STUDIES ON REVERSAL OF THE DISINFECTING ACTION OF MERCURIC CHLORIDE UPON *ESCHERICHIA COLI*

by

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TABLE OF CONTENTS

INTRODUCTION 1

REVIEW OF LITERATURE 2

MATERIALS AND METHODS 6

Preparation of Cell Suspensions and Reversal Procedures 6

Procedures for Electron Microscope Studies 9

EXPERIMENTAL 9

Reversal Effect of Thioglycollic Acid on Mercuric Chloride Treated Cells 9

Reversal Effects of Sulfur Containing Compounds Other Than Thioglycollic Acid on Mercuric Chloride Treated Cells 17

Electron Microscope Studies of Thioglycollic Acid Reversed Cells 20

CONCLUSIONS 22

SUMMARY OF EXPERIMENTAL RESULTS 24

ACKNOWLEDGMENT 26

LITERATURE CITED 27

APPENDIX 29

INTRODUCTION

The value of the mercurials as therapeutic agents has been known for centuries. Arabian physicians first used mercury in a finely divided state and as a vapor for the treatment of skin disorders. Before the germ theory of disease was fully established, it was a known fact that mercury salts would prevent sepsis. After the germ theory of disease was fully established, Koch in 1881 popularized the use of mercuric chloride with his famous string experiment using anthrax spores.

From the time of Koch's experiments until the present day, studies have been conducted in an attempt to ascertain the true value of these compounds. A myriad variety of results have been obtained by different workers. Most of the current work in this field has been directed at discovering the mechanism of mercury poisoning of cells. No definite or uniform conclusions have been reached as to its mode of action.

Several recent workers have indicated that the mode of action probably lies in the destruction or inactivation of an enzyme or enzyme-complex necessary for the normal metabolism of the cell. These studies were undertaken to further substantiate this current hypothesis, and to illustrate the effects of time and the effects of temperature upon the reaction.

Previous workers had indicated that the action of mercuric chloride could be reversed under certain conditions. It was intended to attempt duplication of these results, and at the same time demonstrate a time and a temperature dependence for the reaction.

Some observations will also be made concerning cytological changes within the cell after treatment with mercuric chloride and reversal with thioglycollic acid as revealed by electron microscopy.

REVIEW OF LITERATURE

Shortly after the germ theory of disease was fully established, Koch (1881) popularized the use of mercuric chloride as a disinfectant with his famous string experiment using anthrax spores. He showed that great dilutions of mercuric chloride would kill the spores of the most resistant organisms. Geppert (1889) showed that inorganic mercurial salts possessed very great bacteriostatic properties but that they were actually very poor germicides. With the aid of animals and cultural experiments, Moll (1920) demonstrated that mercuric chloride could not be depended on to kill tetanus spores.

Much of the work done with mercuric chloride not only attempted to ascertain the effectiveness at different concentrations, but also attempted to explain the mechanism of action of mercuric chloride. It had been known for some time that mercuric chloride combined with proteins, precipitating a mercury-proteinate. One of the first ideas concerning the mechanism of action was that the bacterial protein was precipitated because of a direct combination with the mercury. Smith (1869) stated that sepsis was prevented because the mercury combined directly with the flesh, making a permanent compound and keeping out the

contaminating air. Geppert (1889) stated that mercuric chloride formed a chemical bond with the cell but could be broken by converting the mercury to the sulfide. Kronig and Paul (1897) discussed the possibility that the action of mercury in solution was due to the free ions present and not to the molecular concentration.

Engelhardt (1923) stated:

> Mercuric chloride is absorbed by the outer membrane thereby preventing reproduction, and can usually be removed by prolonged washing of the bacteria with water, or, where treatment with mercuric chloride has been more vigorous, by treatment with absorbents such as charcoal, or by decomposition of the mercuric chloride by the action of sulfides. The suggestion that sulfides decompose a mercury-protein compound formed in the interior of the bacterial cell is not feasible. The action of sulfides, like that of charcoal, merely removes adsorbed mercuric chloride. _Staphylococci_ which have been treated for 8 hours with 0.1 percent, or for 2 hours with 1.0 percent solutions of mercuric chloride, and anthrax spores which have been treated at 18 degrees for 14 days with 5.0 percent solutions show signs of life after washing with water; _Staphylococci_ which have been treated for 72 hours with solutions can be rendered capable of reproduction after treatment with sulfides; while anthrax spores which have been treated at 37 degrees for 35 days with 0.1 percent, 25-33 days with 1.0 percent, 13-20 days with 3 percent, or 11-17 days with 5.0 percent solutions, can be revivified by treatment with charcoal or sulfides. Mercuric chloride therefore cannot be described as a disinfectant with a rapid action.

It is well known that enzymes may be reversibly precipitated by heavy metal salts. Duclaux (1900) reasoned that chemical disinfection was a matter of destruction of bacterial enzymes, without which, the bacteria were unable to multiply. This theory is in agreement with the fact that organisms treated with mercuric chloride can be revived with certain neutralizing agents.

