THE ANTAGONISM OF THE ANTIBACTERIAL ACTION OF MERCURY COMPOUNDS

PART IV. QUALITATIVE ASPECTS OF THE ANTAGONISM OF THE ANTIBACTERIAL ACTION OF MERCURIC CHLORIDE

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The stoichiometric relations between mercuric chloride and the sulphydryl antagonists have been examined. In liquid cultures, cysteine, glutathione, dimercaprol and thioglycollate are effective as inactivators of mercuric chloride in quantities close to the theoretical amounts. When used to revive mercuric chloride-treated cells, larger amounts are needed, and horse serum is ineffective. The results obtained with *E. coli* I suggest it is not so resistant to the action of mercuric chloride over long periods as are Gram-positive organisms. Cells treated with mercuric chloride in the presence of a nutrient medium derive some protection from the constituents of the medium. Qualitative experiments show dimercaprol to be the most efficient antagonist and thioglycollate the least.

THE first recorded use of an antagonist was by Geppert in 1889 who used ammonium sulphide to overcome the activity of mercuric chloride. Despite this early work, the literature abounds with reports of the antibacterial activity of mercury compounds in which no antagonist was used, and it is advisable to consider such work with caution. Most workers have used either sulphides or sulphydryl compounds, but sometimes other substances have been used. The introduction of a medium containing thioglycollate for the cultivation of anaerobes[1] also did much to encourage the use of antagonists for the inactivation of mercurial compounds.

EXPERIMENTAL AND RESULTS

Volumetric estimations were made to determine the combining ratios of mercuric chloride with the antagonists, used in Part III[2]. The antagonist solutions were freshly prepared and adjusted to pH 7, with the exception of dimercaprol solution, and each solution was standardised by titration with potassium iodate solution[3]. The antagonist solution was titrated with mercuric chloride solution, using a freshly prepared ammoniacal solution of sodium nitroprusside as indicator. From these results the ratio of the number of molecules of antagonist reacting with one molecule of mercuric chloride was calculated as follows. Cysteine hydrochloride 2·004, glutathione 2·043, thioglycollic acid 2·023, and dimercaprol 1·007. Thus, within the limits of experimental error, it may be stated that one molecule of mercuric chloride reacts with two sulphydryl groups.

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In the first series of experiments to demonstrate antagonism, the procedure was to inoculate peptone water mixtures containing a known bacteriostatic concentration of mercuric chloride and varying concentrations of the antagonists with *E. coli* I and to determine, after incubation, whether bacteriostasis had been achieved. Simultaneously, an accurate determination of the bacteriostatic value of mercuric chloride was made.

In mixtures containing dimercaprol or serum, both of which produced an opacity or precipitate, the indole reaction was used to determine the presence or absence of growth.

Full tables of replicated results for the five antagonists are available but have not been reproduced here. A typical set of results for one

TABLE I

THE EFFECT OF GLUTATHIONE ON THE BACTERIOSTATIC VALUE OF MERCURIC CHLORIDE AGAINST *E. coli* I

Mercuric chloride (μM)	Glutathione (μM)							
	0	10	20	50	100	200	300	400
60	−	−	+	+	+	+	+	+
90	−	−	−	−	+	+	+	+
120	−	−	−	−	−	+	+	+
150	−	−	−	−	−	+	+	+
200	−	−	−	−	−	−	+	+
250	−	−	−	−	−	−	−	+

+ = growth, − = no growth

antagonist and only one replicate is shown in Table I; the bacteriostatic concentration of mercuric chloride against *E. coli* I under the same experimental conditions was 55 μM.

From these results, the following deductions were made. Bacteriostasis is expected if the concentration of mercuric chloride is 55 μM or more, and where growth occurs, the effective concentration of mercuric

