THE ANTAGONISM OF THE ANTIBACTERIAL ACTION OF MERCURY COMPOUNDS

PART II. FACTORS AFFECTING THE ACTION OF MERCURIC CHLORIDE

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Increase in the peptone concentration of the medium causes an increase in the amount of mercuric chloride necessary for bacteriostasis whilst an increase in the sodium chloride content decreases it. The age of the organisms is a factor likely to produce variability in the results and the use of a stored suspension of organisms is suitable for reducing this. Suspensions prepared from plate cultures are less satisfactory than those from slope cultures. The number of viable cells in inocula affects the bacteriostatic concentration of mercuric chloride, an increased concentration of bacteriostat being required with an increased number of organisms. The addition of heat-killed cells appears to make no appreciable difference to the bacteriostatic activity of mercuric chloride.

IN Part I[1] the antibacterial activity of mercuric chloride against *E. coli* I was investigated. The relations between mercuric chloride, the medium and the inoculum have now been investigated.

EXPERIMENTAL

Peptone Concentration in the Medium

A series of peptone waters were prepared containing 1·0 per cent of sodium chloride and 4, 3, 2, 1·5, 1 or 0·5 per cent of peptone. A single batch of Oxoid peptone was used and media were prepared and sterilised under the same conditions. The bacteriostatic value of mercuric chloride against *E coli* I was determined by the liquid dilution method[2] with each of the six samples of peptone water simultaneously and with replication.

Plots relating the concentration of mercuric chloride necessary for bacteriostasis with the peptone concentration of the medium were linear, increased peptone concentration being accompanied by an increase in the amount of mercuric chloride required. The line did not pass through the origin but, at a point corresonding to a peptone concentration of nil, cut the mercuric chloride concentration axis at a value of about 5 μM.

Sodium Chloride Concentration in the Medium

The bacteriostatic value of mercuric chloride against the test organism was determined similarly using a series of peptone waters containing 2·0

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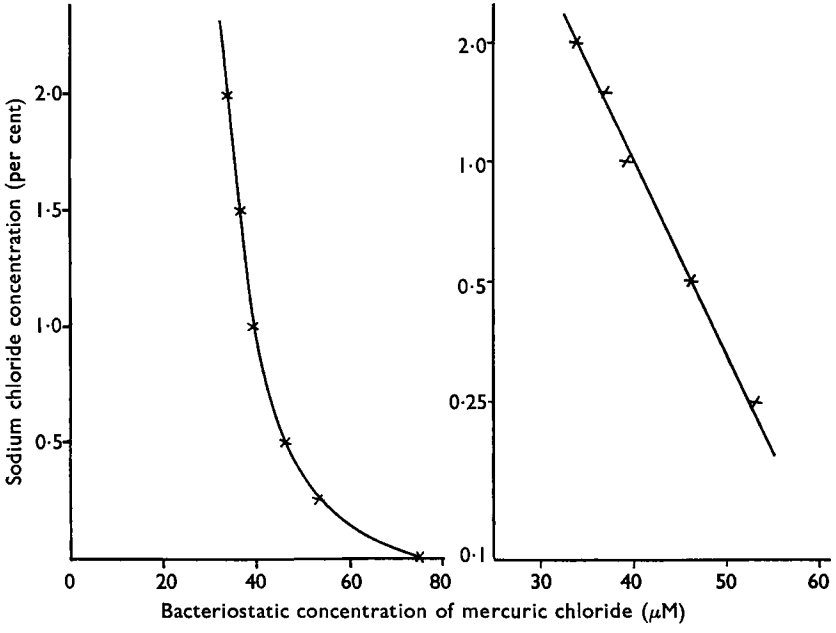
per cent of peptone and 4, 3, 2, 1, 0·5 or 0 per cent of sodium chloride. Figures 1 and 2 show typical results.



FIGS. 1 and 2. Effect of sodium chloride concentration on the bacteriostatic value of mercuric chloride toward *E. coli* I.

