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BIOLOGICAL EFFECTS OF SURFACTANTS

I. INFLUENCE ON THE GROWTH OF ORCHID SEEDLINGS

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Summary

Relative phytotoxicity of purified, biodegradable surfactants was determined by observing their effects on the growth of orchid seedlings *in vitro*. Pronounced phytotoxic effects occurred on culture media containing high concentrations of surfactants. Concentration of ionics above 100 ppm were most damaging and also caused a 90% or greater reduction of interfacial tension. Nonionic ethoxylates reduced growth and viability at lower concentrations than ionics, but larger percentages of seedlings survived at 1000 ppm levels of these agents, which were less efficient in reducing interfacial tension. No correlation was found between biological effects and surface tension data but a coincidence may exist between interfacial tension reduction by ethoxylates and increased phytotoxicity.

A number of purification procedures for surface active agents are presented as an Appendix.

Introduction

Biodegradable surface active agents (surfactants) were developed for the purpose of reducing residual water pollution (Justice and Lamberti, 1964). They are now widely used for household, agricultural or industrial purposes, and large amounts are released into the environment. Some are used to reduce surface tension in a variety of experimental and applied situations, serving as wetting and penetrating agents. Many are applied with herbicides, defoliants, fungicides or insecticides as emulsifying, dispersing and spreading agents, many times markedly enhancing their effectiveness (Parr and Norman, 1965). In view of this, and because the responses of plants to biodegradable surfactants are of considerable interest, we have undertaken to determine the biological effects of selected detergents at several concentrations on orchid seedlings over extended periods of time (Ernst and Arditti, 1968). Furthermore, previous attempts to correlate phytotoxicity of surfactants with some of their physical or chemical properties have been inconsistent (Buchanan, 1965; Furmidge, 1959a, b; Jansen, 1965; Parr and Norman, 1965; Smith and Foy, 1966). Hence, our aim was to identify possible surfactant properties which could affect growth and viability of orchid seedlings.

During our investigations we became aware of the fact that chemical purification procedures for specific surfactants had either not been devised, were implied only through analytical procedures, or were scattered through the literature, often in journals or patents not readily available to plant scientists. On the assumption that others might wish to purify surfactants for use in biological research, this paper includes in an appendix procedures to accomplish this end.

MATERIALS AND METHODS

Surface active agents. Biodegradable, linear surfactants (including one branched chain compound) chosen to represent all ionogenic classes (Table 1) were purified by pre-existing or newly devised methods (see Appendix). They were added to Knudson C medium (Knudson, 1946) at concentrations of 10, 100 and 1000 ppm based on active matter. The pH of culture media was adjusted to 5.1 ± 0.2 prior to autoclaving at 15 lb/in^2 for 20 minutes.

Hydrolytic stability of surfactants containing an amide linkage was determined in autoclaved, agar-free nutrient solutions. Acyl N-methyl taurate and acyl sarcosinate showed no discernible breakdown as measured by cationic titration (Epton, 1948; Iwasenko, 1954) and analysis for free fatty acids by petroleum ether extraction (Appendix, p. 468). Only 1.69% of acyl (coco-fatty) mono (2-hydroxyethyl) amide and 2.1% of lauroyl/myristoyl di (2-hydroxy-ethyl) amide were hydrolysed as calculated from the free fatty acid (soap) content. The resulting purity was deemed sufficient to test these compounds.

Plant material. Culture flasks were inoculated with seedlings of *Phalaenopsis* cv. Alice Gloria × *Phalaenopsis* cv. Francine or seeds of *Epidendrum* cv. O'Brienianum. *Phalaenopsis* seedlings were maintained in a glasshouse under natural daylight conditions, a maximum of 600 ft-candles and a temperature range of 20–26° C. *Epidendrum* seedlings were grown at 22° C under 12-hour days and a light intensity of 293 W/cm^2 (120 ft-candles) produced by Gro-Lux lamps. Seedling survival and fresh weight of *Phalaenopsis* at the end of 5 months are expressed as per cent of control.

Epidendrum seedlings were assayed by the growth index method (Mariat, 1952; Spoerl, 1948) and the data are expressed as per cent of control after 80 and 270 days of growth. The growth index increment between 80 and 270 days is expressed as per cent of the first measurement (Δ 80 days, per cent).

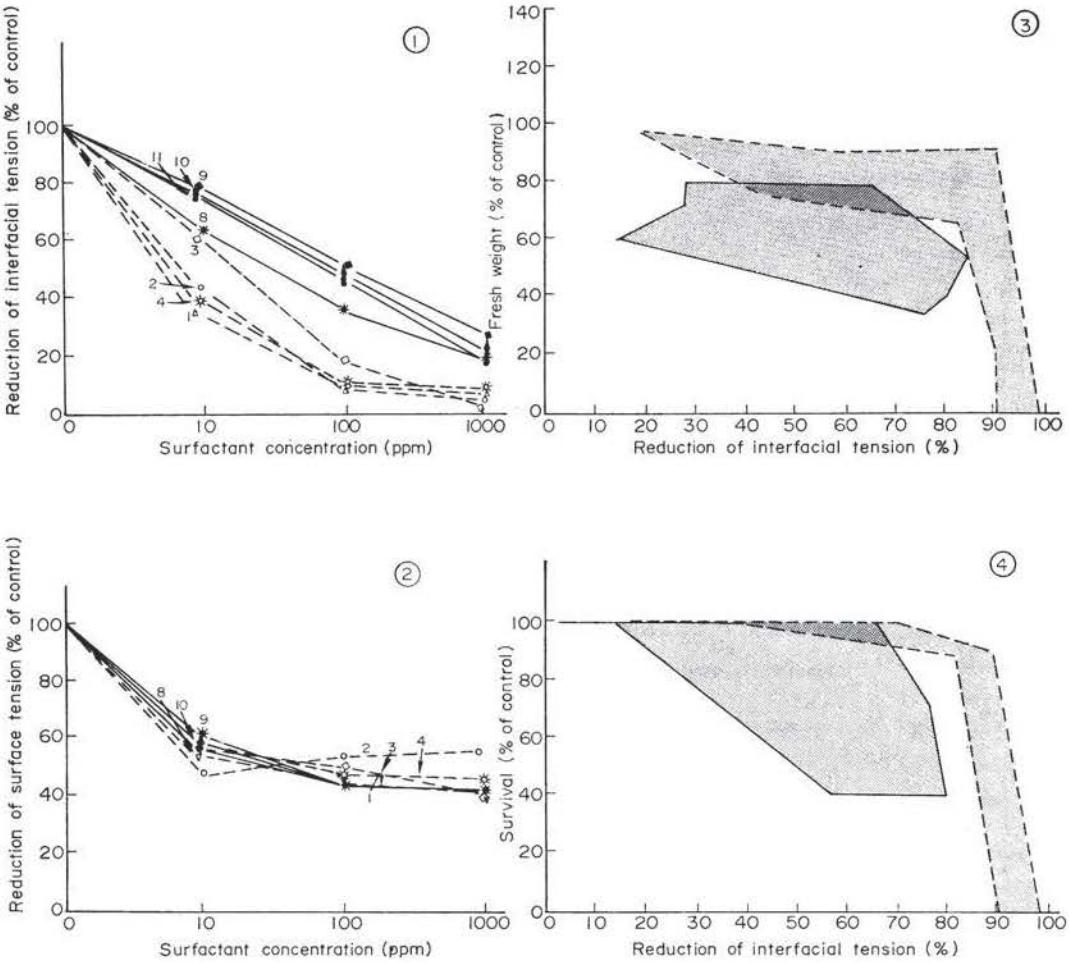
Tension measurement. Surface and interfacial tension (ST and IT) values were obtained for sterilized, agar-free Knudson C medium employing a Cenco duNouy model 70530, surface tensiometer and a Cenco duNouy, model 70540, interfacial tensiometer. Because ST and IT vary with different media (Harkins and Zollman, 1926; Powney and Addison, 1938; Wan and Poon, 1969) Knudson C medium was used as solvent for the surfactants, in preference to distilled water, to ensure measurements which more accurately reflect the culture conditions.

