THE ACTION OF CHLORINE UPON UREASE AND ITS RELATION TO THE BACTERICIDAL ACTION OF CHLORINE

Thesis for the Degree of Ph. D.

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Milton Silverman

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This is to certify that the

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THE ACTION OF CHLORINE UPON UREASE AND ITS RELATION TO THE BACTERICIDAL ACTION OF CHLORINE

by

Milton Silverman

A THESIS

Submitted to the School of Graduate Studies of Michigan State College of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

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To Dr. W. L. Mallmann who suggested the problem and who continued to direct it, and to render aid and encouragement to the student who pursued it.

And to all others who in their own way helped him.

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AN ABSTRACT

Submitted to the School of Graduate Studies of Michigan State College of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

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The thesis problem was based upon the distinction between chlorine that is effective against bacteria, bactericidal chlorine, and chlorine concentration that is measured chemically. An attempt was made to evaluate the action of chlorine upon urease in the hope that such action would parallel the bactericidal action of chlorine more closely than a chemical determination of chlorine concentration.

The methods used in the pursuit of this investigation involved:

1. The maintenance of conditions which provided a reagent of free chlorine.

2. A method for the evaluation of the bactericidal activity of free chlorine at different pH values against Escherichia coli, strain C198. Non-clumping vegetative bacterial cells were washed from agar slants. Small volumes of reagents were used in a repetitive, serological type test at various contact times. Survivors were determined by plate counts. Percentage survival logarithms were plotted against logarithmic time intervals for the ratios of bacterial nitrogen to the dosage of chlorine used.

3. A parallel method for the evaluation of the inhibitory action of chlorine upon urease. Crystalline urease was used. Percentage activity remaining was determined by the action of the inhibited enzyme upon a urea substrate. The measurement of the ammonia nitrogen evolved was by means

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of a Nesslerisation procedure. The logarithms of the percentage activity remaining were plotted against logarithmic time intervals for the ratios of urease nitrogen to chlorine dosage used.

The major findings were:

1. The action of chlorine upon bacteria became more efficient as the pH was lowered. In the presence of chlorine at pH 4, bacterial numbers were reduced to .01 percent of their original value in 40 minutes when the ratio of bacterial nitrogen to chlorine was .1. In the presence of chlorine at pH 9, bacterial numbers were still present to the extent of 100 percent with the same nitrogen to chlorine ratio.

2. The action of chlorine upon urease became more efficient as the pH was raised. In the presence of chlorine at pH 4, urease activity was reduced to .01 percent of the original activity in 100 minutes when the ratio of urease nitrogen to chlorine was 3. In the presence of chlorine at pH 9, urease activity was reduced to .01 percent of its original activity in seven minutes with the same nitrogen to chlorine ratio.

3. The effect of chlorine in the concentrations used for water disinfection upon the viability of a bacterium would not necessarily be duplicated by its effect upon the activity of any one single enzyme. On the basis of this test procedure, the action of chlorine upon urease could not be used to assay directly the bactericidal activity of chlorine.

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I. INTRODUCTION

The experimental work of this thesis concerns itself with the action of a disinfectant, chlorine, upon a bacterium, Escherichia coli and upon an enzyme, urease. It originated from a suggestion of Dr. W. L. Mallmann who referred this student to a previous doctoral thesis by N. H. Ozgumus entitled "Evaluation of Chemical and Bacteriological Methods of Determining Germicidal Activity of Chlorine" (1). In the summary of this thesis it was stated that in the presence of certain forms of organic material the values for free chlorine obtained by chemical tests were in excess of the germicidal chlorine determined bacteriologically. Thus the simple chemical tests used to determine free chlorine did not correctly indicate germicidal activity under certain conditions. However, the bacteriological test is of such a complicated nature that it is not practical as a routine method for determining the germicidal nature of the chlorine present. It would be necessary to obtain standard time-survivor curves for a standard strain of micro-organism in the presence of different concentrations of chlorine for each set of determinations. Time-survivor curves in the presence of test amounts of chlorine would then have to be interpolated onto the standard curves and the equivalent germicidal concentration would thereby be determined. The test is tedious, time consuming, and complicated. It was

suggested that a simpler biochemical method might be sought. Might not the inhibitory action of chlorine upon an enzyme duplicate the destructive action of chlorine upon the bacterial cell? And might not the degree of inhibition be used to measure the amount of germicidal chlorine?

II. REVIEW

Bacteriology as a subject of scientific study may be dated from the middle of the nineteenth century and may be considered as a direct outcome of the work of Pasteur. Studies on fermentation were undertaken by him during 1855 to 1860. He was able to demonstrate that the fermentation of various organic fluids was always associated with the presence of living cells and that different types of fermentation were associated with different micro-organisms. Such investigations of fermentation led to the study of the behavior of various kinds of natural organic fluids, and of simple synthetic media, as substrates for micro-organisms. Different media were found to be suitable for different micro-organisms to a certain extent. Thus, environmental factors exercised a selective effect on the type of naturally occurring bacterial flora. By extension similar ideas were applied to infection resulting from micro-organisms.

Both desirable and undesirable fermentations were produced by micro-organisms through the agency of "organised" ferments. Since such reactions took place only in the presence of living micro-organisms and were not due to the presence of certain chemicals as postulated by Liebig, then the micro-organisms had to be destroyed in order to eliminate any unwanted reaction. Such destruction by heat could be utilized

for sterilising fermentation media. Lister was struck by the analogy between changes which occurred in fermenting organic material and in putrefaction occurring in wounds. The underlying cause was assumed, analogously, to be living micro-organisms. The undesirable reactions could be and were eliminated by the use of chemicals. Carbolic acid sprays were utilized successfully as an antiseptic during surgery by Lister as early as 1867.

The massive figure of Koch dominated the bacteriological scene for the last two decades of the nineteenth century and purely morphological studies dominated this field. The advances in the study of enzymes, as ferments were designated by Kuhne in 1878, were made in the field of chemistry largely through investigations upon the so-called "unorganized" ferments which although secreted by living cells, acted in their absence. However, this distinction of "organized" and "unorganized" ferments was broken down by Buchner (2) who discovered that cell free extracts of yeast could be made to produce alcoholic fermentation. Pasteur and Liebig had both been correct. Living micro-organisms definitely degraded organic substances, respired, and built up cell substance by the use of enzymes. But, such enzymes could be isolated and in the presence of suitable substrates and menstrua could be shown to act as a catalyst independently of the cell. Within a short space of time a partial scheme of glycolysis was formulated by Harden and Young (3) complete with

phosphorylation of sugars and the presence of co-enzymes. Glycolysis came to be regarded as part of the process by which a cell made energy available for its needs by means of intramolecular oxidations not involving oxygen. How could such oxidations be performed? Were these enzyme reactions of such a critical nature that their inhibition resulted in the death of the cell?

The concept of enzymes remained a rather obscure one until 1926 despite the huge amount of work performed upon them. Also despite the impure nature of the usual enzyme preparations huge encylopedic volumes were written ascribing specific properties and names to such preparations as in von Euler (4), Oppenheimer (5), Willstätter (6), and Buchanan and Fulmer (7). Willstätter (8) summarized this obscure viewpoint when he stated that enzymes did not belong to the ordinary proteins or carbohydrates nor to any of the known, large groups of organic compounds with a complex structure. Yet for the quarter century preceeding 1926 and for about fifteen years thereafter the work on bacterial enzymes and the theories of disinfection involving inhibition of such enzymes was based largely on work with impure preparations that produced certain specific reactions.

Wieland (9) provided a theory and a mechanism that was eagerly seized upon by the investigators in this field. The essential mechanism of cellular oxidation was regarded as hydrogen activation and consequent hydrogen transport. The

removal of hydrogen from a molecule could be preceded by the addition of a molecule of water or the hydrogen could be removed directly. In either case a suitable hydrogen acceptor had to be provided. The enzyme that activated the hydrogen in the substrate to be oxidized and which then acted as a reversible hydrogen acceptor and donator, thus transporting the hydrogen, was called a dehydrogenase. When Thunberg (10) provided a simple technique for the measurement of dehydrogenase activity, the school of English biochemists under G. F. Hopkins eagerly adopted it and proceeded to apply it widely to living tissues and cells. Among these were Stephenson, Quastel, Whetham, and Wooldridge. The technique was extensively described by Quastel and Whetham (11). Washed bacterial cells suspended in appropriate buffer solution, inorganic ions, and an indicator dye were placed in a specially constructed tube, by means of which the tube and fluid could be evacuated and a specific substrate could be added without opening the tube to the air. Reactions consisted of reduction of the indicator dye, methylene blue, which was evident by the bleaching of the dyed solution. Concentrations were arranged so that reactions were completed in a short time, 30 minutes or less, to eliminate bacterial multiplication. Change of color of the dye was taken to indicate dehydrogenase activity, due to the enzyme itself. It was believed that any interfering activities due to either living cells or proliferating cells had been eliminated. Cook and Stephenson (12),

and Quastel and Wooldridge (13) reported that the dehydrogenases -- washed, resting cell suspensions -- of Escherichia coli differed in their susceptibility to changes in pH, salt concentration, temperature, and exposure to various chemicals. Thus, Thunberg tube reactions were obtained when the suspensions were subjected to increasing temperature, pH changes, and to exposure to nitrite, benzol, toluol, phenol, ether, chloroform, and propyl alcohol. The most sensitive of the dehydrogenases were those acting on alanine, glycerol, sugars, and glutamic acid. Less sensitive were those acting on lactic, succinic, and fumaric acids. Least sensitive were the formic and acetic dehydrogenases. Potassium cyanide and hydrogen peroxide gave a reversed picture in which the formic and acetic acid dehydrogenases were most sensitive. These investigators pointed out that the effects of such toxic inhibitors, as mentioned above, were of interest for two reasons. They threw light upon the mechanism of action of such inhibitors. Exposure for different periods of time resulted in step by step repression of the various dehydrogenase activities. At least two different groups of inhibitors could be constructed on the basis of this order of suppression. Secondly, information was obtained concerning the properties of enzymes and of bacteria possessing such enzymes.

This work led to the idea that dehydrogenases were critical enzymes for the vital processes of the bacterial cell. Partial or complete destruction of some or all of such enzymes

resulted in the death of the cell. Quastel and Wooldridge (13) had demonstrated that complete inhibition of the sugar dehydrogenases resulted in greatly reduced numbers of organisms or dead ones. Casman and Rettger (14), Edwards and Rettger (15), and Wedberg and Rettger (16) using different species of bacteria, including some thermophiles, performed their experiments in the following manner: the maximum temperature of growth was determined and concomitantly, the heat resistance of various enzymes including succinic dehydrogenase. It was found that the enzymes were inactivated at the same temperature at which the bacteria ceased to grow. However, the exposure of bacteria for viability occurred in nutrient medium while the exposure of bacteria for enzyme inactivation was performed after washing the cells - which process removed co-enzymes. The period of exposure was very long - 24 hours--long enough for the protein of the enzyme to deteriorate. Thus the two parallel tests were not exactly comparable and the results were not valid. Rahn and Schroeder (17) repeated the work of Rettger and his co-workers, but made comparative viability and enzyme tests from cells suspended in buffer. No parallel was found between the two factors of viability and enzyme inactivation. When more than 99.99 percent of the cells were dead only 50 percent of the succinic dehydrogenase was inactivated. These workers concluded that there was no connection between the inactivation of enzymes and the death of microbial cells. Firstly it was demonstrated

that enzyme action persists in dead cells and secondly, it was shown that the logarithmic order of death of bacteria, if such were actually the manner according to which bacteria died, could occur only if death were caused by the effect upon a single molecule. It would have been more correct for Rahn and Schroeder (17) to have said, concerning their first point, that there was no connection between the death of microbial cells and those specific enzyme activities which were studied. The work of Quastel and Wooldridge (13) had demonstrated that toxic substances killed micro-organisms but that enzyme activities are only partially suppressed. Exposure of E. coli to toluene, benzol, ether, chlorform or cyclohexanol was sufficient to eliminate the power of the micro-organism to activate sugars, glycerol, or glutamic acid. The activating mechanisms for succinic, lactic, formic, and alpha glycero-phosphoric, however, remained intact. Acetone acted similarly to toluene, etc., but in addition eliminated the activating mechanisms for polyhydric alcohols. Thus the investigators concluded that dead cells in the sense that they were incapable of proliferation in known media still possessed undiminished activating powers. The action of disinfectants upon cell was a complex one. The death of cells could not be attributed to the inactivation of any one specific enzyme but the dogmatic statement that no enzyme inactivation was involved could not be made. Additional experimental evidence seemed to bear out this conclusion.

Yudkin (18) demonstrated that silver sulphate was lethal to suspensions of E. coli in concentrations much lower than those inhibiting its dehydrogenases. Bucca (19) testing the action of several drugs upon Neisseria gonorrhoeae found that the death of cells occurred long before any significant enzyme inhibition took place. One of the drugs, sulphanilamide, produced practically no enzyme inhibition during an exposure period of 24 hours yet the cells were dead at the end of the period.

Rahn and Schroeder (17) in their second conclusion attempted to refute the contention that enzyme inactivation was the cause of bacterial death on the basis of more theoretical considerations. These were to the effect the time-survivor curve of the action of disinfectants upon micro-organisms could be interpreted only in terms of a monomolecular reaction which followed the formula, $Kt = \log a/a-x$, where t = time, a = initial population of micro-organisms, and x = the number of bacteria killed at time t. This relationship was postulated by Henri (20) for the hemolysis of chicken red blood cells by dog serum. Madsen and Nyman (21) using the data of Krönig and Paul (22) showed that the rate of disinfection followed the same formula. Chick (23) further confirmed the relationship with additional data. Such a relationship, however, was based upon the assumption that the cells of a bacterial culture were of uniform resistance and that death occurred to a

certain proportion in each successive time period, due to a single event. As early as 1889 Geppert (24) had attributed the survival of bacteria to individual variation in the population under study. This concept was in accord with data from the pharmacologic field. Reichenbach (25) also pointed out that organisms varied extensively in regards to the time needed for their destruction. If this variation had a skewed distribution then the approximately linear relation between the logarithm of the survivors and the time might be accounted for. For the action of heat upon bacteria, time-survivor curves similar to those with drugs were obtained. Further cultures of different ages gave time-survivor curves of different shapes, which seemed to indicate that variation in resistance was occurring. It was concluded that chemical molecules were so small compared with bacteria and their number so much greater than the number of bacteria that even in dilute solutions each bacterium would be surrounded by a similar number of molecules. They would, therefore, be equally exposed to the action of the disinfectant. If then, all the bacteria were of equal resistance, why should any one die before the other? Buchanan and Fulmer (26) were of the opinion that the theory of logarithmic death rested on insecure foundations. If the number of cells which died in each equal interval of time were plotted against time, a rate distribution curve would be obtained. The area under such a curve would be proportional to the total number of cells. The area to the

left of any ordinate would be proportional to the total cells dead and to the right of any ordinate to the total still surviving. Such a curve presented a distribution of cell resistances - such resistance being measured by the time required to kill the particular cell. In general, the properties of living things have been found to be distributed according to some form of a probability curve. Such a distribution would be symmetrical if the constants of the equation of such a curve were equal. If however, the constants were unequal, the curve became skewed and if very unequal, so skewed that the major portion of the curve resembled the logarithmic curve. Thus, the logarithmic order of death resulted from data based upon experiments in which rate curves were extremely skewed and in which the variation of resistance was scattered very closely about a mean resistance. In support of this contention data and curves from a paper by Fulmer and Buchanan (27) showing that time survivor curves of yeast cells in the presence of methylene blue were not logarithmic, were presented in (26).

The proponents of the monomolecular theory however, persisted in the belief that their explanation was correct. They received the support of Arrhenius (28) who regarded the single event necessary for such action as the ionization of the protein part of the molecule. Such ionization of but one or two hydrogen and hydroxyl molecules would open the cell to attack and result in destruction of the cell. Yule (29) attempted

to explain the lag period by the occurrence of a few events followed by the logarithmic period due to one event. Chick (30) went so far as to assume that micro-organisms underwent rhythmical changes and were vulnerable only during certain phases. Thus a certain percentage was killed in every time period. But Rahn (31) still insisted that bacterial death was different from that of multicellular organisms and that the data of drug action upon multicellular organisms could not be applied to bacteria. Using arguments based on Yule's probability calculations, it was shown that a theoretical logarithmic order could be obtained only if one definite molecule were affected. Since the order of bacterial death had been found to be logarithmic, then, Rahn concluded that one definite molecule had been affected or that one event had occurred. If curves other than logarithmic ones were presented, they were to be explained away by such assumptions as the occurrence of more than one event due to the clumping of cells. This conclusion has resulted from the commission of a logical error - that of affirming a consequent, and also by disregarding other data.

The case for correlation between bacterial cells and enzyme inactivation was not proved by such simple experiments as those performed upon dehydrogenases, but neither was the possibility of such correlation ruled out by Rahn and Schroeder's two arguments.

Perhaps, then, the experimenters who attributed bacterial death to the inactivation of a single enzyme erred on the side of simplicity but not on the side of a concept. Disinfectants did react with the cell and with the proteins of the cell. This reaction with such complexly arranged organisms would involve reaction with large numbers of enzymes of which more than one would be inactivated in the process of killing one bacterium. Could one say then that the inactivation of one enzyme would be critical? Apparently not, for it had been shown that more than one enzyme was inactivated by any one toxic substance and the selection of any one enzyme for such a critical role was dependent upon the available method of testing rather than upon a complete knowledge of the critical points of the metabolism of the bacterium. Could it be said, then, that inactivation of enzymes was not the cause of bacterial death? Again apparently not, for no studies had been performed with enzyme systems of the entire cell but only with isolated enzymes. Was the assumption of uniform resistance tenable? The evidence that could be presented from disinfectant studies was not conclusive enough either to prove or disprove the above assumption. In a general way however, one would not expect bacteria to act differently from other forms of life and to show a different pattern of resistance. It would seem that the doctrine of uniform resistance followed from the monomorphic presumption of the early bacteriologists that bacteria were perfectly stable, that cell forms remained

constant, that cultural characteristics were immutable, and that cultural reactions were invariable. Variations were dismissed as the result of poor technique. In the light of this assumption differentiation and variation of bacteria would be an impossibility.

Some forms of bacterial variation had been observed to occur spontaneously but these were considered exceptions to the rule of stability. It was difficult to prove in the case of bacteria that such variation was actually an hereditary variation and not merely an adaptation to a changed environment. Luria and Delbrück (32) were the first to prove definitely by a fluctuation test that variations in phage resistance arose as a result of a spontaneous mutation rather than as an adaptation. Newcombe (33) demonstrated that plates cultures which were allowed to incubate and then spread produced a greater number of phage resistant mutants than cultures which were exposed to phage without previous incubation. Since then numerous investigators have demonstrated that spontaneous mutants accumulate during prolonged cultivation. (Stocker, 34; Novick and Szilard, 35; Atwood, Schneider, and Ryan, 36).

Simplified techniques for the detection of such resistant mutants have become routine practice. Some were based on the principle of allowing the growth of mutants but of preventing the growth of the parents. The penicillin method of Lederberg and Zinder (37), and Davis (38) depended upon the destruction

of dividing parent cells by penicillin in a deficient medium in which the mutants persisted. Such mutants were then detected on a complete medium. Gradient plates, (Szybalski and Bryson, 39), easily detected resistant mutants to a variety of chemotheropeutic agents and antibiotics. Other methods were more direct and consisted of the isolation of mutants in the absence of environmental conditions that would favor their establishment. An example of this would be the replica plating technique of Lederberg and Lederberg (40).

The evidence seemed clear that bacterial mutants showing variable resistances did occur. There was, also, evidence concerning the frequency of occurrence of such mutants. The rate of occurrence per bacterium per generation was low, varying from 1×10^{-6} for penicillin resistance of Micrococcus pyogenes var. aureus to 6×10^{-10} for streptomycin resistance of Hemophilus pertussis. (Braun, 41).

It would not be stretching the evidence too far to state that such variation would occur in a bacterial culture during disinfectant testing but that the mechanics of the experiment tended to disregard it. Thus the low mutation rate would account for a low concentration of resistant mutants, and a very high percentage of sensitive forms with a narrow scatter of resistances about the mean. Such a distribution when tested by the ordinary disinfectant testing methods would result in the appearance of logarithmic time-survivor curves. This would then lead the investigator to conclude that the culture was of uniform resistance.

Thus, these early attempts to confirm a theory of disinfection based upon the inactivation of specific enzymes of a cell were not successful. But the counter claims of those who held that such action could not be responsible for death also were not proved. The period from 1926 onward, marked by the first isolation of a pure, crystalline enzyme by Sumner (42) resulted in a change of attitude toward enzymes. They were now known to be proteins. They could be purified of extraneous materials and could be used as relatively pure reagents to study the mechanism of enzyme action. Enzymes were found to be organized into systems, the successful action of which depended not only upon the protein of the enzyme but also upon the presence of such entities as coenzymes, high energy phosphate bonds, inorganic ions, and upon such a vague concept as the organization of the cell. (Dixon, 43).

One of the more successful workers in the field of enzymes attempted a generalization based upon the low concentrations of enzymes found in cells. (Green, 44). This was to the effect that enzymes were present in cells in such low concentrations that they had to be called trace substances. They achieved their effects at such low concentration by acting reversibly at high turnover rates and by being organized into systems that tended to maintain a system in one direction. Consequently, any substance which in low concentration thus affected cells might be conceived of as affecting an enzyme or a system of enzymes. This generalization disregarded the

distinction that was made by pharmacologists between the concentration of drug used and the concentration of drug fixed by the cell. Thus Eichholtz (45) pointed out that although the concentration of copper in a solution may be one part in 10^8 the concentration within algae in such a solution is several parts per 10^4. Despite this oversight, Green and his coworkers (46) proceeded with a demonstration that the action of chlorine upon bacterial cells in water could be explained in terms of the inactivation of enzymes, because of the low concentrations of chlorine used. These investigators insisted that critical inactivation occurred at a single locus -- the sulphydryl group of enzymes possessing such a group. The experimental demonstration was such that a close correlation was first established between bacterial death and the inhibition of glucose oxidation as measured manometrically. Then an examination of isolated enzymes of the glycolytic system established that aldolase was the most sensitive in terms of the amount of chlorine required to inactivate it. But the time-*activity* curve of the inactivation of aldolase never fell below 70 percent inhibition and did not parallel the curve of bacterial death. Despite this, it was concluded that the action of chlorine upon aldolase probably disrupts the balanced system of glucose oxidation resulting in its inhibition and consequently in bacterial death. It seemed that these workers fell into the same error of simplicity as previous workers in trying to

attribute the complex phenomena of bacterial death to the action of chlorine upon a single enzyme in the glycolytic system.

There is another point that had not been taken into consideration. This concerned the criteria involved in disinfectant testing in contradistinction to those involved in the cause of bacterial death. For the purposes of disinfectant testing and for the uses to which disinfectants are put, the failure of micro-organisms to reproduce themselves on the commonly used media is a sufficient and practical criterion of bacterial death. For the purposes of elucidating the mechanism of bacterial death such criteria are insufficient. Thus Heinmets, Taylor and Lehman (47) treated E. coli with chlorine and such treated suspensions were wholly sterile when incubated in nutrient broth. However, survival of from 0.3 percent to 20 percent was obtained when such treated cells were incubated with various metabolites of the tricarboxylic acid cycle either singly or in combination. The evidence of these experiments was such that many enzymes were inactivated by chlorine and not just one enzyme. The provision of several metabolites enabled the damaged organism to resynthesize the enzymes and to re-establish the various cyclic processes more readily than the provision of a single metabolite. It is a question then that the conclusions of Knox, et al., (46) were tenable.

Thus once again an attempt to correlate death of bacteria with enzyme was not successful. But there were several points

in the paper of Knox, et al., (46) that were significant for this thesis. One was the demonstration that sulphydryl enzymes were extraordinarily sensitive to the action of chlorine. The choice of an enzyme for the work of this thesis was thus narrowed down to those containing this group. A purely empirical relationship between bacterial destruction and enzyme inhibition might be established without implying that such enzyme inhibition were the cause of bacterial death. Green and Stumpf (48) had suggested this but did not proceed to that end. Papain - a sulphydryl enzyme - was used and the inhibition of its clotting effect upon milk was used to assay chlorine. But the results were not correlated with bacterial death due to the action of chlorine.

