J. Pharm. Pharmacol. 1990, 42: 790–794
Received April 26, 1990

Cut-off Effect in Antimicrobial Activity and in Membrane Perturbation Efficiency of the Homologous Series of *N,N*-Dimethylalkylamine Oxides†

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Abstract—The antimicrobial activity of the homologous series of *N,N*-dimethylalkylamine oxides (DMAO) was found to be quasi parabolically dependent on alkyl chain length with a maximum at $n \approx 15$ and $n \approx 12$ for *Staphylococcus aureus* and *Escherichia coli*, respectively. The physicochemical properties of DMAOs as characterized by critical micelle concentrations, retention times of 1-alkenes generated from DMAOs by gas-liquid chromatography, R_m values in reversed phase chromatography, and bacterial lipid/aqueous phase partition coefficients were found to correlate with the alkyl chain length. The effect of DMAOs on the structure of the model membrane prepared from isolated lipids from *Escherichia coli* as detected by a spin probe method was maximal for the alkyl chain length $n \approx 10$–12 coinciding with the maximum in the antimicrobial activity observed with *Escherichia coli*. It is suggested that the cut-off in the DMAO antimicrobial activity is caused by the cut-off in the DMAO perturbing effect on the membrane structure.

Biological activities of long chain amphiphilic substances often show a non-linear dependence on chain length that is quasi parabolic. Because of the decrease of activity for the more lipophilic substances within the homologous series it is often called a cut-off effect. This general pharmacological phenomenon has been observed in such homologous series in different pharmacological tests and several hypotheses have been proposed to explain the cut-off effect, as follows.

Limited solubility (Janoff et al 1981; Pringle et al 1981): the partition coefficient between the site of action (e.g. membrane) and aqueous phase, increases less rapidly with the chain length than the aqueous solubility decreases, until a point is reached at which the maximum achievable concentration at the site of action is significantly lower than that required to cause the maximal biological effect.

Limited volume (Franks & Lieb 1986): the compound could act after binding at some site of action which has a limited volume (e.g. hydrophobic pockets on proteins). This volume becomes full with increase in chain length and a decrease in binding occurs.

Compartment theory (Hansch & Fujita 1964; Lien et al 1968; Baláž et al 1988): this relates to the partition in time through several compartments (e.g. a series of lipid bilayers separated by aqueous layers) as the compound gains access to the site of action. Partitioning is influenced both by the lipophilicity of the compound and of each compartment. Short chain amphiphilic substances would be unable to cross the hydrophobic compartments (lipid bilayers) whereas long chain homologues would be unable to penetrate the hydrophilic compartments (aqueous regions). Substances with optimal chain length between these extrema will possess the optimal properties for transport to their site of action and will show maximal biological activity.

† Part VIII of the series "Interaction of Surfactants with Model and Biological Membranes" and part XXIV of the series "Amine Oxides".

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Perturbation theory (Lee 1976; Requena & Haydon 1985): the cut-off effect occurs because long chain amphiphilic substances interact with the site of action in a way which no longer perturbs biological function.

Physical theory (Schoenenberger et al 1977; Devínsky et al 1978): with the increase in chain length the physical properties of amphiphilic substances (e.g. stereochemistry, membrane solubility) could change non-linearly; the cut-off effect might reflect such a physical change.

In our present study, the antimicrobial activity of a homologous series of *N,N*-dimethylalkylamine oxides (DMAOs) has been evaluated, and related to their physicochemical characteristics.

Materials and Methods

The *N,N*-dimethylalkylamine oxides were prepared in our laboratories from the corresponding *N,N*-dimethylalkylamines by hydrogen peroxide oxidation as described by Devínsky et al (1978). The spin label *N*-hexadecyl-*N*-tempoyl-*N,N*-dimethylammonium bromide (CAT-16) was purchased from Technika (Sofia, Bulgaria). Bacterial lipids were isolated from the early stationary phase of growth of *Escherichia coli* Ec 377/79 cells (National Collection of Microorganisms, Prague) using the organic solvent extraction method of Folch et al (1957).

Critical micelle concentration (CMC) was measured using the surface tension method of Devínsky et al (1985b).

The retention times (t_R) in gas-liquid chromatography (GC) are those for the 1-alkenes generated by direct injection pyrolysis GC of *N,N*-dimethylalkylamine oxides. This method has been developed for determination of nanomolar amounts of non-aromatic oxides (Devínsky & Gorrod 1989).

