INTERACTION OF AMINE OXIDES AND QUATERNARY AMMONIUM SALTS WITH MEMBRANE AND MEMBRANE-ASSOCIATED PROCESSES IN E. COLI CELLS: MODE OF ACTION[1]

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1 Part XXV of series Amine Oxides; Part XXVI of series Quaternary Ammonium Salts

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SUMMARY

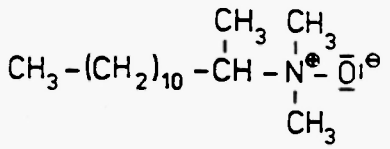
The antimicrobials (1-methyldodecyl)dimethylamine oxide and (1-methyldodecyl)trimethylammonium bromide affect the cytoplasmic membrane of E. coli. The interaction results in release of intracellular material (K^+, 260nm – absorbing material), an effect on dehydrogenase enzyme activity and inhibition of respiration. The final effect of both substances is the same; they differ only in their dynamics. The effect of the membrane was correlated with parameters characterizing these surfactants i.e. critical micelle concentration (c.m.c.) minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) over the concentration range of 10^{-4} to 10^{-1} mmol/dm^3 of active substance. The three stage mode of action model can be summarized as follows: 1 – polar (coulombic) interactions, 2 – polar and hydrophobic interactions, 3 – hydrophobic interactions (extraction and solubilization). The polar and hydrophobic interactions (1st and 2nd stage) are discussed also in relation to model membranes.

I. INTRODUCTION

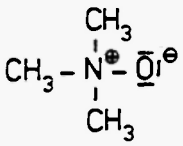
The high adaptability of microorganisms to the environment in connection with the acquired resistance to antimicrobial substances results in persistent searching for new, more effective agents. Many highly effective antimicrobial drugs are well known, e.g. antibiotics, sulphonamides, antimycotics, etc., nevertheless there are in some cases limitations for their use.

The best known and most used disinfectants of the surfactant class type are quaternary ammonium salts (QUATS). These have not lost their importance yet, but are gradually being substituted by compounds with better characteristics. Non-aromatic surface active amine oxides (AOS) /1,2/ are such newer substances, from these (1-methyldodecyl)-dimethylamine oxide (2-ATDNO), Figure 1: (I) belongs to the most effective compounds of this type /3-5/. Structurally very close to this compound is the quaternary salt (1-methyldodecyl)trimethylammonium bromide (2-ATDBr), Figure 1: (II).

The molecules of both compounds have marked similar features. The small differences in structure have, however, a great influence on the properties of the molecules. The positive charge in QUATS has a unit



(I)



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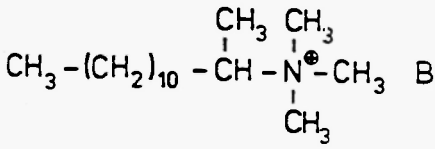
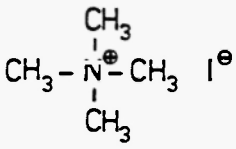


(II)



(IV)

Fig. 1: Structural formulae of tested compounds.

value, although in AOS it is a partial one. 2-ATDBr is a cationic compound dissociated in water, while 2-ATDNO is a compound of non-ionic type which does not dissociate in water.

Considering the structural similarity it is possible also to expect a similar mechanism of antimicrobial action. On the other hand the processes affected by a postively charged nitrogen will surely show dissimilarities.

As recently found /6,7/, the mode of action of N,N-dimethylalkylamine oxides on bacterial cells is the same as with N,N,N-trimethylalkylammonium membrane-active surfactants /8-10/. Non-aromatic amine oxides besides wide use in almost all industry branches /11/ find application also in the pharmacy as constituents of some combined preparations with antimicrobial or disinfectant activity /12-16/. However, only little is known about their mode of action at a cellular or subcellular level /2,17-20/.

The amine oxide (I) is a carrier in a recently developed highly efficient disinfectant /21/ containing iodine. Therefore we focused our attention to study the mode of action of this substance upon bacteria. In this paper we describe the effect of (I) as well as (II) on the metabolic processes of E. coli associated with membrane function, their effect on permeability of native membranes and the contribution of

both parts of the amphiphilic molecules on the quality and quantity of these interactions. To eliminate the hydrophobic interactions we also included in our experiments short-chain analogues lacking a hydrophobic portion: trimethylamine oxide TMAO (III) and tetramethylammonium iodide TMAI (IV), Figure 1.

