Title

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Journal

New Phytologist, 96(2)

ISSN

0028-646X

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Publication Date

1984-02-01

DOI

10.1111/j.1469-8137.1984.tb03556.x

BIOLOGICAL EFFECTS OF SURFACTANTS VII. GROWTH AND DEVELOPMENT OF *BRASSOCATTLEYA* (ORCHIDACEAE) SEEDLINGS

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(*Accepted 27 September 1983*)

SUMMARY

Phytotoxicity of a homologous series of sulphobetaine surfactants, 3-(alkyldimethyl-ammonio)-1-propanesulphonates, on *Brassocattleya* orchids increased with increasing alkyl chain length from eight to 14 carbon atoms of the lipophile, levelled off and then declined with the 18 carbon derivative. Because of its high Krafft point the latter had reduced solubility at the assay temperature. Interfacial tensions of the nutrient solutions, measured against a test lipid, declined in parallel with the observed phytotoxicity. This and previous findings strongly support the suggestion that disruption of cellular membranes by surface active agents plays a key role in their toxicity.

Key words: *Brassocattleya*, amphoteric surfactants, phytotoxicity, sulphobetaines, surface and interfacial tensions.

INTRODUCTION

In earlier work we reported the effects of purified ionic and non-ionic surface active agents (surfactants) of the type used in commercial preparations on orchid seedlings grown *in vitro* (Ernst, Arditti & Healey, 1971). Such surfactants contain largely lipophiles of mixed alkyl or alkylaryl chains.

To assess the role of molecular weight and hydrophilic/lipophilic balance in surfactant toxicity, we prepared homologous series of anionic, non-ionic and amphoteric surfactants with lipophiles ranging from eight to 18 carbon atoms. In case of non-ionics, the hydrophiles ranged from five to 40 ethoxyl units (Bode, Ernst & Arditti, 1978).

Amphoteric surfactants include compounds with zwitterionic structures resembling betaine, (trimethylammonio)-acetate, a compound first isolated from *Beta vulgaris* by Scheibler (Kruger, 1881). The term 'betaines' has also been applied to compounds of related structure (Brühl, 1876).

Recent investigation have shown carboxyalkylbetaines (having ammonium and carboxylate ions) and sulphobetaines (having ammonium and sulphonate ions) to exhibit mild properties toward proteins. Thus, carboxylakylbetaine and sulphobetaine surface active agents were effective in solubilizing membrane lipids without affecting enzymes, which are readily denatured by alkylsulphate surfactants (Allen & Humphries, 1975; Gonenne & Ernst, 1978; Robinson & Manchee, 1979; Hjelmeland, 1980; Malpartida & Serrano, 1980).

Sulphobetaines, unlike their carboxylic analogue do not form external salts. Therefore, they are present as inner salts regardless of solution pH and do not form precipitates in contact with other ionic compounds. These unique properties

0028-646X/84/020197+09 $03.00/0

suggested an investigation of the biological effects of a homologous series of sulphobetaines, higher 3-(alkyldimethylammonio)-1-propanesulphonates, on orchid seedlings grown in axenic cultures, since this prevents changes in concentrations which may occur when these compounds are sprayed on field or greenhouse-grown plants due to evaporation of the solvent.

MATERIALS AND METHODS

Surface active agents

A homologous series of sulphobetaines 3-(alkyldimethylammonio)-1-propanesulphonates having a normal saturated alkyl chain of eight to 18 carbon atoms (Table 1) were prepared and purified as reported previously (Bode *et al.*, 1978).

Table 1. *Composition and properties of 3-(alkyldimethylammonio)-1-propanesulphonates**

n-Alkyl (lipophile)		Molecular weight†	Krafft temperature (°C)
n-Octyl	(C_8)	286	0
n-Decyl	(C_{10})	312	0
n-Dodecyl	(C_{12})	338	0
n-Tetradecyl	(C_{14})	370	16
n-Hexadecyl	(C_{16})	399	27
n-Octadecyl	(C_{18})	423	88

* Sulphobetaines, $R-N^{+}(CH_3)_2-CH_2CH_2CH_2-SO_3^{-}$, R = *n*-alkyl.

† Based on Kjeldahl nitrogen.

The surfactants were added to liquid Knudson C medium (Knudson, 1946) at 0·02, 0·2 and 2·0 mM concentrations and the pH was adjusted to $5{\cdot}4 \pm 0{\cdot}2$.