The R_m values in reversed phase chromatography were determined using silica gel impregnated with 5% silicone oil and using a developing system of 0·1 mol L^{-1} HCl:acetone (1:1) according to Devínsky et al (1987).

As a measure of membrane perturbation effect, an order

parameter, S, of CAT-16 spin probe incorporated in the model lipid bilayer membrane prepared from *E. coli* lipids was used. Ethanolic solutions of CAT-16, bacterial lipids, and DMAO were weighed into polyethylene tubes. After evaporation of the solvent in a stream of nitrogen gas, the samples were dried under vacuum (10^{-2} Pa) at 25°C. Immediately before the experiment, redistilled water was added in a weight ratio of lipid:H_2O, 1:20 and lipid was dispersed by sonication in a UC 005 AJ1 (Tesla, Czechoslovakia) ultrasonic bath sonicator for about 5 min. Final concentrations of the spin label and lipid were $2{\cdot}5 \times 10^{-4}$ mol L^{-1} and 20 g L^{-1}, respectively, and the maximal concentration of DMAO was 5×10^{-2} mol L^{-1}. Electron spin resonance (ESR) spectra were recorded using an ESR-230 X-band spectrometer (ZWG AdW, Berlin, Germany) with a microwave power output of 5 mW and a modulation amplitude of 0·1 mT at a temperature of 23 ± 1°C. The order parameter S was calculated from ESR spectra according to Gordon & Sauerheber (1977) using the formula:

$$S = 0{\cdot}5\{3[(A_{zz} + A_{xx}) - 2A_{\perp}]/(A_{zz} - A_{xx}) - 1\} \quad (1)$$

where $A_{\perp}$ is the time averaged component of the axially symmetric hyperfine splitting tensor A normal to the magnetic field direction, obtained from the inner extrema distance of the experimental ESR spectra, and A_{xx} and A_{zz} are components of the diagonalized A tensor taken from the literature (Gaffney 1976).

Samples used for determination of the partition coefficients, K_p, between the bacterial lipids and the aqueous phase were prepared in the same manner as those for the ESR spectroscopy, with the weight ratio of lipid:H_2O, 1:200 but without spin label. After homogenization and equilibration, the aqueous and lipid phases were separated by ultracentrifugation for 1·5 h at 100 000 *g* and 18°C. The concentration of DMAO in the supernatant was determined by differential pulse polarography (for experimental details see Faith et al (1982)). The concentration of lipid in the supernatant after centrifugation was less than 3% of total lipid so the supernatant concentration of DMAO was assumed to be equal to the aqueous DMAO concentration; the concentration in the lipid phase was calculated from the known total concentration. For simplicity, the densities of the lipid and the aqueous phase were assumed to be 1 g mL^{-1}, and the molecular weight of lipid was assumed to be 800.

The minimum inhibitory concentrations (MIC) were determined using the *Escherichia coli* Ec 377/79 and *Staphylococcus aureus* Oxford Mau cells and the standard dilution technique described previously (Devínsky et al 1985a).

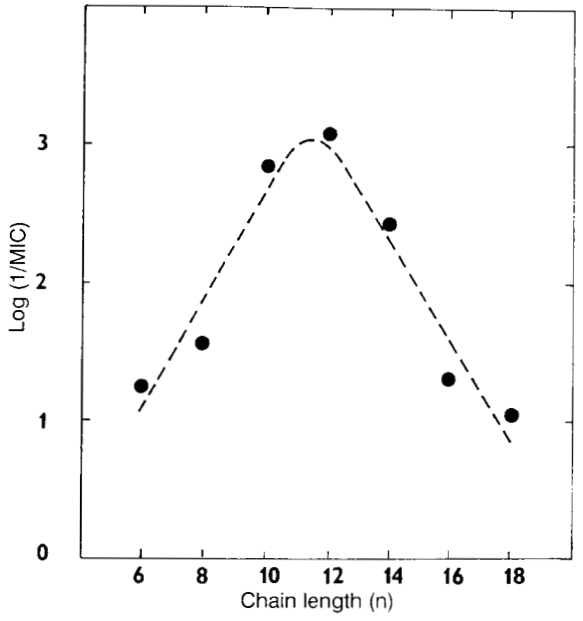

FIG. 1. Antimicrobial activity of DMAO in *E. coli* cells expressed as minimum inhibitory concentration (MIC, mol L^{-1}) as a function of the number of carbon atoms (n) in the DMAO alkyl chain.