And so we come to the point of this thesis. It is not an attempt to locate the cause of death of bacteria. The action of a disinfectant is not to be thought of as localized at some locus in the cell. It may affect permeability, multiplication, and metabolic activities singly or in any possible combination. The means are not available for determining the point of specific activity if such a point does exist. Instead the attempt is to be made to establish a purely empirical relationship between the inactivation of an enzyme - not even present in the bacterial cell - that is sensitive to chlorine, and the death of a bacterium also sensitive to the same chemical. Chlorine will combine with any protein whether it be part of the bacterial cell or not and the use

of a biochemical agent containing extraneous protein as well as active centres may be thought of an paralleling the existing structures of the bacterial cell - containing, likewise, extraneous protein and active centres. In this manner, discrepancies arising from the use of purely chemical tests for the detection of chlorine might be overcome.

End of Review

III. EXPERIMENTAL METHODS - OUTLINE

IV. EXPERIMENTAL METHODS - EXPLANATIONS

A. Protocols

1. Bactericidal Test

Tubes	1	2	3	Control
Chlorine (buffered) ml.	1	0.5	0.1	0
Water (buffered) ml.	0	0.5	0.9	1.0
Bacterial Suspension (unbuffered) ml.	1.0	1.0	1.0	1.0
Variable Contact Time at 30° C.				
Sulfite-Buffer (pH 7.0) ml.	2.0	2.0	2.0	2.0

Plating by a Surface Plating Method
Incubation for 24 hours at 37°C
Counting of Plates

2. Inhibition of Urease Test

Tubes	1	2	3	Control
Chlorine (buffered) ml.	.5	.25	.10	0
Water (buffered) ml.	0	.25	.40	.5
Urease (unbuffered) ml.	.5	.5	.5	.5
Variable Contact Time at 30°C				
Urea-Sulfite Buffer (pH 7.0) ml.	1	1	1	1
Incubation Time at 30°C - 15 minutes				
N-HCl	1	1	1	1

Nesslerization of Portion of Reaction Mixture

Photelometric Measurement of Nessler Reaction

The protocols are presented here to provide an introduction to the nature of the tests used. Following is a more detailed description of the methods used and some of the reasons for using them.

B. Chlorine

Chlorine was prepared as a concentrated solution of chlorine gas by reacting potassium permanganate with concentrated hydrochloric acid in a suitable generator under a hood. The gas so generated was passed through two flasks of distilled water and then collected in a third flask of double distilled water. This latter was transferred to a dark brown, glass stoppered bottle and stored in a refrigerator at about 5°C. Its uses were to prepare test solutions of chlorine in zero demand water and to prepare the zero demand water.

C. Glassware

All the pyrex glassware that was to be used in any test involving chlorine was used only for this purpose and was washed in a prescribed manner. That used to store zero demand water was treated several times with chlorinated water and then it was used for no other purpose. The four ounce bqttles for the preparation of test chlorine solutions were treated similarly. Test tubes and pipettes were washed without detergents in tap and distilled water, autoclaved if necessary, and soaked in chlorinated water before using. The

test tubes were dried in an inverted position in a 37° incubator. The pipettes were dried in a vertical position. In addition any pipette used to deliver a chlorine solution was rinsed several times with the chlorine solution before an actual transfer was made.

Wherever possible glass stoppered bottles were used for all chlorine solutions. If not possible, paper closures secured with rubber bands were used. No cork or rubber stoppers were ever used on a bottle containing a solution of chlorine.

D. Water

The water used in this investigation had to be prepared with the following points in mind: the water had to possess a zero chlorine demand for any test procedure that involved the use of chlorine. The water had to be freeeof any heavy metal ions that have been proved to be inhibitory for urease. (Sumner and Somers, 49). For the urease experiments double distilled water would be needed. It was also used where small amounts of zero demand water were required in the bacterial experiments. Where large amounts of zero demand water were required, singly distilled water was used.

E. Zero Chlorine Demand Water

A method for preparing zero chlorine demand water is described with a minimum of detail in APHA Manual (50a). It was prepared [redacted] in the following manner for both double distilled and singly distilled water: large amounts were

started by chlorinating two and one-half liters of water in three liter Erlenmeyer flasks to give a chlorine residual of 3-5 ppm. The flasks were closed with paper covers and stored in a dark cabinet for several days. For the preparation of buffer solutions, or zero demand water in small, known volumes, this treated water was transferred to smaller bottles, re-chlorinated to give a residual of about 2 ppm. and stored again in a dark cabinet. Prior to the use of water as zero demand water, any excess chlorine was removed by exposing the small bottle to sunlight for about three hours or to germicidal ultra-violet light for about eight hours. Water prepared in this manner was found to possess neither residual chlorine nor chlorine demand.

This rather involved procedure had been followed to remove from the water any extraneous substances with which chlorine might react. By this method the germicidal activity of chlorine could be directed wholly against the experimental objects that were used.

Chlorine can react with many types of substances such as reducing agents; non-nitrogenous, organic compounds; thiocyanates; proteins and peptides; amino acids; and ammonia. The presence of such substances would reduce the amount of chlorine that would be available for action against microorganisms. In this investigation, the distilled water used was presumed to contain ammonia. The amount of chlorine that reacted with this ammonia and that would be removed from the

water as a result of the reaction is called the chlorine demand of the water. This action in an excess of chlorine results in the eventual chlorination of the ammonia to a volatile nitrogen trichloride and in a residual of chlorine called free chlorine. The destruction of this residual by sunlight provides a water free of any ammonia or chlorine. Chlorine may now be added so that its concentration is proportional to the volume of concentrated chlorine used and so that it is present in the form known as free chlorine. This latter is conceived of as existing in water as an equilibrium mixture of hypochlorous acid and hypochlorite ion. This was the form of chlorine sought after by means of the rather involved procedure described above. It is analogous to the use of an analytical, reagent grade chemical. It provides a known factor in the investigation. Free chlorine is expected to act to the full extent of its concentration against the objects presented to it. Chlorine that exists in combination with ammonia or organic nitrogen is called combined chlorine. This is a far less suitable reagent and was not used. (Phelps, 51).

F. Measurement of Chlorine Concentrations

The methods by which chlorine concentration is usually measured is some form of an oxidation-reduction reaction. In this investigation concentrations of chlorine were measured in distilled water. Under these conditions any of the chemical methods would be satisfactory. The one chosen was the

amperometric method which gives values of chlorine concentration accurate to tenths of a part per million (ppm.) and which enabled one to distinguish clearly between free and combined chlorine. The amperometric titrator and the method of its use is completely described by Wallace and Tiernan (52).

Test concentrations of chlorine were made by adding drops of concentrated chlorine to 100 ml. of buffered, zero demand water. Twenty-five ml. of this test solution was diluted to 200 ml. with zero demand water. This diluted chlorine solution was titrated with a prepared phenylarseneoxide solution in the amperometric titrator. At the end point, the volume in milliliters of the arsenic solution used, was equivalent to the ppm. of free chlorine. This was multiplied by eight to compensate for the dilution of the test solution. The dilute solution could be titrated further for the presence of combined chlorine. One drop of five percent potassium iodide was added to the solution whose free chlorine content had just been determined. Phenylarsenoxide was again used to titrate to another end point. If combined chlorine were found to be present in an amount greater than .01 ppm. the test solution was discarded and a new one prepared.

A satisfactory test solution, containing free chlorine in a known concentration was used in either a bactericidal or the enzyme inhibition test within 10 minutes of its preparation and within five minutes of the measurement of its concentration. It was used only once.

G. Buffers

All buffers used in the experimental work were phosphate buffers. In the range of their most effective buffer action the salts, potassium acid phosphate and dipotassium phosphate were used. Each of these was prepared as an M/15 solution, using double distilled, zero demand water. Only 100 ml. of such stock solutions was made up at any one time to eliminate long storage periods and possible mold growth. Usually such an amount lasted for ten days. During that time it was stored at 5°C. For pH values outside the most effective range of the phosphate buffer salts, phosphoric acid was used with potassium acid phosphate for the lower pH range and potassium hydroxide was used with dipotassium phosphate for the higher pH range.

The buffer solutions that were to receive chlorine and that were used as buffered water in the protocols were made up as follows:

pH	H_3PO_4(M)	KH_2PO_4(M/15)	K_2HPO_4(M/15)	KOH(M)	WATER (zero demand double dist.)
4.0	0.1 ml	4 ml	----	----	96 ml
7.0	--	1.9 "	2.1 ml	----	96 "
7.5	--	1.0 "	3.0 "	----	96 "
9.0	--	---	4.0 "	0.1 ml	96 "

The above made buffers were chlorinated to a residual two ppm. to secure zero demand and to disinfect the buffer solutions. Such solutions were stored in the dark for several days. They

were exposed to sunlight or ultraviolet light before use. Measurements for pH were made before chlorination and just before use. Differences of pH amounting to one-tenth of a unit were found usually in the direction of a lower pH. The zero demand buffers were used to prepare test concentrations of chlorine as described under F.

The buffer solutions that were used to neutralise chlorine-bacteria mixtures or to neutralise chlorine-urease mixtures were of a higher buffer index and were of pH 7.0. In view of the dual purpose of such buffers it was deemed simpler to continue the use of zero demand, double distilled water in their preparation.

The sulfite buffer, pH 7.0, for the bactericidal test was made as follows:

Potassium Acid Phosphate (M/15)	10 ml.
Dipotassium Phosphate (M/15	10 "
Sodium Sulfite (0.5 gm/100 ml)	10 "
Water	20 "

Fifty ml. were prepared in glass stoppered bottles. Pipettes used for transferring were sterile. Sodium sulfite solution had previously been autoclaved. Only enough for one day's testing was prepared and was discarded at the end of the day's work.

The urea-sulfite buffer pH 7.0 for the enzyme inhibition test served the additional function of providing a substrate for the enzyme that had not been inactivated by the action of chlorine. It was made up as follows:

Potassium Acid Phosphate M/15	10 ml.
Dipotassium Phosphate M/15	10 "
Sodium Sulfite (0.5 gm/100 ml)	10 "
Urea (20 gms/100 ml)	10 "
Water	10 "

Fifty ml. were prepared in glass stoppered bottles. Pipettes used for transferring were sterile. Sodium sulfite solution had previously been autoclaved. The urea solution had been kept under refrigeration for one month before use. Only enough for one day's testing was prepared and was discarded at the end of the day's work.

H. The Objects of the Action of Chlorine

1. Bacteria. One species of bacterium, E. coli, strain C198, originally obtained from the Environmental Health Center of the U. S. Public Health Service was used. The stock culture was subcultured every three weeks on nutrient agar slants, Difco (53a). The inoculated slants were incubated for twenty-four hours at 37°C. The culture was then sealed by impregnating the cotton plug of the culture tube with paraffin. The sealed culture was stored in the dark at room temperature for three weeks and then subcultured.

A working culture was prepared in nutrient broth, Difco (53b). This was started from one of the slants mentioned above. After twenty-four hours of incubation at 37°C transfers were made by sterile pipette for the purpose of subculturing into nutrient broth and of preparing a culture for use in the bactericidal test. Subculturing was performed every twenty-four hours. Cultures for bactericidal testing

were prepared the day before such tests were to be run. One tenth ml. of the nutrient broth culture was pipetted onto the surface of a sterile, nutrient agar slant in a 16 x 150 mm pyrex tube. The inoculum was distributed over the entire slant by tilting the tube. The inoculated tube was incubated in an horizontal position for 24 hours at 37°C.

Previously a McFarland Nephelometer, (Kolmer, 54), had been prepared. This was a series of tubes containing different concentrations of barium sulfate. The graded turbidities of the successive tubes are taken to indicate different numbers of bacterial cells per ml. Also, some Pasteur capillary pipettes had been plugged, individually wrapped, and sterilized. Finally, 10 ml. of zero demand water, containing no chlorine, had been dispensed in tubes the same size as the nephelometer.

With a capillary pipette, to which a rubber bulb had been attached, some of the water was removed from one of the tubes containing 10 ml. of zero demand water. This water was delivered to a slant containing a 24-hour growth of the test organism. This growth was quickly washed off the slant - within 30 seconds. With the tube held horizontally and the slant uppermost, the capillary pipette was inserted into the small volume of thick suspension and manipulated to take up and expel the suspension rapidly for a two-minute period. This procedure was used in order to break up clumps of cells and prepare a suspension of single cells. The entire suspension was then drawn up into the pipette.

The suspension was added drop by drop to the same tube from which the small amount of water had been withdrawn. Comparison with the proper nephelometer tube was made after the addition of each drop. The process continued until a satisfactory match had been obtained.

This standard suspension of micro-organism was usually between 100 x 10^6 and 300 x 10^6/ml. It was the suspension upon which bacterial nitrogen measurements were made directly. It was also the suspension from which suitable dilutions were made for the bactericidal test. Consequently the bacterial nitrogen content present in the bactericidal test was derived by multiplying the bacterial nitrogen determination figure by certain dilution factors. The usual dilution factor was 1000.

2. Enzyme, Urease. The enzyme used in these experiments was urease. As noted in the review, sulphydryl enzymes are especially sensitive to chlorine. Urease possesses sulphydryl groups and gives tests for such groups, (Sumner and Poland, 55). Crystalline urease was prepared from Arlco jack bean meal by the method of Sumner as described by Sumner and Somers (56). The purification did not proceed further than the first crystallisation due to the sensitivity limits of the method used for the detection of organic nitrogen. As soon as the solution of these crystals had been obtained, dilutions of it were made and these were tested in the following manner:

Urease Dilution	0.5 ml.
Buffer pH 7.0	0.5 "
Urea-Sulfite Buffer	1.0 "
Incubation 30°C 15 minutes	
N-HCl	1.0 "

Nesslerisation of a portion of the acid reaction mixture
Photelometric reading of the Nessler Reaction

That dilution which produced a photelometer reading of between 45-50 with a 0.3 - 0.4 ml. portion of the acid reaction mixture was chosen as satisfactory for the tests to follow. This was found to be a 1/125 dilution. The entire lot of crystalline urease solution was then diluted in 125 ml. batches using zero demand water free of chlorine. The diluted urease was dispensed in six ml. amounts in four inch test tubes. Each tube was corked and the entire lot was quickly frozen and kept frozen at about -5°C. For use, only the required volume of urease was thawed. It was used once and discarded.

The urease nitrogen figure was derived from determination upon various volumes of the diluted preparation. This diluted preparation was also used in the enzyme inhibition tests.

I. Nitrogen Determination

The usual organic nitrogen determinations by the Kjehldahl technique detect 0.5 mg. or more of organic nitrogen. The method desired for this work was the one which would detect at least .05 mg. and possibly .02 mg. The method chosen was that described in Manual TM8-227 (57). This was a colorimetric

method that was described as capable of detecting .05 mg. to 0.2 mg. of nitrogen. In its application it was variously modified.

The ammonia formed by the digestion with sulfuric acid and catalysts of a sample containing 0.05 - 0.2 mg. of nitrogen was estimated from the color obtained by adding Nessler's reagent to the diluted digest. It was recommended that potassium persulfate be used during the final stages of the digestion to hasten the oxidation of organic matter. However, the samples of persulfate that were available contained more nitrogen than the sample being analysed. It was, therefore, omitted. A uniform procedure of digestion with sulfuric acid and copper sulfate was used for all samples. Fifteen minutes from the time of the appearance of the first dense, white fumes was found to give a water clear digest. Silica chips, glass beads or any other form of anti-bump material was eliminated as it proved to be but another source of extraneous nitrogen. Careful heating over a small flame prevented any accidents due to bumping. Large pyrex tubes, 25 x 200 mm., were used.

Nessler's reagent was prepared as described in the APHA Manual (50c). The water clear digest was allowed to cool. Twenty-five ml. of recently boiled and cooled distilled water was added to the digest. Five ml. of Nessler's Reagent was added as the tube was undergoing a swirling motion. Twenty ml. of water made the final volume 50 ml. For organic nitrogen determinations five ml. of Nessler's reagent was found

necessary in order to completely neutralise the acid of the digest.

The mixture was allowed to stand for ten minutes and then examined in a Cenco Photelometer equipped with a number 65 Wratten gelatin film filter. A blank prepared by digesting and Nesslerizing the reagents was run along with the sample.

The procedure described in Manual TM8-227 (57) did not prescribe any definite filter but recommended any suitable one in the range from 450 to 500 millimicrons. Three such filters were tested using Nessler's reagent and standard ammonium chloride. Data for the filters was obtained from "Wratten Light Filters," a data book of the Eastman Kodak Company, and are presented in Table I along with the photelometric determinations. These latter are graphed in Figure 1. Number 49 filter proved to be too sensitive and the plotted points non-linear. Number 47 filter proved to be too insensitive and the plotted points, slightly non-linear. Number 65 filter proved to have a sensitivity intermediate in value and the plotted points resulted in a straight line over the concentration of nitrogen measured. The data of Table 1 for the number 65 filter were replotted in Figures 2 and 3. Assuming a zero value of nitrogen for the value of the blank, a table was created and inserted in each figure giving nitrogen values equivalent to photelometric readings. These standard values were used to obtain all values of organic and urea nitrogen in subsequent tests.

(continue reading on page 41)

TABLE 1

CHOICE OF SUITABLE FILTER

Standard Ammonium Chloride Solution - 0.1mg NH_3-N/ml

Filter		47		49		65		
Transmittance		2.8%		0.7%		10%		
Dominant Wave Length		464 mu		458 mu		497 mu		
Range		400-530 mu		400-500 mu		420-570 mu		
Stability		sl. unstable		rel. stable		rel. stable		
Diaphram Opening		9		14		7		
	Mg. NH_3-N	1 ml N.R.*		1 ml N.R.*		1 ml N.R.*		5 ml. N.R.*
		Read.	Log	Read.	Log	Read.	Log	Read. Log
Reagent Blank	---	96	1.9823	95	1.9777	97	1.9868	97 1.9868
	.005	92	1.9638	92	1.9638	94	1.9731	94 1.9731
	.01	88	1.9445	89	1.9494	92	1.9638	91 1.9590
	.02	84	1.9243	86	1.9345	89	1.9494	
	.025							88 1.9445
	.03	79	1.8976	84	1.9243	87	1.9395	
	.04	74	1.8692	79	1.8976	83	1.9191	
	.05	69	1.8388	76	1.8808	80	1.9031	78 1.8921
	.06	65	1.8129	74	1.8692	76	1.8808	
	.07	61	1.7853	72	1.8573	74	1.8692	
	.075							70 1.8451
	.08	59	1.7709	70	1.8451	72	1.8573	
	.09	56	1.7482	67	1.8261	69	1.8388	
	.10	53	1.7243	66	1.8195	67	1.8265	64 1.8062
	.12	47	1.6721	64	1.8062	61	1.7853	
	.125							55 1.7404
	.15	41	1.6128	61	1.7853	55	1.7404	48 1.6812
	.17	37	1.5682	59	1.7709	52	1.7160	
	.175							42 1.6232
	.20	34	1.5315	57	1.7559	47	1.6721	39 1.5911
	.25	29	1.4624	54	1.7324	42	1.6232	
	.30	21	1.3222	49	1.6902	34	1.5315	

*N.R. = Nessler Reagent

FIG I
CHOICE OF A SUITABLE FILTER

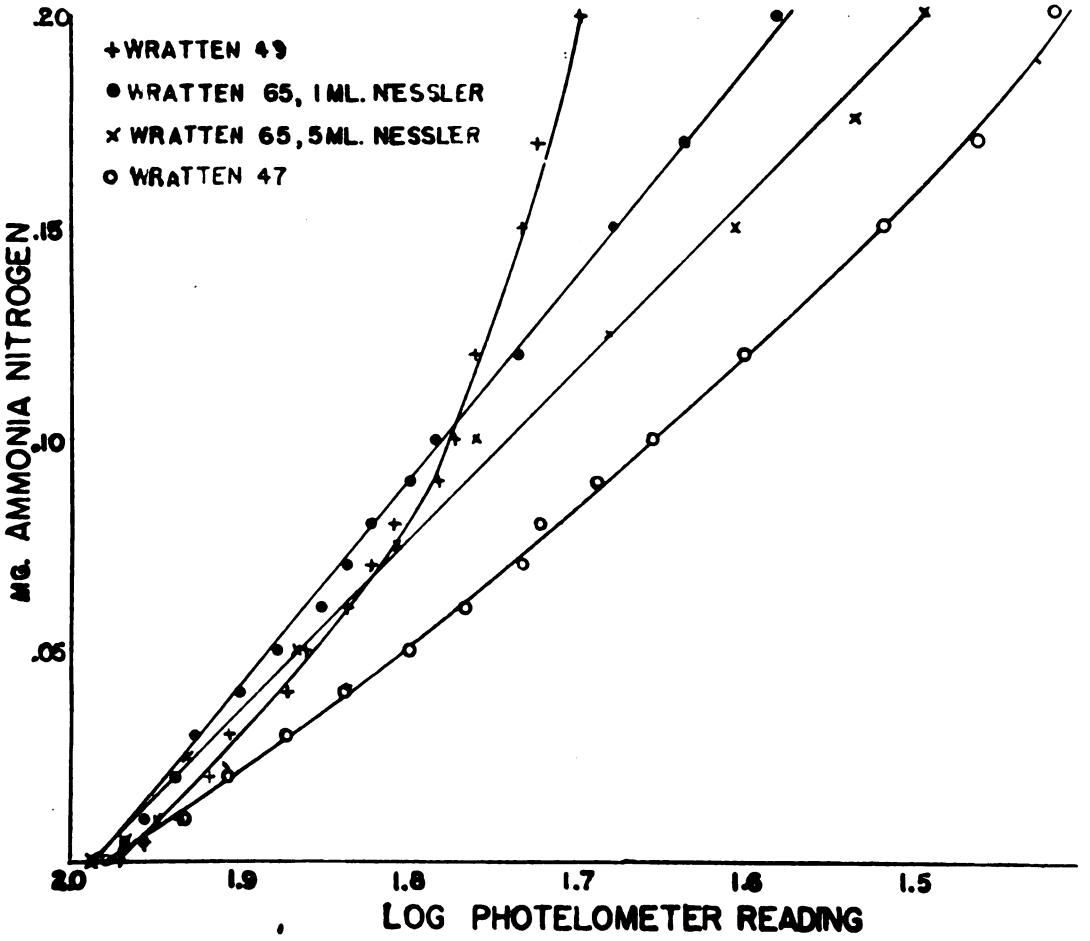


FIG 2

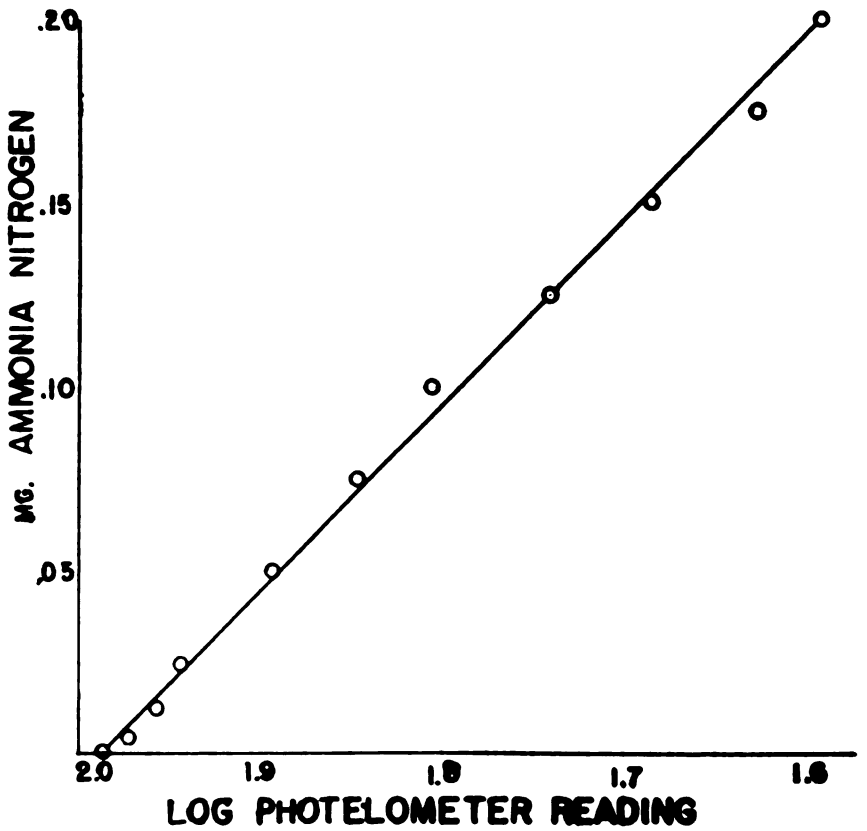
STANDARD CURVE FOR BACTERIAL NITROGEN AND UREASE NITROGEN

AMMONIUM CHLORIDE 0.1 MG. N / ML.

