International Journal of Pharmaceutics, 53 (1989) 241–248
Elsevier

IJP 01808

Influence of non-ionic surfactant structure on motility inhibition of *Tetrahymena elliotti*: a model for surfactant–membrane interactions

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(Received 13 December 1988)
(Accepted 29 January 1989)

Key words: Non-ionic surfactant; TRITON; *Tetrahymena elliotti*; Inhibition of motility; Structure–activity relationship

Summary

The ability of non-ionic surfactants to inhibit the motility of the protozoan *Tetrahymena elliotti* was determined as a minimal inhibitory concentration (MIC), the lowest concentration that stops movement in a cell suspension. A good correlation was found between MIC and the concentration to reduce swimming speed of the protozoan by 50%. The inhibitory activity of the members of a series of *p-t*-octyl phenyl polyoxyethylene ethers (TRITON-X series), nonyl phenyl polyoxyethylene ethers (TRITON-N series), polyoxyethylene alkyl ethers (BRIJ series), poly-oxyethylene (20) sorbitan esters (TWEEN series) and alkanols (methanol to octanol) was compared on the basis of MICs. The system was sensitive and could be used to discriminate between the linear and branched forms of an alkanol, between surfactants of the same series which differed by only a few ethylene oxide (EO) units and between TRITON-X and N surfactants of the same EO content. That is in effect an ability to discriminate between the *n*-nonyl and *p-t*-octyl moieties of the TRITONS. For all of the groups of test substances, inhibitory activity was inversely related to hydrophilicity and some of the most hydrophilic non-ionic surfactants were not inhibitory. Throughout a series of surfactants, MIC was not directly related to the critical micelle concentration and was much more sensitive to the influence of EO content than the latter parameter. The effect of the surfactants was probably mediated via a disruptive action on the cell membrane, the limiting factor being the ability of the surfactant to partition into this lipid domain. Inhibition of protozoan motility may form the basis of a simple means of screening compounds for irritancy.

Introduction

Non-ionic surfactants, used in foodstuffs and pharmaceuticals (Attwood and Florence, 1983), while in general less toxic than ionic surface active agents, are not biologically inert molecules. Some non-ionic surfactants are effective membrane penetration enhancers (Attwood and Florence, 1983: Florence, 1982) and although some structure–activity relationships have been defined (Florence et al., 1984), there is still no clear information on the critical features of surfactant structure which confer membrane fluidizing activity, and there is less information on the relationships between membrane composition and surfactant activity.

As part of a study of these relationships we have investigated the interaction of members of 4

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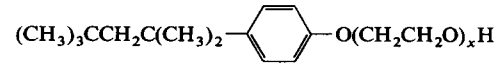
series of non-ionic surfactant with *Tetrahymena elliotti*, a constantly motile protozoan, which has been used here as a living, model, target membrane. *Tetrahymena* spp. have been extensively studied as model eukaryotic cells in biochemical and pharmacological studies. More specifically they have been used to investigate the activity of membrane active agents such as general (Nunn et al., 1974) and local (Cassidy and Henry, 1987) anaesthetics and anti-adrenergic drugs (Cassidy and Henry, 1987). Interaction of agents with the plasma membrane of the cell or cilia interferes with the motility of the organism and so for example, inhibition of motility can be used to provide an indirect measure of such interaction. Recently the ability of some novel local anaesthetics to inhibit protozoan motility was shown to correlate well with their duration of action in the guinea-pig wheal test (Al-Saadi et al., 1981). These novel compounds, in common with local anaesthetics in clinical use, are surface active and their biological activity is related to this property and to their lipophilicity which appears to influence their ability to interact with membranes and subsequently modify membrane function. The inhibitory effects of non-ionic surfactants such as the nonyl-phenoxypolyethyleneoxyethanols (Gadd and Curtis-Prior, 1987) on sperm motility is dependent on their lipophilicity. A greater understanding of the contributions of the hydrophilic head group and lipophilic hydrocarbon moiety of non-ionic surfactants to the ability of such compounds to modify membrane function is required. The effects of TRITON-X and -N surfactants and other non-ionic surfactants, which cover a wide range of solubility characteristics and alkyl moieties, on the motility of *Tetrahymena elliotti* are described, and the results compared with those obtained with a series of alkanols.

