RESEARCH PAPERS

THE ANTAGONISM OF THE ANTIBACTERIAL ACTION OF MERCURY COMPOUNDS

PART V. QUANTITATIVE ASPECTS OF THE ANTAGONISM OF THE ANTIBACTERIAL ACTION OF MERCURIC CHLORIDE

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Received December 8, 1959

Qualitative experiments previously reported showed dimercaprol to be the most, and thioglycollate the least, efficient antagonist of mercuric chloride. Quantitative experiments reversed this order, but the greater susceptibility of dimercaprol to oxidation is responsible for its apparent lack of efficiency. The recovery of mercuric chloride-treated *E. coli* on media containing an antagonist is affected by the incubation conditions especially when thioglycollate is used as the antagonist. Plots of the percentage of recovered cells against the contact time give different shaped curves for glutathione compared with those for cysteine and thioglycollic acid. Glutathione only inactivates mercuric chloride in the system or adsorbed onto the bacterial cells whereas cysteine and thioglycollic acid, in addition, penetrate the cell and antagonise mercuric chloride within. The incomplete recoveries obtained indicate that the action of mercuric chloride is bactericidal, not bacteriostatic. The rate of kill of *E. coli* by mercuric chloride was logarithmic under the experimental conditions used.

IN the previous work[1] on the antagonism of the antibacterial activity of mercuric chloride by sulphydryl compounds no information was obtained on the number of organisms revived. To obtain such information quantitative work was necessary and experiments involving a counting method appeared to be the most suitable.

The results so far obtained show the action of mercuric chloride on *Escherichia coli* type I to be that of a slow acting bactericide, which is reversible during the initial stages. It was therefore necessary to use methods akin to those for estimating bactericidal activity, which fall into two main groups: (*a*) counting methods, which determine the number of surviving organisms by direct colony counts, turbidimetric or respiratory estimations, and (*b*) end point or extinction methods, which measure the time required for the complete death of an inoculum.

Wills[2] discussed the advantages of extinction methods and regarded them as being more realistic than other methods in assessing the value of disinfectants. As such methods give little or no information on the rate of the killing process a method based upon direct colony counts seemed better. But, the measurement of a percentage mortality yields an estimate of bactericidal efficiency based upon something less than the maximal resistance of the test organism, unlike extinction methods which measure the maximal resistance of the organism to the bactericide.

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Experimental and Results

Dilute suspensions of *E. coli* I were treated with mercuric chloride solution and, after varying contact times, samples of the reaction mixture were dropped on to the surface of overdried plates containing a suitable concentration of antagonist incorporated into the medium. After incubation, the resulting colonies could be counted.

The Effect of Incubation Conditions on the Recovery of E. coli *I Treated with Mercuric Chloride*

It was first necessary to determine whether the recovery of the organisms was affected by either or both of two extraneous factors, (i) the time taken for the drops to be absorbed into the medium and (ii) the length of incubation of the plates; and, if so, to decide upon standard conditions for the later experiments.

By means of a standard dropping needle, 20 ml. of water and 20 ml. of 50 μM mercuric chloride solution were each inoculated with 10 drops of a suspension of *E. coli* I such that the number of viable organisms in the final dilution was about $1–2 \times 10^3$ per ml. The reaction mixtures were maintained at 20°. Immediately after inoculation and mixing, a sample of the aqueous dilution was withdrawn and counted by the surface-viable technique, using 10 drops on each of 10 plates. At 5-minute intervals for the first half hour after inoculation and 10-minute intervals for the next half hour, samples were withdrawn from the mercuric chloride solution and dropped on to the surface of peptone agar containing 10 mM thioglycollate.

Of the ten plates for the initial count and those for each of the contact times, half were incubated at 37° immediately and the remainder were allowed to stand at room temperature until drop absorption was complete, and then transferred to the 37° incubator. Incubation was for 7 days, the number of colonies developing being counted each day.

The effect of the incubation conditions upon the recovery of mercuric chloride-treated cells is shown in Figure 1, where the logarithm of the percentage of recovered cells is plotted against the contact time. This shows an approximately linear relationship in each experiment, but the two lines diverge with increasing contact time.

