An Evaluation of Certain Principles of Disinfection

Myers

AN EVALUATION OF CERTAIN PRINCIPLES OF DISINFECTION

by

Gordon Edward Myers

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Department of Bacteriology
and Immunology,
McGill University,
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PREFACE

April the 24th, 1676 has long been considered as the birthday of bacteriology, for on that day Antoni van Leeuwenhoek first described microscopic living creatures which unmistakably belong to the group of organisms now known as bacteria.

"The fourth sort of creatures, which moved through the three former sorts, were incredibly small, and so small in my eye, that I judged that if 100 of them lay (stretched out) one by another, they would not equal the length of a grain of course sand; and according to this estimate, ten hundred thousand of them could not equal the dimension of a grain of such course sand."

If we are to consider this statement as the first written description of bacteria, then the following report of Leeuwenhoek's observation made on May the 26th, 1676 must be considered as the first description of a study of disinfection from a bacteriological point of view.

"After it had stood an hour or two, I took some of the water, before spoken of, wherein the whole pepper lay, and wherein were so many several sorts of little animals; and mingled it with this water, wherein the pounded pepper had lain an hour or two, and observed, that, when there was much of the water of the pounded pepper, with that other, the said animals soon died, but when little, they remained alive."

The quotations given above are excerpts from a letter addressed to Mr. Henry Oldenburgh, Secretary of the Royal Society, London, written by Antoni van Leeuwenhoek, Delft, Holland, October the 9th, 1676.

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AN EVALUATION OF CERTAIN PRINCIPLES OF DISINFECTION

Historical Introduction

It is not the intention to present in this report an extended history of the application of the disinfection process. Chick (1908), and Chick and Martin (1908 a,b) gave an excellent review of this subject. Wyss (1948) reviewed more recent developments. McCulloch (1946) has given an interesting historical account of the origin of the practice of disinfection. Patterson (1932) investigated the origin of terminology commonly associated with disinfection and antisepsis. Our interest lies in the fundamental principles which constitute disinfection. Careful perusal of the literature leads to the conclusion that although the literal meaning of disinfection is relatively well established, the fundamental principles involved in the process or processes remain obscure.

The majority of methods which have been employed for testing disinfectants have been concerned with the action of disinfecting agents in the presence of extraneous matter. Serum, albumen, faecal matter and tissue from various sources have all been employed with the object of simulating conditions encountered in actual practice. Results obtained from such experiments have always been confusing. Ignorance of the fundamental principles involved in disinfection has been the main reason for these confusing results. The presence of

extraneous matter has only served to increase confusion. Uncontrolled or insufficiently controlled experiments have often been used as the basis of theory concerning the process of disinfection. Ill defined terminology or improper usage of well defined terms has served to augment the confusion arising from attempts to interpret experimental results.

The Section of Biology of the New York Academy of Sciences held a conference in October, 1949, for the purpose of : "(a) summarizing and evaluating existing information on the mode and extent of antimicrobial agents in vivo and in vitro, and (b) revealing those avenues of investigation likely to produce more active compounds and more effective application of them." Twenty-four papers presented at this conference have been published in the Annals of the Academy (Davis, 1950). From these and previous publications, which will be cited later, at least three possible principles involved in the process of disinfection have been recognized. These are:

(1) coagulation of protoplasm,

(2) profound alteration in permeability of the membrane surrounding the organism,

(3) poisoning of some essential enzyme system or enzyme substrate within the organism.

The order in which these mechanisms are presented here is not necessarily the order of their importance or of sequence of action. It is highly probable that a single disinfectant may operate against the bacterial cell by

more than one mechanism. It does seem logical that one action will predominate when a given disinfectant is brought in contact with a given test organism. If this is true, a sound classification of disinfecting agents may be established based on the predominating action on the bacterial cell. The methods developed for determining this predominating action may be used subsequently to evaluate and compare the disinfecting efficiency of various agents.

A brief resumé of existing evidence for and against each of the recognized principles involved in the disinfection process will be presented as an introduction for the experimental studies.

RÉSUMÉ OF EVIDENCE REGARDING RECOGNIZED PRINCIPLES OF DISINFECTION

Coagulation of Protoplasm

A great variety of terms appear in the literature describing the effects exerted by disinfectants. Certain of these have been employed because of apparent similarity between the disinfection process and chemical or physical reactions. The phrases "denaturation of protein", "precipitation of protein", "alteration of cell constituents" and "protoplasmic poisoning" have appeared frequently. Used by some authors, these terms are obviously intended to refer to one and the same reaction; used by others, this is not true. Krueger and Nichols (1935) have pointed out the looseness with which the term "protein denaturation" has been employed. They note that the phrase, "has a specific chemical connotation from the biochemists point of view" and that, "it refers to an intramolecular re-arrangement of the protein molecule which is evidenced by certain altered physical and chemical properties, these alterations being detectable by appropriate tests." The term, "denaturation has always been associated with some obscure change from the original protein, which could be detected but could not be explained" (Cook)*. The alteration may be detected by chemical, physical or biological methods. The detection of such alteration may then be used as an indicator of protein denaturation. The appearance of sulfhydryl groups has often been utilized as

*Personal communication to Prof. E.G.D. Murray from Dr. W.H. Cook, Director of Division of Applied Biology, National Research Council, Ottawa.

an indicator of protein denaturation. It must be obvious that this test has limitations, for the native protein must contain the elements necessary to produce these groups. The validity of this test as an indication of protein denaturation has been questioned. Meldrum and Dixon (1930) claim that denatured globin yielded neither sulfhydryl nor disulphide groups, whereas Anson and Mirsky (1931) claimed this observation was erroneous and that they obtained a preparation of denatured globin which gave tests for both groups.

Hardy (1899) and Chick and Martin (1910, 1911) presented evidence that protein coagulation consists of two steps. The first step in the process is known as "denaturation" and makes the protein insoluble. The second step is precipitation of this insoluble protein. Insolubility at the iso-electric point as an indicator of protein denaturation has long been employed (Schmidt, 1938). The test is complicated by secondary effects exerted by certain denaturing agents, thus a protein solution made viscous by alkali denaturation gradually becomes less viscous in the presence of alkali (after denaturation is completed). Anson and Mirsky (1930 a) have pointed out this complicating effect. Another confusing factor with regard to the insolubility of denatured protein at the iso-electric point is the variation in reversibility of this effect. Thus with egg albumen, the reaction is irreversable, whereas with serum albumen, it is reversible (Heidelberger)*. Anson

*Personal communication to Professor E.G.D. Murray from Dr. Michael Heidelberger, Professor of Biochemistry, Columbia University.

and Mirsky (1930 b) claim that completely coagulated haemoglobin can be prepared. This coagulated haemoglobin is soluble and indistinguishable from native protein according to these authors. It is our intention only to point out these conflicting opinions existing with respect to protein denaturation in definition and mechanism.

Krönig and Paul (1897) suggested that certain disinfectants act by altering cell material. These workers did not elaborate on their suggestion. Chick (1908) assumed such an alteration, which she described as, "an alteration in the constituents of the protoplasm (by chemical combination or otherwise) as to render it unfit for continued vitality of the organism". Cooper (1912) using gelatin and egg albumen observed that protein precipitation occurred in the presence of certain concentrations of phenol and meta-cresol. He concluded from his observations that the absorption of phenol by bacteria was the initial stage of disinfection, and that subsequent de-emulsification of the colloidal suspension resulting in precipitation completed the process. Anson (1940) demonstrated lysis of red blood cells and denaturation of haemoglobin by detergents of the Dupanol series. In his experiments, denaturation was detected by solubility and digestibility tests for haemoglobin and spectroscopically for methaemoglobin. Anson suggests that the same effect would result if an excess of detergent was added to a bacterial suspension, intimating that the disinfecting action of these detergents is due to lysis of bacterial

cells with subsequent denaturation of cellular protein. It is worthy of note that the evidence presented has been obtained by the study of protein of other than bacterial origin. The early reports of Wynter Blyth (1906) and Sommerville and Walker (1906, 1907) indicate that different types of protein exert varying effects on the disinfection process. Alteration in the physical state of a given protein (pulverization for example) may have a similar effect. It appears obvious that in order to state conclusively that disinfecting action is due to denaturation of bacterial protein, one must utilize bacterial protein and that this protein should be in the same physical state as exists in the living cell.

Krueger and Nichols (1935) prepared undenatured staphylococcal protein. Taking advantage of the insolubility of denatured protein at the iso-electric point, these workers were able to show a relationship between the amount of protein denatured by heat treatment and the degree of temperature elevation. This method has the advantage that it utilizes bacterial protein. The preparation of undenatured staphylococcal protein was such (grinding by ball mill) that it is highly improbable that the protein exists in the same physical state as that in the living cell. The reaction of this protein to the action of deleterious agents is not necessarily the same as that of unextracted cellular protein. We have no evidence that all chemical agents can pass through the cell wall. Therefore, the mere fact that denaturation of the protein takes place when the protein has been freed from the cell does

not prove that such will be the result when the protein is protected by the intact cell wall. It is indeed possible that denaturation of proteins making up the cell wall may serve to prevent further penetration of the chemical by forming a protective coating surrounding the undenatured metabolically active contents of the cell. The latter theory has been suggested by Hailer (1938) to explain the peculiar disinfecting action attributed to ethyl alcohol.

In the absence of an absolute definition of what actually constitutes protein denaturation and of adequate specific tests for measuring protein denaturation as it progresses within the intact cell, it is extremely difficult to determine the extent to which this mechanism is involved in the process of disinfection.

Alteration in the Permeability of the Membrane Surrounding the Organism

Alteration in the bacterial cell resulting from contact with disinfectant may be manifest by alteration in the permeability of the cell wall. Lamar (1911) suggested that the disinfecting property of alkali soaps was due to action on lipoidal portions of the cell which resulted in altered cell wall permeability. Winslow and Dolloff (1928) discussing results obtained using salts of sodium, potassium and magnesium expressed the opinion that, "cations exert a primary influence upon the bacterial cell which is qualitatively the same for all, but quantitatively different for each". These workers presented evidence that the primary effect was an alteration in cell wall permeability leading to rupture of the cell or irreversable physical change in the cell membrane. Murray (1929) demonstrated microscopically that disorganization of Meningococcus cells results when these are suspended; first, in solutions of sodium chloride and, subsequently in sterile distilled water. He noted that the process is accelerated by temperature elevation. Baker, Harrison and Miller (1941 a) concluded that the effect of detergents on bacteria might be attributed to a two fold action; first, disorganization of the cell membrane by virtue of surface activity and second, denaturation of protein essential to metabolism and growth. Evidence for the first action evolved from the work of Schulman (1937). Using red blood cells and detergent-like compounds, this worker demonstrated

that those compounds which are able to penetrate monolayers of lipoproteins increase surface tension markedly and cause lysis. Compounds, which do not penetrate but are adsorbed, cause agglutination. Hotchkiss (1946 a,b) demonstrated by chemical analysis that, whenever the concentration and the nature of the surface active agent are adequate to be bactericidal, a leakage of nitrogen and phosphorous compounds from the cells is observed. A similar observation was made by Gale and Taylor (1947) using Streptococcus faecalis cells which had been treated with tyrocidin. Leakage of specific amino acids was demonstrated by these workers. Mitchell and Crowe (1947) published electromicrographs supporting this work. The treated cells show disruption of the cell wall. This investigation is of special interest considering the surface activity of tyrocidin.

Poisoning of Essential Enzyme System or Enzyme Substrate Within the Organism

Conn (1892) isolated a rennet-like enzyme from bacterial cultures and proved its activity in culture filtrates. He demonstrated a decrease in the activity when the filtrate was heated. Quastel and Whetham (1925) and Quastel and Wooldridge (1927) using a modified Thunberg technique demonstrated that succinic dehydrogenase of E.coli was partially inhibited by benzene, acetone, toluene and phenol, and was completely inhibited by cyclohexanol and monohydric alcohols above certain concentrations. Bach and Lambert (1937 a,b) demonstrated that succinic dehydrogenase of Staphylococcus aureus was completely inhibited by benzene, toluene and phenol. These investigators also showed that lactic dehydrogenase of this organism was completely or partially inhibited by the same compounds and by cyclohexanol, resorcinol and some alcohols. Sykes (1939) studying the effect of a series of phenols and aliphatic alcohols on the succinic dehydrogenase of E.coli concluded that, "concentrations of the germicide equal to those which kill the whole suspension will considerably or completely inhibit the activity of the enzyme and that, at slightly higher concentrations, the enzyme is in all cases destroyed." Sykes suggested that such a test might be used in conjunction with or as a substitute for the Rideal-Walker test. Ely (1939) determined the effect of germicides on oxygen consumption of E.coli using the Warburg technique.

He observed that complete inhibition of respiration was necessary to render the organism incapable of growth on subculture and that negative subcultures always resulted as soon as respiration reached zero. Bronfenbrenner, Hershey and Doubly (1938, 1939) published a manometric method of evaluating disinfectants. They found the initial effect of disinfectants upon oxygen uptake of both tissue and bacterial cells was much more abrupt than the rate of death of these cells. After five or ten minutes exposure, however, the inhibition of respiration is a function of the concentration of the disinfectant rather than the duration of exposure. A distinct difference was observed in the degree of inhibition of oxygen uptake following exposure to bacteriostatic concentrations of the disinfectant as compared to bactericidal concentration. Greig and Hoogerheide (1941) using a manometric method and the organisms E.coli, Staphylococcus aureus and Proteus vulgaris concluded that inhibition in the normal rate of oxygen uptake corresponds with inhibition of growth. They observed that bacteriostatic concentrations had no effect on metabolic rate but did inhibit cell multiplication. Bucca (1943) studied the effect of certain germicides on the viability and respiratory enzymes of Gonococcus. He found a lack of coincidence between lethal concentrations of the germicides and enzyme inhibition endpoints. He concluded this discrepancy might be due to inhibition of an enzyme system other than that under investigation. Hambas (1945) concluded from study of the

dehydrogenases of Pneumococcus, that, of the alkaloids he tested, the quinine group was the most effective inhibitor. Green and Stumpf (1946) observed that the minimal concentration of chlorine required to give 100 per cent inhibition of glucose oxidation by a bacterial suspension, always completely sterilized the suspension. They noted that the time required for this inhibition was identical with the time required for complete killing. Baker, Harrison and Miller (1941 a,b,c), using surface active agents, noted a general correlation between surface activity and bactericidal activity. These workers reported that, even in high concentrations, the detergents fail to inhibit bacterial metabolism more than 80 to 95 per cent. Knox, Auerbach, Zarudnaya and Spirtes (1949) suggested that this apparent continued cell metabolism was due to traces of other substrate remaining in the test medium. Using a washed cell suspension, they were able to demonstrate complete inhibition of metabolism resulting from the addition of appropriate concentration of cationic detergents. The inhibition of certain metabolic reactions of E.coli was shown to parallel the death of the cell. Lactic acid oxidase of E.coli was cited as an example of a detergent sensitive enzyme. According to these workers, the specific inhibition of this enzyme system can account for metabolic inhibition, death of the cell, and increased permeability.

With regard to the actual mechanism involved in the interference with enzyme systems, very little is known. Fildes (1940) suggested an interference with essential metabolites

by combination, for example, with -SH groups. He believes this to be the mechanism of action of mercury and mercury salts. Klarmann and his co-workers (1929) suggested the formation of additive compounds, with certain free reacting groups, as the mechanism of action of halogen derivatives of resorcinol and phenol. The free reacting groups which may be presented in the cell by cleavage of complex proteins are NH, NH_2, COOH, OH and SH according to these investigators. The so-called "ion antagonism" has recently been suggested as a factor involved in interference with enzyme systems. Falk (1923) emphasized the importance of maintaining a proper ratio of ions for the proper functioning of biological systems. Numerous examples of "antagonistic" and "synergistic" relationships between individual ions in particular biological systems have been observed. Thus, in a suitable potassium-free medium which is also low in sodium and ammonium ions, little or no growth of any of the lactic acid bacteria occurs unless K^+ is added (MacLeod and Snell 1948). In the presence of low concentrations of K^+, Na^+ inhibits growth. This inhibitory action is overcome, by an increase in the concentration of K^+. As the amount of Na^+ present is increased, the amount of K^+ required is correspondingly increased. Using media from which various constituents are deleted, MacLeod and Snell (1950 a) have shown that Lactobacillus arabinosus requires K^+ and Mn^{++} for growth. These are the only metallic ions which must be added to permit growth according to these workers. If, in medium composed of pure

amino acids, glucose, vitamins, appropriate buffers and mineral salts, K^+ is supplied in excess and Mn^{++} is added in only small amounts, relatively low concentrations of Zn^{++} are found to inhibit growth. Such inhibitory effects are prevented completely (at moderate levels of Zn^{++}) if enough Mn^{++} is added. Thus, an antagonistic relationship exists between Zn^{++} and the nutritionally essential Mn^{++}. MacLeod and Snell (1950 b) have presented the following theoretical explanation of their observations: "An ion which suppresses growth frequently does so by interfering with one or more of the essential metabolic roles of an ion required for growth. Since the nutritionally essential trace elements function, at least in part, as necessary components of metabolically essential enzymes, a more exact picture of the mechanism of action of antagonistic ions might be visualized as a competition between the antagonists for an enzyme surface."

Summary of Historical Introduction

The scientific literature to the present yields information supporting at least three basic principles of disinfection. These are: coagulation of protoplasm; alteration in cell wall permeability, and; interference with essential enzyme systems.

"Denaturation" or coagulation of protoplasm through the medium of heat has been successfully demonstrated. This principle of disinfection has been suggested for acids, bases, heavy metals and their salts, chlorine, phenol, cresols, resorcinol, the halogen derivatives of resorcinol and phenol, formaldehyde, alcohols, and surface active agents.

Alteration of cell wall permeability has been demonstrated for salts of sodium, potassium and magnesium and for the surface active agents.

It is evident that a single disinfectant may operate against the bacterial cell through several channels, for example, surface active agents have been shown to interfere with essential enzyme systems and also to cause alteration in cell wall permeability. Phenol has been shown to interfere with essential enzyme systems and it has been suggested that this agent also acts by denaturation of cellular proteins. Denaturation of the protein moiety of the essential enzyme may be the factor connecting the two actions of phenol.

The effects exerted on the bacterial cell by a disinfecting agent may occur simultaneously or in sequence. It has been suggested that the initial action of surface

active agents is disorganization of the cell wall and that this is followed by denaturation of essential proteins.

Object and Purpose of the Present Experimental Investigation

It is obvious from the information presented in the historical introduction, that, in order to evaluate and compare chemical disinfectants, the fundamental principles which constitute the disinfecting action of the various agents must be known. Detailed investigation of these fundamental principles is therefore essential. The necessity for development of suitable methods for the investigation of these principles is obvious.

The experimental investigations presented here have a twofold object: first, the development of suitable methods for the study of the effect of chemical disinfectants on a test organism; second, presentation of results obtained using the methods developed.

It is anticipated that study of the results obtained in this and subsequent investigations will lead to a "new" and satisfactory classification of chemical disinfecting agents based on the fundamental principles predominating in the action of each agent.

Outline of Experimental Work

The experimental investigations reported in this thesis will be limited to the development of methods for studying the effect of various chemical disinfectants on the viable cell count and respiratory enzyme systems of the test organism. The results of experiments using the methods developed will also be presented.

It is anticipated that the methods developed may be utilized for study of chemical disinfectants, test organisms, and enzyme systems other than those reported at this time.

The report of experimental work will consist of five parts:

Part One: The development of a method for determining the effect of disinfectant on the viable cell count of the test organism.

Part Two: The development of a method for determining the effect of disinfectant on the respiratory enzyme systems of the test organism.

Part Three: Section A: The development of a method for simultaneous study of the effect of disinfectant on the viable cell count and the respiratory enzyme systems of the test organism.

Section B: Results of experiments using the method developed for the simultaneous study of the effect of disinfectant on the viable cell count and the respiratory enzyme systems of the test organism.

Part Four: Section A: Experiments demonstrating the effect of heat killed suspensions of test organisms on the respiratory enzyme system (lactic dehydrogenase) of the test organism.

Section B: The effect of disinfectant on heat killed suspensions of the test organism, with respect to the respiratory enzyme system (lactic dehydrogenase) of the test organism.

Section C: Relation existing between Cozymase and heat killed suspensions of the test organism.

Part Five: Final discussion and summary of results.

PART ONE

DEVELOPMENT OF A METHOD FOR DETERMINING THE EFFECT OF DISINFECTANT ON THE VIABLE CELL COUNT OF THE TEST ORGANISM

DEVELOPMENT OF A METHOD FOR DETERMINING THE EFFECT OF DISINFECTANT ON THE VIABLE CELL COUNT OF THE TEST ORGANISM

Introduction

Rahn and Barnes (1933), in an experimental comparison of various criteria of death in yeasts, noted that by definition these are mostly negative. "We call an organism dead when it shows no more the symptoms which we consider characteristic of life". The criteria which these workers studied for yeast cells were: (1) loss of reproductive power, measured by plate count; (2) loss of fermentative ability; (3) change in staining reaction; (4) loss of selective permeability. In experiment, the survivors as measured by the various criteria were determined after varied periods of exposure to harmful influence.

Sykes (1939) studying the effect of germicides on the dehydrogenases of Escherichia coli attempted to demonstrate relation between enzyme inactivation and decrease in viable count. It is our opinion that this approach should yield information of great value in determining the fundamental principles of disinfection contributing to the "death" of the cell.

There are many factors which are known to influence the effect of disinfectants on the bacterial cell. These all must be standardized or excluded before the results of experimental investigation can be reliably interpreted.

Factors involved in any method for testing disinfectants are: choice of test organism, method employed for growing test organism, medium employed for growing test organism, incubation period for growing test organism, preparation of test organism for disinfectant treatment, disinfectant treatment of test organism, removal of disinfectant from contact with test organism when treatment period is ended, and method of determining the number of cells of the test organism surviving. Each of these factors presents many problems, and we have attempted to exclude or entirely avoid these problems by modifying procedures or by introducing new methods.

General Outline of Proposed Method for Determining the Effect of Disinfectant Treatment on the Viable Cell Count of the Test Organism

Test Organism:

It is well known that response to disinfectant treatment varies in degree and character depending upon the genus, species and strain of the test organism employed.

Numerous test organisms have been used for the purpose of evaluating disinfectants. Pringle (1750), in early experimental work on substances resisting putrefaction, tested vinegar, carbon, chalk, sodium chloride, borax, alum and many other substances of both animal and vegetable origin. This work was done before the existence of bacteria was known. The measure of effectiveness of the substances tested was the ability to prevent putrefaction of pieces of lean beef. Clavert (1872) recognized that putrefied matter became "impregnated" with animal life which could be observed by microscope. This worker tested thirty-five substances for the ability to prevent putrefaction. Lack of foul odor and absence of moving animal life was taken as the measure of this ability. Dougall (1872) tested the ability of sixty-seven substances to prevent the appearance of animalcules in organic fluids. Bacteria, vibriones, monads, amoebae and torulae were observed in such fluids by the microscope. The various substances tested were evaluated on the basis of ability to prevent the appearance of moving bodies in

such fluids. Bucholtz (1875) in his study of antiseptics observed the ability of these agents to prevent the appearance of bacteria, "Micrococcus and Microbacterium", in infusions of ordinary smoking tobacco. Jalan de la Croix (1881) made no mention of bacterial species when he investigated the effect of various antiseptics on the behaviour of bacterial infusions obtained by placing peas, egg-white and ergot in tobacco infusion. Koch (1881) introduced the "thread" method of disinfectant testing and for his purpose employed the spores of anthrax. Esmarch (1887) investigated the newly introduced disinfecting agent "Creolin". He used cultures of "Typhus, Cholera and Anthrax" and also "pus in which Staphylococcus pyogenes aureus predominated". Henle (1889(used an emulsion of B.typhosa for testing creolin. Rideal and Walker (1903), Chick and Martin (1908 b) and the United States Public Health Service (1921) recommended Bacillus typhosa for disinfectant testing. Meyer (1906) employed the spores of Bacillus subtilis in determining the effect of temperature on the velocity of the disinfection process.

For our experiments, we have used Staphylococcus aureus (F.D.A. strain 209) which is the organism recommended by Ruehle and Brewer (1931) for use in the United States Food and Drug Administration Methods for Testing Disinfectants.

Brewer (1942) gave a detailed account of advantages and disadvantages of this organism. He states "it is the most generally utilized microorganism for antiseptic tests and at present the only one with a standardized resistance suitable for the purpose". Although Brewer pointed out that certain

variations in resistance have been reported for this organism, we have not detected such variations under the conditions of our experiments.

Method Employed for Growth of the Test Organism:

The possible transference of significant amounts of culture medium along with the test organisms, when the latter are placed in treatment tubes containing disinfectant, has always presented a problem. When the test organism is grown in broth or on the surface of solid medium, there is no doubt that such carry over does take place. Traces of medium carried over in this manner have been suggested as the cause of discrepancies in results. In order to avoid interference in disinfecting action arising from this source, we have utilized the method of Reed and McKercher (1948) growing the test organism on the surface of cellophane. The possibility of medium being carried over is thus reduced materially. We have encountered no difficulty in obtaining luxuriant surface growth of our strain of Staphylococcus aureus using this technique.

Petri plates, 140 mm. in diameter and 20 mm. deep, are employed. A filter mass sheet, 5 mm. thick, is cut so that a circular pad approximately 125 mm. in diameter results. The pad is placed in the bottom of the Petri plate. Peptone broth is poured over this pad until no further absorption takes place and an excess of broth flows into the gutter surrounding the pad. Cellophane is placed over the surface of the pad, folded under and smoothed down so that an even surface results. The plate complete with cover is autoclaved at 15 pounds pressure for 20 minutes. It is important to note that pressure developing beneath

the cellophane during autoclaving may lift it from the surface of the filter mass and even force it against the cover of the plate. To avoid this undesirable situation, the cellophane should be snipped with scissors at several points around its perimeter prior to autoclaving. If this practice is followed, the cellophane will remain tightly adhering to the surface of the filter mass after autoclaving.

After autoclaving and cooling, the upper surface of the cellophane is inoculated.

The cellophane employed by us was "Plain Cellophane" (C.I.L. No.300) listed as 0.00088 inches in thickness, as recommended by Reed and McKercher. We have also employed with equal success the "moisture proof" grades of cellophane. Using this type of cellophane, the procedure was to soak the cellophane in cold water for two hours, wash in cold running water for several minutes and then allow to dry at room temperature. This "leeching" procedure is all that is necessary to remove the so-called plasticizing agents used in "moisture proofing" the cellophane. These plasticizing agents are commonly magnesium sulfate, glycerol and urea and it is necessary to ensure their removal in order to avoid possible inhibiting influence on bacterial growth. We have made it a practice to "leech" all cellophane before use in order to avoid mishaps resulting from confusing "moisture proof" with "plain" cellophane.

Medium Employed for Growth of Test Organism:

Brewer (1942) pointed out the variation in resist-

ance to disinfectant exhibited by Staphylococcus aureus grown in media made with various lots of peptone.

The medium, which we have employed in preparing the cellophane pads, is Peptone Broth. The formula for this medium is that used routinely in this laboratory and may be found in Appendix A of this report.

Employed in the manner described herein, we have not noticed any variation in resistance of the test organism to disinfectant action.

Incubation Periods Employed:

The test organism was maintained on peptone agar slants at room temperature between tests. Prior to testing, the organism was twice subcultured on peptone agar for four hours at 37°C. At the end of the second subculture, a loop-ful of growth was scraped from the surface of the medium and suspended in 9 mil. of sterile distilled water. Three millelitres of this suspension was then spread over the entire surface of the cellophane covered pad. The inoculated plate was then incubated at 37°C. for 18 hours.

The object of the two four-hour subcultures was to obtain a uniform bacterial population consisting of young cells with which to inoculate the cellophane pad. The majority of the bacterial population at the end of the 18 hour incubation period should therefore be of the same age and consequently of the same resistance to deleterious effects.

Preparation of Cell Suspension of Test Organism for Disinfectant Testing:

At the end of the 18 hour incubation period, the surface growth was harvested from the cellophane pad and the cells suspended in a volume of sterile distilled water. One millelitre of this suspension was employed for each disinfectant test.

The suspension was roughly standardized visually by opacity before each test. More rigid methods of standardization employed subsequently will be described later in this report.

Treatment of the Suspension of Test Organisms with Disinfectant:

The treatment was carried out in sterile thick walled centrifuge tubes* of approximately 15 mil. capacity. The tubes were fitted with sterile gum rubber stoppers. One millelitre of the cell suspension of test organisms was placed at the bottom of the tube, one millelitre of aqueous solution of the disinfectant to be tested was then added and the tube was tightly stoppered.

The treatment period arbitrarily chosen was 20 minutes. The temperature for treatment was 37°C.

During the treatment period, tubes were rotated on the "rotating machine" described by Welch and Hunter (1940). Rotation was at 4 revolutions per minute. The rocking motion

*No. 102a Pyrex Tubes 15 mil. capacity, 18 X 100 mm., Ivan Sorvall Inc.

developed by this machine greatly increases the possibility of contact between the cells of the test organism and the disinfectant. Rotating ensures that no part of the inside of the tube escapes contact with the disinfecting solution. Consequently, all test organisms have an equal opportunity of contact with the disinfectant. The possibility of organisms escaping contact with disinfectant was suggested many years ago by Geppert (1889).

Removal of Disinfectant from Contact with Test Organisms at the end of the Treatment Period:

The removal of disinfectant from contact with cells of the test organism has always presented a problem. Davis and Swartz (1920) employed the diluting out method using the centrifuge for this purpose. We are fully aware that this process is relatively slow but if the time taken for the procedure is the same for each experiment, the time the disinfectant remains in contact with the test organisms will always be the same and the whole procedure of testing remains standardized. We are also aware that the diluting out process removes only that portion of the disinfectant which can be easily washed away and that disinfectant which combines with cellular material may not be removed. McCalla (1940) has demonstrated that adsorption of disinfectants such as mercuric chloride does take place. It is our intention in future experiments to further investigate this possibility by including neutralizing agents in the fluid used in the diluting out process. Except where otherwise

noted, sterile distilled water was used as diluting fluid in our experiments.

The centrifuge employed by us is the Serval Superspeed Angle Centrifuge Type SS-I*. This centrifuge was carefully calibrated using the tachometer and was found to attain a speed of 13,500 r.p.m. when the volt adjustor was brought up to 135 volts.

The procedure proposed for diluting out disinfectant was as follows:

At the end of the treatment period, 8 mil. of sterile distilled water is immediately added to the contents of the treatment tube. The total volume of fluid in the treatment tube is then 10 mil. The tube and contents are then centrifuged at 13,500 r.p.m. for 3 minutes. Nine millelitres of supernatant are then removed and replaced by 9 mil. of sterile distilled water. The procedure is repeated three times and following the third centrifugation, 9 mil. of supernatant is removed. The remaining one millelitre contains the cell sediment of test organisms. When nine millelitres of sterile distilled water is added, a one in ten dilution of the cell sediment results. The cell suspension thus obtained is subsequently diluted serially so that a ten fold dilution series results. Aliquots of each dilution of the series are then inoculated to peptone agar plates using calibrated dropping pipettes. The number of viable cells of the test organism surviving disinfectant

*Ivan Sorvall, Inc., 210 Fifth Ave., New York 10, N.Y.

treatment is determined by colony count.

Method of Determining the Number of Cells of the Test Organism Surviving Treatment with Disinfectant:

The "drop plate" method using calibrated dropping pipettes as described by Reed and Reed (1948) was chosen for this purpose. The method is admirably suited to our work for its accuracy has been amply demonstrated by numerous workers. Our own results demonstrate that this method of estimating viable cells is satisfactory for our purpose.

PRELIMINARY EXPERIMENTAL INVESTIGATIONS

Experiments to Establish the Validity of the Proposed Method for Determining the Effect of Disinfectant Treatment on the Viable Cell Count of the Test Organism

Experiment One

A suspension of the test organism was prepared according to the method outlined in the proposed procedure. Serial dilutions of this suspension were then made and plated, using the "drop plate" method.

