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Title STUDIES ON THE BACTERICIDAL ACTION OF IONS OF THE NOBLE METALS

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This investigation was instigated to develop methods of studying quantitatively the effect of the ions of the noble and heavier metals on bacterial cells. Two different methods of study were used. The first consisted of adding the toxic metal ions to a growing culture of bacteria. The second technique determined the time required for the disinfection of suspensions of washed cells.

The possibility of studying the action of metal ions by their effect on cultures of growing Escherichia coli and Pseudomonas fluorescens was investigated and found to be unsatisfactory because of reactions of the metal ions with the nutrient material.

Disinfection studies were made on suspensions of Escherichia coli that had been washed by centrifugation and suspended in either tap water, distilled water, or in redistilled water. Dinsinfection times were measured by an end-point method. The results obtained on suspensions of this type were not always reproducible in successive experiments.

The least variation was obtained in the experiments using cells suspended in tap water.

Experiments on washed cells of Escherichia coli suspended in tap water placed the metal ions studied in the following approximate order of decreasing toxicity: Hg^{++}, Os^{++++}, Pd^{++}, Ag^{+}, Pt^{++++}, Cu^{++}, Au^{+++}, Fe^{+++}, Cs^{+}, Tl^{+}, UO_2^{++}. A concentration of two parts per million of the metal ions was used. Ions of the noble metals showed a greater toxicity than ions of the more active metals. In this concentration di-valent palladium ions and tetra-valent osmium ions were almost as toxic as mercuric ions.

STUDIES ON THE BACTERICIDAL ACTION OF IONS OF THE NOBLE METALS

by

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STUDIES ON THE BACTERICIDAL ACTION OF IONS OF THE NOBLE METALS

PART I

SURVEY OF THE LITERATURE

Introduction

In 1870 a French worker, Raulin (14), found that one part of silver nitrate in 1,600,000 parts of water prevented the germination of spores of Aspergillus niger. His findings were later verified by Nageli (11) who applied the name "oligodynamic action" to the toxicity of certain metals in extreme dilutions. Nageli found that it was sufficient to place a gold coin in water to produce oligodynamic action toward Spirogyra, and that the action was carried over by the glass vessel after the gold had been removed and the flask rinsed. His investigations also included copper and other of the heavier metals. Since that time many studies have been made on the toxic action of metals and metal salts toward microorganisms.

There are numerous explanations of oligodynamic action found in the literature. Nageli (11) makes a distinction between "chemical action" in concentrated solutions and "oligodynamic action" in dilute solutions, but offers no further explanation. A German investigator, Saxl (16),

considers oligodynamic action as due to a form of energy at the surface of the metal which can be transferred, and that the action is not that of the metal itself but of a secondary activated body; he maintains that copper can exert its action through the air. In opposition to Saxl's ideas are the claims of Behring (2) and Messerschmidt (10) that copper has no effect on bacteria unless the copper is in true solution. A relationship between the solubility of copper and silver salts and their toxicity was also noted by Bechhold (1). Other investigators favoring a chemical action of ions rather than a physical explanation are Linden (9), Dreschel (4), Schumacher (17), and Robertson (15).

Dreschel (4) makes an interesting comparison between the amount of toxic substance in solution and the amount of protoplasm attacked. He computed that the amount of copper sulfate necessary to kill Spirogyra is approximately 1/5000 of the weight of the vegetative mass. For a man weighing 50 kg. this would be equivalent to 1 gram of poison; this amount of strychnine would be fatal. Thus the amount of poison is not inconsiderable compared to the weight of the Spirogyra.

Robertson (15) points out that there is no fundamental difference between oligodynamic action and the toxic action

of metal salts in more concentrated solutions other than the rate at which death occurs. In explanation of oligodynamic action he states,

"The phenomenon is not so surprising as it might appear, however, when we recollect that heavy metal ions are protein precipitants and especially tend to form insoluble and non-dissociated compounds with proteins. The effect of this is to reduce the concentration of the heavy metal ions in any region containing protein, and if the protein is surrounded by a medium which still contains free metal ions these will diffuse in to take the place of those precipitated or rendered non-dissociable. These in return will be removed from the solution and so the process will go on until, although the original concentration of metal ions in the external medium may have been very small, in the end the concentration of combined metal in a cell may be considerably greater and quite sufficient to constitute a lethal dosage."

That the toxicity of metal salts in relatively high concentrations depends upon ionization was shown by the investigation of Kronig and Paul (6). They exposed spores of B. anthracis to solutions of various mercury salts and counted the number of colonies that developed on plates after the action of the salts had been interrupted by washing with an ammonium sulfide solution. In every case

there was an increase in the number of survivors as salts of decreasing ionization were used. The same effect was noted whenever the addition of a common anion reduced the mercury ion concentration.

Of the two types of explanation of oligodynamic action, (a) associating it with emanations and imponderable agents, and (b) attributing it to the action of metal ions in solution, the latter is generally accepted.

Metals whose ions have been found to exert bactericidal action in low concentrations are Ti, Be, Al, Nd, Pb, Cu, Tl, Zr, Ni, Cd, Co, Au, Pt, Hg, and Ag. The concentration of a given metal ion required to produce bactericidal action varies with different microorganisms. In the literature there is no general agreement as to the order of toxicity of the metals listed above toward any one organism.

Theories on the Mechanism of the Action of Metal Ions

Although numerous theories have been advanced to explain the bactericidal action of the ions of the heavier and noble metals, no definite explanation has been agreed upon.

That they exert their action by precipitation of proteins is the opinion of Robertson (15) and a number of

other investigators. Schumacher (17) states that his studies show that the ions penetrate and form salts with the nucleic acids of the bacterial nuclei.

Liese and Mendel (8), working with silver nitrate, claim that there is first an adsorption of the metal ion on the surface of the cell. This causes an inhibition of reproduction without necessarily killing the organisms. They believe that actual death does not occur until the silver is demonstrable inside the cell.

Gegenbauer (5), in an attempt to determine the reaction between poison and cell, made a series of experiments on Micrococcus pyogenes using various concentrations of $HgCl_2$. In one series of experiments the bacteria were washed repeatedly with water after disinfection, and in the other they were treated with H_2S to remove or counteract the poison chemically. Cells exposed to 0.01% $HgCl_2$ and washed following removal from the disinfectant showed growth when transferred to a suitable medium at 7 hours but not at 13 hours. Those exposed to a like concentration of $HgCl_2$ but treated with H_2S before transfer, showed growth until 208 hours but were dead at 265 hours. According to the interpretation of Rahn (12), this behavior indicated that there was first some chemical combination of the mercury ion with the protoplasm that prevented reproduction but did not kill the cell. After the addition of

H_2S had precipitated the mercury, the combination was broken up and the cells functioned normally. Following prolonged exposure to the $HgCl_2$, however, the cells could not recover when H_2S was added. Rahn considers that two reactions were going on; the first produced a dormant stage, and the second, a slower process that took place simultaneously, eventually killed the cells.