TABLE II

NUMBER OF MOLECULES OF THE ANTAGONIST WHICH ANTAGONISE ONE MOLECULE OF MERCURIC CHLORIDE

Antagonist	Not antagonistic	Minimum antagonistic
Cysteine	1·7	2·1
Dimercaprol	0·7	1·1
Glutathione	1·9	2·1
Thioglycollic acid	1·9	2·2

chloride is presumed to have been reduced below 55 μM. From a knowledge of the concentration of mercuric chloride and of antagonist in each tube, some approximate value for the combining ratio of the two substances can be calculated. Thus, with 90 μM mercuric chloride, growth occurred in the presence of 100 μM glutathione and so the glutathione has reduced the effective concentration of mercuric chloride by, at least, 90 − 55 = 35 μM, hence one molecule of mercuric chloride has been antagonised by 100 ÷ 35 = 2·86 molecules of glutathione. Similarly, with 90 μM mercuric chloride, no growth occurred in the presence of 50 μM glutathione and so the effective concentration of mercuric

chloride had not been reduced by 35 μM and one molecule of mercuric chloride was not antagonised by 1·43 molecules of glutathione. The results of such calculations for all replicates and concentrations of the antagonists are given in Table II. With horse serum, the concentrations necessary to reduce the effective concentrations of mercuric chloride below their bacteriostatic value were not as constant in replicate experiments as with the sulphydryl compounds. Serum, 20 to 25 per cent, reduced the mercuric chloride concentration by about 100 μM, 10 to 20 per cent by about 60 to 70 μM and 5 to 10 per cent by about 30 to 40 μM.

Dimercaprol showed little antagonistic activity when mixed with the peptone water before addition of the mercuric chloride solution, but if added after the mercuric chloride was present there was good antagonism. This is possibly related to some reaction of the dimercaprol with the medium, the reaction of dimercaprol with proteins having been reported[4]. With glutathione, the results read after 24 hours incubation were usually unchanged by further incubation. With the other antagonists, however, growth often became apparent only after 72 hours. This is believed to be due to the possible role of glutathione in the metabolism or nutrition of the cells rather than to a quicker antagonistic action.

The experimental results obtained were in reasonable agreement with those obtained by titration of mercuric chloride with the antagonists.

Effect of Adding the Antagonist After the Bacteria have been in Contact with Mercuric Chloride

In the previous experiments the antagonist and mercuric chloride were both present in the system before the addition of the bacteria and hence the antagonist was acting in a true antagonistic manner, that is as an inactivator. It is of more value to know whether the antagonist can act as a reviver of organisms which have been in contact with mercuric chloride and, if so, after what time.

In this second series of experiments, the bacteria were added to peptone water containing varying concentrations of mercuric chloride. After 30 minutes contact at 20°, the antagonist was added, the tubes incubated at 37° for 7 days, and then examined for growth. Calculations as before gave the following mean minimum number of molecules of antagonist required to antagonise one molecule of mercuric chloride. Cysteine 3·2, glutathione 2·3, thioglycollate 3·4 and dimercaprol 1·4. With horse serum, only one revival (or recovery) occurred in the series. In view of its virtual inefficiency as a reviver, even after only 30 minutes contact with mercuric chloride, its use was abandoned.

Recovery of Organisms after Longer Exposure to Mercuric Chloride

In the experiments reported the organisms had been in contact with the antibacterial agent for up to 30 minutes before the addition of the antagonist. Such conditions are unlikely to be obtained in practice, for example in testing samples for sterility. The time during which organisms held in a state of bacteriostasis might be expected to survive was now investigated.

A series of tubes containing 10 ml. of peptone water with varying concentrations of mercuric chloride was inoculated with one drop of a suspension of *E. coli* I (equivalent to about 2×10^7 organisms) and incubated at 37° for 24, 48 or 72 hours. After incubation, tubes containing growth were discarded. From each negative tube, half of the contents were transferred to a sterile test-tube as a control. To the remainder, known amounts of antagonist solution were added, and both tubes were re-incubated. The presence or absence of growth was recorded after a further 7 days incubation.

With concentrations of 50 to 100 μM mercuric chloride and 0·2 to 20 mM antagonist solution, only one revival was obtained. This was with 80 μM mercuric chloride and 0·2 mM cysteine added after 24 hours.

Further similar experiments were made but with the addition of an extra 5 ml. of peptone water after the antagonist had been added. In these experiments only 3 recoveries were noted, one each with cysteine, dimercaprol and thioglycollate. No concentration of glutathione produced any recoveries.