II. MATERIALS

2.1 Compounds

(1-Methyldodecyl)dimethylamine oxide and trimethylamine oxide dihydrate were prepared by oxidation of corresponding tertiary amines /22/. (1-Methyldodecyl)trimethyammonium bromide was prepared by methylation of (1-methyldodecyl)dimethylamine with bromomethane following the general procedure as described earlier /23/. Tetramethylammonium iodide, analytical grade, was purchased from Lachema, Czechoslovakia, and purified from ethanol-water mixture 4:1. The values of physico-chemical as well as antimicrobial properties are presented in Table 1. Sodium dodecylsulphate (SDS), pure, was purchased from Serva, FRG. All other chemicals were analytical grade (Lachema, Czechoslovakia) and were used without further purification.

TABLE 1

Physical and antimicrobial characteristics of substances

Substance	M_r	M.P. /^{0}C/	c.m.c.$_1$	c.m.c.$_2$	MIC*	MBC*
			10^3mol/dm^3/			
(I)	243.4	95-97	2.0	0.95	0.29	8.2
(II)	322.4	>300	13	8.00	0.19	1.2
(III)	75.1	198-202	–	–	>900	–
(IV)	201.1	>300	–	–	>500	–

c.m.c.$_1$ – in redistilled water
c.m.c.$_2$ – in the presence of cell extract
* Taken from : Kopecka-Leitmanova, A. et al., 1988; in preparation.

2.2 Microorganism

E. coli strain Ec 377/79 was obtained from the Czechoslovak National Collection of Microorganisms in Prague and was stored on agar plates (pH7.2) at 4°C.

2.3 Cultivation media

Nutrient broth and nutrient agar were purchased from Imuna Czechoslovakia.

2.4 Instrumentation

The ultraviolet-visible spectra were measured on UV-VIS Specord (Zeiss). The measurements were performed in quartz cells of 1.0 and 0.2 cm width, respectively. The samples were prepared in distilled water in acetone-water mixture (5:2).

Potassium ion concentration was determined using an atomic absorption spectrometer AAS-1 (Zeiss) in acetylene-air flame at 766.3 nm. Aqueous solutions of samples were used. Solutions of KCl were used as standard.

The oxygen uptake by bacterial cells was followed using a vibrational gold electrode.

The density of bacterial culture was determined turbidimetrically using the Spekol M (Zeiss) apparatus in 1.0 cm glass cells at 420 nm.

The surface tension of surfactant solutions was measured tensiometrically using the Du Nouy's platinum ring method /24/.

Conductivity of quaternary salt's solutions was measured using a conductometer OK102/1 with platinum bell electrode (Radelkis Hungary) in temperature controlled cells of volume 25 ml.

III. METHODS

3.1 Preparation of Cell Culture

The organisms were grown at 37°C for 6h in nutrient broth and then inoculated onto agar plates. After 12h at 37°C the cells were harvested by triple washing with quarter strength Ringer's solution and

centrifuged. The washed cells were filtered and adjusted turbidimetrically to a required cell concentration using calibration curve $A_{420} = f(n)$, where n is the number of viable cells in 1.0 ml of Ringer's solution. The cell culture was equilibrated before incubation with the test compounds for at least 30 minutes at 4°C.

3.2 Determination of Minimum Inhibitory and Minimum Bactericidal Concentration

The antimicrobial agents were mixed with thioglycollate medium in decreasing concentrations, the tubes were inoculated with culture of the bacteria and examined after incubation at 37°C for 48h. The lowest concentration of antimicrobials completely inhibiting bacterial growth was taken as minimum inhibitory concentration (MIC).

A suspension test was used to determine the minimum bactericidal concentration (MBC). Five ml quantities of the compounds under study in hard water were inoculated by 18h culture of E. coli. After 5, 10, 20, 40min of exposure an aliquot was inoculated into nutrient broth. Bacterial survival was examined after incubation at 37°C for 48h.

3.3 Preparation of Cell Extract

The cell suspension (4×10^9 per ml) prepared by the above mentioned method was autoclaved for 25min at 202kPa and 125°C. After returning to starting volume the cell debris was removed by centrifugation. The total content of released cytoplasmic material was determined and the cell extract used to study drug-cytoplasm interactions.

3.4 Leakage of Intracellular Material

E. coli cells (4×10^9 per ml) were incubated with various concentrations of compounds under study in a shaking water bath at 37°C (shaking rate 60-80 per min). At certain time intervals the suspension was centrifuged and the supernatant was analysed by atomic absorption photometry for potassium ions present, and spectrophotometrically for material absorbing at 260 nm. Quarter strength Ringer's

solution was used as the diluent for cells except for K^+ leakage where normal saline was used.