Krueger and Baldwin (1934) proposed that the action of

mercuric chloride was two fold. The first action was a preliminary stunning of growth function. The second action then was lethal due to increased time of exposure and increased concentration of mercuric chloride. They found that reversal could be readily effected if the cells were treated with a neutralizing agent while in the first phase of action but if the cells were in the second phase of action, reversal could not be effected.

Fildes (1940) stated:

> Certain enzyme reactions can be specifically poisoned by various reagents in a manner which is not clearly understood. It might be due to a specific poisoning of some essential reaction exerted through an interference with the catalyst or essential metabolite. It might combine with essential metabolite and form an inert product or it might compete with the metabolite for the enzyme involved. The addition of mercury salt to a bacterial suspension leads to disappearance of intracellular -SH groups. These groups are essential and without them growth cannot occur. The antibacterial action of mercury might be ascribed to its interference with essential metabolite R-SH. Within limits, the anti-bacterial action of mercury is reversible by -SH containing compounds as well as by hydrogen sulfide. It is concluded that the action of mercury is merely to inactivate the -SH groups without other demonstrable injury to the cell.

McCalla (1940) stated that a bacterial cell possessed approximately 10^8 active sites for adsorption of cations. He suggested that these active spots or sites might account for the presence of a cation adsorption complex in a living cell. He not only showed that bacterial cells adsorb cations but also that the adsorbed cations are exchangeable. He studied the reversal effects of hydrogen sulfide on mercuric chloride treated cells and presented data indicating that he was able to achieve as great as 90 percent reversal.

Selzer and Baumberger (1942) studied the influence of metallic mercury on the respiration of cells. They proposed that a cell was able to restore its enzymatic system by the fresh formation of -SH groups rather than by reduction of the mercury-S-S complex formed when mercury was present. The inhibitory effect of mercury then was the removal of -SH groups exceeding their formation leaving no -SH enzyme system for cell function.

Valko and Dubois (1944) studying the antibacterial action of surface active cations, including mercuric salts, showed that the killing action of surface active cations on bacterial cells could be reversed, under certain conditions, by detoxication with a high molecular weight anion. These workers were in agreement with others that this was a phenomenon of ionic exchange by bacterial cells. Cavillito (1945) postulated that mercuric chloride acted by reacting with -SH groups of enzymes and that the differences in antibacterial action of other agents was dependent, among other factors, upon the ability of the agents to come into contact with the essential -SH groups. Klein et al (1948) noted that analysis of amino acids of influenza A and B showed no presence of any amino acid containing -SH. This is of interest because the inactivation of influenza virus by mercuric chloride could be reversed in vitro with sodium thioglycollate.

Harris et al (1954) found that adsorbed mercuric ions did not affect the electrophoretic mobility of treated cells, and interpreted these results as direct evidence that the site of adsorption was some surface other than the external layer in contact with the suspending medium. This interpretation agreed with

their electron microscope observations. The conclusion reached by these workers was that the site of cation adsorption was the cytoplasmic membrane of the cell.

MATERIALS AND METHODS

Preparation of Cell Suspensions and Reversal Procedures

The organism used throughout these studies was an *Escherichia coli* culture obtained from American Type Culture Collection (#6877). The organism was grown on nutrient agar composed of the following: 8.0 grams sodium chloride, 3.0 grams beef extract (Difco), 5.0 grams peptone (Difco)., 15.0 grams agar agar and 1000.0 ml. distilled water. The pH of the medium was adjusted to 7.0. This medium was used for preparation of stock culture slants as well as being the basal layer for the plating experiments.

An actively growing culture of *E. coli* was used in each experiment, the organism having been transferred to a fresh slant and incubated at 37° C. for 18 hours. A cell suspension was prepared by washing the growth from the slant with 5.0 ml. of 0.1 molar sodium acetate of pH 6.8. This suspension was adjusted to a final concentration of $1 \times 10^3 - 1 \times 10^5$ cells per ml. in sodium acetate, using photometric procedures. All experimental work employed a stock suspension of cells prepared in a similar manner.

A solution of 1×10^{-5} molar mercuric chloride was employed as the killing agent throughout these experiments. The reversal agents employed were cystine, cysteine, sodium thioglycollate,

tartaric acid, sodium sulfide, thioglycollic acid, sodium bicarbonate and hydrogen sulfide. These solutions were all prepared in concentrations of 0.1 molar with the exception of hydrogen sulfide, which was used as a gas. All solutions were stored at 5° C.