Caldwell, Bollen, Bird, and Osler (3) postulate an oxidation-reduction reaction between the metal ions and the bacteria. In such a reaction the bacterial cell would be the reducing agent or electron donator and the metal ion would be the oxidizing agent or electron acceptor. Death of the cell would be caused by oxidation of some of its constituents in the production of free metal atoms from metal ions.

Yet another theory is that of Rahn (12) who believes that chemical poisons in general are catalysts for certain destructive reactions in the cell. He believes that the poison accelerates the constructive process of the cell as well as the destructive ones, but that the latter are affected more strongly.

The Effect of Temperature and Concentration on the Bactericidal Action of Metal Ions.

Measurably slow chemical processes, such as the

reaction between poisons and bacterial cells, are accelerated by an increase in temperature. The ratio between the rates of reaction measured at two different temperatures is called the temperature coefficient, and is usually designated by Q. If the rate of death of the cells is K_1 at an absolute temperature of T, and K_2 at a temperature of T + 10°, the coefficient for a 10° C. increase, Q_{10}, would then be

$$Q_{10} = K_2/K_1$$

If instead of the rates of death, the times required to kill a given number of cells at two different temperatures are measured, the only variable at the different temperatures is time, and the expression becomes

$$Q_{10} = t''/t'$$

in which t' and t'' are the two disinfection times.

The death rate of bacteria exposed to the action of certain toxic agents is proportional to the nth power of the concentration of the poison, i.e., it is assumed that n molecules or ions of the disinfectant must react with one cell before it loses the power of reproduction. This value n is termed the concentration exponent and is given

by the expression

$$n = \frac{\log t_2 - \log t_1}{\log c_1 - \log c_2}$$

where t_2 and t_1 are the disinfection times by concentrations c_2 and c_1 of the poison.

In the comparison of the rates of action of bactericidal substances n-values are of significance. Due to differences in these values, two disinfectants having equal power at a certain concentration may vary widely when both are diluted at the same ratio.

Little work has been done on either the concentration exponents or temperature coefficients of the noble metal ions. Some n-values have been determined for mercury, but these vary widely even when computed from data obtained using the same organism. In an effort to supply some of this lack of information on the bactericidal action of noble metal ions and also to obtain more precise data regarding the effect of mercury, the present investigation was instigated.

A further objective was to develop methods of studying the effect of metal ions on microorganisms in a medium free from organic material and foreign matter that would give reproducible results. In the past most experiments on bactericidal action have been carried out by adding the

toxic agent directly to broth cultures of the organisms. Adsorptions and chemical reactions between the metal ions and foreign material in the substrate complicate the results obtained in such experiments. In this work an attempt has been made to eliminate these errors.

PART II

EXPERIMENTAL WORK

PART II

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Escherichia coli has been frequently used in previous experimental work on disinfection and bactericidal action. There are several reasons for the choice of this organism, one of the principal ones being that it undergoes little variation. It does not tend to clump in suspension but usually grows in pairs. It grows equally suspended throughout the medium eliminating any error from pellicle formation or granular type of sediment. The organism forms a very thin capsule and is easily centrifuged and resuspended, thus facilitating the washing of the cells free of organic material. In its resistance it is similar to some of the pathogenic organisms (*Eberthella typhosa*, etc.) which are a source of danger in contamination of water. These advantages led us to again use *Escherichia coli* in this study.

The strain used in this work was one obtained from Levine (No. 1a) and has been carried on laboratory media for the past 10 years. During this period it has exhibited no appreciable morphological variation, and has shown but slight changes in its resistance to phenol as determined by routine phenol coefficient tests.

Since some of the members of the genus *Pseudomonas*

are pathogenic and are commonly contaminants in water supplies, Pseudomonas fluorescens was used in one experiment. It is usually somewhat less resistant than Escherichia coli.

The Effect of Metal Ions on the Growth of Escherichia coli and Pseudomonas Fluorescens.

In the first experiments an attempt was made to study the inhibition by metal ions of the growth of Escherichia coli and Pseudomonas fluorescens. Solutions of the metal ions Cu^{++}, Fe^{+++}, Hg^{++}, Ag^{+}, and Au^{+++} in distilled water were tried in the following concentrations (expressed as parts per million): 0.005, 0.025, 0.05, 0.1, 0.25, 1, 5, 10, and 20. Chlorides of the metals (c.p. grade) were used for preparing all of the solutions except silver. The extremely low solubility of silver chloride necessitated the use of silver nitrate. To supply nutrient material for the organisms, Bacto peptone (Difco) was added to the metal ion solutions. In order to keep the concentration of organic material as low as possible and to minimize reduction of the metal ions, the concentration of peptone was limited to 0.1%. Both organisms grow fairly well in such a medium. Four tubes of each concentration of each metal ion were prepared and sterilized by autoclaving for 20 minutes at 15 pounds pressure. Two tubes of each were

then inoculated with Escherichia coli and two with Pseudomonas fluorescens. Inoculation was made from 24-hour broth cultures of the organisms by means of a standard loop holding 0.01 ml. The tubes were incubated at 37° C. and observed at 24-hour intervals. Two million or more organisms per ml. produce a noticeable trubidity in the medium, and growth was indicated by the appearance of such turbidity. No attempt was made to estimate the actual number of cells per ml. The results obtained on the duplicate tubes are shown in Tables I and II, in which + indicates growth and - indicates no growth.

TABLE I

THE EFFECT OF METAL IONS ON GROWTH OF ESCHERICHIA COLI

Metal Ion	Incubation Time	Concentration, p.p.m. 0.005	0.025	0.05	0.1	1	5	10	20
Au^{+++}	24 hrs.	++	++	++	++	++	--	+-	
	48 "	++	++	++	++	++	+-	++	
	72 "	++	++	++	++	++	++	++	
Fe^{+++}	24 hrs.	++	++	++	++	++	++	++	+-
	48 "	++	++	++	++	++	++	++	+-
	72 "	++	++	++	++	++	++	++	++
Hg^{++}	24 hrs.	++	++	++	++	--	--	--	
	48 hrs.	++	++	++	++	--	--	--	
	72 "	++	++	++	++	+-	--	--	
Cu^{++}	24 hrs.	++	++	++	++	++	--	--	--
	48 "	++	++	++	++	++	+-	--	++
	72 "	++	++	++	++	++	++	--	++
Ag^{+}	24 hrs.	++	++	++	++	--	--	--	
	48 "	++	++	++	++	+-	++	++	
	72 "	++	++	++	++	++	++	++	