3.5 Determination of Dehydrogenase Activity

The artificial electron acceptor 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium iodide (INT) was added at a concentration of 0.05 mmol/dm^3 to cells (4×10^9 per ml) pre-treated with various concentrations of antimicrobal agents. Simultaneously with the indicator were also added the substrates (glucose, succinate pyruvate) in concentrations of 6 mmol per dm^{-3}. After 45 minutes incubation at 37°C on a shaking water bath cellular metabolism was halted by cooling in an ice bath and by the addition of a 2.5-fold volume of acetone pre-cooled to 0°C. After 10 minutes the cell fragments were centrifuged and the amount of reduced INT in the supernatant was determined spectrophotometrically at 455 nm.

3.6 Respiratory Rate Determination

The cell suspension prepared according to section 3.1. was held in the measuring cell of the oxygraph at 37°C until saturation by oxygen was reached (5-10) minutes. The antimicrobial agent under test was added to the cell in varying concentrations and at certain time intervals (Table 2) the metabolism-inducing substrate was added: glucose or succinate in concentration of 2 mmol/dm^3, and pyruvate in concentration of 10 mmol per dm^3, respectively. The oxygen uptake was registered. The effect of tested compounds was determined indirectly using the equation

$$R = (K_1 / K_2) \times 100 \qquad (1)$$

where R is the degree of respiration (%), K_i = the slope of untreated cells respiration lins, K_2 = the slope of exposed cells respiration line.

3.7 Determination of Interaction Between Surfactants and Cell Extract

To 5.0ml of cell extract different concentrations of tested substances were added. After 30 minutes shaking in a water bath at 37°C the appropriate products of interaction were centrifrugated and the content of cytoplasmic constituents in the supernatant were determined spectrophotometrically as the material absorbing at 260nm. The interaction degree S (%) was calculated from:

$$S = (1 - A/A_0) \times 100 \qquad (2)$$

where A is the absorbance of supernatant caused by cells after active compound action, A_0 is the absorbance of reference cell supernatant.

3.8 C.m.c. Determination of Surfactants in the Presence of Cell Extract

The surfactants (I) and (II) were diluted in redistilled water containing the total cell extract (in concentration corresponding to 20% for N-oxide and 50% for ammonium bromide; extract from 4×10^9 cells per ml). C.m.c. was determined tensiometrically and for 2-ATDBr also conductometrically /24/.

IV. RESULTS

4.1 Effect on Membrane Permeability

Relative values of cytoplasmic constituents released from E. coli cells are related to their content determined in total cell extract. Figure 2 shows the concentration effect of (I) and (II) on leakage of K^+ ions and high molecular weight metabolites determined as 260 nm absorbing material /25/. Both compounds induce a time dependent release of cytoplasmic contents. A maximum release of circa 80% of K^+ pool being achieved in 20 minutes with the MIC of (II). The effect of (I) is substantially less at the MIC although delayed contact results in a gradual increase in leaked material.

By increase of concentration above MIC values of both subtances the

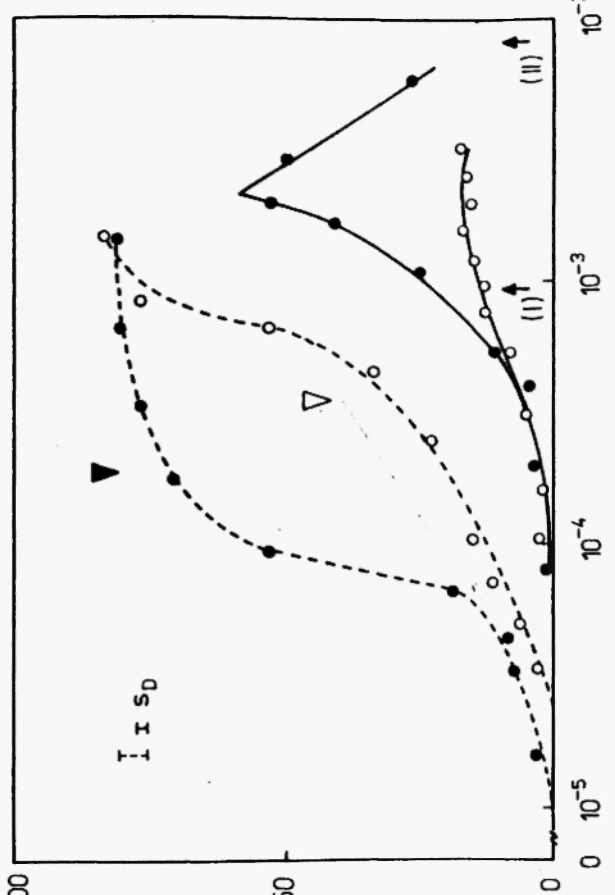


Fig. 2: Leakage of cytoplasmic material from E. coli cells treated with 2-ATDNO (○) and 2-ATDBr (●) for 30 min.
K^+ leakage -----, leakage of 260 nm-absorbing material ————.
Symbols ▽▼ indicate MIC of 2-ATDNO or 2-ATDBr, respectively. Arrows indicate c.m.c.$_2$ values.