The treatment procedure was done as previously outlined with the exception that sterile distilled water was used in place of disinfectant solution. Treatment tubes were rotated on the Welch rotating machine at 4 r.p.m. for 20 minutes at 37°C. The "diluting-out" process for removal of the disinfectant was employed using the high speed centrifuge.

Serial dilutions of the cell sediment, remaining in treatment tubes following the "diluting-out" process, were prepared. Peptone-agar plates were inoculated with aliquots of these dilutions using the "drop plate" technique.

Inoculated plates were incubated 24 hours at 37°C. The number of viable cells contained in one millelitre of the resuspended cell suspensions was calculated based on colony count.

The results of Experiment One are given in Table I.

TABLE I

Experiment to Determine Relation Between Viable Cell Counts Before and After High Speed Centrifugation

TEST NO.	VIABLE CELL COUNT		PERCENT RECOVERED
	Before Centrifugation	After Centrifugation	
1	2.4×10^9	2.2×10^8	9.1
2	1.0×10^9	6.5×10^7	6.5
3	1.5×10^9	8.5×10^7	5.6
4	1.5×10^9	1.0×10^8	6.6
5	6.0×10^9	1.0×10^8	1.6
6	4.4×10^9	2.5×10^8	5.6

For the results given in Table I, an enormous loss of cells apparently takes place during the treatment and subsequent diluting-out processes. Since no disinfectant was employed in these tests, the loss must be a matter of technique.

Experiment Two

In this experiment, the cells of the test organism were washed three times with sterile distilled water using the centrifuge operated at 5,000 r.p.m. for a period of three minutes each time. Washed cells were then resuspended in sterile distilled water by shaking by hand for 5 minutes with sterile glass beads. The resuspended cells were then subjected to the treatment and diluting-out procedures exactly as in Experiment One. The results of these tests are given in Table II.

Table II

Experiment to Determine the Effect of Using Washed Suspension of Test Organisms

TEST NO.	VIABLE CELL COUNT		PERCENT RECOVERED
	Before Centrifugation	After Centrifugation	
1	1.5×10^9	3.2×10^8	21
2	1.5×10^9	5.6×10^8	37
3	1.4×10^9	1.2×10^9	85
4	1.3×10^9	4.4×10^8	33

From a comparison of the results given in Table I and those in Table II, it is evident that by using washed

cells suspended by shaking by hand with glass beads, the percentage of cells recovered following high speed centrifugation is increased.

Experiment Three

Great difficulty had been experienced in resuspending the cell sediment after high speed centrifugation. Results shown in Table II indicate that a great loss of cells apparently takes place during the treatment and subsequent diluting out processes. It was our opinion that this apparent loss was due in part to the failure to break up the large aggregates of organisms formed during high speed centrifugation. In order to test the validity of this opinion, an experiment was designed which was exactly as Experiment Two with the exception that after final centrifugation, the cell sediment was resuspended by shaking by hand for 2 minutes with glass beads. Serial dilutions of resuspended cells were prepared as usual and aliquots of these inoculated to peptone-agar plates. The results of these tests are presented in Table III.

Table III

Experiment to Determine the Effect of Resuspending Cell Sediment by Shaking with Glass Beads Following High Speed Centrifugation

TEST NO.	VIABLE CELL COUNT		PERCENT RECOVERED
	Before Centrifugation	After Centrifugation	
1	3.7×10^9	7.5×10^8	20
2	4.6×10^9	1.2×10^9	26
3	6.0×10^9	6.0×10^9	100
4	2.7×10^9	2.0×10^9	74

Comparison of the results presented in Table II and Table III indicates that the apparent loss of cells during the treatment and diluting out processes can be decreased by resuspending the cell sediment by shaking with glass beads following high speed centrifugation.

Experiment Four

The inconsistency of the results obtained in Experiment Two suggested that a standardized efficient method of resuspending the cell sediment after high speed centrifugation was desirable. Although shaking with sterile glass beads by hand succeeded in resuspending cells, it is readily apparent that the extent of resuspension was not nearly constant. In order that the constancy of suspension might be increased, it was necessary to develop a method of aggitation which would be highly efficient in breaking up cell aggregates and at the same time be readily controlled. The scope and rate of the vibrations obtained when the treatment tube is shaken by hand cannot be rigidly controlled despite the most careful technique. It is obvious that the solution of this problem lies in the use of a machine for producing standardized vibrations. Many devises were tested for this purpose. It is felt that the description of devises which failed to serve the purpose would not add to the value of this report. Therefore, only the apparatus which finally proved successful will be described. The description of this apparatus will be found in Appendix B of this report.

The use of the electric vibrating machine obviates the use of glass beads in the resuspending process. The tube and contents are rotated with a circular motion in both the vertical and horizontal planes. The net result

of this combined motion is that the contents of the tube are swirled upward in a spiralling manner and a turbulence results which very effectively breaks up cell aggregates and uniformly suspends particulate matter. The radius of rotation is approximately 3 mm. The optimum rate of vibration for our purpose was found to be approximately 1300 vibrations per minute. At this rate of vibration, maximum turbulence results. Three minutes vibration using this machine is sufficient to produce satisfactory results.

Table IV gives typical results obtained with this apparatus. In these tests, washed cell suspension was prepared as previously outlined. The suspension was vibrated for 3 minutes at 1300 vibrations per minute before making the serial dilution of the original cell suspension.

Following the treatment and diluting-out processes, the cell sediment was resuspended by vibrating at the same rate for the same period of time before serial dilutions were prepared. Seven aliquots of the original cell suspension were treated in the manner described.

Table IV

Experiments to Determine an Effective and Reliable Method of Resuspending Cells of the Test Organism Following High Speed Centrifugation

TEST NO.	VIABLE CELL COUNT Before Centrifugation	VIABLE CELL COUNT After Centrifugation	PERCENT RECOVERED
1	4.0×10^9	2.6×10^9	65
2	4.0×10^9	2.9×10^9	70
3	4.0×10^9	2.8×10^9	70
4	4.0×10^9	3.2×10^9	80
5	4.0×10^9	2.6×10^9	65
6	4.0×10^9	2.6×10^9	65
7	4.0×10^9	2.7×10^9	67

It is evident from the results presented in Table IV that the apparent loss of cells which occurs during the treatment and diluting-out processes can be nearly eliminated by using a controlled shaking method to resuspend the cell sediment after high speed centrifugation. It is also evident that the use of the electric vibrating machine gives a greater constancy of resuspension than does shaking by hand.

The discrepancy between viable counts of the cell suspension before and after centrifugation which still exists may be attributed to the error inherent in the preparation of serial dilutions and "drop plates" or to clumps of cells which are not broken up despite vibration.

The error inherent in the preparation of serial dilutions and of "drop plates" can be reduced to a minimum by careful adherence to rigid technique.

Experiment Five

The possibility of clumps resisting the effect of vibration can be investigated by the tedious procedure of doing both "viable" and "total" cell counts for each cell suspension resuspended following high speed centrifugation. Provided all clumps are broken up and that each cell gives rise to a single colony, the ratio of viable cells (based on colony count) to total cells (based on microscopic count) should be 1:1.

It is evident that alteration in the ratio of viable to total cell counts will occur when any deleterious influence (such as disinfectant action) is introduced in the system. It was therefore considered that determination of this ratio would be of twofold value; first, as a control for the degree of breaking up of cell clumps following high speed centrifugation and second, as an indicator of the extent of influence of deleterious effect introduced in the system.

In order to establish the constancy of the ratio (viable to total cell count) when no deleterious effect is present, a series of control experiments was set up. In these experiments, the test organism was grown, harvested, washed and suspended as previously outlined. In place of disinfectant solution, sterile distilled water was substituted in the treatment procedure. The high speed centrifuge method of diluting-out was used after the treatment period ended. Cell sediment was resuspended using the

electric vibrating machine as described. Serial dilutions of the resuspended cells were prepared, viable and total cell counts were done for each dilution series. The viable counts were done by the method already described. Total counts were done using the Petroff-Hausser Bacteria Counting Chamber. The accuracy of this method has been investigated by Wilson and Kullman (1931). Table V gives the results of a series of experiments.

Each test in Table V represents a separately prepared suspension of the test organisms.

Table V

Experiment to Determine the Constancy of the Ratio (Viable: Total Cell Count) in the absence of Deleterious Effect

TEST NO.	VIABLE COUNT	TOTAL COUNT	RATIO (VIABLE:TOTAL)
1	1.2×10^6	1.2×10^6	1:1.0
2	3.4×10^6	4.2×10^6	1:1.2
3	1.2×10^7	1.2×10^7	1:1.0
4	2.9×10^7	3.1×10^7	1:1.0
5	3.6×10^7	3.6×10^7	1:1.0
6	2.7×10^8	4.2×10^8	1:1.6
7	1.3×10^8	3.0×10^8	1:2.3
8	1.2×10^9	2.7×10^9	1:2.2
9	2.1×10^9	5.3×10^9	1:2.5

The results given in Table V indicate that in the absence of deleterious effect, the ratio (viable: total cell count) is relatively constant and within the range of 1:1 to 1:2.5

Experiment Six

Having established the constancy of the ratio of viable to total cell counts after the treatment and diluting-out process, it was necessary to investigate the constancy of the total cell counts obtained using a series of aliquots of the same original cell suspension. A series of experiments was done using the same methods of growing, harvesting, washing and suspending cells as previously described. For each experiment, seven or eight aliquots of a washed cell suspension were subjected to the treatment and diluting-out processes. Sterile distilled water was used in the treatment process in place of disinfectant solution. Cell sediment was resuspended following these processes by the use of the electric vibrating machine as described. Serial dilutions of each resuspended cell sediment were prepared and total counts done for each of these. The results of these experiments are given in Table VI.

Table VI

The Reproducibility of Total Cell Counts of Aliquots of the Same Cell Suspension of the Test Organism Following the Treatment and Diluting-out Processes

TEST NO.	TOTAL CELL COUNT FOR ALIQUOT							
	1	2	3	4	5	6	7	8
1	$1.5X10^6$	$1.2X10^6$	$1.3X10^6$	$1.1X10^6$	$1.2X10^6$	$1.5X10^6$	$1.3X10^6$	$1.2X10^6$
2	$2.7X10^6$	$2.0X10^6$	$3.7X10^6$	$3.6X10^6$	$3.3X10^6$	$4.4X10^6$	$4.2X10^6$	
3	$1.0X10^7$	$1.1X10^7$	$1.1X10^7$	$1.0X10^7$	$1.0X10^7$	$1.0X10^7$	$1.2X10^7$	$1.2X10^7$
4	$2.9X10^7$	$2.7X10^7$	$3.0X10^7$	$2.4X10^7$	$2.8X10^7$	$2.4X10^7$	$3.1X10^7$	
5	$4.0X10^7$	$3.7X10^7$	$3.7X10^7$	$4.2X10^7$	$4.1X10^7$	$5.0X10^7$	$3.4X10^7$	$3.6X10^7$
6	$3.6X10^8$	$4.4X10^8$	$3.5X10^8$	$3.8X10^8$	$3.7X10^8$	$3.9X10^8$	$4.1X10^8$	$3.9X10^8$
7	$4.8X10^8$	$4.8X10^8$	$5.1X10^8$	$4.0X10^8$	$4.6X10^8$	$3.9X10^8$	$2.9X10^8$	$3.0X10^8$
8	$3.4X10^9$	$2.9X10^9$	$2.7X10^9$	$3.3X10^9$	$3.7X10^9$	$2.8X10^9$	$4.1X10^9$	$2.7X10^9$

From the results given in Table VI, it is evident that by using careful technique and the controlled methods previously described, a relatively constant total number of cells of the test organism remain in the treatment tubes following the treatment and diluting-out processes.

The results of experiments given in Tables V and VI indicate that in the absence of any deleterious effect, the total number of cells of the test organism, remaining in the treatment tube after the treatment and subsequent diluting-out process, is relatively constant. The ratio of viable to total number of cells also remains relatively constant following these processes. We can therefore consider that, using the procedure outlined, any significant decrease in the number of viable cells must be due to deleterious effect introduced into the system.

It is therefore suggested that the ratio of viable to total cell counts is a satisfactory indicator for deleterious effect. This ratio is in fact the so-called "survivor ratio" which has been employed by many workers (Luckiesh, 1946) to express the results obtained when test organisms are subjected to the germicidal effect of light rays.

The "survivor ratio" has the advantage that no definite end-point need be determined but rather a general trend is indicated. The over-all picture of the result of treating the test organism with various concentrations of disinfectant is admirably expressed using this ratio.

Experiments Seven and Eight

Geppert (1889) using anthrax spores as the test organism established that after treatment with disinfectant, "occasionally one or another isolated anthrax colonies appear on the fourth or fifth day of incubation". For this reason, Krönig and Paul (1897) made a practice of counting the visible colonies on test plates on three successive days following inoculation. In order to determine for ourselves the necessity of such procedure, we have done preliminary experiments using mercuric chloride and silver nitrate as disinfectant.

The preparation of the test organism prior to treatment was exactly as previously outlined. Aqueous solutions of the disinfectants were prepared with care so that a series of weight in volume concentrations of each agent was obtained. Treatment of test organisms and the diluting-out process were as previously outlined. Following the diluting-out process, the cell sediment in the treatment tubes was resuspended using the electric vibrating machine. Serial dilutions of the resuspended cells were prepared and total and viable counts were done according to the procedure already established. For each experiment, a control was prepared in which sterile distilled water was used in place of disinfectant.

Viable counts were estimated on the basis of colony counts done 24, 48, 72 and 96 hours after inoculation of test plates.

Tables VII and VIII give the total and viable cell counts as well as the ratio of viable to total for the various incubation periods. The concentration of disinfectant given in these tables is that which existed in the treatment tube when bacterial suspension and disinfectant solution were mixed.

The results which appear in Tables VII and VIII are presented in graphic form in Figures 1 and 2 respectively. In these figures, the solid line indicates the ratio of viable to total count which persisted despite prolonged incubation. The dotted line indicates the ratio of viable to total count existing at the end of the first twenty-four hour incubation period.

Table VII

Effect of Mercuric Chloride on Viable and Total Cell Counts of the Test Organism Staphylococcus aureus (F.D.A. 209)

Concentration of $HgCl_2$ P.P.M.	Total Count	Viable Count	Ratio:Viable to Total Count (Survival Ratio)
After 24 hour Incubation			
0(control)	5.1×10^8	2.7×10^8	1:1.8
0.50	4.8×10^8	3.2×10^3	$1:1.5 \times 10^5$
0.625	3.9×10^8	0	$<1:3.9 \times 10^8$
1.25	4.1×10^8	0	$<1:4.1 \times 10^8$
2.50	3.8×10^8	0	$<1:3.8 \times 10^8$
5.00	4.9×10^8	0	$<1:4.9 \times 10^8$
After 48 hour Incubation			
0(control)	5.1×10^8	2.7×10^8	1:1.8
0.50	4.8×10^8	7.3×10^4	$1:6.5 \times 10^3$
0.625	3.9×10^8	1.3×10^5	$1:3.0 \times 10^3$
1.25	4.1×10^8	2.6×10^3	$1:1.5 \times 10^5$
2.50	3.8×10^8	2.3×10^2	$1:1.6 \times 10^6$
5.00	4.9×10^8	0	$<1:4.9 \times 10^8$
After 72 hour Incubation			
0(control)	5.1×10^8	2.7×10^8	1:1.8
0.50	4.8×10^8	9.0×10^4	$1:5.3 \times 10^3$
0.625	3.9×10^8	2.0×10^5	$1:1.9 \times 10^3$
1.25	4.1×10^8	2.6×10^3	$1:1.5 \times 10^5$
2.50	3.8×10^8	2.3×10^2	$1:1.6 \times 10^6$
5.00	4.9×10^8	0	$<1:4.9 \times 10^8$
After 96 hour Incubation			
No change in number of colonies given at 72 hour incubation			

Table VIII

Effect of Silver Nitrate on Viable and Total Cell Counts of the Test Organism (Staphylococcus aureus, F.D.A. 209)

Concentration of $AgNO_3$ P.P.M.	Total Count	Viable Count	Ratio: Viable to Total Count (Survival Ratio)
After 24 hour Incubation			
0(Control)	3.6×10^7	3.6×10^7	1:1.0
0.50	5.0×10^7	3.0×10^6	$1:1.6 \times 10$
1.00	4.2×10^7	4.0×10^4	$1:1.0 \times 10^3$
2.50	3.7×10^7	1.4×10^3	$1:2.6 \times 10^4$
5.00	4.0×10^7	0	$<1:4.0 \times 10^7$
After 48 hour Incubation			
0(Control)	3.6×10^7	3.6×10^7	1:1.0
0.50	5.0×10^7	1.5×10^7	1:3.3
1.00	4.2×10^7	2.0×10^5	$1:2.1 \times 10^2$
2.50	3.7×10^7	1.4×10^3	$1:2.6 \times 10^4$
5.00	4.0×10^7	0	$<1:4.0 \times 10^7$
After 72 hour Incubation			
0(Control)	3.6×10^7	3.6×10^7	1:1.0
0.50	5.0×10^7	1.6×10^7	1:3.0
1.00	4.2×10^7	2.0×10^5	$1:2.1 \times 10^2$
2.50	3.7×10^7	1.4×10^3	$1:2.6 \times 10^4$
5.00	4.0×10^7	0	$<1:4.0 \times 10^7$
After 96 hour Incubation			
No change in number of colonies given for 72 hour incubation			

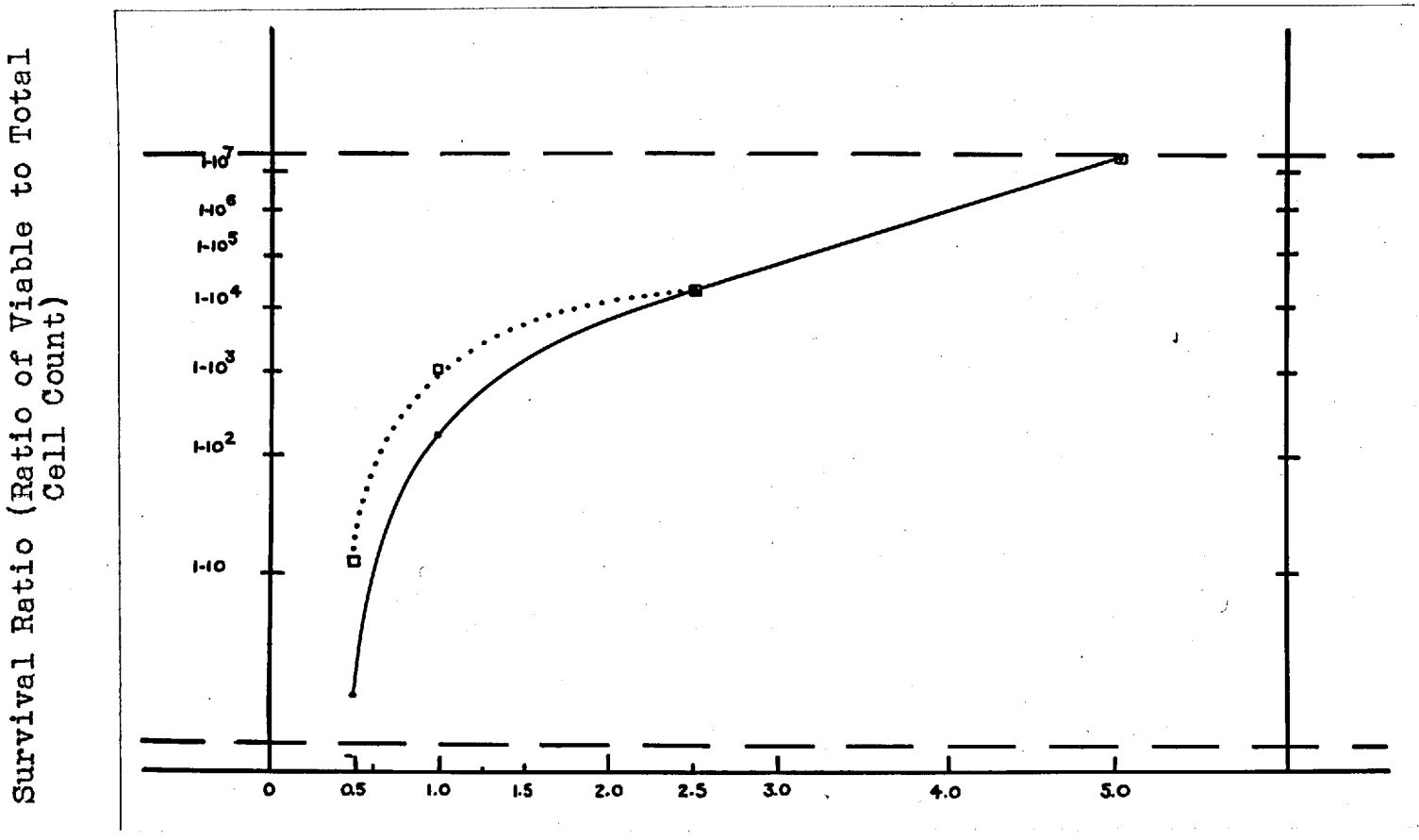


Figure 2: EFFECT OF SILVER NITRATE ON THE VIABLE CELL COUNT OF Staphylococcus aureus (F.D.A209)
A graphic representation of the experimental results given in Table VIII.
Symbols: . . . . . Survival ratio calculated at end of twenty-four hour incubation.
——— Survival ratio calculated at end of seventy-two hour incubation.

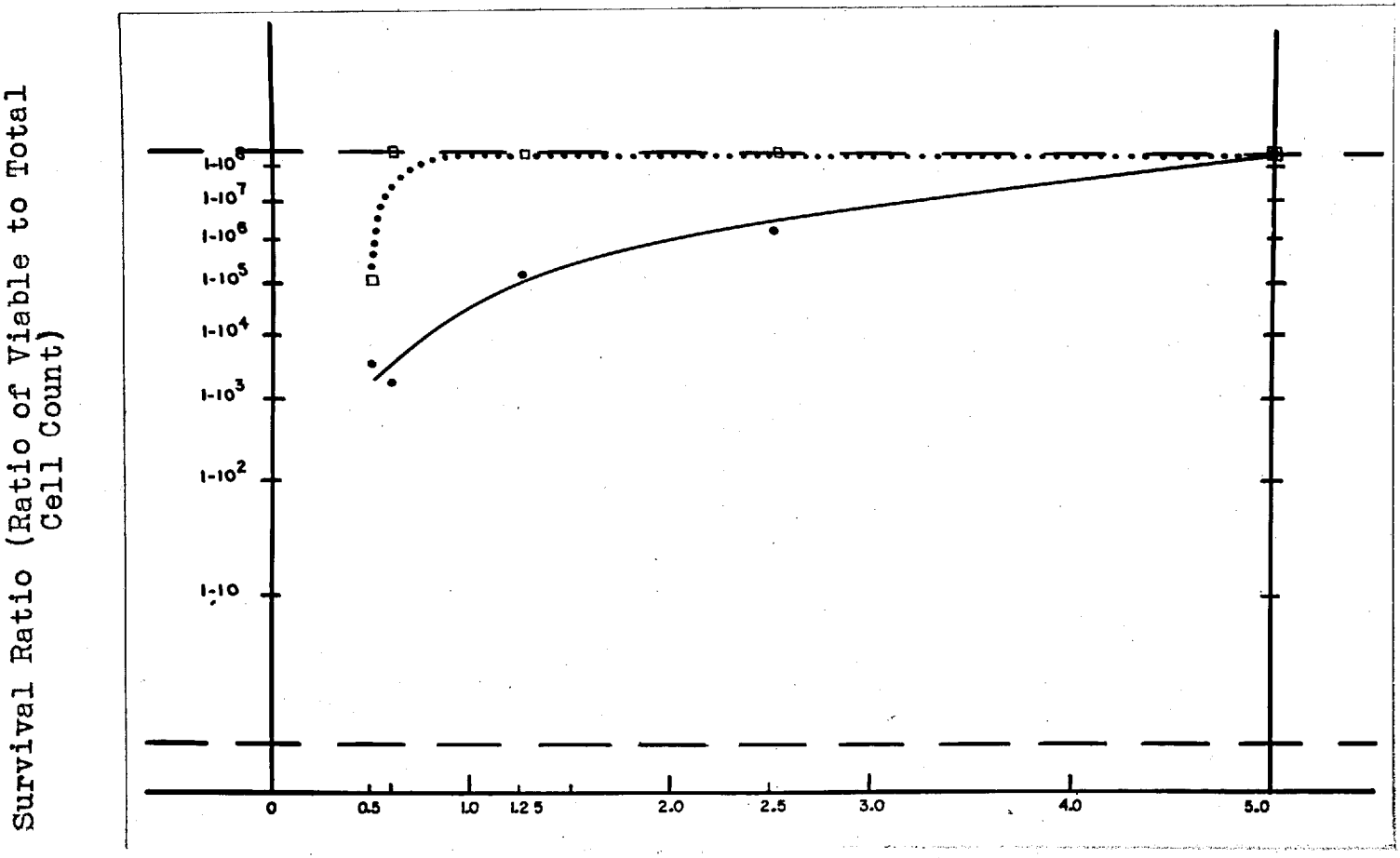


Figure 1: THE EFFECT OF MERCURIC CHLORIDE ON THE VIABLE CELL COUNT OF *Staphylococcus aureus* (F.D.A.209)
A graphic representation of the experimental results given in Table VII.
Symbols: ..... Survival ratio calculated at end of twenty-four hour incubation.
——— Survival ratio calculated at end of seventy-two hour incubation.

It is important to note that the viable cell count of the control test always remained constant after the first twenty-four hour incubation period despite prolonged incubation.

It is evident from these results that the viable cell (Colony) count should be followed carefully by doing such counts 24, 48, 72 and 96 hours after inoculation of the test plates. In this manner, one can obtain an accurate picture of the effect of disinfectant treatment. The increase in viable cell count which occurs following the first twenty-four hour incubation period indicates delayed multiplication of cells of the test organism. Since this delay does not occur with untreated controls, the effect must be the result of disinfectant treatment. This delay in multiplication may well be due to the adsorption of mercury which McCalla (1940) has demonstrated. Gegenbauer (1921) contended that this adsorption does not damage the cells beyond repair. Our experiments appear to substantiate this contention. Schuler (1946) demonstrated that with mercury compounds used in suboptimal concentrations, the respiration of Staphylococcus is immediately decreased following treatment. After standing and without neutralization of the disinfecting agent, the respiration of the cells gradually increases approaching that of normal untreated cells. The time required for recovery and the extent of recovery depend upon the concentration of the disinfectant used. This investigator also found that with a detergent type disinfectant, no

recovery was observable.

In our experiments, recovery from disinfectant treatment as indicated by increasing viable cell counts with prolonged incubation was noted for mercuric chloride and silver nitrate. With phenol and Tincture of Iodine (5%) recovery was occasionally noted but was less obvious and more inconsistent. When ethyl alcohol and "Roccal" were used as disinfecting agents, no recovery was observed.

It has been our practice to do viable cell (colony) counts for all plates at 24, 48, 72 and 96 hours of incubation at 37°C. No increase in colony count has been observed after 72 hours incubation. The constant viable cell count is the count given in all subsequent experiments.

Experiment Nine

Peptone-agar plates used for estimation of viable cells based on colony count must be perfectly dry if the "drop plate" method is to be used. This necessitates that such plates be poured long enough before they are required for use so that all surface moisture will be dried. Our practice has been to pour the plates and when they are solidified place them in the 37°C. incubator for a period of 18 hours. This period is always sufficient to ensure a dry surface for inoculation. The main disadvantage of this procedure is that plates cannot be prepared ahead of time and stored till required for inoculation. If storage did not effect the number of colonies developing on such plates, this procedure would materially facilitate the testing method. It was necessary to investigate this possibility and for this purpose the following experiments were conducted.

In these experiments, the method was exactly as previously outlined. Water was substituted for disinfectant solution during the treatment process. Table IX gives the results of viable cell counts for the same cell suspension inoculated to freshly prepared plates (dried 18 hours at 37°C.) and to stored plates (dried 18 hours at 37°C. then stored at 5°C. for 72 hours). Stored plates were brought out of the ice box four hours prior to inoculation and placed at room temperature until used.

TABLE IX

Comparison of Viable Counts Obtained from Aliquots of the Same Cell Suspension Inoculated to Stored and Freshly Prepared Peptone-Agar Plates

EXPERIMENT NO.	VIABLE COUNT Freshly Prepared Plates	VIABLE COUNT Stored Plates
1	1.0×10^6	1.0×10^6
2	8.0×10^5	8.0×10^5
3	1.4×10^6	1.6×10^6
4	2.4×10^6	2.1×10^6

The results presented in Table IX indicate that the viable count of aliquots of the same cell suspension is not materially altered when stored plates are used.

SUMMARY OF PRELIMINARY EXPERIMENTAL INVESTIGATIONS

The Method Developed for Determining the Effect of Disinfectant Treatment on the Viable Cell Count of the Test Organism

The procedure developed from the results of experiments designed to test the validity of the proposed method for determining the effect of disinfectant on the viable cell count of the test organism is as follows:

Preparation of Suspension of Test Organism:

1. Test organism - Staphylococcus aureus (F.D.A. 209)
2. Test organism subcultured two times at four hour intervals on peptone-agar slants, incubated at 37°C.
3. Surface growth from peptone-agar slant is harvested and suspended in sterile distilled water. This suspension is inoculated to a cellophane covered filtermass pad. The pad of filtermass was soaked with peptone broth prior to inoculation.
4. Inoculated cellophane pad is incubated at 37°C. for 18 hours.
5. Surface growth from the cellophane pad is harvested, washed three times in sterile distilled water using the centrifuge at 5,000 r.p.m. The washed cells are re-suspended in sterile distilled water using the electric vibrating machine at approximately 1,300 vibrations per minute for a period of 5 minutes.

Treatment of Cell Suspension with Disinfectant:

1. One millelitre of cell suspension is placed in a sterile heavy walled centrifuge tube of 15 mil. capacity.
2. One millelitre of aqueous solution of disinfectant is added to the cell suspension. The tube is tightly stoppered with a sterile gum rubber stopper.
3. Treatment tubes and contents are rotated in the Welch rotating machine at four r.p.m. for 20 minutes at a temperature of 37°C.

Removal of Disinfectant from Contact with Cell Suspension:

1. Disinfectant is "diluted-out" at the end of the treatment period by the addition of 8 mil. of sterile distilled water and subsequent centrifugation at 13,500 r.p.m. for three minutes. Nine mil. of supernatant are then removed and replaced by 9 mil. of sterile distilled water. The diluting-out process is repeated three times.
2. Cell sediment remaining in the treatment tubes after the final centrifugation is resuspended in sterile distilled water by vibrating, with the electric vibrating machine, at approximately 1,300 vibrations per minute for three minutes.

Estimation of Viable Organisms Surviving Disinfectant Treatment:

1. Serial dilutions of the resuspended cells are prepared. Peptone-agar plates are inoculated by the "drop plate" method using aliquots from the dilution series. Plates

are incubated at 37°C. Viable counts estimated on the colony counts of such plates are done at 24, 48, 72 and 96 hours following inoculation.

Estimation of Total Numbers of Organisms in Treatment Tubes:

1. Total cell counts are done for each cell suspension after the treatment and diluting-out processes. Total counts are done using the Petroff-Hausser Bacteria Counting Chamber.

Statement of Results:

1. Results presented in Tables contain figures representing:

(a) concentrations of disinfectant existing in each treatment tube;

(b) total cell count;

(c) viable cell count; and

(d) ratio of viable to total cell count.

Control:

1. For each experiment, one control test using sterile distilled water in place of disinfectant solution is subjected to exactly the same procedure.

