.0	13.1 *	3.3
Polyoxyethylene *p-t*-octyl phenol (Triton X-100)		9.5	13.5 *	3.7
Polyoxyethylene sorbitol monooleate (Tween-80)		20.0	15.0 *	100
Polyoxyethylene sorbitol monolaurat (Tween-20)		20.0	16.7 *	100
Polyoxyethylene *p-t*-octyl phenol (Triton X-305)		30.0	17.3 *	100
Polyoxyethylene *p-t*-octyl phenol (Triton X-405)		40.0	17.9 *	100

* The hydrophile-lipophile balance values from the literature [23].

hemolytic activity of the detergents and their hydrophile-lipophile balance values found here but just emphasises the differences in the sorbtion stage and the lytic one. As is already known [27], the adsorption of many lytic agents on the erythrocyte membrane may be accompanied by an increase of the cell resistance to various physicochemical effects. The correlations between the hydrophile-lipophile balance values of the nonionic surfactants and their membrane action power found [1,2,9] may be explained in all cases using the approach which regards the experimentally observed effect as the one accompanying the agent adsorption and not the one resulting from the disaggregation of the membrane.

The process of membrane lysis under the amphiphilic treatment is much toc complicated and too little understood for a detailed analysis. The data obtained in this study evidently are not enough to suggest a model of the mechanisms of this process. On the basis of our results it seems possible to make the following conclusions however. It is essential to distinguish the absorption and the disaggregation stages in the process of cell lysis under the surfactant treatment. The hydrophile-lipophile balance values of the detergents determine their capacities to be bound to the membrane but not their lytic activities. The lytic power of the surfactant is effected by the sizes of its hydrophilic and

hydrophobic moieties and it seems that the volume of the lipophilic group particularly may compensate for its lack in length. The role of the hydrophilic fragment size in the lytic activity of the compound may be due to its effect on the total dipole moment of the agent or on its relative lipophilicity or due to the participation of this molecule fragment in the interaction with the membrane components when the agent is bound to the membrane; this question remains unclear. In the case of ionic detergents, the charge role may also be its influence on the dipole moment and the relative lipophility of the surfactant or in its possible contribution in ionic interactions of the agent with the membrane as the part of the total interaction including the agent sorbtion and the lysis of the membrane.

From the correlation found in this study it follows that the mechanisms of membrane interaction with surfactants in vitro is similar to that with pharmacologically active agents in vivo. It is evident that it does not simplify the investigation of the mechanisms of this interaction but just emphasizes the importance of the research in this field.

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