The experimental procedure consisted of setting up five sterile 125 ml. Ehrlenmeyer flasks, to each of which was added 8.0 ml. of the cell suspension. To the first flask (A) which was to serve as the untreated cell control was added 4.0 ml. of 0.1 molar sodium acetate. To the second, third and fourth flasks (which were labeled B, C, and D respectively) was added 2.0 ml. mercuric chloride solution. To the fifth flask (E) was added 2.0 ml. mercuric chloride and 2.0 ml. sodium acetate. Flask E was the mercuric chloride killed cells control. The flasks were then incubated at the temperature appropriate to the experiment. Five experimental temperatures were used: 5° C., 25° C., 30° C., 37° C., and 45° C. After five minutes of incubation flask B was removed and 2.0 ml. of reversal agent was added and the flask reincubated. Similarly, after ten minutes flask C was removed and 2.0 ml. of reversal agent added and the flask reincubated, and after thirty minutes flask D was removed and 2.0 ml. of reversal agent added and the flask reincubated. Flasks B, C, and D were allowed to incubate for a total of thirty minutes after the addition of the reversal agent, after which time samples were taken from each flask for determining the number of viable organisms present. Samples were taken for plating from flask A at time zero (the time at which the flasks were placed in the incubator

for the first time), and thirty minutes. Samples were taken for plating from flask E at five, ten and thirty minutes.

The plating procedure followed was to make three serial 1:10 dilutions of the sample to be plated in sterile distilled water. From each one of these dilutions 0.1 ml. was pipetted onto the surface of a dried nutrient agar plate on a rotary turntable and spread with a sterile glass rod. The spread plate technique was employed because surface colonies appear more rapidly than sub-surface colonies, and contaminants can be readily separated from the culture organism. Also, in preliminary comparisons between poured plates and spread plates, duplicate counts of spread plates were in closer agreement than poured plates. All platings were done in duplicate. The plates were incubated 24 hours at 37° C. after which time the plates were examined, and those having 30 - 300 colonies were counted and recorded.

When hydrogen sulfide was used as the reversing agent it was bubbled for five minutes into the appropriate flasks. Bubbling was accomplished by means of sterile connections between the flasks and a hydrogen sulfide tank.

An experiment using fluid thioglycollate medium without dextrose (Difco) as a reversal agent was also performed. This differed from the previously described experiments in that 1.0 ml. of the mercuric chloride treated cells was added directly to a tube of fluid thioglycollate medium and immediately serially diluted 1:10 through nine tubes of fluid thioglycollate medium. These tubes were incubated at 37° C. for 24 hours, after which time the tubes were examined. Results were recorded as growth or no growth.

Procedures for Electron Microscope Studies

The same experimental procedure was followed as outlined previously for the preparation and treatment of cells. At the end of the 30 minute incubation period following the addition of the reversal agent, the contents of each flask were removed to centrifuge tubes and centrifuged. The cells were washed three times in distilled water, and then following the standard procedures for preparation of collodion films for electron microscopy, each of the samples was photographed under 90,000 x magnification.

EXPERIMENTAL

Reversal Effect of Thioglycollic Acid on Mercuric Chloride Treated Cells

Considerable thought was given to the choice of organisms to be used in this experiment and the other experiments in this thesis. *E. coli* was chosen because it is easy to maintain and grow in large quantities and results can be tabulated after short incubation periods. Since disinfectants are commonly used against enteric organisms and because *E. coli* is a common enteric organism, this seemed to be an appropriate choice.

The experimental procedure followed in this experiment has been previously outlined under preparation of cell suspensions and reversal procedures. It was first necessary to determine the concentration of mercuric chloride which would give a minimum number of surviving cells and at the same time permit a maximum

efficiency of reversibility. The term "killed" refers to cells which did not survive treatment with hydrogen chloride. Table 1 summarizes these results.

Table 1. The survival effects on cells exposed for various lengths of time to different concentrations of mercuric chloride at 25° C.

	Concentration of mercuric chloride								
	10^{-3} M.			10^{-4} M.			10^{-5} M.		
Time in minutes	10	30	120	10	30	120	10	30	120
Percent killed	100	100	100	100	100	100	76.0	100	100
Percent reversed	4.7	0.0	0.0	9.2	0.2	0.0	100	97.0	29.0
Control	100	100	100	100	100	100	100	100	100

The concentration of mercuric chloride used in the experimental work was 10^{-5} molar. As can be seen in Table 1, this concentration fulfilled the desired conditions most adequately.

Previous workers have demonstrated that the killing action of mercuric chloride could be reversed under certain conditions by the use of sulfur containing compounds. Fildes (1940) indicated that the anti-bacterial action of mercury could be reversed. He suggested that the anti-bacterial action of mercury was due to the poisoning of an enzyme system. He further stated that the differences he observed using various mercury-antagonistic compounds in their ability to restore or permit cell activity was due to the

chemical behavior, rather than the action that they performed on the cells.