leakage of the 260nm absorbing material takes place, with (II) reaches circa 55% in 30 minutes and with (I) circa 20% in 60 minutes (only 14% in 30 minutes) of its total content in cells (concentrations were 1.5-2 mmol/dm^3). Further increase of concentration leads to non-linear relationship A_{260} = f(c) with a minimum at the c.m.c., which is well shown with (II) (Figure 3).

4.2 Effect upon Dehydrogenase Activity

Figure 4 summarizes the effect of surfactants upon INT reduction during the catabolism of substrates (glucose, pyruvate, succinate); the relationship A_{455} = f(c) is non-linear. A week stimulation of INT reduction is seen after the action of (I) at very low concentrations With

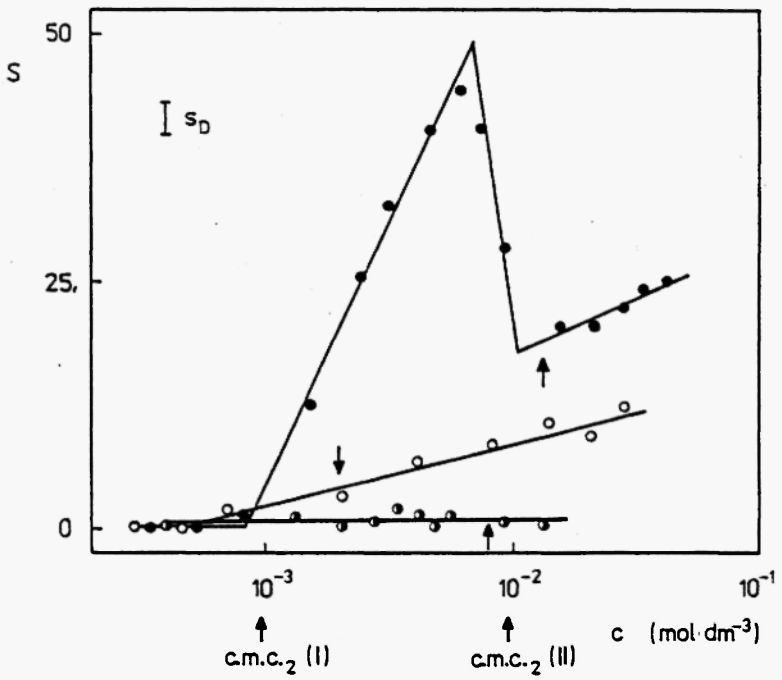


Fig. 3: Interactions of 2-ATDNO (○), 2-ATDBr (●) and SDS (◑) with cells extract's constituent.
Arrows indicate c.m.c.$_1$ values of surfactants.