NESSLER SOLUTION 5 ML.

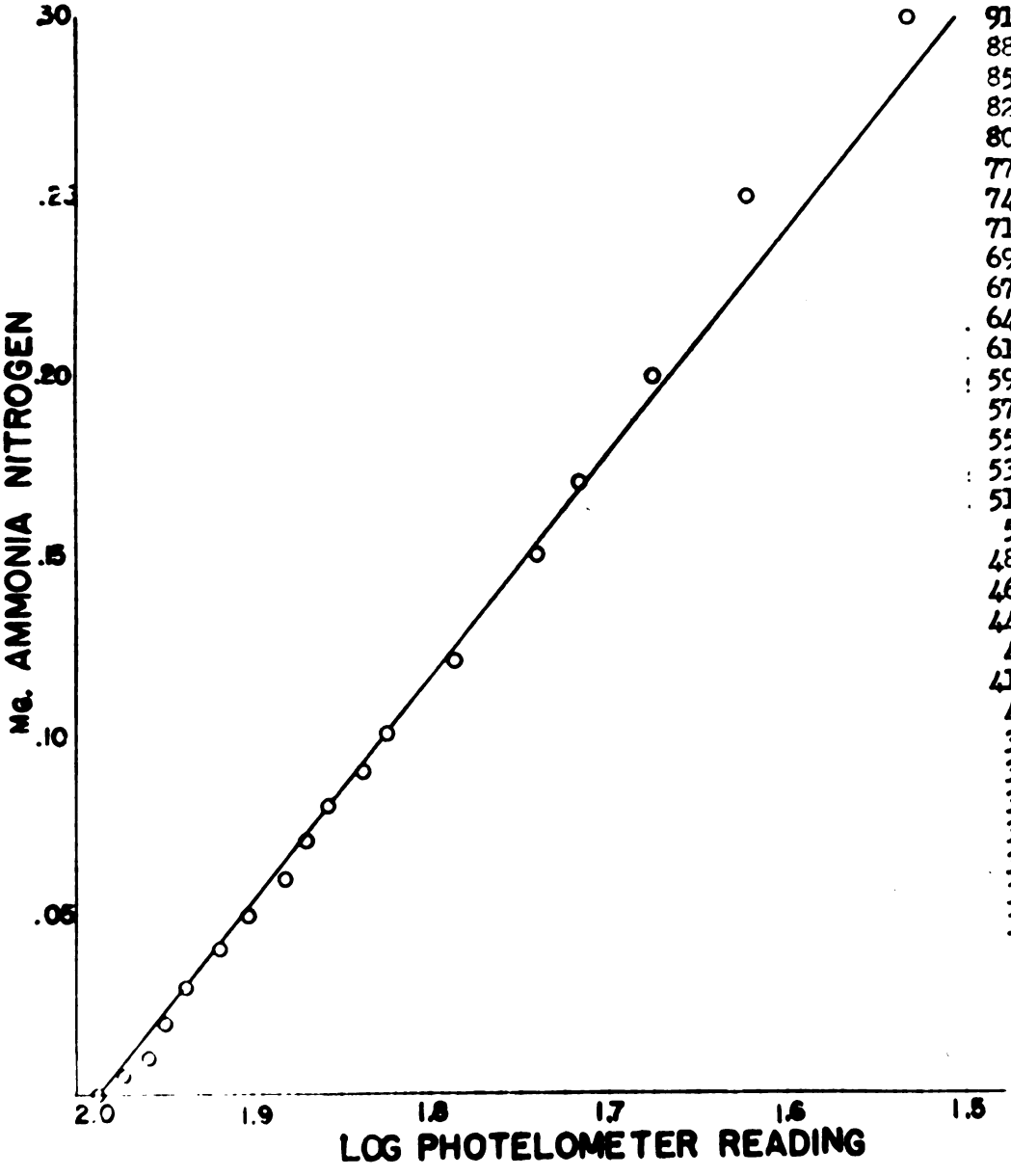
TOTAL VOLUME 50 ML.

PHOTELOMETER WITH WRATTEN 65 FILTER



EQUIVALENTS	
READING	MG.N
97	0
96	.0025
94-95	.005
93	.0075
92	.010
91	.015
89-90	.02
87-88	.025
85-86	.03
83-84	.035
81-82	.04
79-80	.045
77-78	.05
75-76	.055
74-75	.06
72-73	.065
70-71	.07
68-69	.075
67	.08
66	.085
64-65	.09
62-63	.10
59-61	.11
56-58	.12
54-55	.13
51-53	.14
49-50	.15
47-48	.16
45-46	.17
43-44	.18
41-42	.19
39-40	.20

FIG 3
STANDARD CURVE FOR UREA NITROGEN
AMMONIUM CHLORIDE 0.1 MG. N / ML.
NESSLER SOLUTION 1 ML.
TOTAL VOLUME 50 ML.
PHOTELOMETER WITH WRATTEN 65 FILTER



EQUIVALENTS READING	MG. N
97	0
93–96	.005
91–92	.01
88–90	.02
85–87	.03
82–84	.04
80–81	.05
77–79	.06
74–76	.07
71–73	.08
69–70	.09
67–68	.10
64–66	.11
61–63	.12
59–60	.13
57–58	.14
55–56	.15
53–54	.16
51–52	.17
50	.18
48–49	.19
46–47	.20
44–45	.21
43	.22
41–42	.23
40	.24
39	.25
38	.26
37	.27
36	.275
35	.28
34	.29
33	.30

The two determinations for organic nitrogen were those for bacterial nitrogen and urease nitrogen. Five ml. of Nessler's reagent was used. The determination of urea nitrogen as a result of the action of urease upon urea was performed using one ml. of Nessler's reagent.

J. The Bactericidal Test Procedure

Upon completion of the preparatory work described in sections A through I above, the bactericidal and enzyme inhibition tests were set up according to the Protocols A1 and A2. The bactericidal test involved the action of variable chlorine concentrations acting upon a constant number of bacterial cells during six different time periods - 1, 2.5, 5, 10, 20, and 30 minutes. At the end of each time period the action of chlorine was stopped by the addition of the sulfite buffer, pH 7.0. This buffer also changed any adverse pH to a favorable one. As rapidly as possible the contents of each reaction tube were suitably diluted and plated out - five .02 ml. drops of each dilution onto the surface of a dry Eosin Methylene Blue Agar Plate. The medium used was that described in Difco (53c). The inoculated plates were allowed to stand at room temperature for about one hour until the surface inoculated drops dried down. The plates were then inverted and incubated at 37°C for 24 hours. At the end of this period the plates were counted. The numbers of cells were recorded. The numbers of cells present in the medication tubes surviving the action of chlorine were calculated. Log time - log survivor curves

were plotted for different ratios of bacterial nitrogen to chlorine dosage used. This ratio of N/Cl bore the units, micrograms of ammonia nitrogen per micrograms of chlorine.

Suitable dilutions of the medication tubes were based upon the method of counting and the contact time interval of the action of chlorine. The method of plating by drops upon the surface of a plate was described sketchily by Pomales-Lebron and Fernandez (58) and more fully and critically by Reed and Reed (59). Two hundredths of a ml. was the volume dropped upon the plate surface and five such drops were used from each dilution tube. The dilution sought after was one which with a drop volume of .02 ml. would produce five to 75 colonies. In order to be sure to obtain numbers within this range, three dilutions were made from each medication tube. Tubes that had been exposed to low concentrations chlorine for short periods of time were diluted as were the controls. Dilutions became progressively lower as the concentration of chlorine increased and as the time of contact increased. Reed and Reed (59) pointed out that the drop plate method could satisfactorily replace pour plates used for counting micro-organisms from milk and water if sufficient attention was paid to details of the test and if a sufficient number of micro-organisms was present. Further advantages were that it was somewhat more accurate than conventional pour plates, that it gave higher counts, that differential media could be used, and that it was less

laborious. Snyder (60) in a relative study of plate counting methods concluded that it was possible to recommend more extensive use of surface plate counting methods which were conducive to a greater replication of plates. The drop plate method was considered a satisfactory procedure for this research problem as it gave a consistent body of data. It was felt that a greater accuracy was achieved by counting one plate with five drops than would have been achieved by counting two pour plates. And this feeling was no doubt accentuated by the fact that less work was involved.

The incubation of plates for 24 hours at 37°C was considered standard practice. The drop plate procedure, however, produced countable colonies in 12 hours. If contamination could be ruled out these counts were as valid as those at 24 hours. However, the colonial appearance of the early colonies was not distinctive enough for this purpose. Only the results of the count at 24 hours were presented in the data tables. Drops that presented between 5 and 75 separate colonies were counted. The average of the five drop-counts was entered into the data sheet. These counts were then corrected for various dilutions to give the actual number of organisms present during the bactericidal test.

Once the data were obtained, how could they be presented significantly? The usual method of plotting the results of experiments, involving the killing of micro-organisms, would be to use arithmetic time units as abscissae and logarithmic

percentage survival units as ordinates. This method of presenting data from disinfectant testing originated with Madsen and Nyman (21) who used the data of Krönig and Paul (22). Chick (23) confirmed this exponential relationship and postulated it as a universal law of disinfection. For micro-organisms at least, death was supposed to occur to a definite percentage of the survivors in each succeeding time period. The multitude of investigations that followed only partly confirmed this relationship. However, the tendency of the investigators was to assume that the exponential relationship was the correct one and that the discrepancies observed were due to the conditions of performing the experiment, the nature of the bacterial suspension, or the peculiar nature of the bacterial cell at a certain period. The types of curves found by these investigations fell into three classes:

Exponential time-survivor curves;

Sigmoid time survivor curves;

Exponential time-survivor curves with an initial lag phase.

These classes of curves were obtained with individual data or with data representing the means of large numbers of determinations. Thus, it was not just the experimental data or the conditions of the experiment that were at fault. The basic assumptions made by those who postulated an exponential relationship of bacterial death were that the organisms of a culture were essentially uniform in resistance; that their

death was due to one event; and that the times of survival were not normally distributed. Could the data of the non-exponential relationships be explained away by such simple assertions that the organisms were clumped together or by such complex assertions that the nature of such reactions changes under the experimental conditions or that the resistance of the organism presents different aspects during the killing process?

Data from other sources than bacteriology indicated that variation is a rather fundamental attribute of living matter. Therefore the postulation that a culture of bacterial cells was of uniform resistance would run counter to the expected variation of living organisms. In line with this viewpoint, the resistances exhibited by a culture of micro-organisms would be a variable. The length of time that any one organism could survive in a bactericide would be proportional to its resistance. But the objection was raised that if such survival times were plotted as frequency distributions then extremely skewed curves of resistance are obtained. Thus it was argued by the proponents of the exponential relationship that there was no normal distribution of resistances but rather, there was only one uniform resistance. However, this argument supposed that when any attribute were measured then this attribute itself had to be normally distributed. That this was not necessarily so had been pointed out by Gaddum (61). In many cases it was not the attribute itself but the logarithm

of the attribute that was distributed normally. Withell (62) proceeded to show that by adopting this point of view all of the previous conflicting data could be harmonized and the dynamics of bacterial death was brought into conformity with that of other forms of life. The plot of log time-survivor curves from all types of data produced but one type of symmetrical S-shaped curve from which, normally distributed frequency diagrams of survival times could be plotted. Thus, resistances of a culture were not essentially uniform but variable. The degree to which these varied would be expressed by the slope of the S-shaped curve. When the slope of this curve was steep then the variation in the resistance of the culture would be small. The bulk of the resistances would be clustered about the mean. This type of distribution in a culture had provided the exponential curves of the experimenters. If, however, the slope of this curve was flat, then the variation in resistance would be great. A much larger percentage of organisms would possess resistances far from the mean. Such cultures would give sigmoid curves and curves with a lag phase preceeding the logarithmic phase.

For the plotting of curves derived from the data of the bactericidal test a logarithmic time scale was used to conform with the above point of view. A logarithmic survivor scale was used as a matter of convenience. Its use enabled the plotting of four decade cycles of survival from 100

percent to .01 percent on one piece of paper. The significance attached to the log time scale could be given to the log survivor scale.

L. The Enzyme Inhibition Test

The protocol A.2. indicates the analogy between the bactericidal and enzyme inhibition tests as performed in this work.

At the outset, though, a difference in the manner that enzyme inhibition test was used in this investigation from the usual enzyme inhibitor tests might be stressed. Enzyme chemists provide optimal conditions for the activity of the enzyme and note inhibition under such conditions. (Hellerman, 63). The enzyme inhibition test used here subjected the enzyme to adverse conditions and then restored optimal conditions for the measurement of the activity remaining. Thus, it was not analogous to the tests chemists have performed but it was analogous to the tests that bacteriologists have performed. It was the latter point of view which was held to be significant for this thesis.

Concerning the test itself, similar precautions were taken in so far as zero demand, double distilled water was involved. Precautions concerning sterility were relaxed during the final phases of the test which lasted about an hour. There was less flexibility in this test in so far as organic nitrogen was concerned, since once the enzyme concentration had been standardized it could not be altered. Thus,

although one was working with a simpler system, the data obtained were not so widely distributed as that of the bactericidal test.

Instead of 24 hour incubation of the bactericidal test, 15 minute incubation with a urea substrate took place. To insure that measurements of activity remaining occurred only during this 15 minute interval, normal hydrochloric acid was added to stop the enzyme-substrate reaction at the end of this period.

The degree to which the activity of the enzyme had been impaired by its exposure to chlorine was measured indirectly. After a stated contact period, optimal conditions were restored and the enzyme was allowed to act upon its substrate. The amount of ammonia nitrogen liberated from the substrate was taken to be proportional to the percent activity remaining in the enzyme preparation. The method for obtaining ammonia nitrogen values was based upon the standard curve for one ml. of Nessler's reagent (Figure 3) from which an uncorrected value for ammonia nitrogen was obtained. This was corrected for the volume of reaction mixture and for the portion of the sample used in the determination. Finally the value was converted to mg. of ammonia nitrogen per ml. of urease:

$$\text{Uncorrected mg.}NH_3\text{-N} \times \frac{\text{volume of test (ml.)}}{\text{portion analysed (ml.)}} \times \frac{1}{\text{urease (ml.)}} \qquad \text{....1}$$

Comparison with the control value provided the figure for the percent activity remaining. Again, the logarithms of these values were used to provide the plotting of four decade cycles from 100 percent to .01 percent activity remaining. In accord with the discussion under the bactericidal test the logarithms of the time intervals were plotted. These curves were plotted for different values of the ratio of organic nitrogen in the urease preparation to the dosage of chlorine used. This was expressed as an N/Cl ratio.

End of Experimental Methods

V. EXPERIMENTAL DATA - EXPLANATIONS

A. Explanation of the Tables Containing the Data of the Bactericidal and Enzyme Inhibition Tests as Well as Calculations Made From Such Data

The tables which follow are in three parts - the left hand side contains experimental data, the right hand side contains derived data, the lower part of the tables of the bactericidal test contains additional experimental data.

The left hand side of tables for the bactericidal test uses chlorine concentration as the main horizontal heading and contact time as the main vertical heading. For each contact time there is a subdivision of dilutions to which each medication tube was subject. The figures within the body of this portion are averages of actual counts made. The lines of data in the lower part give the control count, and the amount of bacterial nitrogen. The main horizontal headings of the right hand portion are the N/Cl ratios of the corresponding medication tubes of the experimental data. The vertical headings summarize the experimental data for each contact time by providing the count in each medication tube during the actual contact time; the percent survival that such a count represents when compared to the control count; and the logarithm of the percent survival. The general plan of the enzyme inhibition test table is similar to that of the bactericidal test

but the details that are inserted are different. The main difference to be noted is the subdivision of the contact time. Here in place of dilutions there are the volume of acid digest mixture used for Nesslerization (V), the photelometer reading (R), the uncorrected nitrogen equivalent of the reading (Un), and the corrected nitrogen equivalent of the reading (Corr). The right hand portion of the table summarizes the observed data under N/Cl headings. Percent Activity Remaining is obtained by dividing each corrected nitrogen value by the control value. Log_{10}%AR is the logarithm of this number.

The sole organism used in the bactericidal test was E. coli, strain C198.

The sole enzyme used in the enzyme inhibition test was once crystallised urease.

B. Explanation of the Graphs that Were Constructed From The Tables of the Bactericidal and Enzyme Inhibition Tests

For both tests the abscissae are logarithms of the contact time interval expressed in minutes. For the bactericidal test the ordinates are logarithms of the percent survival. For the enzyme inhibition test the ordinates are logarithms of the percent activity remaining. Each line represents the complete vertical summary data under one N/Cl ratio. This ratio is printed near each curve.

Negative numbers have been derived by subtracting the positive mantissa from the negative characteristic of the logarithm.

Extrapolations were made from the curves that actually fitted the points. Such extrapolations are represented by dashed lines. Two such dashed lines are appended to each solid curve wherever such extrapolation falls in with the observed data. One is to the line of .01 percent survival (99.99 percent kill). The other is to the point of 100 percent survival (0 percent kill). These extrapolations will be treated further in the discussions.

VI. BACTERICIDAL TEST DATA

TABLE 2

BACTERICIDAL TEST pH 4.0 30°C

Tubes			1	2	3	4	Tubes	1	2	3
Chlorine ppm			1	.05	0.1	---	N/Cl	.010/1	.010/.5	.010/.1
							N/Cl	.01	.02	.1
Contact Times		Count Dilutions								
1 min.		10^0	20	59			Count x 10^{-5}	.02	.06	3.5
		10^{-1}	3	6	>100	>100	% Surv.	.6	1.7	100.
		10^{-2}	0	0	35	29	Log_{10}%S	-.22	.23	2.
		10^{-3}	-	-	3	4				
2.5	"	10^0	3	11			Count x 10^{-5}	.003	.01	3.5
		10^{-1}	0	2	>100		% Surv.	.09	.3	100.
		10^{-2}			36		Log_{10}%S	-1.05	-.52	2.
5	"	10^0	5	26			Count x 10^{-5}	.005	.03	3.1
		10^{-1}	0	0	>100		% Surv.	.14	.8	90.
		10^{-2}			31		Log_{10}%S	-.85	-.10	1.95
10	"	10^0	0	7	>100		Count x 10^{-5}	0	.007	2.7
		10^{-1}	0	1	>100	>100	% Surv	0	.2	77.
		10^{-2}			27	34	Log_{10}%S	>2.0	-.70	1.88
20	"	10^0	0	2	>100		Count x 10^{-5}	0	.002	.15
		10^{-1}	0	0	15		% Surv.	0	.06	4.3
		10^{-2}			2		Log_{10}%S	>2.0	-1.22	.63
30	"	10^0	0	0	34		Count x 10^{-5}	0	0	.04
		10^{-1}		0	4	>100	% Surv.	0	0	1.
		10^{-2}			0	35	Log_{10}%S	>2.0	>2.0	0.0

Bacterial numbers used for bactericidal test (control count) = 3.5×10^5

Bacterial Nitrogen a) Determinations

Volume of Suspension	Photelometer Reading			$MgNH_3$-N		$MgNH_3$-N/ml	
2 ml	84	83	81	.035	.04	.018	.02
4 ml		66		.085		.021	

b) Calculated Amount Present during bactericidal test

.02 mg/ml x 10^3 x .5 x 10^3ug/mg = .010 ug/ml

TABLE 3

BACTERICIDAL TEST pH 4.0 30°C

Tubes			1	2	3	4	Tubes	1	2	3
Chlorine ppm			0.4	0.2	.04	—	N/Cl	.009/.4	.009/.2	.009/.04
							N/Cl	.022	.044	.22
Contact Times		Count Dilutions								
1	min.	10^0	22	48			Count x 10^{-5}	.02	.05	2.8
		10^{-1}	0	3	>100	>100	% Surv.	.7	2.	93.
		10^{-2}			28	26	Log_{10}%S	-.15	.30	1.97
		10^{-3}				2				
2.5	"	10^0	5	12			Count x 10^{-5}	.005	.01	2.8
		10^{-1}	1	0	>100		% Surv.	.15	.3	93.
		10^{-2}			28		Log_{10}%S	-.82	-.52	1.97
5	"	10^0	0	0			Count x 10^{-5}	0	0	3.0
		10^{-1}	0	0	>100		% Surv.	0	0	100.
		10^{-2}			30		Log_{10}%S	>2.0	>2.0	2.0
10	"	10^0	0	0			Count x 10^{-5}	0	0	3.0
		10^{-1}	0	0	>100	>100	% Surv.	0	0	100.
		10^{-2}			30	32	Log_{10}%S	>2.0	>2.0	2.0
20	"	10^0	0	0			Count x 10^{-5}	0	0	2.3
		10^{-1}		0	>100		% Surv.	0	0	77.
		10^{-2}			23		Log_{10}%S	>2.0	>2.0	1.85
30	"	10^0	0	0			Count x 10^{-5}	0	0	1.2
		10^{-1}		0	>100	>100	% Surv.	0	0	40.
		10^{-2}			12	29	Log_{10}%S	>2.0	>2.0	1.6

