THE EFFECT OF DILUENT ON THE BACTERIAL REACTION TO DISINFECTANTS

by

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A thesis submitted in partial fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in the

Department of Dairy Science

Faculty of Agriculture

University of Natal

Pietermaritzburg

1973

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CHAPTER I

TERMINOLOGY

Before discussing the subjects under review, it is necessary to clarify some of the terms used in this work. Confusion has arisen with some of the terms due to incorrect, loose usage and partly because of their somewhat historical derivation. Some meanings have been modified by time.

A. Sterilization: This is an absolute term denoting the complete destruction or removal of all forms of life within the environment under discussion. It is achieved by a limited number of agents including high temperatures, especially when induced by flame, saturated steam under pressure, and certain filtration procedures. Certain chemical and radiation treatments may also be considered as true sterilising agents.

"Sterilization", together with its derivatives, ("sterilant" and "sterilizer"), is a word often loosely and erroneously applied. These terms do not denote a sense of relativity. The use of these terms in their erroneous context, is especially true in the medical field and in the food industry where disinfection or sanitization is usually implied. Any application of these terms in any but the absolute sense can only lead to abuse and misunderstanding (Sykes, 1965; Beloian & Stuart, 1968).

B. Disinfection: A process of eliminating or destroying "infection". According to Sykes (1965) the term "was introduced before the establishment of the germ theory of infection, and because disease was associated with foul odours", the term "tended to infer primarily the destruction or masking of these odours". Some authorities prefer to confine the use of the word to the treatment of inanimate objects but, strictly, the word should not be used with reference to non-pathogens. The term "disinfectant" is most commonly used to designate chemicals that kill the vegetative forms of bacteria, but not necessarily the spores which are more resistant. In a specific context it may infer action against bacterial spores and viruses

(Lawrence, 1968b). "Germicide" is usually used as a synonym for "disinfectant", while "bactericide" describes an agent which is (usually) active against vegetative cells of both pathogenic and non-pathogenic bacteria, but not necessarily other micro-organisms. "Antiseptic" conveys a meaning similar to that of "disinfectant" with the tendency to use the term specifically in relation to preparations applicable to living tissue, particularly in surgery and hygiene.

C. Sanitizer: According to Lawrence (1968b), a sanitizer is an agent that reduces microbial contamination to safe levels as determined by public health requirements. Schaffer and Stuart (1968) comment that the sanitizing process usually involves some measure of cleaning as it has been held that sanitization can only be achieved on surfaces that are physically clean. Sykes (1965) refers to this 'awkward' word which means the process of "rendering sanitary" or "promoting health". Although like disinfection it infers removal of infection, it also carries the further inference of cleansing, and involves elimination of non-pathogenic micro-organisms as well.

This term is particularly applicable in the food and dairy industries where complete elimination of vegetative organisms may not be essential, but reduction to safe low levels is of great importance.

D. Diluent: As used in this work, a diluent denotes a liquid in which bacterial cells are suspended for various purposes. Before this can be done, they must be harvested from the medium on or in which they have been grown. This may be done by either washing the cells off a solid medium or centrifuging a broth. If the cell concentrates are used in this form, some of the culture medium will be transferred with the cells. A preferred method is to centrifuge and wash the cells in the diluent before final suspension in fresh diluent.

The objects of suspending bacterial cells in a diluent are varied, but invariably it is inferred that the cells shall remain viable, and in an unchanged condition, with neither excessive growth nor death during the period of suspension so that a true assessment may be made of the condition of the bacterial population (Jayne-Williams, 1963).

E. Bacterial Nomenclature: In the Review of Literature no change has been made in the names of bacterial species. They are quoted as identified by the original authors.

F. Bacterial Media: In the context of this work certain bacterial media are discussed and described. A chemically defined medium is considered to be one which contains only substances of known purity added in defined quantities. The substances added are normally of inorganic origin. This type of medium is also known as a synthetic medium. An enriched medium is one containing a 'natural product' such as tomato juice or yeast extract.

