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BIOLOGICAL EFFECTS OF SURFACTANTS, IV. EFFECTS OF NON-IONICS AND AMPHOTERICIS ON HeLa CELLS

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SUMMARY

Surfactants, representing 3 non-ionic and 1 amphoteric series of homologs exhibited marked differences in their lethal and non-toxic effects on HeLa cells. Toxicity of non-ionics generally decreased inversely with increasing hydrophilic chain length and increased with increasing size of the lipophile. Lethal levels of the surfactants coincided with surface tension reduction of the media to 45 dynes cm^{-1} or below. Surface tensions of non-toxic concentrations were substantially higher than those for toxic levels. Non-toxic doses were, therefore, below the critical micelle concentration of the surfactants evaluated. The data suggest that physical properties are a principal cause of the toxic effects of these detergents on HeLa cells.

INTRODUCTION

Limited information is available on the relationship between chemical structure, physical properties and toxicological effects of surface active agents (surfactants) despite their wide industrial, household and agricultural use. Therefore, several homologous series of surfactants with varying charges, molecular weights, and hydrophilic/lipophilic balance (HLB) were prepared and purified. In our prior work [1] with these agents, employing the fresh water coelenterate *Hydra attenuata* as bioassay organism, lethal concentrations were always coincident with surface tensions of 49 ± 4 dynes of the culture solutions. It was possible to establish toxicity trends with respect to the lipophilic radicals of the ionic species screened. However, the

Abbreviations: CMC, critical micelle concentrations; HLB, hydrophilic/lipophilic balance, ST, surface tensions.

commercially important non-ionic surfactants were generally lethal at the same concentration.

HeLa cells were selected as a potentially more sensitive bioassay organism because of the absence of a protective tissue. No work with homologous series of surfactants has been conducted with this cell line. A single non-ionic compound, polyoxyethylene sorbitan monooleate (Tween 80), was evaluated but had no significant effect on the number of cells at concentrations up to 0.5% [2]. In contrast, higher fatty alcohol ethoxylates, widely employed as detergents, emulsifiers, wetting and solubilizing agents, proved lethal to *Hydra attenuata* at levels as low as 8–20 ppm [1]. When HeLa cells were exposed to the anionic sodium dodecylsulfate the surfactant showed selective action against thymidine kinase and uridine kinase activity, [3]. Ono et al. [4] found sodium dodecylbenzenesulfonate to be more toxic to HeLa cells than sodium dodecylsulfate while a detergent grade α -olefinsulfonate proved to be the least damaging member of these 3 anionics.

Only non-ionic and sulfobetaine surfactants were employed in our study in order to avoid misleading results due to ionic interactions between anionic or cationic agents and amino acids or proteins contained in the culture medium. Although sulfobetaines, 3-(alkyldimethylammonio)-1-propanesulfonates, contain both an acidic and a basic radical, they are not retained by anion-, cation-, or mixed bed exchange resins, a unique property among these amphoteric surfactants. Unlike the powerful denaturing effect of sodium dodecylsulfate and other anionic detergents, it has been found that enzymatic activity is not lost in contact with sulfobetaines [5,6] and that the latter can provide protection against denaturation by alkylsulfate detergents [7].

MATERIALS AND METHODS

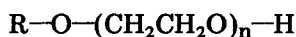
Cell cultures

HeLa cells were grown in uncoated, but oxygen treated polystyrene (personal communication from Mr. C. Higgins, Falcon Plastics, Oxnard CA) Falcon T-25 culture flasks (Becton Dickinson and Company, Oxnard, CA) in 5 ml nutrient consisting of Dulbecco's modification of Eagle's medium (Flow Laboratories, Inc., Rockville, MD, Cat. No. 10-331) plus 20% fetal calf serum [8,9] and the surfactant being screened. All surfactants were added before the introduction of cells.

Following the introduction of 5×10^5 HeLa cells, the cultures were maintained at 37°C for 72 h with periodic visual inspections.