RESULTS OF EXPERIMENTS USING THE METHOD DEVELOPED FOR DETERMINING THE EFFECT OF DISINFECTANT TREATMENT ON THE VIABLE CELL COUNT OF THE TEST ORGANISM

The following Tables and Figures represent the results obtained using the method developed for determining the effect of disinfectant treatment on the viable count of the test organism.

Six chemical disinfecting agents were studied. These were: mercuric chloride, silver nitrate, tincture of iodine,(5% w/v), phenol, ethyl alcohol, and a 10% weight in volume solution of alkyl dimethyl benzyl ammonium chloride (Roccal).

In each Figure, the survivor ratio of the test organism is plotted on semi-logarithmic paper against the concentration of the disinfectant which existed in the treatment tube during the treatment procedure.

EXPERIMENT TEN

Table X

Effect of Mercuric Chloride on Viable and Total Cell Counts of the Test Organism Staphylococcus aureus (F.D.A.209)

Concentration of $HgCl_2$ P.P.M.	Total Count	Viable Count	Ratio:Viable to Total Count (Survival Ratio)
0(Control)	1.2×10^6	1.2×10^6	1:1
0.50	1.3×10^6	2.1×10^4	$1:6.1 \times 10$
0.625	1.5×10^6	4.6×10^3	$1:3.2 \times 10^2$
1.25	1.2×10^6	1.4×10^2	$1:8.5 \times 10^3$
2.50	1.1×10^6	0	$<1:1.1 \times 10^6$
5.00	1.5×10^6	0	$<1:1.5 \times 10^6$

The results given in Table X are presented graphically in Figure 3 which follows immediately.

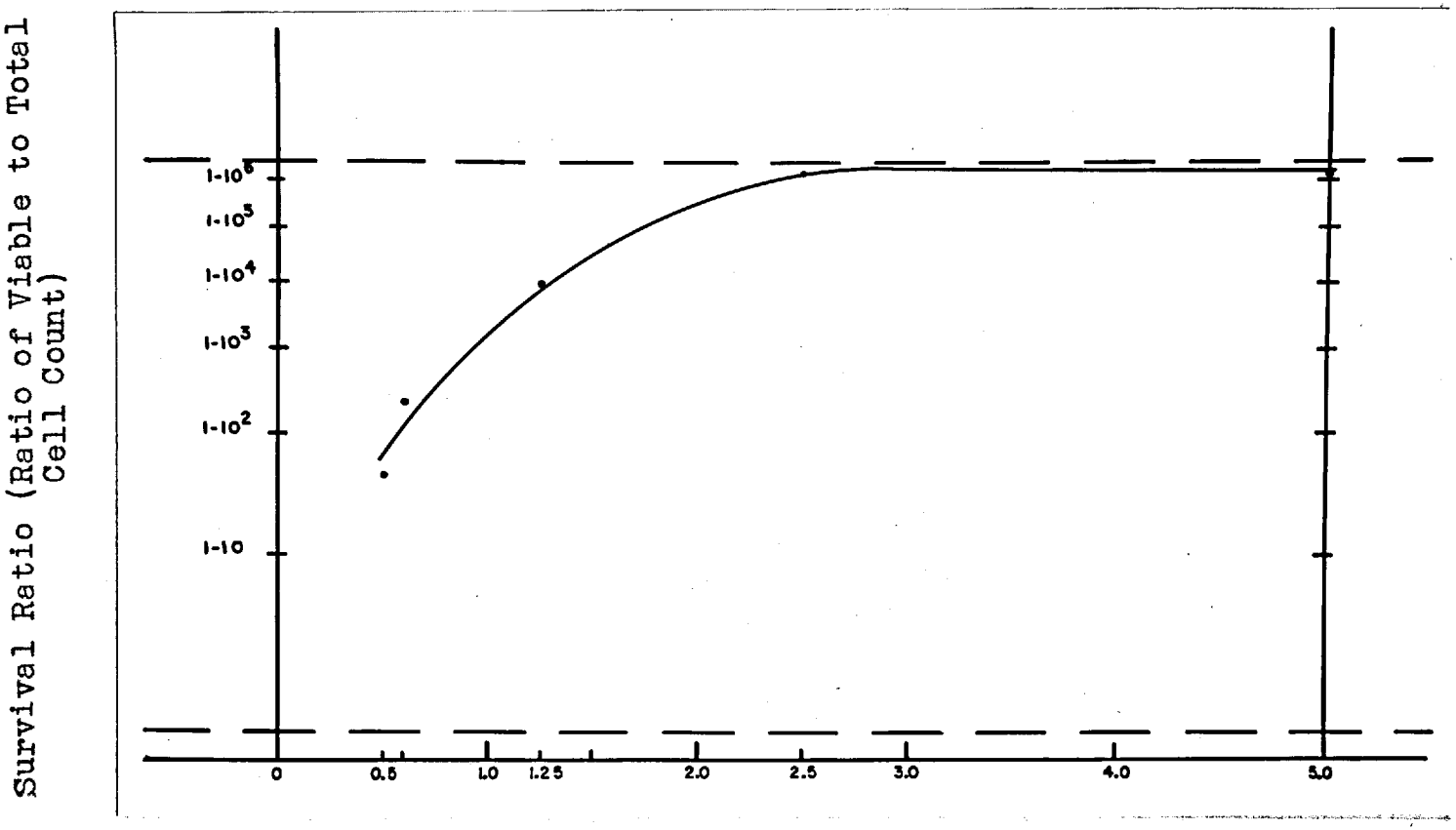


Figure 3: THE EFFECT OF MERCURIC CHLORIDE ON THE VIABLE CELL COUNT OF Staphylococcus aureus (F.D.A.209)
A graphic representation of the experimental results given in Table X

EXPERIMENT ELEVEN

Table XI

Effect of Mercuric Chloride on Viable and Total Cell Counts of the Test Organism Staphylococcus aureus (F.D.A.209)

Concentration of $HgCl_2$ P.P.M.	Total Count	Viable Count	Ratio:Viable to Total Count (Survival Ratio)
0(Control)	5.1×10^8	2.7×10^8	1:1.8
0.50	4.8×10^8	9.0×10^4	$1:5.3 \times 10^3$
0.625	3.9×10^8	2.0×10^5	$1:1.9 \times 10^3$
1.25	4.1×10^8	2.6×10^3	$1:1.5 \times 10^5$
2.50	3.8×10^8	2.3×10^2	$1:1.6 \times 10^6$
5.00	4.9×10^8	0	$< 1:4.9 \times 10^8$

The results given in Table XI are presented graphically in Figure 4 which follows immediately.

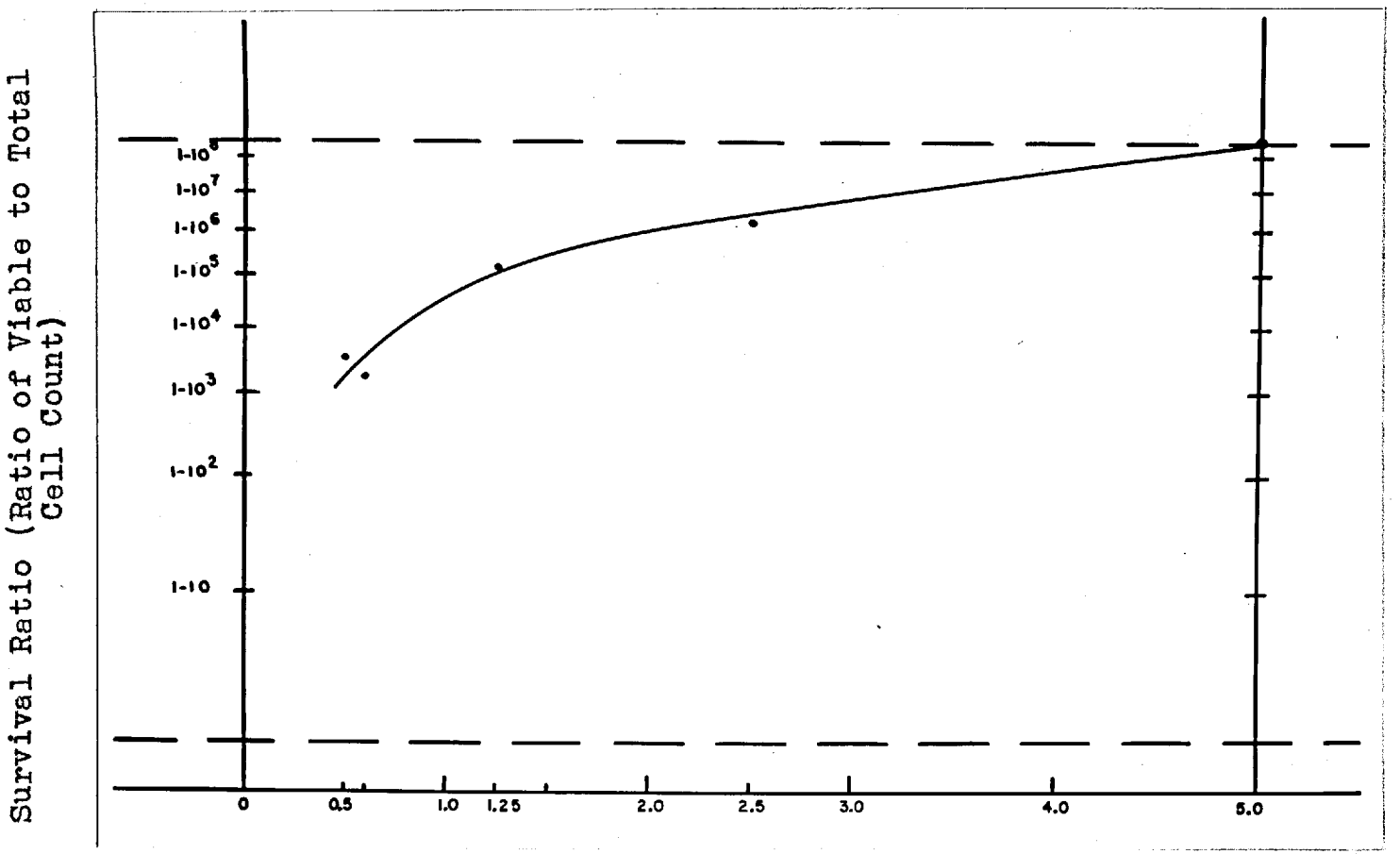


Figure 4: THE EFFECT OF MERCURIC CHLORIDE ON THE VIABLE CELL COUNT OF Staphylococcus aureus (F.D.A.209).
A graphic representation of the experimental results given in Table XI.

EXPERIMENT TWELVE

Table XII

Effect of Silver Nitrate on Viable and Total Cell Counts of Test Organism Staphylococcus aureus (F.D.A. 209)

Concentration of $AgNO_3$ P.P.M.	Total Count	Viable Count	Ratio:Viable to Total Count (Survival Ratio)
0(Control)	4.4×10^7	3.3×10^7	1:1.3
0.625	5.2×10^7	4.7×10^6	$1:1.1 \times 10$
1.25	5.1×10^7	4.0×10^4	$1:1.2 \times 10^3$
2.50	5.3×10^7	2.0×10^4	$1:2.6 \times 10^3$
5.00	5.0×10^7	0	$<1:5.0 \times 10^7$

The results given in Table XII are presented graphically in Figure 5.

EXPERIMENT THIRTEEN

Table XIII

Effect of Silver Nitrate on Viable and Total Cell Counts of Test Organism Staphylococcus aureus (F.D.A.209)

Concentration of $AgNO_3$ P.P.M.	Total Count	Viable Count	Ratio:Viable to Total Counts (Survival Ratio)
0(Control)	$3.6 \bar{X} 10^7$	3.6×10^7	1:1.0
0.50	5.0×10^7	1.6×10^7	1:3.0
1.00	4.2×10^7	2.0×10^5	$1:2.1 \times 10^2$
2.50	3.7×10^7	1.4×10^3	$1:2.6 \times 10^4$
5.00	4.0×10^7	0	$<1:4.0 \times 10^7$

The results given in Table XIII are presented graphically in Figure 5.

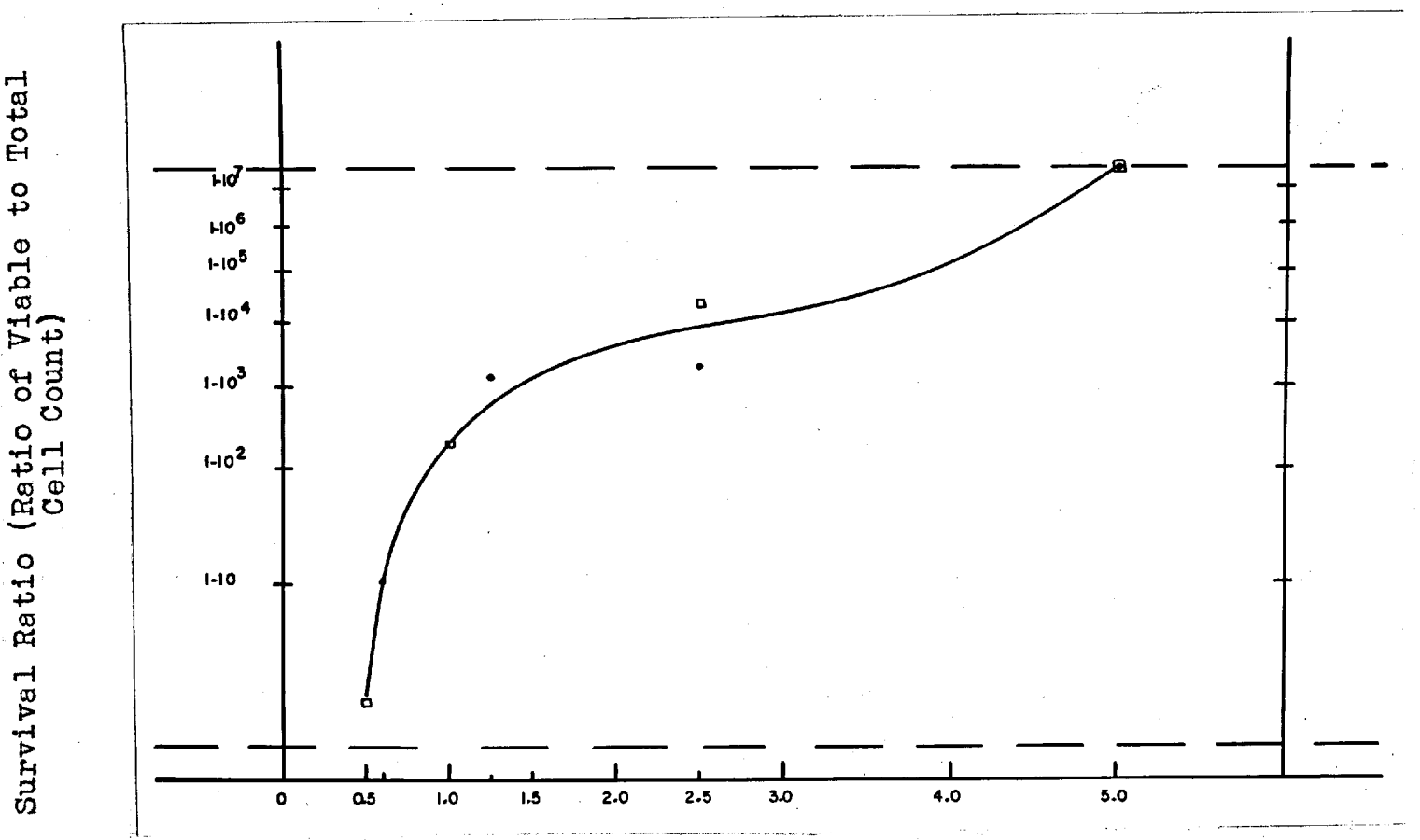


Figure 5: THE EFFECT OF SILVER NITRATE ON THE VIABLE CELL COUNT OF *Staphylococcus aureus* (F.D.A.209)
A graphic representation of the experimental results given in Tables XII and XIII.
Symbols: ● Results from Table XII.
□ Results from Table XIII.

EXPERIMENT FOURTEEN

Table XIV

Effect of Tincture of Iodine (5%w/v) on Viable and Total Cell Count of Test Organism *Staphylococcus aureus* (F.D.A. 209)

Concentration of 5% w/v Tincture of Iodine	Total Count	Viable Count	Ratio:Viable to Total Count (Survival Ratio)
0(Control)	3.9×10^7	1.6×10^7	1:2.4
1-60,000	4.1×10^7	9.0×10^6	1:4.5
1-50,000	3.9×10^7	2.0×10^5	$1:2.0 \times 10^2$
1-40,000	3.7×10^7	2.7×10^3	$1:1.3 \times 10^4$
1-30,000	3.8×10^7	2.7×10	$1:1.6 \times 10^6$
1-20,000	3.5×10^7	0	$<1:3.5 \times 10^7$
1-10,000	4.4×10^7	0	$<1:4.4 \times 10^7$
1-5,000	3.6×10^7	0	$<1:3.6 \times 10^7$

The results given in Table XIV are presented graphically in Figure 6 which follows immediately.

Note: The Tincture of Iodine 5% w/v was assayed for iodine and potassium iodide content. The results of this assay are presented in Appendix C.

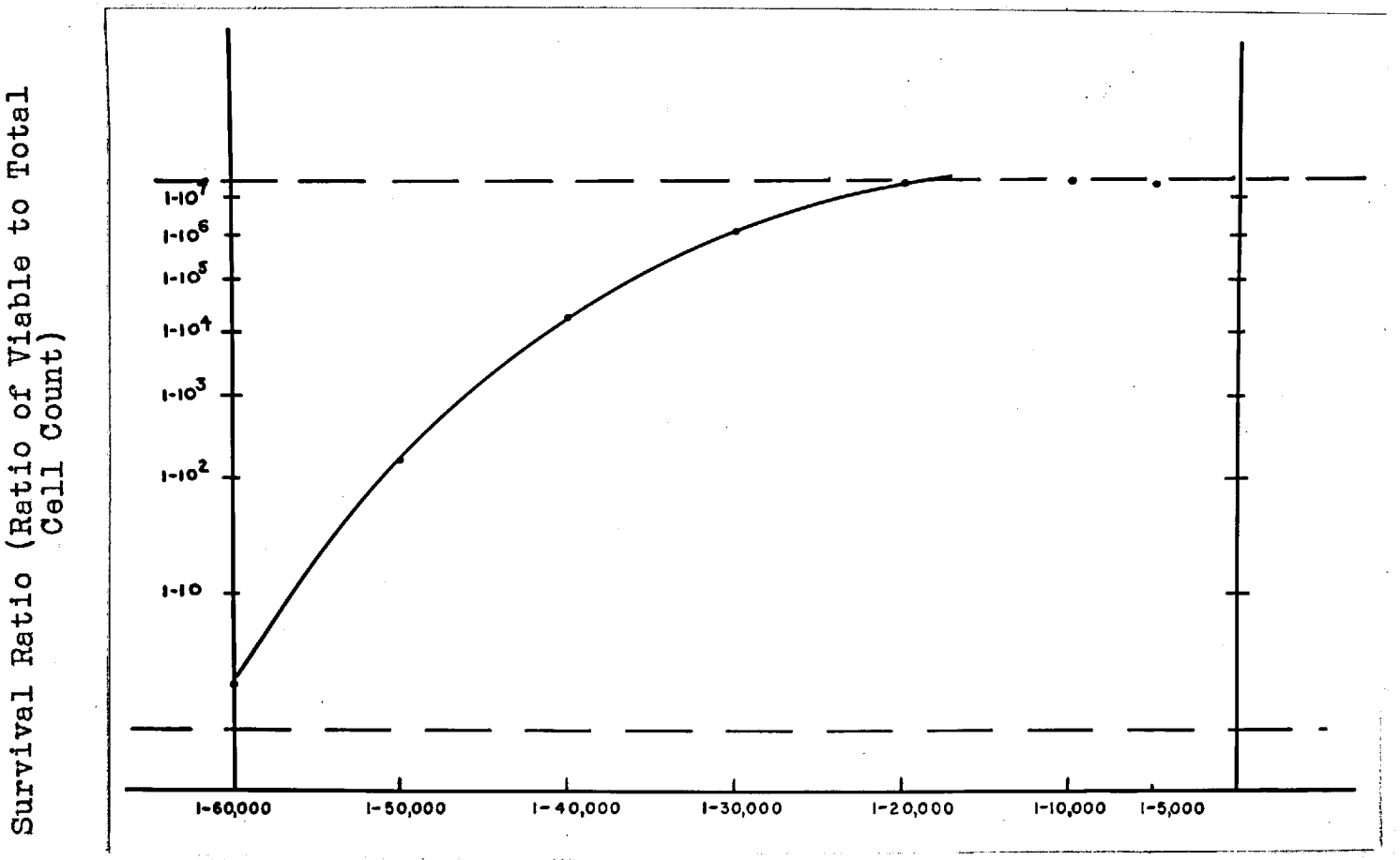


Figure 6: THE EFFECT OF TINCTURE OF IODINE (5% w/v) ON THE VIABLE CELL COUNT OF Staphylococcus aureus (F.D.A.209)
A graphic representation of the experimental results given in Table XIV.

EXPERIMENT FIFTEEN

Table XV

Effect of Tincture of Iodine (5% w/v) on Viable and Total Cell Counts of Test Organism *Staphylococcus aureus* (F.D.A. 209)

Concentration of 5% w/v Tincture of Iodine	Total Count	Viable Count	Ratio:Viable to Total Count (Survival Ratio)
0(Control)	4.2×10^8	2.7×10^8	1:1.6
1-60,000	5.0×10^8	1.3×10^8	1:3.8
1-50,000	5.3×10^8	5.0×10^6	$1:1.0 \times 10^2$
1-40,000	4.8×10^8	1.3×10^5	$1:3.7 \times 10^3$
1-30,000	4.0×10^8	7.3×10^2	$1:5.4 \times 10^5$
1-20,000	4.7×10^8	1.3×10^2	$1:3.6 \times 10^6$
1-10,000	4.9×10^8	0	$<1:4.9 \times 10^8$
1-5,000	5.3×10^8	0	$<1:5.3 \times 10^8$

The results given in Table XV are presented graphically in Figure 7 which follows immediately.

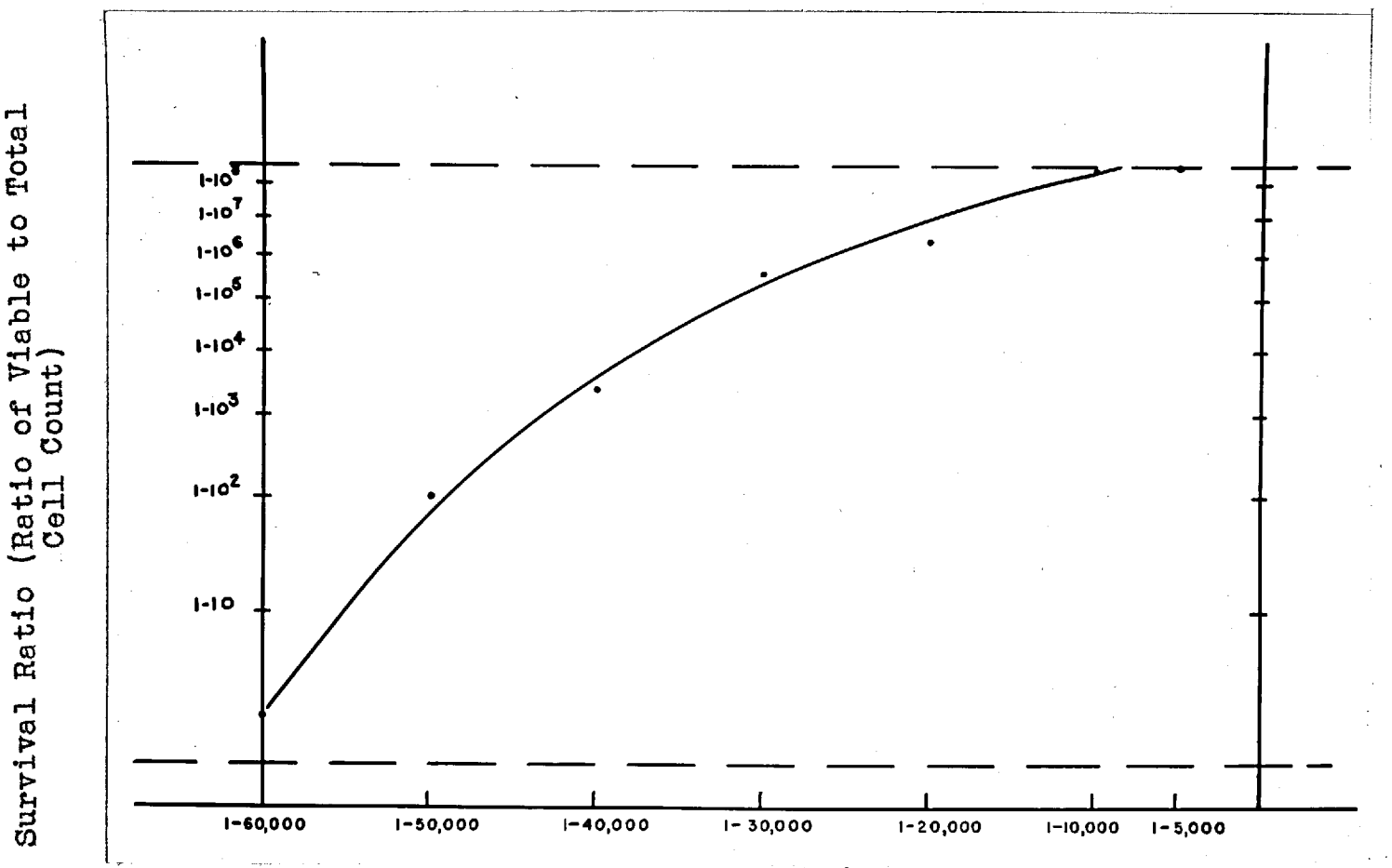


Figure 7: THE EFFECT OF TINCTURE OF IODINE (5% w/v) ON THE VIABLE CELL COUNT OF Staphylococcus aureus (F.D.A.209)
A graphic representation of the experimental results given in Table XV.

EXPERIMENT SIXTEEN

Table XVI

Effect of Phenol on Viable and Total Cell Counts of Test Organism Staphylococcus aureus (F.D.A.209)

Concentration of Phenol w/v	Total Count	Viable Count	Ratio:Viable to Total Count (Survival Ratio)
0 (Control)	4.9×10^7	3.3×10^7	1:1.2
1-600	4.0×10^7	1.3×10^7	1:3.0
1-400	3.0×10^7	1.3×10^7	1:2.3
1-300	3.6×10^7	2.0×10^7	1:1.8
1-200	5.9×10^7	5.3×10^7	1:1.1
1-150	6.1×10^7	1.3×10^3	$1:4.6 \times 10^4$
1-100	5.2×10^7	0	$<1:5.2 \times 10^7$

The results given in Table XVI are presented graphically in Figure 8.

EXPERIMENT SEVENTEEN

Table XVII

Effect of Phenol on Viable and Total Cell Counts of Test Organism Staphylococcus aureus (F.D.A.209)

Concentration of Phenol w/v	Total Count	Viable Count	Ratio:Viable to Total Count (Survival Ratio)
0(Control)	4.3×10^7	3.9×10^7	1:1.1
1-600	4.2×10^7	2.1×10^7	1:2.0
1-400	4.8×10^7	2.4×10^7	1:2.0
1-300	3.8×10^7	2.0×10^7	1:1.9
1-200	3.7×10^7	4.7×10^6	1:7.8
1-150	3.6×10^7	0	$< 1:3.6 \times 10^7$
1-100	4.4×10^7	0	$< 1:4.4 \times 10^7$

The results given in Table XVII are presented graphically in Figure 8 which follows immediately.

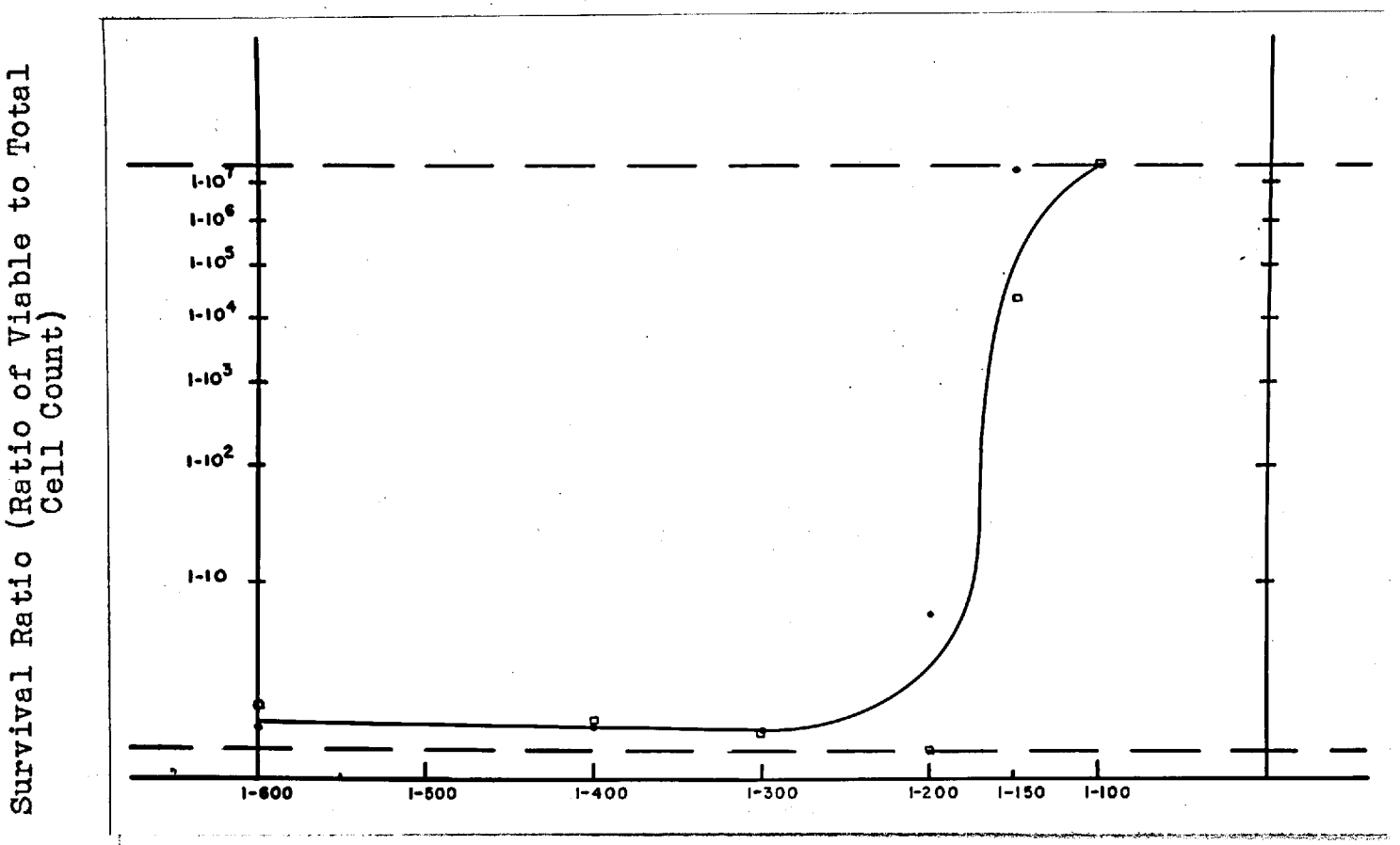


Figure 8: THE EFFECT OF PHENOL ON THE VIABLE CELL COUNT OF Staphylococcus aureus (F.D.A.209)
A graphic representation of the experimental results given in Tables XVI and XVII.
Symbols: □ Results from Table XVI.
● Results from Table XVII.