Cold pressed, acid- and additive-free corn oil served as a test lipid for IT determinations. Corn oil, a highly polar lipid, can be expected to give IT values more closely approximating those of biological membranes (containing highly polar lipids) rather than the more usually reported values for non-polar hydrocarbon (mineral) oils (Becher, 1963).

RESULTS

Over the range of experimental concentrations used (10–1000 ppm), the surfactants behaved quite differently with respect to their relative ability to lower ST and IT. When surfactants, dissolved in the culture medium, were tested against air, ST generally levelled off at 100 ppm for most anionics and polyethoxyethanols (Fig. 2). Reduction of IT was linear between 10 and 1000 ppm of ethoxylates and one anionic (sodium tetradecenyl sulphonate). IT for the remainder of the anionics levelled off between 100 and 1000 ppm (Fig. 1).

All *Phalaenopsis* and *Epidendrum* seedlings survived at concentrations of 10 ppm with only minimal inhibition (Tables 1 and 2; Figs. 5–8). Some enhancement was noted with certain ionics, a nonionic amide and a polyethoxyethanol (Tables 1 and 2; Figs. 5–8) in the range of 10–100 ppm. However, most surfactants were inhibitory at 100 ppm (Tables 1 and 2; Figs. 5–8). At concentrations of 1000 ppm they were invariably deleterious. There were no survivors in cultures of anionics and cationics which also exhibited extremely low IT values. Nonionic higher alcohol polyethoxyethanols (containing an average of 8 ± 1.5 ethylene oxide units) reduced IT much less than ionic compounds and

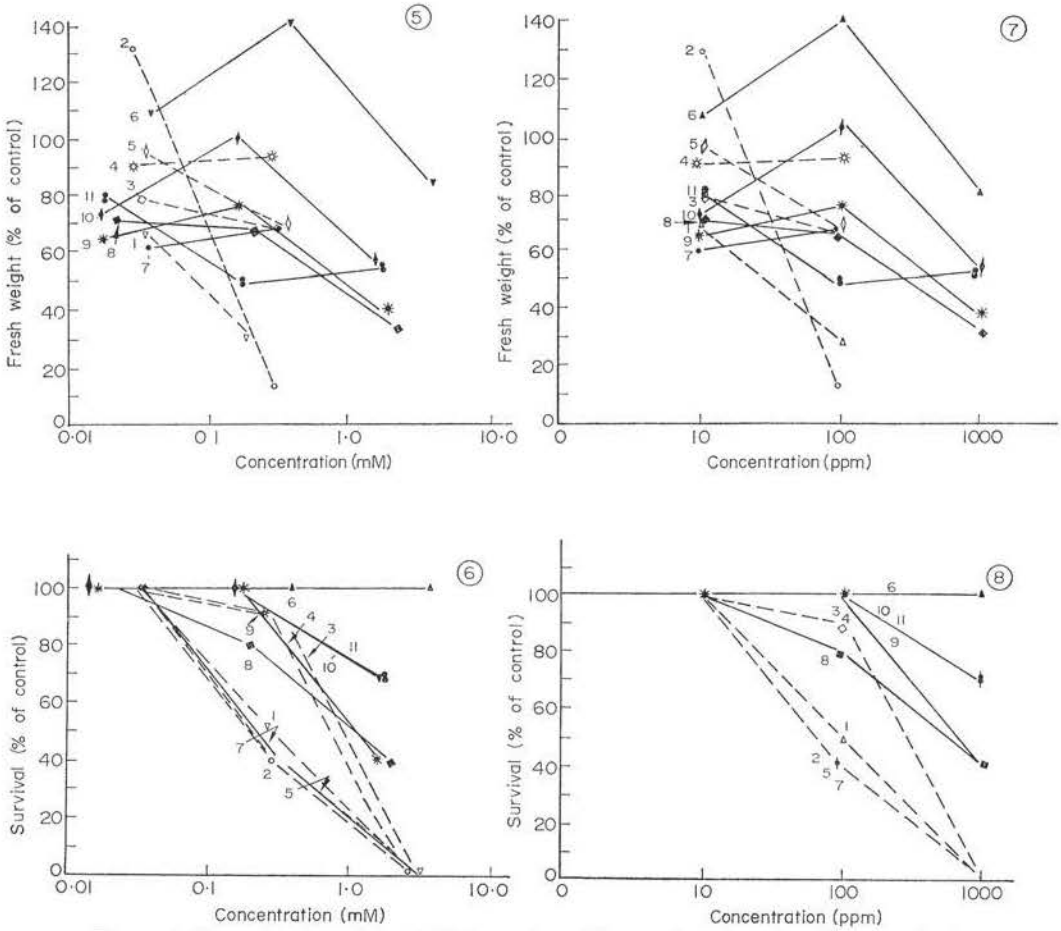


Figs. 1–4. Fig. 1. Reduction of interfacial tension as function of surfactant concentration. Fig. 2. Reduction of surface tension as function of surfactant concentration. Fig. 3. Fresh weight of *Phalaenopsis* seedlings as function of interfacial tension reduction. Fig. 4. Survival of *Phalaenopsis* seedlings as function of interfacial tension reduction. Solid line and symbols as well as fine stipple, nonionics; broken line and open symbols as well as coarse stipple, anionics. Numbers and symbol description in parentheses refer to compounds: 1, sodium (linear) dodecylbenzene sulphonate (triangle); 2, ammonium (linear) dodecylbenzene sulphonate (circle); 3, sodium tetradecenyl sulphonate (square); 4, sodium acyl (coco-fatty)-N-methyl taurate (star); 5, sodium lauroyl sarcosinate (rhomboid); 6, acyl (coco-fatty) mono (2-hydroxyethyl) amide (triangle); 7, lauroyl/myristoyl di (2-hydroxyethyl) amide (circle); 8, n-Alkanol (C_{12}-C_{14}) polyethoxyethanol (square); 9, linear primary alkanol (C_{12}-C_{15}) polyethoxyethanol (star); 10, linear secondary alkanol (C_{11}-C_{15}) polyethoxyethanol (rhomboid); 11, isotridecanol polyethoxyethanol (two circles).

were more readily tolerated by both *Phalaenopsis* and *Epidendrum* seedlings (Tables 1 and 2; Figs. 3–8).