Age of the Organisms in the Inoculum

The bacteriostatic value of mercuric chloride was determined by the liquid dilution method using, as inocula, unwashed suspensions prepared from slope cultures of *E. coli* I incubated for varying periods and a stored suspension[3] prepared from 24-hour slope cultures. The number of viable organisms in each inoculum whilst not identical was approximately constant. The results are shown in Table I from which it can be seen

TABLE I

INFLUENCE OF THE AGE OF THE CULTURE OF *E. coli* I UPON THE BACTERIOSTATIC ACTIVITY OF MERCURIC CHLORIDE

Inoculum age (hours)	Bacteriostatic concentration of $HgCl_2$ μM	Inoculum age (hours)	Bacteriostatic concentration of $HgCl_2$ μM	Storage (days)	Bacteriostatic concentration of $HgCl_2$ μM
19	57	24	58	0	60
20·5	57	,,	60	1	60
23·5	60	,,	55	2	60
24·5	60	,,	57	3	60
25	57	,,	65	4	60
28	83	,,	55	5 (Stored suspension)	60

that with the exception of the inoculum prepared from a 28-hour culture, the bacteriostatic values all fall within the range of 55 to 65 μM.

Further experiments showed that stored suspensions of *E. coli* I maintained their resistance to mercuric chloride for at least one week and such suspensions were used for the remainder of the work recorded in this series of papers, except where otherwise stated. Fresh suspensions were prepared at the beginning of each week and were stored at 4° when not in use.

It was noted that suspensions prepared from plate cultures[4] did not maintain their resistance as did those prepared from slope cultures, and typical results for the bacteriostatic value of mercuric chloride against a suspension of *E. coli* I prepared from a plate culture are shown in Table II.

TABLE II

BACTERIOSTATIC VALUE OF MERCURIC CHLORIDE AGAINST A STORED SUSPENSION OF *E. coli* I PREPARED FROM A PLATE CULTURE

Storage (days)		0	1	2	3	4	6
Bacterostatic concentration of $HgCl_2$ as μM solution	Liquid method	75	85	90	95	100	100
	Solid method	150	175	150	175	200	200

Effect of Inoculum Size

Preliminary experiments indicated that there was some relation between the number of organisms in the inoculum and the concentration of mercuric chloride necessary for bacteriostasis, an increased concentration of bacteriostat being required with increased number of cells in the inoculum. Table III shows typical results.

TABLE III

EFFECT OF NUMBER OF CELLS IN THE INOCULUM ON THE BACTERIOSTATIC CONCENTRATION OF MERCURIC CHLORIDE

Expt.	No. of viable cells in inoculum	Bacteriostatic concn. of $HgCl_2$ μM
A	2×10^7	50
	2×10^6	50
	2×10^5	45
	2×10^4	40
B	4×10^7	65
	4×10^6	60
	4×10^5	57
	4×10^4	47
	4×10^3	43

Effect of the Addition of Dead Cells

From the above results it is apparent that the presence of a small number of dead cells in the inoculum would make little or no difference to the concentration of mercuric chloride necessary for bacteriostasis. It was decided therefore to investigate the possible effects of large numbers of dead cells.

A suspension of *E. coli* I was prepared and divided into four portions, one of which was used as the living cell suspension whilst the other three

portions were heat-treated, in different ways, to kill the cells. The heat-treatments used and the results of microscopical examination of the killed cells are recorded in Table IV. The heated suspensions were tested for sterility and no growth occurred in any test.

