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Biphasic interaction of Triton detergents with the erythrocyte membrane

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Octylphenoxy polyoxyethylene ethers (Triton detergents) interact with the erythrocyte membrane in a biphasic manner, i.e. they stabilize erythrocytes against hypo-osmotic haemolysis at low concentrations (0.0001–0.01%, v/v), but become haemolytic at higher concentrations. This biphasic behaviour was demonstrated with Triton X-114, Triton X-100 and Triton X-102. However, a critical chain length is a prerequisite for the haemolytic effect, because Triton X-45, which differs from the other Tritons only by the shorter chain of the polyoxyethylene residue, does not exhibit this biphasic behaviour, but goes on protecting against osmotic rupture up to saturating concentrations. Even a 1% solution of Triton X-45 does not cause haemolysis. This structural specificity of Triton X-45, namely the lack of haemolysis and efficient stabilization against osmolysis even at higher concentrations of the detergent, is exhibited at 0° and 37 °C as well as at room temperature. Three conclusions are reached: (i) a critical chain length of the octylphenoxy polyoxyethylene ethers is required for the haemolytic effect; (ii) the different structural requirements would suggest that different mechanisms are responsible for the haemolytic and the stabilizing effect of amphiphilic substances; (iii) the results suggest that haemolysis is not caused simply by dissolution of the membrane by the detergent but is a rather more specific process.

INTRODUCTION

A number of lipophilic anaesthetics (Seeman, 1966*a*), phenothiazine tranquilizers (Seeman & Weinstein, 1966) and also non-esterified fatty acids are known to stabilize erythrocytes against hypo-osmotic stress (for reviews, see Seeman, 1966*b*, 1972; Beutler, 1969).

Low concentrations of these drugs increase the osmotic resistance of erythrocytes, whereas higher concentrations lead to promotion of haemolysis. The molecular mechanism of this biphasic behaviour is not known. Unsaturated C_{18} fatty acids exhibit the stabilizing and haemolytic effects with pronounced structural and configurational dependence (Csordas & Schauenstein, 1984). We recently showed, comparing oleic and elaidic acids, that the configurational specificity of the protecting and haemolytic effects exhibited by elaidic acid is strongly temperature-dependent (Csordas & Schauenstein, 1986).

The purpose of the present investigation was to test a number of other amphiphilic substances and compare their effects on the erythrocyte membrane with those of non-esterified unsaturated C_{18} fatty acids, in order to learn more about the structural features of the amphiphilic molecules that have influence on the stabilizing and the haemolytic effect respectively. To that end we selected a number of non-ionic detergents that differed from each other only in their chain length.

The result of the present investigation is that the Triton detergents tested are extremely good at protecting human erythrocytes against hypo-osmotic lysis and become haemolytic above a certain concentration range (0.01%, v/v). The striking observation is that Triton X-45, with a polyoxyethylene chain length shorter than those of the other Tritons, did not cause haemolysis even at the highest concentrations. Thus one can conclude that there is a critical chain length required for the haemolytic effect which has no influence on the protection against haemolysis and, hence, different mechanisms would seem to be responsible for the two effects.

The stabilizing effect of Triton X-45, non-haemolytic up to even the highest concentrations, is not a function of temperature. This is in contrast with the behaviour of elaidic acid, which was shown to cause haemolysis above a critical temperature and did not exhibit any kind of effect at 0 °C (Csordas & Schauenstein, 1986). The specificity of the Triton X-45 effect did not change at 0 °C or at 37 °C.

EXPERIMENTAL

Materials

Heparin, Triton X-102, Triton X-114, Triton X-45, Nonidet P40 and SDS were from Sigma. Triton X-100 was from Loba Chemie, Fischamend, Austria. The salts used for the buffer solutions were from Merck. All reagents were of the highest purity available.