TABLE II

THE EFFECT OF METAL IONS ON THE GROWTH OF PSEUDOMONAS FLUORESCENS

Metal Ion	Incubation Time	Concentration, p.p.m. 0.005	0.025	0.05	0.1	1	5	10	20
Au^{+++}	24 hrs.	++	++	++	++	++	--	+-	
	48 "	++	++	++	++	++	++	++	
	72 "	++	++	++	++	++	++	++	
Fe^{+++}	24 hrs.	++	++	++	++	++	++	++	--
	48 "	++	++	++	++	++	++	++	--
	72 "	++	++	++	++	++	++	++	+-
Hg^{++}	24 hrs.	++	++	++	++	++	--	--	
	48 "	++	++	++	++	++	--	+-	
	72 "	++	++	++	++	++	--	+-	
Cu^{++}	24 hrs.	++	++	++	++	++	++	--	--
	48 hrs.	++	++	++	++	++	++	--	++
	72 "	++	++	++	++	++	++	+-	++
Ag^{+}	24 hrs.	++	++	++	++	+-	--	--	
	48 "	++	++	++	++	++	++	++	
	72 "	++	++	++	++	++	++	++	

The data show that apparently Escherichia coli was more sensitive to the effects of the mercuric ion than was Pseudomonas fluorescens; with the other metals essentially

no difference was observed. Under these conditions mercury was the most toxic of the metals used. With nutrient material in the solution it is probable that the concentrations of metal ions given does not represent effective amounts because of possible reduction and adsorption by the organic matter. This is borne out by the fact that after autoclaving, the highest concentration of gold showed a noticeable purple color indicating some reduction to colloidal gold, while the silver solutions in higher concentrations had a yellowish color. A considerable amount of ferric hydroxide precipitated in the 20 p.p.m. iron solutions. In the other solutions no visible changes took place, so it was impossible to tell if any reaction between the metal ion and the peptone had taken place.

The Bactericidal Action of Metal Ions on Suspensions of Washed Cells of Escherichia Coli.

It was apparent from the previous experiments that the presence of organic matter in the solution made it difficult to study the precise effect of the metal ions on the bacteria alone. In an attempt to overcome this difficulty suspensions of washed cells of Escherichia coli were used and the time required to kill the organisms by exposure to the metal ions was measured. The criterion of death taken was the loss of power of reproduction by the organisms when

they were transferred to standard nutrient broth.

The suspensions were prepared by centrifuging a 24-hour broth culture, suspending the organisms in sterile water, centrifuging a second time, and then re-suspending them in water. Sterile tap water from ordinary 99 ml. water blanks was used for re-suspension. Suspensions containing approximately the same number of cells per ml. were prepared for the experiments by matching their turbidities with the turbidity of a suspension containing a known number of organisms.

The solutions of the metal ions were prepared in such a manner that when 1 ml. of bacterial suspension was added to 9 ml. of metal ion solution, the desired concentration of metal ion was had. After addition of the organisms to the solution containing the metal ion, 0.01 ml. was withdrawn at measured intervals by means of a standard loop and transferred to tubes of nutrient broth (0.5% peptone and 0.3% beef extract). The tubes were incubated at 37° C. for 48 hours and growth was judged by the turbidity of the medium.

In these experiments no attempt was made to use an antidote to counteract the effect of the metal ions on the bacterial cells after exposure. There is no known antidote that will function to the same degree for all the metal ions used. If antidotes that function to various

degrees are used, results obtained using different metal ions could not be compared. Inasmuch as 0.01 ml. of solution containing metal ions was transferred to 10 ml. of sterile broth, this large dilution (1-1,000) was depended upon to minimize subsequent effects the ions might have on the organism.

Table III shows results obtained by using two parts per million of various metal ions on several different suspensions prepared as outlined. The disinfection times indicate the time interval in which the last organisms were killed as measured by this technique. The number of cells per ml. was determined by using the standard plate count method. All experiments were carried out at room temperature.

TABLE III

DISINFECTION TIMES FOR SUSPENSIONS OF WASHED CELLS OF ESCHERICHIA COLI

Metal Ion	Concentration	Disinfection Time	Cells per cc.
Hg^{++}	2 p.p.m.	4-6 min.	2,500,000
Pd^{++}	"	4-6 "	"
Ag^{+}	"	10-15 "	"
Cu^{++}	"	45-60 "	"
Au^{+++}	"	1½-2 hrs.	"
Fe^{+++}	"	6-24 "	"
Hg^{++}	"	2-4 min.	2,400,000
Os^{++++}	"	2-4 "	"
Pd^{++}	"	4-6 "	"
Ag^{+}	"	6-8 "	"
Pt^{++++}	"	20-25 "	"
Au^{+++}	"	2-3 hrs.	"
UO_2^{++}	"	24 "	4,800,000
Tl^{+}	"	24 "	"
Hg^{++}	"	8-10 min.	"
Cs^{+}	"	7-24 hrs.	"

It will be noticed that there was some fluctuation in disinfection time when using the same concentration of the same metal ion in different suspensions, and that apparently this was not a function of the number of organisms in the suspension. Each suspension was prepared and treated in the same manner insofar as these conditions could be controlled.

This technique would serve to place the metal ions tested in the following approximate order of decreasing toxicity: Hg^{++}, Os^{++++}, Pd^{++}, Ag^{+}, Pt^{++++}, Cu^{++}, Au^{+++}, Fe^{+++}, Cs^{+}, Tl^{+}, UO_2^{++}.

Determination of the Concentration Exponents of Mercury and Silver Ions.

In order to determine the relationship between change in the concentration of metal ions and change in disinfection time, concentration exponents were determined using a suspension of washed cells. The suspension was prepared in the manner previously described. All tubes were held at 30° C. by the use of a water bath. The data is given in Table IV, and is plotted in figures 1 and 2. The plate count was 6,760,000 cells per ml.

The break in the curve, figure 2, may indicate that a concentration of silver ion tolerated by the organisms is being approached as the concentration of metal ion

decreases.

Calculations made from the slopes of the curves give a concentration exponent of 2.1 for the Hg^{++} ion and 2.3 for the Ag^{+} ion, i.e., the death rate of the organisms in the concentrations studied is proportional to a little more than the square of the metal ion concentration.