Surfactants

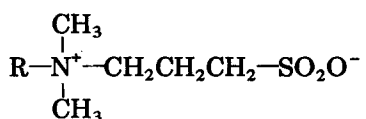
A total of 13 non-ionic surfactants, consisting of fatty alcohol ethoxylates and 6 amphoteric (sulfobetaines) were evaluated. The non-ionics conformed to the following general structure:



where R stands for an n-alkyl group having from 10 to 18 carbon atoms and n equals 5–40 ethoxyl groups. The normal alkyl groups and ethoxyl radical were selected to yield 3 homologous series with constant:

- (a) lipophilic chain and increasing hydrophilic polyethylglycol group;
- (b) hydrophilic/lipophilic balance (HLB);
- (c) hydrophilic group and increasing lipophilic chain, ranging from 10 to 18 carbon atoms.

The sulfobetaines were 3-(alkyldimethylammonio)-1-propanesulfonates with the following conformation:



where R is a normal alkyl chain having from 8 to 18 carbon atoms.

Surfactants (Table I) were prepared and purified as previously reported [1].

Surface tension measurements

Surface tensions (ST) of the nutrients were determined for every surfactant and each concentration at 37°C by the du Nouy surface tension (or ring) method [10] using a Fisher Model 20 Tensiomat and platinum ring of 6 cm circumference. Measurements were made in triplicates and the values shown were averages thereof.

RESULTS

Effects of surfactants on cells

HeLa cells in surfactant-free medium approached a confluent monolayer within 48 h of growth, were angular in shape and retained their healthy appearance during the 72-h observation period. Cultures containing surfactant concentrations yielding growth equal to those of the controls were termed “non-toxic”. Between the concentration levels giving “non-toxic” or “lethal” results, the cells were affected deleteriously and were round or granular in appearance, forming clusters frequently.

Toxicity of non-ionic tetradecyl alcohol ethoxylates decreased with increasing numbers of ethoxyl units, with lethal concentrations ranging from 16 to 1000 μM (Fig. 1) or 7–2000 ppm (Table I). The non-toxic concentrations ranged from 2 to 160 μM (Fig. 1) or 1–318 ppm (Table I).

When the HLB or non-ionic alcohol ethoxylates was constant, toxicity decreased with the increase in carbon atoms of the alkyl chain (lipophile).

TABLE I

NON-TOXIC AND LETHAL LEVELS OF NON-IONICS AND SULFOBETAINE SURFACTANTS TO HeLa CELLS

Surfactants	Ethoxyl units	HLB	Mol. wt.	Effects			
				Non-toxic (conc.)		Lethal (conc.)	
				(ppm)	(μ moles)	(ppm)	(μ moles)
<i>Alcohol ethoxylates</i>							
Lipophile:							
Tetradecyl ^a	5.16	36.9	441	1.8	0.004	7.1	0.016
Tetradecyl ^a	7.11	51.0	527	2.1	0.004	10.7	0.02
Tetradecyl ^a	10.70	76.4	685	1.4	0.002	11.0	0.016
Tetradecyl ^a	18.14	129.6	1012	20.2	0.02	81.0	0.08
Tetradecyl ^a	40.35	288.2	1989	318.0	0.16	1988.0	1.0
Decyl	5.07	50.7 ^b	381	7.6	0.02	61.0	0.16
Dodecyl	6.30	52.5 ^b	464	3.7	0.008	9.3	0.02
Tetradecyl	7.11	51.0 ^b	527	2.1	0.004	10.7	0.02
Hexadecyl	8.18	51.1 ^b	602	1.2	0.002	9.6	0.016
Octadecyl	9.32	51.8 ^b	680	1.4	0.002	5.4	0.008
Decyl	6.70 ^c	67.0	453	4.5	0.01	36.3	0.08
Dodecyl	7.18 ^c	59.8	502	5.0	0.01	20.1	0.04
Tetradecyl	7.11 ^c	51.0	527	2.1	0.004	10.7	0.02
Hexadecyl	7.00 ^c	43.8	550	2.8	0.005	22.0	0.04
Octadecyl	7.00 ^c	38.9	578	2.9	0.005	23.1	0.04
<i>Sulfobetaines</i>							
Lipophile:							
Octyl			286	572.0	2.0		
Decyl			312	312.0	1.0		
Dodecyl			338	6.8	0.02	108.0	0.32
Tetradecyl			370	3.7	0.01	29.6	0.08
Hexadecyl			399	1.6	0.004	16.0	0.04
Octadecyl			423	1.7	0.004	6.8	0.016

^aConstant lipophile. ^bConstant HLB. ^cConstant hydrophile.

