THE ANTAGONISM OF THE ANTIBACTERIAL ACTION OF MERCURY COMPOUNDS

PART I. THE ANTIBACTERIAL ACTIVITY OF MERCURIC CHLORIDE

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E. coli I was the most resistant of nine organisms tested to the bacteriostatic action of mercuric chloride. Incubation of peptone water or Lemco broth containing high concentrations of mercuric chloride produced a greyish-black sediment. The inclusion of dextrose in the medium increased the amount of mercuric chloride necessary for bacteriostasis. The bacteriostatic values of the liquid and solid dilution methods differed, and the results of the liquid dilution method could be varied by alteration of the experimental technique. The bacteriostatic activity of mercuric chloride was greater at 37° than at 20°. The presence of culture medium has a protecting action on the organisms. Mercuric chloride prolongs the lag phase of *E. coli* I but its mechanism has not been investigated.

As few quantitative data are available on the antagonism of the antibacterial action of mercury compounds, a study of their antagonism by various sulphydryl-containing materials was undertaken. The mode of action of mercury compounds on bacteria and some of the factors which could affect this were first investigated. Although several mercury compounds, both inorganic and organic, are in use, the one selected for the work described in this series of papers was the simplest, mercuric chloride. Preliminary experiments showed that of the nine organisms used by Cook and others[1], *E. coli* I was the most resistant to the bacteriostatic action of mercuric chloride.

EXPERIMENTAL

Mercuric chloride. Analytical reagent was used. Stock solutions were prepared with freshly boiled and cooled distilled water, and stored in the dark. These were assayed by the method of the British Pharmacopoeia 1953. Dilutions for use were prepared when required.

Dropping pipettes. These were of the type described by Cook and Yousef[3]. Four needles were calibrated and the 95 per cent confidence limits of the weight of single drops of water delivered were 17·14 to 17·20 mg., thus the volume of one drop of water from any of the needles may be taken as 1/58 ml. The possible change in drop-weights delivered by the needles after prolonged usage may be neglected since after 24 months use, during which period they had been sterilised numerous times by autoclaving, boiling and dry heat, the confidence limits of a single drop of water delivered from any of the needles were 17·08 to 17·14 mg.

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Media. The liquid medium (peptone water) contained 1 per cent of Oxoid peptone and 0·5 per cent of sodium chloride. It was prepared with water distilled from a stainless steel still, and the reaction of the medium was adjusted to pH 7·2. For some experiments the medium was prepared double strength. The solid medium (peptone agar) was of the same composition, being gelled by the addition of 1·5 per cent of Davis bacteriological agar. The Lemco broth consisted of peptone water fortified by the addition of 1 per cent of Lab-Lemco. All media used during the course of this work was prepared from the same batch of peptone.

Test organism. A laboratory strain of *Escherichia coli* type I, formerly N.C.T.C. No. 5933 was used. It was maintained by the continuous daily subculture method described by Cook and Wills[4].

Methods. The liquid and solid dilution methods for evaluating bacteriostatic activity were used as described by Cook[5].

Both methods were carried out simultaneously in replicate with controls. Typical results for the bacteriostatic value of mercuric chloride gainst *E. coli* I were 70 and 150 μM respectively by the liquid and solid dilution method.

No significant difference in the bacteriostatic value of mercuric chloride was noted between the results obtained by the liquid dilution method when peptone water or Lemco broth was used. At high concentrations of mercuric chloride (about 0·1 per cent) a greyish-black sediment appeared in the tubes of media, both inoculated and uninoculated, after incubation; precipitation occurred to a greater extent with the Lemco broth than with the peptone water.

Comparative determinations of the bacteriostatic value using peptone water with and without 1 per cent of dextrose gave results of 75 and 60 μM of mercuric chloride respectively for the two media.

To keep the systems as simple as possible, without resorting to chemically defined media, peptone water was used as the medium for all the work involving *E. coli* I.

It was noted that by modification of the liquid dilution method considerably different results for the bacteriostatic value of mercuric chloride could be obtained. As usually performed, the liquid dilution method involves mixing double strength culture medium with an equal volume of bacteriostat solution before addition of the inoculum. By addition of the inoculum to the bacteriostat solution and maintaining for a period before adding the culture medium, an apparently much lower concentration of mercuric chloride was required for bacteriostasis. The experiments described below were carried out simultaneously and with replication, with the following results for mean bacteriostatic concentrations. A. The liquid dilution method[5]: 63 μM. B. The inoculum was added to 5 ml. of mercuric chloride solution and allowed to stand at 20° for 1 hour before adding the double strength culture medium and incubating at 37°: 17 μM. C. as in B but keeping the reaction mixture at 37°: 10 μM. D. The inoculum was added to 1 ml. of mercuric chloride solution and allowed to stand at 20° for 1 hour before adding 5 ml. of double strength culture medium and 4 ml. of water, and incubating at 37°: 5 μM. E. As in D but keeping the reaction mixture at 37°: < 1 μM.

In the light of these findings that the bacteriostatic value of mercuric chloride against *E. coli* I varied with the method of test used, the liquid dilution technique for determination of bacteriostatic value was carried out by the published method[5], except where specifically indicated otherwise.

Effect of Mercuric Chloride on the Lag Phase of E. coli *I*

One drop of a 24-hour culture of the organism was added to each of a series of tubes of peptone water containing increasing concentrations of mercuric chloride, and the inoculated mixtures incubated at 37°. The time taken for visible growth to occur was noted and the mean values for six replicates are shown in Table I.