TABLE IV

METHODS OF KILLING SUSPENSIONS OF *E. coli* I, AND MICROSCOPICAL EXAMINATION OF THE KILLED CELLS

Method of killing	Microscopical examination
Heating at 60° for 1 hour	Rods, with no signs of disruption and very little clumping
Heating at 98 to 100° for 20 minutes	Many of the cells appeared normal but some clumps were present and some cells showed signs of disruption
Heating in an autoclave at 115 to 116° for 10 minutes	Few intact cells present, some cells were visibly swollen and many aggregates of disrupted cells were present

The bacteriostatic value of mercuric chloride against *E. coli* I was determined by the liquid dilution method in two sets of experiments: With inocula containing a constant total number of cells, but varying in the ratio of living and dead cells, and with inocula containing a constant number of living cells and varying numbers of dead cells in the system. Control determinations without added dead cells were performed simultaneously. The suspensions used as inocula were prepared by serial dilution of the living cell suspension with killed suspension or, for the control determinations, with sterile water.

TABLE V

EFFECT OF THE PRESENCE OF HEAT-KILLED CELLS (60°/1 HOUR) ON THE BACTERIOSTATIC ACTIVITY OF MERCURIC CHLORIDE AGAINST *E. coli* I

Number of living cells in inoculum	Percentage of killed cells in inoculum	Bacteriostatic concentration of $HgCl_2$ (as micromolar solution) in		Ratio of B'static concentrations PRESENCE:ABSENCE of killed cells
		ABSENCE of killed cells	PRESENCE of killed cells	
$3{\cdot}5 \times 10^7$	0	78	—	—
" 10^6	90	72	68	0·94
" 10^5	99	60	56	0·93
" 10^4	99·9	46	46	1
" 10^3	99·99	40	40	1
" 10^2	99·999	30	34	1·13
$3{\cdot}6 \times 10^7$	0	80	—	—
" 10^6	90	70	70	1
" 10^5	99	67	67	1
" 10^4	99·9	50	53	1·06
" 10^3	99·99	43	53	1·23
$4{\cdot}1 \times 10^7$	0	83	—	—
" 10^4	99·9	52	47	0·90
" 10^3	99·99	38	43	1·13
" 10^2	99·999	31	32	1·03
$4{\cdot}16 \times 10^7$	0	83	—	—
" 10^4	99·9	48	47	0·98
" 10^3	99·99	36	35	0·97
" 10^2	99·999	30	30	1

Typical results for these two series of experiments are recorded in Tables V and VI respectively. It should be noted that in both Tables the "number" of killed cells is a nominal value based on the count before

heat treatment. The actual number of intact killed cells will be much lower, especially in the autoclaved suspension where microscopical examination showed only a few intact cells (Table IV).

TABLE VI

EFFECT OF KILLED CELLS IN THE SYSTEM ON THE BACTERIOSTATIC CONCENTRATION OF MERCURIC CHLORIDE AGAINST *E. coli* I

Number of live cells in inoculum	"Number" of killed cells in the system					Temperature at which cells killed
	0	$9{\cdot}3\times10^7$	$1{\cdot}86\times10^8$	$4{\cdot}65\times10^8$	$9{\cdot}3\times10^8$	
$9{\cdot}3\times10^6$	72	78 (1·08)	76 (1·06)	78 (1·08)	71 (0·99)	60°
"	72	72 (1)	76 (1·06)	78 (1·08)	78 (1·08)	98–100°
"	72	73 (1·01)	80 (1·11)	79 (1·10)	79 (1·10)	115–116°
$1{\cdot}1\times10^7$	79	86 (1·09)	80 (1·01)	74 (0·94)	76 (0·96)	60°
"	79	75 (0·95)	75 (0·95)	78 (0·99)	82 (1·04)	98–100°
"	79	72 (0·91)	73 (0·92)	71 (0·90)	79 (1)	115–116°

Bacteriostatic concentration expressed as μM mercuric chloride solution.

Figures in parentheses $= \frac{\text{B'static concentration in presence of dead cells}}{\text{" " " absence " " "}}$

DISCUSSION

That the amount of nitrogenous material present in a medium affects the apparent efficiency of mercuric chloride as an antibacterial agent was shown by Baumgartner and Wallace[5]. In bactericidal tests they demonstrated that an increase of the nitrogenous material above that in ordinary broth increased the death time, whilst a decrease in content decreased it; that is, with more peptone in the recovery medium, either more mercuric chloride or a longer exposure time were necessary to produce the same effect.