Buffer solutions

Phosphate-buffered saline solution was 155 mM-NaCl with 50 mM-sodium phosphate, pH 7.4. Hypo-osmotic buffer solution was either 50 mM-sodium phosphate without NaCl, pH 7.4, or a 1:3 dilution of the buffered saline solution with distilled water. Two different hypo-osmotic buffers were used in order to test the effect of detergents on the erythrocyte membrane under the

Abbreviation used: CMC, critical micellar concentration.

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conditions of low and high hypo-osmotic stress respectively.

Erythrocyte preparation

Heparinized blood was drawn from male volunteers. The cell pellet was washed three times in 10–20 vol. of phosphate-buffered saline (155 mM-NaCl/50 mM-sodium phosphate buffer, pH 7.4); the plasma and buffy coat were removed by aspiration. After three washings with the phosphate-buffered saline solution (centrifugation for 5 min at 700 *g*), the erythrocytes were suspended at a haematocrit of 50%.

Haemolysis assay

Haemolysis was measured, unless otherwise stated, at ambient temperature after an incubation period of 30 min. A 5 ml portion of haemolysis buffer was taken, to which the different concentrations of the detergents were added in volumes ranging from 5 to 15 μl. Haemolysis was started by adding 50 μl of the erythrocyte suspension. At the end of the 30 min incubation period the suspension was centrifuged for 10 min at 700 *g* in a Hettich Roto Silenta/K centrifuge and the A_{540} of the supernatant was determined.

RESULTS

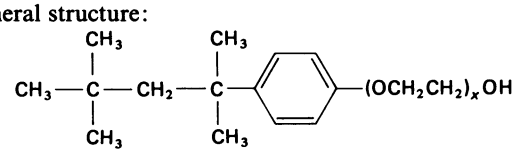
Chemical structure of the Triton detergents

Table 1 shows the structures of the Triton detergents (octylphenoxy polyoxyethylene ethers), which differ only in the number of the ethylene oxide groups and thus in their average M_r values. Triton X-45, with five ethylene oxide groups, has a total chain length of 18 carbon atoms, which gives a structure comparable with that of the C_{18} fatty acids. The benzene ring within the aliphatic chain can be considered as being analogous to the double bonds of unsaturated C_{18} fatty acids. These common features might explain why two classes of compounds apparently quite different from each other have similar effects on the erythrocyte membrane. Triton X-100 with nine to ten ethylene oxide groups has a chain length of 26–28 carbon atoms, and the largest molecule tested, Triton X-102, with 12–13 ethylene oxide groups, is a chain of 32–34 carbon atoms. Thus the analogy to C_{18} fatty acids is very limited, but it should be noted that fatty acids above the chain length of 18 carbon atoms have not yet been tested for their effect on the erythrocyte membrane. On the other hand, chlorpromazine (Kwant & Seeman, 1969; Kwant & van Steveninck, 1968), a large number of structurally unrelated anaesthetics (Seeman, 1966*a*; Seeman & Weinstein, 1966) and other compounds (Seeman, 1966*c*) were found to have a stabilizing effect on the erythrocyte membrane.

Table 1. Structure of the amphiphilic compounds

(*a*) Non-ionic detergent	No. of ethylene oxide groups (*x*)
Triton X-45	5
Triton X-114	7–8
Triton X-100	9–10
Triton X-102	12–13

General structure:



(*b*) Anionic detergent	Structure
SDS	CH_3–$(CH_2)_{11}$–$SO_4^-Na^+$

Biphasic interaction of the higher-M_r Triton detergents with the erythrocyte membrane

Fig. 1 shows the effect of Triton X-100, Nonidet P40, Triton X-102 and Triton X-114 on the osmotic resistance of human erythrocytes. (Nonidet P40 is another trade name for the same product as Triton X-100, i.e. an octylphenoxy oxyethylene condensate containing an average of nine molecules of ethylene oxide per molecule). These non-ionic detergents exhibit the typical biphasic behaviour that is also exhibited by long-chain fatty acids. The non-ionic detergents are extremely good protecting agents over the concentration range 0.0001–0.01% (v/v), but at the upper limit of this concentration range, 0.01%, they become haemolytic, a behaviour for which Triton detergents are better known.