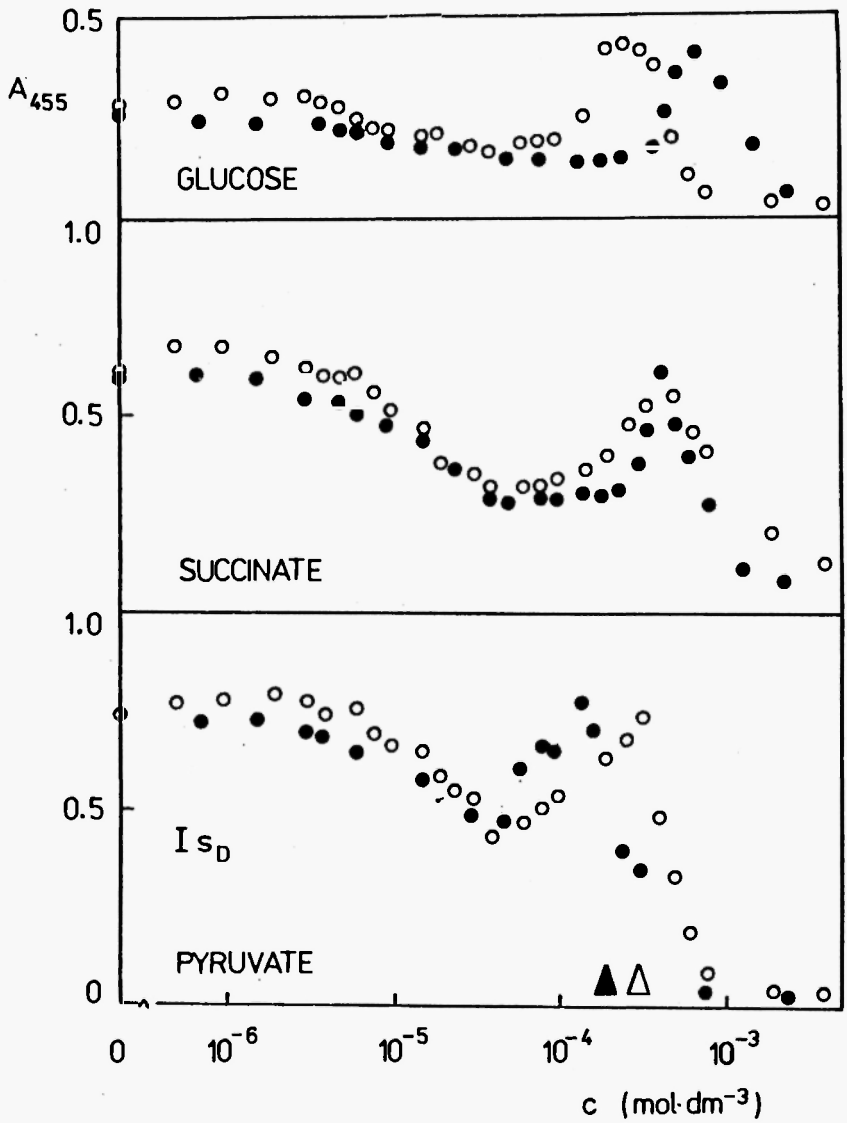


Fig. 4: Effect of 2-ATDNO (○) and 2-ATDBr (●) upon reduction of INT by E. coli cells with using of various substrates.
Symbols △ ▲ indicate MIC of 2-ATDNO or 2-ATDBr, resp.

both substances at subinhibitory concentrations (circa 5 x 10^{-5} mol/dm^3) maximum decrease of this relationship takes place, however by increasing concentration this activity is stimulated with a maximum at the MIC of the tested compounds. The amount of reduced INT with both substances was approximately equal, the differences appeared when different substrates were used. The effect of short-chain analogues (III) and (IV) is shown on Figure 5.

4.3 Effect upon Respiration

The degree of E. coli cells respiration (R) induced by glucose, succinate or pyruvate after exposure to substances was analysed in

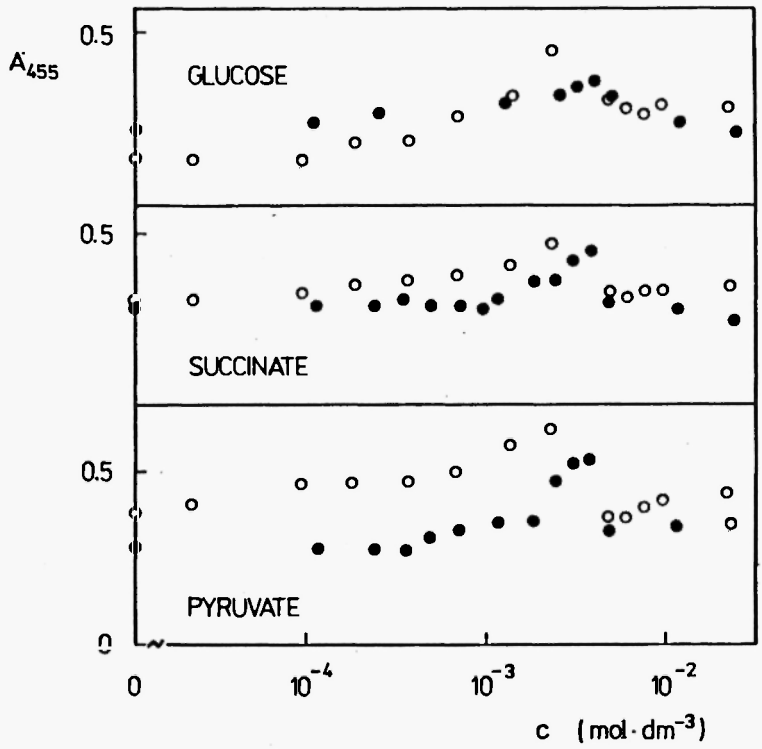


Fig. 5: Effect of short chain analogues TMAO (○) and TMAI (●) upon reduction of INT by E. coli cells.

relation to their concentration. In Table 2 such concentrations of substances are presented, which cause 50% inhibition of respiration (ID_{50}). With glucose, the cells were able to exhibit respiration greater than 50% even in the presence of relatively high drug concentration – in the case of (I) some 20 times higher than with succinate or pyruvate.