TABLE II

SURFACE TENSIONS OF NON-TOXIC AND LETHAL LEVELS OF NON-IONIC AND SULFOBETAINE SURFACTANTS

Surfactants	Ethoxyl units	Surface tension in dynes cm ⁻¹				
		35	40	45	50	55
<i>Alcohol ethoxylates</i>		+ — Lethal o - - - Non-toxic				
Lipophile:						
Tetradecyl ^a	5.16					
Tetradecyl ^a	7.11					
Tetradecyl ^a	10.70					
Tetradecyl ^a	18.14					
Tetradecyl ^a	40.35					
Decyl	5.07 ^b					
Dodecyl	6.30 ^b					
Tetradecyl	7.11 ^b					
Hexadecyl	8.11 ^b					
Octadecyl	9.32 ^b					
Decyl	6.70 ^c					
Dodecyl	7.18 ^c					
Tetradecyl	7.11 ^c					
Hexadecyl	7.00 ^c					
Octadecyl	7.00 ^c					
<i>Sulfobetaines</i>						
Lipophile:						
Octyl						
Decyl						
Dodecyl						
Tetradecyl						
Hexadecyl						
Octadecyl						

^a Constant lipophile.

^b Constant HLB.

^c Constant hydrophile.

The lethal concentrations ranged from 8 to 160 μM (Fig. 2) or 5–61 ppm (Table I). Non-toxic levels were 2–20 μM (Fig. 1) or 1–8 ppm (Table I).

When the number of ethoxyl units was held constant at 7 moles, non-ionics with lower alkyl chains (C_{10} and C_{12}) proved less toxic than those with higher alkyl radicals (C_{14} – C_{18}). The lethal range was from 20 to 80 μM (Fig. 3) or 9–36 ppm (Table I) while 4–10 μM concentrations (Fig. 3) or 2–5 ppm (Table I) proved non-toxic.

The toxicity of amphoteric sulfobetaines increased sharply with increasing lipophilic alkyl chain over a range of 8–18 carbon atoms. Decyl and octyl derivatives were non-toxic at 1000 and 2000 μM , respectively. Lethal concentrations fell within a range of 16–320 μM (Fig. 4) or 7–108 ppm (Table I). The non-toxic weight range spanned 2–572 ppm (Table I).