Interesting differences are evident between cultures containing ionic surfactants and nonionic ethoxylates. Although nonionic surfactants reduce survival and are inhibitory to growth at higher IT values than the ionics, they never cause the degree of ultimate damage which occurs at the higher concentrations of the latter (Figs. 3 and 4). Ionic surfactants which can reduce IT by over 97% at 1000 ppm tend to act over a narrower range (Figs. 3 and 4). In all instances more than 90% of the *Phalaenopsis* seedlings survived treatment with ionics which lowered IT up to 89% (Table 1; Figs. 3 and 4). Yet there were no survivors in cultures where the ionic surfactants lowered IT values by greater than 92% (Figs. 3 and 4).

For the most part *Epidendrum* seedlings behaved like *Phalaenopsis*, although responses to individual compounds differed slightly in a few instances. Higher concentrations of some surfactants either prevented or delayed seed germination (Table 2). Development was inhibited by several compounds and slightly enhanced by others.



Figs. 5–8. Fig. 5. Fresh weight of *Phalaenopsis* seedlings as function of surfactant molarity. Fig. 6. Survival of *Phalaenopsis* seedlings as function of surfactant molarity. Fig. 7. Fresh weight of *Phalaenopsis* seedlings as function of surfactant concentration in parts per million. Fig. 8. Survival of *Phalaenopsis* seedlings as function of surfactant concentration in parts per million. For key to symbols and numbers, see legend to Figs. 1–4.

At 10 ppm the ammonium salt of linear dodecylbenzene sulphonate was less toxic than its sodium counterpart, whereas with 100 ppm the reverse was true (Tables 1 and 2). On the other hand, 10 ppm of ammonium alkyl (coco-fatty)-β-amino propionate were more toxic than an equal concentration of the sodium salt but the situation is reversed at 100 ppm of each (Tables 1 and 2).

Discussion

Reduction of ST or IT; molecular weight, structure or size; osmotic or electrostatic properties; native toxicity; and cation transference number have all been implicated in the biological effects of surfactants (Parr and Norman, 1965). Our growth and viability data show that phytotoxicity cannot be correlated with ST reducing properties of surfactants. Little or no further reduction in ST is observed with surfactant concentrations between 100 ppm and 1000 ppm (Table 1; Fig. 2), yet most damage and death occur at levels above 100 ppm. These findings are in line with previous reports (Buchanan, 1965; Furmidge, 1959a, b; Jansen, 1965; Parr and Norman, 1965; Smith and Foy, 1966) and indicate that correlations must be sought with other surfactant characteristics.

Over the range of concentrations used in these experiments (Table 1) phytotoxicity is related to characteristics of surfactant solutions which are molarity dependent (Figs. 5 and 6, and since in most previous work with surfactants, concentrations are expressed in ppm, also Figs. 7 and 8). The average molarity of all polyethoxyethanols is approximately 56% lower than that of the more deleterious compounds. However, since substantial structural differences exist between the lipophilic and hydrophilic radicals of these surfactants, inherent physical and biochemical properties must also be considered. An interesting inverse correlation between phytotoxicity and ethylene oxide chain length was observed with nonionic and cationic ethoxylates. Toxicity decreased with increasing molecular weight of the hydrophilic polyethyleneglycol radical, i.e. increase in ethylene oxide units (Buchanan, 1965). However, oat seed germination and corn root elongation data on two homologous series of ethoxylates (Buchanan, 1965) can also be related to IT values obtained by us, using corn oil as the test lipid (Table 3). Structural differences also exist, for example, between the more inhibitory alkylaromatic sodium dodecylbenzene sulphonate (LAS) and the less toxic aliphatic sodium tetradecenyl sulphonate (AOS). Furthermore, there is at least limited evidence of differences between ammonium and sodium salts of dodecylbenzene sulphonate and alkyl (coco-fatty)-β-amino propionate.

Molecular structure need not have a direct effect to be an important factor in the biological activity of surfactants since it can influence other features as, for example, electrostatic properties. The latter may, directly or indirectly, determine or modify some biological effects of surface active agents. For example, rather low concentrations of ionics can precipitate many proteins. This phenomenon is probably due to electrostatic attraction between the two types of charged molecules (Parr and Norman, 1965). Ionic radicals have been reported to play an important role in the interaction of surfactants with proteins (Foster, 1960; Putnam, 1948; Renoll and Van Winkle, 1951, 1953). This ability to precipitate proteins is related to their isoelectric point (Putnam and Neurath, 1943, 1944). Two anionics, sodium dodecyl sulphonate and the sodium salts of homologous alkyl sulphates (chain length C_8–C_{18}) can precipitate proteins only in cationic form, i.e. below their isoelectric point (Putnam and Neurath, 1944). On the other hand, precipitation of proteins with a cationic, alkyl (coco-fatty) dimethyl benzyl ammonium chloride, occurred at a pH above (i.e. alkaline to) its isoelectric point (Schmidt, 1943).