Culture vessels

Assays were carried out in duplicate 500 ml Erlenmyer flasks, each containing 120 ml of medium. Glass wool (washed previously with water), $3\frac{1}{2}$ g per flask, served as a platform. The flasks were covered with rubber stoppers, having cotton-plugged holes, and autoclaved at 17 psig for 25 min.

Plant material

Seed of *Brassocattleya* Dèesse × *Cattleya* Mount Shasta were germinated on solid Knudson C medium and grown to the protocorm stage. The protocorms, some bearing one leaf, were rinsed with sterile water and 65 ± 7 of them were transferred to each assay flask.

Growth conditions

The culture flasks were maintained for 9 months at 24 ± 2 °C under 18-h photoperiods and a light intensity of 2600 lx, provided by two 40 W Gro-Lux lamps.

Evaluation of results

Seedlings were blotted to remove surface water and stored in a desiccator prior to the determination of fresh weight and the number and length of leaves and roots.

Surface and interfacial tension measurements

Determinations of surface (ST) and interfacial (IT) tensions were made using the duNouy surface and interfacial tensiometer (ring method, Schwartz & Perry, 1949) with a Fisher Model 20 Tensiomat. Cold pressed, additive free corn oil (Hain Company) was used as a test lipid for IT determinations. Results reported (Fig. 3) are averages of duplicate measurements. Replicates were typically $\pm 2\%$. All measurements were conducted with agar-free Knudson C solution at 24 ± 1 °C.

Results

Surface tensions

Surface tensions were lower than controls for all surfactant-containing media and fell with increasing concentrations and chain length of the alkyl radicals over the range of eight to 16 carbon atoms. The octadecyl derivative gave somewhat higher ST than the C_{12} to C_{16} compounds (Fig. 3).

Interfacial tensions

A steady lowering of IT occurred with increasing concentrations and alkyl chain length over the range of eight to 16 carbon atoms. The C_{18} compound decreased IT's somewhat less at all concentrations in comparison with C_{16} and, at the highest concentration, also with C_{14}. With increased concentrations from 0·2 to 2·0 mM IT was lowered more substantially than ST, particularly within the range of C_{12} to C_{16}.

3-(Octyldimethylammonio)-1-prophanesulphonate

Both seedling survival and fresh weight, as well as leaf and root development, were superior to the controls at 0·02 and 0·2 mM levels (Table 2; Figs 1, 2). At 2·0 mM, survival was equal to the controls but leaf and root numbers and sizes were similar. Fresh weight was reduced (Table 2; Figs 1, 2).

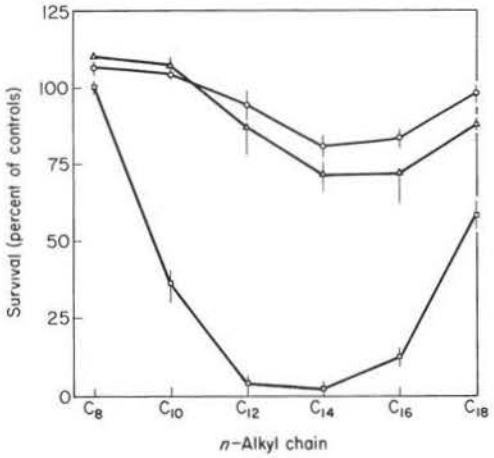

Fig. 1. Survival of orchid seedlings as a function of surfactant concentration (○, 0·02 mM; △, 0·2 mM; □, 2·0 mM) and sulphobetaine alkyl chain length (lipophile).

Table 2. *Growth effects of 3-(alkyldimethylammonio)-1-propanesulphonates on* Brassocattleya *sedlings* in vitro*