Effect of an anionic detergent on erythrocyte stability against hypo-osmotic haemolysis

After the above observations on non-ionic detergents, the question was raised as to whether other types of detergents also exhibit this behaviour and, if so, to what extent. This question is important in the context of whether the dissociated or non-dissociated form of the fatty acid or amphiphilic anaesthetic is responsible for the interaction with erythrocytes. For that purpose the anionic detergent SDS was tested. The result of this experiment is shown in Fig. 2. The protecting effect appears over the same concentration range as was the case with non-ionic detergents. Thus it appears that the mechanisms responsible for the effect exhibited by SDS and the non-ionic detergents are the same. This also holds true for the concentration at which haemolysis starts. Thus one has to conclude that uncharged, but polar, groups of amphiphilic compounds and detergents with a fully dissociated negatively charged group are equally good protecting agents against hypo-osmotic haemolysis.

It has been suggested (Roth & Seeman, 1971) that all lipid-soluble anaesthetics protect erythrocytes against hypo-osmotic haemolysis, whereas water-soluble drugs are not effective. In another study (von Ehrly *et al.*, 1964) it was observed that sodium salts of fatty acids decreased erythrocyte haemolysis in hypo-osmotic solutions, whereas undissociated acids were ineffective. A comparison of the esterified forms of C_{18} fatty acids and alcohols of equal chain length with the corresponding fatty acid with a free carboxy group with respect to their ability to protect erythrocytes against hypo-osmotic stress (Raz & Livne, 1973) led to the conclusion that the protecting effect becomes marginal when there is no negative charge in the molecule. According to this study (Raz & Livne, 1973) alcohols protect much less than the corresponding fatty acids, esters only marginally at low extents of haemolysis and at higher extents of haemolysis not at all. The results with Triton ethers with an alcohol group (the present investigation), however, demonstrate that a