Enzymes have been indicated as being involved in the antibacterial action of mercury. By definition, enzymes are thermolabile biochemical catalysts elaborated by living cells without they themselves being used up or attached to the end products of the reaction. The active enzyme is made up of two parts, an apoenzyme, which is the specific protein alone and in itself has no enzymatic activity, and a coenzyme, which is a specific organic or inorganic compound. Catalysts are usually activated by an increase in temperature. This is also true of enzymes, but enzymes, being protein in nature are sensitive to heat and therefore demonstrate a temperature optimum. The rate of enzyme reactions are proportional to the substrate concentration and time. It should be possible then to demonstrate a time and a temperature dependence interaction on mercury poisoning. Studies were undertaken in an attempt to show this relationship. Fig. 1 is a summarization of data showing the effects of time and temperature as measured by the percentage of cells killed by mercuric chloride. The percentage of cells killed was arrived at by:

$$\frac{\text{untreated cells} - \text{survivors of mercury treatment}}{\text{number of untreated cells}} \times 100 = \text{percent killed cells}$$

As can be seen from Fig. 1, time and temperature have very significant effects on the killing ability of mercuric chloride. It can be seen that up to ten minutes time exposure to mercuric chloride there appears to be a direct linear relationship to killing ability. In a zero order reaction the product concentration

accumulates according to time. Up to ten minutes, with the exception of 37° and 45° C. the velocity of the reaction appears to be going through a zero order reaction. In a first order reaction, the accumulation of products not only is dependent on time but is also proportional to the amount of reactants present. In an enzyme reaction this would mean that with excess substrate the only limiting factors will be the numbers of available sites on the enzyme(s). As the substrate concentration is decreased it eventually reaches a concentration insufficient to saturate the enzyme sites and the rate of action becomes dependent upon how fast the substrate comes in contact with the enzyme. The curves in Fig. 1 beyond ten minutes show a characteristic first order picture. In most complex biochemical systems a reaction does not remain constant for the entire extent of the reaction. The order can vary as the reaction proceeds.

Fildes (1940) in his procedure for reversing the anti-bacterial action of mercuric chloride mixed the mercuric chloride and reversing agent before treating the cells. By doing this the mercuric chloride may have completely combined with the mercury-antagonist thereby having no effect on the cells. In the present experiments a two step procedure was followed as has been previously described so that at no time would the mercury-antagonist (reversal agent) be in contact with mercuric chloride alone.

In these studies, since time and temperature markedly affected the rate of killing of cells, it was thought that possibly there would be an equally marked effect on the ability to reverse the killing action of mercuric chloride. If the

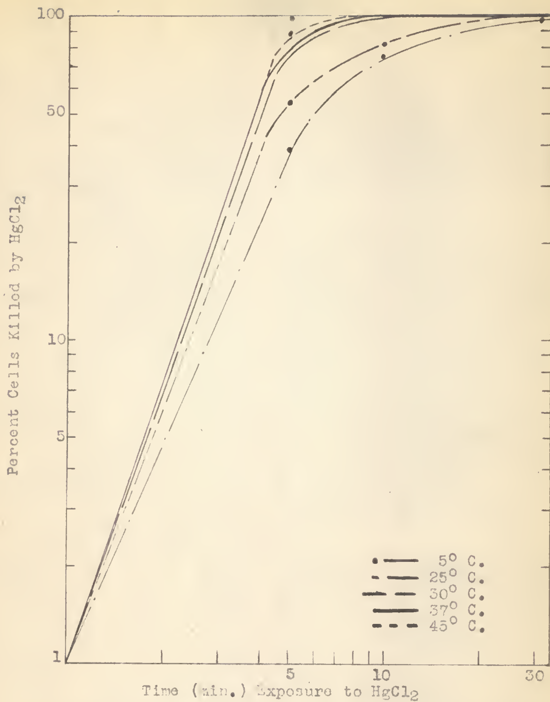

Fig. 1. Effect of time on the killing ability of 10^{-5} molar $HgCl_2$ at 5° C., 25° C., 30° C., 37° C., and 45° C.

killing action of mercuric chloride was simply a surface phenomenon, with the concentration of mercuric chloride that was used, the kinetics of the reaction should remain in zero order. By the same token, with the high concentration of reversal agent added, the velocity of the reversal reaction should never approach zero order but should remain constant in first order. In Fig. 2 are summarized the experimental results obtained using 0.1 molar thioglycollic acid as the reversing agent. It can readily be seen that there is a marked effect on the degree of reversibility, decreasing with higher temperatures, following exposure to mercuric chloride for various time intervals. The maximum velocities of the reversal reactions are achieved at a slower rate. If it were surface phenomena, a velocity approaching the rate of inactivation or even a faster rate would be expected, since better than a thousand fold higher concentration of thioglycollic acid is present than mercuric chloride.

Calculations for percentage of reversed cells were obtained by:

$$\frac{\text{Number of cells reversed}}{\text{Number of untreated cells} - \text{Number of cells surviving mercuric chloride}} \times 100 = \text{percent cells reversed}$$

A small percentage of cells which were resistant to the killing action of mercuric chloride were always observed under these experimental conditions. Their numbers were subtracted from the control counts.

Temperature coefficients (Q_{10}) for killing and reversal were

calculated. They are presented in Table 2.

Table 2. Temperature coefficients expressed as Q_{10} for 5°, 25°, 30°, 37°, and 45° C. for cells killed by mercuric chloride exposure for five minutes and reversed by thioglycollic acid.