Recovery of Mercuric Chloride-treated Organisms in Aqueous Media

Experiments were now made by adding the test organisms to mercuric chloride solution and the antagonist and culture medium after varying periods of contact. The following methods of adding the antagonist solution and the medium were considered possible. First, by allowing a short period for reaction of the antagonist and the mercuric chloride and then adding the medium. Second, by adding the antagonist solution immediately followed by the medium, and third by adding the medium into which the antagonist had been incorporated. Fildes[5] showed the reaction between mercuric chloride and sulphydryl compounds was practically immediate and so the first method was not used. The third method is an easier procedure, involving less manipulations, but the oxidation of sulphydryl compounds has been shown to occur more rapidly in dilute solution, and the second method was finally adopted.

One drop of a suspension of *E. coli* I was added to each of a series of tubes containing (A) 1 ml. of 500 μM and (B) 4 ml. of 125 μM mercuric chloride solution. The tubes were kept at 20°. At 5-minute intervals up to 1 hour, 1 ml. of antagonist solution was added to replicate reaction mixtures followed by 5 ml. of double strength peptone water and, with (A) only, 3 ml. of sterile water. The tubes were then incubated at 37° for 7 days and examined for growth. The concentrations of antagonists were 10, 5, 2·5 and 1 mM for the monothiols and 5, 2·5, 1·25 and 0·5 mM for dimercaprol. These concentrations corresponded, in the final mixtures, to molecular ratios of sulphydryl to mercuric chloride (—SH : Hg ratio) of 20, 10, 5, and 2 : 1 respectively.

The results showed that recovery always occurred where the —SH : Hg ratio was 10 or 20 : 1 and in most instances where it was 5 : 1. Only dimercaprol and thioglycollate caused any recovery at a 2 : 1 ratio. Bearing in mind the variation in resistance of the organism to bactericides, a similar variation in resistance to bacteriostatic action may account for

the few recoveries obtained with thioglycollate at a 2:1 ratio. With dimercaprol at the same ratio, most of the recoveries occurred in the first 20 minutes of testing and these are significant.

As it was possible to obtain recovery after a one hour contact with mercuric chloride by the use of a sufficiently large excess of antagonist, experiments were made to ascertain the time after which recovery was not possible. The experimental procedure used 5 ml. of 50 or 100 μM

TABLE III

RECOVERY OF MERCURIC CHLORIDE-TREATED *E. coli* I FROM AQUEOUS SUSPENSION, USING THIOGLYCOLLATE AS ANTAGONIST

Percentages of Replicates showing Recovery

Contact time, hours	Mercuric chloride concentration			
	50 μM		100 μM	
	Reaction temp.			
	20°	37°	20°	37°
1	100	100	100	50
1·25	100	100	80	40
1·5	100	80	60	30
1·75	100	30	70	10
2	100	40	90	30
2·5	100	20	70	20
3	100	10	50	15
3·5	90	0	30	20
4	70	0	20	0
over 4	0	0	0	0

mercuric chloride solution, inoculating with one drop of a suspension of *E. coli* I and keeping the reaction mixtures at 20 or 37°. After varying contact times, 5 ml. of double strength peptone water, containing a concentration of thioglycollate equivalent to twenty times the mercuric chloride concentration, was added and the mixtures incubated at 37° for 7 days. (The peptone water-thioglycollate mixtures were freshly prepared.) Table III shows the results, expressed as the percentage of replicates showing recovery.

DISCUSSION

The dual action of inactivation and revival has been discussed, in relation to thioglycollate, by Berry[6] who pointed out that not all inactivators would function as revivers. Comparison of the results obtained when the antagonist was present in the reaction mixture (Table II) with those when it was added later shows that a significantly higher amount of antagonist is necessary for revival than for inactivation.

The poor revival obtained with horse serum suggests its lack of specificity. It is believed that when mercuric chloride is mixed with serum before addition of the bacteria, combination of the mercurial compound with serum proteins occurs which reduces the antibacterial activity. When the bacteria have been in contact with mercuric chloride it is possible that adsorption of the mercury on the cells, or its combination with them, is by bonds of such a strength that the serum proteins are incapable of causing its removal. On the other hand, if reversal is brought

about by sulphydryl groups then serum is of little value as a reviver, as its sulphydryl content is very low[7]. It is possible that serum may be of more value when a more specific sulphydryl-reacting mercuric compound is investigated; Davison[8] found normal horse serum to be a suitable antagonist for phenylmercuric nitrate.