TABLE IV

DISINFECTION TIMES BY MERCURY AND SILVER IONS

Metal Ion	Concentration	Disinfection Time	Logarithms of Disinfection Times
Hg^{++}	4 p.p.m.	0-1 min	0.00-----
"	3 "	1-2 "	0.00-0.30
"	2 "	5-6 "	0.69-0.78
"	1 "	10-15 "	1.00-1.18
"	0.5 "	20-25 "	1.30-1.40
"	0.2 "	40-45 "	1.60-1.65
Ag^{+}	6 "	0-1 "	0.00-----
"	4 "	1-2 "	0.00-0.30
"	2 "	6-7 "	0.78-0.85
"	1 "	15-20 "	1.18-1.30
"	0.5 "	55-60 "	1.74-1.78
"	0.2 "	90-120"	1.95-2.01

Figure 1

Change in Disinfection Time With
Change in Concentration of Mercuric Ions

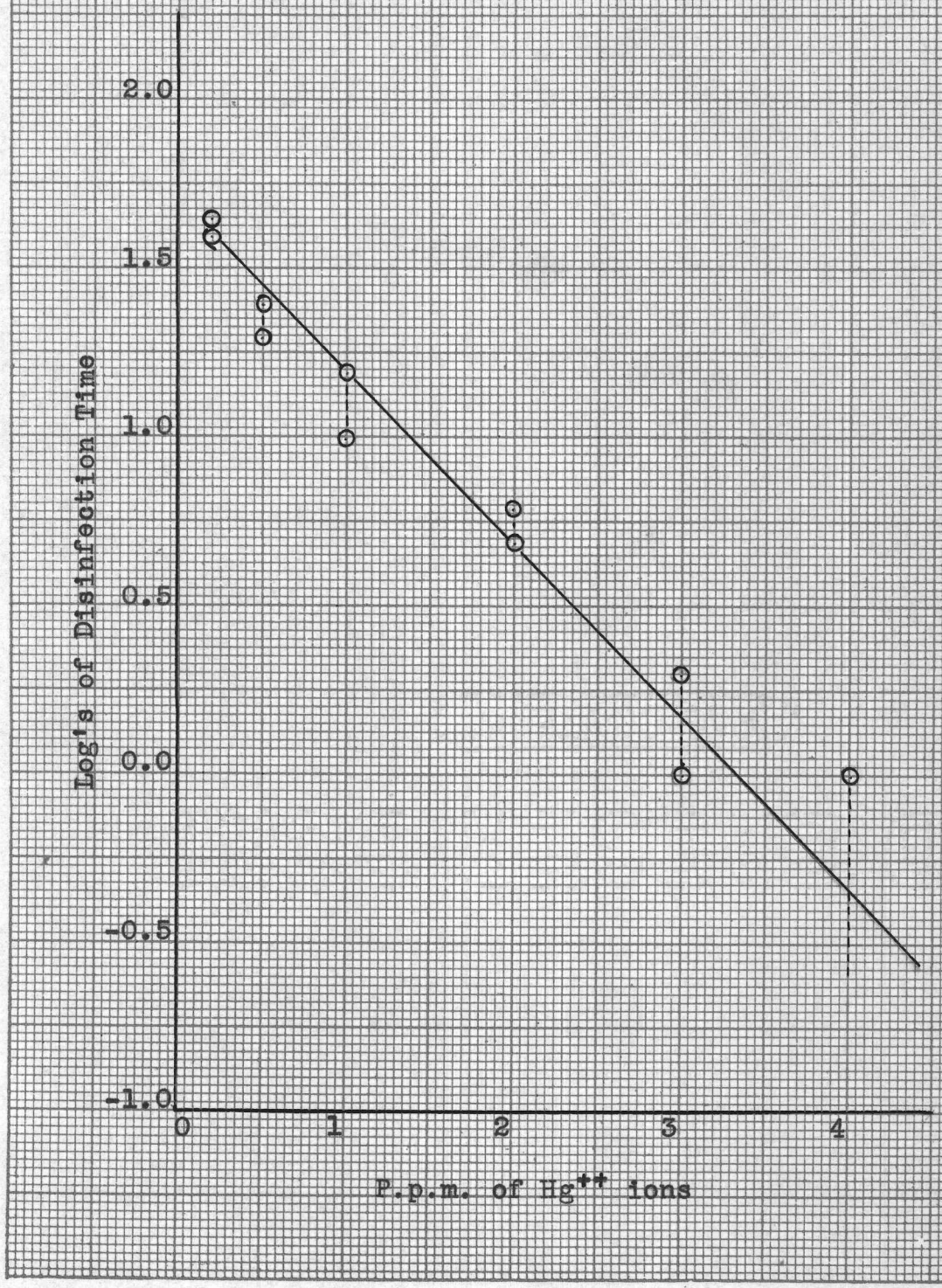
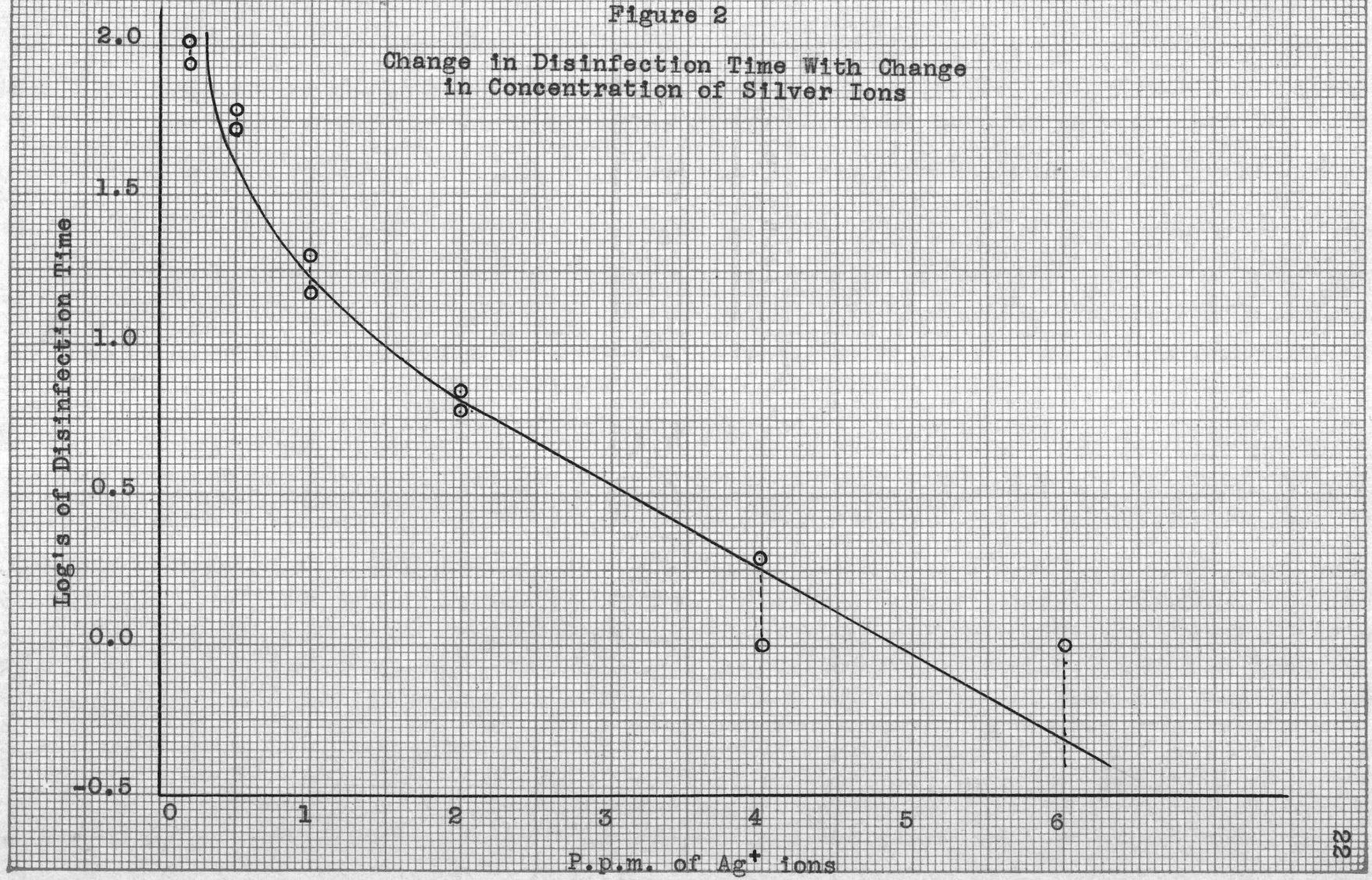