Temperature of incubation	Q_{10}	
	Killed	Reversed
5-25° C.	0.18	0.68
25-30° C.	2.80	2.00
30-37° C.	0.39	2.80
37-45° C.	1.25	1.05

Q_{10} values for monomolecular chemical reactions usually fall within the limits of two and four. Some of the Q_{10} values that were obtained in these experiments do not fall within these limits. This does not negate the fact that the reversal process(es) is chemical in nature. It may be indicative of a two step reaction in which a primary slower adsorptive phase occurs at lower temperature before the secondary, purely chemical reaction, which results in reversal takes place. If it was a single reaction, the Q_{10} values should be the same throughout. The variance of these values indicates that more than one reaction is taking place. They also tend to further substantiate the enzymatic nature of these processes in that there is probably more than one site on the enzyme surface which must be inactivated before the enzyme activity is inhibited.

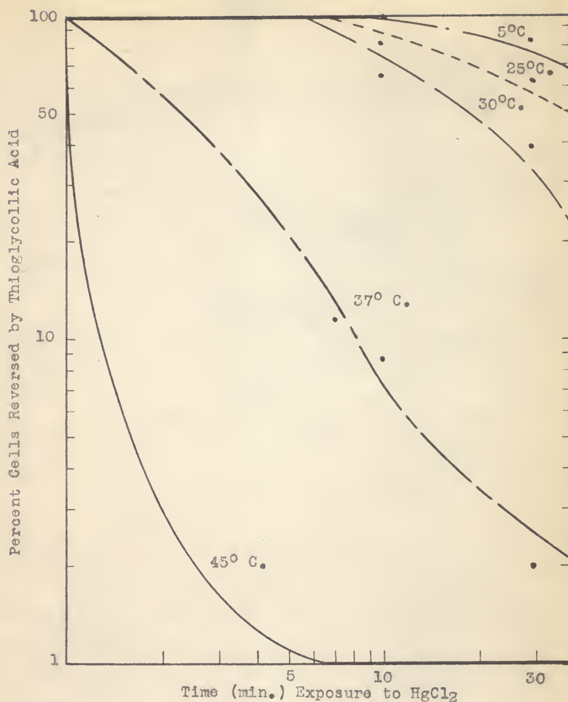

Fig. 2. Effect of time and effect of temperature on the ability of cells to be reversed by treatment with 0.1 M. thioglycollic acid for thirty minutes following exposure to 10^{-5} molar $HgCl_2$.

Reversal Effects of Sulfur Containing Compounds Other Than Thioglycollic Acid on Mercuric Chloride Treated Cells.

In these experiments several different sulfur containing compounds were used in an attempt to find an efficient mercury-antagonist. Some of the compounds such as hydrogen sulfide and sodium sulfide, were used because it was thought it might be possible to precipitate the mercury as an insoluble sulfide. This would occur if there was a greater attraction of mercury for the sulfur of the added inorganic sulfides, than to the sulfur in the enzyme-complex sites which are probably organic. Organic amino acids containing sulfur, such as cystine and cystein were also employed, as it was thought that the addition of a protein containing -SH groups might serve to replace the inactivated -SH groups of the protein portion of the enzyme-complex, or may serve to replace the sulf-hydryl containing amino acid. Under either hypothesis the result would be a restoration of "normal" metabolic pathways and thus effect the phenomenon of reversal.

A weak acid, tartaric acid, and a weak base, sodium bicarbonate, were also used in an attempt to bring about reversal of mercuric chloride treated cells. Tartaric acid was used in the hope that it might precipitate mercury as the insoluble tartarate. Sodium bicarbonate was used because a shift in pH of the mixture containing cells and mercuric chloride might precipitate the mercury. Normally, mercuric chloride solutions tend to give an acidic reaction. Table 3 summarizes the results of these experiments.

Table 3. Reversal effects of sulfur containing compounds other than thioglycollic acid.

Chemical agent	Maximum percent reversal
Hydrogen sulfide ($HgCl_2$0.01)	1.00
Sodium sulfide	0.25
Cystine	0.11
Cystein	0.10
Sodium bicarbonate	0.00
Tartaric acid	0.25
(Thioglycollic acid)	99.9

Harris et al (1954) using hydrogen sulfide reported the presence of a precipitate of mercuric sulfide within the cells. They made no mention of viability of these cells following treatment with hydrogen sulfide. Englehardt (1923) stated that reversal was possible using sulfides as reversing agents. He however suggested that the mercuric chloride was adsorbed on the cell wall, and used as proof the ability to effect reversal by treating the inactivated cells with an adsorbent such as charcoal. McCalla (1940) suggested that since the bacterial cell contains proteins, approaches inorganic colloids in size, and is negatively charged, it seemed logical that it might function as an agent in the adsorption of positively charged ions. Using 4×10^{-4} molar mercuric chloride to inactivate E. coli cells, he was able to effect as much as 99 percent reversal. The mechanism of adsorption that he suggested was that the polar groups of proteins, whether in the cell or on the cell surface, will orient themselves in the aqueous phase whenever possible. This would result in hydration, and the presence of such groups in the cell or on the cell surface

would produce an ionizable H which could be replaced by any cation.