The results obtained for the recovery of organisms after longer exposure to mercuric chloride are not in agreement with reports in the literature on the time organisms can be held in a state of bacteriostasis and still show viability when a suitable antagonist is added. Fildes[5] however reported that *E. coli* could not be revived after 17 hours contact with 2×10^{-6} M mercuric chloride at 38°, but his inoculum was only about 1000 organisms.

The following possible explanations are advanced to account for these results. (i) The organisms of the inoculum were dead by the time the antagonist was added, (ii) insufficient antagonist was added, although in some cases the —SH : Hg ratio was 400 : 1, (iii) nutrients were not available for re-growth of the treated cells; this implies that the combination of mercuric chloride with constituents of the medium was such that the antagonist was incapable of reversal. In experiments of this nature it is not feasible to greatly increase the concentration of antagonist as it might itself have an adverse effect upon the organisms[2].

From the results where additional medium was added after the antagonist, the hypothesis that the medium was "poisoned" by the excess mercuric chloride has not been proved valid. It appears that *E. coli* I is not a suitable organism for experiments of this kind as revival was rarely possible after only 24 hours contact with mercuric chloride. Examination of other organisms should give information whether this phenomenon is peculiar to *E. coli* I or is applicable to other Gram-negative organisms. Most literature reports on the revival of mercurial-treated organisms are confined to Gram-positive ones.

Recovery and growth of injured cells may occur if it is possible for the cells to rid themselves of mercury which has entered into combination with their substance. Wyss[9] believed that cells could recover spontaneously from bacteriostasis produced by limiting concentrations of heavy metals, by the production of more sulphydryl groups which displaced the metal from its attachment on the sulphydryl group in the active site of enzymes or gene proteins. If the organisms are treated with mercuric chloride in the absence of a culture medium, no re-growth will be possible and it is expected that cells held in a state of bacteriostasis will not be revivable, by addition of an antagonist, for as long a period as cells in a nutrient medium.

The experiments show that it is possible to antagonise the effect of mercuric chloride on *E. coli* I after a limited contact time and to cause revival and recovery, by addition of a sulphydryl compound. For recovery, an excess of the sulphydryl compound is required. Under the experimental conditions used, cysteine, glutathione and dimercaprol were effective in ratios of —SH : Hg of 5 or more to 1; thioglycollate was effective at a 10 : 1 ratio. Of these compounds, dimercaprol was the most efficient antagonist and thioglycollate the least. It appears that even the

lowest theoretical concentration of dimercaprol prevented mercuric chloride from exerting its action upon bacteria over a certain time.

Opinions vary on the most suitable antagonist for mercurial compounds, although all agree on the necessity of having an excess of antagonist present. Fildes[5] found the antagonistic action of glutathione was greater than that of thioglycollate, on a molecular basis, whilst Bailey and Cavallito[10] found cysteine to produce more rapid reversal than glycylcysteine, *N*-acetylcysteine or sodium thioglycollate. Brewer[11] showed dimercaprol, mercaptoethanol and sodium formaldehyde sulphoxalate to be no more effective than sodium thioglycollate and Powell[12] reported the reviving power of sodium thioglycollate to be greater than that of dimercaprol, but believed this might be due to decomposition of the latter. Woodbine[13], however, considered dimercaprol and glutathione were more effective than either cysteine or thioglycollate.

The necessity of an excess amount of antagonist over the theoretical quantity may be due to any or all of the following factors. (i) a loss of antagonist by atmospheric oxidation, (ii) the necessity of lowering the oxidation-reduction potential of the system to a level suitable for the growth of the revived cells, (iii) after removal of the mercuric chloride, essential sulphydryl groups contained within the bacterial cells may be in the oxidised state and additional antagonist may be required for their reduction, (iv) the complexes formed between mercuric chloride and the sulphydryl compounds are capable of ionisation[14] and excess antagonist may be necessary to overcome the effects of this ionisation. Sulphydryl antagonism of the antibacterial action of mercuric chloride does not involve competitive inhibition, which is regarded as occurring when two substances, which cannot chemically interact, compete for a common site on an enzyme. It is well known that the antagonists used can combine chemically with mercuric chloride.

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After Dr. Steel presented both papers there was a DISCUSSION.