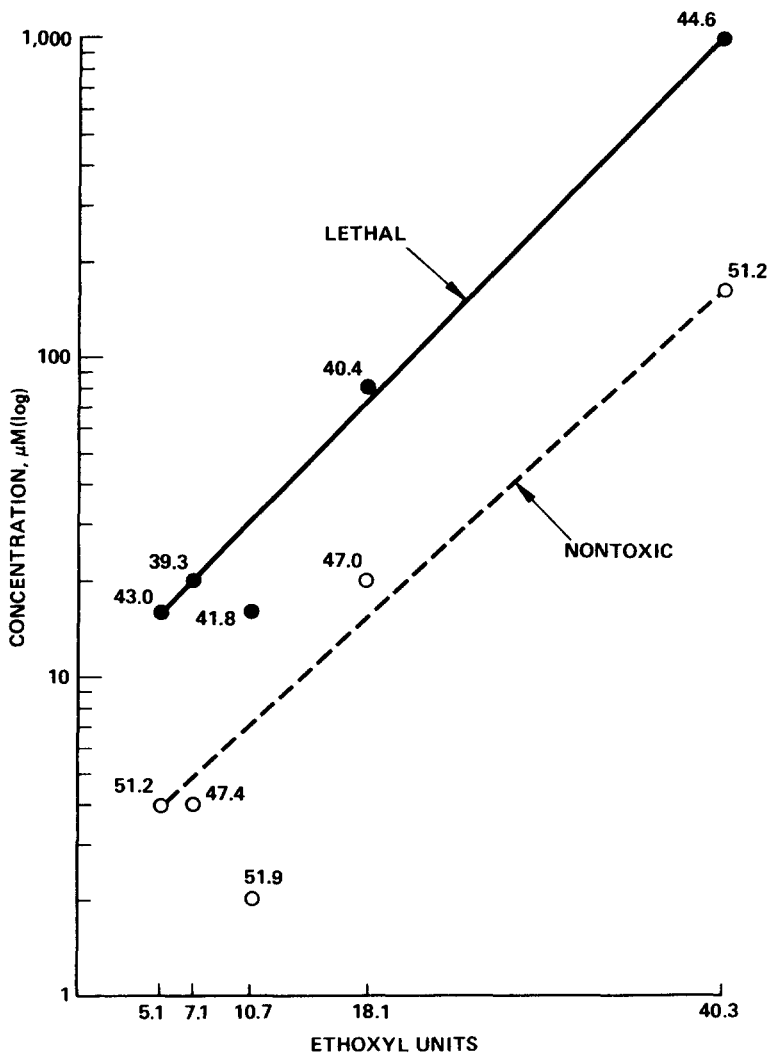


Fig. 1. Effects of non-ionic surfactants with constant lipophile, tetradecyl alcohol ethoxylates, on HeLa cells. —○—, Non-toxic concentrations. —●—, Lethal concentrations. Numbers at symbols are surface tensions in dynes cm^{-1} .

Surface tension data

The surfactant free nutrient had an average ST of 57.9 dynes cm^{-1} , compared to 73.6 dynes cm^{-1} for distilled water. This reduction in surface tension was largely due to the added fetal calf serum, which when dissolved in distilled water reduced the surface tension to 63.4 dynes cm^{-1} .

It is noteworthy that, with a single exception, ST of media containing lethal concentrations of surfactants were always below 45 dynes cm^{-1} , while non-toxic concentrations were above 47 dynes cm^{-1} and generally not greatly lower than those of the control (Figs. 1--4).

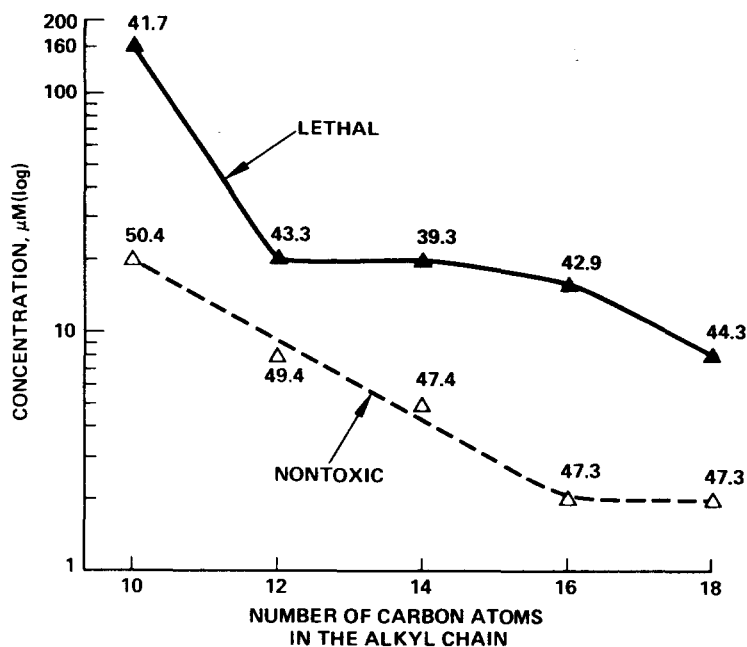


Fig. 2. Effects on non-ionic surfactants with constant HLB, decyl to octadecyl alcohol ethoxylates, on HeLa cells. —△—, Non-toxic concentrations. —▲—, Lethal concentrations. Numbers at symbols are surface tensions in dynes cm^{-1} .

