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HEMOLYSIS CAUSED BY POLYOXYETHYLENE-DERIVED SURFACTANTS

EVIDENCE FOR PEROXIDE PARTICIPATION

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The hemolytic properties of nonionic surfactants of the series $CH_3(CH_2)_{15-17}$-O-$(CH_2CH_2O)_nCH_2CH_2OH$ were investigated and compared to those of saponins, sapogenins and H_2O_2. Antioxidants and anaerobic conditions were shown to inhibit the hemolysis, while glycyrrhizin was found to enhance it. Similar effects were obtained for H_2O_2 hemolysis, but not for saponin and sapogenin hemolysis. It is proposed that peroxides and free radicals are mainly responsible for the polyoxyethylene derived surfactants induced hemolysis.

Introduction

The hemolytic activity of polyoxyethylene derived surfactants is well known and has repeatedly been investigated [1–3], but the mechanism of this process has not yet been elucidated. Surface activity is generally accompanied by hemolysing capacities. In polyoxyethylene-derived surfactants, however, hemolysis does not correlate with any parameter used for evaluation of surface activity, e.g. reduction of surface tension [1], critical micelle concentration [2] or hydrophile-lipophile balance [3].

It was recently reported [4,5] that polyoxyethylene-derived surfactants have a strong tendency to autocatalytic peroxide formation in aqueous solutions. Peroxides and free radicals are known to cause membrane damage and to induce hemolysis [6,7]. The aim of the present study was to find out whether the hemolytic activity of these surfactants may be ascribed to their tendency to form peroxides. Accordingly, the hemolytic activities of various polyoxyethylene surfactants were compared to those of saponins and sapogenins which are surfactants devoid of autocatalytic peroxide formation.

Experimental

Materials. The polyoxyethylene-cetostearyl ethers, Texofor A series, supplied by A.B.M. Chemicals Ltd., Woodley-Stockport Cheshire, U.K. Cetomacrogol refers to polyoxyethylene(24)-cetostearyl ether. All surfactants were solids and thus not sensitive to autooxidation [8]. Solutions of surfactants were freshly prepared before use. Polyethyleneglycol-1000, solid consistency Fluka A.G., Buchs, Switzerland. Cetostearyl alcohol, B.P. Evans Medical Ltd., Liverpool, U.K. Quercetin and rutin, Sigma Chemical Company, St. Louis, MO, U.S.A. Digitonin and hydrogen peroxide (30%) (Perhydrol), Merck, Darmstadt, F.R.G. Vitamin E, D-alpha-tocopheryl acetate N.F. Type 6-100, Distillation Products Industry; Division of Eastman Kodak Co. Rochester, NY, U.S.A. Saponin-A and sapogenin-A were obtained from *Styrax officinalis* [9].

Determination of hemolytic activities. All experiments were performed on fresh citrated blood drawn from albino rats. The erythrocytes were freed of

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plasma by three washings in cold isotonic saline. The erythrocytes were then diluted with isotonic buffer (3.95 g $Na_2HPO_4 \cdot 2H_2O$, 0.76 g KH_2PO_4, 7.2 g NaCl, aquadist. ad 1000 ml; pH adjusted to 7.4) to give 1% suspension. Varying quantities of hemolysin, dissolved in buffer or 80% dimethyl sulfoxide (DMSO) (in case of sapogenins and other insoluble hemolysins), were added to 2 ml of erythrocyte suspension. The volume was made up to 4 ml with buffer. The components were added in the following order: first erythrocyte suspension then the buffer and last the hemolysing agent. The mixtures were incubated for 90 min at 37°C in a shaking bath. Then they were centrifuged at 1 000 × *g* and the absorbance of the supernatant determined at 540 nm, the hemolysing activity was measured as the concentration inducing 50% hemolysis (H_{50}) as described previously [10]. When DMSO was used as solvent its final concentration was kept constant at 16% throughout the whole experiment (including control).