Bacterial numbers used for bactericidal test (control count) = 3.0×10^5/ml

Bacterial Nitrogen a) Determinations

Volume of Suspension	Photelometer Reading		$MgNH_3$-N		$MgNH_3$-N/ml	
2 ml	85	83	.03	.035	.015	.018
4 ml	69	68	.075		.019	

b) Calculated amount present during bactericidal test

.018 mg/ml x 10^{-3} x .5 x 10^3 ug/mg = .009 ug/ml

FIG 4
BACTERICIDAL TEST PH 4.0

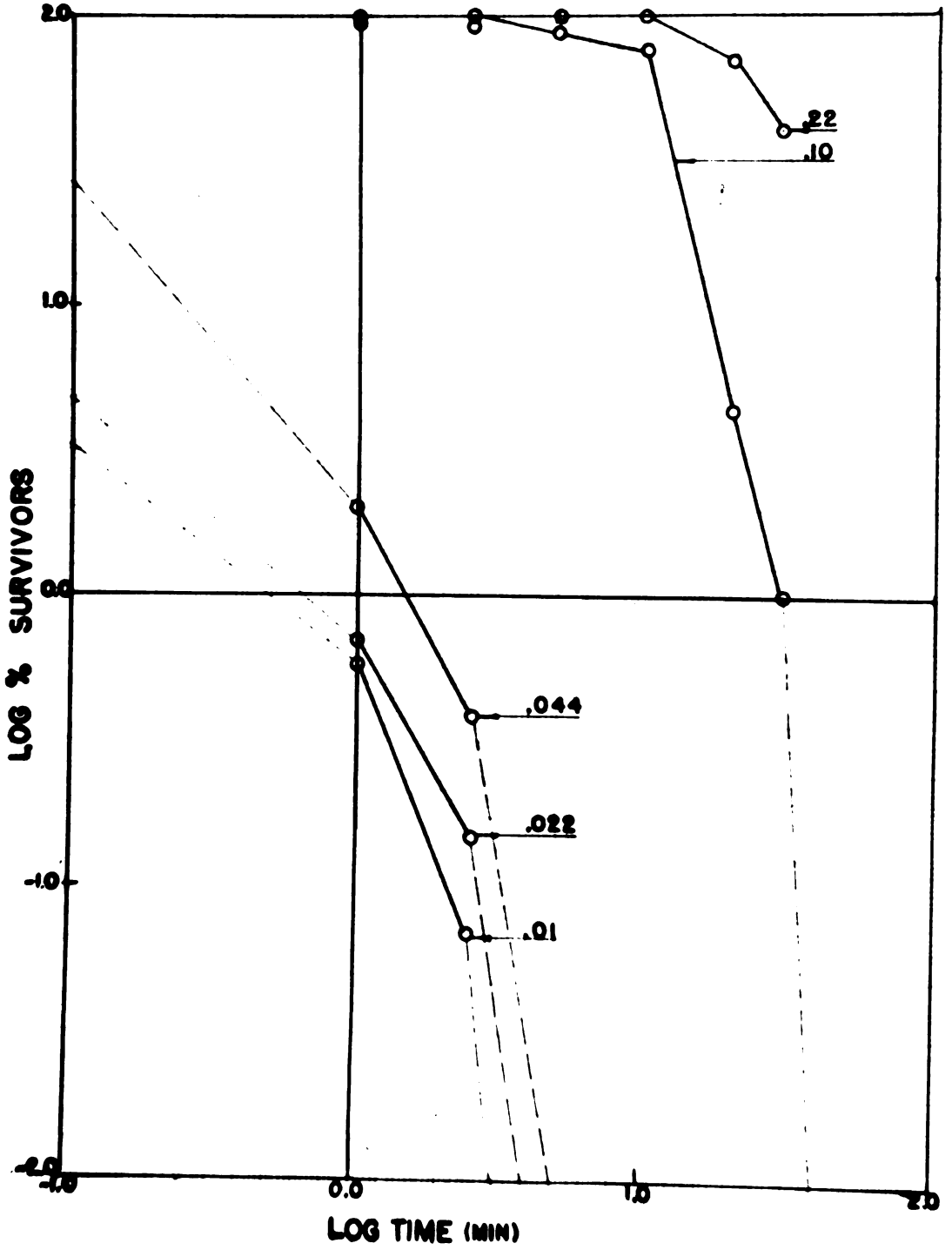


TABLE 4

BACTERICIDAL TEST pH 7.0 30°C

Tubes		1	2	3	4	Tubes	1	2	3
Chlorine ppm		1.2	0.6	.12	0	N/Cl	.008/1.2	.008/0.6	.008/.12
						N/Cl	.007	.014	.07
Contact Times	Count Dilutions								
1 min.	10^0	9	85			Count x 10^{-5}	.09	.1	2.
	10^{-1}		10			% Surv.	4.	5.	100.
	10^{-2}		1	21	22	Log_{10}%S	.6	.7	2.
	10^{-3}			2	3				
2.5 "	10^0	0				Count x 10^{-5}	0	0	1.8
	10^{-1}		0			% Surv.	0	0	90.
	10^{-2}		0	18		Log_{10}%S	>2.0	>2.0	1.95
	10^{-3}			1					
5 "	10^0	0				Count x 10^{-5}	0	0	1.7
	10^{-1}		0	TMTC		% Surv.	0	0	85.
	10^{-2}		0	17		Log_{10}%S	>2.0	>2.0	1.93
10 "	10^0	0				Count x 10^{-5}	0	0	1.5
	10^{-1}		0	TMTC		% Surv.	0	0	85.
	10^{-2}		0	15	20	Log_{10}%S	>2.0	>2.0	1.93
20 "	10^0	0	0			Count x 10^{-5}	0	0	.2
	10^{-1}		0	12		% Surv.	0	0	6.
	10^{-2}			2		Log_{10}%S	>2.0	>2.0	.78
30 "	10^0	0	0	50		Count x 10^{-5}	0	0	.05
	10^{-1}		0	6		% Surv.	0	0	2.5
	10^{-2}				19	Log_{10}%S	>2.0	>2.0	0.4

Bacterial numbers used for bactericidal test (control count) = 2×10^5/ml ($20 \times 10^2 \times 50 \times 2$)

Bacterial nibtogen	a) Determinations	Volume of Suspension	Photelometer Reading		$MgNH_3$-N		$MgNH_3$-N/ml	
		2 ml	86	85	.03		.015	
		4 ml	71	72	.07	.065	.017	.016

b) Calculated nitrogen present during bactericidal test

.016 mg/ml x 10^{-3} x 0.5 x 10^3 ug/mg = .008 ug/ml

TABLE 5

BACTERICIDAL TEST pH 7.0 30°C

Tubes Chlorine ppm		1 0.6	2 0.3	3 0.05	4 -	Tubes N/Cl N/Cl	1 .007/0.6 .001	2 .007/.03 .023	3 .007/.06 0.11
Contact Times	Count Dilutions								
1 min.	10^0	>100				Count x 10^{-5}	1.1	1.8	1.8
	10^{-1}	75	>100			% Surv.	55.	100.	100.
	10^{-2}	11	18	18	19	Log_{10}%S	1.74	2.0	2.0
	10^{-3}			2	1				
2.5 "	10^0	>100	TMC			Count x 10^{-5}	.12	2.0	2.0
	10^{-1}	12	>100	>100		% Surv.	6.	100.	100.
	10^{-2}	3	21	24		Log_{10}%S	.78	2.0	2.0
5 "	10^0	25	TMC			Count x 10^{-5}	.025	2.0	2.0
	10^{-1}	1	>100	>100		% Surv.	1.	100.	100.
	10^{-2}		20	19		Log_{10}%S	0	2.	2.
10 "	10^0	15	TMC			Count x 10^{-5}	.015	2.0	1.8
	10^{-1}	1	>100	>100		% Surv.	0.8	100.	100.
	10^{-2}		20	18	19	Log_{10}%S	-.10	2.0	2.0
	10^{-3}				2				
20 "	10^0	0	>100	TMC		Count x 10^{-5}	0	0.2	2.0
	10^{-1}		20	>100		% Surv.	0	10.	100.
	10^{-2}		1	20		Log_{10}%S	>2.0	1.0	2.0
30 "	10^0	0	5	TMC		Count x 10^{-5}	0.005	.005	1.3
	10^{-1}			>100		% Surv.	0	.3	65.
	10^{-2}			13	20	Log_{10}%S	>2.0	-0.52	1.82
	10^{-3}				2				

Bacterial numbers used for bactericidal test (control count) = 2×10^5/ml ($20 \times 10^2 \times 50 \times 2$)

Bacterial Nitrogen	a) Determinations	Volume of Suspension	Photelometer Readings		$MgNH_3$-N	$MgNH_3$-N/ml
		2 ml	88	88	.025	.013
		4 ml	87		.025	.013

b) Calculated amount present during bactericidal test

.013 mg/ml x 10^{-3} x .5 x 10^3 ug/mg = .007 ug/ml

FIG 5
BACTERICIDAL TEST PH 7.0

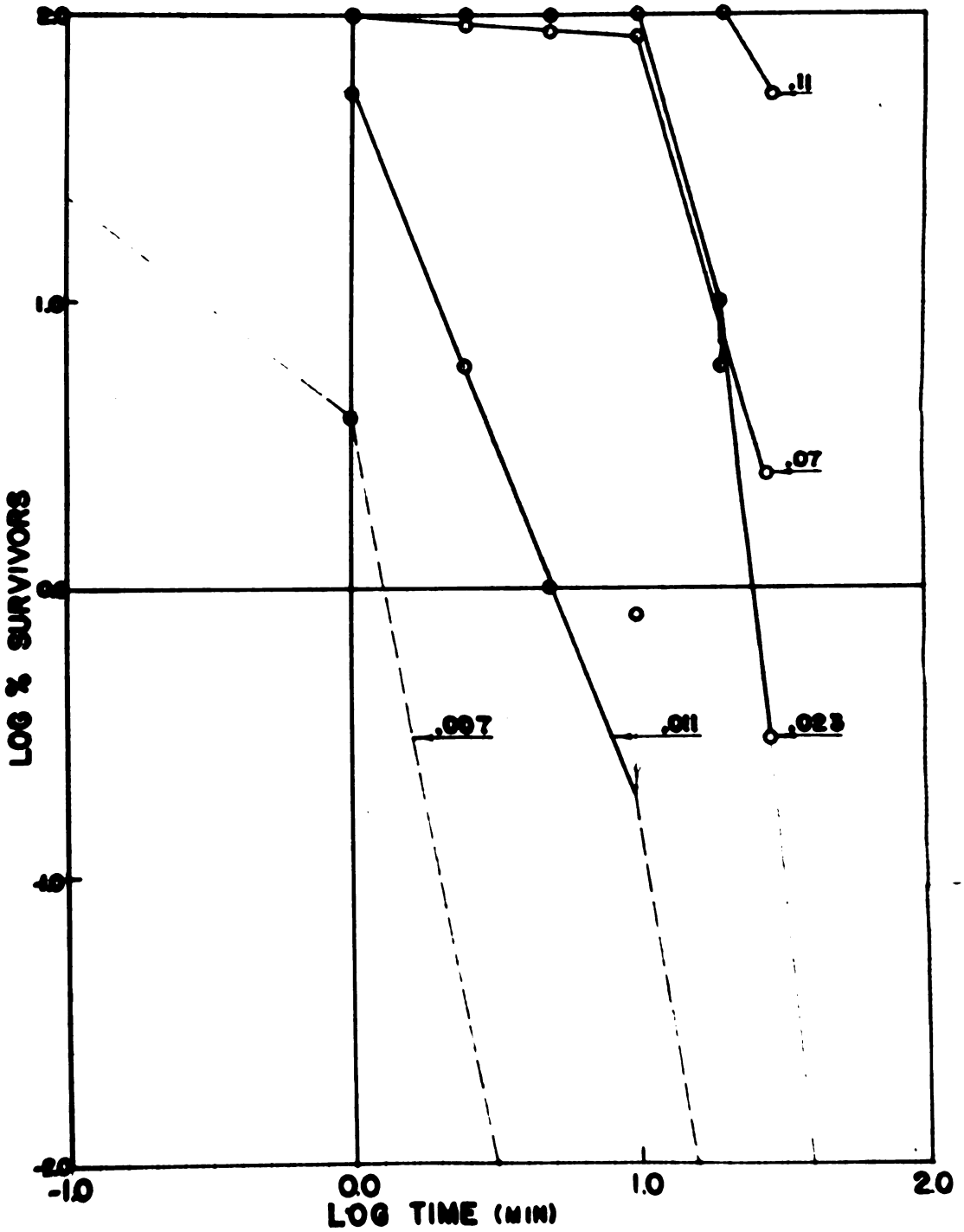


TABLE 6

BACTERICIDAL TEST pH 7.5 30°C

Tubes		1	2	3	4	Tubes	1	2	3
Chlorine	ppm	1.6	0.8	0.16	—	N/Cl	.008/1.6	.008/.8	.008/.16
						N/Cl	.005	.01	.05
Contact Times	Count Dilutions								
1 min.	10^0	12				Count x 10^{-5}	.01	.9	2.
	10^{-1}	1	>100	>100	>100	% Surv.	.4	36.	80.
	10^{-2}	0	9	20	22	Log_{10}%S	-.40	1.56	1.90
2.5 "	10^0	5				Count x 10^{-5}	.005	.6	1.2
	10^{-1}	0	50	>100		% Surv.	.2	24.	50.
	10^{-2}		6	12		Log_{10}%S	-.70	1.38	1.70
5 "	10^0	12				Count x 10^{-5}	.01	.5	1.7
	10^{-1}	0	55	>100		% Surv.	.4	20.	68.
	10^{-2}		5	17		Log_{10}%S	-.40	1.30	1.83
10 "	10^0	3				Count x 10^{-5}	.003	.4	.9
	10^{-1}	0	32	84	>100	% Surv.	.12	16.	36.
	10^{-2}		4	9	26	Log_{10}%S	-.92	1.20	1.56
20 "	10^0	0	20			Count x 10^{-5}	0	.03	.7
	10^{-1}	0	3	67		% Surv.	0	1.2	28.
	10^{-2}			7		Log_{10}%S	>2.0	.08	1.45
30 "	10^0	0	0			Count x 10^{-5}	0	0	.4
	10^{-1}		0	32	>100	% Surv.	0	0	16.
	10^{-2}			4	29	Log_{10}%S	>2.0	>2.0	1.20

Bacterial numbers used for bactericidal test (control count) = 2.5×10^5

Bacterial nitrogen a) Determinations

Volume of Suspension	Photelometer Readings		$MgNH_3$-N	$MgNH_3$-N/ml
2 ml	86	85	.03	.015

b) Calculated amount present during bactericidal test
.015 mg/ml x 10^{-3} x .5 x 10^3 ug/mg = .008 ug/ml

TABLE 7

BACTERICIDAL TEST pH 7.5 30°C

Tubes			1	2	3	4	Tubes	1	2	3
Chlorine ppm			1	0.5	0.1	--	N/Cl	.008/1	.008/.5	.008/.1
							N/Cl	.008	.016	.08
Contact Times		Count Dilutions								
1	min.	10^0	15				Count x 10^{-5}	.015	1.5	2.5
		10^{-1}	0	>100	>100	>100	% Surv.	.6	60.	100.
		10^{-2}		15	25	30	Log_{10}%S	-.22	1.78	2.
2.5	"	10^0	10				Count x 10^{-5}	.01	1.9	2.1
		10^{-1}	0	>100	>100		% Surv.	.4	76.	84.
		10^{-2}		19	21		Log_{10}%S	-.40	1.88	1.92
5	"	10^0	6				Count x 10^{-5}	.006	1.0	2.2
		10^{-1}	0	>100	>100		% Surv.	.25	40.	88.
		10^{-2}		10	22		Log_{10}%S	-.60	1.60	1.94
10	"	10^0	1				Count x 10^{-5}	0	1.1	2.1
		10^{-1}	0	96	>100	>100	% Surv.	0	44.	84.
		10^{-2}		11	21	25	Log_{10}%S	>2.0	1.64	1.92
20	"	10^0	1	15			Count x 10^{-5}	0	.015	2.3
		10^{-1}	0	0	>100		% Surv.	0	.6	92.
		10^{-2}			23		Log_{10}%S	>2.0	-.22	1.96
30	"	10^0	0	0			Count x 10^{-5}	0	0	1.8
		10^{-1}	0	0	>100	>100	% Surv.	0	0	72.
		10^{-2}			18	24	Log_{10}%S	>2.0	>2.0	1.86

Bacterial numbers used for bactericidal test (control count) = 2.5×10^5

Bacterial nitrogen a)	Determinations	Volume of Suspension	Photelometer Readings		$MgNH_3$-N		$MgNH_3$N/ml	
		2 ml	84	86	.035	.03	.017	.015

b) Calculated amount present during bactericidal test
.016 mg/ml x 10^{-3} x 0.5 x 10^3 ug/mg = .008 ug/ml

TABLE 8

BACTERICIDAL TEST pH 7.5 30°C

Tubes		1	2	3	4	Tubes	1	2	3
Chlorine	ppm	0.5	0.25	.05	--	N/Cl	.008/.5	.008/.25	.008/.05
						N/Cl	.016	.032	0.16
Contact Times	Count Dilutions								
1 min.	10^0					Count x 10^{-5}	1.9	2.4	2.5
	10^{-1}	>100	>100	>100	>100	% Surv.	76.	96.	100.
	10^{-2}	19	21	26	25	Log_{10}%S	1.88	1.98	2.0
2.5 "	10^0					Count x 10^{-5}	2.0	2.0	2.4
	10^{-1}	>100	>100	>100		% Surv.	80.	80.	96.
	10^{-2}	20	20	24		Log_{10}%S	1.90	1.90	1.98
5 "	10^0	>100				Count x 10^{-5}	1.1	1.8	2.4
	10^{-1}	80	>100	>100		% Surv.	44.	72.	96.
	10^{-2}	11	18	24		Log_{10}%S	1.64	1.86	1.98
10 "	10^0	>100				Count x 10^{-5}	.7	1.5	2.6
	10^{-1}	50	>100	>100	>100	% Surv.	28.	60.	100.
	10^{-2}	7	15	26	25	Log_{10}%S	1.45	1.78	2.0
20 "	10^0	25	>100			Count x 10^{-5}	.04	.9	2.1
	10^{-1}	4	70	>100		% Surv.	1.6	36.	84.
	10^{-2}	0	9	21		Log_{10}%S	.20	1.56	1.92
30 "	10^0	0	10			Count x 10^{-5}	0	.02	1.6
	10^{-1}	0	2	>100	>100	% Surv.	0	.8	80.
	10^{-2}			20	29	Log_{10}%S	>2.0	-.10	1.90

Bacterial numbers used for bactericidal test (Control count) = 2.5×10^5

Bacterial nitrogen a) Determinations

Volume of Suspension	Photelometer Readings		$MgNH_3$-N		$MgNH_3$-N/ml	
2 ml	83	85	.035	.03	.017	.015

b) Calculated amount present during bactericidal test
.016 mg/ml x 10^{-3} x 0.5 x 10^3 ug/mg = .008 ug/ml

FIG 6
BACTERICIDAL TEST PH 7.5

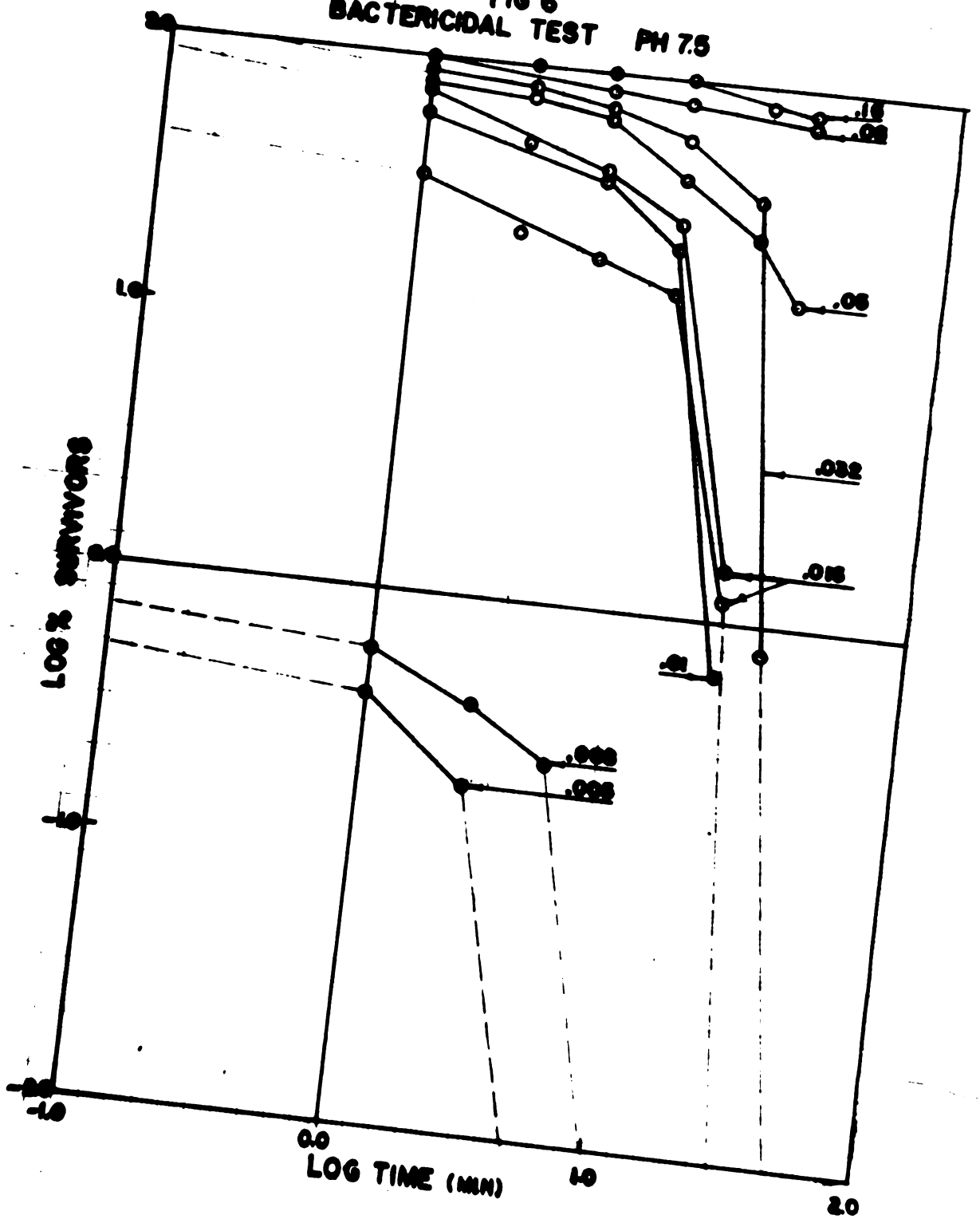


TABLE 9

BACTERICIDAL TEST pH 9.0 30°C

Tubes		1	2	3	4	Tubes	1	2	3
Chlorine	ppm	2.2	1.1	0.22	--	N/Cl	.007/2.2	.007/1.1	.007/.22
						N/Cl	.003	.006	.03
Contact Times	Count Dilutions								
1 min.	10^{-1}	25	>100	>100	>100	Count x 10^{-5}	.25	1.2	2.0
	10^{-2}	0	12	20	20	% Surv.	12.	60.	100.
	10^{-3}				2	Log_{10}%S	1.08	1.78	2.
2.5 "	10^{0}	20				Count x 10^{-5}	.02	.6	1.9
	10^{-1}	2	55	>100		% Surv.	1.	30.	95.
	10^{-2}	0	8	19		Log_{10}%S	0	1.48	1.98
5 "	10^{0}	5				Count x 10^{-5}	.05	.3	1.7
	10^{-1}	0	25	>100		% Surv.	2.5	15.	85.
	10^{-2}		3	17		Log_{10}%S	.40	1.18	1.93
10 "	10^{0}	1	55			Count x 10^{-5}	0	.05	1.7
	10^{-1}	0	5	>100	>100	% Surv.	0	2.5	85.
	10^{-2}		1	17	20	Log_{10}%S	>2.0	.40	1.92
20 "	10^{0}	0	4			Count x 10^{-5}	0	.004	1.7
	10^{-1}	0	0	>100		% Surv.	0	.2	83.
	10^{-2}			17		Log_{10}%S	>2.0	-.70	1.92
30 "	10^{0}	0	0			Count x 10^{-5}	0	0	1.2
	10^{-1}	0	0	>100	>100	% Surv.	0	0	60.
	10^{-2}			12	17	Log_{10}%S	>2.0	>2.0	1.78

Bacterial numbers used for bactericidal test (control count) = 2×10^5

Bacterial Nitrogen a) Determinations

Volume of Suspension	Photelometer Readings		$MgNH_3$-N		$MgNH_3$-N/ml	
2 ml	87	87	.025		.0125	
4 ml	74	71	.06	.07	.015	.017

b) Calculated amount present during bactericidal test

$.014 \times 10^{-3} \times .5 \times 10^{3}$ ug/mg = .007 ug/ml

TABLE 10

BACTERICIDAL TEST pH 9.0 30°C

Tubes		1	2	3	4	Tubes	1	2	3
Chlorine	ppm	1.1	0.55	0.11	--	N/Cl	.009/1.1	.009/.55	.009/.11
						N/Cl	.008	.016	.08
Contact Times	Count Dilution								
1 min.	10^{-1}	>100	>100	>100	>100	Count x 10^{-5}	2.8	3.	3.
	10^{-2}	28	30	29	30	% Surv.	95.	100.	100.
	10^{-3}	3	4	4	5	Log_{10}%S	1.98	2.	2.
2.5 "	10^{-1}	>100	>100	>100		Count x 10^{-5}	2.0	27.	3.
	10^{-2}	20	27	30		% Surv.	67.	90.	100.
	10^{-3}			4		Log_{10}%S	1.83	1.95	2.
5 "	10^{-1}	>100	>100			Count x 10^{-5}	2.1	1.9	2.9
	10^{-2}	21	19	29		% Surv.	70.	63.	100.
	10^{-3}		2	3		Log_{10}%S	1.85	1.80	2.
10 "	10^{0}	TMTC	TMTC			Count x 10^{-5}	.9	9.0	2.9
	10^{-1}	>100	>100	>100	>100	% Surv.	30.	67.	100.
	10^{-2}	9	20	29	35	Log_{10}%S	1.48	1.83	2.
	10^{-3}			4	4				
20 "	10^{0}	TMTC	TMTC	TMTC		Count x 10^{-5}	.37	1.8	3.0
	10^{-1}	37	>100	>100		% Surv.	12.	60.	100.
	10^{-2}	4	18	30		Log_{10}%S	1.08	1.78	2.
30 "	10^{0}	TMTC	TMTC	TMTC		Count x 10^{-5}	.28	1.4	2.9
	10^{-1}	28	>100	>100	>100	% Surv.	9.	47.	100.
	10^{-2}	3	14	29	32	Log_{10}%S	.95	1.67	2.