Table 1. *Growth effect of surface active agents on orchid seedlings* in vitro (Phalaenopsis *cv. Elinor Shaffer* × Phalaenopsis *cv. Francine*)

Compound	Concentration (mmoles)	Concentration (ppm)	Trademark	% survival	Fresh weight (% of control)	Interfacial tension dynes/cm at 25° C	Interfacial tension % reduction	Surface tension dynes/cm 25° C	Surface tension % reduction
Control				100	100	19.2	0	71.8	0
Anionics									
Sodium (linear) dodecyl-benzene sulphonate	0.029	10	Sulphotex LAS	100	69.7	6.4	66.7	38	47.1
	0.291	100		50	30.9	1.8	90.6	32	55.4
	2.906	1000		0	No survivors	1.3	93.2	30	58.2
Ammonium (linear) dodecyl-benzene sulphonate	0.030	10	–	100	130.8	8.2	57.3	38	47.1
	0.295	100		40	13.2	1.6	91.7	32.5	54.7
	2.949	1000		0	No survivors	1.1	94.3	31.0	56.8
Sodium tetradecenyl sulphonate	0.034	10	αOlefin sulphonate	100	79.1	11.7	39.1	40.5	43.6
	0.336	100		90	67.8	3.4	82.3	36	49.9
	3.356	1000		0	No survivors	Below 0.5	97.4	30	58.2
Sodium acyl (coco-fatty)-N-methyl taurate	0.028	10	Igepon TC	100	90.9	7.4	61.5	41	42.9
	0.279	100		90	93.4	2.0	89.6	34.5	52
	2.786	1000		0	No survivors	1.6	91.7	34	52.7
Sodium lauroyl sarcosinate	0.034	10	Sarcosyl NL	100	95.5	15.8	17.7	41	42.9
	0.341	100		40	68.7	–	–	28	61
	3.413	1000		0	No survivors	Below 2	90	27.5	61.7
Amphoterics									
Sodium N-(2-hydroxytetradecyl) sarcosinate	0.031	10	–	100	58.5	9.3	51.6	45.5	36.6
	0.31	100		100	91.3	2.4	87.5	29	59.6
	3.1	1000		60	74.3	1.1	94.3	29	59.6
Sodium alkyl (coco-fatty) β aminopropionate	0.034	10	Deriphat 151	100	120.9	9.4	51.0	34	52.7
	0.341	100		70	38.7	2.6	86.5	30.5	57.5
	3.413	1000		0	No survivors	0.8	95.8	29.5	58.9

Ammonium alkyl (coco-fatty) β amino propionate	0.035	10	Deriphat M21	100	106.4	10.3	46.4	44	38.7
	0.347	100		40	59.4	2.4	87.5	32.5	54.7
	3.472	1000		0	No survivors	0.5	97.4	28.5	60.4
n Alkyl (C_{12}-C_{16}) dimethyl-ammonium propane-sulphonic acid betaine	0.029	10	Sulphobetaine DC	100	52.1	7.0	63.5	38.5	46.4
	0.289	100		100	119.9	2.5	87	35.5	50.6
	2.890	1000		0	No survivors	1.2	93.8	34	52.7
Cationic									
Cetyl pyridinium chloride	0.029	10	Intexsan CPC	100	83.2	4.5	76.6	38.5	46.4
	0.294	100		100	58.2	2.2	88.5	35	51.3
	2.941	1000		0	No survivors	0.7	96.4	34	52.7
Nonionics									
Acyl (coco-fatty) mono (2-hydroxyethyl) amide	0.039	10	Textamide CE	100	108.3	18.4	4.2	38.5	46.4
	0.386	100		100	141.5	–	–	32.5	54.7
	3.861	1000		100	83.0	–	–	32	55.4
Lauroyl/myristoyl di(2-hydroxyethyl) amide	0.034	10	Nitrene L-76	100	60.7	16.4	14.6	37.5	47.8
	0.338	100		40	68.5	7.8	59.4	31.5	56.1
	3.38	1000		0	No survivors	–	–	30	58.2
n Alkanol (C_{12}-C_{14}) polyethoxy-ethanol	0.020	10	Alfonic 1214-6	100	70.8	15.1	21.4	40.5	43.6
	0.201	100		80	67.4	9.8	49	32	55.4
	2.012	1000		40	34.7	5.1	73.4	31.5	56.1
Linear primary alkanol (C_{12}-C_{15}) polyethoxyethanol	0.017	10	Neodol 25–9	100	64.9	11.8	38.5	43.5	39.4
	0.173	100		100	76.5	6.7	65.1	31.5	56.1
	1.733	1000		40	39.2	3.8	80.2	31.5	56.1
Linear secondary alkanol (C_{11}-C_{15}) polyethoxyethanol	0.016	10	Tergitol 15-S-9	100	73.9	14.6	24	43	40.1
	0.1595	100		100	105.1	9.2	52.1	32	55.4
	1.595	1000		70	55.7	4.2	78.1	31	56.8
Iso-tridecanol polyethoxyethanol	0.018	10	Surfonic TD-90	100	80.1	14.6	24	43.5	39.4
	0.177	100		100	48.7	9.1	52.6	33.5	53.3
	1.766	1000		70	54.1	3.7	80.7	30.5	57.5

Table 2. *Germination and growth of* Epidendrum *cv. Obrienianum on surface active agents*

Compound	Concentration (mmoles)	Concentration (ppm)	Growth index (% of control) at age (days) 80	Growth index (% of control) at age (days) 270	Growth index (% increase in 190 days)
Control		*N*	100	100	38
Anionics					
Ammonium (linear) dodecylbenzene sulphonate	0.030	10	81	94	59
	0.295	100	NG	NG	
	2.949	1000	NG	NG	
Sodium tetradecenyl sulphonate	0.034	10	87		
	0.336	100	76		
	3.356	1000	NG	NG	
Sodium acyl (coco-fatty)-N-methyl-taurate	0.028	10	96	89	61
	0.279	100	86	104	67
	2.786	1000	NG	NG	
Sodium lauroly sarcosinate	0.034	10	83	98	61
	0.341	100	NG	NG	
	0.413	1000	NG	NG	
Amphoterics					
Sodium N-(2-hydroxytetradecyl) sarcosinate	0.031	10	106	102	33
	0.31	100	86	107	71
	3.1	1000	NG	NG	
Sodium alkyl (coco-fatty)β amino-propionate	0.034	10	95		
	0.341	100	NG	NG	
	3.413	1000	NG	NG	
Ammonium alkyl (coco-fatty) β aminopropionate	0.035	10	87	91	44
	0.347	100	103	85	15
	3.472	1000	NG	NG	
n Alkyl (C_{12}-C_{16}) dimethyl-ammonium propanesulphonic acid betaine	0.029	10	NG	NG	
	0.289	100	67	93	91
	2.890	1000	NG	NG	
Cationic					
Cetyl pyridinium chloride	0.029	10	70	105	107
	0.294	100	58	NS	
	2.941	1000	NG	NG	
Nonionics					
Acyl (coco-fatty) mono(2-hydroxy-ethyl) amide	0.039	10	108	100	37
	0.386	100	95	100	41
	3.861	1000	115	100	33
Lauroyl/myristoyl di(2-hydroxy-ethyl) amide	0.034	10	108	98	24
	0.338	100	76	108	95
	3.38	1000	NG	NG	
n Alkanol (C_{12}-C_{14}) polyethoxy-ethanol	0.020	10	86	105	67
	0.201	100	NG	101	
	2.012	1000	NG	NG	
Linear primary alkanol (C_{12}-C_{15} polyethoxyethanol	0.017	10	87	110	43
	0.173	100	NG	91	
	1.733	1000	NG	NG	
Linear secondary alkanol (C_{11}-C_{15}) polyethoxyethanol	0.016	10	95	100	41
	0.1595	100	86	92	48
	1.595	1000	NG	NG	
Iso-tridecanol polyethoxyethanol	0.018	10	106	105	37
	0.177	100	103	101	40
	1.766	1000	NG	NG	

NG, no germination; NS, no survivors.

