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Application of the Ferguson principle to the antimicrobial activity of quaternary ammonium salts

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The activity of three quaternary ammonium salts, dodecyltrimethylammonium chloride, dodecyldimethylethylammonium chloride and dodecylpyridinium chloride, against *M. aureus*, *E. coli* and *C. albicans* has been determined and correlated with the surface properties of these compounds. The Ferguson principle has been applied by using surface concentration to estimate thermodynamic activity. Results obtained for the three quaternary ammonium salts and a particular micro-organism species were found to be in good agreement with each other when microbiological and surface studies were conducted under identical conditions.

SINCE the discovery of the antimicrobial activity of alkylbenzyldimethylammonium chlorides by Domagk (1935), quaternary ammonium salts have received wide recognition as effective germicides. They are effective against Gram-positive and Gram-negative bacteria, as well as a wide range of fungi (Kull, Eisman, Sylwestrowicz & Mayer, 1961). The antimicrobial activity of these compounds has been related to their surface activity, as demonstrated by surface tension lowering (Zissman, 1957), and critical micelle concentrations (Cella, Eggenberger, Noel, Harriman & Harwood, 1952). They have also been reported to exert their effect by altering the microbial cytoplasmic membrane (Hotchkiss, 1946, Gale & Taylor, 1947, Gilby & Few, 1957).

We set out to determine whether the Ferguson principle (Ferguson, 1939) is applicable to these systems when the surface properties of the compounds under consideration are used to obtain thermodynamic activities. To quantitatively correlate surface tension data with antimicrobial activities, these should be assessed in the same conditions as those in which the surface properties are measured otherwise a number of changes in the environment of the antimicrobial agent and of the micro-organism would have to be taken into account. For example, the ionic strength of the two media may be different and affect the surface tension as well as the growth of the micro-organism; changes in temperature, besides having an effect on the micro-organisms, may alter the surface properties of the compound under investigation; an ingredient in a nutrient medium, e.g., protein, may complex with the compound under investigation, thereby either antagonising or potentiating the antimicrobial activity (Few, Ottewell & Parreira, 1955), or altering the surface properties of the compound (Smith, Shay & Doorenbos, 1964). To avoid introducing unnecessary variables, microbial experiments were made in 0·1M potassium chloride solutions at 25°, the same conditions used to measure the surface properties of the compounds under investigation.

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Experimental

Quaternary ammonium salts. Three compounds, each having the same chain length and counterion, but differing in their polar group, were chosen. These were: dodecyltrimethylammonium chloride (DTAC); dodecyldimethylethylammonium chloride (DEAC); and dodecylpyridinium chloride (DPC). The methods of preparation and purification have been reported previously (Weiner & Zografi, 1965).

Surface tension measurements. The surface tensions and critical micelle concentrations of these compounds in 0·1M potassium chloride at 25° were measured by the drop-volume method. Surface concentrations, expressed as molecules/cm^2, were calculated from the Gibbs adsorption equation. The details of these experiments have been reported previously (Weiner & Zografi, 1965).

Choice of micro-organisms. Three species of micro-organisms, purchased from American Type Culture Collection, were used in this investigation. These were: *Micrococcus pyogenes* var. *aureus* 209 (ATCC No. 6538); *Escherichia coli* (ATCC No. 9661), and *Candida albicans* (ATCC No. 752). The cultures were examined microscopically at the conclusion of the investigation, and no apparent contaminants were observed.

MICROBIOLOGICAL TECHNIQUES

Micrococcus pyogenes var. *aureus* 209. A test tube containing 10 ml of nutrient broth (Difco) was inoculated with a 4 mm loopful of culture. This tube was incubated at 37° for 24 hr. Five ml of this mixture was then transferred to a 500 ml Erlenmeyer flask containing 250 ml of nutrient broth. The flask was then incubated in a Gyrotory Shaker at 37°. The incubation was continued until the absorbance of the mixture at 600 mμ was 0·400. This absorbance was found to correspond to a concentration of $3{\cdot}2 \times 10^7$ bacteria/ml. The concentration was determined by the use of standard serial dilution and plating techniques (Burrows, 1959).

Twenty ml of the resulting culture ($3{\cdot}2 \times 10^7$ bacteria/ml) was centrifuged for 5 min, and the supernatant liquid discarded. The discarded liquid was replaced by 20 ml of 0·1M potassium chloride, which was then intimately mixed with the bacteria. The mixture was centrifuged again for 5 min and the supernatant liquid discarded. Enough 0·1M potassium chloride was then added to adjust the concentration to $1{\cdot}0 \times 10^7$ bacteria/ml. 0·5 ml of this mixture was then added to each of a series of tubes containing 4·5 ml of 0·1M potassium chloride; one was control, the others contained various concentrations of the three quaternary ammonium compounds. The contents of each tube were mixed and the tubes were placed in a constant temperature water-bath at 25°. After 15 min, 0·1 ml of each mixture was added to a tube containing 9·9 ml neutralising medium (lecithin, 0·5; polysorbate 80, 3·0 g; nutrient broth, 100 ml). This medium neutralises the antimicrobial activity of quaternary ammonium compounds without significantly affecting the growth of the

organism (Kohn, Gerschenfeld & Barr, 1963). A tube containing about 10 ml of melted nutrient agar (Difco) at 40° was then inoculated with 1 ml of each neutralised mixture. The contents of each tube were mixed and poured into petri dishes. The dishes were incubated at 37° for 24 hr and the number of colonies in each plate was counted. All experiments were made in duplicate.