Effect of glycyrrhizin on cetomacrogol- and H_2O_2-induced hemolysis. Two sets of experiments were run in parallel: (a) Erythrocyte suspension (2 ml) was incubated at 37°C with glycyrrhizin solution and buffer to give a final volume of 3 ml. After 1 h, buffer and then hemolysin solution were added to give a final volume of 4 ml. The mixtures were incubated for another 90 min at 37°C and the percentage of hemolysis determined [10]. (b) Erythrocyte suspension (2 ml) was incubated with buffer (1 ml) for 1 h at 37°C, then buffer and hemolysin solution were added, and further processed as outlined under (a). Percentage of augmentation was determined as

$$100 \times \left(\frac{\%\text{ hemolysis with glycyrrhizin}}{\%\text{ hemolysis without glycyrrhizin}} - 1 \right)$$

Effect of quercetin, rutin and Vitamin E on the hemolytic effect of various hemolysins. All the above-mentioned compounds which are water insoluble, were dissolved in DMSO. The solutions were carefully tested to assure that no precipitation occurred on dilution with water; when necessary, the concentration of the DMSO solutions was reduced. Before use, the DMSO solutions were diluted with water in the proportion of 5 : 1 resp. The final concentration of DMSO in any erythrocyte suspension did not exceed 25%. The inhibitory tests were performed exactly as outlined above; however, in the controls (b), the erythrocytes were incubated with 1 ml DMSO buffer mixture. Percentage inhibition was determined as described [11].

The effect of oxygen exclusion on cetomacrogol and saponin hemolysis. A mixture of erythrocyte suspension (1%) and buffer were introduced into a round bottom flask with a side arm closed with a rubber septum. The mixture was flushed with wet N_2 and then evacuated. This procedure was repeated several times. The mixture was then kept under an N_2 atmosphere. Hemolysin solution which was kept under N_2 was injected through the side arm. The mixture was incubated for 90 min at 37°C then centrifuged and percentage hemolysis was determined by the usual methods.

Surface tensions were measured at 25°C by the du Nouy ring method as described [12].

Results

The surfactants tested in this investigation were all ethers of cetostearyl alcohol having the general formula $CH_3(CH_2)_{15-17}\text{-}O\text{-}(CH_2CH_2O)_nCH_2CH_2OH$, *n* being the average of the number of ethylene oxide units in the nonhomogeneous polyethers.

The hemolytic activities of the surfactants were determined in terms of H_{50}, The hemolysin concentration inducing 50% hemolysis after 90 min incubation at 37°C.

Table I summarizes the hemolytic potencies of the surfactants investigated and their surface tension at the H_{50} concentrations. Obviously, there is no correlation between chain length, surface activity and hemolytic potency of the polyoxyethylene cetostearyl derivatives. These findings are in line with the results obtained for other polyoxyethylene-derived surfactants [1,2,3].

The time dependence of surfactant induced hemolysis was tested. Figs. 1 and 2 show the results obtained for various polyoxyethylene-cetostearyl ethers and for saponins, respectively. There is an essential difference between the two types of hemolysis. While saponins produce rapid hemolysis reaching a maximum level, depending on the concentration of the hemolysin, within less than one hour [13], hemolysis produced by the polyoxyethylene-cetostearyl ethers progresses slowly towards 100%, irrespective of the

TABLE I

HEMOLYTIC ACTIVITY AND SURFACE TENSION OF VARIOUS POLYOXYETHYLENE (*n*)-CETOSTEARYL ETHERS

Surfactant name	Average ethylene oxide number (*n*)	M_r	H_{50}		Surface tension at H_{50} (dyn/cm)
			(μg/ml)	M	
Texofor A_{14}	14	872	5.8	$6.6 \cdot 10^{-6}$	45
Texofor A_1 (Cetomacrogol)	24	1 314	6.25	$4.8 \cdot 10^{-6}$	48
Texofor A_{30}	30	1 576	12.5	$7.9 \cdot 10^{-6}$	47
Texofor A_{45}	45	2 235	>2 500	$>1 \cdot 10^{-3}$	–
Texofor A_{60}	60	2 896	50	$17.2 \cdot 10^{-6}$	55
Polyethyleneglycol-1 000	24	1 000	>2 500	$>2.5 \cdot 10^{-3}$	–
Cetostearyl alcohol		246	5 μg/ml gives 11% hemolysis	$6 \cdot 10^{-5}$ M gives 11% hemolysis	–

concentration. A similar curve was shown by Naim et al. [14] for the rate of H_2O_2-induced hemolysis in sheep erythrocytes.

It was previously reported [11] that glycyrrhizin is an effective inhibitor for saponin and sapogenin-induced hemolysis. When the effect of glycyrrhizin on cetomacrogol hemolysis was tested, it was found to have a contrary effect: instead of inhibiting, it markedly enhanced the extent of hemolysis (Table II). Likewise, glycyrrhizin was found to increase H_2O_2-induced hemolysis. Since H_2O_2 by itself gives only slight hemolysis, the up to 12-fold increase in this case is dramatic.