Interactions of nonionics, dodecyl alcohol ethoxylates (Klingmüller and Schroeder, 1953) and octyl phenol ethoxylate (Triton X-100; Dowben, Koehler and Barrieux, 1961) was only observed with very high (10%) concentrations. Release of chlorophyll from chloroplast preparations has been noted, and splitting between protein and prosthetic groups suggested with surface active quaternary ammonium and sulphonium compounds (Kuhn and Bielig, 1940; Kuhn and Dann, 1940). Enzyme activity or structural characteristics of proteins may be modified or lost as a result. If these proteins were plant cellular enzymes or structural components, phytotoxicity would be the probable result.

Several other factors may contribute to the differences in effects observed between nonionic ethoxylate and ionic surfactants. A portion of the charged (i.e. ionic) molecules may become bound to the agar or cell walls (which are not present during IT measurements) by electrostatic attraction or polyvalent bridges. Adsorption studies and dose-response curves with several higher alkyl trimethylammonium bromides revealed inactivation of these and related antibacterial cationic surfactants in seeded agar plate tests (Groves and Turner, 1959). The adsorption isotherms for each of these compounds reached a high point corresponding to the critical micelle concentration (CMC). How-

Table 3. *Interrelationship between surface tension, oat seed germination and corn root elongation*

Compound 0.1% solution in deionized water at 25° C	Ethylene oxide units	Interfacial tension dynes/cm*	Interfacial tension % reduction	Oat seeds germinated (no.)†	Corn roots elongation (cm)†
Igepal CO‡ 630	9	4.7	77.6	3	9.1
Igepal Co 850	20	5.2	75.3	12	10.0
Igepal CO 890	40	9.5	54.8	24	15.2
Igepal CO 990	100	12.4	41	31	15.4
Ethomeen 18§/25	15	2.6	87.6	0	1.3
Ethomeen 18/60	50	8.2	61.4	23	11
Control solution	–	21.0	–	–	–
Control bioassays	–	–	–	36–42	16.7–21.8

* Values obtained for this paper using corn oil.
† Bioassay data by Buchanan (1965).
‡ Nonylphenol polyethoxyethanol.
§ Stearylamine polyethoxyethanol.

ever, the use of adsorption isotherms for a thermodynamic analysis of this phenomenon may prove unsuccessful due to the partial irreversibility of the process (Fujita and Koga, 1966). In any event, if ionic loading takes place with Knudson C agar, effective concentrations of the ionics will be lower under culture conditions and result in IT values which are higher than those measured against corn oil. Binding of ionics to the cell wall is yet another possibility as has been demonstrated with yeast cells and cetyltrimethylammonium bromide (Fujita and Koga, 1966). The cell wall apparently plays a prominent role in binding the surfactant when cells are exposed to it at concentrations less than those required for complete killing. A gradual saturation of wall binding sites by detergent precedes killing of the cells. Ten times the cell surface area must be covered by bound surfactant to produce 100% killing. The retardation of binding caused by the lowering of pH may be taken as indicating competition between detergent cations and hydrogen ions for acid groups on cell surfaces (Fujita and Koga, 1966). So, if orchid cells behave like those of yeast, damage would occur only after their binding sites have been saturated. That this may be so is suggested by the relationship between IT reduction and growth or killing (Figs. 3 and 4).

Another possibility related to electrostatic properties is that nonionics can enter or be bound to cells with greater ease because they are not charged. If so they may reach toxic levels faster than charged (i.e. ionic) molecules which enter the cells more slowly.

The relative size of hydrophilic and lipophilic moieties may also play a role in determining the biological effects of a surfactant. The hydrophilic moiety of the ethoxylates is generally large and frequently exceeds their lipophilic radical in chain length. Hydrophilic sulphonate, sulphate, phosphate carboxylate, or amino salt ions of the ionics are considerably smaller. In our case, the nonionic ethoxylates are about twice the size of the ionics. This may give rise to a greater area of disruption for each molecule present.

An attractive possibility for the primary site of surfactant action is the cytomembrane. The obvious and well known emulsifying and dispersing properties of these molecules may act to break down the integrity of the highly polar lipid structure of all cytomembranes. If the site of surfactant activity were at the level of the cytomembrane, one would expect to find this mirrored in the lipid-culture medium interface of test systems as a concentration-dependent reduction of IT. This correlation has not been reported previously, probably owing to the general use of nonpolar mineral oils (e.g. Nujol and similar paraffinic oils) as test lipids rather than the more polar types (e.g. corn oil). The polar groups of these oils contribute to ionic and hydrogen bonding interactions at the lipid-surfactant solution interface and greatly modify IT properties. This, as well as a coincidence between phytotoxicity and reduced IT, is indeed what we have found for some surfactants (Tables 1–3; Figs. 3 and 4).

Additional evidence that surfactants may act on cell membranes and their lipids comes from studies with animals. Nonionic surfactants extracted the phospholipids, lecithin and cephalin, as well as cholesterol of dog red blood cell membranes at about the same efficiency. The ionics, however, removed the phospholipids more readily, but had no effect on cholesterol (Kondo and Tomizawa, 1968). When the alkyl chain length of cationics and anionics were reduced stepwise (lauryl, decyl and octyl), the molarity required to lyse red blood cells had to be increased logarithmically (Kondo and Tomizawa, 1966). At still lower alkyl chain length (hexyl and butyl) only cationics retained lytic activity and only at extremely high concentrations (Kondo and Tomizawa, 1966). Intravenous injection of the nonionics Tween 80 and Triton A 20 (alkylphenol ethoxylate) into rabbits (Kellner, Correll and Ladd, 1951) and Triton WR 1339 (alkylphenol ethoxylate) into dogs (Scanu *et al.*, 1961) caused striking and sustained elevations of blood cholesterol, phospholipids and neutral fats. This was accompanied by increased opacity of the sera. It suggests that increased emulsification of cellular components, possibly resulting from reduced IT, may be one of the causes of surfactant toxicity.