EXPERIMENT EIGHTEEN

Table XVIII

Effect of Ethyl Alcohol on Viable and Total Cell Counts of Test Organism Staphylococcus aureus (F.D.A.209)

Concentration of Ethyl Alcohol	Total Count	Viable Count	Ratio:Viable to Total Count (Survival Ratio)
0(Control)	4.2×10^6	3.4×10^6	1:1.2
12.50% v/v	4.4×10^6	3.4×10^6	1:1.2
22.50%	3.3×10^6	3.3×10^5	1:1 X 10
25.00%	3.6×10^6	1.2×10^4	$1:3.0 \times 10^2$
30.00%	3.7×10^6	0	$<1:3.7 \times 10^6$
40.00%	3.0×10^6	0	$<1:3.0 \times 10^6$

Results given in Table XVIII are presented graphically in Figure 9 which follows immediately.

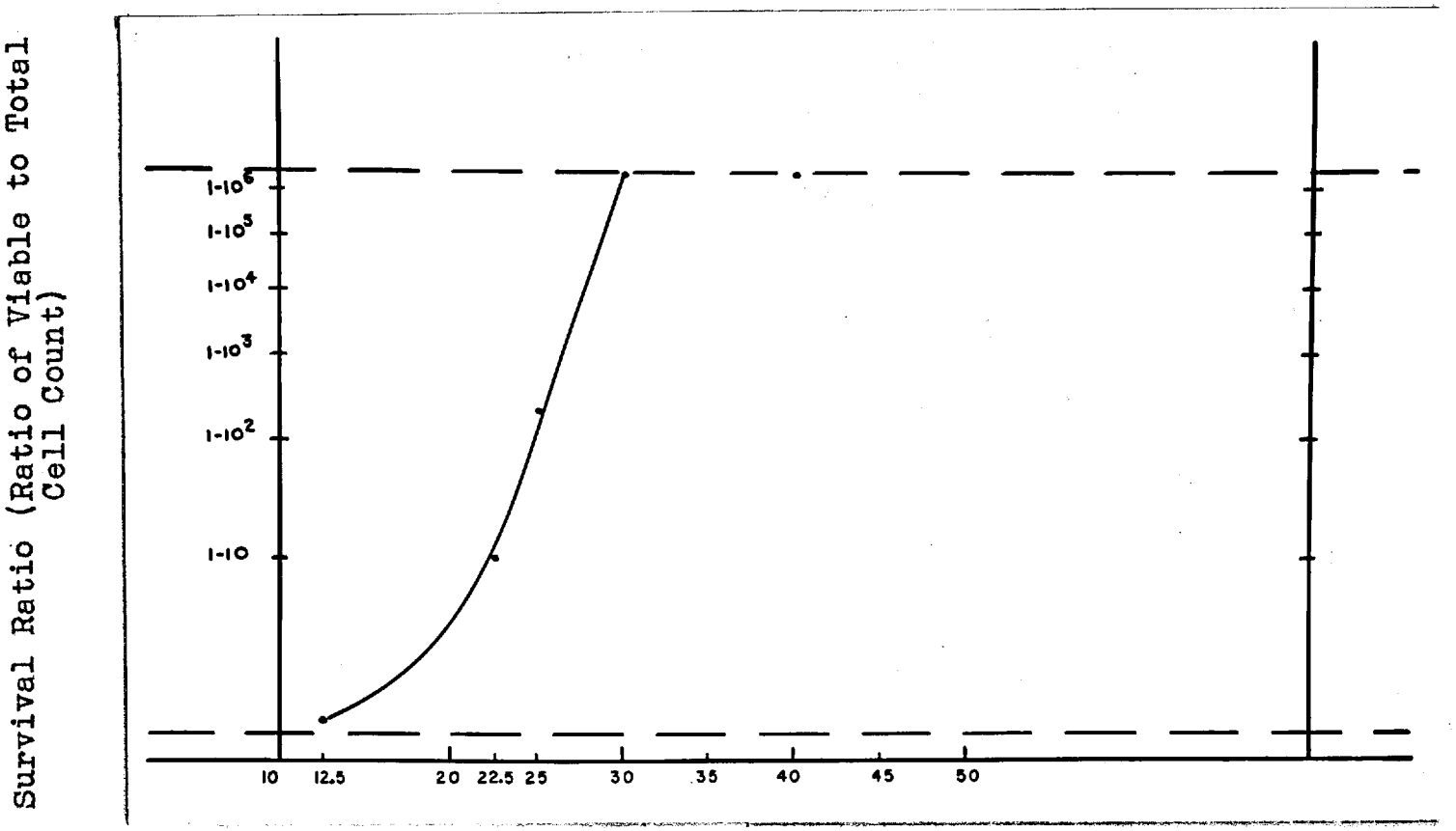


Figure 9: THE EFFECT OF ETHYL ALCOHOL ON THE VIABLE CELL COUNT OF Staphylococcus aureus (F.D.A.209).
A graphic representation of the experimental results given in Table XVIII.

EXPERIMENT NINETEEN

Table XIX

Effect of "Roccal" on Viable and Total Cell Counts of Test Organism Staphylococcus aureus (F.D.A.209)

Concentration of Roccal:10% w/v	Total Count	Viable Count	Ratio:Viable to Total Count (Survival Ratio)
0(Control)	3.8×10^7	2.0×10^7	1:1.9
$1 - 10^8$	4.9×10^7	2.0×10^7	1:2.4
$1 - 10^7$	4.6×10^7	1.3×10^7	1:3.5
$1 - 10^6$	4.8×10^7	5.3×10^6	1:9.0
$1 - 10^5$	5.4×10^7	1.3×10^6	$1:4.1 \times 10$
$1 - 10^4$	4.5×10^7	1.3×10	$1:3.5 \times 10^6$
$1 - 10^3$	4.5×10^7	0	$< 1:4.5 \times 10^7$

The results given in Table XIX are presented graphically in Figure 10.

EXPERIMENT TWENTY

Table XX

The Effect of "Roccal" on Viable and Total Cell Counts of Test Organism Staphylococcus aureus (F.D.A.209)

Concentration of Roccal:10% w/v	Total Count	Viable Count	Ratio:Viable to Total Count (Survival Ratio)
0(Control)	2.2×10^7	2.0×10^7	1:1.1
$1 - 10^8$	2.4×10^7	8.0×10^6	1:3.0
$1 - 10^7$	2.8×10^7	3.3×10^6	1:8.6
$1 - 10^6$	2.7×10^7	4.6×10^6	1:5.8
$1 - 10^5$	2.7×10^7	4.6×10^6	1:5.8
$1 - 10^4$	2.7×10^7	0	$< 1:2.7 \times 10^7$
$1 - 10^3$	2.8×10^7	0	$< 1:2.8 \times 10^7$

The results given in Table XX are presented graphically in Figure 10 which follows immediately.

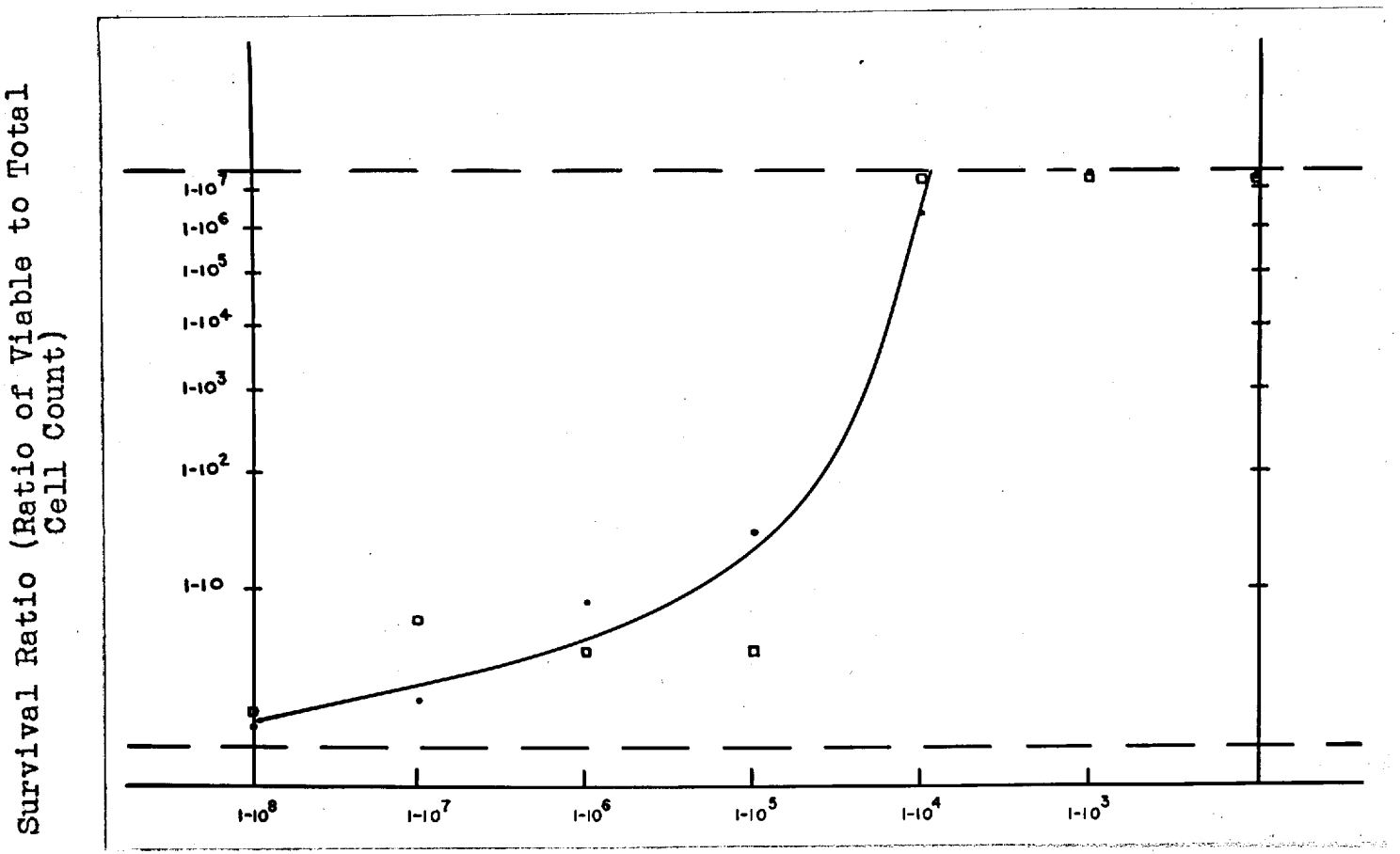


Figure 10: THE EFFECT OF "ROCCAL" ON THE VIABLE CELL COUNT OF Staphylococcus aureus (F.D.A.209)
A graphic representation of the experimental results given in Tables XIX and XX.
Symbols: ● Results from Table XIX.
□ Results from Table XX.

EXPERIMENT TWENTY-ONE

Table XXI

Effect of "Roccal" on the Viable and Total Cell Counts of Test Organism Staphylococcus aureus (F.D.A.209)

Concentration of Roccal:10% w/v	Total Count	Viable Count	Ratio:Viable to Total Count (Survival Ratio)
0(Control)	2.7×10^9	1.2×10^9	1:2.2
$1 - 10^7$	4.1×10^9	8.6×10^8	1:4.9
$1 - 10^6$	2.8×10^9	7.0×10^8	1:4.0
$1 - 10^5$	3.7×10^9	7.0×10^8	1:5.2
$1 - 10^4$	3.3×10^9	2.7×10^8	1:1.2 X 10
$1 - 10^3$	2.7×10^9	0	$< 1:2.7 \times 10^9$
$1 - 10^2$	2.9×10^9	0	$< 1:2.9 \times 10^9$

The results given in Table XXI are presented graphically in Figure 11 which follows immediately.

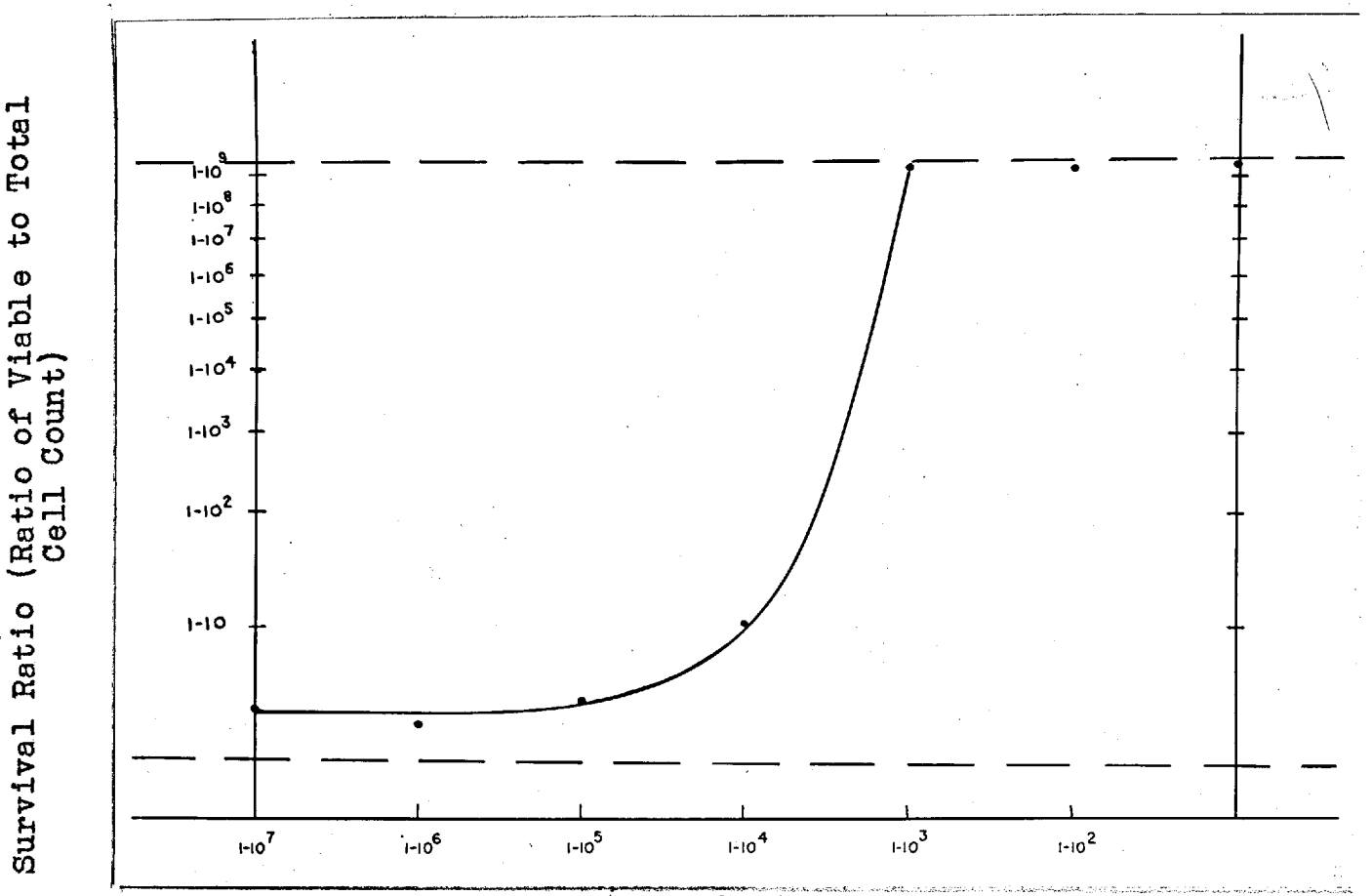


Figure 11: EFFECT OF "ROCCAL" ON THE VIABLE CELL COUNT OF Staphylococcus aureus (F.D.A.209)
A graphic representation of the experimental results given in Table XXI.

Discussion of Results

The experimental results presented in Tables X to XXI and Figures 3 to 11 indicate that the method developed for determining the effect of disinfectant treatment on the viable cell count of the test organisms can be successfully applied. These experiments indicate that reproducible results can be obtained using the method and procedures described.

The method which has been developed makes it possible to follow the effect of various dilutions of a chemical disinfecting agent on the viable cell count of the test organism. Results expressed as the survivor ratio (ratio of viable to total cell count following disinfectant treatment) thus give an overall picture of the general trend of the disinfection process.

The main weakness in the method appears to be lack of uniformity of the total number of cells of the test organism present in cell suspensions prepared at different times. This weakness calls for the application of more exact methods of standardizing such cell suspensions. The problem of standardization has been successfully eliminated. The technique for exact standardization will be dealt with in the experimental work which follows.

PART TWO

DEVELOPMENT OF A METHOD FOR DETERMINING THE EFFECT OF DISINFECTANT ON RESPIRATORY ENZYME SYSTEMS OF THE TEST ORGANISM

DEVELOPMENT OF A METHOD FOR DETERMINING THE EFFECT OF DISINFECTANT ON RESPIRATORY ENZYME SYSTEMS OF THE TEST ORGANISM

Introduction

In the historical introduction, we have briefly stated results of certain earlier experiments concerning the effect of disinfectants on enzyme systems of various test organisms.

The majority of these experiments exployed modifications of the Thunberg technique (1917-18, 1930) in which the reduction of methylene blue in the presence of suitable substrate, phosphate buffer and tissue suspension was studied in a special test tube from which atmospheric oxygen was evacuated. Quastel and Whetham (1924) modified the original Thunberg technique using "resting organisms" which consisted of "organisms grown for two days in tryptic broth, separated by centrifuging, thoroughly washed with normal saline, made up to a thick emulsion with saline and finally well aerated". The reactions of these organisms under these conditions are considered to be the reactions of resting or non-proliferating organisms. Measurements of the velocity of reduction of methylene blue were made under anaerobic conditions in vacuum tubes at 45°C. in carefully buffered solutions of pH 7.2. Braun and Wörderhoff (1933) modified the Thunberg-Quastel methylene blue method for the study of bacterial

dehydrogenations, assuring the absence of oxygen from the liquids under study, not by evacuation but by boiling. Bach (1937) pointed out the disadvantages of boiling and developed yet another modification. His method employs a special vacuum ramp by means of which tubes are evacuated at the water pump and subsequently filled with nitrogen. Tubes are then detached from the ramp, sealed and placed in the 40° incubator. In this manner, atmospheric oxygen is removed, replaced by inert nitrogen and prevented from re-entry by a layer of nitrogen maintained above the surface of the fluid in the tube during the test period. Bach and Lambert (1937 a,b) employed this apparatus in testing the action of certain antiseptics on the lactic, formic and glucose dehydrogenases of Staphylococcus aureus. Sykes (1939) studying the effect of germicides on dehydrogenases of Bact. coli attempted to correlate increases in reduction time following treatment with phenol with decrease in viable cell count. In the Part One of this report, a method for determining the effect of disinfectant treatment on the viable and total cell counts of the test organism has been described. It seemed possible that, by combining this method with the methylene blue reduction method of Bach (1937), results could be obtained indicating the effect of disinfectant treatment on both the viable cell count and respiratory enzyme systems of the test organism. Preliminary experimentation was necessary in order to develop a method for determining the effect of disinfectant on respiratory enzyme systems of the test organism. The method developed must of necessity

be so designed that it could be easily combined with the method for determining the effect of disinfectant on the viable cell count of the test organism. The preliminary experiments which follow describe the development of such a method.

PRELIMINARY EXPERIMENTAL INVESTIGATIONS

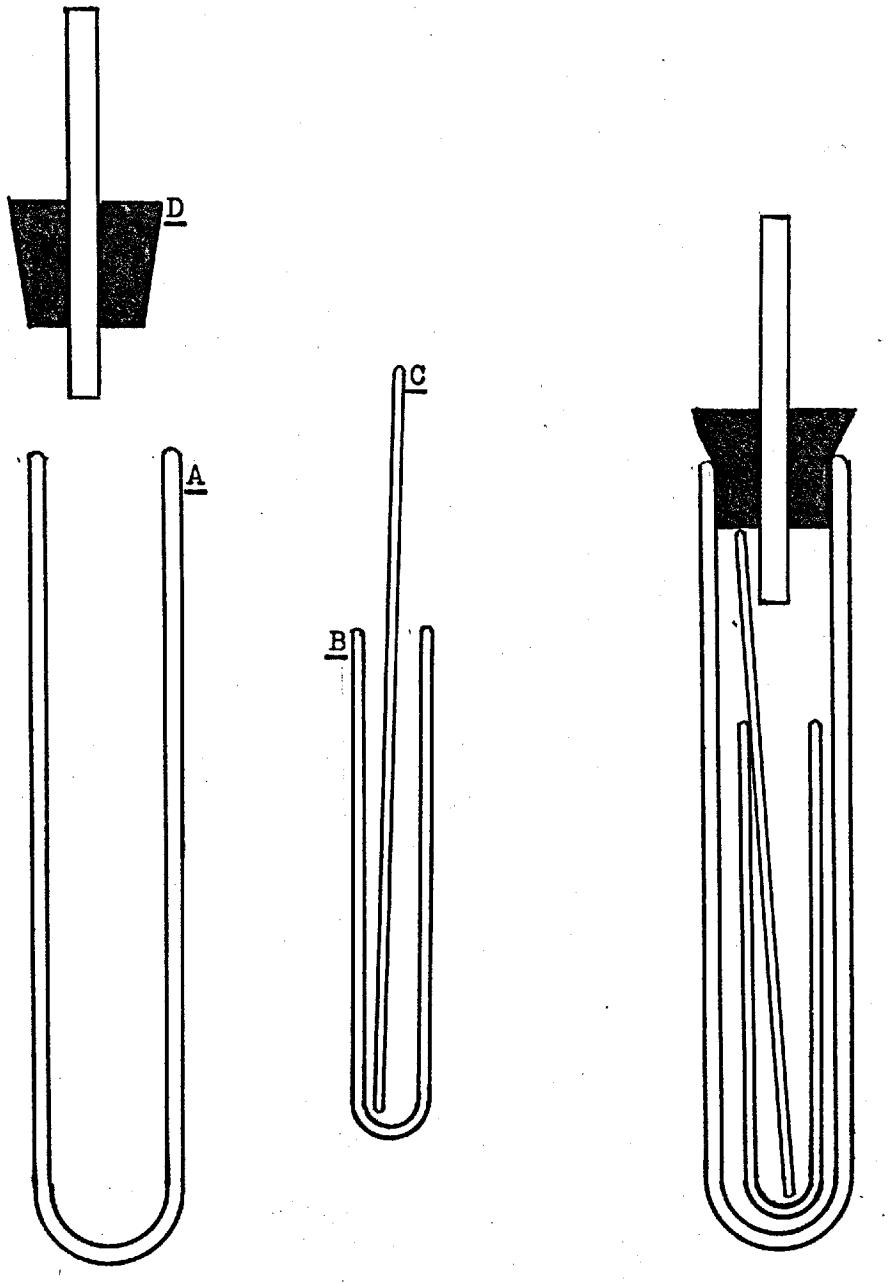
Apparatus:

The apparatus used in these experiments is similar in design to that of Bach (1937). Our apparatus is described in Appendix D. The vacuum ramp was designed to provide attachment for eight test tubes. One modification was introduced by placing a trap between the vacuum ramp and the nitrogen source. This trap is partially filled with a mixture of pyrogallic acid and sodium hydroxide. Nitrogen passing into the vacuum ramp is slowly bubbled through this mixture, the object being to remove traces of oxygen which may exist in the otherwise inert nitrogen. The method of employing alkaline pyrogallol for the purpose of removing oxygen was first suggested by Buchner (1888). This worker demonstrated that 1 gramme of pyrogallic acid crystals with 10 mil. of ("1/10 concentration of NaOH") suffices to completely absorb oxygen present in 100 cubic centimetres air space. Griffin (1932) introduced a modification of the alkaline pyrogallol technique in which the test tube containing the pyrogallic acid-alkali mixture is attached to a second tube by means of a glass U-tube and two one-hole rubber stoppers. By means of this modification, the pyrogallol-alkali mixture is thus contained in a tube separate from the tube from which the oxygen is to be removed. This principle applies to the modification introduced in our apparatus. In the apparatus, the vacuum ramp is suspended

above a water bath in such a position that tubes attached to the ramp may be immersed three quarters of their length in the water. The bath is regulated to give a temperature of 37°C. to 38°C.

"New" Reaction Tubes for the Oxidation-Reduction Test:

Thunberg (1917 - 1918) designed special vacuum tubes for use in his methylene blue reduction test. A popular modification of this tube has been the Hopkins-Dixon tube (1922). The Thunberg tube and the Hopkins-Dixon tube are easily broken even under ordinary conditions of use. Tubes of the same thickness of glass as the Thunberg and Hopkins-Dixon tube were tested in the high speed centrifuge and invariably shattered when the centrifuge speed increased above 9,000 r.p.m. In the method which has been developed for determining the effect of disinfectant treatment on the viable cell count, it is necessary to use the centrifuge at 13,500 r.p.m. The centrifuge tubes designed for use at this speed have extra heavy walls. The logical solution to the problem was to adapt the heavy walled centrifuge tube for use in the methylene blue reduction test. Attempts to design detachable side arms (substrate containers) for these tubes, failed. A very simple device was finally developed which obviates any alteration in the tube. The apparatus is shown in Figure 12. A description of this new reaction tube for oxidation-reduction tests follows immediately.



Disassembled Assembled

FIGURE 12: A "NEW" REACTION TUBE FOR OXIDATION-REDUCTION TESTS

Symbols:
A - heavy walled centrifuge tube (18 X 100 mm.)
B - small substrate tube (8 X 60 mm.)
C - glass rod (1 X 90 mm.)
D - one-hole gum rubber stopper fitted with glass tubing (4 X 50 mm.)

Description of the "New" Reaction Tube for Oxidation-Reduction Tests:

The new reaction tube consists of:

(a) One heavy walled centrifuge tube (18 X 100 mm.)*

(b) One small glass tube (8 X 60 mm.)

(c) One glass rod (1 X 90 mm.)

(d) One single-hole gum rubber stopper to fit the larger tube. This stopper is fitted with a length of glass tubing (4 X 50 mm.) in such a manner that the tubing will project into the reaction tube a distance of 10 mm.

Mechanism of Use of the "New" Reaction Tube for Oxidation-Reduction Tests:

Methylene blue solution, buffer solution and bacterial cell suspension are placed in the heavy walled tube. Nitrogen is bubbled vigorously through the fluid for 30 seconds. Substrate solution is placed in the small tube. The glass rod is now placed in the small tube. Using forceps, the small tube is placed in the heavy walled tube. The gum rubber stopper is then fitted into the larger tube. By means of the glass tubing projecting from the stopper, the tube is now attached to the rubber adapter of the vacuum ramp. The tube thus attached hangs downward from the ramp. All rubber-glass joints are sealed with collodion. The apparatus described can be used successfully only if the dimensions of the constituent parts are as stated and the quantities of reacting fluids are as follows:

*Pyrex tubes, 18 X 100 mm. heavy walled. Ivan Sorvall Inc., New York.

Contents of large tube:

Bacterial suspension: 1.0 mil.

Phosphate Buffer solution: 1.0 mil.

Methylene Blue solution: 1.0 mil.

Contents of small tube:

Substrate solution: 1.0 mil.

The total volume of reacting fluids thus equals 4.0 mil.

When the tube is attached to the vacuum ramp, evacuation of air within the tube is brought about by the water pump. Following evacuation, the valve controlling the suction is closed and that regulating nitrogen supply is opened. Nitrogen is bubbled through the pyrogallol-alkali mixture and passes into the reaction tube. A positive pressure of nitrogen within the tube is thus obtained. If the reaction tube is inverted while this positive pressure exists, the fluid contents of the tube will not pass into the glass tubing leading to the vacuum ramp. In this inverted position, the substrate flows freely from the small inner tube and mixes with the contents of the larger tube. The glass rod resting inside the smaller tube serves a double purpose. First, it supports the smaller tube when it is inverted; second, the fluid contents of the smaller tube, which would otherwise remain trapped within the small tube when inverted, flow freely from this tube along the glass rod. It has been our practice to repeat this inversion of the tubes three times in order to ensure complete washing out of all substrate and adequate mixing of substrate with the content of the large tube.

After the substrate solution has been mixed with the contents of the large tube, this tube is then allowed to return to its original position hanging downward from the vacuum ramp.

Evacuation and Refilling of Reaction Tube with Nitrogen:

In all experiments, tubes were evacuated by water pump three times for three minute periods. The degree of evacuation was determined by the mercury manometer, 18 cm. of mercury being the negative pressure obtained. During each evacuation period, reaction tubes were tapped gently against an inclining rubber apron which extends downward from the vacuum ramp into the water bath. Following evacuation, nitrogen was allowed to flow into the reaction tubes until a positive pressure of 2.0 cm. was registered by the mercury manometer. Following the third evacuation, this positive pressure of 2.0 cm. mercury was maintained throughout the entire test period.

Estimation of Methylene Blue Reduction:

Tam and Wilson (1938, 1941) described a method for estimating methylene blue reduction using the Evelyn photometer. This method involves the use of optically standardized Thunberg tubes. The method would obviously be of value in our work. We are at present using the heavy walled reaction tubes described and since these tubes are not optically standardized, the method is not applicable at this time. Future investigations employing this method may be possible if optically standardized heavy walled reaction tubes can be obtained.

Using the apparatus described, it has been found that, in control experiments, the reduction of methylene blue progresses until only a very thin layer (approximately 0.5 mm. deep) of unreduced methylene blue remains at the upper surface of the fluid in the tubes. It is therefore unnecessary to employ methods of estimating the extent of methylene blue reduction. In the present experiments, the reduction time given is the time taken to obtain reduction of methylene blue which is as complete as that obtained in a control tube.

Reaction tubes are not aggitated or disturbed in any manner throughout the entire test period. The tubes are not detached from the vacuum ramp as in the method described by Bach (1937) but remain attached and under a positive pressure of nitrogen (2.0 cm. mercury) throughout the entire test period. This method possesses the advantage that, any leakage which may occur during the test period, will be a leakage of nitrogen out of the tube rather than air into the tube. Such leakage can be immediately detected by alteration in the reading of the mercury manometer.

Observation of reduction of methylene blue is facilitated by the background of the white rubber apron hanging from the vacuum ramp behind the tubes. A sheet of white rubber placed beneath the tubes in the water bath prevents interference in observations due to reflection from the interior of the water bath.

Experiment Twenty-two

A preliminary experiment was designed to test the efficiency of the new reaction tubes and compare the results obtained with those obtained with the modified type of Thunberg tube available in this department. In this experiment, a bacterial suspension was prepared which represented 10 mgm. of bacterial cells (based on wet weight) per mil. of suspension. One mil. of this suspension was placed in each tube; 1.0 mil. of phosphate buffer solution (pH 7.3) and 1.0 mil. of methylene blue solution (1-20,000 w/v) were added to each tube. The substrate used was 1.0 mil. of sodium lactate solution (0.1% w/v). Evacuation and nitrogen replacement was as described previously. The water bath temperature was 38°C. The results of this experiment are given in Table XXII.

Table XXII

Comparison of Velocity of Reduction of Methylene Blue in "New" Reaction Tubes and in Thunberg Tubes

TEST NUMBER	REDUCTION TIME OBTAINED	
	"New" Reaction Tubes	Thunberg Tubes
1	10 min.	15 min.
2	10 min.	15 min.

The results presented in Table XXII indicate that the reduction of methylene blue proceeds with greater velocity in the "new" reaction tubes than in the modified Thunberg tubes.

Buffer Solution Employed in the Methylene Blue Reduction Tests:

It has been noted by several groups of workers that methylene blue and other dyes used as indicator of oxidation-reduction tests often exert toxic effects on the enzymes of bacteria and tissues. Quastel and Wheatley (1931), Yudkin (1933) and Tam and Wilson (1941) noted that phosphates protect against this effect. The use of phosphate buffer has therefore become routine for experimentation in this field. In all experiments, molar fifteen solutions of Na_2HPO_4 and KH_2PO_4 were prepared and buffer solution made by mixing appropriate volumes of these. Buffer solution was autoclaved and the pH checked after autoclaving. The buffer solution employed in the tests was always of pH 7.25 to pH 7.35.