Escherichia coli. The same technique was used for *E. coli* as for *M. aureus* with the following exceptions. One ml, instead of 5 ml, of the mixture was transferred to a 500 ml Erlenmeyer flask containing 250 ml of nutrient broth. An absorbance of 0·400 at a wavelength of 600 mμ corresponded to a concentration of $9{\cdot}2 \times 10^7$ bacteria/ml. However, the final concentration was also adjusted to $1{\cdot}0 \times 10^7$ bacteria/ml.

Candida albicans. The same technique was used for *C. albicans* as for *M. aureus* with the following exceptions. Sabouraud agar and broth (Difco) were used, the incubation temperature was 25°, and 10 ml of the mixture was transferred to a 500 ml Erlenmeyer flask containing 250 ml of Sabouraud broth. An absorbance at 600 mμ of 0·400 corresponded to a concentration of $1{\cdot}1 \times 10^7$ organisms/ml; the final concentration was adjusted to $1{\cdot}0 \times 10^7$ organisms/ml.

All experiments were made during the logarithmic growth phase of the micro-organism, as previously determined by standard procedures (Burrows, 1959).

Since the antimicrobial activities of the quaternary ammonium compounds were tested in 0·1M potassium chloride solution, it was necessary to determine the effects of this salt solution on the growth of the micro-organisms. Broth-free micro-organisms were inoculated into 0·1M potassium chloride solution and the concentration of the micro-organisms determined at 15 min intervals by standard serial dilution and plating techniques (Burrows, 1959). Over a 30 min interval, potassium chloride did not kill any of the micro-organisms.

Results and discussion

The number of colonies per plate for each concentration of quaternary ammonium compound tested is shown in Table 1. The lowest concentration of each compound that resulted in less than 10 colonies per plate was termed the "minimum effective concentration" (MEC) for that particular organism. This corresponds to a concentration that kills at least 99·9% of the micro-organisms.

The surface activities of the three quaternary ammonium compounds tested are in the same order as the activities against the micro-organisms tested, i.e., DPC>DEAC>DTAC. The interfacial tensions, as well as the corresponding surface concentrations of the quaternary ammonium compounds tested, are compared with their minimum effective concentration values in Table 2. A comparison of these values for the quaternary ammonium compounds for each species of organism, with surface tension, supports Zissmann's findings (1957) that solutions having equal antimicrobial activity have surface tension values of the same order of magnitude. Similarly, solutions having equal antimicrobial activity against a

TABLE 1. ANTIMICROBIAL ACTIVITY OF QUATERNARY AMMONIUM SALTS

Micro-organism	Concentration (molarity) of quaternary ammonium compound	Number of colonies/plate					
		DPC		DTAC		DEAC	
M. aureus	$1{\cdot}0 \times 10^{-4}$	+	+	+	+	+	+
	$1{\cdot}5 \times 10^{-4}$	7	9	+	+	+	+
	$2{\cdot}5 \times 10^{-4}$	0	2	+	+	+	+
	$3{\cdot}0 \times 10^{-4}$	0	0	+	+	0	0
	$4{\cdot}0 \times 10^{-4}$	0	0	+	+	0	0
	$5{\cdot}0 \times 10^{-4}$	0	0	10	5	0	0
	Control	+	+	+	+	+	+
E. coli	$7{\cdot}5 \times 10^{-5}$	+	+	+	+	+	+
	$1{\cdot}0 \times 10^{-4}$	12	7	+	+	+	+
	$1{\cdot}5 \times 10^{-4}$	0	1	+	+	+	+
	$2{\cdot}5 \times 10^{-4}$	0	0	+	+	10	10
	$3{\cdot}0 \times 10^{-4}$	0	0	+	+	0	0
	$4{\cdot}0 \times 10^{-4}$	0	0	0	1	0	0
	Control	+	+	+	+	+	+
C. albicans	$4{\cdot}0 \times 10^{-4}$	+	+	+	+	+	+
	$5{\cdot}0 \times 10^{-4}$	0	1	+	+	+	+
	$6{\cdot}5 \times 10^{-4}$	0	0	+	+	+	+
	$7{\cdot}5 \times 10^{-4}$	0	0	+	+	+	+
	$1{\cdot}0 \times 10^{-3}$	0	0	+	+	+	+
	$1{\cdot}5 \times 10^{-3}$	0	0	+	+	0	0
	$2{\cdot}5 \times 10^{-3}$	0	0	10	3	0	0
	Control	+	+	+	+	+	+

+ Represents 100 colonies or more per plate.