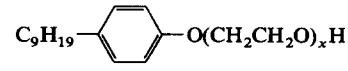
Materials and Methods

Materials

Tetrahymena elliotti (from The Culture Centre of Algae and Protozoa, Cambridge, U.K.) was maintained in static axenic culture at 27 °C in 100 ml of medium (autoclaved 2.0% Difco neopeptone and 0.1% Oxoid neutralised liver digest in water) in a cotton wool plugged 500 ml flask. The cells were harvested after 3 days, in the log phase of growth, by centrifuging at 900 *g* for 15 min and twice washing the pellet with distilled water. Cells were finally suspended in water and diluted to a standardised (Coulter Counter) absorbance of 0.5 at 600 nm which corresponded to a cell density of some 1.8×10^4 ml^{-1}. Cells were used in experiments ≤ 24 h after centrifuging.



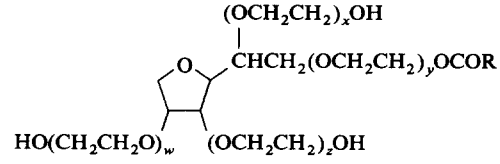
Poly(oxyethylene) *p*-t-octylphenyl ethers



Poly(oxyethylene) *n*-alkylphenyl ethers

$C_nH_{2n+1}O(CH_2CH_2O)_xH$

Poly(oxyethylene) *n*-alkyl ethers



Poly(oxyethylene) anhydrohexitol fatty esters

Scheme 1.

The *p-t*-octyl phenyl polyoxyethylene ethers (TRITON-X series), the polyoxyethylene *n*-alkyl ethers (BRIJ series), the polyoxyethylene sorbitan (anhydrohexitol) esters and the alkanols were obtained from Sigma, the nonyl phenyl polyoxyethylene ethers (TRITON N series) from the Rohm and Haas (U.K.) Ltd. All were used as received.

Methods

Determination of Tetrahymena swimming speed. Equal volumes (0.1 ml) of *Tetrahymena* cell suspension were mixed with equal volumes of water (control) or of serial dilutions of selected TRITON X surfactants (X-35, X-45, X-100, X-165 and X-705) on the surface of a microscope slide. The cells in the drop were examined using a 10 ×

objective without a coverslip, under dark-field illumination on a Polyvar research microscope (Cambridge Instruments). At 90 min after mixing cells and surfactant solution, cell movement was video-recorded using a camera with a gridded lens. The grid was calibrated, 90 μm side, using a stage micrometer (Graticules Ltd.) to allow frame by frame (0.04 s) analysis of movement. The change in position of a cell after a known number of frame shifts (typically 20–25), plotted on an acetate overlay on the monitor, was used to calculate swimming speed (μm/s) and a mean speed was obtained from the observation of 10–20 cells. A plot of swimming speed against surfactant concentration was used to determine the surfactant concentration (C_{50}) for a 50% reduction in swimming speed.

Determination of minimal inhibitory concentration (MIC). Equal volumes (0.1 ml) of *Tetrahymena* cell suspension and surfactant solution were mixed in the flat-bottomed wells of a microtitre plate (Flow Laboratories). A range of surfactant concentrations was used and after 90 min at room temperature, examination of the wells under a dissecting microscope allowed identification of that concentration of any particular surfactant which stopped all cell movement in a well. The lowest concentration to give complete cessation of movement was taken as the MIC.

Results and Discussion

The effect of alkanols on *Tetrahymena* motility (MIC, mM) is shown in Fig. 1 where it can be seen that, as expected, increasing the number of carbon atoms (n) in the series was associated with a decrease in MIC, that is an increase inhibitory activity. The regression of log MIC with n, obtained from the slope of the best linear fit to the data in Fig. 1, was 0.44 per CH_2 which is in good agreement with published values for the dependence of partition coefficient on the addition of carbon atoms to non-aromatic compounds. These results also indicate the importance of partitioning in the mechanism of inhibition of the motility of this organism. It was found that for the two examples investigated, butan-2-ol and pentan-2-ol, that these branched alcohols were slightly less active