Hydrogen peroxide induced hemolysis is known to be inhibited by various antioxidants, e.g. vitamin E, phenols and flavonoids [13]. Our study has shown that vitamin E, rutin and quercetin effectively inhibit the hemolytic activity of cetomacrogol ($n = 24$), chosen to represent the polyoxyethylene-cetostearyl ether surfactants. On the other hand, when these were tested on saponins and sapogenins the following results were obtained.

(a) Vitamin E had no inhibitory effect on saponins. (It could not be tested on sapogenins because they coprecipitated with the vitamin E.)

(b) Rutin and quercetin had no effect at high levels of hemolysis and only a slight effect at low levels. It should be noted that the final concentrations of rutin and quercetin could not exceed 330 μM and 160 μM, respectively, because of their restricted solubility. Oxygen devoid atmosphere also inhibited only the cetomacrogol-induced hemolysis. The results of these experiments are summarized in Table III.

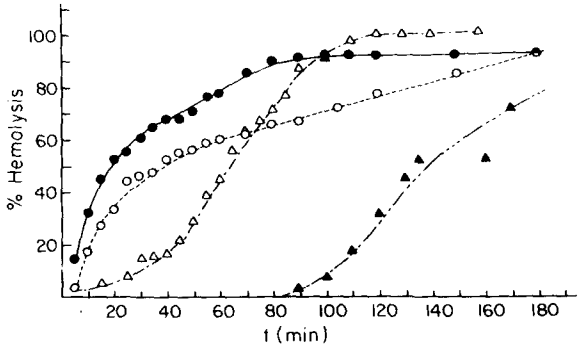

Fig. 1. Time dependence of the hemolysis caused by polyoxyethylene(*n*)-cetostearyl ethers. o- - - - -o, $n = 14$ (8.5 μM); •———•, $n = 24$ (8 μM); ▲- - - -▲, $n = 45$ (1.1 mM); △- - - - -△, $n = 60$ (90 μM).

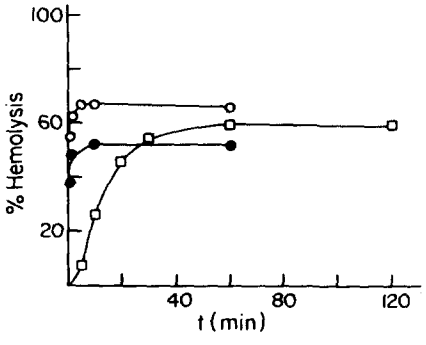

Fig. 2. Time dependence of saponin induced hemolysis. •———•, tomatin (10 μM); □———□, styrax saponin A (0.3 μM); o———o, digitonin (3 μM).

TABLE II

EFFECT OF GLYCYRRHIZIN ON CETOMACROGOL- AND H_2O_2-INDUCED HEMOLYSIS

For percentage hemolysis the number of experiments in parenthesis.

Hemolysin	Hemolysin cocn.	% Hemolysis (Mean ± S.E.)	Glycyrrhizin Concn. (mM)	% Hemolysis (Mean ± S.E.)	% Augmentation
Cetomacrogol	8 μM	59 ± 0.3 (10) [a]	3	68 ± 0.2 (10) [a]	15
Cetomacrogol	6.6 μM	46 ± 0.3 (10) [a]	3	60 ± 0.2 (10) [a]	30
H_2O_2 (batch I)	0.5%	3 ± 0.15 (8) [b]	3	36 ± 1.7 (8) [a]	1 200
(batch II)	0.5%	3.5 ± 0.15 (8) [b]	3	43 ± 2 (8) [b]	1 200
(batch I)	0.05%	4 ± 0.15 (8) [b]	3	16 ± 0.8 (8) [b]	400
Cetostearyl alcohol	60 μM	11 ± 0.4 (6) [b]	3	6 ± 0.3 (6) [b]	−45 [c]

[a] $P < 0.01$ compared to average hemolysis under equal conditions.
[b] $P < 0.02$ compared to average hemolysis under equal conditions.
[c] i.e. 45% inhibition.

TABLE III

THE INHIBITORY EFFECT OF VITAMIN E, QUERCETIN, RUTIN AND N_2 ON THE HEMOLYSIS OF VARIOUS HEMOLYSINS

For percentage hemolysis the number of experiments in parenthesis.