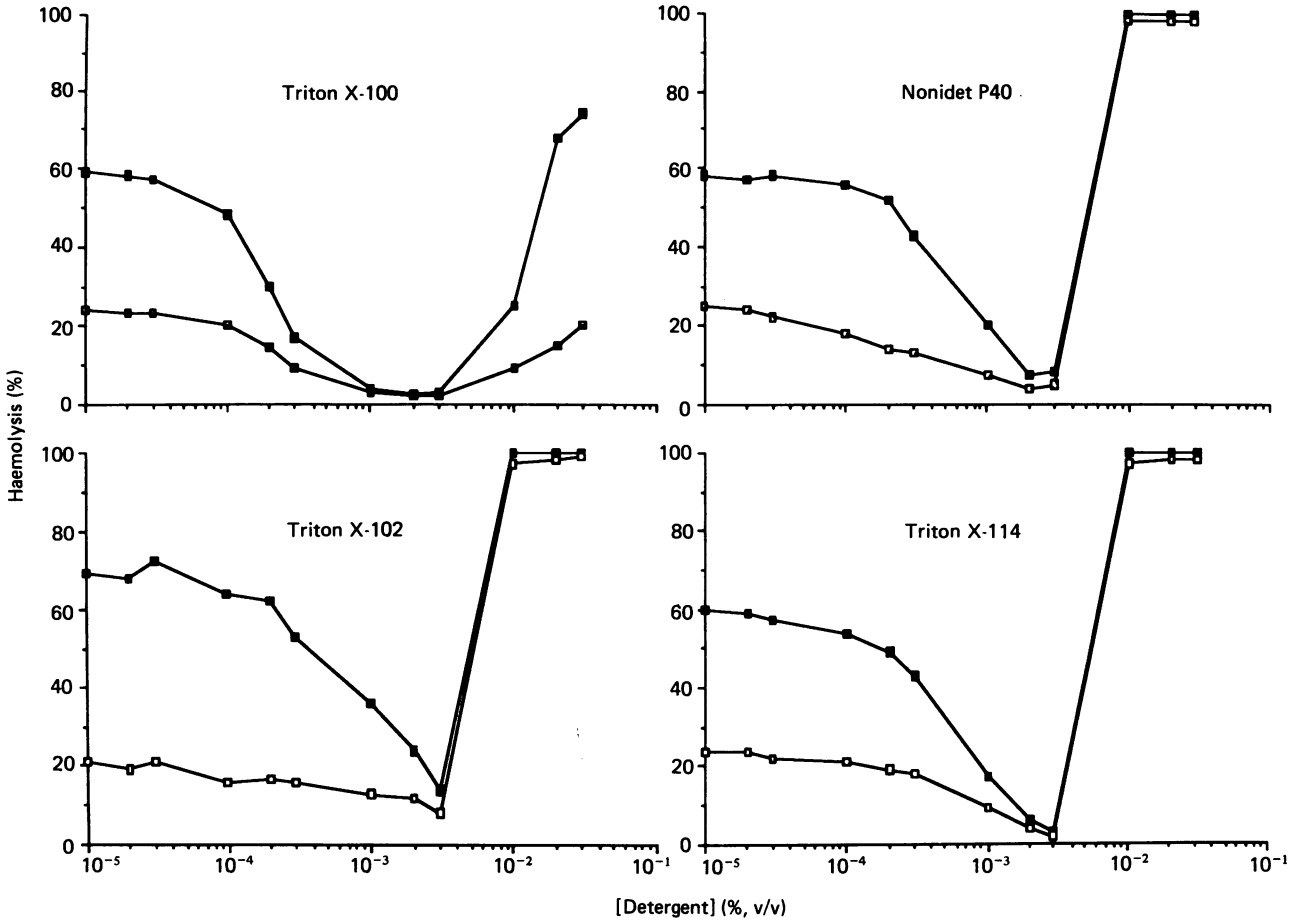


Fig. 1. Effects of hypo-osmotic treatment with the non-ionic Triton detergents of various chain lengths on the osmotic resistance of human erythrocytes

For each detergent the effects were examined at two different levels of haemolysis. First the aqueous solution of the detergent and then 50 μl of the erythrocyte suspension were added to 5 ml of the hypo-osmotic buffer solution. After 30 min of incubation, the samples were centrifuged (10 min at 700 g) and the A_{540} of the supernatant was determined. The degree of haemolysis is plotted against the concentration of the detergent (%, v/v).

negative charge is not in itself a prerequisite for the efficient protection of the erythrocyte membrane against hypo-osmotic stress.

Lack of haemolysis, and efficient protection against osmotic rupture, by Triton X-45

Examining the effect of Triton X-45 on the osmotic resistance of erythrocytes the striking observation was made that, even at the highest concentrations tested, there was no promotion of haemolysis, but only protection from haemolysis. This is shown in Fig. 3. Thus there is a very sharply defined critical chain length (more than five ethylene oxide groups) that is a requirement for haemolysis in the case of octylphenoxy polyoxyethylene ethers. However, the protecting effect is not affected by this chain length. Therefore one can speak of an uncoupling of the two effects by Triton X-45. As the molecular basis of the two effects, namely protection against osmotic rupture and haemolysis promotion, is not known, the structural similarity of Triton X-45 and Triton X-114 and the correlation of the haemolytic effect with a specific structural feature are of considerable interest in elucidating the mechanism of the biphasic behaviour.