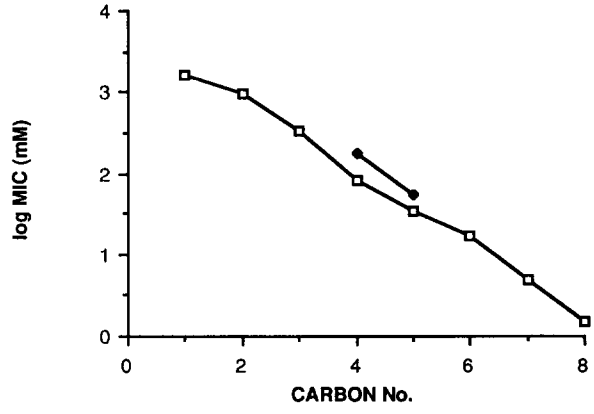


Fig. 1. The effect of alkyl chain length (carbon no.) of *n*-alkanols (□) and 2-ol alkanols (◆) on their ability to inhibit motility of *T. elliotti*. Inhibitory activity is expressed as the MIC (mM), the lowest concentration to just inhibit protozoan motility in a suspension of the cells.

than their linear isomers. As a presumptive test of the importance of partitioning, it is possible to calculate expected MIC values for the 2-ols, on the basis of the MIC values found for the 1-ols and the published values (Leo et al., 1971) for the partition coefficients of the 1-ols and 2-ols. The results, for butan-2-ol: calculated 0.15 mM, found 0.17, and for pentan-2-ol: calculated 0.065, found 0.056, provide strong evidence for the important role that partitioning of the alkanols into the protozoan membrane plays in the mechanism of inhibition since it is recognised that branching decreases the oil/water partition coefficient. These results also give a good indication of the sensitivity of the system.

The effects of the TRITON surfactants on *Tetrahymena* motility determined as MIC are shown in Table 1 and Fig. 3 and as that concentration to reduce mean swimming speed by 50% (C_{50}) in Fig. 2. For those TRITON-X surfactants whose C_{50} was determined, there was a good correlation between the quantal C_{50} and the all-or-nothing measurement inherent in MIC (Fig. 3) as demonstrated by the constancy of the MIC/C_{50} ratio (Fig. 2). However, the determination of MIC was much simpler than C_{50} and MIC was therefore used more frequently in this work. The C_{50} values were all well below the surfactant CMC (Table 1)

TABLE 1

MIC of some TRITON surfactants for the inhibition of T. elliotti motility

Surfactant	EO *	MIC (mM)
X-15	1	0.162
35	3	0.089
45	5	0.065
114	7–8	0.117
100	9–10	0.25
102	12–13	0.33
165	16	0.55
305	30	8.18
405	40	> 200 *
705	70	19.01
N-42	4	0.32
57	5	0.57
60	6	0.52
101	9–10	2.39
111	11	2.22
150	15	7.10

EO, Manufacturers mean value for the number of ethylene oxide units in the hydrophilic domain of the surfactant.
* Cells still motile at this (200 mM) concentration.

and although for some TRITON-X surfactants, values for CMC and MIC were close, the C_{50} values show that the inhibitory activity against protozoan motility was not dependent on micelle formation and that it apparently results from association of surfactant monomer with the cell membrane.

Table 1 and Fig. 3 also reveal the dependence of MIC on the ethylene oxide chain length (x) of the TRITON-X and TRITON-N surfactants. The X series covered a range of average chain length from 1 to 70 EO units and perhaps for this reason the dependence of MIC on EO appeared more complex for these surfactants than for the N series, of which fewer were studied. In Fig. 3 three regions can be described. In the first, for the lipophilic members X-15 to X-45, there was an increase in activity with x such that X-45 was the most active of these surfactants. From X-45 to beyond X-305, the second region was one of decreasing activity with increasing x. This second region encompassed the range of ethylene oxide chain length of the N series and the decrease of MIC per ethylene oxide unit, 0.33 mM, was lower than with the N series, 0.57 mM. Over the range of some 35 EO residues there was an order of magnitude change in activity. For very hydrophilic members of the TRITON-X series, X-405 and 705, there appeared to be a different relationship between activity and EO. In this third region, activity was low and for X-405, MIC > 0.2 M, an end-point was not obtained. These values were the lowest for any of the TRITON surfactants tested.

The TRITON-N surfactants encompassed a

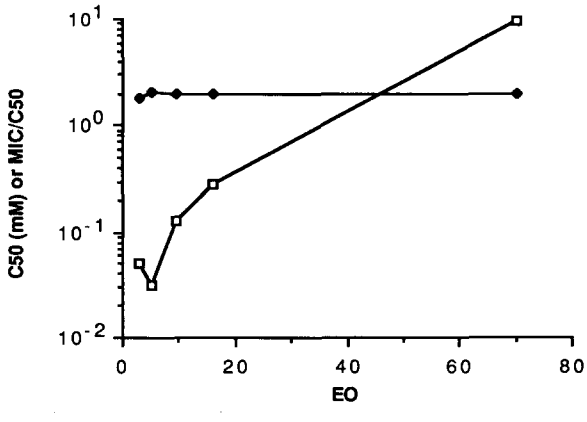


Fig. 2. The influence of poly-oxyethylene chain length (EO) on the inhibitory activity of selected TRITON-X series surfactants. The surfactant concentration (mM) required for the inhibition of protozoan swimming speed by 50% (C_{50}, □) is shown, and the ratio MIC/C_{50} (◆).