Methylene Blue Solution Used in Reduction Tests:

As has been previously mentioned, the toxic effect of methylene blue solutions has been demonstrated for certain enzyme systems. The concentration of methylene blue commonly employed has been 1-5,000, Thunberg (1930), Quastel and Whetham (1924), Yudkin (1933), Sykes (1939), Bambas (1945). The salt used was methylene blue chloride. Bach and Lambert (1937 a) have suggested that, in the case of bacteria, very sensitive to the antiseptic action of methylene blue, it is of value to use concentrations of this dye as low as possible. These workers therefore reduced the concentration of dye to one-fourth of that used by Quastel. The concentration they employed was therefore 1-20,000. In the experiments reported here

methylene blue chloride* solution, concentration of 1-20,000 w/v, has been used. The solution is autoclaved in amber glass bottles. Following autoclaving, the bottles are tightly stoppered and subsequently stored in the dark. Using this concentration of methylene blue, no toxic action towards enzyme activity during a test period of three hours was demonstrable. Tests to check this possibility were done by determining successive reduction times for the same sample of bacterial cell suspension allowing the methylene blue to re-oxidize between tests.

*Methylene Blue 1% (Oxidation-Reduction Indicator), Hartmann-Leddon Company, Philadelphia, Pa. Sold by Will Corporation, 594 Broadway, New York 12, N.Y.

Experiment Twenty-three

Use of Indicators Other than Methylene Blue for Reduction Test: 2, 3, 5- Triphenyl Tetrazolium Chloride

Triphenyl tetrazolium chloride has been suggested for use as an indicator in oxidation reduction tests by Jerchel and Möhle (1944). This chemical is extremely sensitive to reducing agents. In its oxidized form, it is colorless but when reduced, the highly colored triphenyl formazan is formed. A sample of this chemical was obtained from the Montclair Research Corporation, New Jersey. For the purpose of the investigation, the chemical was made up to a 1% w/v aqueous solution. One mil. of this solution was substituted for the methylene blue solution in each reaction tube. The procedure for testing was as we have previously outlined. In a comparative series of experiments using 1-20,000 w/v methylene blue solution and 1% w/v tetrazolium chloride solution, the following results were obtained when samples of the same bacterial cell suspension were tested.

Table XXIII

Comparison of Triphenyl tetrazolium chloride and Methylene Blue as indicator for the Reduction Test

TEST NUMBER	REDUCTION TIME OBTAINED	
	Methylene Blue	Triphenyl Tetrazolium Chloride
1	15 min.	Could not be estimated
2	16 min.	" " " "
3	16 min.	" " " "

With reference to the results presented in Table XXIII, it is necessary to note that the change of color which occurs when tetrazolium chloride solution is reduced involves an alteration from colorless to red with a variety of intervening shades of pink. It was absolutely impossible to determine any exact end-point under these conditions. The reduction was allowed to continue for 16 hours and at no time during that period was it possible to establish a definite end-point. Kun and Abood (1949) developed a method for estimating the reduction of this chemical. The method involves concentration of the reduced dye using acetone and subsequent colorimetric evaluation of the concentrate. This method could not be employed for our purpose since the introduction of acetone would interfere with the interpretation of subsequent viable cell counts.

For the above reasons, attempts to employ triphenyl tetrazolium chloride as a reduction indicator were abandoned.

Methylene Blue Thiocyanate:

Acting on the recommendation of Thornton and Sandin (1935), the American Public Health Association adopted, as a standard for the methylene blue reduction test for quantitative estimation of bacteria in milk, a concentration of 1 part of methylene blue thiocyanate to 300,000 parts of milk. At the time this chemical was adopted, it was found to lend itself to preparation in a state of purity not practically obtainable with the formerly used methylene blue chloride. Standard tablets of methylene blue thiocyanate are now on the market. The two salts are said, by the above mentioned authors, to give similar results as oxidation-reduction indicators in milk. In a single experiment, we endeavored to determine whether or not the thiocyanate salt would offer any improvement over the chloride for our purpose. A sample of methylene blue thiocyanate was obtained* and prepared in aqueous solution according to the method of Thornton and Sandin. The volume used in each reduction test was 1.0 mil of 1-300,000 solution.

When duplicate reaction tubes were set up using methylene blue chloride in one set of tubes and methylene blue thiocyanate in the other, all tubes showed complete reduction of dye in exactly the same time when aliquots from a suspension of test organisms were tested. The testing procedure was according to the method already described. For our purpose, methylene blue thiocyanate did not show any advantage over methylene blue chloride.

*Methylene Blue Thiocyanate. Certified for use in: Reduction Testing in Milk. National Aniline Division, Allied Chemical and Dye Corporation, New York, N.Y.

Concentration of Bacterial Cells in Suspension Used for Reduction Test:

It has been the practice of many workers to prepare a thick emulsion of washed cells of the test organism. This emulsion is then diluted with buffer until a standard reduction time is obtained and this diluted cell suspension is then used for subsequent tests (Quastel and Whetham, 1925). The standard existing using such a procedure is therefore the reduction time. It has been amply demonstrated that the concentration of bacterial cells treated by disinfectant has a great influence on the result of such treatment. Rideal and Walker (1903) recognized that the time required to disinfect was dependant upon the number of organisms initially present. Chick (1908) established by her investigations that the initial number of bacteria must be the same if it is desired to use results of disinfectant testing for comparison. Phelps (1911) attempted to explain mathematically the effect which results by varying the initial concentration of the cells of the test organism. Regardless of the mechanism involved, the fact remains that for the purpose of evaluating disinfectant action, the number of cells of the test organism must be standardized if we are to attempt to correlate the effect on viable cell count with the effect on enzyme systems. It is necessary therefore for our purpose to use as a standard the number of cells of test organism rather than the reduction time.

Experiment Twenty-four

Preliminary experiments indicated that a washed cell suspension of the test organism which represented a concentration of cells of 5 mgm. per millilitre (based on wet weight of the organisms) gave a reduction time varying from 13 to 27 minutes. Recognizing that wet weight is not an extremely accurate method of standardizing the number of cells present, this has been used only as a rough preliminary procedure. Suspensions prepared in this manner were further standardized by adjusting to a standard opacity as indicated by the Evelyn photometer. A series of experiments were done using such standardized cell suspensions, Reduction time, total cell count and viable cell count were determined for each experiment and the results are presented in Table XXIV. Total and viable cell counts were done according to the method described in Part One of this report.

Table XXIV

Comparison of Reduction Time, Total Cell Count and Viable Cell Count for Standardized Suspensions of *Staphylococcus aureus* (F.D.A.209)

SUSPENSION NUMBER	REDUCTION TIME	TOTAL CELL COUNT	VIABLE CELL COUNT
1	27 min.	6.2×10^9	5.3×10^9
2	13 min.	4.6×10^9	4.6×10^9
3	15 min.	5.2×10^9	4.6×10^9
4	22 min.	4.6×10^9	4.6×10^9
5	20 min.	4.4×10^9	3.3×10^9
6	22 min.	4.0×10^9	2.6×10^9
7	20 min.	4.3×10^9	2.6×10^9
8	25 min.	4.2×10^9	2.6×10^9
9	18 min.	3.3×10^9	3.3×10^9
10	15 min.	3.9×10^9	3.3×10^9

From the results presented in Table XXIV, it appears that the reduction time is not exactly standard when total and viable cell count is standardized.

Experiment Twenty-five

Quastel and Wooldridge (1927) demonstrated that the activities of formic, lactic and succinic dehydrogenase are not entirely dependent on the proportion of viable to total cells of the test organism. Wooldridge and Glass (1937) showed similar results for the activities of sugar and amino-acid enzymes of Bact. coli. If the number of cells of the test organism is standard, the enzyme activity of such standardized suspension can be conveniently expressed by an arbitrary value of 100 which represents the reduction time of the undiluted suspension (Yudkin, 1933). In the subsequent work involving disinfectant treatment, the reduction time of untreated cell suspension is always determined. This time is given an arbitrary value of 100. The reduction times obtained for aliquots of the same cell suspension which have been treated with disinfectant are expressed as a fraction of 100 which is obtained by dividing the reduction time of the untreated cell suspension by that of the treated cell suspension. This procedure can only be justified if aliquots of a standardized suspension of untreated cell will give a constant reduction time. To determine that this is true, Experiment Twenty-five was done.

Table XXV

Comparison of Reduction Times Obtained using Aliquots of a Standardized Suspension of Staphylococcus aureus (F.D.A.209)

ALIQUOT NUMBER	VIABLE CELL COUNT	REDUCTION TIME
1	3.3×10^9	19 min.+ *
2	2.6×10^9	19 min.+ *
3	2.6×10^9	19 min.+ *
4	3.2×10^9	20 min.
5	3.6×10^9	20 min.
6	3.5×10^9	20 min.

*These results are expressed to the nearest minute.

The results presented in Table XXV indicate that reduction times given by aliquots of a standardized suspension of the test organism are relatively constant.

Growth of the Test Organism and the Preparation of Standardized Cell Suspensions:

In order to conform with the method for determining the effect of disinfectant on the viable and total cell count which is described in Part One of this report, the test organism was always grown on cellophane covered filtermass pads. Pads were soaked with peptone broth according to the method previously described. After 18 hours incubation at 37°C., the surface growth was harvested and washed. Washing was done three times with sterile phosphate buffer (pH 7.25 to 7.35) using the centrifuge operated at 5,000 r.p.m. Washed cells were weighed, and suspended in sufficient sterile phosphate buffer to give a concentration of 5 mgm. per mil. This suspension was vibrated with the electric vibrating machine at approximately 1300 vibrations per minute for five minutes. The opacity of a 1 in 4 dilution of this suspension was then checked using the Evelyn photometer with filter number 660. The undiluted suspension was adjusted by the addition of sterile phosphate buffer so that a 1 in 4 dilution gave a standard light transmission of 40 per cent. The undiluted standardized cell suspension was stored at 5°C. for 18 hours. Immediately prior to testing, the suspension was once more vibrated, the opacity checked and adjusted if necessary. One mil. of this suspension was used in each test. Except where otherwise indicated, all cell suspensions mentioned in this report were prepared according to this procedure.

Storage of Standardized Cell Suspension Prior to Testing:

It would considerably facilitate disinfectant testing if it were practical to prepare the cell suspension ahead of time and store it until required for use. In order to determine whether or not such a procedure was valid, the following experiments were done.

Experiment Twenty-six

Quastel and Woolf (1926) stated that the enzyme activity of a resting cell suspension of B. coli was apparently unimpaired during storage in the ice-chest for at least two months. Wooldridge and Glass (1937) investigated the effect of storage of such a suspension at 0°C. for a period of 50 days. These workers demonstrated that a decline in enzyme activity occurred under these conditions. In our experiments, resting cell preparations were stored in 150 mil. Erlenmeyer flasks plugged with cotton. Storage was at three temperatures, namely, 28°C., 5°C. and -20°C. Cell suspensions stored at -20°C. were allowed to thaw at room temperature before testing. All suspensions were vibrated for five minutes at 1300 vibrations per minute immediately before aliquots were removed for testing. Suspensions were tested for lactic dehydrogenase activity by the method outlined. The results of these experiments are given in Table XXVI.

Table XXVI

Comparison of Reduction Times Obtained using Stored Resting Cell Suspension of Staphylococcus aureus (F.D.A.209)

DAYS OF STORAGE	REDUCTION TIME OF PREPARATIONS STORED AT: 28°C.	5°C.	-20°C.
0	20 min.	20 min.	20 min.
1	47 min.	20 min.	23 min.
2	114 min.	30 min.	31 min.
4	>180 min.	39 min.	>120 min.
7		40 min.	>180 min.

The results given in Table XXVI represent the average of duplicate experiments. From these results, it would appear that an apparent loss of enzyme activity does occur when the resting cell suspension is stored at 28°C., 5°C., and -20°C. It is also obvious that loss of activity is less when the preparation is stored at 5°C.

In all subsequent experiments, resting cell preparations were stored at 5°C. for 18 hours following preparation and then tested.

SUMMARY OF PRELIMINARY EXPERIMENTAL INVESTIGATIONS

Method for Determining the Effect of Disinfectants on the Respiratory Enzyme Systems of the Test Organism

Preparation of Suspension of Test Organism:

1. The test organism is Staphylococcus aureus (F.D.A.209)
2. The test organism is subcultured twice at four hour intervals on a peptone agar slant, incubated at 37°C.
3. Surface growth from the peptone agar slant is harvested and suspended in distilled water. This suspension is inoculated to a cellophane covered filtermass pad. The pad of filtermass was previously soaked with peptone broth.
4. The inoculated cellophane pad is incubated at 37°C. for 18 hours.
5. Surface growth from the cellophane pad is harvested and washed three times in sterile phosphate buffer (pH 7.25 to 7.35) using the centrifuge at 5000 r.p.m. The washed cells are weighed and then resuspended in sufficient sterile buffer to give a suspension representing 5 mgm. of organisms per mil. (based on wet weight).
6. The suspension of test organisms is further standardized by opacity as measured by the Evelyn photometer. The suspension is vibrated at 1300 vibrations per minute for 5 minutes prior to opacity measurment. Adjustment to standard opacity is effected by the addition of sterile buffer solution.

7. Standardized resting cell suspension is stored at 5°C. for 18 hours.

Treatment of Standardized Resting Cell Suspension with Disinfectant:

1. Immediately prior to treatment with disinfectant, the resting cell suspension is removed from storage and vibrated at 1300 vibrations per minute for five minutes. Aliquots of the suspension are removed, the opacity is checked and if necessary adjusted to standard.
2. One millelitre of the standardized cell suspension is placed in a sterile heavy walled centrifuge tube of 15 mil. capacity.
3. One millelitre of aqueous solution of disinfectant is added to the cell suspension. The tube is tightly stoppered with a sterile gum rubber stopper.
4. Treatment tubes and contents are rotated on the Welch rotating machine at 4 r.p.m. for 20 minutes at temperature of 37°C.

Removal of Disinfectant from Contact with Cell Suspension:

1. Disinfectant is "diluted-out" at the end of the treatment period by the addition of sterile buffer solution and subsequent centrifugation at 13,500 r.p.m. for three minutes. Nine mil. of supernatant are then removed and replaced by 9.0 mil. of sterile buffer. The diluting-out process is repeated three times.

2. Following the removal of 9.0 mil. of supernatant at the end of the third centrifugation, 1.0 mil. of fluid containing the cell sediment remains in the centrifuge tube.

Estimation of Activity of Respiratory Enzyme Surviving Disinfectant Treatment:

1. One mil. of 1-20,000 methylene blue chloride solution and 1.0 mil. of phosphate buffer (pH 7.25 to 7.35) are added to the 1.0 mil. of cell sediment remaining in each centrifuge tube.
2. Nitrogen is bubbled through the contents of each tube for 30 seconds.
3. One mil. of 0.1% sodium lactate is placed in a small glass tube (8 X 60 mm.). A glass rod 1 X 90 mm. is placed in the small tube. The small tube complete with contents is then placed within the tube containing the methylene blue bacterial mixture.
4. A one-hole gum rubber stopper fitted with glass tubing is tightly fitted in the mouth of the larger tube.
5. Tubes are attached to the vacuum ramp and immersed three quarters of their length in the 37°C. water bath.
6. Evacuation of air and replacement with nitrogen is carried out as has been described.
7. Admixture of the substrate and subsequent estimation of methylene blue reduction is done according to the methods previously described.

8. A control tube in which sterile buffer solution is substituted for disinfectant during the treatment process is included in each experiment.

Statement of Results:

1. The time taken for the reduction of methylene blue by the untreated control is stated by the arbitrary value of 100. The effect of disinfectant treatment on the respiratory enzyme being studied is represented by the fraction of 100 which is given by dividing the reduction time of the untreated control by the reduction time of the treated specimen. The figure thus obtained is called the "enzyme activity coefficient" in the statement of results.

Experiment Twenty-seven

In order to test the validity of the proposed procedure, the following experiment was done. In this experiment, phenol was used as the disinfectant. The concentration of phenol which existed in the treatment tube is given in the table of results.

Table XXVII

Effect of Phenol on the Respiratory Enzyme System (Lactic Dehydrogenase) of the Test Organism Staphylococcus aureus (F.D.A.209)

CONCENTRATION OF PHENOL: W/V	REDUCTION TIME IN MINUTES	ACTIVITY COEFFICIENT
0 (Control)	22 min.	100
1-400	48 min.	45
1-300	60 min.	36
1-200	110 min.	20
1-150	>180 min.	<12
1-100	∞	0

From the results presented in Table XXVII, it is evident that the enzyme activity (lactic dehydrogenase) decreases when the resting cell suspension is treated with increasing concentrations of the disinfectant (phenol).

Experiment Twenty-eight

The procedure followed in this experiment was exactly the same as that previously described and employed in Experiment Twenty-seven. In this experiment, mercuric chloride was the disinfectant used. The concentrations of mercuric chloride given in the table of results are those existing in the treatment tubes during the treatment process. The results of this experiment are given in Table XXVIII.

Table XXVIII

The Effect of Mercuric Chloride on the Respiratory Enzyme System (Lactic dehydrogenase) of the Test Organism Staphylococcus aureus (F.D.A. 209)

CONCENTRATION OF $HgCl_2$:W/V	REDUCTION TIME IN MINUTES	ENZYME ACTIVITY COEFFICIENT
0(Control)	21 min.	100
0.625 P.P.M.	55 min.	38
1.25 P.P.M.	135 min.	16
2.50 P.P.M.	184 min.	11
5.00 P.P.M.	$>$184 min.	$<$11
10.00 P.P.M.	∞	0

The results given in Tables XXVII and XXVIII indicate that the procedure as outlined can be successfully employed to determine the effect of disinfectant treatment on the respiratory enzyme systems of the test organism.

PART THREE

Two Sections

SECTION A: THE DEVELOPMENT OF A METHOD FOR SIMULTANEOUS STUDY OF THE EFFECT OF DISINFECTANT TREATMENT ON THE VIABLE CELL COUNT AND RESPIRATORY ENZYME SYSTEMS OF THE TEST ORGANISM.

SECTION B: RESULTS OF EXPERIMENTS USING THE METHOD DEVELOPED.

SECTION A

THE DEVELOPMENT OF A METHOD FOR SIMULTANEOUS STUDY OF THE EFFECT OF DISINFECTANT TREATMENT ON THE VIABLE CELL COUNT AND RESPIRATORY ENZYME SYSTEMS OF THE TEST ORGANISM

Introduction

The purpose of the experimental investigations reported in Parts One and Two was to establish the validity of methods whereby the effect of disinfectant treatment on both the viable cell count and respiratory enzyme systems of the test organism could be studied. It is desirable that such studies be done on the same specimen of cells of the test organism in order that the course of the two effects may be studied in parallel and possible correlation be established.

The results given in Parts One and Two indicate that the methods employed are valid. By comparison of the two methods, a similarity can be seen which suggests that the methods may be combined making possible the simultaneous study of the effect of disinfectant treatment on the viable cell count and respiratory enzyme systems of the same specimen of cells of the test organism. The development of this method and the results obtained using it will be next described.

Proposed Method for Simultaneous Study of the Effect of Disinfectant Treatment on the Viable Cell Count and Respiratory Enzyme Systems of the Test Organism

Apparatus and Technique:

All apparatus employed is sterile and asceptic technique is followed throughout the entire procedure.

Effect on Respiratory Enzyme Systems:

The preparation of standardized suspension of the test organism, treatment with disinfectant, removal of disinfectant from contact with cells of the test organism, and estimation of enzyme activity are done exactly as described in Part Two of this report.

Effect on the Viable Cell Count:

The test period for enzyme activity has been arbitrarily set at 3 hours. At the end of this period, viable and total cell counts are done on the contents of the reaction tubes. Each reaction tube contains, at the end of the enzyme activity test -

1.0 mil. bacterial cell suspension,

1.0 mil. buffer solution,

1.0 mil. methylene blue solution,

1.0 mil. substrate solution.

A 1 in 10 dilution of the bacterial cells present in the reaction tube is prepared by adding 6.0 mil. of sterile distilled water. The reaction tube complete with contents

(small inner tube, glass rod, and fluid) is then vibrated with the electric vibrating machine at 1,300 vibrations per minute for 3 minutes. An aliquot of the suspension thus obtained is used to make further 1 in 10 serial dilutions. Viable and total cell counts are done according to the procedure given in Part One of this report.

PRELIMINARY EXPERIMENTAL INVESTIGATIONS

It has been shown that certain concentrations of methylene blue commonly employed as oxidation reduction indicator possess toxic properties toward the enzymes of bacteria and tissue (Quastel and Wheatley, 1931; Yudkin, 1933; Tam and Wilson, 1941). The concentration of methylene blue employed as indicator in the present experiments is much less than that studied by these workers. In addition, phosphate buffer is employed which protects against the toxic effect. It still might be possible that some toxicity exists and that viable cell counts done at the end of the enzyme activity test might be significantly less than the viable cell count at the beginning of the test. On the other hand, it is possible that cells of the test organism which are presumed to be "resting" or non-proliferating might actually multiply during the enzyme activity test so that the viable cell count taken at the end of the test would be greater than the count at the beginning of the test. In order to determine that no toxic influence is exerted during the test period and that no multiplication of cells occurs during that period, the following experiments were done.

Experiment Twenty-nine

In this experiment, each test represents a separately prepared suspension of the test organism which was treated according to the proposed method for simultaneous study of the effect of disinfectant on the viable cell count and respiratory enzyme systems. Double volumes of the standardized resting cell suspension were placed in each treatment tube and an equal volume of sterile distilled water was added in place of disinfectant. This made it possible to remove 1.0 mil. of cell suspension for a viable cell count before the enzyme activity test, leaving the required 1.0 mil. of volume of cell suspension for enzyme activity testing and subsequent viable cell count. The results of these tests are given in Table XXIX.

Table XXIX

Comparison of Viable Cell Counts of Test Organism Before and After Enzyme Activity Test

TEST NUMBER	VIABLE CELL COUNT BEFORE ENZYME TEST	VIABLE CELL COUNT AFTER ENZYME TEST
1	5.3×10^9	6.0×10^9
2	4.6×10^9	4.7×10^9
3	4.3×10^9	4.0×10^9
4	4.6×10^9	6.6×10^9
5	3.6×10^9	2.2×10^9
6	3.3×10^9	3.0×10^9
7	2.1×10^9	1.8×10^9
8	3.3×10^9	2.6×10^9
9	1.9×10^9	2.6×10^9
10	2.2×10^9	3.3×10^9
11	2.0×10^9	1.8×10^9
12	2.0×10^9	2.6×10^9
13	2.6×10^9	2.6×10^9

The results presented in Table XXIX indicated that very little change in the viable cell count occurs during the period of three hours required for the enzyme activity

test. Differences between viable counts before and after the test period which do appear in Table XXIX are within the realm of the inherent error present in all counting methods. From these results, we may safely assume that the toxic effect of the concentration of methylene blue used as an oxidation-reduction indicator in the method is negligible during the three hour test period and that the multiplication of cells of the test organism during the same period is likewise negligible.

Experiment Thirty

It has been established that the viable cell count does not change appreciably during the test period for enzyme activity. It is still conceivable that the total number of cells (dead and living) may change although the number of viable cells does not. In order to investigate this possibility, an experiment was set up exactly as Experiment Twenty-nine. Total cell counts were done before and after the test period for enzyme activity. The results are presented in Table XXX. Each test given in this table represents a separately prepared suspension of the test organism.

Table XXX

Comparison of Total Cell Counts of the Test Organism Before and After Enzyme Activity Test

TEST NUMBER	TOTAL CELL COUNT BEFORE ENZYME TEST	TOTAL CELL COUNT AFTER ENZYME TEST
1	6.2×10^9	6.0×10^9
2	4.6×10^9	4.6×10^9
3	5.2×10^9	5.3×10^9
4	3.9×10^9	4.6×10^9
5	4.8×10^9	4.0×10^9
6	4.4×10^9	5.0×10^9
7	4.0×10^9	4.2×10^9
8	4.3×10^9	4.3×10^9
9	4.5×10^9	4.6×10^9
10	3.9×10^9	4.5×10^9
11	4.1×10^9	4.0×10^9
12	4.3×10^9	4.2×10^9

Results presented in Table XXX indicate that no appreciable change in the total cell count of the suspension of test organisms occurs during the three hour enzyme activity test.

From the results of experiments given in Tables XXIX and XXX, it is evident that the procedure of doing total and viable cell counts at the end of the enzyme activity test is valid and that counts done at that time are the same as counts done at the beginning of the test period.

It has been shown in Table VI that for aliquots of the same cell suspension, total cell counts done after the treatment and diluting-out processes are reproducible. In all subsequent experiments, the practice of doing one total count following the enzyme activity test period for each experiment was followed. This total count serves as a check on the viable count of the untreated control. The total cell count also serves as a basis for the survivor ratio of the control and the treated aliquots of the same cell suspension.

Experiment Thirty-one

In order to check on the reproducibility of enzyme activity and viable cell counts of aliquots of a standardized cell suspension, the following experiment was done.

Aliquots of the standardized cell suspension were treated exactly as outlined in the proposed procedure with the exception that sterile distilled water was substituted for disinfectant in the treatment procedure. Enzyme activity was tested according to the proposed method. Viable cell counts were done for each aliquot. A single total cell count was done after the enzyme activity test.

The results of this experiment are presented in Table XXXI.

Table XXXI

Reproducibility of Enzyme Activity (Lactic Dehydrogenase) and Viable Cell Counts Obtained with Aliquots of a Standardized Suspension of the Test Organism

	TOTAL CELL COUNT AFTER ENZYME ACTIVITY TEST 4.2×10^9		
Aliquot Number	Viable Cell Count After Enzyme Activity Test	Ratio: Viable to Total Cell Count	Reduction Time for Methylene Blue
1	3.3×10^9	1:1.2	19 min (+)
2	2.6×10^9	1:1.6	19 min.(+)
3	2.6×10^9	1:1.6	19 min.(+)
4	3.2×10^9	1:1.3	20 min.
5	3.6×10^9	1:1.1	20 min.
6	3.5×10^9	1:1.2	20 min.

+ indicates that reduction occurred between 19 and 20 minutes. Estimation in seconds was not attempted.

The results presented in Table XXXI indicate that viable cell counts and enzyme activity obtained from aliquots of a standardized suspension of the test organism are reproducible.

SECTION B

RESULTS OF EXPERIMENTS USING THE METHOD DEVELOPED FOR THE SIMULTANEOUS STUDY OF THE EFFECT OF DISINFECTANT TREATMENT ON THE VIABLE CELL COUNT AND RESPIRATORY ENZYME SYSTEMS OF THE TEST ORGANISM

The tables which follow give the results obtained using the method which has been developed for the simultaneous study of the effect of disinfectant treatment on the viable cell count and respiratory enzyme systems of the test organism.

Six chemical disinfectants were studied. These were: mercuric chloride, silver nitrate, tincture of iodine (5% w/v), phenol, ethyl alcohol and a 10% w/v solution of alkyl dimethyl benzyl ammonium chloride (Roccal).

The test organism was Staphylococcus aureus (F.D.A.209).

In each Table the concentration of disinfectant which existed in the treatment tube during the treatment procedure, the total cell count and the viable cell count after the enzyme activity test, the survivor ratio, the time taken for complete reduction of methylene blue and the enzyme activity coefficient is given.

EXPERIMENT THIRTY-TWO

Table XXXII

Effect of Mercuric Chloride Treatment on the Viable Cell Count and Respiratory Enzyme System (Lactic Dehydrogenase) of the Test Organism Staphylococcus aureus (F.D.A.209)

TOTAL CELL COUNT: 4.0×10^9				
Concentration of $HgCl_2$ w/v P.P.M.	Viable Cell Count	Reduction Time in Minutes	Survivor Ratio	Enzyme Activity Co-efficient
0(Control)	2.6×10^9	21 min.	1:1.5	100
0.625	2.6×10^9	55 min.	1:1.5	38
1.25	6.6×10^8	136 min.	1:6.0	15
2.50	1.8×10^6	180 min.	$1:2.2 \times 10^3$	11
5.00	1.6×10^5	∞	$1:2.5 \times 10^4$	0
10.00	1.3×10^3	∞	$1:3.0 \times 10^6$	0

EXPERIMENT THIRTY-THREE

Table XXXIII

Effect of Mercuric Chloride Treatment on the Viable Cell Count and Respiratory Enzyme System (Lactic Dehydrogenase) of the Test Organism Staphylococcus aureus (F.D.A.209)

TOTAL CELL COUNT 4.4×10^9				
Concentration of $HgCl_2$ w/v P.P.M.	Viable Cell Count	Reduction Time in Minutes	Survivor Ratio	Enzyme Activity Coefficient
0(Control)	3.3×10^9	20 min.	1:1.3	100
0.625	6.0×10^8	56 min.	1:7.3	36
1.25	3.6×10^8	126 min.	$1:1.2 \times 10$	15
2.50	2.8×10^6	180 min.	$1:1.5 \times 10^3$	11
5.00	8.6×10^4	∞	$1:5.1 \times 10^4$	0
10.00	2.0×10^3	∞	$1:2.2 \times 10^6$	0

EXPERIMENT THIRTY-FOUR

Table XXXIV

Effect of Mercuric Chloride Treatment on the Viable Cell Count and the Respiratory Enzyme System (Lactic Dehydrogenase) of the Test Organism Staphylococcus aureus (F.D.A.209)

TOTAL CELL COUNT: 4.6×10^9				
Concentration of $HgCl_2$ w/v P.P.M.	Viable Cell Count	Reduction Time in Minutes	Survivor Ratio	Enzyme Activity Co-efficient
0(Control)	4.6×10^9	22 min.	1:1.0	100
0.0625	2.0×10^9	22 min.	1:2.3	100
0.125	2.0×10^9	28 min.	1:2.3	79
0.25	2.6×10^9	29 min.	1:1.7	76
0.50	2.0×10^9	37 min.	1:2.3	59
1.00	2.6×10^9	57 min.	1:1.7	39

EXPERIMENT THIRTY-FIVE

Table XXXV

Effect of Silver Nitrate Treatment on the Viable Cell Count and Respiratory Enzyme System (Lactic Dehydrogenase) of the Test Organism Staphylococcus aureus (F.D.A.209)

TOTAL CELL COUNT: 3.3×10^9				
Concentration of $AgNO_3$ w/v P.P.M.	Viable Cell Count	Reduction Time in Minutes	Survivor Ratio	Enzyme Activity Coefficient
0(Control)	3.3×10^9	18 min.	1:1.0	100
0.50	3.3×10^9	34 min.	1:1.0	52
1.00	3.3×10^9	180 min.	1:1.0	10
2.50	2.0×10^9	>180 min.	1:1.6	<10
5.00	4.0×10^8	∞	1:8.2	0
10.00	2.0×10^5	∞	$1:1.6 \times 10^4$	0

EXPERIMENT THIRTY-SIX

Table XXXVI

Effect of Silver Nitrate Treatment on the Viable Cell Count and Respiratory Enzyme System (Lactic Dehydrogenase) of the Test Organism Staphylococcus aureus (F.D.A.209)

TOTAL CELL COUNT: 4.2×10^9				
Concentration of $AgNO_3$ w/v P.P.M.	Viable Cell Count	Reduction Time in Minutes	Survivor Ratio	Enzyme Activity Co-efficient
0(Control)	2.6×10^9	25 min.	1:1.6	100
0.50	2.6×10^9	55 min.	1:1.6	45
1.00	2.6×10^9	180 min.	1:1.6	13
2.50	9.3×10^8	∞	1:4.5	0
5.00	2.0×10^6	∞	$1:2.1 \times 10^3$	0
10.00	2.0×10^4	∞	$1:2.1 \times 10^5$	0

EXPERIMENT THIRTY-SEVEN

Table XXXVII

Effect of Tincture of Iodine Treatment on the Viable Cell Count and Respiratory Enzyme System (Lactic Dehydrogenase) of the Test Organism Staphylococcus aureus (F.D.A.209)

TOTAL CELL COUNT: 4.0×10^9				
Concentration of Tr.Iodine (5%) *	Viable Cell Count	Reduction Time in Minutes	Survivor Ratio	Enzyme Activity Co-efficient
0(Control	2.8×10^9	29 min.	1:1.4	100
1-40,000	1.8×10^9	61 min.	1:2.2	47
1-30,000	1.3×10^9	120 min.	1:3.0	24
1-20,000	9.3×10^7	180 min.	$1:4.3 \times 10$	16
1-10,000	2.3×10^5	∞	$1:1.7 \times 10^4$	0
1-5,000	5.3×10^3	∞	$1:7.5 \times 10^5$	0

*The Tincture of Iodine used in these experiments was assayed for iodine and potassium iodide content by the method of the British Pharmacoepeia, 1932. The results of this assay are given in Appendix C.