Slightly higher recoveries occurred when the plates were allowed to stand at room temperature until drop absorption was complete than when they were immediately incubated. This effect was more marked with samples after longer contact times, where the number of colonies developing was much lower. Colonies which had developed within 24 hours were larger on the plates incubated immediately than those on the other plates; on further incubation however there was no appreciable difference in colony size.

The effect of prolonged incubation time on the number of colonies developing on thioglycollate agar is shown in Figure 2. This shows that the number of colonies developing increased over the first 72 hours of incubation, remaining approximately constant on further incubation.

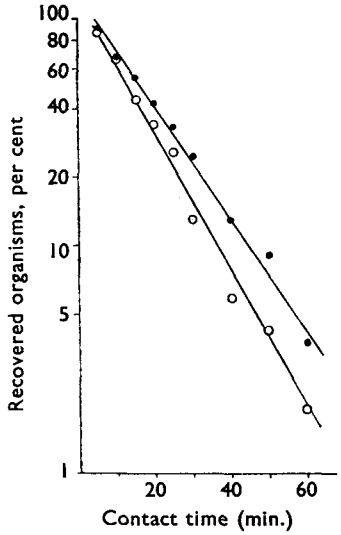

FIG. 1. Effect of incubation conditions on the recovery of mercuric chloride treated *Escherichia coli* developing on thioglycollate agar.

○—○ Plates immediately incubated.
●—● Plates kept at 20° until drops were absorbed.

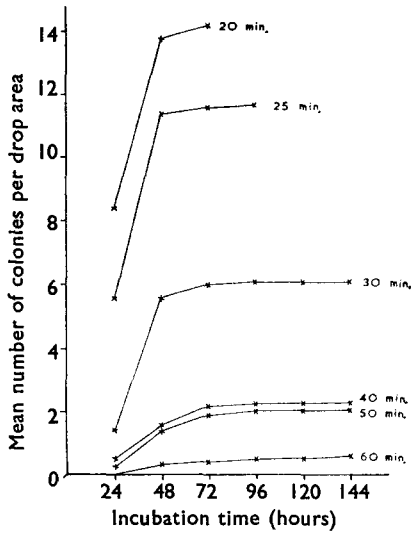

FIG. 2. Effect of prolonged incubation upon mercuric chloride treated *Escherichia coli* growing on thioglycollate agar (6 contact times).

In counting the number of colonies arising in each drop area it was often impossible to repeat the counts after 48 hours. This usually occurred with samples exposed to mercuric chloride for up to 15 minutes, where the number of colonies developing was such as to make growth confluent on prolonged incubation. Repetition of the experiments with a smaller number of organisms showed that the increased count occurring on incubation after 48 hours was negligible with these shorter contact times, and thus any error attached would be small.

With contact times of 20 minutes, or more, the counts obtained after 24 hours incubation bore little resemblance to those obtained after 96, or even 72, hours incubation. In these cases with a smaller number of colonies in the drop areas, the development of one or two additional colonies might make a considerable difference when the results are expressed as percentage recoveries.

The experiments were repeated using peptone agar containing 15 mM cysteine or 10 mM glutathione. Plates incubated immediately at 37° developed fewer colonies, of only slightly if any larger size, than those developing on plates incubated after drop absorption. Neither phenomenon was as marked as with thioglycollate agar.

The counts obtained with cysteine agar were almost complete after 24 hours incubation, with few colonies developing between 24 and 72 hours and none thereafter. The use of glutathione agar resulted in the rapid recovery and growth of the treated organisms, practically no increase in

the colony count occurring after 24 hours incubation. The more rapid growth of mercuric chloride-treated organisms in the presence of glutathione has already been noted[1]. The colonies developed on glutathione agar were much larger than those occurring on cysteine or thioglycollate agar.

Comparison of the Efficiency of the Antagonists in Reviving Mercuric Chloride-treated E. coli *I*

The experiments described above were repeated using peptone agar containing the following concentrations of antagonist, which approximate to the highest concentrations shown[3] to be without adverse effect on the viability of *E. coli* I: cysteine 15 mM, dimercaprol 5 mM, glutathione 10 mM and thioglycollic acid 10 mM. In addition, a second concentration of cysteine (10 mM) was used so that direct comparison of the antagonists at the same concentration could be made. (As the dimercaprol molecule has two sulphydryl groups, a 5 mM solution may be considered as 10 mM with respect to sulphydryl.)