On further incubation the inoculated mixtures all eventually reached approximately the same optical density, except those containing a bacteriostatic concentration of mercuric chloride.

Discussion

The bacteriostatic value of mercuric chloride against *E. coli* I as determined by the solid dilution method was approximately double that by the liquid dilution method. Cook[5] showed a correlation of the two methods for evaluating bacteriostats, but his experiments were conducted with ten-fold dilutions of the bacteriostats and not such close concentrations as used here.

The reason for the discrepancy in the values obtained with *E. coli* I by the two methods remains obscure and it is possible that it might not have been noted if a wider range of dilutions had been used. Several explanations appear feasible: (*a*) organisms on the surface of the medium in the solid method are not in such intimate contact with the bacteriostat as they are in the liquid method and hence a higher concentration of bacteriostat may be required for bacteriostasis; (*b*) the work of Bean and Walters[6] on the release of nitrogenous materials from killed cells of *E. coli* which were capable of supporting the growth of living cells may be pertinent in this phenomenon; cells in immediate contact with the bacteriostat in the solid medium may release these materials which serve to increase the resistance of cells in not such close contact with the bacteriostat; (*c*) combination of mercuric chloride with constituents of the medium may occur to a greater extent in the solid medium, which could be significant by reducing the effective concentration of mercuric chloride or by rendering essential nutrients unavailable to the organisms; (*d*) the absorption of a drop of liquid medium into the surface of solid medium containing bacteriostat may cause a local decrease in the effective concentration of the bacteriostat in the drop area; (*e*) volatilisation of the mercuric chloride[2] may occur on mixing the bacteriostat solution with the molten peptone agar in the solid dilution method.

The precipitation occurring in peptone water containing mercuric chloride was noted by Hotchkiss[7] and is believed to arise from the reaction of the mercuric chloride with constituents of the medium. Greater reaction with Lemco broth would therefore be expected.

Dextrose has been reported to be without effect upon the antibacterial activity of mercuric chloride[8]. The decrease in the bacteriostatic activity of mercuric chloride observed in the presence of dextrose can be simply explained on the grounds that inclusion of a fermentable carbohydrate provides a more nutrient medium for the growth of the organisms and one in which they are less susceptible to adverse conditions. The efficiency of mercuric chloride is increased by a reduction in pH[9]; fermentation of dextrose will reduce the pH of the medium to about pH 4 to 5 which should increase the activity of the mercuric salt. That more mercuric chloride is required in the presence of dextrose to produce bacteriostasis implies either that mercuric chloride is less active against actively multiplying cells or that the inoculum has increased to a value greater than the critical value for a given concentration of mercuric chloride[10].

Some lowering of the oxidation-reduction potential of the system may occur in the presence of dextrose but this factor was not investigated. No antagonism of the mercuric chloride by the dextrose is envisaged.

TABLE I

TIME TAKEN FOR VISIBLE GROWTH OF *E. coli* I IN PEPTONE WATER CONTAINING MERCURIC CHLORIDE

Concentration of mercuric chloride	Time for visible growth
Nil	2·5 hours
5 μM	2·5 "
10 "	2·6 "
15 "	3·0 "
20 "	3·0 "
25 "	4·4 "
30 "	4·8 "
35 "	5·5 "
40 "	8·0 "
45, 50, 55 "	>8 "
60 " (b'static concn.)	∞

The varied bacteriostatic values for mercuric chloride when determined by different methods have been interpreted as follows.

(i) In methods B, C, D and E the organisms do not have the protecting influence of the medium constituents until after the mercuric chloride has been adsorbed to them.

(ii) In the modified methods, there is no chance of reaction of the mercuric chloride with the medium constituents before the bacteria are "coated" with the mercurial salt, and thus the effective concentration of the mercuric chloride is not decreased until after it has begun to exert its antibacterial action.

(iii) In the absence of the medium in the modified methods, the mercuric chloride can enter into a stable combination with the organisms which is not reversed upon subsequent addition of the medium.

(iv) The increased efficiency of mercuric chloride in methods C and E as compared with that in B and D is merely a function of the temperature at which the reaction mixtures were held; indicating that mercuric chloride is a more efficient bacteriostat at 37° than at 20°.

(v) The increased efficiency of mercuric chloride in methods D and E over that in B and C may be explaind by the fact that the binding of sulphydryl groups by mercury is a second order reaction[11]; from this it is inferred that the speed of reaction decreases with increasing dilution and hence a higher concentration of mercuric chloride is required to produce bacteriostasis in a given time.

The long lag period after the use of mercurial compounds has been noted by many workers. In demonstrating the effect of mercuric chloride

on the lag phase of the organism (Table I) the whole interval between inoculation and the first visible signs of growth is not the lag period, but these results may be regarded as an indication that mercuric chloride prolongs the lag phase or retards the rate of multiplication of *E. coli* I, or both. To show which of these two factors is mainly responsible the experiment could be carried out quantitatively, following the growth by a series of total and viable counts.

In later work, the isolation of a strain of *E. coli* I more resistant to the antibacterial action of mercuric chloride was attempted. The procedure consisted of subculturing the organism daily, or on alterante days, into peptone water containing increasing concentrations of mercuric chloride. The time taken for visible growth to occur increased with increasing concentration of mercuric chloride, but once the organisms had become adapted to growth in a particular concentration of the mercuric salt, further subculture into the same concentration resulted in growth appearing more rapidly.

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