The dynamics of respiration inhibition caused by (I) and (II) is different; the rate of amine oxide action is slower.

The short-chain analogues (III) and (IV) do not cause inhibition of respiration even at a concentration of 10 mmol/dm^3 and 60 minutes exposure.

V. DISCUSSION

From results of this study we can infere that the mode of action of quaternary ammonium salts and amine oxides upon bacteria, determined

TABLE 2

ID_{50}/mmol/dm^3/ of substances for Escherichia coli respiration (t=37^{0}C)

Substance	Substrate	τ /min/ 5	10	30	60
(I)	a	>10	6	1.3	0.84
	b	0.56	–	0.45	0.38
	c	0.78	–	0.54	0.40
(II)	a	0.56	0.44	0.28	0.26
	b	0.26	0.22	0.14	–
	c	0.16	0.14	0.11	–
(III)	a	–	–	–	>10
(IV)	a	–	–	–	>10

a – glucose; b – succinate; c – pyruvate

in *in vitro* studies may be considered in three stages: The first stage is characterised by the rate of onset of the action for which the polar interactions of molecules with bacterial membrane are responsible. In the second stage the polar and hydrophobic interactions are involved – this stage means the destructive effect on membrane. The third stage is represented by hydrophobic interactions which lead to death of cells.

The permeability changes of the cytoplasmic membrane caused by action of surface active and antimicrobially effective substances leads to leakage of intracellular material from cells /9,10/, which may by regarded as a consequence of primary damage of the membrane. Damage of the bacterial membrane may also be detected indirectly by following some biochemical processes mediated by membrane-associated enzymes.

Ability of chemicals to cause considerable and immediate leakage of potassium ions from cells is regarded as evidence of modification of membrane structure and function by direct physico-chemical interactions. At the same time specific effects on biochemical reactions catalyzed by enzymes are not necessarily involved /26/.

2-ATDNO and 2-ATDBr cause K^+ leakage from cells already in sub-inhibitory concentrations although in different amounts (Figure 2). This is possibly a consequence of affecting the surface potential of external bacterial membrane, i.e. changes in potential gradient between intracellular and extracellular medium /27-30/ as well as changes of external osmotic conditions takes place. The fact that the negative charge of the membrane is decreased as well as the membrane potential being changed by cationic surfactants was proved on living cells /31/ and on model lipid bilayers /32,33/. The process of K^+ leakage caused by both tensides reflects the dynamics of their action on E. coli cells, because while 2-ATDBr completes this effect at concentrations corresponding to the MIC (0.25 mmol/dm^3) in a relatively short time, this process shows a different course with 2-ATDNO. However finaly the same effect with both substances was reached (Figure 2). This is caused by a difference in the positive charge size on the nitrogen (QUAT $\gg$ NO), which is responsible above all for polar interactions, i.e. for bonding of the corresponding compound on negatively charged constituents of the membrane surface by coulombic interactions (1st stage) and at the same time for faster penetration of the surfactant's hydrophobic tail into the hydrophobic interior of the membrane and damage of its integrity.

Leakage of high molecular weight cytoplasmic constituents has been observed only after such cell membrane damage which leads as a rule to total cell destruction. Helenius and Simon /34/ ascribe this effect caused by some amphiphilic surface active substances to their solubilization effect.

For the leakage of high molecular weight cell constituents, different features in action of (I) and (II), dependent on their concentration, were observed. With the amine oxide, which releases less 260nm-absorbing material than the ammonium salt the concentration causing the maximum leakage always approaches the c.m.c.; the maximum amount depends on time. In case of 2-ATDBr the maximum amount of released material is circa 2.5 times higher than with 2 ATDNO after using concentrations one order lower than the c.m.c.

With both substances, after having reached A_{260} maximum, a temporary absorbance decrease takes place (Figure 2), this being the consequence of the concentration decrease of cytoplasmic material in the analyzed supernatant. This is caused by having reached the concentration at which high molecular weight aggregate formation between the surfactant and 260nm-absorbing material takes place approximately in 1:1 molar ratio and higher, which after a further increase of tenside concentration will be solubilized by known mechanisms /35,36/.