Table 1. Regression coefficients for bilinear relationships between antimicrobial activity (log(1/MIC)), critical micelle concentration (log(CMC)) and structure for *N,N*-dimethylalkylamine oxides obtained by fitting eqn 2 to experimental data. I: relationship log(1/MIC) = f(n), x = n; II relationship log(1/MIC) = f(log(CMC)) x = log(CMC).n_o: optimum chain length; CMC_o: optimum CMC. The values of CMC and MIC are assumed to be in mol L^{-1} units; F: value of Fisher–Snedecor's F-test.

Coefficient		*E. coli*	*Staph. aureus*
a	I	0·411 ± 0·066	0·458 ± 0·024
	II	0·883 ± 0·132	1·260 ± 0·198
b	I	−0·783 ± 0·108	−0·849 ± 0·092
	II	−1·849 ± 0·266	−2·291 ± 0·236
c	I	−1·422 ± 0·599	−1·817 ± 0·260
	II	5·646 ± 0·550	10·831 ± 0·970
logβ	I	−11·452	−15·006
	II	2·456	4·306
N	I	7	7
	II	7	7
r_i	I	0·965	0·995
	II	0·962	0·996
F	I	26·7[a]	210·5[c]
	II	24·6[b]	234·4[c]
n_o	I	11·5	15·1
$\log CMC_o$	II	−2·497	−4·219

[a] $F_{2,4;\,0.005}$; [b] $F_{2,4;\,0.01}$; [c] $F_{2,4;\,0.001}$.

Results and Discussion

The antimicrobial activity of DMAOs expressed as a reciprocal minimum inhibitory concentration is quasi parabolically dependent on alkyl chain length with a maximum at $n \approx 15$ and $n \approx 12$ for *Staphylococcus aureus* and *Escherichia coli*, respectively (Fig. 1, Table 1). In QSAR studies this type of dependence is usually approximated by a parabolic or better, by a bilinear equation to find the optimal chain length (Kubinyi 1984). Fitting of our data to the bilinear equation

$$\log(1/\mathrm{MIC}) = ax - b\log(\beta 10^{x} + 1) - c \quad (2)$$

where x = n, the alkyl chain length and a, b, c and β are constants, gave numerical values shown in Table 1. Statistical evaluation of equation 2 shows high significance (data points n = 7, correlation indices $r_i > 0{\cdot}96$, standard deviations from equation 2 s.d. < 0·3).

Critical micelle concentrations (CMC), retention times (t_R) in gas chromatography and R_m values in reversed phase chromatography of the DMAOs increased with increase of alkyl chain length. Fitting of experimental data to the linear equation

$$y = An + B \quad (3)$$

Table 2. Regression coefficients for linear relationships between critical micelle concentrations (log(CMC)), retention times (t_R) and R_M values, and the number of carbon atoms x = n in the DMAO alkyl chain, obtained by fitting eqn 3 to experimental data. r: correlation coefficient; N: number of data points; s: standard deviation from the equation; F: value of Fischer–Snedecor's F-test.

Y	A	B	N	r	s	F
log(CMC)	−0·462 ± 0·004	2·837 ± 0·052	7	0·999	0·044	12517·2[a]
t_R	4·448 ± 0·230	−20·379 ± 2·908	7	0·993	2·433	374·4[a]
R_M	0·103 ± 0·002	−1·004 ± 0·028	8	0·999	0·030	2000·8[b]

[a] $F_{1,5;\,0·001}$; [b] $F_{1,6;\,0·001}$.

where n is the alkyl chain length, A and B are constants, and y is t_R, R_m or log CMC gave numerical values shown in Table 2. The slope of the least squares line fitted to the data gives a value for the incremental free energy of transfer for DMAO methylene group $\Delta(\Delta G^\circ)_{CH_2} = -1·026 \pm 0·006$ RT from the aqueous phase to the micelle interior. This value is typical for amphiphile micelle formation (Anacker 1970). The data in Table 2 indicate that the measured physicochemical properties of DMAOs do not display any anomaly at some particular chain length which could be responsible for the cut-off in their antimicrobial activity. However, there is a possibility that these parameters might not be sensitive enough to show fine differences in e.g. stereochemistry of individual compounds in the homologous series.