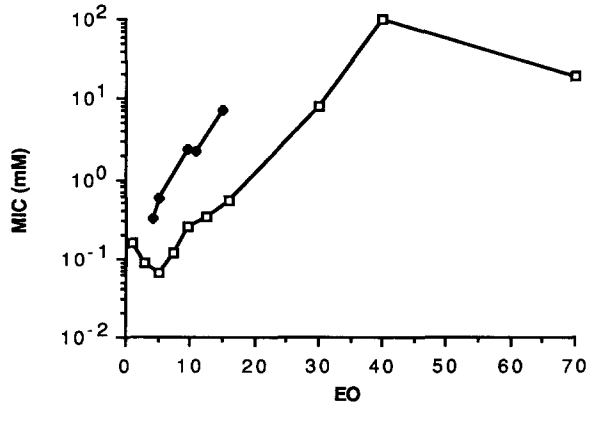


Fig. 3. The influence of poly-oxyethylene chain length (EO) on the inhibitory activity of TRITON-X (□) and TRITON-N (◆) series surfactants. Inhibitory activity is expressed as the MIC (mM), the lowest concentration to just inhibit protozoan motility in a suspension of the cells.

more restricted range of EO chain length but within this range, there was a monotonic decrease in activity with increasing x. The decrease in activity was calculated to be 0.57 mM/EO and amounted to an order of magnitude change in activity over the range of 11 EO residues. Overall however, the general result seen with the TRITON-X series that MIC increased (i.e. activity decreased) with increasing EO chain length, was also apparent with the N series. For equivalent EO values, TRITON-N surfactants were less active than their X counterparts, a result not anticipated by the data for the linear and branched alkanols. An arrangement of a series of TRITON-N surfactants (EO = 4–30) into 3 groups on the basis of their antimicrobial activity, similar to that based on MIC described here for the TRITON-X surfactants, has been described (Cserhati et al., 1982).

The spermicidal activity of the closely related non-ionic surfactant nonylphenoxypoly(ethyleneoxy)ethanols, the nonoxynols, decreases with increasing hydrophilic chain length (Gadd and Curtis-Prior, 1987). Levin (1988), working with a much broader range of nonoxynols, to investigate structure-activity relationships on rat vaginal bioelectric activity, showed that the most potent structures were those with 8 or 9 ethylene oxide residues (x). These observations were explained largely on the basis of solubility characteristics, the more hydrophilic members of the series ($x > 15$) being too water-soluble to attain an effective intramembrane concentration and the more lipophilic members ($x < 6$) being so poorly water-soluble that access to the cell membrane was restricted, although their low molar volume may also contribute to their lack of activity. This phenomenon of optimum solubility characteristics (partition coefficient) is a theme common to the activity of diverse drugs which act at the level of the membrane in cells or subcellular organelles (Albert, 1985).

CMC's for both series of TRITONS are of course also closely related to EO chain length; the increment of CMC per EO was 0.017 mM for the X series, and 0.002 mM for the N series surfactants. As a result, MIC and CMC values were similar only for a limited number of surfactants, X-114, X-100 and X-102. For the more lipophilic surfactants, MIC was < CMC and for more hydrophilic surfactants, MIC ≫ CMC.