Hemolysin	Hemolysin concn. (μM)	% Hemolysis (Mean ± S.E.)	Inhibitor	Inhibitor concn. (μM)	% Hemolysis (Mean ± S.E.)	% Inhibition
Cetomacrogol	7.6	42 ± 0.3 (10) [a]	Vitamin E	24	11 ± 0.2 (10) [a]	75
	11	94 ± 0.4 (10) [a]		24	61 ± 0.2 (10) [a]	36
Saponin A	0.8	60 ± 0.3 (10) [a]	Vitamin E	24	60 ± 0.3 (10) [a]	–
	1.2	85 ± 0.3 (10) [a]		24	85 ± 0.3 (10) [a]	–
Digitonin	1.3	42 ± 0.4 (10) [a]	Vitamin E	24	42 ± 0.4 (10) [a]	–
	2	95 ± 0.4 (10) [a]		24	91 ± 0.4 (10) [a]	–
Cetomacrogol	7.6	42 ± 0.3 (10) [a]	Quercetin	330	1.9 ± 0.4 (10) [a]	55
	8.8	76 ± 0.3 (10) [a]		330	42 ± 0.6 (10) [a]	45
Saponin A	0.8	60 ± 0.3 (10) [a]	Quercetin	330	52 ± 0.4 (10) [a]	14
	1.2	85 ± 0.3 (10) [a]		330	86 ± 0.5 (10) [a]	–
Digitonin	1.3	42 ± 0.4 (10) [a]	Quercetin	330	32 ± 0.4 (10) [a]	24
	2	91 ± 0.4 (10) [a]		330	76 ± 0.6 (10) [a]	17
Sapogenin A	0.6	77 ± 0.5 (6) [a]	Quercetin	330	77 ± 0.5 (6) [a]	–
Cetomacrogol	7.6	42 ± 0.2 (10) [a]	Rutin	160	17 ± 0.3 (10) [a]	60
	8.8	76 ± 0.2 (10) [a]		160	33 ± 0.3 (10) [a]	57
Saponin A	0.8	60 ± 0.3 (10) [a]	Rutin	160	50 ± 0.3 (10) [a]	17
	1.2	85 ± 0.3 (10) [a]		160	85 ± 0.3 (10) [a]	–
Digitonin	1.3	42 ± 0.4 (10) [a]	Rutin	160	33 ± 0.7 (10) [a]	21
	2	91 ± 0.4 (10) [a]		160	79 ± 0.8 (10) [a]	14
Sapogenin A	0.6	77 ± 0.3 (8) [a]	Rutin	160	77 ± 0.3 (8) [a]	–
Cetomacrogol	9.6	90 ± 0.1 (8) [a]	N_2		72 ± 3.3 (8) [c]	20
	8	57 ± 0.3 (8) [a]			36 ± 1.5 (8) [b]	34
Saponin A	0.8	60 ± 0.3 (6) [a]	N_2		60 ± 0.3 (6) [a]	–
Cetostearyl alcohol	60	10 ± 0.4 (6) [b]	N_2		10 ± 0.4 (6) [b]	–

[a] $P < 0.01$ compared to average hemolysis under equal conditions.
[b] $P < 0.02$ compared to average hemolysis under equal conditions.
[c] $P < 0.04$ compared to average hemolysis under equal conditions.

The time dependence of quercetin inhibited cetomacrogol hemolysis is represented by Fig. 3. While cetomacrogol hemolysis always tends towards 100% hemolysis, the presence of quercetin effects a lag in the onset of the hemolysing process, and hemolysis reaches final levels below 100% depending on its concentration. Similar results were obtained for vitamin E inhibition [15].

The possibility that one of the two parts composing the surfactant molecule (i.e. the alcohol or the polyoxyethylene part) is responsible for the hemolytic activity was considered. Consequently the hemolytic properties of cetostearyl alcohol and of polyethyleneglycol-1000 (equivalent in the polyoxyethylene moiety to cetomacrogol) were tested. While the polyethylene glycol caused no hemolysis up to 2.5 mM, cetostearyl alcohol induced hemolysis. Because of the low solubility of this alcohol, the concentration could not be increased beyond 60 μM. At this concentration about 11% hemolysis was obtained. This activity is in accordance with our previous results concerning the hemolytic properties of steroids and other non-ionic hemolysins [16]. Nevertheless cetomacrogol-induced hemolysis cannot be ascribed to the cetostearyl part for the following reasons:

(a) Cetomacrogol is by far more hemolytic than cetostearyl alcohol (Table I).

(b) Glycyrrhizin enhances cetomacrogol hemolysis but inhibits cetostearyl alcohol and saponin hemolysis (Table II).