The maximum percent reversal that could be effected using hydrogen sulfide in these present experiments was 1.0 percent as indicated in Table 3. This appears to be discrepant with the results obtained by McCalla (1940) as outlined previously. An explanation for this may rest in the fact that inactivation by mercuric chloride in these experiments was accomplished using 1×10^{-2} molar mercuric chloride, whereas McCalla used 4×10^{-4} molar mercuric chloride. He estimated that there were 1×10^{8} active sites per bacterial cell. If this is true, the concentration of mercuric chloride that McCalla employed would just barely suffice to saturate all the sites available. The increased concentration of mercuric chloride used in these present experiments would provide an excess of 2×10^{4} molecules per bacterial cell of mercuric chloride. The 10^{8} active sites as calculated by McCalla was based on the maximum number of hydrogen ions that would be adsorbed to cells and still maintain a one hundred percent viable count of bacteria. The results obtained in these present experiments would seem to indicate that if there are 10^{8} active sites per bacterial cell, it is a minimal or threshold number, and that there are a great many more active sites whose saturation results in irreversible death of the cell. Thus, the excess mercury ions used in these experiments surpass the threshold amount making reversal impossible.

An experiment using fluid thioglycollate medium as a reversal agent was performed. This experiment differed from the others

in that instead of adding the reversal agent to the mercuric chloride treated cells, an aliquote of mercuric chloride treated cells was added to fluid thioglycollate medium and ten-fold serial dilutions were made through nine tubes of fluid thioglycollate medium. Growth was observed after 48 hours incubation at 37° C. in the ninth tube. This agreed with the untreated control series. A mercuric chloride treated cells control suspension, run simultaneously, showed 100 percent inactivation. This may indicate that the mercuric chloride affects one of the aerobic systems of metabolism such as the cytochrome system, but that under anaerobic conditions a different pathway, not affected by mercuric chloride may be in operation. It is also possible that the presence of a nutritionally complete medium at the time of reversal such as fluid thioglycollate medium may have had a stimulatory reversal effect. Reversal procedures using the other reversal agents were carried out in the absence of nutrients.

Electron Microscope Studies of Thioglycollic Acid Reversed Cells

These studies were carried out to determine whether a mercury-thioglycollate crystalline precipitate was formed within the cells, and if so, to determine where within the cell it was formed, and the fate of the precipitate after cell division. The cells were treated in the same manner as for reversal. After removing a sample for a plate count, a sample was removed for microscopy. A sample was also transferred to nutrient broth and

allowed to incubate until four generations had been produced. From this population a sample was removed for microscopy and a second sample transferred to nutrient broth. After four generations a sample was taken from this population for electron microscopy.

All cell samples were washed three times in distilled water prior to preparation of the films for electron microscopy. In almost every case the centrifuged pellet of the reversed cells appeared brownish-black in color in contrast to the grey color of the pellets of the other cell samples. The proposed scheme was to observe the different samples of cells and determine whether or not the crystals formed were divided equally among two dividing cells or all the crystals retained by the parent. The results obtained were not as expected. No structures at any stage of growth of the cells which could be identified as mercuric crystals were found. However, in almost all cases, there was a definite increase in density resulting in lower refractivity of the cells treated with mercuric chloride, and then reversed with thioglycollic acid. The control cells, thioglycollic acid treated cells (not treated with mercuric chloride), and mercuric chloride treated cells were similar in appearance.

Harris et al (1954) showed that cells treated with mercuric chloride and then exposed to hydrogen sulfide had a crystalline precipitate of mercuric sulfide on the cytoplasmic membrane. The results of the present studies indicate that the increased density of the cells that was observed, and the brownish-black color of the centrifuged pellet, may be due to the formation of

a semi-soluble or only slightly soluble mercuric glycollate within the cell. This would seem probable since most organic mercury compounds are soluble.

CONCLUSIONS

The results of these experiments seem to substantiate the concept that the primary nature of mercuric chloride inactivation of cells is due to an enzyme inactivation within the cell, and not a purely surface phenomenon. It was demonstrated that there is a direct relationship between the rate of killing by mercuric chloride, the time of exposure to it, and temperature. There appears to be a saturation of the enzyme(s) at temperatures 30° C. and above after five minutes exposure to 1×10^{-5} molar mercuric chloride. That is, there is no apparent increase in the numbers of organisms inactivated at these elevated temperatures after five minutes. The ability of mercuric chloride treated cells to be revivified seems to be inversely proportional to the length of exposure and the temperature at which they are exposed to 1×10^{-5} molar mercuric chloride, in that, above 30° C. the maximum reversal that can be obtained is approximately ten percent. At the lower temperatures virtually 100 percent reversal could be effected.