The concentration of mercuric chloride solution used was 50 μM and the inoculum contained approximately 2×10^6 organisms per ml. The reaction temperature was 20°. After sampling and plating, the drops were absorbed at room temperature before transferring the plates to an incubator at 37°. The number of colonies developing on each plate was counted daily and the results were based on the number of colonies present after 4 days incubation.

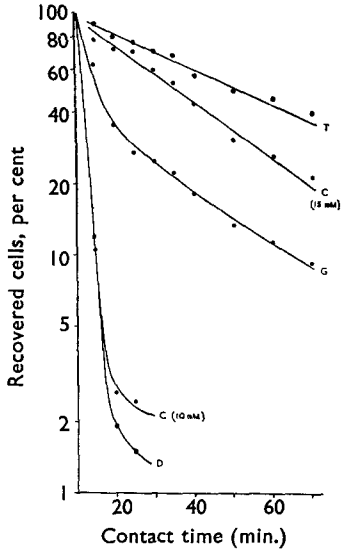

FIG. 3. Recovery of mercuric chloride treated *Escherichia coli* on media containing the antagonists.

T = Thioglycollate.
C = Cysteine.
G = Glutathione.
D = Dimercaprol.

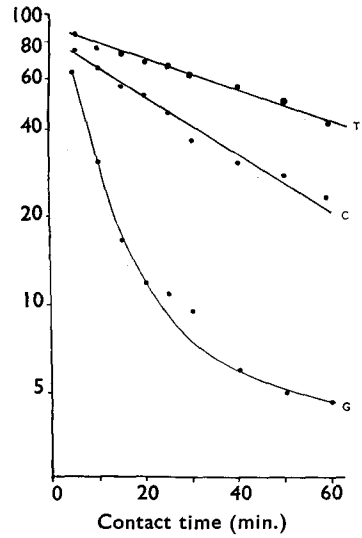

FIG. 4. Recovery of mercuric chloride treated *Escherichia coli* on media containing the antagonists.

T = Thioglycollate.
C = Cysteine.
G = Glutathione.

Typical results are shown in Figure 3, where the logarithm of the percentage of recovered cells is plotted against the contact time.

Media containing the higher concentration of cysteine were found to contain innumerable small particles of cystine, after 24 hours incubation. This tended to make counting difficult, especially with very small colonies; this precipitation did not occur with media containing 10 mM cysteine.

Figure 3 shows that when cysteine (15 mM) or thioglycollate was used as the antagonist, plots of the logarithm of the percentage of recovered cells against the contact time approximated to straight lines, whereas with glutathione as the antagonist an exponential curve was obtained.

Further experiments produced similar results of which Figures 4 and 5 are examples. These are plots of the logarithm of the number of recovered cells against the contact time, or its logarithm respectively. Whilst the plots of Figure 4 closely resemble those of Figure 3, those of Figure 5 are exponential for cysteine and thioglycollate but linear for glutathione.

In experiments of this type where the number of organisms involved is small, the apparent "kill" by the mercuric chloride will be enhanced by the numbers of organisms dying in the aqueous suspension from effects other than those of the antibacterial agent. Control determinations were made in all experiments and these showed that after a period of 1 hour, the number of viable organisms in aqueous suspension varied from 58 to 94 per cent of the original number, with in only one experiment a slight increase (0·5 per cent) in the viable count. The average viability in these controls after 1 hour was about 80 per cent.

Figure 6 shows a typical log survivor : time plot for the aqueous control and a log recovery : time plot for the reaction mixture. In all experiments the slope of the reaction mixture curve was much steeper than that of the aqueous control.