The polyoxyethylene alkyl ethers (BRIJ type surfactants) tested were as a group, apart from the C12 series, much less active than the TRITONS, and an end point was only obtained for 3 members of the BRIJ series. For these surfactants, the negative influence of the hydrophilic head group size on surfactant inhibitory activity, found for the X and N series of the TRITONS (Table 1 and Fig. 3), was also apparent. Thus BRIJ 30, $n = 11$; $x = 4$; MIC = 0.08 mM, was some 16 times more active than BRIJ 35, $n = 11$; $x = 23$; MIC = 1.24 mM. BRIJ 30 and 36T were as active as the most potent TRITON surfactants, X-30 and -45 which contained a comparable number of ethylene oxide residues. For some other BRIJ surfactants e.g. BRIJ 52, 72, 82 and 76, aqueous solubility limited the MIC determination and the cells were still motile at the highest concentrations tested as shown in Table 2. These concentrations are all

TABLE 2

MIC of some BRIJ type surfactants, general formula, $CH_3(CH_2)_nO(CH_2CH_2O)_xH$, for the inhibition of T. elliotti motility

Surfactant	C	EO (x)	MIC (mM)
n-alkyl	C_4	20	6.55
n-alkyl	C_8	10	10.96
		20	24.75
n-alkyl	C_{12}		
BRIJ 30		4	0.08
36T		10	0.08
35		23	1.24
n-alkyl	C_{16}		
BRIJ 52		2	> 87.2
56		10	> 71.8
58		20	> 176.1
n-alkyl	C_{18}		
BRIJ 72		2	> 80.6
92 *		2	> 81.1
76		10	> 138.1
96 *		10	> 277.0
78		20	> 128.9
98 *		20	~ 172.1
721		21	> 165.6
700		100	> 16.0

* Unsaturated.

TABLE 3

MIC of some polyoxyethylene (20) sorbitan ester surfactants for the inhibition of T. elliotti motility

Sorbitan ester	C	MIC (mM)
Monolaurate (TWEEN 20)	C_{12}	>130.6
Monopalmitate (TWEEN 40)	C_{16}	19.5
Monostearate (TWEEN 60)	C_{18}	57.3
Monooleate (TWEEN 80)	C_{18} *	>122.4

* Unsaturated

much higher than the MIC's determined for the more hydrophilic TRITONS (with the exception of X-405). The shorter chain alkyl (butyl and octyl) ethers were also active, but less so than the "30" series BRIJ dodecyl ethers.

A comparison of the data in Table 1 with that in Table 2 confirms the finding evident from a comparison of the TRITON X and N data (Table 1 and Fig. 3) that the alkyl moiety also influences the activity of the surfactants against *Tetrahymena*. It would appear that the C-16 and -18 alkyl moieties of the inactive BRIJ surfactants contribute to their lack of activity, and that the dodecyl group of the "30" BRIJ's was optimal for inhibitory activity here, as has been frequently observed in other systems (Attwood and Florence, 1983; Walters et al., 1982; Dominguez et al., 1977; Ferguson and Prottey, 1976). The polysorbates tested (Table 3) had low activity against *Tetrahymena* motility, lower than TRITON 705, and for two of these (TWEEN 20 and 80) the cells were still motile in 16% solutions. The optimum alkyl chain length was C16.

Thermodynamics

Calculation of the change in free energy involved in the micellization (self-association) of the TRITON surfactants, ΔG_{CMC}, and that involved in their association with the lipids of the protozoan cell, ΔG_{MIC}, gave the values shown in Table 4. The ratio of these two parameters (Table 4) indicates that the thermodynamics of the two types of association were similar for most of the surfactants except for the more hydrophilic species, TRITON X-305, 405 and 705. If we assume that the free energy change can be separated into the

TABLE 4

Values for free energy change (kJ/mol) on micelle formation (ΔG_{CMC}) and inhibition (ΔG_{MIC}) of TRITON surfactants

TRITON	$-\Delta G_{CMC}$	$-\Delta G_{MIC}$	ΔG ratio
X-15	31.71	29.03	0.92
X-35	30.64	30.40	0.99
X-45	29.92	31.11	1.04
X-114	28.55	29.78	1.04
X-100	28.14	28.05	1.03
X-165	26.81	26.25	0.98
X-305	25.87	20.10	0.78
X-405	25.37	<12.38	<0.49
X-705	24.51	18.18	0.74
N-42		27.48	
N-57	31.46	26.17	0.83
N-60		26.38	
N-101	31.26	22.90	0.73
N-111		23.07	
N-150	30.64	20.42	0.67

$\Delta G_{CMC} = RT \cdot \ln$ CMC (mol fraction); $\Delta G_{MIC} = RT \cdot \ln$ MIC (mol fraction).