n-Alkyl group		Concentration (mM)	Survival (%)	Fresh weight† (g)	Leaves		Roots	
					Number	Length (mm)	Number	Length (mm)
Octyl	(C_8)	0·02	96·1/0·9	5·77/0·18	5·1/1·3	21·0/9·5	4·1/1·1	17·9/4·71
		0·2	100·0/0	5·57/0·37	4·5/1·7	16·7/7·6	4·2/1·3	19·0/6·5
		2·0	90·4/0·9	3·69/0·44	3·3/1·0	11·5/3·5	3·6/1·4	13·1/4·3
Decyl	(C_{10})	0·02	94·4/0·5	5·68/0·35	3·6/0·9	15·4/4·8	4·0/1·2	15·6/4·2
		0·2	96·6/2·3	3·91/0·40	4·2/1·0	12·7/3·5	4·2/3·7	14·9/4·3
		2·0	31·7/5·5	0·95/0·13	3·6/1·1	6·0/2·0	1·3/1·1	5·4/4·8
Dodecyl	(C_{12})	0·02	84·7/5·2	5·10/0·39	4·4/1·2	10·5/3·2	3·4/1·1	16·2/5·4
		0·2	77·9/7·4	3·04/0·60	3·6/1·3	10·4/2·7	2·6/1·2	13·4/4·0
		2·0	2·4/0·8	0·34/0·02	3·3/1·5	4·3/1·5	2·3/1·2	3·0/2·7
Tetradecyl	(C_{14})	0·02	73·0/3·8	3·91/0·18	4·0/1·0	13·0/2·7	4·0/0·8	19·0/5·6
		0·2	64·8/5·4	2·68/0·15	4·3/1·1	8·5/3·3	2·5/1·0	12·7/7·0
		2·0	2·5/1·2	0·15/0·03	3·0/0	5·0/1·6	1·0/0	4·7/1·2
Hexadecyl	(C_{16})	0·02	75·2/2·8	3·84/0·37	3·5/1·0	10·3/3·9	3·4/1·0	19·8/6·4
		0·2	65·6/6·9	2·68/0·24	2·3/1·0	6·5/2·9	2·0/1·0	12·1/6·2
		2·0	10·8/3·3	0·29/0·10	2·7/1·0	5·2/1·1	1·4/0·6	5·0/1·5
Octadecyl	(C_{18})	0·02	88·5/2·0	3·64/0·54	3·7/1·1	12·1/3·4	2·6/1·1	16·1/6·1
		0·2	79·1/0·9	3·23/0·58	3·7/1·3	12·1/4·5	3·1/1·3	16·8/5·4
		2·0	48·5/3·7	3·04/0·29	3·8/1·0	12·7/3·9	3·3/1·3	16·7/6·1
Controls		—	90·3/3·4	5·06/0·32	3·8/1·1	14·0/3·2	3·5/1·1	19·2/5·2

* Data from two replicates, average/standard deviation.
† Total weight of all seedlings in culture vessel.

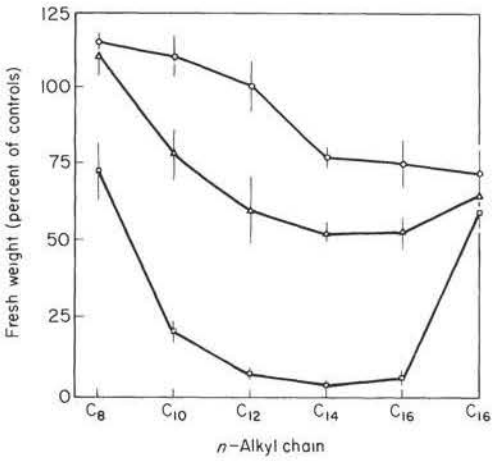

Fig. 2. Fresh weight of orchid seedlings as a function of surfactant concentration and sulphobetaine alkyl chain length (lipophile). Symbols as in Figure 1.

3-(Decyldimethylammonio)-1-propanesulphonate

At concentrations of 0·02 and 0·2 mM survival percentage was similar to that of the controls, but fresh weight at 0·2 mM was substantially lower (Table 2; Figs 1, 2). A notable reduction in fresh weight as well as root number and length occurred at the 2·0 mM level (Table 2; Figs 1, 2). The number of leaves at 2·0 mM was not affected, but their length was reduced.

3-(Dodecyldimethylammonio)-1-propanesulphonate

On 0·02 mM surfactant, seedling survival, fresh weight and leaf and root development were in the range of the controls (Table 2; Figs 1, 2). Survival and fresh weight were lower at 0·2 mM. Only three seedlings survived on 2·0 mM (Table 2; Figs 1, 2). They were chlorotic and of greatly reduced size.

3-(Tetradecylidimethylammonio)-1-propanesulphonate

This homologue reduced survival and fresh weight at all three concentrations (Table 2; Figs 1, 2). Only three small and chlorotic plantlets survived at the highest concentration (Table 2; Figs 1, 2).