Bacterial numbers used for bactericidal test (control count) = 3×10^5

Bacterial Nitrogen a) Determinations

Volume of Suspension	Photelometer Readings		$MgNH_3$-N	$MgNH_3$-N/ml
2 ml	85	84	.035	.017
4 ml	71		.07	.017

b) Calculated amount present during bactericidal test

.017 mg/ml x 10^{-3} x .5 x 10^3 ug/mg = .009 ug/ml

FIG 7
BACTERICIDAL TEST PH 9.0

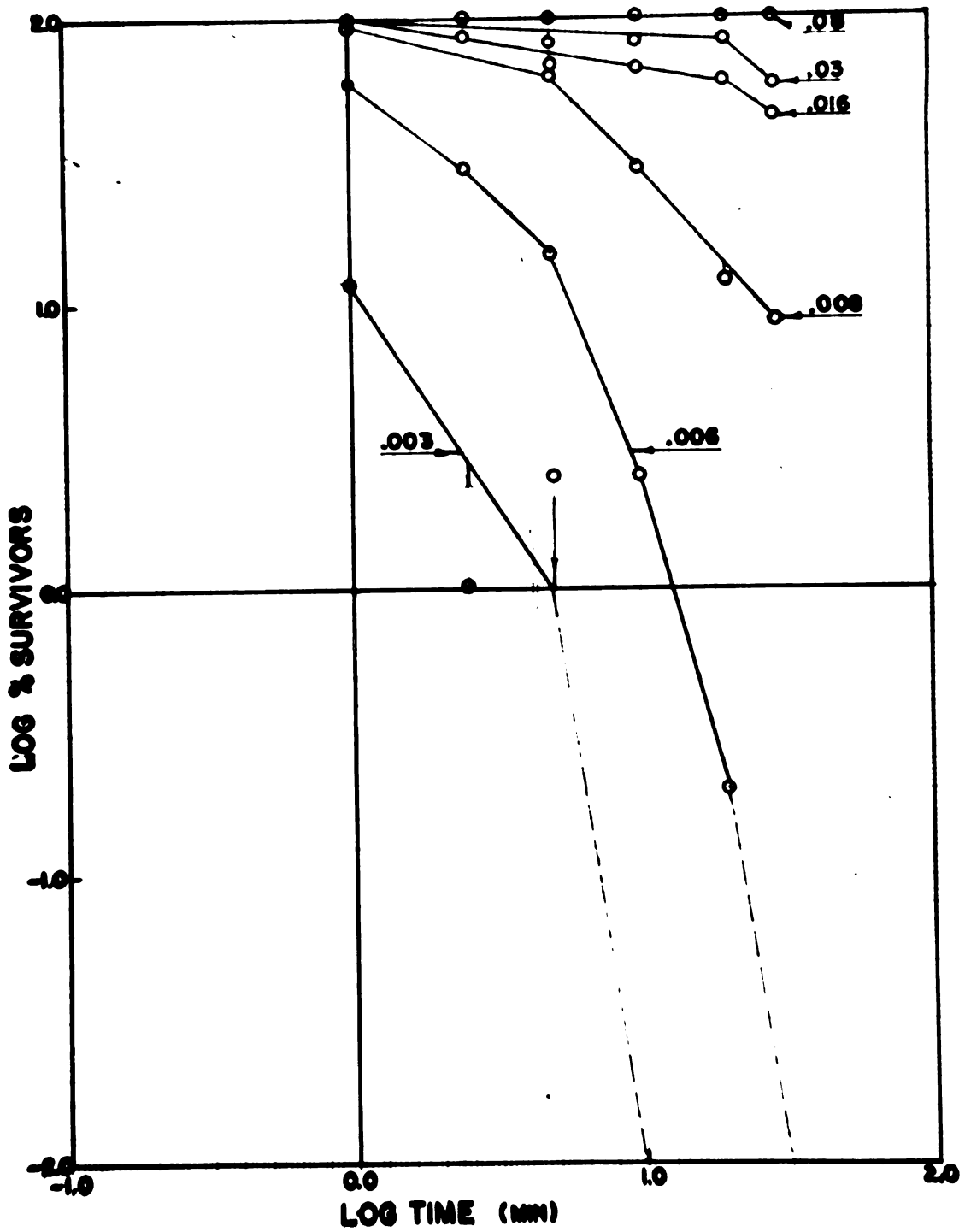


TABLE 11

BACTERICIDAL TEST Uncontrolled pH 30°C

Tubes		1	2	3	4	Tubes	1	2	3
Chlorine	ppm	1.0	0.5	0.1	--	N/Cl	.011/1	.011/.5	.011/.1
						N/Cl	.011	.022	.11
Contact Times	Count Dilutions								
1 min.	10^0	0	TMTC	TMTC	TMTC	Count x 10^{-5}	0	.1	.5
	10^{-1}	0	10	50	55	% Surv.	0	16.	83.
	10^{-2}	0	1	2	2	Log_{10}%S	>2.0	1.20	1.92
2.5 "	10^0	0	50	TMTC		Count x 10^{-5}	0	.05	.45
	10^{-1}	0	1	45		% Surv.	0	8.	75.
	10^{-2}			4		Log_{10}%S	>2.0	.90	1.88
5 "	10^0	0	30	TMTC		Count x 10^{-5}	0	.03	.30
	10^{-1}	0	0	30		% Surv.	0	5.	50.
	10^{-2}			0		Log_{10}%S	>2.0	.70	1.70
10 "	10^0	0	10	TMTC	TMTC	Count x 10^{-5}		.01	.20
	10^{-1}	0	0	20	60	% Surv.		1.6	33.
	10^{-2}			1	3	Log_{10}%S		.20	1.52
20 "	10^0	0	1	50		Count x 10^{-5}		.001	.05
	10^{-1}	0	0	5		% Surv.		.16	8.
	10^{-2}			1		Log_{10}%S		-.80	.90
30 "	10^0	0	0	25	TMTC	Count x 10^{-5}		0	.025
	10^{-1}	0	0	1	60	% Surv.		0	4.2
	10^{-2}				7	Log_{10}%S		>2.00	.62

Bacterial numbers used for bactericidal test (control count) = $.6 \times 10^5$ 60,000

Bacterial Nitrogen a) Determinations

Volume of Suspension	Photelometer Readings			$MgNH_3$-N	$MgNH_3$-N/ml
2 ml	80	80	79	.045	.023
4 ml	67			.08	.020

b) Calculated amount present during bactericidal test
$.022 \text{ mg/ml} \times 10^{-3} \times .5 \times 10^3 \text{ ug/mg} = .011 \text{ ug/ml}$

TMTC = Too many to count

TABLE 12

BACTERICIDAL TEST Uncontrolled pH 30°C

Tubes		1	2	3	4	Tubes	1	2	3
Chlorine	ppm	.5	.25	.1	--	N/Cl	.009/.5	.009/.25	.009/.1
						N/Cl	.018	.036	.09
Contact Times	Count Dilution								
1 min.	10^0	5	TMTC	TMTC	TMTC	Count x 10^{-5}	.005	.60	.80
	10^{-1}	0	60	80	95	% Surv.	.5	63.	84.
	10^{-2}	0	8	10	4	Log_{10}%S	-.30	1.80	1.92
2.5 "	10^0	1	TMTC	TMTC		Count x 10^{-5}	.001	.40	.65
	10^{-1}	0	40	65		% Surv.	.1	42.	68
	10^{-2}		7	10		Log_{10}%S	-1.0	1.62	1.83
5 "	10^0	0	TMTC	TMTC		Count x 10^{-5}	0	.20	.70
	10^{-1}	0	20	70		% Surv.	0	21.	73.
	10^{-2}		7	11		Log_{10}%S	>2.00	1.32	1.86
10 "	10^0	0	50	TMTC	TMTC	Count x 10^{-5}		.05	.50
	10^{-1}	0	2	50	90	% Surv.		5.0	52.
	10^{-2}			6	10	Log_{10}%S		.70	1.72
20 "	10^0	0	20	TMTC		Count x 10^{-5}		.02	.30
	10^{-1}	0	6	30		% Surv.		2.	31.
	10^{-2}			8		Log_{10}%S		.30	1.49
30 "	10^0	0	5	60	TMTC	Count x 10^{-5}		.005	.06
	10^{-1}	0	0	9	95	% Surv.		.5	6.
	10^{-2}				12	Log_{10}%S		-.301	.78

Bacterial numbers used for bactericidal test (control count) = .95 x 10^5 95,000

Bacterial Nitrogen a) Determinations

Volume of Suspension	Photelometer Readings	$MgNH_3$-N	$MgNH_3$-N/ml
2 ml	84 84 83	.035	.018
3 ml	76	.055	.017
4 ml	71	.070	.018

b) Calculated amount present during bactericidal test
.018 mg/ml x 10^{-3} x .5 x 103 ug/mg = .009 ug/ml

FIG 8
BACTERICIDAL TEST
PH UNCONTROLLED

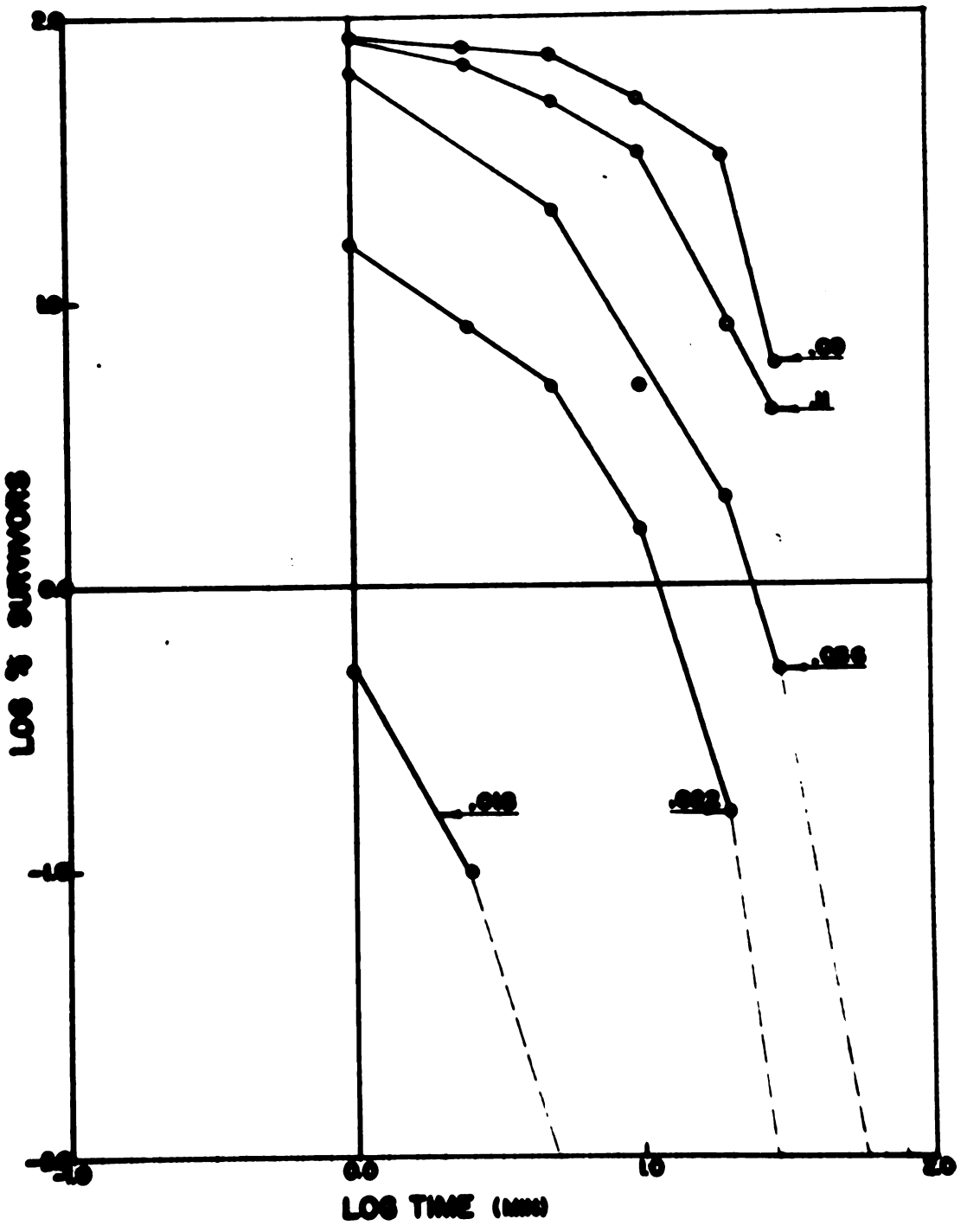


TABLE 13

BACTERICIDAL TEST Uncontrolled pH 30°C

Tubes / Chlorine ppm	1 / 1.0	2 / 0.5	3 / 0.1	4 / --	Tubes / N/Cl / N/Cl	1 / .08/1 / .08	2 / .08/.5 / .16	3 / .08/.1 / .8
Contact Times / Count Dilution								
1 min. 10^{-1}	TMTC	TMTC	TMTC	TMTC	Count x 10^{-5}	2.5	6.5	7.5
10^{-2}	25	65	75	72	% Surv.	33.	87.	100.
10^{-3}	1	10	8	7	Log_{10}%S	1.52	1.94	2.0
2.5 " 10^{-1}	TMTC	TMTC	TMTC		Count x 10^{-5}	2.0	6.0	7.5
10^{-2}	20	60	75		% Surv.	27.	80.	100.
10^{-3}		9	10		Log_{10}%S	1.43	1.90	2.0
5 " 10^{-1}	TMTC	TMTC	TMTC		Count x 10^{-5}	1.5	2.5	7.0
10^{-2}	15	15	70		% Surv.	20.	33.	94.
10^{-3}		0	1		Log_{10}%S	1.30	1.52	1.97
10 " 10^{0}	70	TMTC			Count x 10^{-5}	.25	.85	6.0
10^{-1}	25	85	TMTC	TMTC	% Surv.	3.	11.	80.
10^{-2}	3	9	60	74	Log_{10}%S	.48	1.04	1.90
10^{-3}			7	8				
20 " 10^{0}	50	TMTC			Count x 10^{-5}	.05	.40	3.5
10^{-1}	1	40	TMTC		% Surv.	.07	.5	47.
10^{-2}	0	5	35		Log_{10}%S	-1.15	-.30	
10^{-3}			6					
30 " 10^{0}	50	65	TMTC		Count x 10^{-5}	.05	.07	.85
10^{-1}	8	7	85	TMTC	% Surv.	.07	.09	11.
10^{-2}	0	1	9	75	Log_{10}%S	-1.15	-1.05	1.04

Bacterial numbers used for bactericidal test (control count) = 7.5×10^5 750,000

Bacterial Nitrogen a) Determinations

Volume of Suspension	Photelometer Readings			$MgNH_3$-N		$MgNH_3$-N/ml
2 ml	84	84	85	.035	.03	.017
3 ml		78			.05	.016

b) Calculated amount present during bactericidal test

.016 mg/ml x 10^2 x .5 x 10^3 ug/mg = .08 ug/ml

TABLE 14

BACTERICIDAL TEST Uncontrolled pH 30°C

Tubes		1	2	3	4
Chlorine	ppm	1	.5	.1	--
Contact Time	Count Dilutions				
1 min.	10^{-2}	TMTC			
	10^{-3}	10	30	30	28
	10^{-4}		4	5	3
2.5 "	10^{-2}	75	TMTC	TMTC	
	10^{-3}	0	20	28	
	10^{-4}			3	
5 "	10^{-1}	TMTC	TMTC		
	10^{-2}	55	TMTC	TMTC	
	10^{-3}		20	25	
10 "	10^{-1}	TMTC	TMTC		
	10^{-2}	50	60	TMTC	
	10^{-3}		10	25	30
	10^{-4}			4	2
20 "	10^{0}	TMTC			
	10^{-1}	10	TMTC		
	10^{-2}	1	40	TMTC	
	10^{-3}		2	20	
30 "	10^{0}	20	TMTC		
	10^{-1}	2	80		
	10^{-2}		10	TMTC	
	10^{-3}			20	

Tubes	1	2	3
N/Cl	16/1	.16/.5	.16/.1
N/Cl	.16	.32	1.6
Count x 10^{-5}	10.	30.	30.
% Surv.	33.	100.	100.
Log_{10}%S	1.52	2.00	2.00
Count x 10^{-5}	7.5	20.	28.
% Surv.	25.	67.	93.
Log_{10}%S	1.40	1.83	1.97
Count x 10^{-5}	5.5	20.	25.
% Surv.	18.	67.	83.
Log_{10}%S	1.26	1.83	1.92
Count x 10^{-5}	5.	10.	25.
% Surv.	16.	33.	83.
Log_{10}%S	1.20	1.52	192.
Count x 10^{-5}	.1	4.	20.
% Surv.	.3	13.	67.
Log_{10}%S	-.52	1.11	1.83
Count x 10^{-5}	.02	1.	20.
% Surv.	.07	3.	67.
Log_{10}%S	-1.15	.48	1.83

Bacterial numbers used for bactericidal test (control count) = 30×10^5 3,000,000

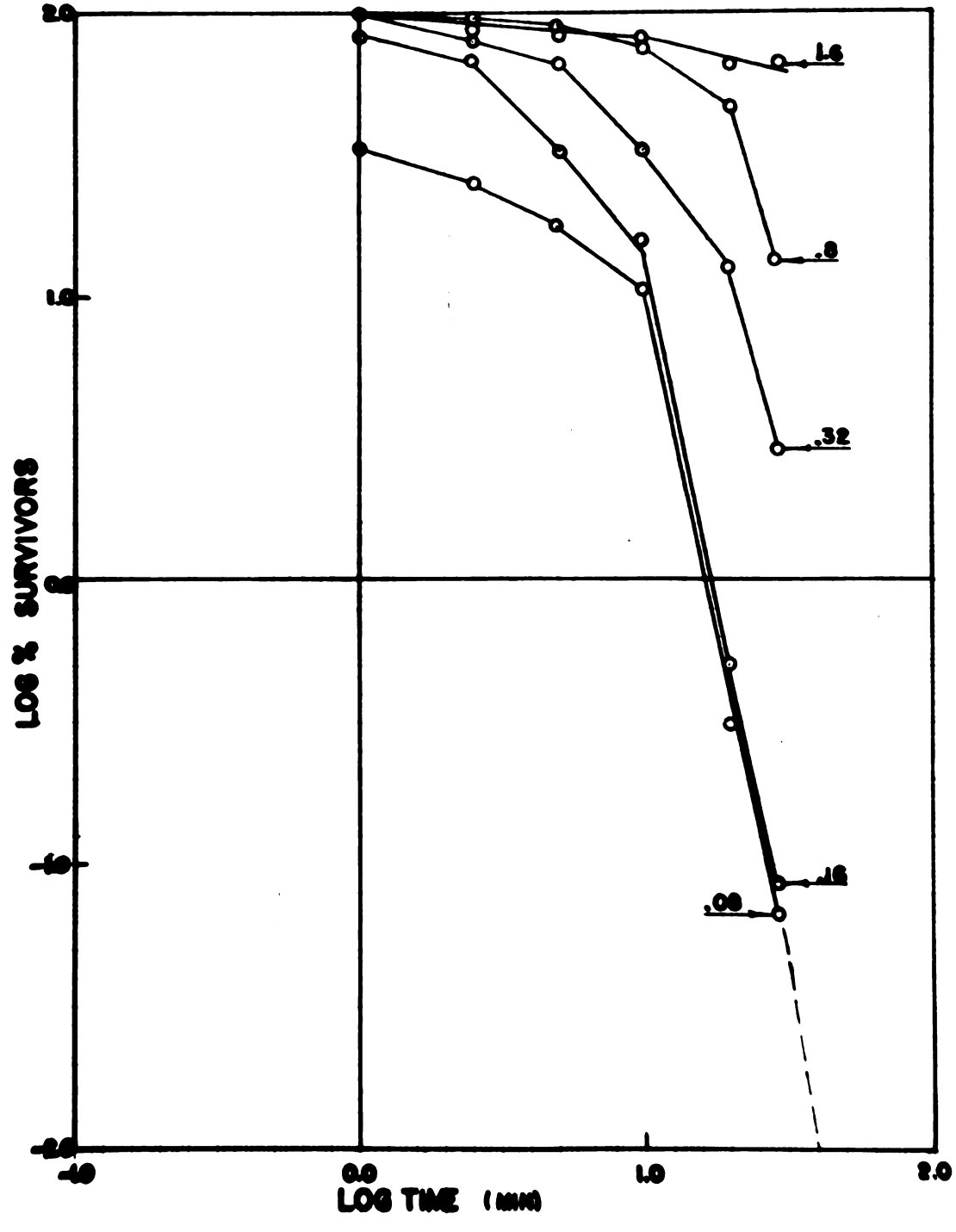
Bacterial Nitrogen a) Determination

Volume of Suspension	Photelometer Readings		$MgNH_3$-N	$MgNH_3$-N/ml
2 ml	72	71	.065	.032

b) Calculated amount present during bactericidal test

.032 mg/ml x 10^3 ug/mg x 10^{-2} x .5 = .16 ug/ml

FIG 9
BACTERICIDAL TEST
PH UNCONTROLLED



Interpretation of the Graphs Derived from Bactericidal Test Data

1. Individual Curves from any one Figure.

At least one curve in each figure could be plotted through the six points of the contact periods. This determined the general shape of the curve given by the data. Data for any N/Cl ratio which provided fewer points were plotted to conform to this general shape. Extrapolations were also drawn to conform to this shape to the points of .01 percent and 100 percent survival.

For any one N/Cl ratio, an increase in contact time resulted in a decrease of the number of survivors.

2. Groups of Curves from any one Figure.

In any one figure, an increase of the N/Cl ratio resulted in a shift of the group of curves to the right and upwards. Since an increase of the N/Cl ratio could mean either an increase of the micrograms of bacterial nitrogen or a decrease of the micrograms of chlorine, then it could be concluded:

That in the presence of an increased number of bacterial cells as represented by an increase in bacterial nitrogen or in the presence of a decreased concentration of chlorine, the time to obtain a specified percentage survival of bacteria was increased (rightward movement) and the percentage survival of bacteria at a specified contact time was greater (upward movement).

On any one figure the extrapolated values for .01 percent survival could be obtained. These values were in accord

with the above conclusion. The times for .01 percent survival increased with an increase in the N/Cl ratio.

On any one figure the extrapolated values for 100 percent survival could be obtained. These values correspond to the values obtained for .01 percent survival in the sense that a short 100 percent survival time was followed by a short .01 percent survival time and a long 100 percent survival time by a long .01 percent survival time.

3. Groups of Curves from all the Figures for Controlled pH Values.

From all these figures together a comparison of corresponding N/Cl ratios provided comparative data for the bactericidal activity of chlorine at different pH values.

It was noted that as the pH increased in value any one N/Cl ratio provided a curve that was shifted to the right and upwards. Thus with increasing pH value but in the presence of relatively comparable amounts of bacterial nitrogen and chlorine, the percent survival of bacteria was greater and the time to obtain a specified percent survival was increased.

At high pH values lower amounts of bacterial nitrogen or increased chlorine concentrations were required to secure percentage survivals and time intervals comparable to those at low pH values.

4. Curves obtaines with uncontrolled conditions of pH.

Figure 8 represented the data obtained with bacterial numbers that were comparable to those that were used to

obtain Figures 4 to 7. From Figure 8 it could be concluded:

a. That discrepancies in the grading of the curves may arise due to failure to control pH. Thus N/Cl .09 is further to the right and higher than N/Cl .11.

b. That the curves presented values of N/Cl ratios that fitted best onto the curves with values of N/Cl ratios obtained at pH 4.0 and pH 7.0.

Figure 9 represented data obtained with bacterial numbers that were much greater than those used to obtain Figures 4 to 7.

If these curves were compared to Figures 4 to 7 it could be seen that they could not be fitted even roughly to any of the groups of curves for controlled pH values. Thus the use of N/Cl ratios is restricted to the comparison of groups of data that have been derived from tests performed with comparable amounts of bacterial nitrogen.