It is interesting to note, as was shown in Table 2 that the Q_{10} values for 25°-30° C. of the reversed cells was only about 70 percent of the Q_{10} value for killed cells for the same temperature interval. At the temperature range 30°-37° C. the reversed

cells have a Q_{10} value approximately seven times as great as the killed cells in the same temperature interval. At temperatures above 37° C. the Q_{10} values for killed and reversed cells are essentially the same. This strongly suggests that there is a threshold response to inactivation and reversibility, there being many sites on the enzyme surface for the mercury to inactivate (-SH groups), but the inactivation of a given number of these sites, though not necessarily all, would result in killing of the cell. This hypothesis is further strengthened by the increased ability to effect the larger magnitude of reversal at lower temperatures. Saturation of the enzyme not being as complete at lower temperatures, less mercury has to be removed by thioglycollic acid from a given cell to restore activity, and therefore results in an increased percentage of reversal. The results obtained in these experiments are in general agreement with Krueger and Baldwin (1934). They suggested that the action of mercuric chloride was two fold, a preliminary stunning of growth function, with subsequent increase in time of exposure and concentration of mercuric chloride giving lethal action. The results reported in this thesis seem to indicate that this primary stunning of growth is due to adsorption, while the killing or lethal action is due to a chemical interaction.

In these experiments, having been unable to effect a significant percentage of reversal with compounds other than thioglycollic acid, it would seem to indicate that the reversal process was specific. None-the-less, it is significant that even a small percentage of reversal could be achieved with other

compounds. A 100 percent reversal could be achieved under the anaerobic conditions of the fluid thioglycollate reversal treatment. These two facts seem to be indicative of the presence of an alternate metabolic pathway which will permit survival of the organism although a supposedly vital enzyme(s) has been inactivated. This would presuppose that a small percentage of the reversal effected by thioglycollic acid was due to the action of the above suggested alternate metabolic pathway.

Electron microscope studies carried out in conjunction with the reversal studies failed to reveal the presence of any well-defined crystalline structures within the cells. The reversed cells, however, after repeated washing appeared brownish-black in color indicating that a sulfide was present. The compound formed may have been semi-soluble or only slightly soluble, and probably not carried on the cell surface.

SUMMARY OF EXPERIMENTAL RESULTS

The concentration of mercuric chloride which would give a minimum number of surviving cells and at the same time permit a maximum efficiency of reversibility was determined experimentally. This was 1×10^{-5} molar mercuric chloride.

The effects of length of exposure to mercuric chloride at various temperatures and the ability to effect reversibility of the mercuric chloride treated cells using various mercury-antagonists was investigated. Of the compounds used, thioglycollic acid was the most efficient reversing agent. The

experimental results demonstrated that the killing process as well as the reversal process is due to enzymatic destruction of inactivation for the former, and reactivation for the latter. It was indicated that reversal is dependent upon the degree of saturation of active enzyme sites.

Q_{10} values were calculated for the killing process and the reversal process. Their values are indicative of a two step reaction in which a primary slower adsorptive phase occurs at lower temperature before the second, purely chemical reaction, which results in reversal, takes place.

The reversal effects of sulfur containing compounds other than thioglycollic acid were investigated. Hydrogen sulfide, sodium sulfide, cystine, cystein, sodium bicarbonate, and tartaric acid were not effective as reversal agents, the maximum percent reversal achieved by any of these compounds being one percent.

An experiment using fluid thioglycollate medium as a reversal agent was performed. The results of this experiment indicate that the action of mercuric chloride may be on one of the aerobic systems of metabolism, such as the cytochrome system.

Electron microscope studies on mercuric chloride treated cells reversed with thioglycollic acid failed to show the presence of a crystalline precipitate. However, an increased optical density of the cells was observed.

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APPENDIX

Table 4. Raw data representing averages of five typical experiments for determination of killing effectiveness of 10^{-5} M. $HgCl_2$. Data represents numbers of colonies per plate calculated to the same dilution.

	Length of time exposure to $HgCl_2$ in minutes		
	5	10	30
	Temperature of incubation 5° C.		
Control	54	54	54
Survivors	33	13	2
Killed	21	41	52
	Temperature of incubation 25° C.		
Control	128	128	128
Survivors	57	24	0
Killed	71	104	128
	Temperature of incubation 30° C.		
Control	133	133	133
Survivors	3	6	1
Killed	130	127	132
	Temperature of incubation 37° C.		
Control	240	240	240
Survivors	6	1	0
Killed	234	239	240
	Temperature of incubation 45° C.		
Control	72	72	72
Survivors	3	2	0
Killed	69	70	72

Table 5. Raw data representing averages of five typical experiments for determination of reversal percentage 10^{-5} M. $HgCl_2$ treated cells, using 0.1 M. thioglycollic acid at the reversing agent. Data represents numbers of colonies per plate calculated to the same dilution.