3-(Hexadecyldimethylammonio)-1-propanesulphonate

Seedling survival and fresh weight were similar to those observed on 0·2 and 0·2 mM tetradecyl derivative but somewhat higher on the 2·0 mM (Table 2; Figs 1, 2). Fourteen chlorotic plantlets survived at the highest concentration.

3-(Octadecyldimethylammonio)-1-propanesulphonate

Survival was similar to the controls but fresh weight was lower at 0·02 mM. Both survival and fresh weight declined with increasing concentrations but were higher than on the C_{12} to C_{14} compounds (Table 2; Figs 1, 2). Approximately half of the seedlings survived the highest surfactant concentration, and were not chlorotic.

Effects on leaves and roots

The number of roots was more substantially reduced by these surfactants than

the number of leaves. Both leaf and root length were, however, greatly affect by the highest concentration of surfactants within the range of the dodecyl-to hexadecyl-sulphobetaines (Table 2).

DISCUSSION

The critical micelle concentration (CMC) of sulphobetaines decreases logarithmically with each increase of two methylene groups in their lipophilic alkyl chain (Herrmann, 1966). This in turn is accompanied by a substantial increase in micellar molecular weight or monomers per micelle (Herrmann, 1966; Ernst & Miller, 1982). Chain length of the lipophile affected solution properties, both ST and IT dropping with increasing molecular weight (Fig. 3). The 18 carbon homologue was

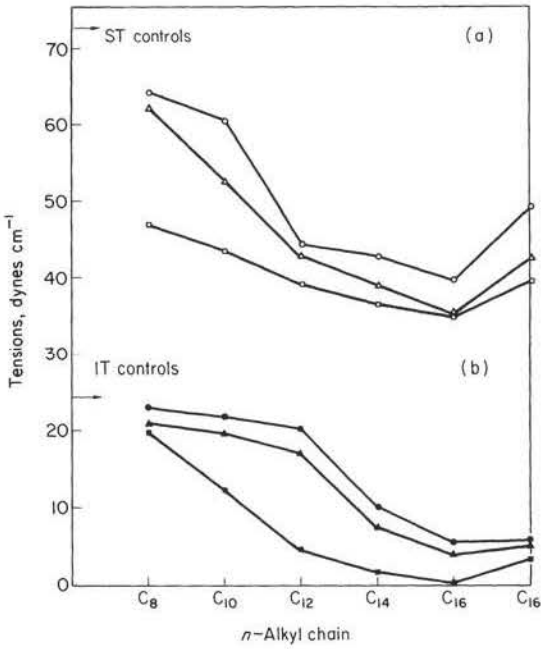

Fig. 3. (a) Surface (ST) and (b) interfacial (IT) tensions as a function of surfactant concentration and sulphobetaine alkyl chain length (lipophile). Symbols as in Fig. 1.

anomalous because of the high Krafft point (88 °C) of its aqueous solutions, which prevented micelle formation at the culture temperature. The Krafft temperature of the hexadecyl homologue (27 °C) which is slightly above the culture temperature employed (24 ± 2 °C) could also be responsible for its slightly lower phytotoxicity.

Growth inhibition of orchid seedlings by sulphobetaine surfactants increased with increasing alkyl chain length within the range of eight to 14 carbon atoms, and was most severe at the 2·0 mM concentration where the higher molecular members were in the micellar range. The octyl and decyl derivatives do not reach their CMC at this concentration and were substantially less damaging to the seedlings. Among the sulphobetaines, the 16 carbon alkyl compound was only marginally less inhibitory to orchid seedlings than the 14 carbon homologue. This confirms previous reports that inhibition of callus cultures of *Dimorphotheca*

sinuata (Cape marigold) by sulphobetaines increased with increasing lipophile from eight to 14 carbon atoms. The inhibition levelled off at 16 carbon atoms of the lipophile and declined with the less water soluble octadecyl derivative (Ernst, Ball & Arditti, 1982).

The same homologous series of sulphobetaines inhibited the growth of the green alga *Chlamydomonas reinhardi* at micellar concentrations when the lipophile contained 12 or more carbon atoms. In this study as well, toxcity increased with increasing alkyl chain length, reaching a plateau with the hexadecyl derivative (Ernst, Gonzales & Arditti, 1983).