Figure 2

Change in Disinfection Time With Change in Concentration of Silver Ions



Experiments on Suspensions of Escherichia Coli in Distilled Water.

The suspensions in the previous experiments had been prepared using sterile tap water; in order to eliminate any possible errors from this source, distilled water (from a block tin still) that had been sterilized by autoclaving was substituted in the subsequent experiments. Otherwise the suspensions of organisms were prepared as previously described.

Some disinfection times using osmium and palladium ions on distilled water suspensions are given in Table V. The experiments were run at 30° C.

The data show very erratic results and no correlation between concentration of metal ions and disinfection time. Another experiment using osmium was carried out under apparently identical conditions. The results obtained showed no agreement with those of the previous experiment.

The data obtained from an attempt to determine the temperature coefficients of disinfection (Q_{10}) of mercury and silver ions on a distilled water suspension are shown in Table VI. A plate count on this suspension showed 2,000,000 cells per ml.

TABLE V

DISINFECTION TIMES OF DISTILLED WATER SUSPENSIONS BY OSMIUM AND PALLADIUM IONS AT 30° C.

Metal Ion	Concentration	Disinfection Time	Cells per cc.
Os^{++++}	4 p.p.m.	6-8 min.	1,250,000
"	3 "	10-12 "	"
"	2 "	10-12 "	"
"	1 "	15-20 "	"
"	0.5 "	10-15 "	"
"	0.2 "	5-10 "	"
Pd^{++}	4 "	0-1 "	1,930,000
"	3 "	0-1 "	"
"	2 "	0-1 "	"
"	1 "	0-1 "	"
"	0.5 "	55-60 "	"
"	0.2 "	95--- "	"

TABLE VI

DISINFECTION TIMES OF A DISTILLED WATER SUSPENSION BY MERCURY AND SILVER IONS

Metal Ion	Concentration	Disinfection Time @ 20° C.	Disinfection Time @ 30°C.	Q_{10}
Hg^{++}	2 p.p.m.	5-6 min.	under 1 min.	--
Hg^{++}	3 "	4-5 "	" " "	--
Ag^{+}	2 "	12-13 "	over 20 min.	--
Ag^{+}	3 "	2-4 "	4½-5 "	0.63

It was impossible to calculate the exact temperature coefficients for the two concentrations of mercury since disinfection times at 30° C. were unknown, but they would have been approximately 5 or more. The coefficient calculated for silver in the one case was less than 1; in the other case the value could not be calculated because the organisms exposed to the Ag^{+} ions at 30° C. were not killed in the period of observation, but the data show that it also would have been less than 1.

Such diverse results as those obtained on mercury, silver, osmium, and palladium ions using distilled water suspensions of organisms apparently indicate that some change in the suspension was taking place during the course of an experiment. There was also the possibility that in

spite of the rigidly standardized procedure for the preparation of the suspensions, some unknown or unrecognized variable was still left uncontrolled. For this reason most of the subsequent experiments were attempts to determine the cause of this variability in disinfection times, and to develop a method of obtaining duplicative and quantitative results.

The fact that the suspensions were unbuffered suggested the possibility that this might have had some bearing on the variation in disinfection times, although it does not seem probable that any very great changes in pH could have occurred in the suspension in the duration of an experiment. A comparison of disinfection times on organisms suspended in an M/15 phosphate buffer solution and in distilled water showed that those in the buffer solution were much more resistant to the action of 2 p.p.m. of Hg^{++} ion. This might have been due to the formation of some insoluble $Hg_3(PO_4)_2$ with a consequent reduction in the amount of mercury in the active ionic form. Both suspensions were prepared from the same 24-hour broth culture of Escherichia coli. Since there was no available system buffering at a pH of 7 which would neither react with the metal ions nor cause harmful effects on the organisms, no further experiments using buffered suspensions were tried.

Death Curves of Suspended Bacteria

In order to determine if any rapid decrease in the number of viable cells in the suspension itself was taking place during the course of an experiment, death curves were run on Escherichia coli suspended in distilled water and in an M/15 phosphate buffer solution. These suspensions were prepared from a 24-hour broth culture by centrifugation and washing as previously described. Table VII shows results obtained over a period of 27 hours on the suspension in the phosphate buffer solution; Table VIII shows results over a period of 75 hours on the organisms suspended in distilled water.

TABLE VII

VIABILITY OF ESCHERICHIA COLI SUSPENDED IN AN M/15 PHOSPHATE BUFFER SOLUTION

Time Interval	Average number per ml.	Log number per ml.
0 hrs.	65,300,000	7.81
3 "	55,400,000	7.74
18.5 "	52,600,000	7.72
27 "	50,400,000	7.70