EXPERIMENT THIRTY-EIGHT

Table XXXVIII

Effect of Tincture of Iodine Treatment on the Viable Cell Count and Respiratory Enzyme System (Lactic Dehydrogenase) of the Test Organism Staphylococcus aureus (F.D.A.209)

	TOTAL CELL COUNT: 4.2×10^9			
Concentration of Tr. Iodine (5%)	Viable Cell Count	Reduction Time in Minutes	Survivor Ratio	Enzyme Activity Co-efficient
0(Control)	2.6×10^9	16 min.	1:1.6	100
1-40,000	2.6×10^9	32 min.	1:1.6	50
1-30,000	3.0×10^8	89 min.	1:1.4 X 10	17
1-20,000	6.6×10^7	180 min.	1:6.3 X 10	8
1-10,000	1.0×10^6	∞	$1:4.2 \times 10^3$	0
1-5,000	1.7×10^3	∞	$1:2.4 \times 10^6$	0

EXPERIMENT THIRTY-NINE

Table XXXIX

Effect of Phenol Treatment on the Viable Cell Count and Respiratory Enzyme System (Lactic Dehydrogenase) of the Test Organism Staphylococcus aureus (F.D.A.209)

TOTAL CELL COUNT: 6.0×10^9				
Concentration of Phenol w/v	Viable Cell Count	Reduction Time in Minutes	Survivor Ratio	Enzyme Activity Co-efficiant
0(Control)	5.3×10^9	27 min.	1:1.1	100
1-200	3.3×10^9	180 min.	1:1.8	15
1-150	1.1×10^9	180 min.	1:5.4	15
1-100	1.6×10^6	∞	$1:3.7 \times 10^3$	0
1-75	1.0×10^4	∞	$1:6.0 \times 10^5$	0
1-50	5.3×10^3	∞	$1:1.1 \times 10^6$	0

EXPERIMENT FORTY

Table XL

Effect of Phenol Treatment on the Viable Cell Count and Respiratory Enzyme System (Lactic Dehydrogenase) of the Test Organism Staphylococcus aureus (F.D.A.209)

TOTAL CELL COUNT: 4.6×10^9				
Concentration of Phenol w/v	Viable Cell Count	Reduction Time in Minutes	Survivor Ratio	Enzyme Activity Co-efficient
0(Control)	4.6×10^9	13 min.	1:1.0	100
1-200	4.6×10^9	81 min.	1:1.0	16
1-150	4.0×10^9	81 min.	1:1.1	16
1-100	8.6×10^5	∞	$1:5.3 \times 10^3$	0
1-75	4.0×10^3	∞	$1:1.1 \times 10^6$	0
1-50	3.3×10^3	∞	$1:1.3 \times 10^6$	0

EXPERIMENT FORTY-ONE

Table XLI

Effect of Ethyl Alcohol Treatment on the Viable Cell Count and Respiratory Enzyme System (Lactic Dehydrogenase) of the Test Organism Staphylococcus aureus (F.D.A.209)

TOTAL CELL COUNT: 4.6×10^9				
Concentration of Ethyl Alcohol v/v %	Viable Cell Count	Reduction Time in Minutes	Survivor Ratio	Enzyme Activity Coefficient
0(Control)	2.8×10^9	20 min.	1:1.6	100
12.50	2.0×10^9	20 min.	1:2.3	100
25.00	1.5×10^7	∞	$1:3.0 \times 10^2$	0
35.00	1.3×10^4	∞	$1:3.5 \times 10^5$	0
40.00	1.3×10^4	∞	$1:3.5 \times 10^5$	0
45.00	1.3×10^3	∞	$1:3.5 \times 10^6$	0

EXPERIMENT FORTY-TWO

Table XLII

Effect of Ethyl Alcohol Treatment on the Viable Cell Count and Respiratory Enzyme System (Lactic Dehydrogenase) of the Test Organism Staphylococcus aureus (F.D.A.209)

TOTAL CELL COUNT: 4.5×10^9				
Concentration of Ethyl Alcohol w/v %	Viable Cell Count	Reduction Time in Minutes	Survivor Ratio	Enzyme Activity Coefficient
0(Control)	3.3×10^9	15 min.	1:1.3	100
12.50	2.2×10^9	15 min.	1:2.0	100
25.00	6.6×10^6	∞	$1:6.9 \times 10^2$	0
35.00	6.6×10^2	∞	$1:6.9 \times 10^6$	0
40.00	0	∞	$<1:4.5 \times 10^9$	0
45.00	0	∞	$<1:4.5 \times 10^9$	0

EXPERIMENT FORTY-THREE

Table XLIII

Effect of Alkyl Dimethyl Benzyl Ammonium Chloride (Roccal) Treatment on the Viable Cell Count and Respiratory Enzyme System (Lactic Dehydrogenase) of the Test Organism Staphylococcus aureus (F.D.A.209)

	TOTAL CELL COUNT: 4.2×10^9			
Concentration of Roccal 10% w/v	Viable Cell Count	Reduction Time in Minutes	Survivor Ratio	Enzyme Activity Co-efficient
0(Control)	2.6×10^9	22 min.	1:1.6	100
$1\text{-}10^6$	2.0×10^9	22 min.	1:2.1	100
$1\text{-}10^5$	2.2×10^9	22 min.	1:1.9	100
$1\text{-}10^4$	1.2×10^9	∞	1:3.5	0
$1\text{-}10^3$	0	∞	$<1:4.2 \times 10^9$	0
$1\text{-}10^2$	0	∞	$<1:4.2 \times 10^9$	0

EXPERIMENT FORTY-FOUR

Table XLIV

Effect of Alkyl Dimethyl Benzyl Ammonium Chloride (Roccal) Treatment on the Viable Cell Count and the Respiratory Enzyme System (Lactic Dehydrogenase) of the Test Organism Staphylococcus aureus (F.D.A.209)

		TOTAL CELL COUNT: 4.3×10^9		
Concentration of Roccal 10% w/v	Viable Cell Count	Reduction Time in Minutes	Survivor Ratio	Enzyme Activity Co-efficient
0(Control)	2.6×10^9	20 min.	1:1.6	100
$1\text{-}10^6$	2.6×10^9	20 min.	1:1.6	100
$1\text{-}10^5$	2.6×10^9	20 min.	1:1.6	100
$1\text{-}10^4$	6.0×10^8	∞	1:7.1	0
$1\text{-}10^3$	0	∞	$<1:4.3 \times 10^9$	0
$1\text{-}10^2$	0	∞	$<1:4.3 \times 10^9$	0

DISCUSSION OF RESULTS

The results presented in Tables XXXII to XLIV demonstrate that two distinct types of action are obtained when the cells of the test organism are treated with the chemical disinfectants employed in this investigation. These actions are: first, inhibition of the enzyme (lactic dehydrogenase) by concentrations of the disinfectant which are less than that required to effect appreciably the viable cell count; second, no inhibition of the enzyme (lactic dehydrogenase) by concentrations of the disinfectant below that required to cause appreciable decrease in the viable cell count. Mercuric chloride, silver nitrate, tincture of iodine (5% w/v) and phenol inhibit the enzyme in concentrations which have no apparent effect on the viable cell count. Ethyl alcohol and alkyl dimethyl benzyl ammonium chloride (Roccal) show no inhibition of enzyme activity until the concentration of disinfectant is sufficient to cause appreciable decrease in the viable cell count of the test organism.

The summary of results presented above indicates the type of information which can be obtained by using the procedure, methods and apparatus which have been described. The procedure which has been developed makes it possible to study simultaneously the effect of various concentrations of disinfectant on the viable cell count and enzyme activity of aliquots of a standardized suspension of test organisms. The methods and apparatus employed are relatively simple compared

to other techniques (e.g., the Barcroft-Warburg apparatus) which might be employed to obtain similar information.

The versatility of the apparatus and techniques which have been described is further demonstrated by the experiments which follow in Part Four of this report.

PART FOUR

Three Sections

SECTION A: EXPERIMENTS DEMONSTRATING THE EFFECT OF HEAT KILLED SUSPENSIONS OF THE TEST ORGANISMS ON THE RESPIRATORY ENZYME SYSTEM (LACTIC DEHYDROGENASE) OF THE TEST ORGANISM.

SECTION B: THE EFFECT OF DISINFECTANT ON HEAT KILLED SUSPENSIONS OF TEST ORGANISMS WITH RESPECT TO THE RESPIRATORY ENZYME SYSTEM (LACTIC DEHYDROGENASE) OF THE TEST ORGANISM.

SECTION C: RELATION EXISTING BETWEEN COZYMASE AND HEAT KILLED SUSPENSIONS OF THE TEST ORGANISM.

SECTION A

EXPERIMENTS DEMONSTRATING THE EFFECT OF HEAT KILLED SUSPENSIONS OF TEST ORGANISMS ON THE RESPIRATORY ENZYME SYSTEM (LACTIC DEHYDROGENASE) OF THE TEST ORGANISM

Introduction

Yudkin (1933) demonstrated that the glucose dehydrogenase activity of washed suspensions of Bact. coli falls off disproportionately with dilution. This observation led to a detailed investigation of each of several factors involved in the methylene blue reduction technique. Yudkin reasoned that the product of enzyme activity and the reciprocal of the cell suspension dilution should be constant. He found that this was true for the formic and succinic dehydrogenase enzyme systems but not for glucose dehydrogenase. None of the factors investigated could explain this striking lack of proportionality between activity and dilution. The results of these investigations suggested the existence of some glucose dehydrogenase co-factor which is diffusable and consequently is diluted out. Further investigation demonstrated that there exists a thermostable co-enzyme in the bacteria which is probably washed out on dilution. On the addition of heated suspension of bacteria, the effect of dilution is that which would be expected and direct proportionality exists between enzyme activity and the dilution of the suspension. Yudkin (1934) in a subsequent investigation presented evidence that the

glucose dehydrogenase coenzyme of Bact. coli is identical with cozymase of yeast fermentation. An investigation of lactic dehydrogenase activity of Bact. coli demonstrated that this dehydrogenation also involves a coenzyme which is replaceable by heated bacterial suspension and by cozymase (Yudkin, 1937 a). When the cells of Bact. coli were lysed by repeated freezing and thawing, lactic dehydrogenase activity was found to decrease by more than 50 per cent. Enzyme activity could not be restored by the addition of coenzyme in the form of heated bacteria or a solution of cozymase. These observations led Yudkin (1937 b) to suggest that in this case the enzyme activity was in some way linked with the structure of the cell.

Gale and Stephenson (1938)and Gale (1943) noted that the serine deaminase of E. coli decays rapidly on standing of washed cell suspensions. These workers suggested that the loss of activity appears to be associated with the diffusion of some co-enzyme-like factor out of the cell, as it can be prevented by the addition of boiled organisms.

Kocholaty and Hoogerheide (1938) have reported that the dehydrogenases of Cl. sporogenes appreciably lose activity during the washing of the cells.

Woods and Trim (1942) demonstrated that the activity of Cl. welchii on serine in growth medium lagged behind growth in early stages of the lag phase. This delay in activity was associated with a loss of diffusible coenzyme. Dilution of the cell suspension caused a disproportionate

fall in enzyme activity, approaching zero when the suspension was diluted 1 in 10.

Weil, Kocholaty and Smith (1939) and van Heyningen (1940) have made observations with Cl. histolyticum which again suggest the existence of diffusable co-enzyme.

In preliminary experiments concerning the lactic dehydrogenase of Staphylococcus aureus (F.D.A.209), a lack of proportionality between enzyme activity and cell suspension dilution was noted. Further investigation showed that the addition of heated suspensions of the test organisms restored this proportionality. The experimental reports which follow present the results of these investigations.

EXPERIMENTAL INVESTIGATIONS

Experiment Forty-five

In order to investigate the various factors involved in the estimation of lactic dehydrogenase activity of Staphylococcus aureus (F.D.A.209) by the methylene blue reduction test, the following experiment was done.

Standardized "resting" cell suspension of the test organism was prepared according to the method outlined in Part Two of this report. The concentration of this cell suspension was 5 mgm. per mil. based on wet weight of organisms.

Seven oxidation-reduction reaction tubes were set out so that four of these served as "test" tubes and the remaining three as "controls". The content of the various tubes was as follows:

"Test" tubes contained 1.0 mil. of "resting" cell suspension, 1.0 mil. of phosphate buffer solution (pH 7.35), 1.0 mil. of methylene blue chloride solution (1-20,000 w/v), and 1.0 mil. of substrate solution (sodium lactate 0.1% w/v).

"Control" tube number one contained 1.0 mil. of substrate solution, 1.0 mil. of methylene blue chloride solution and 2.0 mil of cell suspension which had been boiled for 10 minutes prior to testing.

"Control" tube number two contained 1.0 mil. of methylene blue chloride solution, 1.0 mil. of phosphate buffer solution, and 2.0 mil. of "resting" cell suspension.

"Control" tube number three contained 1.0 mil. of methylene blue chloride solution, 1.0 mil. of substrate solution, and 2.0 mil. of phosphate buffer solution.

The total volume in each tube was thus four millelitres.

Lactic dehydrogenase activity was tested for by the method previously described in Part Two of this report.

Table XLV gives the results obtained in this experiment. The activity of lactic dehydrogenase is represented in this table by the actual reduction times obtained.

Table XLV

Experiment Investigating Various Factors Involved in the Estimation of Lactic Dehydrogenase Activity of Staphylococcus aureus (F.D.A.209)

Test Number	Reduction Time Obtained
1	15 minutes
2	15 minutes
3	14 minutes
4	14 minutes
Control Number	**Reduction Time Obtained**
1	No reduction in 18 hours
2	No reduction in 18 hours
3	No reduction in 18 hours

Experiment Forty-six

This experiment was designed as a duplicate of Experiment Forty-five. The resting cell suspension of the test organism was prepared in the manner which we have described. The ingredients in the "test" tubes and the "control" tubes were the same as in Experiment Forty-five.

Results obtained in this Experiment are given in Table XLVI which follows immediately.

Table XLVI

Experiment Investigating Various Factors Involved in the Estimation of Lactic Dehydrogenase Activity of Staphylococcus aureus (F.D.A.209)

Test Number	Reduction Time Obtained
1	14 minutes
2	15 minutes
3	14 minutes
4	15 minutes
Control Number	**Reduction Time Obtained**
1	No reduction in 18 hours
2	No reduction in 18 hours
3	No reduction in 18 hours

The results presented in Tables XLV and XLVI may be summarized as follows: in the presence of sodium lactate substrate 1.0 mil. of the standardized resting cell suspemsion of Staphylococcus aureus (F.D.A.209) completely reduced 1.0 mil. of methylene blue chloride solution (1-20,000 w/v) in 14 to 15 minutes. Two millelitres of the same resting cell suspension, which had been boiled for 10 minutes, did not reduce the dye in the presence of the substrate in 18 hours. Two millelitres of unboiled resting cell suspension failed to reduce methylene blue in the absence of sodium lactate substrate. Methylene blue was not reduced by buffer and substrate in the absence of resting cell suspension.

From these results, the following conclusions may be drawn: aliquots of standardized suspension of Staphylococcus aureus (F.D.A.209) show equal lactic dehydrogenase activity. The enzyme activity is specific for the substrate sodium lactate. Boiled cell suspensions even in double concentration demonstrated no specific enzyme activity during the test period. The substrate and buffer were unable to reduce methylene blue in the absence of "resting" cell suspension.

Experiment Forty-seven

In order to investigate more fully the effect of resting cell concentration on the lactic dehydrogenase activity, the following experiment was done.

The standardized resting cell suspension used in this experiment was exactly double the concentration of that used in Experiments Forty-five and Forty-six (10 mgm. per millelitre based on wet weight of organisms). The content of "test" tubes and "control" tubes was as described in Experiment Forty-five. Lactic dehydrogenase activity was estimated by the methods previously described.

The results obtained in this experiment are presented in Table XLVII.

Table XLVII

Experiment to Determine the Effect of Resting Cell Concentration on the Lactic Dehydrogenase Activity of Staphylococcus aureus (F.D.A.209)

Test Number	Reduction Time Obtained
1	7 minutes
2	8 minutes
3	8 minutes
4	8 minutes
5	8 minutes
Control Number	**Reduction Time Obtained**
1	No reduction in 18 hours
2	No reduction in 18 hours
3	No reduction in 18 hours

Comparing the results presented in Tables XLV and XLVI with those given in Table XLVII, it can be seen that the effect of doubling the concentration of the "resting" cell suspension of the test organism is to halve the reduction time. From these results, it appears that the lactic dehydrogenase activity of the test organism Staphylococcus

aureus (F.D.A.209) is directly proportional to the concentration of the resting cell suspension. Quastel and Whetham (1924) showed that this proportionality existed in the case of the succinic dehydrogenase of Bact. coli. This was the only dehydrogenase studied and the dilution of resting cell suspension was made over only a small range. Yudkin (1933) studied the effect of dilution on resting cell preparations of Bact. coli over an extended range of dilutions. He was able to demonstrate in this manner that a disproportionality existed between dilution and the glucose dehydrogenase activity of this organism.

Experiment Forty-eight

In order to investigate more fully the effect of dilution on the lactic dehydrogenase activity of the resting cell suspension of the test organism, the following experiment was designed covering a wider range of dilutions.

In this experiment, the concentration of the standardized resting cell suspension of the test organism was the same as that employed in Experiment Forty-seven (10 mgm. per mil. based on wet weight of organisms). The content of "test" and "control" tubes was the same as in Experiment Forty-five with the exception that the resting cell suspension was diluted as indicated.

The results of this experiment are given in Table XLVIII.

Table XLVIII

Experiments to Determine the Effect of Dilution on the Lactic Dehydrogenase Activity of Staphylococcus aureus (F.D.A. 209)

Test Number	Dilution of Resting Cell Suspension	Reduction Time Obtained
1	Undiluted	9 minutes
2	1 in 2	18 minutes
3	1 in 4	63 minutes
4	1 in 8	Not reduced in 90 min.
Control Number		
1		Not reduced in 90 min.
2		Not reduced in 90 min.
3		Not reduced in 90 min.

From the results presented in Table XLVIII, it is apparent that the relation between enzyme activity (lactic dehydrogenase) and dilution of the resting cell suspension is disproportionate when the dilution is greater than 1 in 2.

Expression of Enzyme Efficiency of Resting Cell Preparations

If the actual reduction time is known for an undiluted resting cell preparation, then the theoretical reduction time for any particular dilution of the same preparation may be calculated. This theoretical reduction time should equal the product of the reciprocal of dilution multiplied by the actual reduction time obtained for the undiluted material. Thus, if the undiluted preparation reduces methylene blue in 12 minutes, then a 1 in 4 dilution of the same preparation should require 48 minutes to reduce the same amount of methylene blue . If the actual reduction time of the diluted preparation is obtained by experiment, then the enzyme efficiency of the diluted preparation may be expressed by dividing the actual reduction time by the calculated reduction time and multiplying the result by 100. If the enzyme activity of a resting cell preparation is directly proportional to the degree of dilution then the enzyme efficiency should always be 100. In Experiment XLVIII, we have given results which indicate that this is not true. In the experiments which follow, the enzyme efficiency of resting cell preparations will be expressed as outlined above.

Experiment Forty-nine

Yudkin (1933) demonstrated that the addition of heated cell suspension restored the direct proportionality between enzyme activity and dilution of the resting cell preparation for glucose dehydrogenase of Bact. coli. The following experiment was designed to determine if a similar effect could be demonstrated for the lactic dehydrogenase of Staphylococcus aureus (F.D.A.209).

The experiment may be divided into two parts. In the first part, the time required for reduction of methylene blue was determined for various dilutions of a preparation of resting cells. Dilutions of resting cells were prepared in phosphate buffer (pH 7.35). In the second part of the experiment, dilutions of the same resting cell preparation were tested by exactly the same technique with the exception that 1.0 mil. of heated cell suspension (heated in a boiling water bath for 10 minutes) was added to each reaction tube prior to testing.

The results of this experiment are presented in Table XLIX. The undiluted resting cell suspension was prepared so as to have a concentration of 5 mgm. per millelitre based on wet weight of organisms.

In order to provide evidence that the heated cell suspension did not contain living cells, the suspension was inoculated to peptone agar and incubated for 96 hours at 37°C. In this experiment and all subsequent experiments involving heated cell suspensions, this practice was followed.

Heated cell suspensions were always prepared in the manner outlined above, i.e. heated in a boiling water bath for 10 minutes. Heated cell suspensions prepared and tested as described never gave evidence of colonial development.

Table XLIX

Experiment to Determine the Effect of the Addition of Heated Cell Suspension on the Lactic Dehydrogenase Activity of Various Dilutions of a Resting Cell Suspension of *Staphylococcus aureus* (F.D.A.209)

Absence of Heated Cell Suspension			
Dilution of Resting Cell Suspension	Actual Reduction Time obtained	Calculated Reduction Time	Enzyme Efficiency
Undiluted	12 minutes		100
1 in 2	26 minutes	24 minutes	92
1 in 4	>18 hours	48 minutes	<4.4
Presence of Heated Cell Suspension			
Dilution of Resting Cell Suspension	Actual Reduction Time obtained	Calculated Reduction Time	Enzyme Efficiency
Undiluted	9 minutes		100
1 in 2	18 minutes	18 minutes	100
1 in 4	39 minutes	36 minutes	92
1 in 8	74 minutes	72 minutes	97

The results given in Table XLIX are presented graphically in Figure 13.

Experiment Fifty

A duplicate experiment was done in the same manner as Experiment Forty-nine. The only difference involved in this experiment was that a new resting cell suspension was employed. The concentration of this cell suspension was the same as that used in Experiment Forty-nine.

The results of this experiment are presented in Table L.

Table L

Experiment to Determine the Effect of the Addition of Heated Cell Suspension on the Lactic Dehydrogenase Activity of Various Dilutions of a Resting Cell Suspension of Staphylococcus aureus (F.D.A.209)

Absence of Heated Cell Suspension			
Dilution of Resting Cell Suspension	Actual Reduction Time obtained	Calculated Reduction Time	Enzyme Efficiency
Undiluted	32 minutes		100
1 in 2	80 minutes	64 minutes	80
1 in 4	>18 hours	128 minutes	<12
Presence of Heated Cell Suspension			
Dilutions of Resting Cell Suspension	Actual Reduction Time obtained	Calculated Reduction Time	Enzyme Efficiency
Undiluted	19 minutes		100
1 in 2	38 minutes	38 minutes	100
1 in 4	76 minutes	76 minutes	100
1 in 8	159 minutes	152 minutes	95

The results given in Table L are presented graphically in Figure 13.

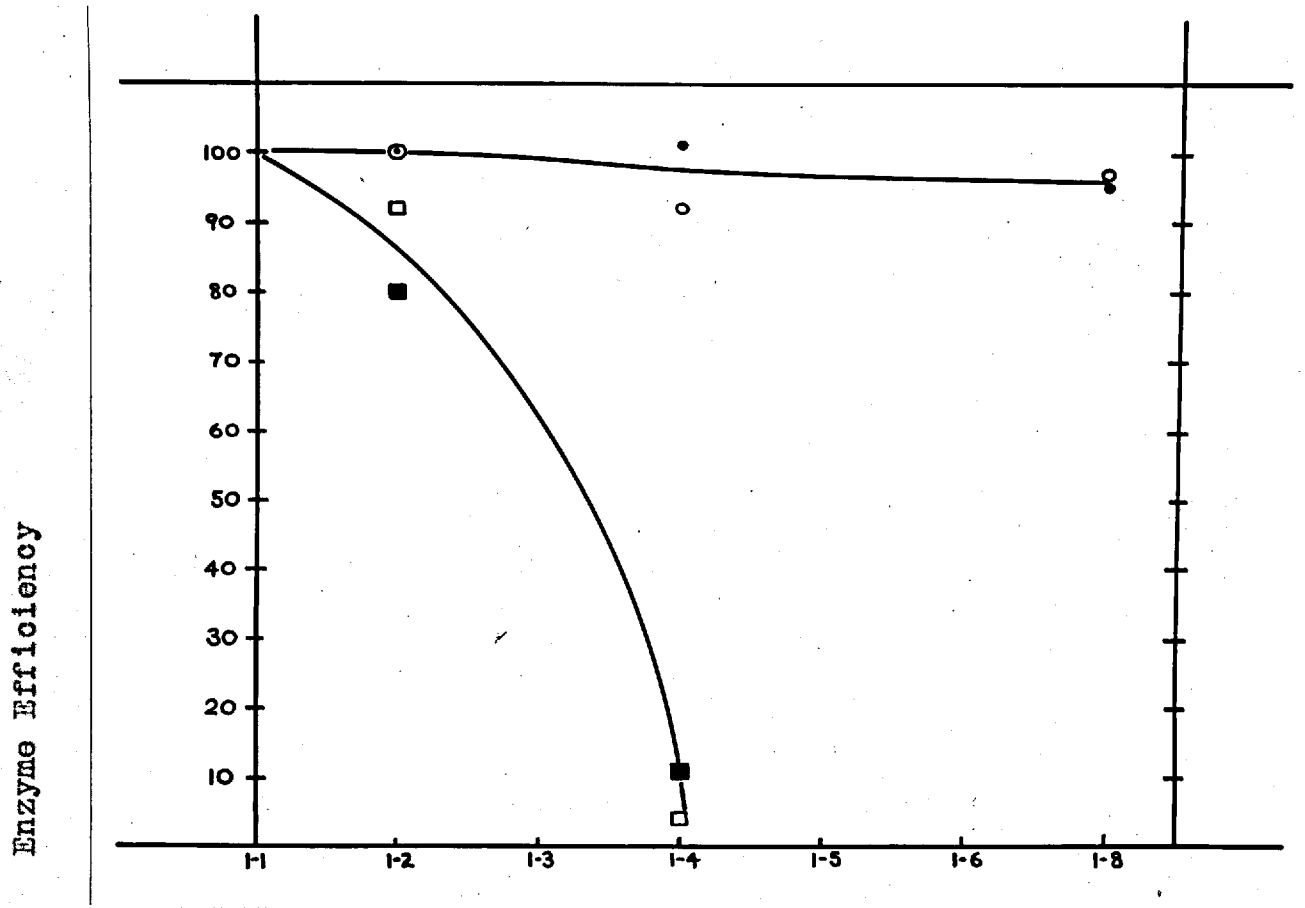


Figure 13: THE EFFECT OF THE ADDITION OF HEATED CELL SUSPENSION ON THE LACTIC DEHYDROGENASE ACTIVITY OF Stephylococcus aureus (F.D.A.209).
A graphic representation of the experimental results given in Tables XLIX and L.

Symbols: Results given -	TABLE XLIX	TABLE L
Enzyme activity (absence of heated cells)	□	■
Enzyme activity (presence of heated cells)	○	●

Experiment Fifty-one

It is possible that the heated cell suspension employed in Experiments Forty-nine and Fifty acted as a hydrogen donor supplementing the specific substrate and thus increasing the velocity of reduction of methylene blue. In order to investigate this possibility, the following experiment was performed using the undiluted resting cell preparation employed in Experiments Forty-nine and Fifty.

For each undiluted resting cell suspension, two tests were done. In test number one, the reacting ingredients were 2.0 mil. of heated cell suspension, 1.0 mil. of substrate solution (sodium lactate 0.1% w/v) and 1.0 mil. of methylene blue solution. In test number two, the ingredients were 1.0 mil. of unheated undiluted resting cell suspension, 1.0 mil. of heated cell suspension, 1.0 mil. of phosphate buffer solution (ph 7.35) and 1.0 mil. of methylene blue solution.

The results of this experiment are presented in Table LI

Table LI

Experiment to Investigate the Role of Heated Cell Suspension as Hydrogen Donor in the Lactic Dehydrogenase Enzyme System of Staphylococcus aureus (F.D.A.209)

Tests with Cell Suspension Employed in Experiment Forty-nine	
Test Number	Reduction Time Obtained
1	Not reduced in 18 hours
2	Not reduced in 18 hours

Tests with Cell Suspension Employed in Experiment Fifty	
Test Number	Reduction Time Obtained
1	Not reduced in 18 hours
2	Not reduced in 18 hours

The following conclusions may be derived from the results presented in Table LI. First, the heated cell suspension (even when present at double the concentration employed in Experiments Forty-nine and Fifty) is not capable per se of activating the specific substrate, sodium lactate. Second, the heated cell suspension does not act as hydrogen donor (replacing the specific substrate) in the absence of specific substrate.

Discussion

From the results obtained in Experiments Forty-nine to Fifty-one, it can be seen that the lactic dehydrogenase activity of diluted resting cell preparations of Staphylococcus aureus (F.D.A.209) is disproportional to the dilution of the preparation. The addition of heated cell suspension restores direct proportionality between lactic dehydrogenase activity and dilution. Heated cell suspension does not act as a hydrogen donor in the absence of the specific substrate (sodium lactate) under the test conditions prevailing and is not capable per se of activating that substrate in the absence of resting cells. Therefore, the heated cell suspension must provide some co-factor necessary for lactic dehydrogenation which co-factor is diluted out when the resting cell preparation is diluted.

SECTION B

THE EFFECT OF DISINFECTANT ON HEAT KILLED SUSPENSIONS OF TEST ORGANISMS WITH RESPECT TO THE RESPIRATORY ENZYME SYSTEM (LACTIC DEHYDROGENASE) OF THE TEST ORGANISM

Introduction and Experimental Investigations

Combination of disinfectant treated heated cell suspension with untreated resting cells of the same standardized suspension should make it possible to determine whether or not the disinfectant acts on the co-factor. For this purpose, the following experiments were done.

Experiment Fifty-two

A standardized resting cell suspension was prepared according to the method described in Part Two of this report. Part of this suspension was heated in a boiling water bath for ten minutes. One millelitre volumes of the heated cell suspension were then treated with the various disinfecting agents according to the treatment method outlined in Part Two of this report. The concentration of each disinfectant used was that which, according to the experiments recorded in Part Two of this report, showed appreciable effect on both lactic dehydrogenase activity and viable cell counts. Following disinfectant treatment, disinfectant was diluted out according to the method previously described. One millelitre of untreated resting cell suspension was then added to each tube along with 1.0 mil. of methylene blue chloride solution and 1.0 mil. of substrate solution (sodium lactate). Two control tubes were prepared. Control number one contained 1.0 mil. of resting cell suspension, 1.0 mil. of sodium lactate solution, 1.0 mil. of methylene blue solution and 1.0 mil. of phosphate buffer (pH 7.35), thus heated cells were omitted

from this tube. Control number two contained the same ingredients with the exception that 1.0 mil. of untreated heated cell suspension was included in place of the buffer solution.

In this case, as in all other experiments involving heated cell suspensions, the suspension was inoculated to a peptone agar plate and incubated for 96 hours to establish that heat treatment had killed the cells.

The results of the oxidation-reduction tests done with the aforementioned materials are given in Table LII.