Under the experimental conditions used, a linear relation existed between the concentration of peptone in the medium and the amount of mercuric chloride necessary for bacteriostasis. Two explanations are advanced to account for these results. Firstly, the increased peptone content may merely serve as a physical protectant for the organisms or, secondly, combination of mercuric chloride with the peptone would be more marked with higher peptone concentrations and this would reduce the antibacterial efficiency of the mercuric salt. Comparison of the behaviour of mercuric chloride with that of other mercury compounds (e.g. an organomercurial salt) with an extended range of peptone concentrations might shed some light on the nature and mechanism of this phenomenon.

It is not known whether the linear relation would hold over a wider range of peptone concentrations or whether it would prove to be exponential. Much smaller concentrations of peptone could be used as

it has been reported that 0·0125 per cent will support the growth of *E. coli*[5].

Paul and Prall[6] were among the first to report the diminution in activity of mercuric chloride in the presence of sodium chloride, this being attributed to decreased dissociation of the mercuric salt. Krahé[7] considered a complex was formed which decreased the concentration of mercuric ions and also decreased the solubility of mercuric chloride in lipids. The formation of complex anions by mercuric chloride is well known, but Gay and others[8] regarded the complex formed with sodium chloride as undissociated. Some workers[9,10] however considered the addition of sodium chloride increased the antibacterial activity of mercuric chloride. Italian workers[10] assumed the complex formed was more active than mercuric chloride alone.

The shape of the graph (Fig. 1) resembles half of a parabola having its vertex at a point corresponding to a mercuric chloride concentration of 75 μM and a sodium chloride content of zero. If however it was half of a true parabola the curve would extend to infinity. It is reasonable to assume that the sodium chloride itself would be inibitory at some concentration and hence the bacteriostatic activity of mercuric chloride under these conditions would be zero and the curve could not extend to infinity. Plotted on a logarithmic axis, the relation is linear (Fig. 2). Extrapolation of this line back to the abscissa gives a value for the sodium chloride content of about 55 to 60 per cent. As the solubility of sodium chloride in water is only about 1 in 3, it is not possible to prepare media having such high sodium chloride contents. If the experiments were repeated with a range of concentrations from 0 to 30 per cent it is believed that the true shape of Figure 1 would be a sigmoid.

From the results however it appears that an increase in the sodium chloride content of the medium increases the bacteriostatic activity of mercuric chloride against *E. coli* I. The presence of 0·25 per cent of sodium chloride greatly enhances the bacteriostatic activity when compared with that in its absence, but the effect of further increasing the sodium chloride concentration up to 2 per cent causes a less marked increase in the bacteriostatic activity of the mercuric salt.

The following explanations are advanced to account for these observations. The increased bacteriostatic efficiency of mercuric chloride in the presence of sodium chloride may be due to the formation of complex anions (e.g. $HgCl_4^{--}$) as mercuric compounds form such complex anions with chlorides of the alkali metals[11]. It is well known that when solutions of two electrolytes having a common ion are mixed, the dissociation of each is diminished. As the bacteriostatic activity of mercuric chloride is increased in the presence of sodium chloride, the theory that the activity of mercuric salts is dependent upon the free mercuric ions in solution[12] appears to be contra-indicated. The apparent increased efficiency of mercuric chloride in the presence of sodium chloride may not be connected with the mercurial compound *per se* but may be due to osmotic effects on the organism, caused by the sodium chloride and rendering the cells more susceptible to the action of mercuric chloride. It is unlikely however

that the relatively low sodium chloride concentrations used in these experiments would exert much influence on the cells by way of simple osmotic pressure effects. A possibly more valid explanation lies in the fact that the cell surfaces of bacteria possess a net negative charge at physiological pH[13]. As the concentration of monovalent cations increases this potential is decreased, although neither monovalent nor divalent cations of the alkaline earth metals can reverse the charge on the bacterial surface[14]. It is believed that with a decreased surface potential the bacteria would be more sensitive to mercuric chloride, and thus the surface potential effect of sodium chloride might potentiate the antibacterial action of mercuric chloride.