Ultrastructural evidence (Healey, Ernst and Arditti, 1971) as well as reports of reduced ^{32}P (Swanson and Whitney, 1953) and potassium uptake (Parr and Norman, 1965) in plants, following treatments with Tween 80 (polyoxyethylene sorbitan monooleate) and Tween 20 (polyoxyethylene sorbitan monolaurate), all seem to support the idea of surfactant mediated membrane changes.

Some of the surfactants tested here actually stimulate growth of *Phalaenopsis* seedlings, resulting in fresh weights which are higher than the control (Table 1). Surfactants eliciting this effect include, particularly, nitrogen containing anionics, amphoterics and an alkanol amide. The nonionic amides also enhance the development of *Epidendrum* seedlings (Table 2). Stimulation occurs in the range of 10–100 ppm, but higher concentrations are inhibitory. Good growth obtained with 1000 ppm alkyl (coco-fatty) mono (2-hydroxyethyl) amide may be due to its limited solubility in the nutrient solution.

The causes of growth stimulation are still unclear. Such effects have been reported by a number of investigators, for nonionic fatty esters (Beal, Christensen and Colby, 1954; Parr and Norman, 1964) and surfactants of all four major classes (Jansen, 1961). It may be that at lower concentrations the growth-stimulating features of these, or similar, compounds simply overcome inhibitory characteristics for a net increase in fresh weight. These agents may interact with cell components causing increased water or nutrient uptake, or could serve as metabolic stimulants. Since orchid seedlings seem to benefit from reduced nitrogen compounds (Arditti, 1967) the presence of such groups in the agents, yielding growth stimulation at low concentrations, may also be a factor. It is also possible that, as in microfungi (Lee, 1970), some surfactants may act as carbon sources. Use of the growth index to evaluate *Epidendrum* seedlings also suggests possible growth enhancing properties of some surfactants. The growth index (Mariat, 1952; Spoerl, 1948) is designed to take into account orderly growth and differentiation in a seedling population. Its use here suggests that at low concentrations most surfactants do not prevent germination and inhibit development only slightly (Table 2). Further, it appears that seedlings which survive on surfactant media may develop faster than controls. At least the growth index between 80 and 270 days is in most instances higher or equal to the controls (Table 2). Such growth enhancing effects, described as 'hormonal' have been reported previously for low concentrations of a number of alkyl lipids and surfactants (Stowe, 1958, 1960).

Clearly, surfactants and lipids can have profound effects on plants. These include inhibition and phytotoxicity (Ernst and Arditti, 1968; Parr and Norman, 1965), disruption of ultrastructure (Healey, Ernst and Arditti, 1971) as well as growth enhancing effects (Parr and Norman, 1965; Stowe, 1958) which '. . . seem most likely to be hormonal in nature . . .' (Stowe, 1960). It is equally clear that different ionogenic classes of surface active agents have dissimilar modes of action (Kondo and Tomizawa, 1966, 1968).

In summary, toxicity of polyethoxyethanols increases with molar concentration (Figs. 7 and 8) but their effect may be due to the reduction of IT (Figs. 1–3) and a subsequent membrane–lipid emulsification. Another factor which must be considered is the number of ethylene oxide units (Buchanan, 1965) or other moieties in surfactant molecules since they would affect the molecular weight of a compound and, therefore, actual molarities when solutions are prepared on a parts-per-million basis. Most of the damage by anionics is caused between 100 and 1000 ppm (Figs. 5–8) when further reduction of IT in the test lipid-surfactant system is only marginal (Figs. 1–4). However, IT values and, thus, phytotoxic effects of the nutrient media may have been modified by ionic interactions as previously outlined. The amides may have growth enhancing as well as toxic properties which tend to be independent of, and antagonistic to, each other with the balance between the two functions being concentration-dependent. Activity of the ionics, limited as it is to a narrower concentration range, may be dependent on their charges as well as other properties.

The different effects of the various ionogenic classes may reflect differences in interactions between certain surfactants and cell or culture media components. These, as well as the mode of action of surfactants in growth, physiology and ultrastructure of cells are currently being investigated.

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APPENDIX

Preparation and purification procedures

Biodegradable ionic and nonionic surfactants were selected from compounds having a linear chain of 10–16 carbon atoms in the lipophilic group. This range is typical for organic detergents in general use. Each of the nonionic polyethoxyethanols (ethoxylates) contained 8 ± 1.5 ethylene oxide units in the hydrophilic ether chain, corresponding to the most widely employed hydrophilic/lipophilic balance (HLB). The purification procedures employed were designed to remove unreacted starting materials, catalysts and products of side reactions. Precise activities of these agents were also established.

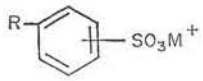
Average molecular weights are based on the distribution of the lipophilic or, with the ethoxylates, also of the hydrophilic moieties. In LAS (I) there is a further distribution of isomers resulting from the position of the phenyl group on the alkane chain (Swisher,

1963). Compounds not standardized by volumetric analytical procedures were also examined and corrected for moisture content by the Karl Fischer reagent titration method (ASTM D 1364), except the amphoterics, which were oven dried at 105° C to constant weight. It is hoped that these procedures will prove helpful to other investigators.

Anionics

These surfactants ionize in solution, with the lipophilic group carrying a negative charge.

Sodium (linear) dodecylbenzene sulphonate, LAS, I (Sulfotex LAS Slurry, Textilana Corporation)



(I)

R = linear alkyl group having 10–14 carbon atoms
$M^+ = Na^+$, molecular weight 344
$M^+ = NH_4^+$, molecular weight 339 (Ia)

Linear alkylbenzene sulphonates (LAS) are produced by sulphonation of linear alkylbenzene with sulphur trioxide or oleum, followed by neutralization with alkali hydroxide solution. The commercial product contains free oil composed of unreacted alkylbenzene as well as sulphones produced by a side reaction. Salt content (Na_2SO_4) varies with the sulphonation process.

For purification the LAS is adjusted to a pH of 7.5–8.5 and dried at 80° C. About 10 g of the dried material is boiled with 100 ml neutral anhydrous ethanol. Upon settling the supernatant is filtered with suction through a Gooch crucible. The salt-free filtrate is diluted with 100 ml of deionized water and the aqueous/alcoholic solution is extracted three times with 100 ml petroleum ether or n-pentane.

Following evaporation of the aqueous/alcoholic (lower) layer, a standard stock solution is prepared. Activity is determined by methylene blue-cationic titration (Epton, 1948; Barr, Oliver and Stubbings, 1948) with 0.004 M Hyamine 1622 (Rohm and Haas Company) or other suitable standardized quaternary ammonium compounds.