Table LII

The Effect of Disinfectant on Heat Killed Suspensions of Test Organisms with Respect to the Respiratory Enzyme System (Lactic Dehydrogenase) of the Test Organism Staphylococcus aureus (F.D.A.209)

Resting Cell Suspension	Heated Cell Suspension	Disinfectant Treatment of Heated Cell Suspension	Reduction Time Obtained
1.0 mil. +	1.0 mil.	5.00 P.P.M. $HgCl_2$	34 minutes
1.0 mil. +	1.0 mil.	5.00 P.P.M. $AgNO_3$	15 minutes
1.0 mil. +	1.0 mil.	1-10,000 Tr.Iodine (5% w/v)	7 minutes
1.0 mil. +	1.0 mil.	1-100 Phenol	7 minutes
1.0 mil. +	1.0 mil.	30% v/v Ethyl Alcohol	7 minutes
1.0 mil. +	1.0 mil.	1-10,000 Roccal (10% w/v)	7 minutes
1.0 mil. +	1.0 mil.	Phosphate Buffer (pH 7.35)	7 minutes
1.0 mil.	No heated Cells		14 minutes

Experiment Fifty-three

This experiment is a duplicate of Experiment Fifty-two. The resting cell preparation used was the same as that used in Experiment Fifty-two but had been stored at 5°C. for twenty-four hours.

Table LIII gives the results obtained in Experiment Fifty-three.

Table LIII

The Effect of Disinfectant on Heat Killed Suspensions of Test Organisms with Respect to the Respiratory Enzyme System (Lactic Dehydrogenase) of the Test Organism Staphylococcus aureus (F.D.A.209)

Resting Cell Suspension		Heated Cell Suspension	Disinfectant Treatment of Heated Cell Suspension	Reduction Time Obtained
1.0 mil.	+	1.0 mil.	5.00 P.P.M. $HgCl_2$	28 minutes
1.0 mil.	+	1.0 mil.	5.00 P.P.M. $AgNO_3$	18 minutes
1.0 mil.	+	1.0 mil.	1-10,000 Tr.Iodine (5% w/v)	11 minutes
1.0 mil.	+	1.0 mil.	1-100 Phenol	11 minutes
1.0 mil.	+	1.0 mil.	30% v/v Ethyl Alcohol	11 minutes
1.0 mil.	+	1.0 mil.	1-10,000 Roccal (10% w/v)	11 minutes
1.0 mil.	+	1.0 mil.	Phosphate Buffer (pH 7.35)	11 minutes
1.0 mil.		No heated Cells		18 minutes.

From the results presented in Tables LII and LIII, it is evident that concentrations of 5.00 P.P.M. of $HgCl_2$ and of $AgNO_3$ destroy the activity of the co-factor for lactic dehydrogenase which is provided by heated cell suspensions. Concentrations of the other disinfectants (Tincture of Iodine, Phenol, Ethyl Alcohol, Roccal) which demonstrate an appreciable effect on the viable cell count and the lactic dehydrogenase activity of the test organism apparently do not effect the co-factor.

Experiment Fifty-four

Further Investigation of the Effect of Heat Killed Suspensions of Test Organisms on the Respiratory Enzyme System (Lactic Dehydrogenase) of the Test Organism Staphylococcus aureus (F.D.A.209)

It was previously mentioned that the lactic dehydrogenase activity of resting cell preparations of the test organism decreased markedly upon storage at various temperatures (see Table XXVI). It was considered of interest to determine the effect of adding heated cell suspension to resting cell suspensions which had been stored. The results given in Table XXIV were actually derived from the experiment the results of which are now given in full in Table LIV.

Table LIV

The Effect of the Addition of Heated Cell Suspension on the Lactic Dehydrogenase Activity of Resting Cell Suspensions of Staphylococcus aureus (F.D.A.209) Stored at Various Temperatures

DAYS OF STORAGE	REDUCTION TIME OBTAINED (IN MINUTES)					
	STORED AT 28°C.		STORED AT 5°C.		STORED AT -20°C.	
	Resting Cells	Resting Cells + Heated Cells	Resting Cells	Resting Cells + Heated Cells	Resting Cells	Resting Cells + Heated Cells
0	20		20		20	
1	47	21	20		23	
2	114		30		31	
4	>180	60	39	23	>120	38

The results presented in Table LIV indicate that the addition of heated cell suspension at least partially restores the lactic dehydrogenase activity of resting cell suspensions which have been stored at various temperatures. Restoration appears to be most effective in the case of resting cell suspension stored at 5°C. It must be noted that the loss of lactic dehydrogenase activity resulting from storage was also less at this temperature.

Further Investigation of the Effect of Disinfectant on Heat Killed Suspensions of the Test Organisms with Respect to the Lactic Dehydrogenase Activity of Staphylococcus aureus (F.D.A.209)

In Experiments Fifty-two and Fifty-three, it was demonstrated that certain disinfectants effect the co-factor of lactic dehydrogenase of Staphylococcus aureus whereas others do not. The concentration of disinfectant employed in these experiments was that which was previously found to effect appreciably both the viable cell count and the lactic dehydrogenase activity of the organism. In the following series of experiments, further investigations of the effect of disinfectants on both the co-factor (provided by heated cell suspensions) and the enzyme itself (provided by resting cell preparation) will be presented.

Standardized resting cell suspensions were prepared by the method described in Part Two of this report. Preparations were stored at 5°C. for 4 to 7 days prior to testing. Part of the resting cell suspension was heated in a boiling water bath for ten minutes. One millelitre volumes of the resting cell preparation and of the heated cell suspension were then treated with disinfectant as previously described. Untreated resting cells, untreated testing cells plus untreated heated cells, and various combinations of these were tested for lactic dehydrogenase activity. The tables related to the following experiments provide the details of the combinations of ingredients tested.

Experiment Fifty-five

In this experiment, the disinfectant employed was mercuric chloride. The concentration of disinfectant used (5.00 P.P.M.) had previously been shown to effect appreciably both the viable cell count and the lactic dehydrogenase activity.

Table LV gives the results of this experiment. Tests A and B differ only in that a different resting cell preparation was employed.

Table LV

Experiment to Investigate the Effect of Mercuric Chloride on the Co-factor of Lactic Dehydrogenase (Heated Cell Suspension) and the Lactic Dehydrogenase Activity (Resting Cell Suspension) of *Staphylococcus aureus* (F.D.A.209)

Ingredients of Test	MERCURIC CHLORIDE CONCENTRATION: 5.00 P.P.M. Reduction Time Obtained
TEST A	
Untreated Resting Cells	25 minutes
Untreated Resting Cells plus Untreated Heated Cells	12 minutes
Untreated Resting Cells plus Treated Heated Cells	27 minutes
Treated Resting Cells	No reduction in 16 hours
Treated Resting Cells plus Untreated Heated Cells	128 minutes
Treated Resting Cells plus Treated Heated Cells	No reduction in 16 hours
TEST B	
Untreated Resting Cells	46 minutes
Untreated Resting Cells plus Untreated Heated Cells	25 minutes
Untreated Resting Cells plus Treated Heated Cells	46 minutes
Treated Resting Cells	No reduction in 16 hours
Treated Resting Cells plus Untreated Resting Cells	97 minutes
Treated Resting Cells plus Treated Heated Cells	No reduction in 16 hours

From the results presented in Table LV, it can be seen that 5.00 P.P.M. of mercuric chloride effects both the heated cell suspension (co-factor) and the resting cell preparation (lactic dehydrogenase) of the test organism. Addition of untreated heated cell suspension (co-factor) partially restores the lactic dehydrogenase activity of resting cells which have been treated with 5.00 P.P.M. of mercuric chloride.

Experiment Fifty-six

In this experiment, the disinfectant was silver nitrate and the concentration employed was 5.00 P.P.M. In all other respects, the experiment was the same as Experiment Fifty-five.

The results of Experiment Fifty-six are given in Table LVI.

Table LVI

Experiment to Investigate the Effect of Silver Nitrate on the Co-factor of Lactic Dehydrogenase (Heated Cell Suspension) and the Lactic Dehydrogenase Activity (Resting Cell Suspension) of Staphylococcus sureus (F.D.A.209)

SILVER NITRATE CONCENTRATION: 5.00 P.P.M.	
Ingredients of Test	Reduction Time Obtained
TEST A	
Untreated Resting Cells	35 minutes
Untreated Resting Cells plus Untreated Heated Cells	17 minutes
Untreated Resting Cells plus Treated Heated Cells	34 minutes
Treated Resting Cells	No reduction in 180 min.
Treated Resting Cells plus Untreated Heated Cells	34 minutes
Treated Resting Cells plus Treated Heated Cells	No reduction in 180 min.
TEST B	
Untreated Resting Cells	38 minutes
Untreated Resting Cells plus Untreated Heated Cells	22 minutes
Untreated Resting Cells plus Treated Heated Cells	38 minutes
Treated Resting Cells	No reduction in 180 min.
Treated Resting Cells plus Untreated Heated Cells	38 minutes
Treated Resging Cells plus Treated Heated Cells	No reduction in 180 min.

The results presented in Table LVI indicate that silver nitrate at a concentration of 5.00 P.P.M. effects both the co-factor (heated cell suspension) and the lactic dehydrogenase (resting cell preparation) of the test organism. The addition of untreated heated cell suspension partially restores the lactic dehydrogenase activity of treated resting cell preparations.

Experiment Fifty-seven

In this experiment, the disinfectant employed was phenol in a concentration of 1 in 100 w/v. In all other respects, the experiment was the same as Experiment Fifty-five.

The results obtained in this experiment are given in Table LVII.

Table LVII

Experiment to Investigate the Effect of Phenol on the Co-factor of Lactic Dehydrogenase (Heated Cell Suspension) and the Lactic Dehydrogenase Activity (Resting Cell Suspension) of Staphylococcus aureus (F.D.A.209)

PHENOL CONCENTRATION: 1 in 100w/v	
Ingredients of Test	Reduction Time Obtained
TEST A	
Untreated Resting Cells	18 minutes
Untreated Resting Cells plus Untreated Heated Cells	10 minutes
Untreated Resting Cells plus Treated Heated Cells	11 minutes
Treated Resting Cells	No reduction in 180 min.
Treated Resting Cells plus Untreated Heated Cells	No reduction in 180 min.
Treated Resting Cells plus Treated Heated Cells	No reduction in 180 min.
TEST B	
Untreated Resting Cells	20 minutes
Untreated Resting Cells plus Untreated Heated Cells	11 minutes
Untreated Resting Cells plus Treated Heated Cells	13 minutes
Treated Resting Cells	No reduction in 180 min.
Treated Resting Cells plus Untreated Heated Cells	No reduction in 180 min.
Treated Resting Cells plus Treated Heated Cells	No reduction in 180 min.

The results presented in Table LVII indicate that phenol in a concentration of 1 in 100 w/v effects the lactic dehydrogenase activity (resting cell suspension) of the test organism, but has little effect on the co-factor supplied by heated cell suspensions. The addition of untreated heated cell suspension did not restore the lactic dehydrogenase activity of treated resting cell suspensions.

Experiment Fifty-eight

The disinfectant employed in this experiment was ethyl alcohol. The concentration of disinfectant existing in the treatment tubes was 25% v/v. In all other respects, this experiment was the same as Experiment Fifty-five.

Table LVIII

Experiment to Investigate the Effect of Ethyl Alcohol on the Co-factor of Lactic Dehydrogenase (Heated Cell Suspension) and the Lactic Dehydrogenase Activity (Resting Cell Suspension) of *Staphylococcus aureus* (F.D.A.209)

ETHYL ALCOHOL CONCENTRATION: 25% v/v	
Ingredients of Test	Reduction Time Obtained
TEST A	
Untreated Resting Cells	17 minutes
Untreated Resting Cells plus Untreated Heated Cells	6 minutes
Untreated Resting Cells plus Treated Heated Cells	6 minutes
Treated Resting Cells	No reduction in 180 min.
Treated Resting Cells plus Untreated Heated Cells	17 minutes
Treated Resting Cells plus Treated Heated Cells	47 minutes
TEST B	
Untreated Resting Cells	27 minutes
Untreated Resting Cells plus Untreated Heated Cells	12 minutes
Untreated Resting Cells plus Treated Heated Cells	12 minutes
Treated Resting Cells	No reduction in 180 min.
Treated Resting Cells plus Untreated Heated Cells	27 minutes
Treated Resting Cells plus Treated Heated Cells	70 minutes

The results presented in Table LVIII indicate that treatment with 25% v/v ethyl alcohol effects the lactic dehydrogenase activity (resting cell suspension) but does not effect the co-factor (heated cell suspension) for lactic dehydrogenase of the test organism. The addition of untreated heated cells partially restores the lactic dehydrogenase activity of treated resting cell suspensions. The addition of treated heated cell suspension likewise restores part of the lactic dehydrogenase activity of treated resting cell suspensions. The fact that the restoration brought about by the addition of treated heated cells is not as great as that brought about by untreated heated cells (even though treatment apparently has no effect on the heated cell suspension) may possibly be due to interference with the transfer of hydrogen from enzyme to co-factor.

Experiment Fifty-nine

Roccal (10% w/v alkyl dimethyl benzyl ammonium chloride) was the disinfectant used in this experiment. The concentration of the 10% w/v stock solution which existed in the treatment tubes was 1 in 10,000. In all other respects, this experiment was the same as Experiment Fifty-five.

Table LIX

Experiment to Investigate the Effect of Roccal on the Co-factor of Lactic Dehydrogenase (Heated Cell Suspension) and the Lactic Dehydrogenase Activity (Resting Cell Suspension) of Staphylococcus aureus (F.D.A.209)

ROCCAL CONCENTRATION (10% w/v); 1 in 10,000	
Ingredients of Test	Reduction Time Obtained
TEST A	
Untreated Resting Cells	28 minutes
Untreated Resting Cells plus Untreated Heated Cells	17 minutes
Untreated Resting Cells plus Treated Heated Cells	18 minutes
Treated Resting Cells	No reduction in 180 min.
Treated Resting Cells plus Untreated Heated Cells	60 minutes
Treated Resting Cells plus Treated Heated Cells	Approximately 180 min.
TEST B	
Untreated Resting Cells	31 minutes
Untreated Resting Cells plus Untreated Heated Cells	20 minutes
Untreated Resting Cells plus Treated Heated Cells	20 minutes
Treated Resting Cells	No reduction in 180 min.
Treated Resting Cells plus Untreated Heated Cells	77 minutes
Treated Resting Cells plus Treated Heated Cells	Approximately 180 min.

The results presented in Table LIX indicate that treatment with Roccal (10% w/v) in a concentration of 1-10,000 effects the lactic dehydrogenase activity (resting cell suspension) but does not effect the co-factor (heated cell suspension) for lactic dehydrogenase. The addition of treated or untreated heated cell suspension partially restores the lactic dehydrogenase activity of treated resting cell suspensions.

Summary of Results of Experiments Designed to Investigate the Effect of Disinfectants on the Co-factor of Lactic Dehydrogenase (Heated Cell Suspension) and the Lactic Dehydrogenase Activity (Resting Cell Suspension) of Staphylococcus aureus (F.D.A.209)

The results presented in Tables LV to LIX may be summarized as follows: the co-factor of lactic dehydrogenase (heated cell suspension) of Staphylococcus aureus (F.D.A.209) is effected by mercuric chloride (5 P.P.M.) and silver nitrate (5 P.P.M.). Phenol (1 in 100 w/v), ethyl alcohol (25% v/v), Roccal (10% w/v) 1 in 10,000 show no effect on the co-factor. The disinfectants mentioned above in the concentrations indicated apparently destroy the lactic dehydrogenase activity (resting cell suspension) of the test organism as demonstrable under the conditions of these experiments. The addition of untreated co-factor restores (at least partially) the lactic dehydrogenase activity of resting cells which have been treated with the various disinfecting agents with the exception of those treated with phenol. The addition of treated co-factor fails to restore the lactic dehydrogenase activity of resting cells treated with mercuric chloride, silver nitrate and phenol. The addition of treated co-factor restores (at least partially) the lactic dehydrogenase activity of resting cells treated with ethyl alcohol and Roccal.

SECTION C

RELATION EXISTING BETWEEN COZYMASE AND HEAT KILLED SUSPENSION OF THE TEST ORGANISM

Introduction

Yudkin (1934) investigated the various properties of the coenzyme of glucose dehydrogenase which he had demonstrated in heat killed suspensions of E.coli. He concluded that this factor is identical with the cozymase of yeast.

Gale and Stephenson (1939) established the existence of malic dehydrogenase in E.coli and studying its properties, they demonstrated that this enzyme is most active in the presence of coenzyme 1. They further demonstrated that the coenzyme is essential for the action of malic dehydrogenase with methylene blue. The coenzyme is present in cozymase preparations from yeast. Boiled bacteria will act as a source of the co-dehydrogenase as will also untreated cozymase solution. These workers believe that the active substance in boiled bacteria is identical with coenzyme 1. The active substance can be extracted in the supernatant of boiled bacteria.

It has been established in the present work that the co-factor (heated cell suspension) for lactic dehydrogenase of Staphylococcus aureus (F.D.A.209) is effected by certain chemical disinfectants. The possibility that this coenzyme is likewise identical with cozymase is of interest.

The experiments described here are strictly of a preliminary nature and are included at this time to indicate the course of proposed future investigation.

EXPERIMENTAL INVESTIGATIONS

Experiment Sixty

Cozymase (coenzyme 1)* was prepared in sterile phosphate buffer solution (pH 7.35) so that a concentration of 1.0 mgm. per millelitre was obtained.

A standardized resting cell suspension of Staphylococcus aureus (F.D.A.209) was prepared according to the method previously outlined. Preliminary standardization of this suspension was based on the preparation of a suspension containing 5.0 mgm. (wet weight) of organisms per mil. Final standardization was by adjustment of opacity as previously described.

The heated cell suspension was prepared by heating the above mentioned resting cell preparation in a boiling water bath for 15 minutes. The sterility of this suspension was then proved by inoculation to peptone agar plates. Such plates were incubated at 37°C. for 96 hours.

It has been demonstrated that dilution of standardized resting cell suspension of Staphylococcus aureus (F.D.A.209) results in disproportional falling-off in lactic dehydrogenase activity (see Tables XLVIII, XLIX and L). In the following experiments, undiluted resting cell suspension was tested in Tubes 1 and 2. Resting cell suspension diluted 1 in 2 in phosphate buffer was tested in Tubes 3 and 4.

It has been shown that the addition of heated cell suspension restores direct proportionality between lactic dehydrogenase activity and dilution of resting cell suspension

*Nutritional Biochemicals Corp., Cleveland, Ohio.

(see Tables XLIX and L). The effect of the addition of heated cell suspension is tested in Tubes 5 and 6. In these tubes, the resting cell suspension is diluted 1 in 2 in phosphate buffer and 1.0 mil. of heated cell suspension is added.

The possibility that cozymase may replace heated cell suspension in the lactic dehydrogenase system is tested in Tubes 7 and 8. In these tubes, the resting cell suspension is diluted 1 in 2 in phosphate buffer and 1.0 mil. of cozymase preparation (1.0 mgm.) is added.

The results of this experiment are presented in Table LX.

Table LX

Experiment Investigating Substitution of Cozymase for Heated Cell Suspension in the Lactic Dehydrogenase Enzyme System of *Staphylococcus aureus* (F.D.A.209)

TUBE NO.	CONTENT OF TEST TUBE	REDUCTION TIME OBTAINED
1	Undiluted resting cell suspension	18 minutes
2	Undiluted resting cell suspension	18 minutes
3	Resting cell suspension diluted 1 in 2	96 minutes
4	Resting cell suspension diluted 1 in 2	96 minutes
5	Resting cell suspension diluted 1 in 2 plus heated cell suspension	34 minutes
6	Resting cell suspension diluted 1 in 2 plus heated cell suspension	34 minutes
7	Resting cell suspension diluted 1 in 2 plus cozymase (1.0 mgm.)	28 minutes
8	Resting cell suspension diluted 1 in 2 plus cozymase (1.0 mgm.)	28 minutes

Experiment Sixty-one

This experiment is an exact duplicate of Experiment Sixty with the exception that the standardized resting cell suspension employed had been stored.

The results of this experiment are given in Table LXI.

Table LXI

Experiment Investigating Substitution of Cozymase for Heated Cell Suspension in the Lactic Dehydrogenase Enzyme System of Staphylococcus aureus (F.D.A.209)

TUBE NO.	CONTENT OF TEST TUBE	REDUCTION TIME OBTAINED
1	Undiluted resting cell suspension	35 minutes
2	Undiluted resting cell suspension	35 minutes
3	Resting cell suspension diluted 1 in 2	>120 minutes
4	Resting cell suspension diluted 1 in 2	>120 minutes
5	Resting cell suspension diluted 1 in 2 plus heated cell suspension	68 minutes
6	Resting cell suspension diluted 1 in 2 plus heated cell suspension	68 minutes
7	Resting cell suspension diluted 1 in 2 plus cozymase (1.0 mgm.)	68 minutes
8	Resting cell suspension diluted 1 in 2 plus cozymase (1.0 mgm.)	68 minutes

Cozymase solution (1.0 mgm. per mil.) was tested and failed to reduce methylene blue in the absence of resting cell suspension.

From the results presented in Tables LX and LXI, it is evident that small concentrations of cozymase (1.0 mgm. per mil.) are able to replace heated cell suspension in the lactic dehydrogenase system of Staphylococcus aureus (F.D.A.209). These results are in accord with those of Yudkin (1934, 1937) who demonstrated that the bacterial coenzyme for glucose and lactic dehydrogenases of E.coli may be replaced by very small concentrations of cozymase.

Although the results presented here suggest that the coenzyme for lactic dehydrogenase of Staphylococcus aureus (F.D.A.209) which is present in heat killed cell suspensions is similar to cozymase (Coenzyme 1), further investigation will be necessary to prove that these substances are identical.

Experiment Sixty-two

Yudkin (1933, 1934, 1937 a) demonstrated that the coenzyme of the lactic and the glucose dehydrogenases of E. coli was soluble and could be obtained in the supernatant of boiled cell suspensions. Gale and Stephenson (1939) likewise showed that the coenzyme of malic dehydrogenase of E.coli can be extracted in the supernatant of boiled bacteria.

The following experiment was designed to investigate the possibility that the coenzyme for lactic dehydrogenase of Staphylococcus aureus is soluble.

A standardized suspension of resting cells of Staphylococcus aureus (F.D.A.209) was prepared in the manner previously described.

Heat killed suspension was prepared by heating the resting cell preparation in a boiling water bath for 15 minutes. The sterility of this preparation was verified by inoculation to peptone agar plates and subsequent incubation at 37°C. for 96 hours.

The lactic dehydrogenase activity of the standardized resting cell suspension was tested in Tubes 1 and 2. These tubes contained 1.0 mil. of standardized resting cell suspension, plus buffer solution, methylene blue solution and substrate solution in the amounts previously indicated.

The effect of addition of heated cell suspension was tested in Tubes 3 and 4. These tubes contained 1.0 mil. of heated cell suspension in place of buffer solution, otherwise the ingredients were the same as in Tubes 1 and 2.

One millelitre volumes of heat killed cell suspension were centrifuged at 5000 r.p.m. The supernatant was removed and the cell sediment resuspended in an equal volume of fresh buffer solution. The effect of the addition of resuspended heat killed cells was tested in Tubes 5 and 6.

The supernatant previously obtained by centrifuging heated cell suspension was tested in Tubes 7 and 8. These tubes contained 1.0 mil. of supernatant plus buffer solution, methylene blue solution and substrate in the amounts previously indicated.

The results of this experiment are given in Table LXII.

Table LXII

Experiment to Determine the Solubility of the Coenzyme of Lactic Dehydrogenase of Staphylococcus aureus (F.D.A.209)

TUBE NO.	CONTENT OF TUBES	REDUCTION TIME OBTAINED
1	Resting cell suspension	14 minutes
2	Resting Cell suspension	14 minutes
3	Resting cell suspension plus heated cells	8 minutes
4	Resting cell suspension plus heated cells	8 minutes
5	Resting cell suspension plus resuspended heated cells	7 minutes
6	Resting cell suspension plus resuspended heated cells	7 minutes
7	Resting cell suspension plus supernatant from heated cells	7 minutes
8	Resting cell suspension plus supernatant from heated cells	7 minutes

Experiment Sixty-three

This experiment is the duplicate of Experiment Sixty-two with the exception that the resting cell suspension employed had been stored.

The results of this experiment are given in Table LXIII.

Table LXIII

Experiment to Determine the Solubility of the Coenzyme of Lactic Dehydrogenase of Staphylococcus aureus (F.D.A.209)

TUBE NO.	CONTENT OF TUBES	REDUCTION TIME OBTAINED
1	Resting cell suspension	44 minutes
2	Resting Cell suspension	44 minutes
3	Resting cell suspension plus heated cells	22 minutes
4	Resting cell suspension plus heated cells	22 minutes
5	Resting cell suspension plus resuspended heated cells	22 minutes
6	Resting cell suspension plus resuspended heated cells	22 minutes
7	Resting cell suspension plus supernatant from heated cells	15 minutes
8	Resting cell suspension plus supernatant from heated cells	15 minutes

The results presented in Tables LXII and LXIII indicate that the coenzyme of lactic dehydrogenase of Staphylococcus aureus (F.D.A.209) is present in the supernatant of boiled cell suspensions.

PART FIVE

FINAL DISCUSSION AND SUMMARY OF RESULTS

GENERAL DISCUSSION

Throughout this report, the results of specific experiments have been briefly discussed. The resumé which follows consists of a compilation of these discussions with possible interpretations of these.

Part One of this report described the development of a method for investigating the effect of various chemical disinfectants on the viable and total cell counts of the test organism Staphylococcus aureus (F.D.A.209). The results presented indicate that mercuric chloride and silver nitrate used in suboptimal doses exert a delaying effect on cell multiplication. Viable cell counts done after disinfectant treatment show a progressive increase during an incubation period of 72 hours. Viable cell counts done for untreated test organisms did not show this increase. The increase in viable cell counts is demonstrable when no neutralizing agent is employed and disinfectant is removed from contact with bacterial cells merely by diluting out with sterile distilled water.

A similar but much less evident increase in viable cell counts was occasionally observed for organisms treated with phenol and tincture of iodine. No such increase in viable cell counts was noted when organisms were treated with ethyl alcohol or Roccal (alkyl dimethyl benzyl ammonium chloride). It has therefore been demonstrated that mercuric chloride and silver nitrate and to a lesser extent phenol and

tincture of iodine used in suboptimal doses exert a "stunning" effect as regards the multiplication of cells of the test organism. This observation bears a marked resemblance to that of Schuler (1946) who demonstrated that mercuric chloride used in suboptimal doses causes a logarithmic falling-off in respiration of Staphylococcus aureus followed by a period of recovery during which respiration returns to nearly normal. This worker also demonstrated that no respiratory recovery occurred when the bacterial cells were treated with disinfectant of the detergent type.

Parts Two and Three of this report describe the development of a method for the simultaneous study of the effect of disinfectant treatment on the viable cell count and the lactic dehydrogenase activity of the test organism. The results presented illustrate two distinct types of action for the chemical disinfectants tested. Mercuric chloride, silver nitrate, phenol and tincture of iodine show marked inhibition of lactic dehydrogenase activity in concentrations far below those necessary to appreciable effect the viable cell count. This observation is in accord with that of Braun and Vasarhelyi (1940) who demonstrated that phenol and mercuric chloride inactivate the lactic dehydrogenase of Proteus OX19 in concentrations which lie far below those necessary to kill the organisms. Roberts and Rahn (1946) reported a similar effect for mercuric chloride with E.coli respiration. Ethyl alcohol and Roccal, on the other hand,

do not effect the lactic dehydrogenase activity until the concentration of disinfectant reaches that which causes appreciable decrease in the viable cell count. These results are similar to those of Sevag and Ross (1944) who demonstrated that Zephiran (a highly refined solution of alkyl dimethyl benzyl ammonium chloride) failed to effect the respiration of yeast cells in concentrations which effected the growth of such cells.

Part Four of this report presents experimental evidence that mercuric chloride and silver nitrate exert a deleterious effect on a co-factor of lactic dehydrogenase which has been demonstrated in heat killed suspensions of Staphylococcus aureus. This effect could not be demonstrated for tincture of iodine, phenol, ethyl alcohol or Roccal.

It has been suggested that bacterial cells brought in contact with deleterious agents may utilize alternate enzyme pathways and so continue to metabolize under adverse conditions (Sevag and Shelburne, 1942 a,b,c; Sevag and Green, 1944 a,b; Sevag and Ross, 1944). It has also been suggested that deleterious agents may interfere with the function of essential metabolites by combining with certain reactive groups (Fildes, 1940; Klarmann et al, 1929). Other workers have suggested that interference with bacterial enzyme systems may result from the formation of catalytically inactive metalloprotein which is incapable of taking its proper part in metabollic processes essential for growth of the cell. (MacLeod and Snell, 1948; MacLeod and Snell, 1950 a,b).

In the present investigation, it has been demonstrated that suboptimal doses of mercuric chloride and silver nitrate and to a lesser extent tincture of iodine and phenol exert a "stunning" effect on cell multiplication of Staphylococcus aureus. These disinfectants adversely influence lactic dehydrogenase activity of the cells of the test organism in concentrations which are far below those necessary to appreciable effect the viable cell count. It has been demonstrated also that mercuric chloride and silver nitrate exert an inhibitory effect on a co-factor of lactic dehydrogenase which is present in heat killed suspensions of the test organism. These observations may all be reconciled if we assume that, following treatment with suboptimal concentrations of these disinfectants, the bacterial cell continues to metabolize utilizing an alternate or less efficient (cell multiplication being decreased or absent) enzyme pathway. Co-factor which is necessary in the original enzyme system is destroyed or damaged by treatment with mercuric chloride or silver nitrate. During the period of metabolism when an alternate enzyme pathway is being used, the cell gradually replenishes the co-factor. When a sufficient concentration of co-factor is available, cell metabolism is again directed through the original enzyme pathway. Thus, temporary falling-off in respiratory enzyme activity accompanied by decrease in cell multiplication can be followed by recovery of both these functions. With tincture of iodine and phenol which have no effect on the

co-factor of lactic dehydrogenase, we can only suggest that the action may be of such low intensity that we have been unable to detect it or the action may take place entirely within the enzyme-protein complex itself.

It has been noted that ethyl alcohol and Roccal effect lactic dehydrogenase only when the concentration of these disinfectants is great enough to effect the viable cell count. It has been observed that these disinfectants do not effect the co-factor of lactic dehydrogenase for our test organism. These results would appear to indicate that killing of the cell by these agents is not due to interference with the lactic dehydrogenase enzyme system. It is possible that our methods are not sufficiently delicate to determine critical interference by these chemicals. It is likewise possible that death of the cell may result from interference with enzyme systems other than that tested or by some mechanism other than interference with enzyme systems.

SUMMARY OF RESULTS

The development of a standardized method suited to the simultaneous study of the effect of disinfectant treatment on the viable cell count and the respiratory enzyme systems of a test organism has been described.

Results of experiments employing the method which has been developed with a test organism (Staphylococcus aureus F.D.A.209) may be summarized as follows:

1. A delay in multiplication of the cells of the test organism has been demonstrated following treatment of the cells with suboptimal concentrations of mercuric chloride or silver nitrate. A similar but much less evident delay has been noted occasionally following treatment with tincture of iodine or phenol whereas the effect has not been observed when cells were treated with ethyl alcohol or Roccal.
2. The lactic dehydrogenase activity of "resting" cells of the test organism is inhibited by mercuric chloride, silver nitrate, tincture of iodine and phenol in concentrations far below those necessary to effect appreciably the viable cell count.
3. Ethyl alcohol and Roccal do not effect the lactic dehydrogenase activity of "resting" cells until the concentration of the disinfectant has reached the level which appreciably effects the viable cell count.
4. A co-factor for the lactic dehydrogenase enzyme system of Staphylococcus aureus (F.D.A.209) has been demonstrated.

The co-factor is present in heat killed suspensions of the test organism and may be demonstrated in the supernatant of such suspensions.