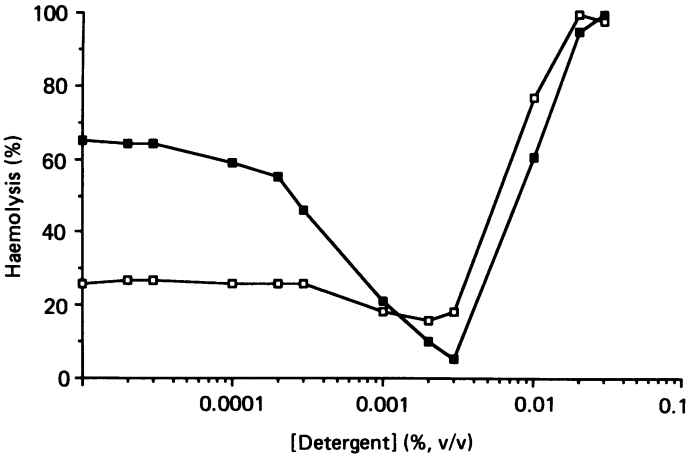


Fig. 2. Biphasic behaviour of SDS examined under the same experimental conditions as for the Triton detergents in Fig. 1 (see also the Experimental section)

Protection against osmolysis by Triton X-45 at different temperatures

Previous studies in this laboratory (Csordas & Schauenstein, 1986) have shown that lack of haemolysis

and efficient protection are characteristic for elaidic acid at room temperature. However, the interaction of elaidic acid with the erythrocyte membrane proved to be a strongly thermotrophic effect. At 37 °C, elaidic acid becomes a powerful haemolytic agent, even under iso-osmotic conditions. Interestingly, at 0 °C, elaidic acid showed no effect whatsoever, i.e. no protection and no haemolysis. On the contrary, oleic acid showed extremely good protection, even at 0 °C (Csordas & Schauenstein, 1986).

The results depicted in Fig. 4 show that the lack of biphasic behaviour exhibited by Triton X-45 is not a thermotrophic effect. Protection against hypo-osmotic lysis and lack of haemolysis can be observed at 0 °C, at room temperature and at 37 °C. Thus different mechanisms must be responsible for the effects exhibited by elaidic acid and Triton X-45 respectively.

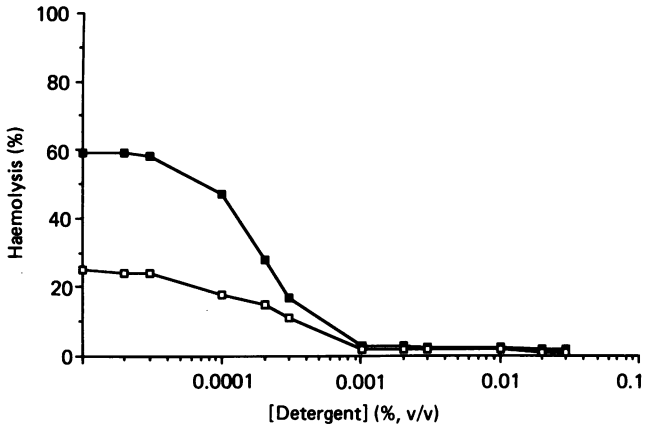


Fig. 3. Effect of the non-ionic detergent Triton X-45 on the resistance of human erythrocytes to hypo-osmotic stress

Triton X-45 does not show the biphasic behaviour shown by the other Tritons, but continues to protect against hypo-osmotic lysis up to the highest concentrations of the detergent. This concentration-dependent effect of Triton X-45 is shown at two different levels of hypo-osmoticity.

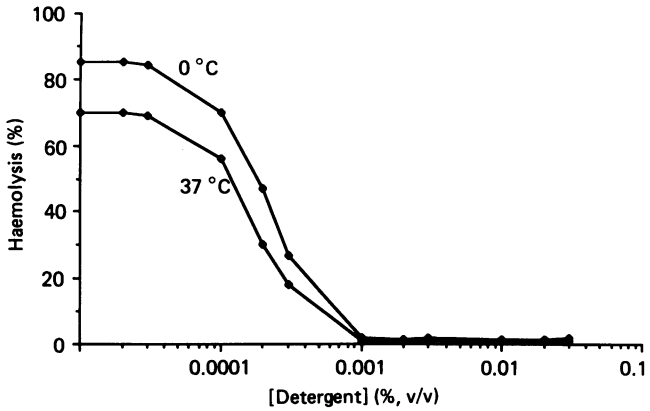


Fig. 4. Protecting effect of Triton X-45 at 0 °C and at 37 °C after an incubation period of 30 min

The experimental set-up was as described for Fig. 1. The protecting effect of Triton X-45 occurs at the same concentration, and to the same extent, at the two different temperatures.