Comparison of values of MIC and of CMC indicates that the micelle formation of DMAOs might be responsible for the cut-off in antimicrobial activity. The MIC and CMC values were correlated using equation 2 with x = logCMC. The results are shown in Table 1. It is clear from these results as well as from comparison of Fig. 1 and Table 2 that the decrease of antimicrobial activity occurs at chain lengths n for which MIC ≥ CMC. Consequently, the cut-off effect could be explained by the limited solubility theory. To test this particular point, the partition coefficients K_p between the bacterial lipid and aqueous phase were determined using bacterial lipid liposomes and ultracentrifugation and pulse polarography techniques (Table 3). The concentration of DMAOs in these experiments (5×10^{-3} mol L^{-1}) was higher than the CMC for those compounds where MIC > CMC, i.e. for compounds where the cut-off effect occurs. If the micelle formation were responsible for the low membrane concentration of DMAOs, we should observe some type of anomaly on the log K_p vs n plot. However, the values of log K_p are linearly dependent on the alkyl chain length n (not shown). The value of the incremental free energy of transfer for a DMAO methylene group $\Delta(\Delta G^\circ)_{CH_2} = -1·034 \pm 0·080$ RT from the aqueous phase to the lipid phase calculated from the data in Table 3 corresponds well to that found for the micelle formation process (see above) and for the binding of different amphiphilic substances to the bilayer of phospholipid liposomes (Zaslavskii et al 1980; Requena & Haydon 1985; Matsumura et al 1986; Franks & Lieb 1986). Two conclusions follow from the results in Table 3. First, the DMAO partitioning seems not to be influenced significantly by the micelle formation, so that in this case the limited aqueous solubility theory does not explain the cut-off effect. Second, the value of $\Delta(\Delta G^\circ)_{CH_2}$ indicates that the main motive force of the binding of DMAO molecules to the lipid bilayer is the hydrophobic interaction, and that the binding environment for the hydrocarbon chains of different amphiphilic compounds in the lipid bilayer is very similar.

Table 3. DMAO partition coefficients (K_p) between *Escherichia coli* lipids and aqueous phase, as a function of the number of carbon atoms of the DMAO alkyl chain.

n	6	8	10	12	14	16
K_p	4·5	10·7	45·8	212·6	450	3880

In our recent paper (Šeršeň et al 1989) we observed that the order parameter S of spin probes located in different bilayer depths decreases with the increase of the amphiphilic substance concentration. The same effect has been observed in the present study for DMAOs and CAT-16 spin probe with the paramagnetic group located in the polar region of the lipid bilayer. The order parameter decreased nearly linearly with the increase of DMAO concentration up to the maximal DMAO concentrations (0·05 mol L^{-1}). Similarly, Podolak et al (1987) observed a decrease of the order parameter S of the fatty acid spin probes (with the paramagnetic groups located in the hydrophobic region of the bilayer) in phosphatidylcholine liposomes in the presence of *N*-trimethylalkyloxymethylammonium chlorides up to molar ratios of amphiphilic substance: lipid as high as 1:2 and alkyloxy chain lengths up to n = 16. Our findings as well as those of Podolak et al (1987) suggest that the perturbation of the bilayer structure can occur at concentrations well above the CMC. This is also in agreement with the partitioning of DMAO into the lipid bilayer described above. Fig. 2 shows the dependence of the order parameter S of CAT-16 spin probe on the number of carbon atoms n in the alkyl chain of the DMAO at a given concentration. The effect of the DMAO on the bilayer structure of the model membrane prepared from lipids of *E. coli* is maximal for the alkyl chain