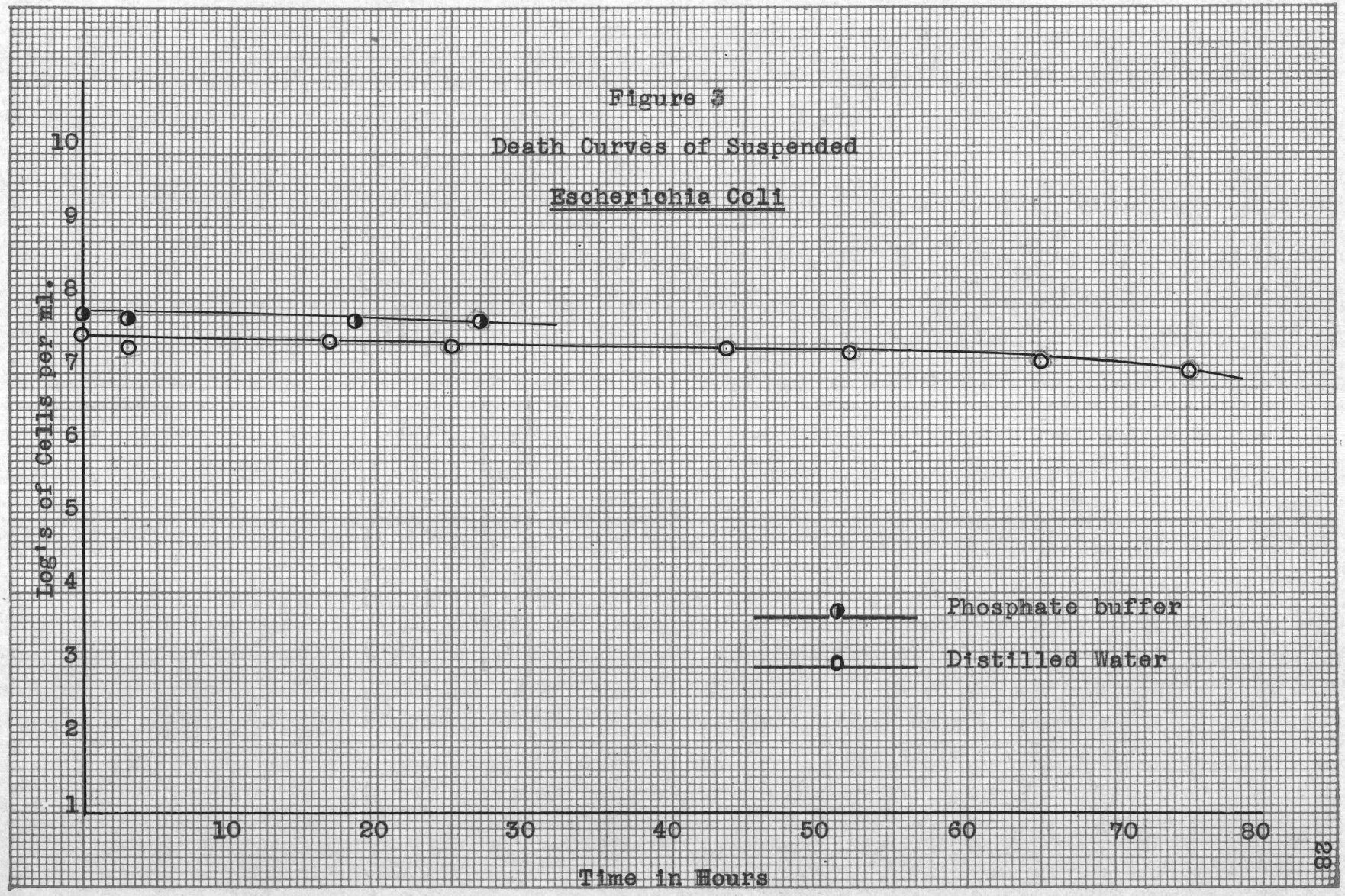


Figure 3
Death Curves of Suspended
Escherichia Coli
log's of cells per ml.
10
9
8
7
6
5
4
3
2
1
10
20
30
40
50
60
70
80
Time in Hours
Phosphate buffer
Distilled Water

TABLE VIII

VIABILITY OF ESCHERICHIA COLI SUSPENDED IN DISTILLED WATER

Time Interval	Average number per ml.	Log number per ml.
0 hrs.	33,000,000	7.52
3 "	29,100,000	7.46
16.7 "	27,400,000	7.44
25 "	23,300,000	7.38
43.6 "	20,800,000	7.32
52 "	17,950,000	7.25
65 "	12,800,000	7.11
75 "	9,450,000	6.98

There was no rapid change in the number of viable cells during the period of observation in either of the two suspensions; therefore this could not be considered as a factor contributing to the variabilities in disinfection times recorded.

Experiments on Escherichia Coli Washed and Suspended in Re-distilled Water.

In an attempt to reduce the number of possible vari-

able factors, several refinements of procedure were introduced in these experiments. In place of ordinary distilled water, distilled water that had been re-distilled from a Pyrex-glass still was employed in preparing the suspensions. All glassware used was of Pyrex that had been cleaned by means of chromic acid cleaning solution, trisodium phosphate, and nitric acid; following treatment with each of the above agents it was rinsed six times in distilled water and finally six times in re-distilled water. In the previous experiments the bacterial suspensions had been held in soft glass dilution bottles. To minimize any effects dissolved substances from the glassware might have had, new Pyrex test tubes were used for this part of the work. With the exception of the above changes, the procedure was the same as before.

To test the effects of these changes of procedure, disinfection times at 20° C. and 30° C. on three different suspensions prepared in this way were determined using several concentrations of Hg^{++} ions. Essentially the results were no better than those obtained in the experiments using ordinary distilled water. Disinfection times could not be duplicated in successive experiments and the temperature coefficients calculated from the data varied. In one case the disinfection time measured at 30° C. was greater than the corresponding one at 20° C., thus making

the temperature coefficient less than 1. The other temperature coefficients calculated were much lower than normally would be expected. Again there was no apparent connection between the disinfection time observed for a given concentration of Hg^{++} ions and the number of cells per ml. The data are shown in Table IX.

TABLE IX

DISINFECTION TIME BY MERCURY IONS ON SUSPENSIONS OF ESCHERICHIA COLI IN RE-DISTILLED WATER

Concentration Hg^{++} ions	Cells per ml.	t @ 20°C.	t @ 30°C.	Q_{10}
0.2 p.p.m.	2,500,000	20-22 min.	16-18 min.	1.23
0.5 "	"	12-14 "	14-16 "	0.87
0.8 "	"	7-8 "	5-6 "	1.36
0.8 "	11,100,000	2-4 "	0-2 "	--
0.8 "	"	2-4 "	0-2 "	--
0.8 "	8,100,000	12-14 "	10-11 "	1.24

Experiments on Suspensions Prepared From Agar Slants

In the previous experiments the organic material was washed free of the cells by centrifugation. The effect which such treatment might have had upon their resistance

was unknown. In order to prepare a suspension of cells without the use of centrifugation, and yet one that would be reasonably free of organic material, the following method was employed: The organisms were grown on nutrient agar slants at 37° C. for 48 hours; the surface growth was then gently scraped off with a platinum loop and suspended by shaking in re-distilled water. Disinfection times by Hg^{++} ions at 30° C. on three suspensions of this type are shown in Table X. In the first case transfers were made at five minute intervals, and in the second at two minute intervals. Since the organisms were killed before the first transfer in each experiment, no definite disinfection times could be recorded. In the third suspension some of the cells were viable when the experiment was terminated, and here again the time of disinfection is undetermined.

Disinfection times observed on the suspensions prepared by re-suspending the growth from agar slants showed discrepancies even greater than those found using suspensions of cells that had been centrifuged. For this reason no further experiments following this procedure were performed.

TABLE X

DISINFECTION TIMES OF AGAR SLANT SUSPENSIONS BY MERCURY IONS AT 30° C.