The use of increased amounts of bacterial nitrogen and corresponding increases of chlorine concentration which maintained a numerically similar ratio resulted in a shift of N/Cl curves downward and to the left. Thus, the somewhat paradoxical result was obtained that with large bacterial numbers and with higher chlorine concentrations, smaller percentage survivals and shorter time intervals were obtained.

It must be stated, however, that this conclusion is not too reliable since it was based on results obtained with uncontrolled pH data.

A summary table of the points of .01 percent survival is presented in Table 32 and of the points of 100 percent survival in Table 34.

VII. ENZYME INHIBITION TEST DATA

TABLE 15

ENZYME INHIBITION TEST pH 4.0 30°C

Tubes		1	2	3	4	Tubes	1	2	3
Chlorine ppm		1.7	.85	.34	--	N/Cl	5/1.7	5/.85	5/.34
						N Cl	3	6	15
Contact Times									
1 min.	V	.4	.4	.4	.4	% AR*	7.	20.	87.
	R	92.	82.	52.	46.	Log AR**	.85	1.30	1.94
	Un	.01	.04	.17	.20				
	Corr	.2	.6	2.6	3.0				
2.5 "	V	.4	.4	.4		% AR*	0	26.	70.
	R	97.	80.	58.		Log AR**	>2.00	1.41	1.84
	Un	0	.05	.14					
	Corr	0	.8	2.1					
5 "	V	1.0	.4	.4		% AR*	4.	20.	57.
	R	90.	82.	64.		Log AR**	.60	1.30	1.76
	Un	.02	.04	.11					
	Corr	.12	.6	1.7					
10 "	V	1.0	.4	.4	.4	% AR*	4.	10.	50.
	R	90.	88.	67.	45.	Log AR**	.60	1.00	1.69
	Un	.02	.02	1.0	.21				
	Corr	.12	.3	1.5	3.2				
20 "	V	1.0	.4	.4		% AR*	1.	2.6	40.
	R	95.	95.	72.		Log AR**	0	.42	1.60
	Un	.005	.005	.08					
	Corr	.03	.08	1.2					
40 "	V	2.0	1.0	.4	.4	% AR*	.3	2.	30.
	R	95.	92.	77.	48.	Log AR**	-.52	.30	1.48
	Un	.005	.01	.06	.19				
	Corr	.01	.06	.9	2.8				

% AR* - Percent Activity Remaining Log AR** - Logarithm of precent activity remaining

V = Volume of acid digest used for Nesslerisation
R = Photelometer reading of the Nesslerised digest
Un = Uncorrected nitrogen equivalent of the photelometer reading
Corr = Corrected nitrogen equivalent per ml. of urease

TABLE 16

ENZYME INHIBITION TEST pH 4.0 30°C

Tubes		1	2	3	4	Tubes	1	2	3
Chlorine ppm		0.9	0.45	0.18	00	N/Cl	5/.9	5/.45	5/.18
						N/Cl	5.5	11	28
Contact Times									
1 min.	V	.4	.4	.4	.4	% AR	20.	87.	100.
	R	84.	52.	47.	47.	$Log_{10}AR$	1.30	1.94	2.
	Un	.04	.17	.20	.20				
	Corr	.6	2.6	3.0	3.0				
2.5 "	V	.4	.4	.4		% AR	16.	57.	80.
	R	85.	65.	54.		$Log_{10}AR$	1.20	1.76	1.90
	Un	.03	.11	.16					
	Corr	.5	1.7	2.4					
5 "	V	.4	.4	.4		% AR	13.	47.	67.
	R	86.	70.	59.		$Log_{10}AR$	1.11	1.67	1.83
	Un	.03	.09	.13					
	Corr	.4	1.4	2.0					
10 "	V	.4	.4	.4	.4	% AR	7.	20.	63.
	R	92.	82.	60.	45.	$Log_{10}AR$	.85	1.30	1.79
	Un	.01	.04	.13	.21				
	Corr	.2	.6	1.9	3.2				
20 "	V	.8	.4	.4		% AR	3.	20.	50.
	R	91.	84.	68.		$Log_{10}AR$	.48	1.30	1.09
	Un	.01	.04	.10					
	Corr	.08	.6	1.5					
40 "	V	1.0	.4	.4	.4	% AR	1.	10.	33.
	R	95.	89.	76.	46.	$Log_{10}AR$	0.0	1.0	1.52
	Un	.005	.02	.07	.20				
	Corr	.03	.3	1.0	3.0				

TABLE 17

ENZYME INHIBITION TEST pH 4.0 30°C

Tubes		1	1	2	3	4	Tubes	1	2	3
Chlorine ppm			0.5	0.25	0.1	0	N/Cl	5/5	5/.25	5/0.1
							N/Cl	10	20	50
Contact Times										
1 min.		V	.4	.4	.4	.4	% AR	77.	100.	100.
		R	55.	47.	46.	45.	$Log_{10}AR$	1.88	2.	2.
		Un	.15	.20	.20	.21				
		Corr	2.3	3.0	3.0	3.2				
2.5	"	V	.4	.4	.4		% AR	57.	90.	100.
		R	66.	50.	46.		$Log_{10}AR$	1.76	1.95	2.
		Un	.11	.18	.20					
		Corr	1.7	2.7	3.0					
5	"	V	.4	.4	.4		% AR	50.	70.	100.
		R	68.	58.	47.		$Log_{10}AR$	1.69	1.85	2.
		Un	.10	.14	.20					
		Corr	1.5	2.1	3.0					
10	"	V	.4	.4	.4	.4	% AR	30.	67.	100.
		R	77.	59.	46.	46.	$Log_{10}AR$	1.48	1.83	2.
		Un	.06	.13	.20	.20				
		Corr	.9	2.0	3.0	3.0				
20	"	V	.4	.4	.4		% AR	27.	57.	97.
		R	80.	65.	49.		$Log_{10}AR$	1.43	1.76	1.98
		Un	.05	.11	.19					
		Corr	.8	1.7	2.9					
40	"	V	.4	.4	.4	.4	% AR	16.	33.	87.
		R	85.	75.	52.	46.	$Log_{10}AR$	1.20	1.52	1.94
		Un	.03	.07	.17	.20				
		Corr	.5	1.0	2.6	3.0				

FIG 10
INHIBITION OF UREASE
PH 4.0

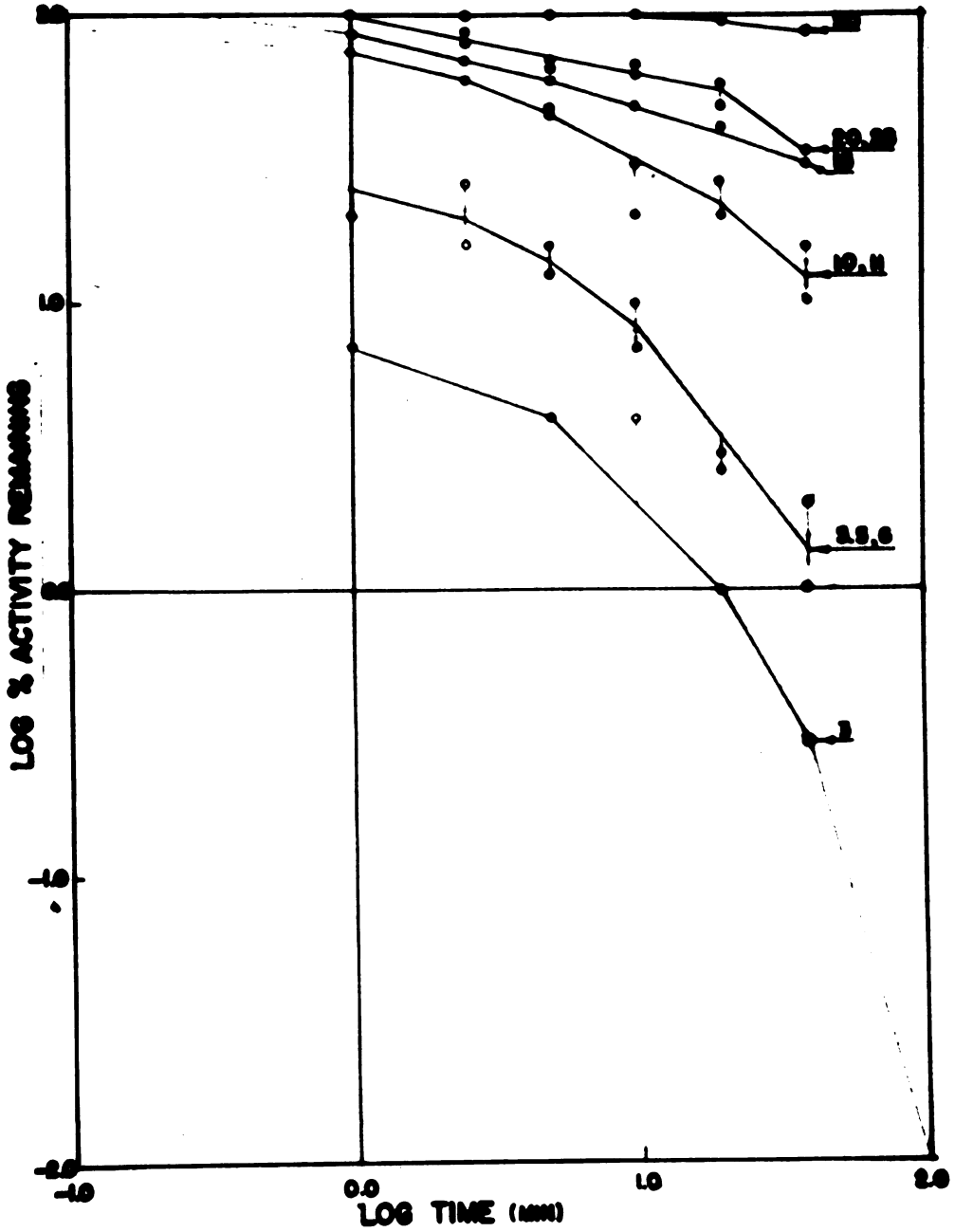


TABLE 18

ENZYME INHIBITION TEST pH 7.0 30°C

Tubes		1	2	3	4	Tubes	1	2	3
Chlorine ppm		1.5	.75	.3	0	N/Cl	5/1.5	5/.75	5/.3
						N/Cl	3	6.5	16
Contact Times									
1 min.	V	1.0	.8	.4	.4	% AR	10.	27.	90.
	R	81.	65.	50.	46.	$Log_{10}AR$	1.00	1.43	1.95
	Un	.05	.11	.18	.20				
	Corr	0.3	0.8	2.7	3.0				
2.5 "	V	1.	1.	.4		% AR	1.	6.	83.
	R	95.	86.	51.		$Log_{10}AR$	0.0	.78	1.92
	Un	.005	.03	.17					
	Corr	.03	.18	2.5					
5 "	V	1.	1.	.4		% AR	1.	4.	70.
	R	96.	89.	57.		$Log_{10}AR$	0.0	.60	1.85
	Un	.005	.02	.14					
	Corr	.03	.12	2.1					
10 "	V	2.	1.	.4	.4	% AR	.7	1.	47.
	R	95.	95.	69.	45.	$Log_{10}AR$	-.15	0.0	1.67
	Un	.005	.005	.09	.21				
	Corr	.02	.03	1.4	3.1				
20 "	V	2.	2.	.4		% AR	0	.7	33.
	R	97.	96.	75.		$Log_{10}AR$	>2.00	-.15	1.52
	Un	0	.005	.07					
	Corr	0	.02	1.1					
40 "	V	2.	2.	.4	.4	% AR	0	0	30.
	R	96.	96.	78.	46.	$Log_{10}AR$	>2.00	>2.00	1.48
	Un	.005	.005	.06	.20				
	Corr	.02	.02	.9	3.0				

TABLE 19

ENZYME INHIBITION TEST pH 7.0 30°C

Tubes Chlorine ppm		1 .75	2 .38	3 .15	4 0
Contact Times					
1 min.	V	.4	.4	.4	.4
	R	84.	52.	46.	46.
	Un	.04	.17	.20	.20
	Corr	.6	2.3	3.0	3.0
2.5 "	V	1.0	.4	.4	
	R	82.	56.	48.	
	Un	.04	.15	.19	
	Corr	.24	2.2	2.9	
5 "	V	1.0	.4	.4	
	R	90.	62.	49.	
	Un	.02	.12	.19	
	Corr	.12	1.8	2.8	
10 "	V	1.0	.4	.4	.4
	R	96.	75.	54.	47.
	Un	.005	.07	.16	.20
	Corr	.03	1.0	2.4	3.0
20 "	V	2.0	.4	.4	
	R	96.	83.	55.	
	Un	.005	.04	.15	
	Corr	.02	0.6	2.2	
40 "	V	2.0	.4	.4	.4
	R	96.	89.	60.	47.
	Un	.005	.02	.13	.2
	Corr	.02	.3	1.9	3.0

Tubes N/Cl N/Cl	1 5/.75 6.5	2 5/.38 13	3 5/.15 33
% AR	20.	76.	100.
Log_{10}AR	1.30	1.88	2.
% AR	8.	73.	97.
Log_{10}AR	.90	1.86	1.98
% AR	4.	60.	93.
Log_{10}AR	.60	1.78	1.97
% AR	1.	33.	80.
Log_{10}AR	0.	1.52	1.90
% AR	.7	2.0	73.
Log_{10}AR	-.15	1.30	1.86
% AR	.7	10.	63.
Log_{10}AR	-.15	1.00	1.80

TABLE 20

ENZYME INHIBITION TEST pH 7.0 30°C

Tubes			1	2	3	4	Tubes	1	2	3
Chlorine ppm			.35	.18	.07	0	N/Cl	5/.35	5/.18	5/.07
							N/Cl	14	28	70
Contact Times										
1 min.		V	.4	.4	.4	.4	% AR	84.	90.	100.
		R	50.	48.	45.	46.	$Log_{10}AR$	1.92	1.95	2.
		Un	.18	.19	.21	.20				
		Corr	2.7	2.9	3.2	3.2				
2.5	"	V	.4	.4	.4		% AR	81.	93.	100.
		R	52.	47.	45.		$Log_{10}AR$	1.91	1.97	2.
		Un	.17	.20	.21					
		Corr	2.6	3.0	3.2					
5	"	V	.4	.4	.4		% AR	56.	90.	100.
		R	61.	48.	45.		$Log_{10}AR$	1.75	1.95	2.
		Un	.12	.19	.21					
		Corr	1.8	2.9	3.2					
10	"	V	.4	.4	.4	.4	% AR	37.	78.	100.
		R	72.	52.	45.	45.	$Log_{10}AR$	1.57	1.89	2.
		Un	.08	.17	.21	.21				
		Corr	1.2	2.5	3.2	3.2				
20	"	V	.4	.4	.4		% AR	28.	66.	100.
		R	78.	58.	45.		$Log_{10}AR$	1.45	1.82	2.
		Un	.06	.14	.21					
		Corr	.9	2.1	3.2					
40	"	V	.4	.4	.4	.4	% AR	18.	1.6	3.2
		R	83.	64.	45.	45.	$Log_{10}AR$	1.26	1.69	2.
		Un	.04	.11	.21	.21				
		Corr	.16	1.6	3.2					

FIG 11
INHIBITION OF UREASE
PH 7.0

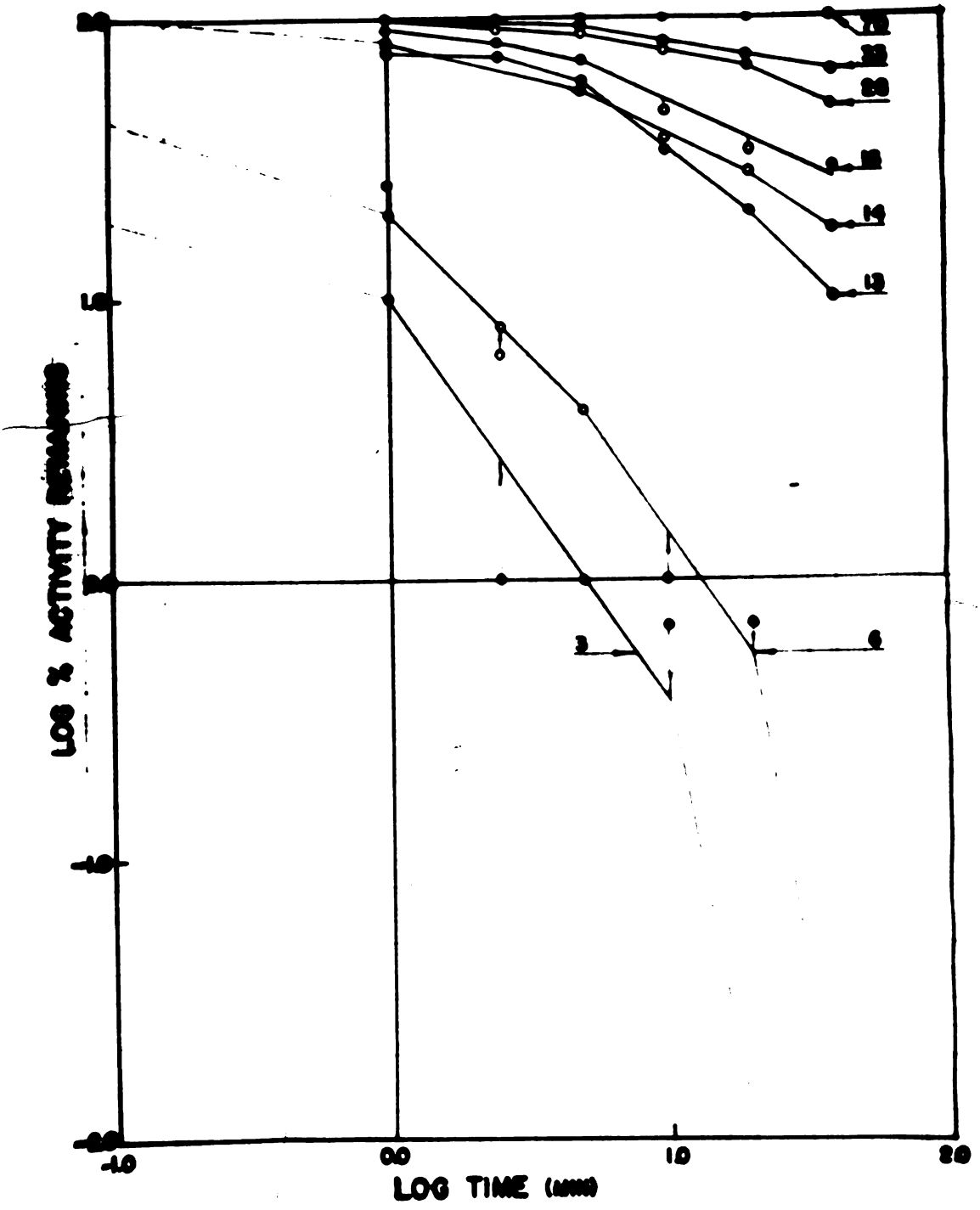


TABLE 21

ENZYME INHIBITION TEST pH 7.5 30°C

Tubes			1	2	3	4	Tubes	1	2	3
Chlorine ppm			1.8	0.9	0.36	0	N/Cl	5/1.8	5/.9	5/.36
							N/Cl	2.8	5.5	15
Contact Times										
1 min.		V	1.0	.5	.4	.4	% AR	1.	4.	70.
		R	96.	92.	60.	47.	$Log_{10}AR$	0.0	.60	1.84
		Un	.005	.01	.13	.20				
		Corr	.03	.12	2.1	3.0				
2.5	"	V	2.0	1.0	.4		% AR	1.	4.	50.
		R	91.	90.	67.		$Log_{10}AR$	0.0	.60	1.69
		Un	.01	.02	.10					
		Corr	.03	.12	1.5					
5	"	V	2.0	1.0	.4		% AR	.3	1.	20.
		R	95.	93.	82.		$Log_{10}AR$	-.53	0.0	1.30
		Un	.005	.005	.04					
		Corr	.01	.13	.16					
10	"	V	2.0	2.0	.4	.4	% AR	0	.3	10.
		R	97.	94.	90.	48.	$Log_{10}AR$	>2.00	-.53	1.00
		Un	0	.005	.02	.9				
		Corr	0	.01	.3	3.0				
20	2	V	2.0	2.0	.4		% AR	0	.3	2.5
		R	96.	96.	96.		$Log_{10}AR$	>2.00	-.53	.39
		Un	.005	.005	.005					
		Corr	.01	.01	.07					
40	"	V	2.0	2.0	1.0	.4	% AR	0	0	1.0
		R	96.	97.	95.	47.	$Log_{10}AR$	>2.00	>2.00	0.0
		Un	.005	0	.005	.20				
		Corr	.01	0	.03	3.0				

TABLE 22

ENZYME INHIBITION TEST pH 7.5 30°C

Tubes		1	2	3	4	Tubes	1	2	3
Chlorine ppm		1.0	0.5	0.2	0	N/Cl	5/10	5/.5	5/.2
						N/Cl	5	10	25
Contact Time									
1 min.	V	.4	.4	.4	.4	% AR	10.	50.	100.
	R	90.	67.	48.	47.	$Log_{10}AR$	1.00	1.69	2.00
	Un	.02	.10	.19	.20				
	Corr	.3	1.5	3.0	3.0				
2.5 "	V	1.0	.4	.4		% AR	10.	30.	70.
	R	80.	75.	60.		$Log_{10}AR$	1.00	1.48	1.85
	Un	.05	.07	.13					
	Corr	.3	1.0	2.1					
5 "	V	1.0	.4	.4		% AR	6.	20.	58.
	R	85.	83.	68.		$Lg_{10}Ar$	.78	1.30	1.76
	Un	.03	.04	.10					
	Corr	.18	.6	1.5					
10 "	V	1.0	.5	.4	.4	% AR	4.	8.	33.
	R	88.	88.	74.	47.	$Log_{10}AR$	.60	.90	1.52
	Un	.02	.02	.07	.2				
	Corr	.12	.24	1.0	3.0				
20 "	V	1.0	.6	.4		% AR	1.	2.	20.
	R	96.	95.	84.		$Log_{10}AR$	0.0	.30	1.30
	Un	.005	.005	.04					
	Corr	.03	.05	.6					
40 "	V	1.0	.4	.4	.4	% AR	0	1.	10.
	R	95.	95.	90.	47.	Log AR	>2.0	0.0	1.00
	Un.	.005	.005	.02	.2				
	Corr	.02	.03	.3	3.0				

TABLE 23

ENZYME INHIBITION TEST pH 7.5 30°C

Tubes			1	2	3	4	Tubes	1	2	3
Chlorine ppm			.4	.2	.08	0	N/Cl	5/.4	5/.2	5/.08
							N/Cl	12	25	60
Contact Times										
1	min.	V	.4	.4	.4	.4	% AR	62.	83.	100.
		R	62.	54.	48.	48.	Log_{10}AR	1.79	1.92	2.
		Un	.12	.16	.19	.19				
		Corr	1.8	2.4	2.9	2.9				
2.5	"	V	.4	.4	.4		% AR	51.	62.	79.
		R	67.	62.	55.		Log_{10}AR	1.71	1.79	1.89
		Un	.10	.12	.15					
		Corr	1.5	1.8	2.3					
5	"	V	.4	.4	.4		% AR	24.	55.	66.
		R	81.	65.	59.		Log_{10}AR	1.38	1.74	1.82
		Un	.05	.11	.13					
		Corr	.17	1.6	1.9					
10	"	V	.4	.4	.4	.4	% AR	10.	24.	55.
		R	88.	76.	65.	48.	Log_{10}AR	1.0	1.38	1.74
		Un	.02	.07	.11	.19				
		Corr	.3	.7	1.6	2.9				
20	"	V	.4	.4	.4		% AR	2.8	20.	51.
		R	95.	84.	68.		Log_{10}AR	.45	1.30	1.71
		Un	.005	.04	.10					
		Corr	.05	.6	1.5					
40	"	V	.6	.4	.4	.4	% AR	1.7	2.8	45.
		R	96.	94.	70.	48.	Log_{10}AR	.23	.45	1.65
		Un	.005	.09	.19					
		Corr	.05	.08	1.3	2.9				