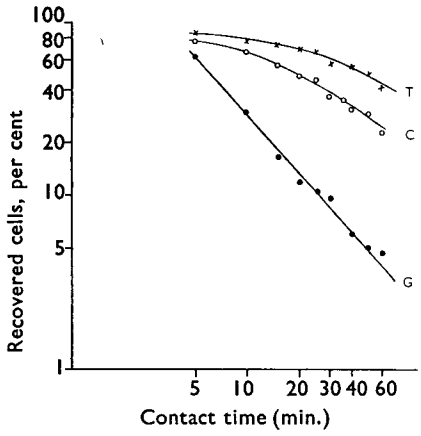

FIG. 5. Recovery of mercuric chloride treated *Escherichia coli* on media containing the antagonists.

T = Thioglycollate.
C = Cysteine.
G = Glutathione.

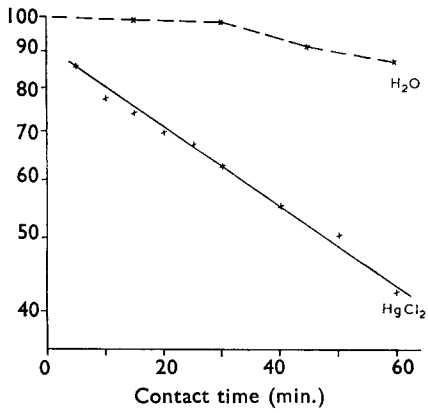

FIG. 6. Effect of mercuric chloride and water on the viability of *Escherichia coli*.

× – – × Aqueous control.
×——× Mercuric chloride reaction mixture.

Discussion

There is little published information on the effects of incubation conditions on the results of a count of organisms surviving the action of an antibacterial substance. Wills[4], investigating the resistance of *E. coli* I to moist heat, found that agar plates kept at room temperature for a time after inoculation before transfer to the incubator gave a lower count than those transferred immediately after inoculation. This effect which was not noted with unheated control suspensions is contrary to our findings. In our experiments, prolonged incubation did not increase the count when there were a large number of survivors, but with smaller numbers the count increased with increase of incubation time. Wilson and others[5] noted a similar effect in plate counts on milk samples.

In attempt to explain the discrepancy in the recoveries obtained by the different incubation conditions, the following points were noted.

If drops of water are placed on the surface of overdried plates containing one of the antagonists, and immediately removed, the liquid gives a positive reaction for sulphydryl. From this it is inferred that some antagonism of mercuric chloride will occur as soon as the treated organisms are placed on the surface of the medium, by diffusion of the antagonist into the drop of reaction mixture.

Drops are absorbed more rapidly at 37° than at 20° and hence the organisms will be in contact with the mercuric chloride for a shorter time at 37° than at 20° before complete absorption ensures intimate mixing with the antagonist.

The antibacterial action of mercuric chloride is more rapid at 37° than at 20°.

Oxidation of sulphydryl compounds occurs more rapidly at 37° than at 20°, and possibly more of the antagonist will be oxidised, rendering it useless as an antagonist, before the treated organisms come into contact with it.

Viable counts of untreated organisms gave identical results whether incubated before or after drop absorption.

From these experiments it is concluded that in work of this nature the absorption of the drops should be allowed to occur at room temperature before the plates are incubated for a period of up to 96 hours, depending upon the particular antagonist used.

From the results shown in Figure 3 it was concluded that neither 5 mM dimercaprol nor 10 mM cysteine in peptone agar were suitable media for the determination of the number of organisms recovering from mercuric chloride treatment. This was believed to be due primarily to oxidation of the sulphydryl compounds rather than to their chemical inefficiency as antagonists. The rapid oxidation of these concentrations of cysteine and dimercaprol when incorporated into solid media has been shown[6], and overdrying of the plates was the main factor in accelerating the oxidation. The oxidation occurring leads to the belief that little if any sulphydryl compound remained to act as an antagonist. In such experiments drops of the reaction mixture on the surface of the medium will still be subjected to the action of the mercuric chloride. Glutathione,

thioglycollate and the higher concentration of cysteine were shown to be less susceptible to oxidation, and this is reflected in their efficiency in allowing the recovery of large numbers of the treated organisms.

From the results obtained it appears that thioglycollate was the most efficient antagonist and reviver of mercuric chloride-treated *E. coli* I. Its main disadvantage is the length of incubation necessary to ensure that the majority of the cells which have been inhibited, are revived and develop.