Concentration Hg^{++} ions	Disinfection Time	Cells per ml.
2 p.p.m.	under 5 min.	32,200,000
5 "	"	"
8 "	"	"
1 "	under 2 min.	16,300,000
2 "	"	"
0.5 "	"	"
0.5 "	over 6 min.	18,400,000
0.2 "	" 10 "	"
0.1 "	" 10 "	"

The Preparation of Suspensions by Filtration

As an alternative procedure for freeing the cells of organic material, filtration of the cells and washing on the filter was substituted for centrifugation in these experiments. Although the effect of filtration on the organisms was unknown, this method was tried in an effort to obtain better results. The suspension was prepared by filtering 10 ml. of a 24-hour broth culture of Escherichia

coli on a sintered glass (Jena) filter, washing the cells on the filter with three 10 ml. portions of re-distilled water, and then suspending them in re-distilled water. Various concentrations of Hg^{++} ions on suspensions of this type gave the results shown in Table XI.

TABLE XI

DISINFECTION TIMES OF FILTERED SUSPENSIONS BY MERCURY IONS AT 30° C.

Concentration Hg^{++} ions	Disinfection Time	Cells per ml.
2 p.p.m.	1-2 min.	6,010,000
2 "	2-3 "	7,600,000
2 "	3-4 "	"
2 "	2-3 "	"
1* "	12-14 "	"
1* "	22-24 "	"
1 "	11-12 "	"
2 "	2-2½ "	6,040,000
1 "	14-16 "	"

* These were run simultaneously at two-minute intervals.

In general the results given by this procedure were better than those obtained on suspensions prepared from

agar slant cultures. There was a great variation in disinfection time by two of the solutions of 1 p.p.m. Hg^{++} ions on the same suspension. This is noted in Table XI. In this case the time required to kill the organisms in one solution of Hg^{++} ions was almost double that required in an identical solution. The fact that the two were run simultaneously made this result impossible of interpretation. It shows, however, that the procedure is open to error.

The Bactericidal Action of Mercuric Ions on Cultures of Escherichia Coli Grown in Dilute Peptone Solutions

Since the previously used methods of study were not capable of giving results which could be duplicated, further work on suspensions of cells was discontinued. Instead, some experiments were performed using cultures of Escherichia coli grown in dilute peptone solutions. In order to have the source of organisms a constant factor, the same cultures held at a temperature of 2°C. were used for all the work. To eliminate possible injury centrifugation or washing might have had on the cells, the cultures were used without any preliminary treatment.

A concentration of peptone giving a suitable number of cells per ml. was determined by making plate counts on a series of peptone solutions of different concentrations.

These had been inoculated with a strain of Escherichia coli re-isolated from Levine's strain (1a), and incubated at 37° C. for 48 hours. It was found that in 0.1% peptone the number of cells was 209,000,000 per ml. and in 0.05% peptone, 117,000,000 per ml. A ten-fold dilution of either culture gave a suitable number of cells, i.e., approximately ten or twenty million per ml. It also served to lower the concentration of organic material.

Two flasks of media, one containing 0.1% peptone and the other containing 0.05% peptone were prepared using redistilled water. Following sterilization in the autoclave, each flask was inoculated with 0.1 ml. of a suspension of Escherichia coli (re-isolated strain) that had been prepared by scraping the surface growth from a 48-hour agar slant culture and suspending the growth in water. This means of inoculation was used to prevent the transfer of organic material to the medium with the inoculum. After incubation at 37° C. for 48 hours, each culture was covered with a three-quarter inch layer of sterile paraffin oil and held at 2° C. in the refrigerator.

The procedure used in the experiments on these cultures was similar to that previously used on the suspensions of cells. Instead of adding 1 ml. of a suspension to 9 ml. of solution containing metal ions, 1 ml. of the culture was added directly. Before the beginning of an

experiment a portion of the culture was withdrawn from beneath the surface of the paraffin oil by means of a pipette and transferred to a sterile test tube. It was then held in a 30° incubator for 1 hour, and in a 30° water bath for ½ hour before use. The technique of measuring the disinfection time was the same as that used before. In all previous work the metal ion solutions had been sterilized in the autoclave. In these experiments the solutions were prepared aseptically diluting a strong solution of mercuric chloride that had stood until it had become sterile. Disinfection times on two cultures are given in Table XII. The plate count on the 0.05% peptone culture was 12,800,000 cells per ml., and that on the 0.1% culture 15,700,000 per ml.

It required almost twice as long for disinfection of the culture grown in the 0.1% peptone solution. To determine if this was due to a better nutritional condition of the cells, or whether it was caused by reaction of organic material with the metal ions, the following experiment was performed: Disinfection times using 2 p.p.m. Hg^{++} ions were determined on three portions of the culture grown in 0.05% peptone. Peptone was added to three other portions of the same culture until the total concentration of each was 0.1%. Disinfection times on these were then immediately

TABLE XII

DISINFECTION TIMES OF CULTURES OF ESCHERICHIA COLI GROWN IN DILUTE PEPTONE SOLUTIONS

Concentration of Peptone	Concentration Hg^{++} ions	Disinfection Time
0.05%	2 p.p.m.	3-4 min.
0.05%	2 "	3-4 min.
0.05%	2 "	2-3 min.
0.05%	2 "	3-4 min.
0.10%	2 "	7-8 min.
0.10%	2 "	8-9 min.

determined. The results of this experiment are shown in Table XIII.

The data show that when the amount of organic material was slightly increased by the addition of peptone, disinfection time was appreciably lengthened. From this it was evident that the effective concentration of the metal ions had been decreased by reaction with the added organic material. The longer disinfection time observed on the culture grown in 0.1% peptone (Table XII) was probably due to a similar reaction between the metal ions and residual nutrient material or secretory products of the cells. Since it was probable that the results obtained using the

culture grown in 0.05% peptone were influenced by the same factor, this procedure was considered unsuitable as a method of studying the action of metal ions on bacterial cells alone. It was noticeable, however, that results could be duplicated in successive experiments.

TABLE XIII

THE EFFECT OF ADDED ORGANIC MATERIAL ON DISINFECTION TIME

Treatment of Culture	Concentration Hg^{++} ions	Disinfection Time
Culture alone	2 p.p.m.	3-4 min.
" "	2 "	3-4 "
" "	2 "	3-4 "
Culture + added peptone	2 "	6-7 "
" " "	2 "	4-5 "
" " "	2 "	5-6 "

PART III

DISCUSSION, CONCLUSIONS, AND SUMMARY

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Discussion

The possibility of studying the action of metal ions by observing their effect upon growing bacterial cells in their medium was investigated and found to give unsatisfactory results. Metal ions added to a culture medium undergo reactions with the constituents of the medium, and the initial concentration of metal ions does not represent the amount available to react with the organisms. It is possible that the nutrient material in the medium reacts with the ions by either reduction, adsorption, or both. The metals low in the activity series would be more easily reduced than those high in the series. This factor alone would be a cause of error in any comparison of the toxic action of different metal ions using growth studies. It is impossible to determine the amount of adsorption taking place in such a study.