Phytotoxicity of all sulphobetaines tested increased with higher concentrations. This increase was surprisingly small over the concentration range of 0·02 to 0·2 mM, despite the fact that the hexadecyl derivative is in the micellar range at the 0·2 mM level, based on ST data (Table 2; Figs 1 to 3). Solubility and therefore toxicity may, however, have been reduced due to a Krafft point above the culture temperature. In contrast, a further tenfold increase in concentration of all sulphobetaines containing 10 or more carbon atoms in the lipophile resulted in a sharp decline of seedling survival and fresh weight (Table 2; Figs 1, 2).

Of the surfactant solution properties examined, IT, measured against a test lipid, was the only parameter which paralleled the growth inhibitions. The test lipid chosen is substantially less hydrophilic than membrane lipids and therefore can only serve as a rough indicator for the action of sulphobetaine surfactants on cell membranes. Nevertheless, it is interesting to note that when these surfactants were employed in the extraction of proteins from 3T6 mouse fibroplast membranes, efficiency increased with increasing alkyl chain length, reaching a plateau at the tetradecyl sulphobetaine (Gonenne & Ernst, 1978). Resistance of lipid membrane bilayers to cationic surfactants (alkytrimethylammonium chlorides with four, nine, 12 and 16 carbons in the alkyl chain) was also affected by increasing alkyl chain length (Grupe *et al.*, 1978). Haemolytic action of homologous anionics (sodium alkyl sulphates) and cationics (alkylpyridinium iodides) increased almost logarithmically with each increase of two carbons in the alkyl chain with the range of four to 12 carbon atoms (Kondo & Tomizawa, 1966). Limited surfactant properties could be expected of members below eight carbon atoms in the lipophile, but tension-reducing properties do increase sharply from the octyl to the dodecyl derivatives.

Orchid seedlings treated with micellar concentration of an anionic surfactant show drastic ultrastructural changes (Healey, Ernst & Arditti, 1971). These include (1) plasmolysis, (2) swelling of thylakoids and mitochondrial cristae, (3) gross changes in chloroplast morphology including the formation of dense osmophilic granules and (4) appearance of unidentified vesicles in the cytoplasm. The effects are indicative of lysis caused by the surfactant. Interaction with proteins may also be involved in surfactant toxicity (Reynolds *et al.*, 1967; Steck & Fox, 1972; Helenius & Simons, 1975; Tanford & Reynolds, 1976; Schwuger & Bartrik, 1980).

Recent studies of erythrocyte membranes exposed to cetyltrimethyl (1-^{14}C) ammonium bromide (a cationic surfactant) revealed only very small amounts of radioactivity associated with membrane proteins, suggesting that the latter play no major role in the lytic events (Isomaa, Bergman & Sandberg, 1979). Toxicity of sulphobetaines to animal tissue was distinctly related to increasing molecular weight of the lipophile radical from eight to 18 carbon atoms. In an assay with *Hydra attenuata* (Bode *et al.*, 1978) and HeLa cells (Ernst & Arditti, 1980) lethal

concentrations were lower than those which had phytotoxic effects on orchids. The toxicity of lower concentrations and a correlation between ST-reducing properties and toxicity, noted in both cases, may be due to the absence of cell walls and cuticle. These may provide protection against the physical effects of surface active agents.

Disorganization of cell membranes by surface active agents has long been suggested as cause of their bactericidal action (Baker, Harrison & Miller, 1941). Indeed, the lethal level of cationic and anionic surfactants on staphylcoccii is always accompanied by release of large amounts of phosphorus and nitrogen into the surrounding medium (Hotchkiss, 1946). This does not occur with surfactants having low or no lethal effect on this organism. Such a process is analogous with the release of haemoglobin into the suspending medium by surfactant solutions (Kondo & Tomizawa, 1966; Pethica & Schulman, 1977). An hypothesis was therefore proposed that cytolytic injury is the principal cause of the bactericidal property of surfactants (Hotchkiss, 1946). Our present and previous data (Ernst *et al.*, 1971; Healey *et al.*, 1971) strongly supports the view that disruption of cellular membranes also plays a key role in the phytotoxicity of surfactants.

ACKNOWLEDGEMENTS

This study was supported in part by grant R01 ES 60726-02 from NIH.

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