A temperature-dependence of specificity would indicate whether a lipid layer with a critical transition point is affected, or rather a membrane area which is not temperature-sensitive. The results in Fig. 4 show that, whatever temperature-dependent transitions occur in the membrane between 0 °C and 37 °C (Morariu *et al.*, 1981), they do not have an influence on the specific interaction with Triton X-45. This behaviour is in contrast with that of elaidic acid (Csordas & Schauenstein, 1986), which becomes haemolytic above a critical temperature.

DISCUSSION

It has been suggested that amphiphilic substances interfere with the physical stability of biological membranes because of their 'detergent-like activities' (Katz & Messineo, 1981). According to this hypothesis, the erythrocyte membrane is dissolved by micelles formed at the critical micellar concentration (CMC) of the amphiphiles.

We cannot defend or refute this hypothesis by comparing the CMC values published by others (Helenius & Simons, 1975; Mukerjee & Mysels, 1971; Ray & Nemethy, 1971; Becher, 1967), because they were not determined exactly under the same salt and temperature conditions of the buffers of the haemolysis assay used in the present study, and a systematic investigation of the possible correlation between haemolytic concentrations and CMC values is still lacking. The published CMC values for the detergents used in the present study are not correlated with the haemolytic concentrations and with the distinctly different behaviour of Triton X-45.

The CMC values (Helenius & Simons, 1975) are: for Triton X-45, 0.11 mM; Triton X-114, 0.20 mM; Nonidet P40, 0.29 mM; Triton X-100, 0.240 mM; Triton X-102, 0.3–0.4 mM; SDS in water, 8.2 mM. Since Triton X-45 forms micelles at a lower concentration than do the other detergents, the haemolytic concentration does not seem to be correlated with the CMC value. The lack of haemolysis cannot be explained by the lack of micelles attributable to shorter ethylene oxide chains in this compound.

Non-ionic detergents are widely used for the solubilization of integral membrane proteins which interact directly with the hydrophobic core of the lipid bilayer (Rubin & Tzagoloff, 1973; Tanford & Reynolds, 1976; Helenius *et al.*, 1979). It has been demonstrated for a number of detergents that it is the monomeric species that is bound, and not the micellar form, when both ligand and protein are present at relatively low concentration (Reynolds & Tanford, 1970; Makino *et al.*, 1973; Nozaki *et al.*, 1974; Le Maire *et al.*, 1983).

As an explanation for an altered osmotic resistance, a direct effect solely on the membrane or, alternatively, a mechanism via signal transduction involving the cytoskeleton by glycophorin–spectrin interaction is conceivable (Anderson & Lovrien, 1984; Bodine *et al.*, 1984; Podgorski & Elbaum, 1985; Forte *et al.*, 1985).

From the fact that a chain-length difference of a few carbon atoms determines whether a non-ionic detergent is haemolytic, one has to conclude that either micelles of a certain critical size are required for the rupture of the erythrocyte membrane or that, for a certain type of binding, the length of the molecule is crucial. Since

Triton X-45 markedly increases the osmotic resistance of erythrocytes, it is evident that an interaction does take place between Triton X-45 and the erythrocyte membrane, and it is known that Triton X-45, in principle, does form micelles at an even lower concentration than do the other Tritons tested. However, this type of interaction is not related to haemolysis. Thus Triton X-45 causes a structural distortion that, even at maximum loading with the detergent, does protect against hypo-osmotic lysis and is not severe enough to cause haemolysis.

Further studies using liposomes will be required to determine whether the protein moiety of the membrane is a prerequisite for the biphasic behaviour of non-ionic detergents and the specific effect exhibited by Triton X-45.

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