Hotchkiss[15] observed that *E. coli* grew in peptone water containing 1·0 per cent of sodium chloride but was inhibited by 2·0 per cent. In our experiments, the organism grew equally well, as determined by turbidity and the time necessary to give a strong indole reaction, in all concentrations of sodium chloride tested, in its absence and in all peptone concentrations tested. Matsuyama[16] reported the growth of *E. coli* in 1 per cent peptone solution was most favourable with sodium chloride concentrations between about 0·58 and 1·75 per cent; increasing inhibition was observed above 1·75 per cent with complete inhibition at about 17·5 per cent.

Table I shows the reduction in variation in bacteriostatic evaluations when a stored suspension from a slope culture is used as the inoculum. Such results support previous work[3].

From the results in Table II it is seen that the sensitivity of a suspension of *E. coli* I from a plate culture to mercuric chloride decreased on storage. A rapid decrease in the viable counts on such suspensions on storage has been shown to occur[17] and it is concluded that suspensions prepared from plate cultures are not as satisfactory as those derived from slope cultures.

A possible explanation of the phenomenon is that in plate cultures, the ratio of surface to depth of the medium is considerably increased over that in a slope culture. Brewer[18] noted an increased resistance to phenol was shown by *Staph. aureus* when the surface area of the medium, on which it was cultured, was increased.

At the outset of this investigation it was hoped to establish a relation between the number of organisms in the inoculum and the bacteriostatic concentration of mercuric chloride, from which conclusions might have been reached about the mechanism of the antibacterial action of mercuric chloride. This correlation was not found although there was a trend for an increase in the number of organisms to be paralleled by an increase in the bacteriostatic concentration of mercuric chloride (Table III). Plots relating the number of organisms in the inoculum with the bacteriostatic concentration of mercuric chloride showed a pronounced scatter. The poor reproducibility of replicate results obscures any relationship, but in a few experiments an approximately linear relation was found between the logarithm of the number of cells in the inoculum and the bacteriostatic concentration of mercuric chloride. During their studies of *Aerobacter aerogenes*, Poole and Hinshelwood[19] demonstrated that for a

given inoculum size there was a critical concentration of mercuric chloride above which no growth occurred, and, conversely, for a given concentration of mercuric chloride there was a critical inoculum size below which no growth occurred.

It was believed that any effect of adding killed cells to a mercuric chloride-bacteria reaction mixture would be one towards increasing the concentration of bacteriostat required, for the following reasons. The dead cells would offer a large competitive surface area for adsorption of the bacteriostat; a similar adsorptive capacity between heat-killed and live cells of *E. coli* has been demonstrated[20]. The dead cells might contribute cell constituents and essential nutrients to the system which could afford protection to the living cells either by adsorption onto the cell surface or by promoting subsequent recovery and growth of cells damaged by the bacteriostat. The dead cells would contribute a large number of sulphydryl groups to the system, if these groups were unaffected by the conditions necessary to kill the cells.

From the results in Table V it is seen that the presence of dead cells in the inoculum makes little difference to the concentration of mercuric chloride necessary to produce bacteriostasis of the living cells. The high value of 1·23, for the ratio of the bacteriostatic concentrations in the presence and absence of dead cells, occurring in the 11th line of the Table appears suspiciously large but, statistically, the means from which this ratio was calculated were not significantly different ($P = 0{\cdot}95$).

From an analysis of variance of the results recorded in Table VI there is no evidence to suggest that either the different heat treatments used in killing the cells or the different concentrations of dead cells in the systems made any significant difference to the amount of mercuric chloride required to produce bacteriostasis of the living cells of *E. coli* I.

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