Ammonium (linear) dodecylbenzene sulphonate, LAA, Ia

Three grams of the compound purified as LAS (I) are dissolved in 27 ml deionized water and passed through a 25 mm × 300 mm column of Dowex W-X12 resin (Dow Chemical Company) in the H^+ form. The eluted free sulphonic acid is neutralized with dilute NH_4OH. Activity is determined by methylene blue-cationic titration as with the sodium salt.

Sodium tetradecenyl sulphonate (α-olefin sulphonate), AOS, II (Gulf Oil Corporation)

$R\text{-}CH=CH\text{-}SO_3^-Na^+$
R = n-dodecyl (II)
Average mol. wt 308

AOS is obtained by sulphonation of α-tetradecene with air-diluted sulphur trioxide. Tetradecenyl sulphonates and tetradecanesultones are produced as co-products of sulphonation. The latter are hydrolysed with sodium hydroxide at elevated temperature to hydroxytetradecane sulphonates and tetradecenyl sulphonates (Kaiser and Püschel, 1964). α-Olefin sulphonates (AOS) are therefore a mixture of two species of compounds having commercial importance as biodegradable surfactants. The product of commerce contains free oil as unsulphonated olefins, unhydrolysed sultones and Na_2SO_4 and, when bleached, also NaCl.

For purification, the slightly alkaline mixture is dried at 80° C and about 10 g of the dried substance is placed in a Whatman fat extraction thimble, 33 × 80 mm, interspersed and covered with glass beads. Extraction is carried out in a soxhlet apparatus for 12 hours using 100 ml anhydrous ethanol. The residue in the extraction thimble is discarded and the anhydrous ethanol solution containing the surfactant is diluted with 100 ml deionized water. After three extractions with petroleum ether the solution is dried and standardized as shown for LAS (I).

Sodium acyl (coco-fatty)-N-methyltaurate, SAT, III (Igepon TC, GAF Corporation)

$$RCON(CH_3)CH_2CH_2SO_3^-Na^+$$

RCO = cocoyl (III)

Average mol. wt 359

This compound is a reaction product of coconut fatty acid chloride and methyltaurine in aqueous sodium hydroxide solution (Schotten-Baumann reaction). The commercial product contains coconut fatty acid soap, free methyltaurine and sodium chloride as impurities.

It was dried and about 20 g dissolved in 200 ml ethanol–deionized water (1:1, v/v). The pH was adjusted to 4.5–5.0 with 0.5 N HCl and the solution was extracted with petroleum ether as shown for LAS(I). The aqueous/alcoholic (lower) layer was dried and the residue redissolved in 200 ml boiling methanol, filtered with suction through a Gooch crucible and the filtrate added under agitation to 2000 ml acetone. Crystallized surfactant was collected on a Buchner filter and washed with methanol–acetone (1:10, v/v). A Nujol mulled sample (Silverstein and Bassler, 1967) of the dried product showed no carboxylate ion band at 6.4 μ (1563 cm^{-1}) by infrared spectroscopy. A thin layer chromatogram (TLC) on silical gel G employing n-propanol–water (7:3, v/v) as the developer and ninhydrin–collidine–cupric nitrate as visualizer (Brenner and Niederwieser, 1960) showed the absence of free methyltaurine. Activity of the purified product was determined by methylene blue-cationic titration.

Sodium lauroyl sarcosinate, SLS, IV (Sarcosyl NL-30, Geigy Chemical Corporation)

$$RCON(CH_3)CH_2COO^-Na^+$$

RCO = lauroyl (IV)

Mol. wt 293

Lauroyl chloride is reacted with sarcosine in aqueous sodium hydroxide to produce SLS (Orthner and Mayer, 1936). Impurities of commercial products are sodium laurate (soap), sarcosine and sodium chloride.

For purification the surfactant was extracted with petroleum ether and with methanol like SAT (III) and the methanol solution evaporated. Ten grams of the compound were then dissolved in 25 ml deionized water, adjusted to a pH of 8 and crystallized by addition to 190 ml acetone. Crystals were collected on a Buchner filter washed with three 50 ml portions of water–acetone (1:9, v/v) and dried at about 50° C with a stream of nitrogen. No sarcosine was detected by TLC using the method shown for SAT (III). Activity of the soap-free surfactant was determined by cationic titration using dichlorofluorescein as indicator (Iwasenko, 1954).

Amphoterics

Amphoteric surfactants have anionic and cationic groups. They assume an anionic charge in basic media and a cationic one under acidic conditions. Water solubility is lowest in the isoelectric (zwitterion) range. Betaine type compounds, although generally grouped with amphoterics, show good solubility in water over the entire pH range.

Sodium alkyl (coco-fatty) β-aminopropionate, SAP, V (Deriphat 151, General Mills, Inc.)

$$RNHCH_2CH_2COO^-M^+ \quad (V)$$

R = coco alkyl

M = Na^+, average mol. wt 293

M = NH_4^+, average mol. wt 288 (Va)

Appendix Table 1. *Higher alcohol ethoxylates having* 8 ± 1.5 *ethylene oxide units*

Polyethoxyethanol of	Source		Hydroxyl no. (mg KOH/g)	Mol. wt (calculated)	Polyethylene glycols (found)
n-Alkanol ($C_{12} = 55\%$; $C_{14} = 43\%$; $C_{10}+C_{16} = 2\%$)	Alfonic 1214-6, Continental Oil Co.	(XI)	112.9	497	1.45%
Linear primary alkanol ($C_{12} = 20\%$; $C_{13} = 30\%$; $C_{14} = 30\%$; $C_{15} = 20\%$)	Neodol 25-9, Shell Chemical Co.	(XII)	97.2	577	1.3%
Linear secondary alkanol ($C_{11} = 15\%$; $C_{12} = 22\%$; $C_{13} = 23\%$; $C_{14} = 17\%$; $C_{15} = 15\%$; $C_{16} = 7\%$)	Tergitol 15-S-9, Union Carbide Corp.	(XIII)	89.5	626.8	1.0%
Iso-tridecanol (mostly tetramethyl-nonanols), a 'hard' surfactant	Surfonic TD-90, Jefferson Chem. Co.	(XIV)	99.1	566.1	2.2%

Surfactants of this type are produced by addition of methylacrylate to primary coco alkylamine, followed by saponification with aqueous sodium hydroxide (Isbell, 1949). The commercial product contains free alkylamine and acrylic side reaction products. Purification was by modification of a previous method (Nordgren, 1959).

Twenty grams of the dry amphoteric surfactant were dissolved in 35 ml of methanol–water (1:1, v/v) and stirred into 190 ml acetone. The crystals formed were collected on a Buchner filter and washed three times with 50 ml portions of water–acetone (1:9, v/v). Upon drying, analysis of the compound showed the presence of 0.7% petroleum ether soluble matter. The surfactant was therefore redissolved in water–ethanol (1:1, v/v) and extracted with petroleum ether as in LAS (I). Kjeldahl nitrogen content of the dried material was 4.68%, in good agreement with the calculated average molecular weight.