Marked changes in the concentration region 1 to 3 mmol/dm^3 which were observed with 2-ATDBr (moderate with amine oxide) (Figure 3) are again caused by a different size of positive charge localized on the nitrogen atom, which is responsible for polar intermolecular interactions These interactions were proven by measurement of UV-absorbing material at 260 nm in the cell extract after treatment with surfactants. The ability to coagulate the intracellular material decreases in the order: ammonium salt > N-oxide > sulphate, i.e. with a decrease of the positive charge size of the hydrophilic group in the above mentioned surfactants. The relationship correlates well with theoretical assumptions on repeated solubilization of cell constituents after having reached the c.m.c. value. However, certain disagreement exists between concentrations, at which a break in relationship of S = f(c) appears (Figure 3) this disagreement is only apparent. It is known /37,38/ that addition of salts to surfactant solutions causes a decrease in c.m.c. values. And, as we found, also in case of (I) and (II) the c.m.c. value determined in

presence of cell extracts (c,m.c.$_2$) has been shifted to cut-off values (Table 1).

The dehydrogenase activity decrease (Figure 4) with a minimum at a concentration of 0.06 mmol/dm^3, is probably due to bonding of substances on the surface of bacterial cells as a consequence of polar interactions. To eliminate the hydrophobic effects at this process, we tested also the short-chain analogues (III) and (IV) (Figures 1 and 5). Here the concentration increase leads only to a moderate influence upon dehydrogenase activity (at concentration = 4 mmol/dm^3), where the decrease begins as a consequence of osmotic changes. With regard to the hydrophilic character of these substances and their excellent solvation in aqueous solution, the transport processes towards membranes are slowed. This explains also the results obtained by Sarapuk et al. /39/ with a series of alkylcarboxyethylammonium salts on black lecithin membranes for short-chain homologues. From this follows that the first stage of action – i.e. formation of coulombic interactions – is affected by transport of substances to the interface boundary water-membrane, which only such AOS and QUATS (as include in their molecule certain hydrophobic part) are capable. With regard to knowledge about the action of antimicrobial AOS and QUATS /40/, one may say that the hydrophobicity index of the non-polar part must be in region of values corresponding to approximately 6-8 carbon atoms in the alkyl chain to produce an observable effect.

Stimulation of metabolic activity by action of amphiphilic molecules (Figure 4) in the concentration region of 0.09-0.2 mmol per dm^3 means, as in the case of K^+ leakage, the activation of protective mechanisms of the cell, whose gradual degradation begins at a critical concentration, which is for both surfactants practically identical with the MIC. Such activation of defense mechanisms has been observed by Mlynarcik et al. /18/, which was demonstrated by stimulation of ATP synthesis by bacteria using subinhibitory concentrations of amine oxide and ammonium salt. Stimulation of respiration and other metabolic activities by QUATS used in subinhibitory concentrations was observed by different authors mentioned by Hugo /41/. This second stage of action is connected not only with polar interactions, but above all with hydrophobic processes, as was proved also by ESR techniques with model bacterial membranes /7/. The modification of proton-motive forces in membranes of S. aureus cells after action of cetyltrimethyl-

ammonium bromide observed by Denyer & Hugo /42/ can also be explained in this way.

With regard to observed changes in dehydrogenase activity in relation to surfactant concentration and other metabolic activities with bacteria, the assumption rose that also changes in respiration would take place. We found, however, that the respiration of cells is not significantly affected by low concentrations of substances (under the MIC). Its strong decrease takes place only after using higher concentrations of surfactants (mainly in the case of 2-ATDNO). Differences between amine oxide and ammonium bromide appear again from the point of view of quantity and dynamics of action (Table 2). From this it follows that the surfactants in their action do not affect specifically the enzymes involved in these processes, i.e. the observed phenomena are only secondary consequences of primary physical damage of membrane, consisting in consecutiveness of interactions already mentioned.

By the use of three substrates – glucose, succinate and pyruvate (as by dehydrogenase activity examination, so by respiration of native cells) – the following differences were observed. Amount of reduced INT increases in order of glucose $\ll$ succinate $<$ pyruvate. We explain the very low utilization of glucose in comparison to the other two substrates by the catabolic repression (glucose effect) /43/. Respiration of cells is affected to the smallest extent by increasing concentrations of surfactants in the case of glucose, however, with pyruvate and succinate the respiration is more sensitive. This may be caused by the fact that the metabolism of pyruvate and succiante is closely associated with the cytlplasmic membrane which is the site of the surfactants action, while the glycolysis proceeds in the cytoplasm. Utilization of substrates is not influenced by the tested surface active compounds, which means that metabolic pathways of E. coli cells are not specifically affected by these surfactants.