5. Mercuric chloride and silver nitrate effect the activity of the co-factor of lactic dehydrogenase whereas tincture of iodine, phenol, ethyl alcohol and Roccal show no apparent effect.

6. Preliminary experiments suggest a relationship between the co-factor of lactic dehydrogenase of Staphylococcus aureus (F.D.A.209) and cozymase (Coenzyme 1) of yeast.

CLAIM OF ORIGINALITY

CLAIM OF ORIGINALITY

The results presented in this report constitute an original contribution to the knowledge of the process of disinfection in that the following facts have been established:

A. RESULTS OF EXPERIMENTAL INVESTIGATIONS:

1. The lactic dehydrogenase activity of "resting" cells of Staphylococcus aureus (F.D.A.209) is inhibited by mercuric chloride, silver nitrate, tincture of iodine and phenol in concentrations far below those necessary to effect appreciably the viable cell count.
2. Ethyl alcohol and Roccal do not effect the lactic dehydrogenase activity of "resting" cells of the test organism until the concentration of the disinfectant has reached the level which appreciably effects the viable cell count.
3. A co-factor for the lactic dehydrogenase enzyme system of Staphylococcus aureus (F.D.A.209) has been demonstrated. The co-factor is present in heat killed suspensions of the organism and may be demonstrated in the supernatant of such suspensions.
4. Mercuric chloride and silver nitrate effect the activity of the co-factor of lactic dehydrogenase whereas tincture of iodine, phenol, ethyl alcohol and Roccal show no apparent effect.
5. Preliminary experiments suggest a relationship between the co-factor of lactic dehydrogenase of Staphylococcus

aureus (F.D.A.209) and cozymase (Coenzyme 1) of yeast.

. TECHNIQUE AND METHODS:

.. A method has been developed which is suited to the simultaneous study of the effect of disinfectant treatment on the viable cell count and the respiratory enzyme systems of a test organism.

2. Techniques have been developed for the study of the effect of disinfectant treatment on a co-factor of the lactic dehydrogenase enzyme system of the test organism.

C. APPARATUS:

1. A new apparatus suitable for producing uniform controlled dispersion of bacterial cells in fluid has been developed. The apparatus is described in Appendix B of this report. A similar but larger apparatus suitable for a variety of laboratory uses is also described.

2. A modification of the apparatus designed by Bach (1937) is described in Appendix D of this report. This apparatus makes it possible to study oxidation-reduction reactions under a known positive pressure of nitrogen which can be rigidly controlled throughout the entire test period.

3. A "new" oxidation-reduction reaction tube has been devised. The tube has all the advantages of the original Thunberg tube and the Hopkins-Dixon modification. In addition, it has the advantage of simplicity and can be assembled from materials readily available in any well equipped bacteriological laboratory. The "new" reaction tube makes possible the

treatment of test organisms with disinfectant, subsequent removal of disinfectant, testing for effect on respiratory enzyme systems and testing for effect on viable cell count using the same cells of the test organism contained in the same test tube throughout the entire procedure.

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APPENDICES

APPENDICES

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APPENDIX A

Formula and Method for Preparation of Peptone Broth

Finely minced fresh beef heart, freed of fat, is added to distilled water. One pound of minced heart is required for each litre of distilled water. The mixture is heated at 75° to 80°C. for one and a half to two hours. The fluid extract is siphoned off and filtered through several layers of gauze.

The following ingredients are added to the filtered beef heart extract; proteose peptone (Difco) 1%, sodium chloride 0.25%, potassium chloride 0.02% and calcium chloride 0.01%.

The reaction of the mixture is adjusted to pH 8.4 using 10 N sodium hydroxide. Phenol red is the indicator solution employed in this procedure. The adjusted solution is heated at 120°C. for 20 minutes. Precipitated phosphates are then removed by filtration through filter paper pulp.

Following filtration, the reaction of the medium is adjusted to pH 7.2 using N hydrochloric acid and phenol red as the indicator. The mixture is then heated to 120°C. for 20 minutes. Precipitated phosphates are again removed by filtration through filter paper pulp using slight suction.

The clear filtrate is adjusted to pH 7.2, bottled and sterilized by autoclave at 120°C. for 20 minutes.

APPENDIX B

Electric Vibrating Machine

During the course of experiments with various devices designed to produce controlled dispersion of bacterial cells in fluid, an electric vibrating machine was tested and found very satisfactory. The machine which will now be described was used in all experiments where resuspension of bacterial sediment following high speed centrifugation was desired. This machine consists of a Hamilton Beach Vibrator, Type C, 115 volts A.C.* which has been slightly modified. The machine was formerly used for massage and for that purpose was equipped with a rubber suction cup which forms the contact between the vibrator and the skin surface being massaged. In order to adapt the apparatus for the vibration of test tubes and small bottles, the rubber suction cup has been replaced by an adjustable rubber lined clamp. The whole apparatus is mounted on a heavy wooden base which in turn is mounted on a foam rubber pad. The apparatus is shown in Figure 14.

The speed of the electric motor is controlled by means of a Variable Transformer.** In all experiments described in this report, the motor speed has been adjusted so as to give approximately 1,300 vibrations per minute.

Between the drive shaft of the motor and test tube

*Hamilton-Beach Manufacturing Co., Racine, Wisconsin, U.S.A.
**Variable Transformer, "Adjust-A-Volt". Type PA-5. Standard Electrical Products Co., Dayton, Ohio, U.S.A.

clamp are two working parts which are responsible for the particular type of vibrations obtained; first, an eccentric ball and socket coupling and second, a shaft which pivots universally about a ball and socket fulcrum.

In operation, the test tube clamp moves through a circle of 5-6 mm. diameter in both the horizontal and vertical planes. The net result of this motion is that the contents of the test tube continually swirls upward in a spiralling manner against the inner wall of the tube, while the fluid from the crest of the spiral falls downward toward the centre of the tube. A turbulence results which uniformly disperses particulate matter throughout the fluid.

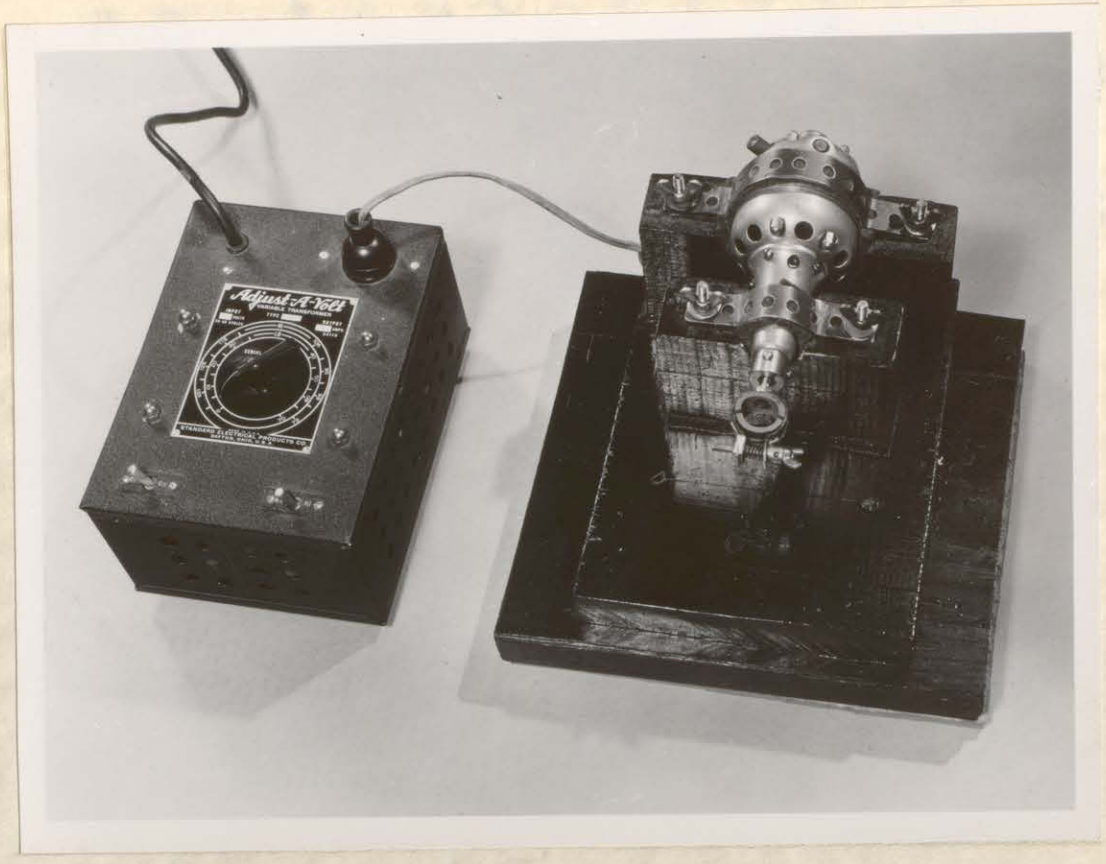

Figure 14: ELECTRIC VIBRATING MACHINE

Modified Electric Vibrating Machine

A modified version of the electric vibrating machine previously described has been manufactured*. This machine is designed to vibrate one to eight test tubes, flasks or bottles, the maximum load being 1000 grammes. The apparatus is shown in Figure 15.

The apparatus consists of a circular duraluminum head around the circumference of which are eight adjustable clamps for the attachment of the various cohtainers. The head is attached to a quarter horsepower electric motor by means of an eccentric coupling. In operation, the head is moved vertically through a circle of diameter 5 - 6 mm. The particular type of eccentric coupling used in this apparatus imparts a second motion to the head which causes the outer edge to tilt up and down through a distance of approximately 3 mm. The combination of these movements causes the contents of attached containers to spiral upward in a similar manner to that previously described.

Motor speed is controlled by means of a built in rheostat and is indicated by a pointer which passes over a curved scale.

The adjustable clamps with which this machine is equipped makes it possible to attach containers of various shapes and sizes and also to vibrate such containers at any angle desired. Most efficient operation is obtained when containers are balanced with regard to weight and attached

* Designed on the basic principles of the apparatus previously described. The Modified Electric Vibrating Machine was manufactured by Mr. Milos Srb, Optical Engineer, Montreal,

equidistant about the circumference of the head.

Preliminary experiments conducted in this laboratory indicate that the apparatus can be used with considerable success for numerous purposes (e.g. suspending bacteria in fluids, grinding bacteria in a dry or wet state, extraction of bacterial cells with various solvents, preparing emulsions of oil in water).

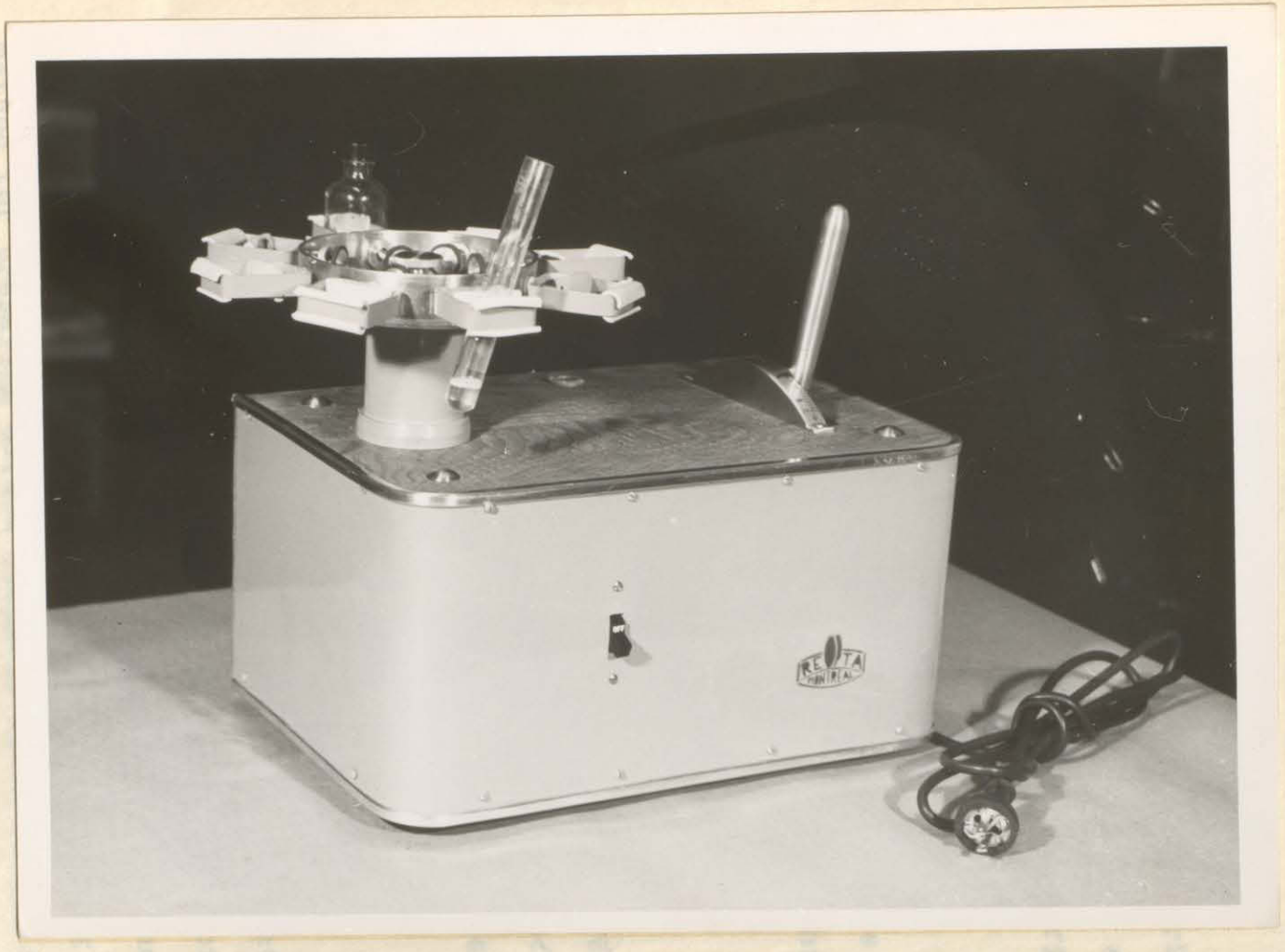

Figure 15: MODIFIED ELECTRIC VIBRATING MACHINE

APPENDIX C

Chemical Disinfectants Employed in the Experimental Investigations

Mercuric Chloride

Weight in volume solutions of mercuric chloride ($HgCl_2$) were prepared. The mercuric chloride used was manufactured by British Drug Houses Limited, Certified Chemical (Lot 209474).

Silver Nitrate

Weight in volume solutions of silver nitrate ($AgNO_3$) were employed. The silver nitrate used was that manufactured by Johnson Mattley and Company (Batch No.525).

Phenol

Weight in volume solutions of phenol (C_6H_5OH) were prepared. Phenol used in all experiments was manufactured by British Drug Houses Limited and was labelled "Laboratory Reagent (Lot No.48213)".

Ethyl Alcohol

The ethyl alcohol employed in all experiments was obtained from Commercial Alcohols Ltd. The product was labelled "Absolute Ethyl Alcohol". Volume in volume solutions of ethyl alcohol were prepared according to the method for preparing dilute alcohols which is set down in the British Pharmacopoeia (1932) pages 45 and 46.

Roccal

Roccal employed in all experiments was manufactured

by Winthrop Chemical Company Inc., Industrial Division (Lot AG 385). As stated on the label, Roccal is a brand of higher molecular alkyl dimethyl benzyl ammonium chlorides. The commercial product consists of a 10% weight in volume solution.

Tincture of Iodine

Tincture of Iodine employed in all experiments was a commercial preparation which was labelled Tincture of Iodine (5%) B.P. The produce was assayed according to the procedures outlined in the British Pharmacopoeia (1932) pages 265 and 266. The iodine and potassium iodide content of the preparation tested was as follows:

Iodine

Titration with N/10 sodium thiosulphate

Test Number	Percentage of Iodine
1	4.18
2	4.18
3	4.11
4	4.33
5	4.41
Average	4.24

Potassium Iodide

Titration with M/20 potassium iodate

Test Number	Percentage Potassium Iodide
1	4.00
2	3.98
3	3.88
4	3.91
5	3.96
Average	3.94

The Canadian Formulary (1935) states that Tinctura Iodi 5 per centum shall contain iodine between the limits 4.8 to 5.2 per cent and potassium iodide between the limits 3.1 to 3.5 per cent.

APPENDIX D

A Modified Oxidation-Reduction Apparatus

Bach (1937) designed an apparatus for the study of oxidation-reduction reactions. His apparatus consisted of a vacuum ramp which permitted the simultaneous operation of six tubes. Movement of a single valve permitted the operation of extraction of air from the tubes and refilling with nitrogen. A mercury manometer made it possible to determine at any instant the extent of vacuum existing within the tubes and subsequently the positive pressure of nitrogen. When tubes had been evacuated and refilled with nitrogen, they were removed from the ramp, sealed with vaseline and placed in a 40°C. water bath for oxidation-reduction determinatic

The apparatus which will now be described is a modification of that designed by Bach. A pyrogallol-alkali reservoir has been introduced between the nitrogen source and the vacuum ramp. This permits "washing" of nitrogen to remove traces of oxygen before the nitrogen enters the reaction tubes. In operation, reaction tubes are not removed from the vacuum ramp (as in Bach's procedure) but once attached are sealed to the rubber nipples of the ramp with collodion. The tubes when attached to the ramp are immersed in a thermoregulated 37°C. water bath and remain so throughout the test period. Figure 16 shows the apparatus in operation with reaction tubes attached and immersed in the water bath. In Figure 17, the water bath has been temporarily removed to illustrate the method of attachment

for reaction tubes. A stiff white rubber apron extending downward from the lower edge of the vacuum ramp makes an excellent background for observing the reduction of methylene blue when the reaction tubes are hanging in the water bath. A sheet of white rubber placed on the tray in the water bath prevents reflection from the interior of the bath.

Figure 18 is a diagrammatic representation of the vacuum ramp, the pyrogallol-alkali reservoir, the mercury manometer and the valves which are concerned in the operation of the apparatus.

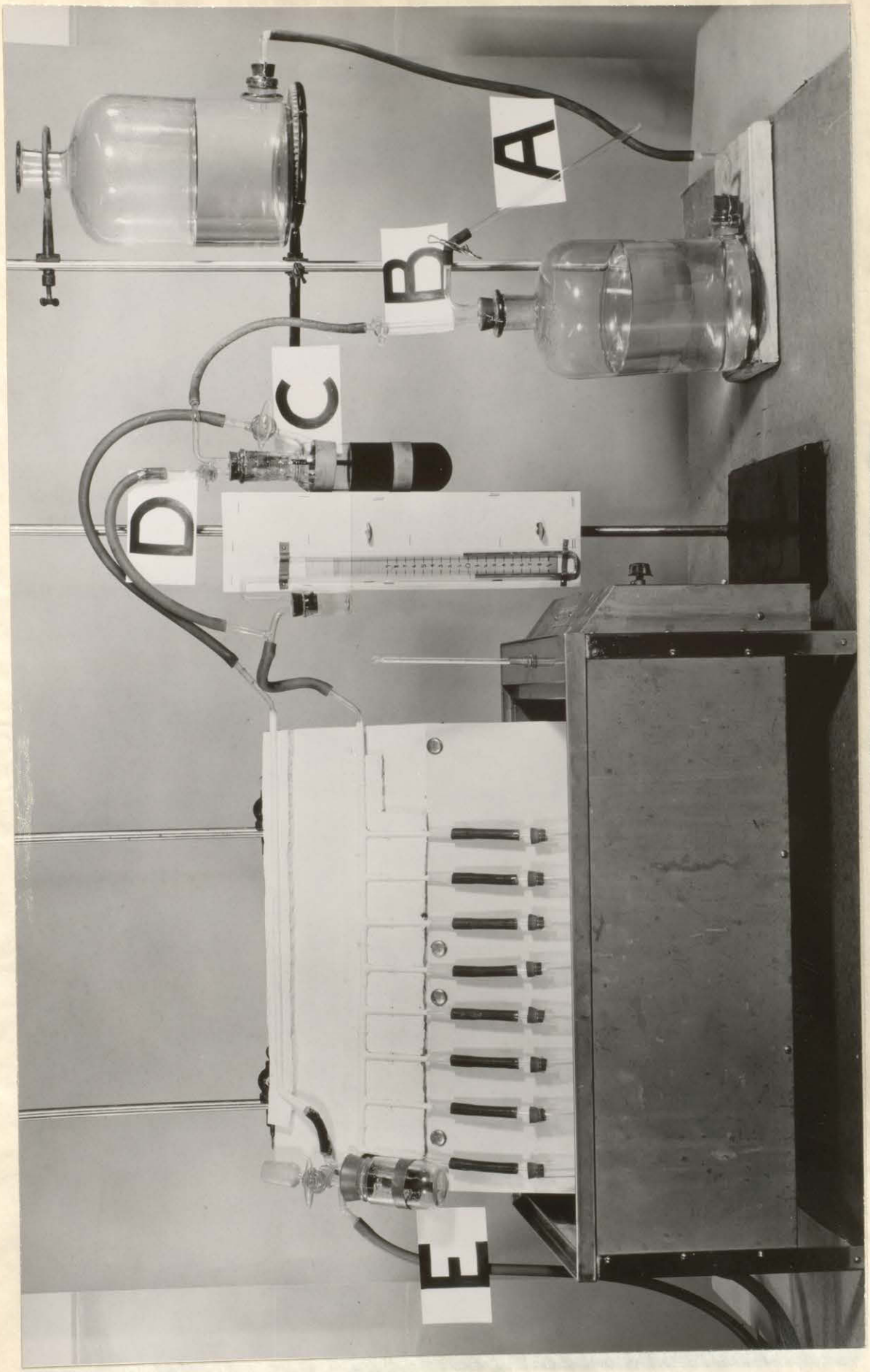

Figure 16: OXIDATION-REDUCTION APPARATUS

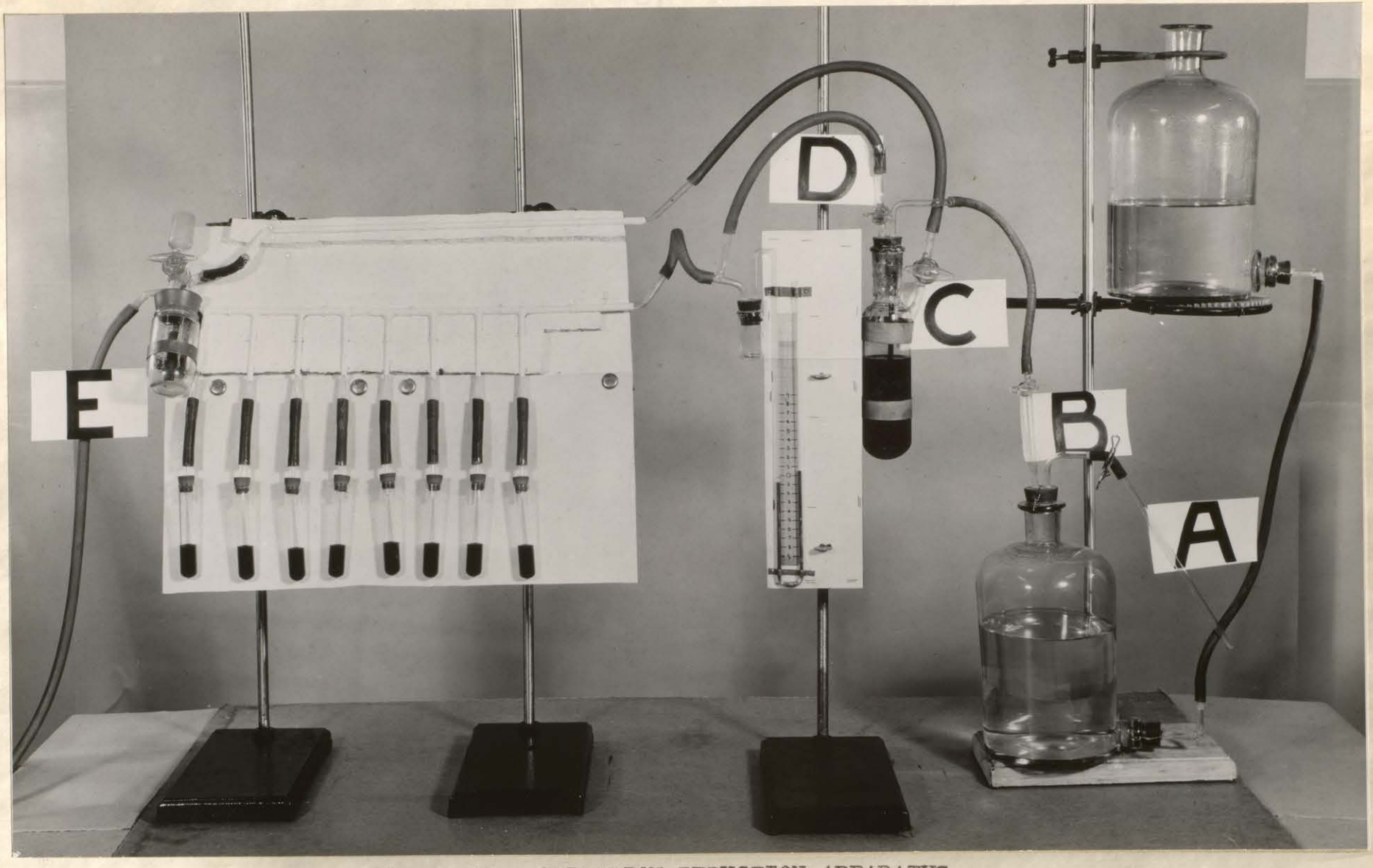

Figure 17: OXIDATION-REDUCTION APPARATUS

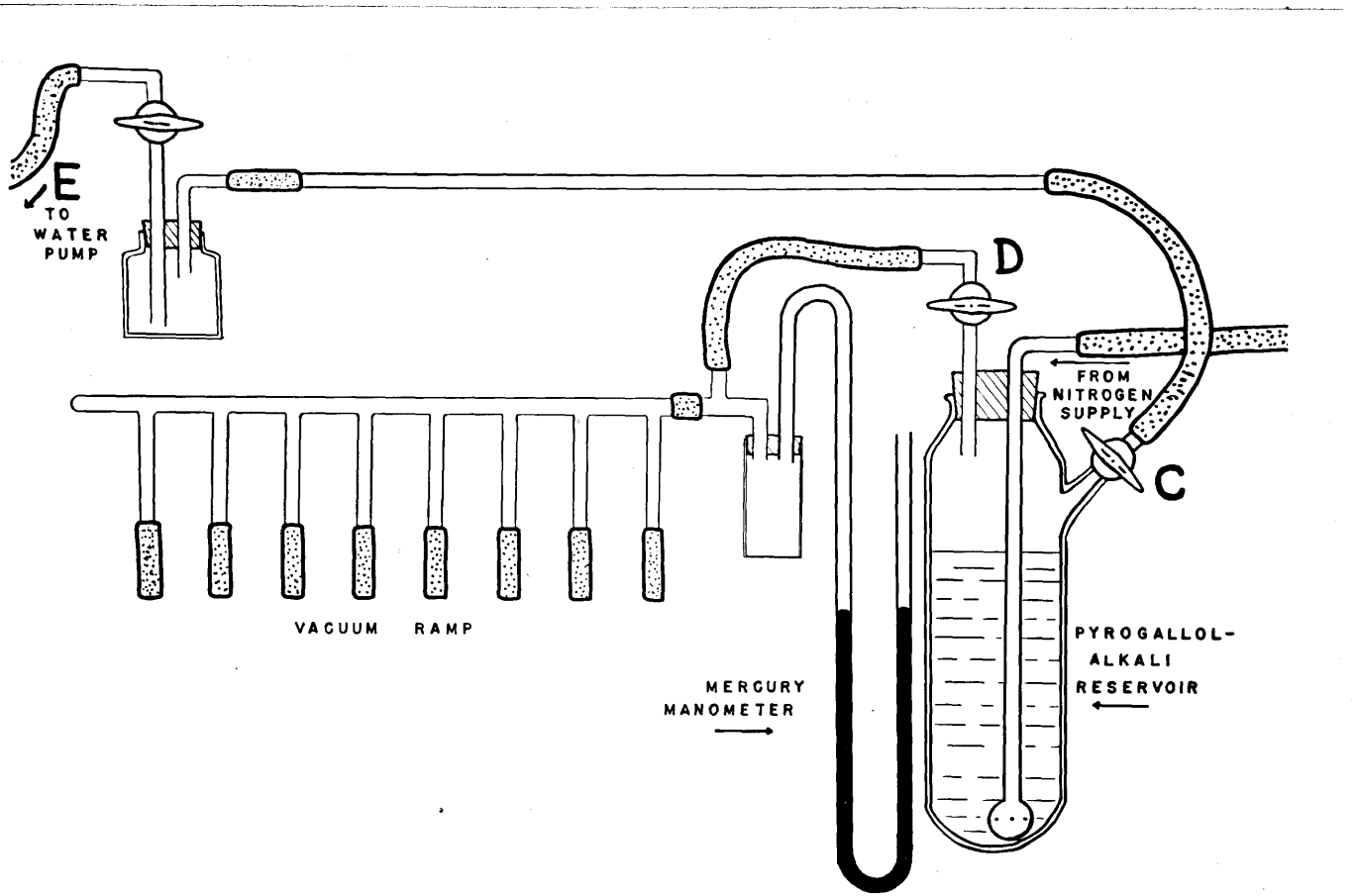

Figure 18: DIAGRAMMATIC REPRESENTATION OF OXIDATION-REDUCTION APPARATUS

OPERATION OF THE OXIDATION-REDUCTION APPARATUS

The modified apparatus which has been employed in all oxidation-reduction experiments described in this report is operated as follows:

Evacuation of Reaction Tubes:

Reaction tubes are attached to the rubber nipples of the vacuum ramp and all connections are sealed with collodion.

Valve B is closed, Valve C and D are opened and suction applied by water pump by way of the tube E. The extent of vacuum obtained in the reaction tubes and in the pyrogallol-alkali reservoir is registered by the mercury manometer. In all experiments, 18 cm. of mercury was the negative pressure employed. Tubes are evacuated for three minutes during which time they are aggitated by gentle tapping against the white rubber apron. Following evacuation, Valve C is closed.

Refilling Reaction Tubes with Nitrogen:

Nitrogen is originally supplied from a tank of the compressed gas. Bach (1937) has pointed out the advantage of having an intermediary nitrogen reservoir which forms a gasometer and permits the refilling of reaction tubes under an exact known positive pressure of nitrogen. The reservoir is represented in Figures 16 and 17 by two bottles, each of approximately three litre capacity. The bottles are joined at their lower extremities by rubber and glass tubing. The lower bottle is closed at its upper end with a two-hole

rubber stopper. Prior to use, the valve B is closed.

The lower bottle is completely filled with water by way of the tubing leading from the upper bottle. Nitrogen is permitted to enter the lower bottle through the tubing labelled A. With valve B closed, water is forced from the lower to the upper bottle. When valve B is opened, nitrogen flows from the lower bottle under the pressure exerted by the head of water present in the upper bottle. The flow of nitrogen from the bottle is controlled by adjustment of valve B. Nitrogen is bubbled slowly through the pyrogallol-alkali mixture. When valve C is closed and valve D is opened, nitrogen flows from the pyrogallol reservoir into the vacuum ramp and attached reaction tubes. The positive pressure of nitrogen present in the reservoir and reaction tubes is registered by the mercury manometer. In all experiments reported, the positive pressure of nitrogen employed has been 2 cm. of mercury. Valve B is closed following the operation of filling the reaction tubes with nitrogen.

Evacuation and refilling of reaction tubes is repeated three times as described. Following the third refilling, the valve B is adjusted so that a positive pressure of nitrogen equivalent to 2 cm. of mercury is maintained in the reaction tubes throughout the entire oxidation-reduction test period.

REFERENCES FOR APPENDICES

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