Ammonium alkyl (*coco-fatty*) *β-aminopropionate*, *AAP*, Va (Deriphat M21, General Mills, Inc.)

Ten grams Deriphat M21 (inner salt) was dissolved in methanol–water (1:1, v/v) to a 25% solution (w/v), then neutralized with NH_4OH and stirred into 380 ml acetone. The pasty precipitate was redissolved in 20 ml of methanol–acetone (1:1, v/v) and reprecipitated by addition to 280 ml acetone. This was repeated until the resulting dried granular material was free of acrylic odour and showed no presence of primary coco-fatty amine by TLC, using the method outlined for SAT (III). The acid value found was 192, corresponding to an equivalent molecular weight of 292.

Sodium N-(*2-hydroxytetradecyl*) *sarcosinate*, *SHS*, VI

$$CH_3(CH_2)_{11}CH(OH)CH_2N(CH_3)CH_2COO^-Na^+ \quad \text{(VI)}$$

Mol. wt 323

Preparation of SHS, especially for this project, was by modification of an amino acid adduct process (Ulsperger, 1966). One mole (223.15 g, based on oxirane O_2, 7.17%) of 1, 2 epoxytetradecane and 1.1 mole (122.1 g) sodium sarcosinate in 330 ml water were heated in a Parr autoclave under pressure of 15 lb/in^2 N_2 and 106° C$\pm$2° C for 6 hours. The white paste obtained on cooling contained 54.4% solids including 3.42% free sodium sarcosinate. The dried product showed no oxirane absorption by infrared spectroscopy at 910 cm^{-1} and 830 cm^{-1}. To purify, 5 g of the paste were dissolved in methanol–chloroform–0.1 N H_2SO_4 (38:160:2, v/v/v) and passed through a 25 × 300 mm column of silica gel. The eluate was dried, redissolved in an equal weight of water and recrystallized from acetone and shown to be homogeneous by TLC (see SAT, III).

n-Alkyl (C_{12}-C_{16}) *dimethylammonium propanesulphonic acid betaine*, *ADPS*, VII

$$RN^+(CH_3)_2CH_2CH_2CH_2SO_3^- \quad \text{(VII)}$$

R = n-alkyl; C_{12} = 65%; C_{14} = 25%; C_{16} = 10%

Average mol. wt 346

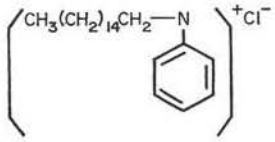
The betaine is prepared by reaction of the corresponding tertiary dimethylamine with propanesultone in aqueous alcohol (Ernst, 1966). Unreacted amine and hydroxypropane sulphonate occur as impurities.

To remove these, 25 g of the dried product are dissolved in 30 ml isopropanol and stirred into 1000 ml of acetone. Crystals formed on standing are collected on a Buchner Filter and dried. Kjeldahl nitrogen was 4.05% nitrogen, in good agreement with theory. No petroleum ether soluble matter was found by the extraction process shown for LAS (I).

Cationics

Cationic surfactants ionize in solution, with the lipophilic group carrying a positive charge.

Cetyl pyridinium chloride, CPC, VIII (Intexsan CPC, Washine Chemical Co.)



(VIII)

Mol. wt 340

CPC may be produced by reaction of cetyl chloride and pyridine (Fawcett and Gibson, 1934). Cetyl chloride and cetyl alcohol are the principal impurities.

Twenty grams of the dry material were dissolved in 200 ml absolute ethanol and filtered. The filtrate was diluted with 100 ml deionized water and extracted three times with petroleum ether. On drying the activity of the compound was 98.6% by argentimetric titration, using dichlorofluorescein as the indicator (DuBois, 1945).

Nonionic amides (alkanolamides)

Nonionic surfactants do not ionize in solution. Hydration of the hydrophilic (polar) moiety contributes to the solubility of these agents.

Acyl (coco-fatty) mono (2-hydroxyethyl) amide, CMA, IX (Textamide CE, Textilana Corporation)

$RCONHCH_2CH_2OH$
RCO = Cocoyl (IX)
Average mol. wt 259

Methyl coconate is reacted with monoethanolamine in the presence of an alkali metal alcoholate to produce this amide. CMA may also be produced by condensing coco-fatty acid with monoethanolamine. Unreacted starting material, soap, amino esters and amido esters may be found in the commercial products (Dow Chemical Company, 1964; Kroll and Nadeau, 1957).

A solution of 20 g CMA dissolved in 400 ml methanol was passed through a column (25 × 750 mm) of Amberlite MB I resin. The eluate was evaporated at 50° C and found to be free of ionic impurities. The product showed no absorption for ester, or ionized acid carbonyl by infrared spectroscopy.

Lauroyl/myristoyl di(2-hydroxyethyl) amide, LDA, X (Nitrene L76, Textilana Corporation)

$RCON(CH_2CH_2OH)_2$ (X)
RCO = 70% lauroyl; 30% myristoyl
Average mol. wt 296

Prepared from methyl laurate/methyl myristate and diethanolamine in the presence of an alkali metal alcoholate. Impurities and their removal were as with CMA (IX), but solvent evaporation was carried out at 40° C under reduced pressure to prevent amide/ester transformation (Ernst, 1962). On cooling, the wax-like substance was free of ionic materials and contained 0.3% ester by infrared spectroscopic method (optical, density at 1725 cm^{-1} based on amide mono ester standard).

Nonionic ethoxylates

$RO(CH_2CH_2O)_xH$ (XI–XIV)

These surfactants (Table 1) are produced by reaction of higher alcohols with ethylene oxide in the presence of a basic catalyst and under pressure. The catalyst left in the adduct is then neutralized. These salts and polyethyleneglycols are the contaminants found.

Removal of polyethyleneglycols is based upon a gravimetric analytical method (Weibull, 1960). Twenty grams of the ethoxylate dissolved in 100 ml ethyl acetate were extracted twice with 100 ml aliquots of 5 N NaCl and the aqueous (lower) layer was drawn off. The ethyl acetate solution containing the surfactant is evaporated and the residue dissolved in anhydrous ethyl or isopropyl alcohol, agitated for 30 minutes with 60 g Amberlite MB I resin, filtered and dried. Polyethyleneglycol content of the unrefined product may be established from the sodium chloride layer by the analytical method cited. The equivalent weight of the nonionic ethoxylate may be determined by uncatalysed acetic anhydride acetylation (Ogg, Porter and Willits, 1945). Use of β-picoline–acetic anhydride (4:1, v/v) at 113–116° C for 20 minutes is a preferred modification.