Almost all poisons in minute amounts exert a stimulative effect on the growth of microorganisms. This is an additional complicating factor when the toxic action of metal ions in very low concentrations is studied.

For these reasons the data showing the effect of metal ions on the growth of Escherichia coli and Pseudomonas

fluorescens can not be taken as an indication of the actual toxicities of the metal ions used. Reaction of the organic material with the metal ions was noticeable in the higher concentrations of gold and silver. The appearance of a purple color in the highest concentration of gold indicated that some reduction of the auric ion to metallic gold had occurred. Here the product of reduction was present in sufficient concentration to be visible. There was no corresponding change in the appearance of the solutions containing a lower concentration of gold, but there is no reason to believe that a similar reaction had not occurred. In the solutions of other metal ions the extent of any reactions that might have taken place was undeterminable. These difficulties made it necessary to seek other methods for studying the action of metal ions on bacterial cells.

Experiments were then performed in which suspensions of washed cells were exposed to the action of the metal ions, and the time required to kill the organisms measured. By means of centrifugation, cells of Escherichia coli from broth cultures were washed free from organic material coming either from nutrient material or from secretions of the cell. Suspensions of these cells in tap water, distilled water, and in water re-distilled from glass, were then used for disinfection studies. In each of these

cases it was found that results obtained in one experiment could not be duplicated in succeeding experiments carried out under apparently identical conditions. Variations in disinfection times as great as 100% were obtained in some instances. No satisfactory explanation can be offered for this phenomenon. It occurred to some degree in all types of experiments using centrifuged cells, and, therefore, this method of investigation had to be abandoned. It is well known that centrifuging and washing change the physical and chemical properties of the cell (13), and it was thought that perhaps this might be the cause of the observed variations.

A similar technique was developed whereby the organic material was removed from broth culture of the cells by successive washings with re-distilled water on a sintered glass filter. This eliminated the use of centrifugation in the preparation of the suspensions, but here again great variations were obtained in apparently identical experiments using the same metal ion solution.

Another type of suspension was prepared by scraping the cells from the surface of an agar slant and suspending them in re-distilled water. This technique allows very little contamination with organic material from the substrate, but it removes little of the secretory products of the bacterial cells, i.e., the capsules, etc. (7).

Successive experiments on these suspensions, too, showed great discrepancies in disinfection times. It is conceivable that differences in the age of the cells on the surface growth might have been responsible for the results obtained. This is not borne out, however, by the results of the previous experiments using broth cultures in which the cells were of a more uniform age. The same type of variation was observed in both cases.

Since any treatment designed to remove organic material from the cells caused variable results in the experiments, an attempt was made to use directly cultures grown in very dilute peptone solutions. It was hoped that the very low concentration of peptone would not appreciably change the concentration of metal ions by reaction with them. It was found, however, that any concentration of peptone supporting the growth of the culture would, following growth of a complete population, contain sufficient residual organic matter to lower the effective metal ion concentration. This was shown when disinfection times were lengthened by slightly increasing the concentration of peptone. It is noticeable, though, that fairly consistent disinfection times could be obtained in identical experiments using cultures prepared and treated in this manner. The reaction between the metal ions and organic material (loc. cit.) made it impossible to obtain precise

data on the toxicity of metal ions toward bacterial cells by this procedure.

In making this study two types of discrepancies were noted in the disinfection experiments. The first of these was the variation in disinfection times obtained using the same solution of metal ions on suspensions prepared in an apparently identical manner. The second type of variation was that resulting from the method of treatment to which the cells had been subjected in the preparation of the suspension. The average disinfection time by mercury ions at a level of 2 p.p.m. of suspensions of Escherichia coli prepared by the procedures investigated is shown in Table XIV. A wide variation is shown in the disinfection time of cells prepared by different procedures.

TABLE XIV

DISINFECTION OF SEVERAL TYPES OF SUSPENSIONS BY 2 P.P.M. Hg^{++} IONS AT 30° C.

Type of Suspension	Disinfection Time	Cells per ml.
Centrifuged, tap water	5-6 min.	6,760,000
Centrifuged, distilled water	under 1 min.	2,000,000
Centrifuged, re-distilled water	under 1 min.	8,100,000
From agar slant, re-distilled water	under 2 min.	16,300,000
Filtered, re-distilled water	2-3 min.	7,600,000
Cells grown in 0.05% peptone, used directly	3-4 min.	12,800,000
Cells grown in 0.1% peptone, used directly	8-9 min.	15,700,000

Conclusion

None of the methods investigated were entirely satisfactory for studying quantitatively the precise effect of noble and heavy metal ions on bacteria. Consistent results could not be obtained by using any procedure in which the metal ions acted upon the bacterial cells in a medium free of organic material. Under these conditions it was not possible to make any very accurate comparison of their toxicities by the determination of concentration exponents or temperature coefficients.

Variations of a greater or lesser degree were observed in all experiments using suspensions of washed cells. The least variation was obtained in the experiments on suspensions of cells that had been washed by centrifugation and suspended in sterile tap water. By this means it was possible to place the metal ions studied in an approximate order of toxicity at concentrations of two parts per million. Ions of the noble and heavier metals were shown to be much more toxic than ions of the more active metals. At a concentration of two parts per million, di-valent palladium ions and tetra-valent osmium ions were almost as toxic toward Escherichia coli as were mercuric ions.

Summary

1. This investigation was instigated to develop methods of studying quantitatively the precise effect of the ions of the noble and heavier metals on bacterial cells.

2. Two different methods of study were used. The first consisted of adding the toxic metal ions to a growing culture of bacteria. The second technique determined the time required for the disinfection of suspensions of washed cells.

3. The possibility of studying the effect of metal ions by their action on cultures of growing Escherichia coli and Pseudomonas fluorescens was investigated and found to be unsatisfactory because of reactions of the metal ions with the nutrient material.

4. Disinfection studies on suspensions of Escherichia coli that had been washed by centrifugation and suspended in either tap water, distilled water, or in re-distilled water gave results that were not reproducible in successive experiments.

5. Experiments on washed cells of Escherichia coli suspended in tap water placed the metal ions studied in the following approximate order of decreasing toxicity: Hg^{++}, Os^{++++}, Pd^{++}, Ag^{+}, Pt^{++++}, Cu^{++}, Au^{+++}, Fe^{+++}, Cs^{+}, Tl^{+}, UO_2^{++}.

6. Ions of the noble and heavier metals showed a greater toxicity than ions of the more active metals. In concentrations of 2 p.p.m. Pd^{++} ions and Os^{++++} ions were almost as toxic toward washed cells of Escherichia coli as Hg^{++} ions.

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