TABLE 2. COMPARISON OF INTERFACIAL PROPERTIES AND THE MINIMUM EFFECTIVE CONCENTRATIONS OF THE QUATERNARY AMMONIUM COMPOUNDS

Micro-organism	Quaternary ammonium compounds	MEC (molarity)	Surface tension (dynes/cm)— Air— 0·1M KCl	Surface concentration $\times 10^2$ (molecules/A^2)— Air— 0·1M KCl
M. aureus	DPC	$1{\cdot}5 \times 10^{-4}$	11·5	1·46
	DTAC	$5{\cdot}0 \times 10^{-4}$	11·4	1·36
	DEAC	$3{\cdot}0 \times 10^{-4}$	9·2	1·25
E. coli	DPC	$1{\cdot}0 \times 10^{-4}$	9·0	1·31
	DTAC	$4{\cdot}0 \times 10^{-4}$	10·2	1·28
	DEAC	$2{\cdot}5 \times 10^{-4}$	8·4	1·19
C. albicans	DPC	$5{\cdot}0 \times 10^{-4}$	19·7	1·83
	DTAC	$2{\cdot}5 \times 10^{-3}$	21·8	1·79
	DEAC	$1{\cdot}5 \times 10^{-3}$	19·1	1·68

particular species of micro-organism have surface concentration values of the same order of magnitude. For example, although there is a four-fold difference in the minimum effective concentration values for DPC and DTAC against *E. coli*, there is less than a 25% difference in any of the corresponding interfacial tension values or surface concentration values. Thermodynamic activities based on critical micelle concentrations and surface concentrations are shown in Table 3. The approach using critical micelle concentrations was previously reported by Ecanow & Siegel (1963), using the data of Cella & others (1952). They considered the thermodynamic activity of a solution that kills 99·99% of the micro-organisms equal to c/CMC, where c is the concentration of the solution, and CMC is the critical micelle concentration. Unfortunately, the microbiological data were not standardised since the time required to kill 99·99% of the micro-organisms tested ranged from 0·35 to 25 min.

TABLE 3. THERMODYNAMIC ACTIVITY NECESSARY FOR 99·9% KILLING OF MICRO-ORGANISMS BY QUATERNARY AMMONIUM COMPOUNDS

Micro-organism	Quaternary ammonium compound	CMC (molarity)	MEC/CMC	$\Gamma_{CMC} \times 10^2$ (molecules/A²)	$\Gamma_{MEC}/\Gamma_{CMC}$
M. aureus	DPC	$2{\cdot}3 \times 10^{-3}$	0·065	2·02	0·72
	DTAC	$7{\cdot}5 \times 10^{-3}$	0·067	1·94	0·70
	DEAC	$7{\cdot}0 \times 10^{-3}$	0·043	1·81	0·69
E. coli	DPC	$2{\cdot}3 \times 10^{-3}$	0·043	2·02	0·65
	DTAC	$7{\cdot}5 \times 10^{-3}$	0·053	1·94	0·66
	DEAC	$7{\cdot}0 \times 10^{-3}$	0·036	1·81	0·66
C. albicans	DPC	$2{\cdot}3 \times 10^{-3}$	0·22	2·02	0·91
	DTAC	$7{\cdot}5 \times 10^{-3}$	0·33	1·94	0·92
	DEAC	$7{\cdot}0 \times 10^{-3}$	0·21	1·81	0·93

The thermodynamic activities necessary for 99·9% killing of the micro-organisms, based on surface concentrations, are expressed as $\Gamma_{MEC}/\Gamma_{CMC}$, where Γ_{MEC} is the surface concentration at the air-0·1M potassium chloride interface at a bulk concentration corresponding to the minimum effective concentration, and Γ_{CMC} is the surface concentration at the CMC The two thermodynamic activities are not necessarily the same since the standard states employed are different. Whereas Γ_{CMC} represents a saturated "surface solution", the CMC may represent an unsaturated bulk solution.

From the results in Table 3 it is apparent that, whereas thermodynamic activities for a particular species of micro-organism based on bulk concentration are not always in good agreement, activities based on surface concentration data are in excellent agreement.

Conclusions

It appears from these results that the Ferguson principle may be applied to soluble surface-active quaternary ammonium salts when surface concentrations are used and when both microbiological and surface studies are made under identical conditions. It would seem that the mechanism of action of these compounds, although complex, depends primarily on a physical relationship between an external phase and the biophase, e.g., the cytoplasmic membrane.

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