hydrophobic (HØ) and hydrophilic contributions thus:

$$\Delta G_{CMC} = \Delta G_{H\O} + \Delta G_{hydrophile}$$

and $$\Delta G_{MIC} = \Delta G_{H\O} + \Delta G_{hydrophile}$$

we can obtain from plots of ΔG_{MIC} (Fig. 4) or ΔG_{CMC} (Fig. 5) against EO chain length, $\Delta G_{H\O}$ values (intercept on ordinate) from the MIC data of -33.7 kJ/mol for the TRITON-X series and -29.7 kJ/mol for the TRITON N series and $\Delta G_{H\O}$ values of -31.6 kJ/mol and -31.9 kJ/mol respectively from the CMC data for these surfactants. This indicates that the lipophilic portion of the surfactants is involved, within the limits of experiment, to equal extents in the process self-association and insertion into the cell membrane. As might be anticipated, the comparable *n*-nonyl phenyl ethers of the TRITON-N series have lower CMC's and thus greater values of ΔG_{CMC} than the TRITON-X surfactants. Their lower biological activity requires explanation. It would seem that the EO residues make a greater negative contribution to MIC than CMC so that for the hydrophilic

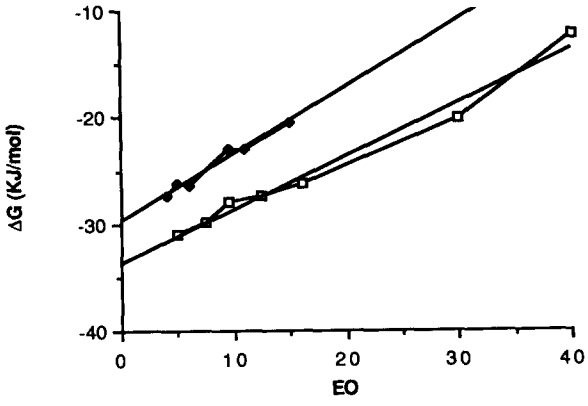


Fig. 4. The appropriate data of Table 4 plotted as ΔG_{MIC} against EO for TRITON-X (□) and TRITON-N (◆) surfactants. The equations of the linear fits to the data are, TRITON-X, $y = -33.7 + 0.5x$ and TRITON N, $y = -29.7 + 0.63x$.

surfactants, the MIC values are very much greater than those for CMC. The (poly)ethylene oxide head group would appear to be more able to prevent the access of the alkyl portion of the surfactant to the lipid domain of the protozoan membrane.

As far as the difference in activity between the TRITON-X and the TRITON-N series is concerned it would appear that several series of equivalent EO content have an optimum hydrocarbon moiety. In the *n*-alkyl series the order of activity is generally C8 < C12 > C16. It would seem that the tertiary octyl phenyl group more closely "resembles" the C12 moiety than does the *n*-nonyl phenyl group of the N series. However, the influence of the nature of the target membrane on the optimum HLB of the active surfactant is also of crucial importance and will be discussed in a following paper.

In spite of these mechanistic considerations, it would seem likely that *Tetrahymena* motility is inhibited by the membranolytic activity of these surfactants, some of which, e.g. TRITON X-100, are used for the solubilisation of membrane proteins. We did not observe any evidence for membrane-stabilising effects (Cassidy and Henry, 1986). It is interesting to speculate that the effects of the surfactants on the protozoan are an indication of their ability to cause tissue damage in higher organisms. The system described here could then form the basis of a simple, rapid screen for the irritant effects of surface-active agents and drugs.

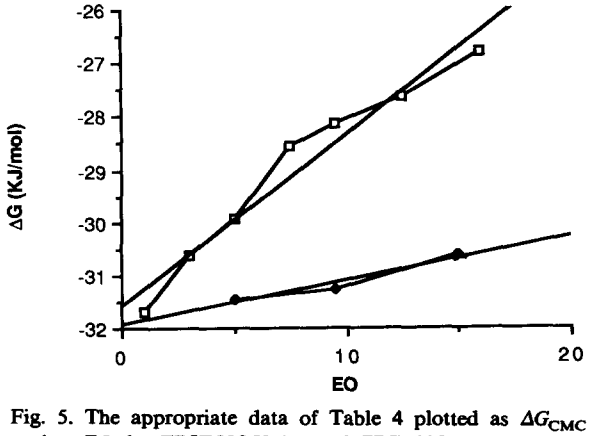


Fig. 5. The appropriate data of Table 4 plotted as ΔG_{CMC} against EO for TRITON-X (□) and TRITON-N (◆) surfactants. The equations of the linear fits to the data are TRITON-X, $y = -31.6 + 0.32x$ and TRITON-N, $y = -31.9 + 0.087x$.

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