DISCUSSION

Toxicity of surfactants can relate to their molecular structure, their reactivity and their many solution properties. Anionic and cationic surfactants are powerful protein denaturants and readily react with other ionic components (nutrients, organisms, ionically charged culture surfaces, etc.). The compounds selected for this essay were therefore non-ionic, and in the case of surface active betaines, ionic-neutral substances, which were purified by passing them through mixed-bed ion exchange resin columns. This rules out the ionic interactions mentioned above. Further, the removal of ionic impurities from non-ionic and sulfobetaine surfactants gave neutral compounds which had no effect on the pH of the media. Therefore the results were not influenced by changes in pH.

The 3 non-ionic and 1 amphoteric series of homologous surfactants showed marked differences their lethal and non-toxic effects on HeLa cells (Figs. 1–4, Tables I and II). While other physico-chemical properties can not be ruled out, there was a striking correlation between surface tension reduction and toxicity of all surfactants tested. Although the control medium had a relatively low ST of about 58 dynes cm^{-1} , substantial further reductions to levels below 45 dynes cm^{-1} coincided with surfactant concentrations causing death to the cells. Conversely, concentrations which were non-toxic to HeLa

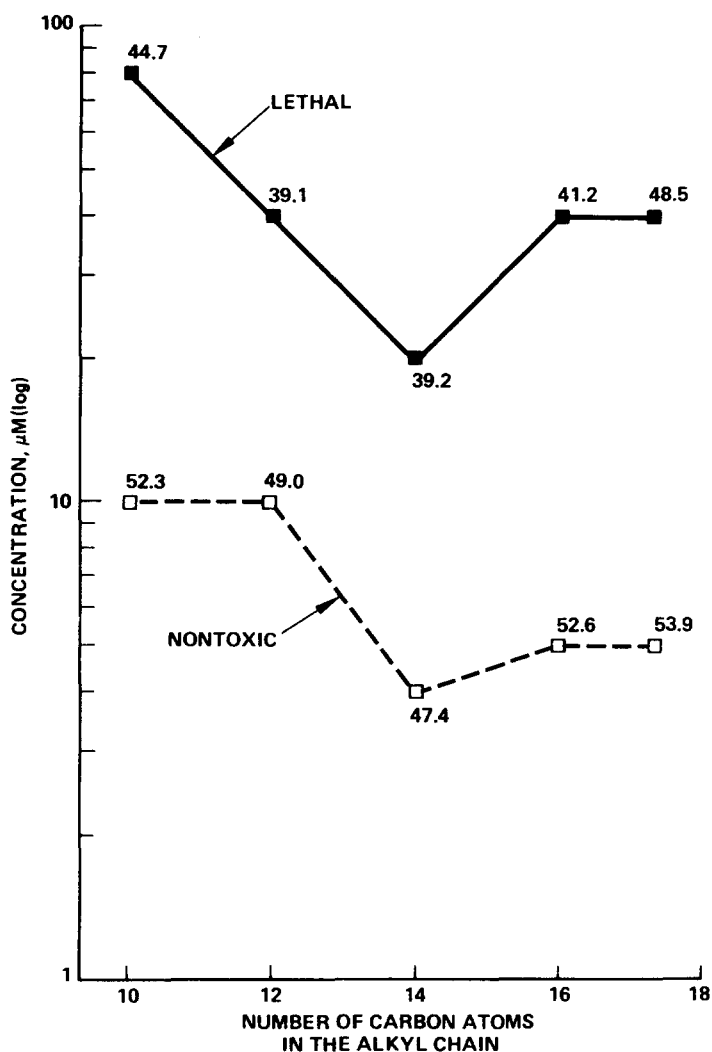


Fig. 3. Effects of non-ionic surfactants with constant hydrophile, decyl to octadecyl alcohol ethoxylates, on HeLa cells. —□—, Non-toxic concentrations. —■—, Lethal concentrations. Numbers at symbols are surface tensions in dynes cm^{-1} .

cells gave ST measurements above 47 dynes cm^{-1} (Figs. 1-4). A single exception was the octadecyl alcohol ethoxylate with 7 ethoxyl units. This may be due to marginal water solubility which causes this surfactant to stratify in solution.