The third stage of action which finally leads to complete lysis and death of cells is related practically to pure hydrophobic interactions, above all to solubilization processes. Because the positive charge of nitrogen at 2-ATDBr is substantially larger that with 2-ATDNO, fast extraction of membrane substructures by QUATS proceeds the solubilization. This process has a great similarity to liquid-liquid phase transfer catalysis, and therefore the minimum bactericidal concentration (MBC) of 2-ATDBr is lower than its c.m.c.. With the amine oxide the

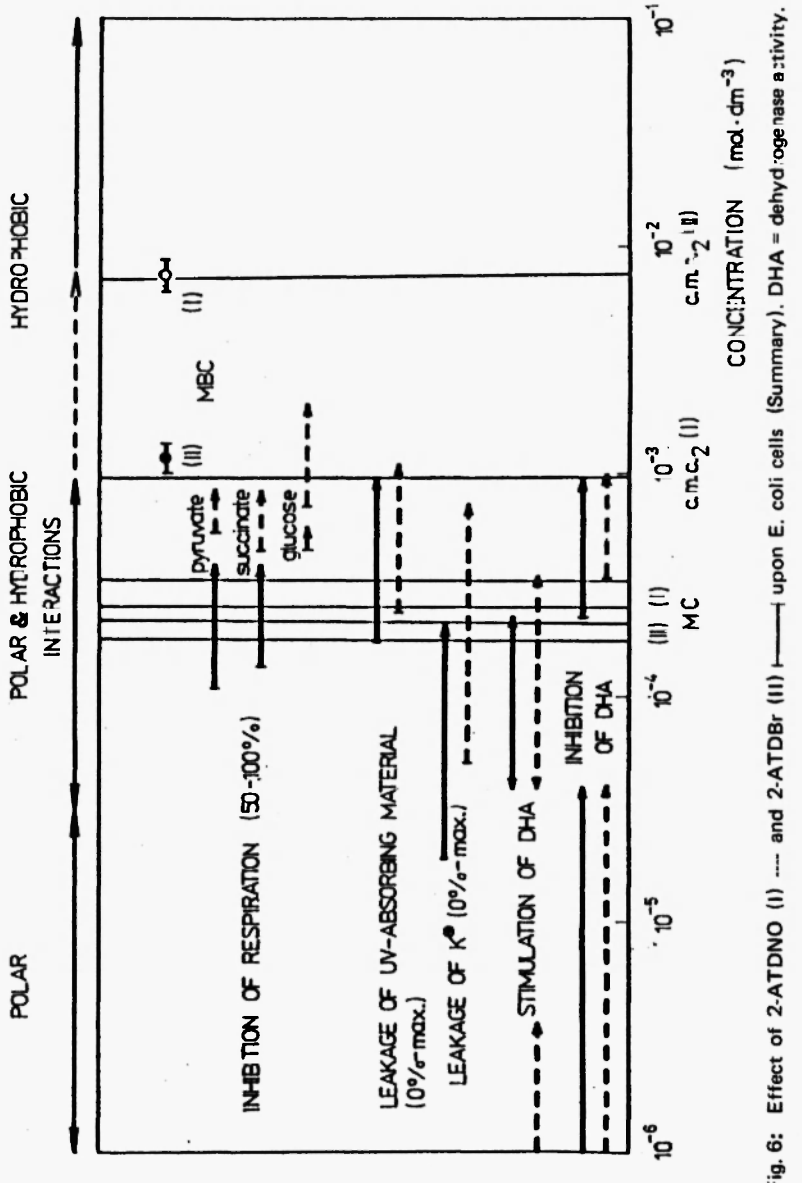


Fig. 6: Effect of 2-ATDNO (I) --- and 2-ATDBr (II) ——— upon E. coli cells (Summary). DHA = dehydrogenase activity.

extraction phase is absent, i.e. the final membrane destruction takes place exclusively by solubilization processes and therefore the MBC value of 2-ATDNO is approximately identical with its c.m.c.

This three stage mechanism as well as the dynamics of action of 2-ATDNO and 2-ATDBr upon E. coli cells are summarized in Figure 6. The first stage of polar (coulombic) interactions represents concentration region of 0.001-0.1 mmol/dm^3, the second stage is affected also by polar, but above all hydrophobic interactions, is down in limits of concentrations of 0.1-1 mmol/dm^3, and the third stage of hydrophobic interactions is in region of concentration higher than 1 mmol/dm3.

Further examination of similar substances (Devinsky et al., in preparation) allows us to say that this mode of action of 2-ATDNO and 2-ATDBr, as a consequence of their interaction with the bacterial cell, is general for the whole group of surface active non-aromatic organic ammonium salts and amine oxides.

VI. ACKNOWLEDGEMENTS

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