	Length of time exposure to $HgCl_2$ in minutes		
	5	10	30
	Temperature of incubation 5° C.		
Control	54	54	54
Surv. ($HgCl_2$)	33	13	2
Kill. ($HgCl_2$)	21	41	54
Reversed	21	42	50
	Temperature of incubation 25° C.		
Control	128	128	128
Surv. ($HgCl_2$)	57	24	0
Kill. ($HgCl_2$)	71	104	128
Reversed	71	79	81
	Temperature of incubation 30° C.		
Control	133	133	133
Surv. ($HgCl_2$)	3	6	1
Kill. ($HgCl_2$)	130	127	132
Reversed	127	71	60
	Temperature of incubation 37° C.		
Control	240	240	240
Surv. ($HgCl_2$)	6	1	0
Kill. ($HgCl_2$)	234	239	240
Reversed	24	27	0.4

Table 5. (concl.)

	Length of time exposure to $HgCl_2$ in minutes		
	5	10	30
	Temperature of incubation 45° C.		
Control	72	72	72
Surv. ($HgCl_2$)	3	2	0
Kill. ($HgCl_2$)	69	70	72
Reversed	0	0	0

The calculations of Q_{10} values were performed as follows:

$$k = \frac{2.3}{t} \log \frac{P}{Po} \quad ; \quad Q_{10} = \frac{10}{dT} \log \frac{k_2}{k_1}$$

k = velocity constant of a given reaction at a given time interval.

P = cells killed by $HgCl_2$ for calculation of Q_{10} for kill, or cells reversed by thioglycollic acid following exposure to $HgCl_2$ for calculation of Q_{10} for reversal.

Po = original number of organisms for calculation of Q_{10} for kill, or number of organisms killed by exposure to $HgCl_2$ for calculation of Q_{10} for reversal.

t = time exposure to $HgCl_2$.

dT = temperature change.

k_1 = velocity constant for lower temperature.

k_2 = velocity constant for higher temperature.

STUDIES ON REVERSAL OF THE DISINFECTING ACTION OF MERCURIC CHLORIDE UPON *ESCHERICHIA COLI*

by

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B. S., Kansas State College
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Koch in 1881 popularized the use of mercuric chloride as a disinfectant with his famous string experiment. The use of various other mercury compounds were subsequently investigated by many workers. A myriad of results were obtained. Most of the current work in this field has been directed at discovering the mechanism of mercury poisoning of cells. Several recent workers have indicated that the mode of action probably lies in the destruction or inactivation of an enzyme or enzyme-complex necessary for the normal metabolism of the cell. Previous workers had also indicated that the action of mercuric chloride could be reversed under certain conditions. These studies were undertaken to further substantiate these current hypotheses, and to illustrate the effects of time and the effects of temperature upon the reactions. Some observations will also be made concerning cytological changes within the cell after treatment with mercuric chloride and reversal with thioglycollic acid as revealed by electron microscopy.

The organism used throughout these studies was an Escherichia coli culture obtained from the American Type Culture Collection (6877). The procedure followed was to suspend an actively growing culture of the organism (18 hrs. old) in 0.1 M sodium acetate (pH 6.8). These cells were then exposed to 1×10^{-5} M mercuric chloride for various lengths of time, after which time they were treated with a reversing agent and incubated. Five experimental incubation temperatures were used; 5° C., 25° C., 30° C., 37° C., and 45° C. The reversal agents employed were cystine, cysteine, sodium thioglycollate, tartaric acid, sodium sulfide,

thioglycollic acid, sodium bicarbonate, and hydrogen sulfide, all of which were prepared in concentrations of 0.1 M. Plate counts were made from appropriately treated suspensions to determine percent kill, and percent reversal of the cells. The standard procedure was followed for preparation of collodion films for electron microscopy of the cell suspensions.

Preliminary experiments revealed that a 1×10^{-5} M concentration of mercuric chloride permitted the maximum efficiency of reversability at the same time effecting a maximum percent kill. Of the compounds used, thioglycollic acid was the most efficient reversing agent. The experimental results demonstrated that the killing process as well as the reversal process was due to enzymatic destruction or inactivation for the former, and reactivation for the latter. It was indicated that reversal is dependent upon the degree of saturation of active enzyme sites.

Q_{10} values were calculated for the killing and reversal processes. Their values are indicative of a two-step reaction, in which a distinguishable adsorptive phase occurs at lower temperatures before the second, purely chemical reaction which results in reversal, takes place. The reversal effects of the sulfur-containing compounds other than thioglycollic acid were of small magnitude, the maximum percent reversal achieved by any of these compounds being one percent. It was possible to achieve as much as one hundred percent reversal with thioglycollic acid.

An experiment using fluid thioglycollate medium as a reversal agent was performed. The results of this experiment seemed to indicate that the action of mercuric chloride was on one of

the aerobic systems of metabolism, such as the cytochrome system.

Electron microscope studies on mercuric chloride treated cells reversed with thioglycollic acid failed to show the presence of a crystalline precipitate. However, an increased optical density of the cells was observed.