Surface tensions of non-toxic concentrations of these detergents were substantially higher than those for toxic levels. Since surface tension values level off at their critical micelle concentrations (CMC), the non-toxic concentrations were always below the CMC.

Considerable differences in toxicity occur with most homologs of each

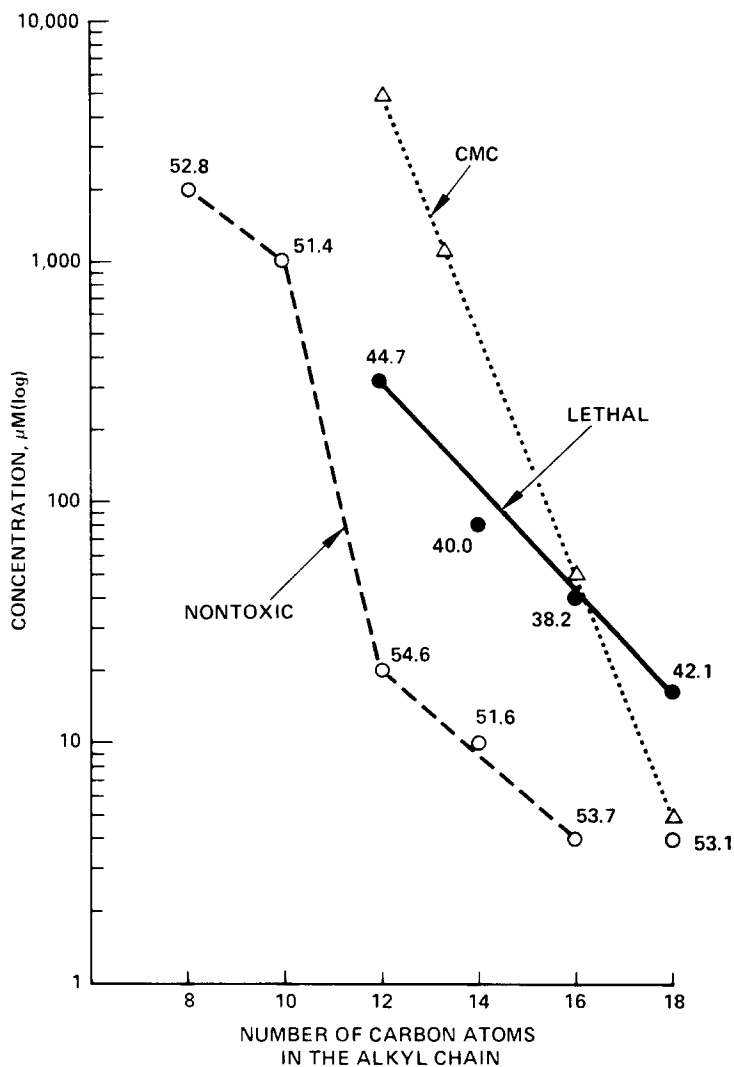


Fig. 4. Effects of sulfobetaines, 3-[alkyl(C_8 - C_{18})-dimethylammonio]-1-propanesulfonates, on HeLa cells. —○—, Non-toxic concentrations. —●—, Lethal concentrations. ---△---, Critical micelle concentrations.

series of surfactants employed. A steep slope results when toxic concentrations of tetradecyl alcohol ethoxylates are plotted logarithmically against their water solubilizing ethoxyl units. Toxicity falls sharply with increasing hydrophilicity (Fig. 1).

A trend among the other non-ionic series of compounds is generally increased toxicity with increasing alkyl chain length over the range of 10–18 carbon atoms. This is true even when the HLB is held constant (Figs. 2,3).

Toxicity of the sulfobetaines increases very sharply with increased mole-

cular weight due to the increased length of their lipophilic group. This correlates well with the reported [11] logarithmic decrease of their CMC (Fig. 4).

Data presented here and in prior work from our laboratory [1,12,13] point to the physical (surface active) effects of detergents as a principal cause of their toxicity. Furthermore, these results show that HeLa cells are a sensitive cell line for assay of surfactant effects.

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