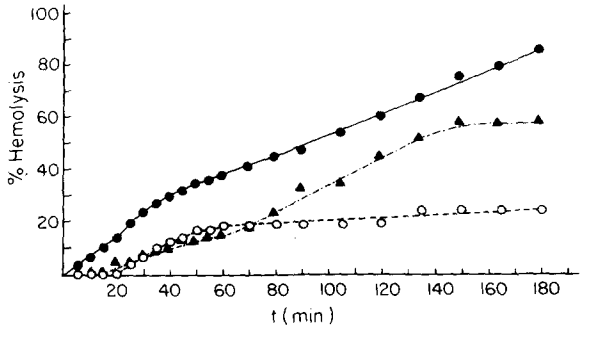

Fig. 3. Time dependence of cetomacrogol hemolysis in the presence of quercetin: ●——●, cetomacrogol (4.8 μM) without quercetin; ▲- - - -▲, cetomacrogol (4.8 μM) with quercetin (210 μM); ○- - - - -○, cetomacrogol (4.8 μM) with quercetin (330 μM).

(c) N_2 inhibits cetomacrogol hemolysis but has no effect on cetostearyl alcohol and saponin hemolysis (Table III). The influence of quercetin and vitamin E could not be tested since the quantity of DMSO needed for these experiments exceeded the permissible.

Discussion

The results of our investigation indicate that there exists great similarity between the hemolytic properties of polyoxyethylene-derived surfactants and H_2O_2. The similarity is reflected both in the kinetics of the process and in the effect of various inhibitors. The rate of hemolysis obtained by the polyoxyethylene-cetostearyl ethers tested is slow when compared to that of saponins, (Figs. 1 and 2). It proceeds in an S shape curve tending slowly to 100% similar to what has been reported for H_2O_2 [13]. Saponins on the other hand reach their final hemolytic level within a few minutes; the extent of this level depending on the concentration of the hemolysin [12]. Furthermore, glycyrrhizin, a potent inhibitor for saponin hemolysis [14], greatly enhances H_2O_2 and cetomacrogol hemolysis, while various antioxidants such as quercetin, rutin, and especially vitamin E, have a strong inhibitory effect not only on H_2O_2 hemolysis [13] but also on that of cetomacrogol (Table III). These compounds have no such effects on saponins and sapogenins. (Whatever slight effect of the quercetin and rutin could be observed, may be due to some non-specific protective interaction of these compounds with either the red blood cell membrane or the hemolysin). Although in general phenols are known to interact with polyoxyethylene-derived surfactants [17,18], the quercetin- and rutin-induced inhibition of hemolysis cannot solely be attributed to this phenomenon. The time dependence curve of hemolysis in the presence of quercetin does not show a reduction in the rate of attaining 100% hemolysis, as would be expected if part of the hemolysin was neutralized by interaction with the inhibitor, but reveals a plateau at a fixed level, apparently due to the interference in the hemolytic process.

Peroxide induced hemolysis is a well known process [7,8] and is obtained by various drugs, all of which have been shown to generate H_2O_2 in vitro [19,20]. The damage to the red blood cell membrane

in this type of hemolysis is generally ascribed to peroxidation of the unsaturated fatty acids which are components of the erythrocyte membrane [21]. Peroxide formation in aqueous solutions of polyoxyethylene-derived surfactants was recently reported [4,5]. However their hemolysing capacities have not as yet been associated with this property.

We suggest that autoxidation of polyoxyethylene-derived surfactants comprises a major component in their hemolysing capacity. The fact that anaerobic conditions inhibit only cetomacrogol hemolysis further supports our assumption.

Since the susceptibility of a given commercial compound to auto-oxidation is dependent on numerous uncontrollable factors (e.g. age, conditions of storage and packaging, source of raw materials, etc.) the diversity in hemolysing capacity of various surfactants (Table I) would not be surprising.

The examination of the hemolytic properties of each of the cetomacrogol components clearly shows that although one of them, namely the cetostearyl alcohol, is hemolytic by itself, it changes its hemolytic properties when it is bound to the polyoxyethylene moiety. While by itself, it resembles saponins and sapogenins but it acquires hemolysing properties characteristic of H_2O_2 when bound to the polyoxyethylene part. Undoubtedly, the polyoxyethylene moiety of the surfactant, though non hemolytic in itself, is responsible for the 'peroxide' mechanism of polyoxyethylene surfactants hemolysis. Although surface activity could not be correlated to the hemolytic properties of these surfactants, it seems that surface activity is necessary for obtaining hemolysis. Therefore polyethyleneglycol-1000, not being a surfactant, did not produce hemolysis. The influence of surface activity on peroxide induced hemolysis is now under investigation.

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