The revival and growth of mercuric chloride-treated cells caused by glutathione was the most rapid and this might indicate its efficiency as an antagonist. The much smaller number of organisms recovered, however, compared with thioglycollate, point to the fact that antagonism is incomplete.

Cysteine (15 mM) appeared to occupy an intermediate position between the other two antagonists, in both the number of organisms recovered and the time required for their recovery.

The difference in shape of the recovery: time plots (Figs. 4 and 5) for glutathione compared with those for cysteine and thioglycollate may point to some differing mechanism of antagonism. It is known that thioglycollic acid is miscible with both water and many organic solvents whereas glutathione is water-soluble only; cysteine again occupies an intermediate position being soluble in water and some organic solvents.

A possible explanation of the differing antagonistic efficiency of these three compounds may lie in their differing lipid solubility. Glutathione may act by mere chemical combination with mercuric chloride in the reaction mixture and adsorbed on the bacterial surface. Its marked loss of activity after prolonged contact of the organisms with mercuric chloride may be an indication of its inability to antagonise the antibacterial agent which has penetrated the interior of the bacterial cell. Cysteine and thioglycollic acid having some degree of lipid solubility, may be able to enter the cells and antagonise the mercuric chloride which has penetrated.

This is contrary to the theory of Hess and Speiser[7], who believe that the action of mercurial compounds is irreversible once they have penetrated the cytoplasmic membrane.

Qualitative experiments[1] showed dimercaprol to be the most efficient reviver of mercuric chloride-treated cells, and thioglycollate the least. These quantitative experiments have reversed the order.

Consideration of the acid strength of sulphydryl groups raises an important question whether the antagonism of the antibacterial action of mercuric chloride proceeds by molecular or ionic reactions. Danielli and Davies[8] stated that in biological systems, the pH is such that the sulphydryl group is practically unionised. They distinguished two types of sulphydryl compounds: nonionogenic, which are without ionising groups other than sulphydryl and ionogenic, which have ionising groups other than sulphydryl. Of the sulphydryl compounds used as antagonists in this investigation, only dimercaprol is nonionogenic; cysteine, glutathione and thioglycollic acid being ionogenic. Benesch and Benesch[9] reported ionisation of the sulphydryl group of the latter three compounds to occur at pH 7·4 to an extent of 6, 1 and 0 per cent, respectively. If

antagonism proceeds through the mercaptide ion, then cysteine would be expected to be the most efficient.

Until the antagonism of the antibacterial action of mercuric chloride has been studied more fully it is felt desirable to refer to organisms recovered from the mercuric chloride reaction mixtures as "recoveries" rather than "survivors."

Figure 6 showed the divergence between the number of organisms which could be recovered from a reaction mixture compared with those recovered from aqueous suspension. If the action of mercuric chloride was that of a bacteriostat, then the number of organisms recoverable should be the number originally present, less the smaller number which had died in the mixture from other causes. As this was not found to be the case, it implies that mercuric chloride acts as a bactericide towards *E. coli* I rather than as a bacteriostat. On the basis of these observations with only one antibacterial agent and one test organism, it is not proposed to cite this in favour of Price's hypothesis[10] that there is no such thing as a state of bacteriostasis. It is reasonable, however, to assume the revival : time curves obtained are analogous to survivor : time curves of disinfection studies.

If it is assumed that, in the case of thioglycollate, revival of the mercuric chloride-treated organisms was complete, then the results obtained give some information on the bactericidal action of mercuric chloride. For example, under the conditions of the experiments, the rate of kill of *E. coli* I appeared to be logarithmic.

It is possible, however, that other sulphydryl-containing compounds could be found which would produce greater recoveries from the reaction mixtures, and so any conclusions drawn as to the bactericidal activity of mercuric chloride must of necessity only be provisional. For this reason, no attempts have been made to discuss the distribution of resistances of the bacteria which could be deduced from plots of the percentage recoveries against the logarithm of the contact times[11].

Most reports in the literature dealing with revival of mercuric chloride-treated organisms refer to Gram-positive organisms and, even so, few quantitative data are given. McCalla and Foltz[12], however, claimed 100 per cent revival of mercuric chloride-treated *E. coli* after an hour's contact by the use of a sulphide as antagonist. Such high recoveries have not been obtained in this investigation.

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