FIG 12
INHIBITION OF UREASE
PH 7.5

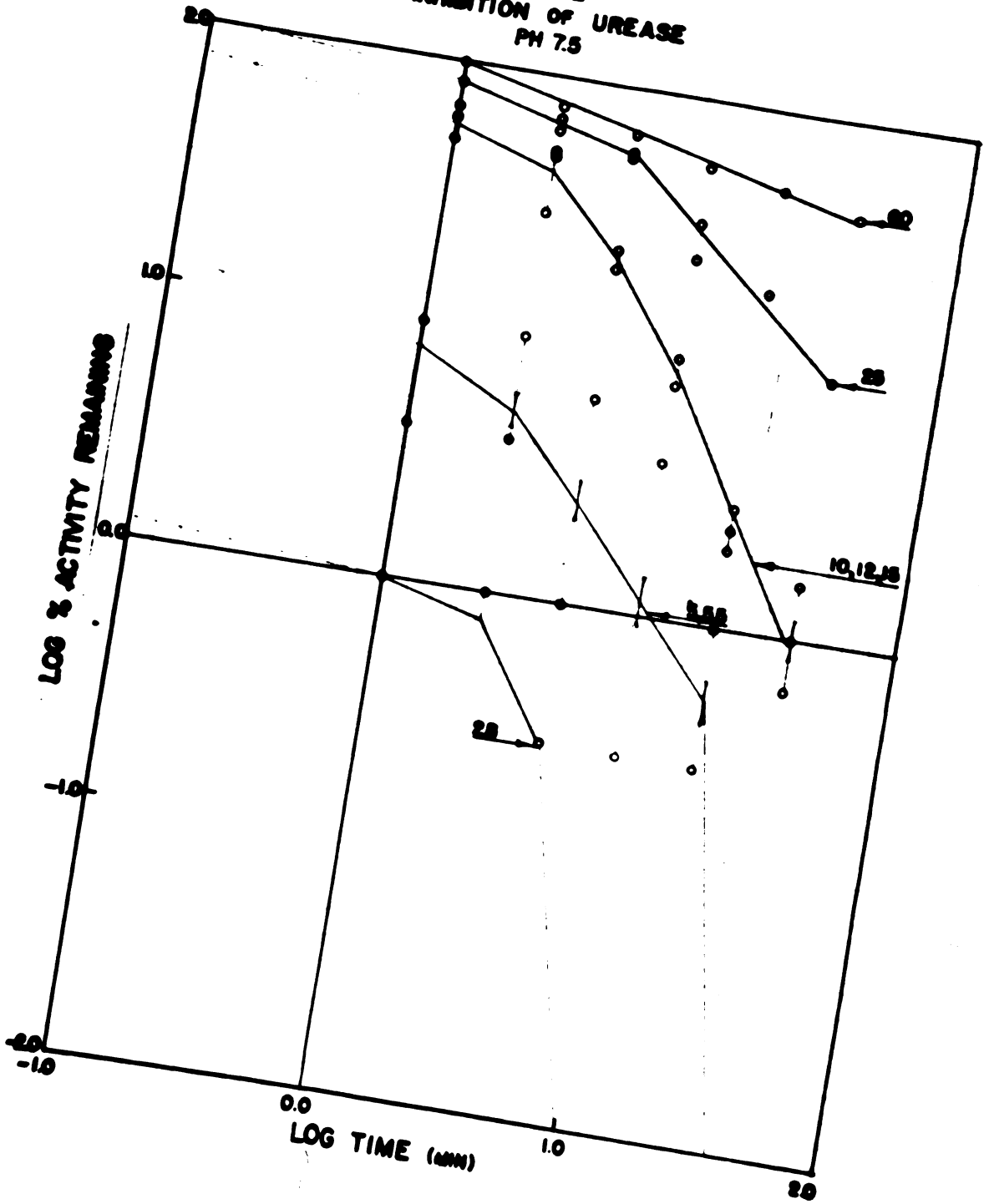


TABLE 24

ENZYME INHIBITION TEST pH 9.0 30°C

Tubes			1	2	3	4	Tubes	1	2	3
Chlorine ppm			1.6	0.8	.32	0	N/Cl	6/1.6	5/.8	5/.32
							N/Cl	3	6	16
Contact Times										
1 min.		V	1.0	.5	.4	.4	% AR	1.1	18.	52.
		R	96.	82.	70.	48.	$Log_{10}AR$	.04	1.26	1.72
		Un	.005	.04	.09	.19				
		Corr	.03	.5	1.4	2.9				
2.5	"	V	2.0	1.0	.4		% AR	.3	2.2	18.
		R	94.	91.	85.		$Log_{10}AR$	-.52	.34	1.26
		Un	.005	.01	.03					
		Corr	.01	.06	.5					
5	"	V	2.0	1.0	.4		% AR	0	1.1	7.4
		R	95.	95.	92.		$Log_{10}AR$	>2.00	.04	.87
		Un	.005	.005	.01					
		Corr	.01	.03	.2					
10	"	V	2.0	2.0	1.0	.4	% AR	0	.6	1.2
		R	95.	97.	96.	50.	$Log_{10}AR$	>2.00	-.22	.04
		Un	.005	0	.005	.18				
		Corr	.01	0	.03	2.7				
20	"	V	2.0	2.0	2.0		% AR	0	0	.3
		R	96.	95.	93.		$Log_{10}AR$	>2.00	>2.00	-.52
		Un	.005	.005	.005					
		Corr	.01	.01	.01					
40	"	V	2.0	2.0	2.0	.4	% AR	0	0	0
		R	96.	95.	95.	50.	$Log_{10}AR$	>2.00	>2.00	>2.00
		Un	.005	.005	.005	.18				
		Corr	.01	.01	.01	2.7				

TABLE 25

ENZYME INHIBITION TEST pH 9.0 30°C

Tubes		1	2	3	4	Tubes	1	2	3
Chlorine ppm		1.1	.55	.21	0	N/Cl	5/1.1	5/.55	5/.21
						N/Cl	4.5	9	25
Contact Times									
1 min.	V	.4	.4	.4	.4	% AR	3.	30.	60.
	R	93.	79.	61.	46.	$Log_{10}AR$	.48	1.48	1.78
	Un	.005	.06	.12	.20				
	Corr	.08	.9	1.8	3.0				
2.5 "	V	1.0	.4	.4		% AR	.4	3.	30.
	R	89.	94.	79.		$Log_{10}AR$	.60	.48	1.48
	Un	.02	.005	.06					
	Corr	.12	.08	.9					
5 "	V	1.0	1.0	.4		% AR	.3	.6	13.
	R	95.	87.	87.		$Log_{10}AR$	-.52	.78	1.11
	Un	.005	.03	.03					
	Corr	.01	.18	.14					
10 "	V	2.0	1.0	.4	.4	% AR	0	.6	3.
	R	95.	96.	93.	47.	$Log_{10}AR$	>2.00	-.22	.48
	Un	.005	.005	.005	.20				
	Corr	.01	.02	.08	3.0				
20 "	V	2.0	2.0	1.0		% AR	0	.3	.6
	R	97.	95.	95.		$Log_{10}AR$	>2.00	-.52	-.22
	Un	0	.005	.005					
	Corr	0	.01	.02					
40 "	V	2.0	2.0	2.0	.4	% AR	0	0	.3
	R	96.	97.	96.	49.	$Log_{10}AR$	>2.00	>2.00	-.52
	Un	.005	0	.005	.19				
	Corr	.01	0	.01	3.0				

TABLE 26

ENZYME INHIBITION TEST pH 9.0 30°C

Tubes		1	2	3	4	Tubes	1	2	3
Chlorine ppm		.4	.2	.08	0	N/Cl	5/.4	5/.2	5/.08
						N/Cl	12	25	60
Contact Times									
1 min.	V	.4	.4	.4	.4	% AR	33.	60.	70.
	R	75.	62.	58.	47.	$Log_{10}AR$	1.52	1.78	1.85
	Un	.07	.12	.14	.20				
	Corr	1.0	1.8	2.1	3.0				
2.5 "	V	.4	.4	.4		% AR	13.	30.	53.
	R	85.	78.	65.		$Log_{10}AR$	1.11	1.48	1.72
	Un	.03	.06	.11					
	Corr	.4	.9	1.6					
5 "	V	.4	.4	.4		% AR	3.	1.3	23.
	R	96.	87.	80.		$Log_{10}AR$	.48	1.11	1.36
	Un	.005	.03	.05					
	Corr	.08	.4	.7					
10 "	V	1.0	.4	.4	.4	% AR	2.	0	3.
	R	92.	97.	93.	47.	$Log_{10}AR$	.30	>2.00	.48
	Un	.01	0	.005	.20				
	Corr	.06	0	.08	3.0				
20 "	V	2.0	1.0	1.0		% AR	.3	.6	.6
	R	96.	95.	95.		$Log_{10}AR$	-.52	-.22	-.22
	Un	.005	.005	.005					
	Corr	.01	.02	.02					
40 "	V	2.0	2.0	2.0	.4	% AR	0	.3	.3
	R	95.	96.	96.	49.	$Log_{10}AR$	>2.0	-.52	-.52
	Un	.005	.005	.005	.19				
	Corr	.01	.01	.01	2.9				

FIG 13
INHIBITION OF UREASE
PH 9.0

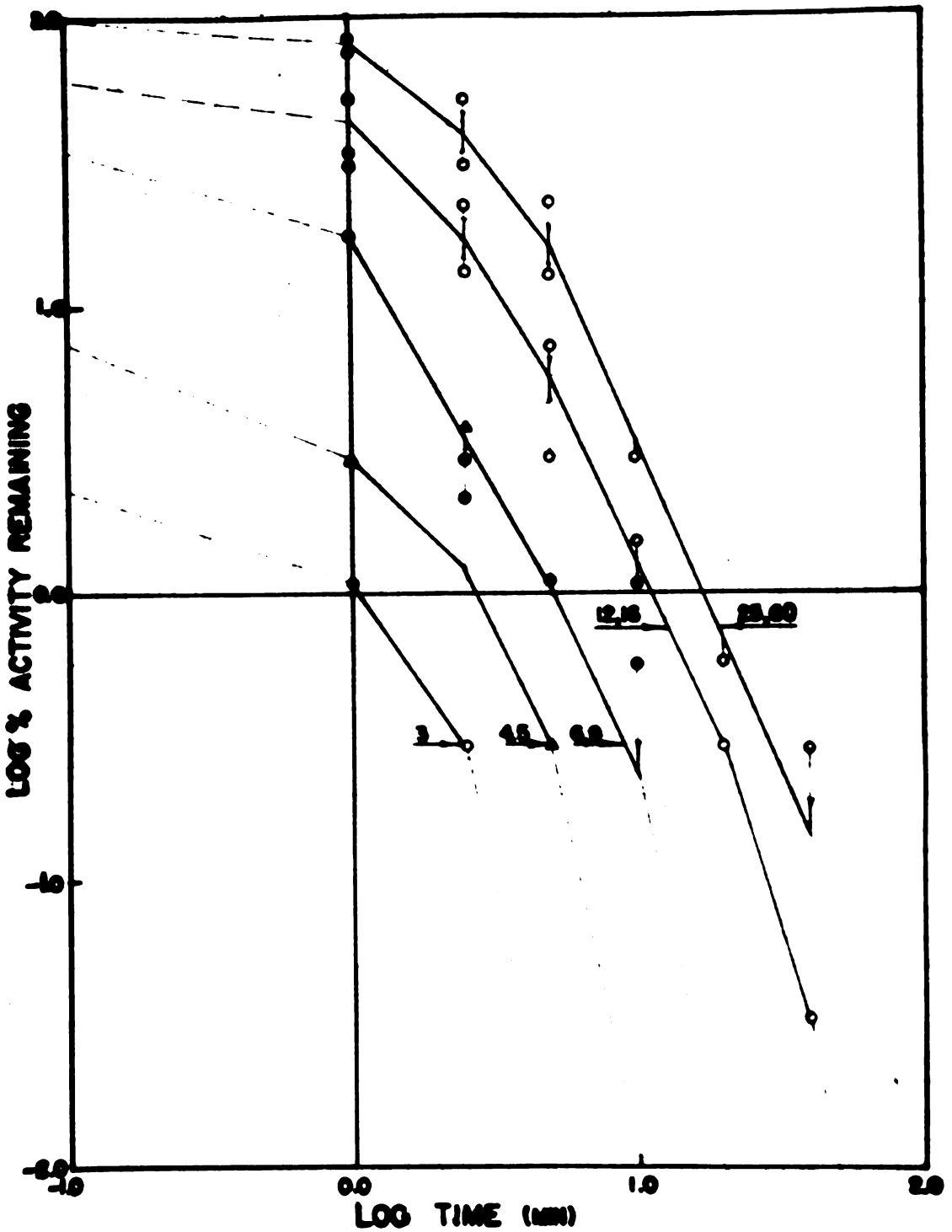


TABLE 27

ENZYME INHIBITION TEST Uncontrolled pH 30°C

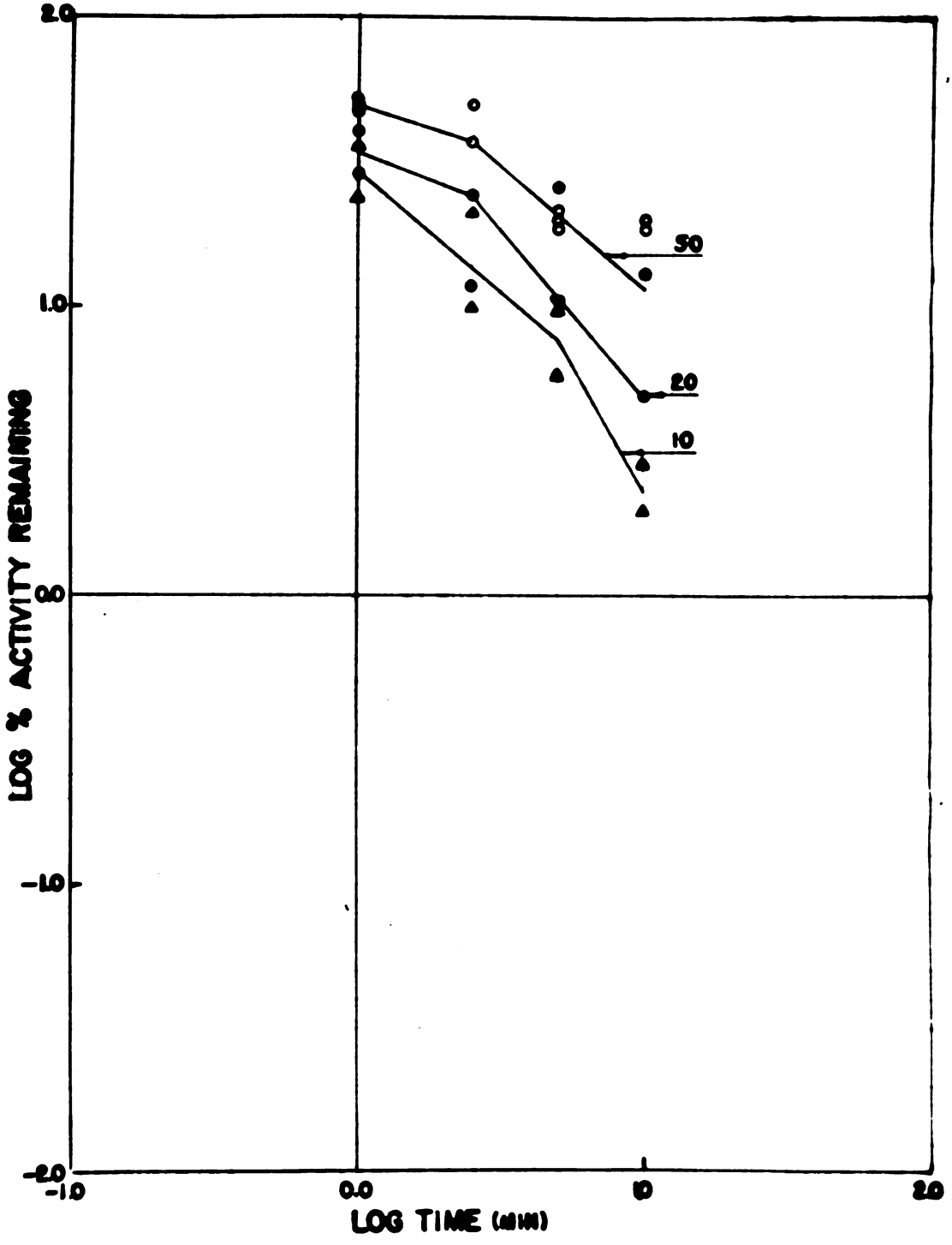
Tubes		1	2	3	4	Tubes	1	2	3
Chlorine ppm		1	.5	.2	0	N/Cl	10/1	10/.5	10/.2
						N/Cl	10	20	50
Contact Times									
1 min.	V	1.	.6	.4	.3	% AR	36.	45.	48.
	R	50.	48.	59.	45.	$Log_{10}AR$	1.56	1.65	1.68
	Un	.18	.19	.3	.21				
	Corr	1.48	1.90	1.95	4.2				
1 "	V	1.	.6	.4	.3	% AR	30.	40.	50.
	R	45.	51.	57.	45.	$Log_{10}AR$	1.48	1.60	1.70
	Un	.20	.6	.3	.20				
	Corr	1.2	1.6	2.0	4.0				
1 "	V	1.	1.	.5	.2	% AR	30.	40.	50.
	R	51.	54.	49.	58.	$Log_{10}AR$	1.38	1.48	1.73
	Un	.16	.20	.18	.13				
	Corr	.96	1.2	2.2	3.9				
2.5 "	V	1.	1.	.4	.2	% AR	10.	25.	37.
	R	73.	48.	65.	56.	$Log_{10}AR$	1.	1.40	1.57
	Un	.08	.19	.11	.15				
	Corr	.48	1.14	1.65	4.5				
2.5 "	V	.8	.6	.4	.2	% AR	22.	40.	50.
	R	64.	59.	65.	62.	$Log_{10}AR$	1.34	1.60	1.70
	Un	.11	.12	.10	.11				
	Corr	.75	1.2	1.5	3.3				
2.5 "	V	1.	1.	.5	2.	% AR	10.	15.	24.
	R	70.	61.	67.	54.	$Log_{10}AR$	1.	1.18	1.38
	Un	.09	.12	.10	.16				
	Corr	.48	.66	1.08	4.5				

TABLE 28

ENZYME INHIBITION TEST Uncontrolled pH 30oC

Tubes		1	2	3	4	Tubes	1	2	3
Chlorine ppm		1	.5	.2	0	N/Cl	10/1	10/.5	10/.2
						N/Cl	10	20	50
Contact Times									
5 min.	V	1.	1.	1.	.2	% AR	6.	11.	22.
	R	82.	75.	58.	60.	$Log_{10}AR$	.78	1.04	1.34
	Un	.04	.07	.14	.13				
	Corr	.24	.42	.84	3.9				
5 "	V	1.	.6	.4	.2	% AR	10.	14.	21.
	R	71.	74.	75.	55.	$Log_{10}AR$	1.0	1.45	1.32
	Un	.08	.06	.06	.14				
	Corr	.42	.60	.90	4.2				
5 "	V	1.	1.	.5	.2	% AR	6.	10.	20.
	R	86.	81.	80.	65.	$Log_{10}AR$	.78	1.0	1.30
	Un	.03	.05	.05	.11				
	Corr	.18	.30	.60	3.0				
10 "	V	1.	1.	1.	.2	% AR	3.	11.	18.
	R	90.	76.	65.	63.	$Log_{10}AR$	.48	1.04	1.26
	Un	.02	.07	.11	.12				
	Corr	.12	.42	.66	3.6				
10 "	V	1.0	.8	.5	.2	% AR	5.	13.	20.
	R	80.	70.	69.	53.	$Log_{10}AR$	.70	1.11	1.30
	Un	.04	.08	.09	.15				
	Corr	.24	.60	.96	4.5				
10 "	V	1.	1.	.5	.2	% AR	2.	5.	11.
	R	91.	86.	85.	67.	$Log_{10}AR$	.30	.70	1.04
	Un	.01	.02	.025	.09				
	Corr	.06	.12	.30	2.7				

FIG 14
INHIBITION OF UREASE
PH UNCONTROLLED



Interpretation of Graphs derived from Enzyme Inhibition Test Data

The remarks under the interpretation of graphs derived from bactericidal test data are applicable to the tests for enzyme inhibition insofar as points 1 and 2 of that discussion are concerned.

3. Groups of Curves from all the Figures for Controlled pH Values.

From all of these figures together a comparison of corresponding N/Cl ratios provided comparative values for the enzyme inhibitory action of chlorine at different pH values.

It was noted that as the pH decreased in value any one N/Cl ratio provided a curve that was shifted to the right and upwards. Thus with decreasing pH value but in the presence of relatively comparable amounts of urease nitrogen and chlorine, the percent activity remaining of urease was greater and the time to obtain a specified percent activity remaining was increased. This was the reverse of the result obtained with the bactericidal test.

At low pH values lower amounts of urease nitrogen or increased chlorine concentrations were required to secure percentages activity remaining and time intervals comparable to those at high pH values.

4. Curves obtained under Uncontrolled conditions of pH.

The curves of Figure 14 present values of N/Cl ratios that fitted best on to the curves with values of N/Cl ratios obtained at pH 9.0.

A summary table of the points of .01 percent activity remaining is presented in Table 32 and of the points of 100 percent survival in Table 34.

A discussion of the behavior of urease in the presence of chlorine is presented under section D of the discussion.

VIII. EXPERIMENTAL DATA - ERRORS

Discussion of the Errors Involved in the Data as Represented by the Deviations of Points Shown on the Graphs

Deviations on the graphs reflect back upon the experimental data and the methods used for obtaining such data. The sources of error may be divided into two categories for the purpose of this discussion. One was the counting method which gave the numbers on the data sheet. The other was all other experimental procedures. It will be assumed that these procedures provided a constant source of error which affected the entire body of data in a uniform manner. The Nesslerisation procedure which corresponded to the counting procedure was not considered to be a source of error of any magnitude. In comparison with a biological method, any chemical quantitative procedure is to be considered a marvel of precision. Instead the wide variations of the enzyme inhibition test data were considered to be due to an inherent inconstancy of the action of chlorine in alkaline media. This role of the chloramines will be discussed more fully towards the end of the discussion.

In general the bacterial count data provided points that could be used to fit curves of a certain form. There was one discrepancy in Figure 7 which can be attributed to an error of recording more readily than to an error of method. The other two serious discrepancies occur in Figure 5.

For the curve representing the N/Cl value of .07, the point at the 20-minute time interval is about 1 percent lower than the line itself. The data from which the point was derived showed a count of 12 colonies from a 1/10 dilution of the medication tube. This represented six percent survival. If, however, only three more organisms had been counted, the percentage survival becomes 7.5 and the point falls in the curve. This difference of 3 in 12 is a 25 percent error which is about within the accuracy of any plate counting procedure.

For the curve representing the N/Cl value of .011, the point at the 20-minute interval is about .6 percent higher than the line itself. The data from which the point was derived showed a count of 15 colonies from an undiluted medication tube. This represented .8 percent survival. In order to fit a point to the line, the figure for the percentage survival would have to be .2. The colony count for this figure would be 4, which is below the limit of accuracy of the counting method. The error of 11 in 15 is a 70 percent error. This was possible because of the low numbers of organisms and the small size of inoculum used for counting. It was one of the limitations of the method used. It led to the most careful attention to those medication tubes that contained low numbers of organisms and which would be expected to yield low counts.

It should also be recognized that the method of presenting data as logarithms actually provided four uneven intervals of percentage survival as follows:

-2.0 to -1.0,	.01% - .1%	or a .09% interval;
-1.0 to 0.0,	.10% - 1%	or a .9% interval;
0.0 to 1.0,	1% - 10%	or a 9% interval;
1.0 to 2.0,	10% - 100%	or a 90% interval.

In the regions of high numbers of organisms or high survival values, any error in the plating and counting procedure made a slight difference in the percentage figure. In the region of low numbers of organisms or low survival values, errors in the plating and counting procedures tended to be higher and differences in the percentage figures larger. However the use of logarithms tended to make such differences less apparent.

End of Experimental Data

IX. DISCUSSION

A. Measurement of Bacterial Nitrogen

Although it was stated that a uniform procedure was used in the determination of bacterial nitrogen and that therefore the results were relatively significant, other evidence is provided below concerning the correctness of the results of this procedure.