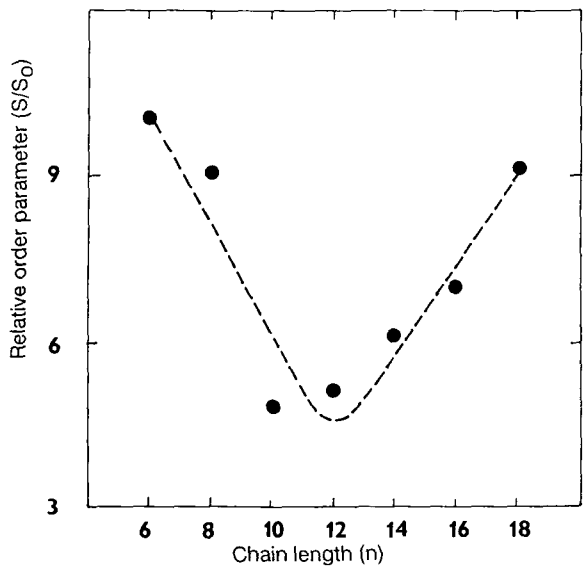

FIG. 2. Relative order parameter S/S_0 of CAT-16 spin probe embedded in model membranes prepared from lipids of *E. coli* in the presence of DMAO, as a function of the number of carbon atoms (n) in the DMAO alkyl chain. Order parameter of control sample (without DMAO) was $S_0 = 0·41$, DMAO concentration was 0·05 mol L^{-1}, and the measurements were made at 23°C.

length $n \approx 10$–12 which coincides with the maximum in the antimicrobial activity observed with *E. coli*.

The insertion of DMAO molecules into the lipid part of the membrane is anisotropic. DMAOs bear a non-dissociable polar group with the positive charge on the nitrogen. This group can interact with various molecular fragments of lipids in the polar region in the membrane (e.g. phosphate, carbonyl, etc). The alkyl chain of the DMAO will orient parallel to the hydrocarbon chains of lipid molecules. At this location the packing density of lipids will be influenced due to lateral expansion of the membrane and formation of free volume below the DMAO alkyl chain end. If the alkyl chain is short, the free volume created below its end will be large, but its interaction with the lipid hydrocarbon chains will be weak and the partition coefficient K_p small, so that the total free volume created in the membrane hydrophobic region will be small at a given total DMAO concentration. As alkyl chains of DMAOs become comparable with lipid hydrocarbon chains the partition coefficient will increase but the free volume will decrease to zero. Substances with alkyl chains between these extrema will thus induce maximal free volume in the membrane. The free volume can be filled in because of high flexibility of hydrocarbon chains. The effective shape of DMAO molecules in the bilayer can thus be described as an inverted cone. After insertion into the bilayer they should deform it. With the increase of the length of alkyl substituent the conical asymmetry of DMAO molecules decreases and, consequently, the bilayer deformation should decrease. Most probably, the changes in the bilayer polar region structure as detected by CAT-16 spin probe are due to this deformation.

Energy of elastic deformation of the bilayer is the main factor determining the bilayer stability: after reaching some critical value of the free volume, the bilayer collapses and a new non-bilayer structure (e.g. micelle) forms in the membrane at a minimum energy of elastic deformation (Charvolin & Mely 1978; Derzhanski & Bivas 1979). In our previous study we observed the formation of non-bilayer structures in model lipid membranes in the presence of *N*-alkyltrimethylammonium iodides with chain lengths of $n = 6$ and 9, but not with shorter or longer alkyl chains (Balgavý et al 1984). Since the mode of membrane interaction with different types of long chain substances is similar as shown by the $\Delta(\Delta G^{\circ})_{CH_2}$ values, we would also predict formation of non-bilayer structures in *E. coli* lipids for DMAOs with maximum of efficiency at $n \approx 10$–12 as observed for membrane structure perturbation detected by the CAT-16 spin probe. Under physiological conditions the formation of domains of non-bilayer structures in the membrane is prevented by the cell's adjustment of the membrane lipid composition (Gruner 1985; Goldfine et al 1987), but the presence of large amounts of amphiphilic substance in the membrane can drive this feedback mechanism out of control. As a result, fragmentation of cell membrane and leakage from its interior may occur leading eventually to the cell death. Indeed, the fragmentation of the cell membranes in the presence of non-aromatic *N*-oxides has been observed by electron microscopy in *E. coli* cells. The critical chain length at which the free volume in the membrane is maximal and at which the bilayer structure collapses is dependent on the properties of the membrane lipids (chain length, degree of unsaturation, fluidity). Therefore, it is not surprising that the chain length at which the maximal biological effect occurs is different for *E. coli* and for *Staph. aureus* (see Table 1) or for bacterial cells cultivated under different conditions (Wright & Gilbert 1987).

The results presented in our study together with the data of other workers therefore support the free volume mechanism of antimicrobial action of amphiphilic substances and the perturbation theory of the cut-off effect.

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