The value for the weight of a bacterial cell will be calculated from the data of this thesis and compared with a calculation made from data presented by Clark (64) in a table "On the Dimensions of Cells." For E. coli these dimensions are:

a) linear dimensions	3 x 1.2 micra;
b) surface area	11.6 square micra;
c) volume	0.26 cubic micra.

Using this last figure the following calculation was made on the assumption that the density of bacterial cells is approximately equal to that of water:

$$0.26 \frac{u^3}{cell} \times \frac{1\ gm}{cm^3} \times 10^6 \frac{ug}{gm} \times \frac{cm^3}{10^{12} u^3} = 2.6 \times 10^{-7}\ ug/cell \qquad ....(2)$$

From my data in this thesis the average figure of .007 ug. NH_3-N/ml for 2 x 10^5 cells/ml was chosen. Further it was assumed that the percent of nitrogen in bacterial cells was approximately 16. Thus:

$$.007 \frac{\text{ugNH}_3\text{-N}}{\text{ml}} \times \frac{1}{16} \frac{\text{ug (cell)}}{\text{ug NH}_3\text{-N}} \times \frac{\text{ml}}{2 \times 10^5 \text{ cells}} = 2.1 \times 10^{-7} \text{ ug/cell} \quad \ldots.(3)$$

B. The Urease Preparation

The following calculations are provided to indicate that the urease preparation conformed to data reported in the literature for other urease preparations.

One pound or approximately 450 grams of Arlco jack bean meal was used as the starting material. From this an unweighed amount of urease crystals was obtained. This was dissolved in 12 ml. of sterile water and this was further diluted 1/125 to give the urease test preparation. The organic nitrogen determination of this diluted preparation was found to be 5 micrograms/ml. Sumner (65) reported that the percent nitrogen in urease that had been crystallised once was 15.4 percent. Assuming an approximate factor of 6 then the amount of urease in the diluted preparation was 30 ug/ml. The total amount of urease obtained was

$$30 \text{ ug/ml} \times 125 \times 12 \text{ ml} = 45000 \text{ ug} = 45 \text{ mg} \quad \ldots.(4)$$

The percent of urease in the original jack bean meal was

$$\frac{45 \text{ mg}}{450 \text{ gms}} \times \frac{\text{gms}}{10^3 \text{ mg}} \times 100 = .01 \text{ percent} \quad \ldots.(5)$$

This was somewhat low in comparison with the figures given by Sumner who quotes figures ranging from .07 - .13 percent.

For the determination of the activity of this preparation a urea-phosphate buffer of pH 7 was used. The concentration of urea in this solution was .66M or 660uM/ml. The hydrolysis of urea was performed at 30°C giving the following results.

TABLE 29

Time (Min.)	$\frac{mgNH_3\text{-}N}{ml.urease}$	$\frac{mg.Urea\ decomp.}{ml.urease}$	$\frac{uM.Urea\ decomp.}{ml.urease}$	$\frac{uM.Urea\ decomp.}{ml/min.}$	% Urea Hydrolysed
1	.20	.43	7.1	7.1	1.08
2.5	.38	.83	13.9	5.5	2.1
5	.88	1.88	31.3	6.2	4.8
10	1.65	3.50	58.3	5.8	8.9
20	3.80	8.00	133.3	6.6	20.
30	5.70	12.00	200.	6.6	30.
				Av.= 6.3	

From this table it could be seen that the rate of hydrolysis of urea by urease was linear at a rate of 6.3 micromoles of urea per milliliter per minute. Such a relationship for a reaction indicated that the rate of reaction was constant and was not influenced by the concentration of reactants. This result had been previously reported by Sumner and Hand (66) for crystalline urease.

The same authors had also expressed urease activity in units. A unit of urease activity was that amount of urease which would form 1 mg. of ammonia nitrogen from a urea-phosphate buffer at pH 7.0 at 20°C in five minutes. Since the above hydrolysis was run at 30°C it will be assumed that half the amount of ammonia nitrogen would have been formed

at 20°C. This amounted to .44 mg. NH_3-N/ml. Since the amount of urease was 30 micrograms/ml or .03 milligrams/ml, then:

$$\frac{.03 \text{ mg. Urease/ml}}{.44 \text{ mg. } NH_3\text{-N}} = \frac{.066 \text{ mg. Urease}}{1 \text{ mg. } NH_3\text{-N}} \quad \ldots.(6)$$

Thus the unitage was .066 per milligram or 66 per gram. But this was the diluted preparation. Therefore the original crystalline precipitate possessed:

$$66 \times 125 \times 12 = 99{,}000 \text{ units per gram} \quad \ldots.(7)$$

Sumner (65) reported upon a once crystallized urease preparation as having 115,000 units per gram. The result of this extraction and crystallisation was considered to be in good agreement with Sumner's values when it was recalled that the percentage figure for urease in jack bean meal for this preparation was lower than the amount usually present.

C. N/Cl Ratios

The use of this ratio was suggested in the paper of Knox, et al. (46). These investigators, however, reversed the fraction and used it as a chlorine:nitrogen ratio. It was claimed that although the bactericidal concentration of chlorine varied with the number of bacteria, the ratio of chlorine to bacteria was constant. This dependence of bactericidal action upon the amount of chlorine for a given amount of bacteria was experimentally useful since comparable

results could be obtained under widely different conditions. It also provided a value for the bactericidal amount of chlorine which could be applied to bacteria and to enzyme proteins.

The same reasoning may be applied to the use of the reversed ratio, nitrogen:chlorine, with the note that the ratio now indicates the amount of organic nitrogen present per unit of free chlorine concentration. It was expected that curves for similar nitrogen:chlorine ratios would coincide closely and that there would be an orderly stepwise arrangement of such curves. For the controlled pH experiments, this was generally true. There was only one serious discrepancy occurring in the group of bactericidal tests at pH 4 (Table 2, N/Cl .02). However, this curve was not plotted as another curve with the same N/Cl ratio was available. When it was realized that these ratios represent microgram quantities of nitrogen per microgram quantities of chlorine and that the method of counting organisms involved about a 25 percent error then it was apparent that the ratio was not a precise one but rather a representation of a tendency. Similar comments might be applied to the enzyme inhibition curves. It was noted that the curves for the uncontrolled pH experiments could be presented in an orderly progression only if numbers of bacteria fell between certain levels. Thus in Figure 8 the data were based on bacterial numbers that did not exceed 100,000, and in Figure 9 the data were based on

numbers that varied from $7.5 \times 10^5 - 3 \times 10^6$/ml. If, however, the two groups are combined then serious discrepancies were noted and there was no orderly progression of curves in terms of N/Cl ratios. It would seem then that the significance of these ratios is dependent upon the manner of obtaining them - pH had to be controlled, and the bacterial nitrogen could not vary too greatly. The statement in Knox, et. al. (46) appeared to have been based upon very dense suspensions of E. coli that were used for manometric determinations. Such suspensions possessed high nitrogen values. Two sets of data were presented and from them the concentrations of chlorine could be calculated as 10 ppm. and 40 ppm. Such high concentrations of chlorine were well beyond those used in the disinfection of water. This leads one to cast additional doubt upon the applicability of these data toward this specific problem.

Returning to the work of this thesis and remembering the possible limitations of such ratios, what has been found? It should be noted that the N/Cl ratios for the enzyme inhibition test were much larger than those for the bactericidal test. It was mentioned in the Experimental Methods that this followed from the nature of the reagents used. A standard amount of enzyme had to be present in all test procedures in order for any comparisons to be made. Once set, this could not be varied. The criterion for the bactericidal test was more flexible -- that of viability -- and some variation in

the numbers of organisms was permissible and was even inevitable.

Yet despite this difference in the magnitude of the N/Cl ratios the data of both tests appeared to be comparable. The curves obtained in both tests were of similar shape, and they were arranged in a stepwise progression of N/Cl values. The ranges of the N/Cl ratios in both tests as well as the excess of that of the enzyme inhibition test over that of the bactericidal test follow:

TABLE 30

pH 4	Bactericidal Range of N/Cl	.01 - .2
	Enzyme Inhibition Range of N/Cl	3 - 50
	Ratio of E/B	300 - 250
pH 7	Bactericidal Range of N/Cl	.007 - .11
	Enzyme Inhibition Range of N/Cl	3 - 70
	Ratio of E/B	400 - 700
pH 7.5	Bactericidal Range of N/Cl	.005 - .16
	Enzyme Inhibition Range of N/Cl	2.8 - 60
	Ratio of E/B	600 - 400
pH 9.0	Bactericidal Range of N/Cl	.003 - .08
	Enzyme Inhibition Range of N/Cl	3 - 60
	Ratio of E/B	1000 - 800

What is the meaning of this excessive ratio of the enzyme inhibition test to the bactericidal test?

Consider the data of the two tests at pH 7.0:

TABLE 31

	N/Cl	Cl	Molecules of hypochlorite	Molecules chlorine / bacterial cell	Time for .01% survival or Activity Rem.
B	.011	.6 ug/ml	6×10^{15}	3×10^{10}	15 min.
				Molecules chlorine / molecules urease	
E	3	1.5 ug/ml	1.5×10^{16}	5×10^{2}	20 min.

The excess of the ratio of enzyme N/Cl over that of bacterial N/Cl arises from relative figures of organic nitrogen for the enzyme preparation and for the bacterial suspension. Although bacteria are giants compared to the molecules of urease there are more molecules of the latter than there are cells of the former. In the case of the enzyme it is known that the chlorine is acting upon a particle of known weight which for urease is 483,000. (Sumner, Gralen, Eriksson, and Quensel, 67). In the case of the [redacted] bacteria it can only be assumed that all the chlorine molecules are distributed equally among the cells. If the figure for the distribution of chlorine molecules per molecule of urease were taken as the approximate distribution of chlorine per large protein molecule and if the same distribution were assumed for the bacterial cell then the 3×10^{10} molecules of chlorine would be distributed among 10^{8} protein molecules of the bacterium. Assuming the volume of a protein molecule to be about 5×10^{-7} Å^3, then the total volume of 10^{8} protein molecules would be:

$$\frac{5 \times 10^{-7}\text{Å}^3}{\text{protein molecule}} \times 10^{8} \text{ protein molecules} \times \frac{1\ u^3}{10^{12}\text{Å}^3} = 5 \times 10^{-11} u^3 \qquad \ldots.(8)$$

And since the volume of the E. coli cell was $2.6 \times 10^{-1} u^3$ the percent fraction of the bacterial cell occupied by the affected protein molecules is

$$\frac{.00000005}{2.6} = \text{ca}.0000002\% \qquad \ldots.(9)$$

This would further assume that chlorine acted by diffusing through the cell as a neutral hypochlorous acid molecule and that it acted upon many loci within the cell some sensitive and some not. The large N/Cl ratio for urease might then be taken to indicate that many molecular centers of one specific enzymatic activity are present. The nature of the experimental test for its detection was such that only a few needed to be inactivated to reduce the activity of the enzyme and to lower its activity towards a specific substrate. The small N/Cl ratio for bacteria might be taken to indicate that many molecular centers of one specific enzyme were affected or that many molecular centers of diverse enzymes were affected. It has already been indicated in the review that the latter seems more probable. Chlorine is not a specific reagent and it would tend to oxidize any protein with which it came in contact. The nature of the test for the detection of its activity, i.e. viability of living organisms, was such that complete disruption of enzyme systems would be necessary to detect its effect. The small percentage of such centers affected in relation to the entire cell is reflected in the small N/Cl ratio.

Some confirmation of the amount of chlorine required to kill a bacterial cell comes from the work of Heinmets, Taylor and Lehman (47) whose measurements indicated that over 10^6 hypochloride molecules are required to kill one organism,,

E. coli, strain B/r. Since the calculation in Table 31 had provided a figure of 3×10^{10} hypochloride molecules per cell then there would be 10^{6} such molecules available for the destruction of the cell. Such correspondence of conclusions should be considered good in view of the different techniques involved and the different strains of organism used.

This result also lends support to a theme of the review. Bacterial death is not the simple result of the inactivation of one enzyme.

D. The Relationship of the Enzyme Inhibition Test to the Bactericidal Test

The following table summarizes the results of both tests in terms of the average time required to reach the point of .01 percent survival or activity remaining.

TABLE 32

EXTRAPOLATIONS TO THE .01 PERCENT SURVIVAL POINT

N/Cl	Cl/N	Time of .01 Percent Survival (min) pH 4	pH 7	pH 7.5	pH 9.0	Uncontrolled pH
.005 .006 .007	200 170 140	--	2	5	30	
.01	100	3	15	30	100	5 - 60
.02 .03 .04	50 30 25	4	45	50	>200	
.1	10	40	>100	>100	>200	60 ->100
.2	5	>100				
.8 1.6						100 ->100
		Time of .01 Percent Enzyme Activity Remaining (min.)				
3	.33	100	20	10	7-10	100
6	.16	ca500	40	40	20	
10	.1	beyond 500	100-500	100	20	beyond 500
15	.06	"	beyond 500	100	40	
25	.04	"	"	beyond 500	100	
50	.02	"	"	"	100	beyond 500

The data of the bactericidal test seemed to fit well with the prevailing concept of the bahavior of chlorine as a water disinfectant. (Fair, Morris, Chang, Weil, and Burden, 68).

Chlorine in water has been considered to act primarily as an oxidizing agent. When chlorine is dissolved in water it undergoes hydrolysis as follows:

$$Cl_2 + H_2O \longrightarrow HOCl + H^+ + Cl^- \qquad ....(10)$$

At the concentrations employed in water disinfection this hydrolysis is complete and rapid, in a very few seconds at ordinary temperatures. The hypochlorous acid undergoes a further reaction with water as follows:

$$HOCl \rightleftharpoons H^+ + OCl^- \qquad ....(11)$$

This process is reversible giving the equilibrium expression:

$$\frac{(H^+)(OCl^-)}{(HOCl)} = K \qquad ....(12)$$

At 25°C this ionization constant has a value of 3.7×10^{-8}. The significant part of this equation is considered the ratio of $(OCl^-)(HOCl)$. The equation can be rearranged to give:

$$(OCl^-)(HOCl) = K/(H^+) \qquad ....(13)$$

Thus the ratio is dependent inversely upon the hydrogen ion concentration and directly upon the pH. Since it has been found that chlorine in acid solution is more bactericidal than in alkaline solutions a relationship can be established between the decrease of hydrogen ion concentration and the increase of the concentration of undissociated hypochlorous acid.

TABLE 33

pH	Percent HOCl	Free Chlorine Required to give an Equivalent (HOCl)
4	100	1.0
7	75	1.3
7.5	50	2.
9	3	34.

Also since the measurement of free chlorine by a chemical test is the sum of both the undissociated hypochlorous acid and the hypochlorite ion concentration, such measurement does not express bactericidal efficiency which is supposed to depend upon the concentration of hypochlorous acid alone. Thus, with a pH greater than 4 more free chlorine is necessary to give a concentration of undissociated acid equivalent to the concentration of undissociated acid at pH 4. Up to pH 8 this amount is no more than four times that required at pH 4. However beyond pH 8 the amount becomes very great. The disinfectant action of solutions of chlorine may be further modified by the reaction of hypochlorous acid with ammonia to give chloramines. However since precautions were taken to eliminate this type of reaction in this investigation this subject will not be discussed here. It will suffice to mention that the chemical tests will measure a chlorine residual due to chloramines with the same efficiency that they measure chlorine residual due to hypochlorous acid and hypochlorite.

Yet the disinfectant efficiency of chloramines is much less than that of hypochlorous acid.

The data in the paper of Fair, et. al. (68) demonstrate the marked decrease in the disinfection efficiency of chlorine with increase of pH, and the marked increase of contact time required to reduce the numbers of micro-organisms as the chlorine concentration was lowered. In the first instance at pH 7, .03 ppm of free chlorine resulted 1 percent survival in 4 minutes; at pH 8.5, .03 ppm required 30 minutes for the same survival figure and at 9.8 about 150 minutes were required. Second, to secure a 1 percent survival at 4 minutes, about .15 ppm of chlorine had to be used at pH 8.5, and 1 ppm had to be used at pH 9.0. Similar results have been noted in this investigation except that N/Cl ratios were used. The ratio provided a missing factor from the above data -- the effect of organic material upon the efficiency of the germicidal activity of chlorine. At an N/Cl value of .02 the time for .01 percent survival increased from 4 minutes at pH 4, to 45 minutes at pH 7.0, to 50 minutes at pH 7.5, and to more than 200 minutes at pH 9.0. To secure an equivalent survival time to that of pH 4 the N/Cl had to be reduced to .007 for 7.0 and .005 for pH 7.5 which meant either that the chlorine concentration had to be increased 4 times or that the amount of organic nitrogen had to be reduced 4 times. This agreed with the efficiency figures for HOCl cited at the beginning of this section, Table 32. Thus the data of the bacteriological test are confirmed by reliable reports in the literature.

The results of the enzyme inhibition tests were, however, contrary to the results of the bactericidal test. The times to reach points of .01 percent Activity Remaining are much greater and the results at the ends of the pH scale were actually the inverse of the bactericidal test. Thus there was the result that chlorine at pH 9 is more inhibitory than at pH 4. This result, of course, ruled out any possibility of the use of this enzyme as a biochemical reagent for the assay of bactericidal chlorine. It was the result of attempting to set up an empirical relationship. There was no generalization at the start of the work that could have predicted the occurrence of such a finding. There was also little or no indication for an explanation of the failure to find a direct relationship. However, one might be attempted.

Urease possesses an isoelectric point of pH 5.1. (Sumner and Hand, 69). Thus, at pH 4 the reactive groups should show a different distribution of charges than at the other experimental pH values and it might be possible that under such conditions the molecule of urease becomes more resistant to the action of hypochlorous acid. Similar arguments could not be applied to bacteria. The proteins of the cell are more complex and their isoelectric points differ. Further, the inactivation of a few enzymes under adverse conditions, might entirely disrupt a delicately balanced, multi-enzyme system. Sumner and Myrbäck (70)

presented uncited data of the action of an acid pH upon urease. At pH 4 in water the unitage of urease activity was reduced to 0.8 of its value after 15 minutes exposure of the enzyme but within five minutes after neutralization the unitage rose to 0.9 of its original value. Comparative values at other pH values were lacking. Chlorine at pH 4 did not act destructively upon urease in the short time periods allowed. It should also be remembered that the urea-phosphate buffer that was added to the chlorine-enzyme mixture contained sulfite which not only reduced the hypochlorous acid to chloride ion but also provided a poised potential of +200 mv. According to Sizer and Tytell (71) urease showed a maximum activity when the potential of the medium was poised at +150 mv. Thus a chance possible combination of a change in the isoelectric point and a quick re-establishment of optimal conditions might account for the persistence of urease activity at pH 4.

For the behavior of urease in the presence of chlorine at pH 9 another explanation might be offered for the increased sensitivity of the enzyme to chlorine. At alkaline pH values some ammonia would be liberated from the protein. This would combine with the free chlorine present to form monochloramine:

$$NH_3 + HOCl \rightleftharpoons NH_2Cl + H_2O \qquad \ldots.(14)$$

This would be formed very rapidly in the neighborhood of pH 9. Although monochloramine is a very inefficient disinfectant

for bacteria it may or it may not affect the enzyme at this stage. However, when the pH is lowered some of the monochloramine would change to dichloramine

$$2NH_2Cl + H^+ \rightleftharpoons NH_4^+ + NHCl_2 \qquad \ldots.(15)$$

At pH 7, 35 percent of the chloramine would be present as this form. The dichloramine in the presence of the sulfite of the buffer would be reduced but slowly to chloride ion and ammonia. Thus the actual contact period for the action of chlorine as chloramine would not be known but it may well extend for some irregular time interval into the fifteen minute incubation period. A combination of the action of chloramines during both the contact period and the incubation period would result in a prolonged period of activity of chlorine against the sensitive loci of urease. The result would be an apparent increase in the sensitivity of the enzyme at pH 9.0. Now with low N/Cl ratios the concentration of dichloramine per urease nitrogen would be relatively greater than with high N/Cl ratios. Thus in the former case a greater number of the active centers would be affected and the time to reach .01 percent activity remaining would be short. In the latter case the relatively greater concentration of urease in proportion to chlorine would provide many more active centers. A lesser percentage of these would be inactivated and the time to reach .01 percent activity remaining would be extended. This also has

been observed and has been summarised in tables at the beginning of this section. (Table 32). The irregular action of the chloramines during the contact and incubation periods would also account for the deviations of the points of the N/Cl curves presented for the enzyme inhibition test. The observation that the deviations were greater at alkaline pH values might also be accounted for by the loss of ammonia from the reaction mixtures at the temperature (30°C) of the test procedure. In order to explain why the action upon bacteria is so different one would be forced to fall back upon the argument that more than one enzyme would be involved and that the slow disinfectant action of chloramines would be balanced by the ability of bacteria to replace any of the inactivated centers more rapidly than they would be destroyed.

E. Extrapolation of Curves in the Direction of Decreasing Time

This was done merely as a matter of interest and it really did bear upon any of the significant points of the thesis. The action of chlorine is so rapid that measurements could not usually be made at such short intervals. By following the general shape of the curves they may be extrapolated back without unnecessarily forcing the data from which they have been derived. When this was done the intercept at the 100 percent survival line would indicate the time to which the entire inoculum maintained itself completely. The graded series of times thus obtained paralleled the points of .01

percent survival for curves of similar N/Cl value. Short 100 percent survival times corresponded with short .01 percent survival times. Long 100 percent survival times corresponded with long .01 percent survival times.

TABLE 34

EXTRAPOLATIONS TO THE 100 PERCENT SURVIVAL POINT

N/Cl	Cl/N	Maintenance Time of 100 Percent Survival pH 4	pH 7	pH 7.5	pH 9.0
.005	200			1 sec.	20 sec.
.006	170				
.007	140		1 sec.		
.01	100	.012 sec.	25 "	1 "	1 min.
.02	50	.06 "		15 "	1 "
.03	30		1 min.		
.04	25	1 "			
.07			1 "	1 min.	
.1	10	2.5 "	20 "	10 "	30 "
.2		10 "			
		Maintenance Time of 100 Percent Enzyme Activity			
3	.33	.03 sec.	.001 sec.	.0001 sec.	<.0001 sec.
6	.16	.06 "	.06 "	.006 "	<.006 "
10	.1	2 "		.06 "	<.06 "
15	.06	15 "	6 "	.6 "	.06 "
25	.04	1 min.	1 min.	10 "	.6 "
50	.02	20 "	20 "	1 min.	.6 "

End of Discussion

X. SUMMARY

A comparison was made between the action of chlorine upon an enzyme urease and upon a bacterium, E. coli, strain C198.

The experimental method was set up to conform with the procedures of a bactericidal test in which bacteria were treated with a bactericide over different contact periods and in which the survival of bacteria was determined by a plate counting method. A parallel enzyme inhibition test was developed and used. The activity of the enzyme was estimated by a Nesslerisation procedure.

The experimental procedure used differed from the previously reported bactericidal tests in two respects. Small volumes were used for each combination of chlorine concentration and contact time. Meticulous attention to such details as cleanliness of glassware and pipettes and the use of reagents that possessed no chlorine demand provided a body of experimental data that was consistent and was presumed to be reliable.

The action of chlorine upon urease at different pH values provided results that differed from the action of chlorine upon the complex bacterial enzyme system.

In the presence of chlorine at pH 4, urease activity was reduced to .01 percent of the original activity in 100

minutes when the ratio of urease nitrogen to chlorine was three. In the presence of chlorine at pH 9, urease activity was reduced to .01 percent of its original activity in 7 minutes with the same N/Cl ratio. Intermediate values were obtained for the intermediate pH values used. Corresponding results were obtained at different N/Cl ratios. Urease showed its highest rate of inactivation at pH 9.

In the presence of chlorine at pH 4 bacterial numbers were reduced to .01 percent of their original value in 40 minutes when the ratio of bacterial nitrogen to chlorine was .1. In the presence of chlorine at pH 9, bacterial numbers were still present to the extent of 100 percent with the same N/Cl ratio.

The results of the bactericidal test are similar to those reported in the literature.

The results of the enzyme inhibition test were inversely related to the results of the bactericidal test. These results could not be confirmed directly but there was no reason to expect close correspondence between the activity and reactions of a single enzyme and the multiple enzyme activity of a complex, living cell.

On the basis of these studies the urease inhibition test could not be recommended for the assay of bactericidal chlorine.

End of Summary

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