G. General: It is now customary in the Republic of South Africa to adopt the S.I. units. Further, in place of the decimal point, ".", the comma, "," is the required decimal symbol and the comma previously used to simplify the reading of thousands and multiples of thousands is no longer in favour and is replaced by a space, thus 1 000,00 replaces 1,000·00.

CHAPTER II

INTRODUCTION

A major problem in disinfectant tests is ensuring that the resistance of the micro-organism used in a particular test remains constant. Further, that the resistance of the suspension shall remain constant for a period long enough to carry out the tests on the disinfectants or sanitizers presented for assessment.

Factors affecting the resistance of a micro-organism are manifold but the following are perhaps of more significance than others:

a. The maintenance of the stock culture. Micro-organisms kept in continuous subcultured conditions are liable to develop variations in resistance to disinfectants probably due to mutation. For this reason test cultures are prepared from stock cultures; the test cultures being maintained by sub-culturing at least three or four and not more than fourteen times from the same stock culture.

b. Incubation temperature. The metabolic characteristics and growth rate of the micro-organisms may be affected by variations in the incubation temperature leading to a loss in viability of the cells on suspension in a diluent.

c. The age of the culture. Young growing cells are often more susceptible than older stationary phase cells to stress.

d. The type of diluent used to suspend the bacterial cells. Several diluents are recommended by various authorities for the suspension of micro-organisms during disinfectant testing.

Examples of diluents required by various authorities for disinfectant test procedures include: water, milk or water and milk, quarter strength Ringer's solution, distilled water, nutrient broth and phosphate buffer.

Except in the case of the South African Bureau of Standards test procedure, no publication studied has stipulated the period during which the suspension remains viable and thus apparently not under stress.

Trials prior to this study, intended to investigate the viability of bacteria in various diluents, indicated that viable numbers decreased rapidly when suspensions were held for periods of three hours or more, particularly when population densities were relatively small. In fact small populations of Pseudomonas aeruginosa decreased by over ninety per cent viable organisms when suspended in distilled water for three hours.

Stress induced prior to the test period may affect the resistance of the micro-organism to disinfectants; the last disinfectant tested might appear to be superior to the first merely due to a decrease in the resistance of the micro-organisms as a result of the stress imposed by the diluent.

Should the effect of diluent not be of significance, then a simple diluent should be suitable for all test methods, and yet the test procedures studied stipulate that a number of different suspension media be used.

These facts suggested an avenue of investigation: the influence of diluent on the survival of bacterial cells and their retention of resistance to detergent sanitizers commonly used in the dairy industry.

CHAPTER III

REVIEW OF LITERATURE

A. Introduction

The bacterial cell is, subject to favourable conditions, capable of orderly growth and multiplication by virtue of its enzymic constitution. Gale (1943) contends that a micro-organism is a cell with many potential activities governed by the endogenous enzymic constitution. Herbert (1961) states that the chemical composition of a bacterial cell is more directly affected by its environment than any other of its characteristics. Changes in the chemical environment may have either qualitative or quantitative effects. The most striking examples of qualitative changes in cell composition are found in connection with adaptive enzymes - produced in some environments but completely absent in other environments. Quantitative changes may be represented by a sixteen-fold variation in the ribonucleic acid (RNA) content of a single bacterial strain under different conditions of growth (Herbert, 1961). Thus it is meaningless to discuss the chemical composition of a bacterial cell without also defining the environmental conditions which produced it.

Reproduction in the bacterial cell, being normally a process of asexual fission, will continue as long as a suitable environment exists; in fact, Koch (1959) considers that, under normal conditions, cell death rarely occurs. When changes which are somewhat unfavourable occur in the growth environment, cell division becomes retarded or ceases, resulting in survival without cell division or growth of individual cells. These changes may involve moisture content, osmotic pressures, pH, nutrient supply and many other factors (Hawker & Linton, 1971). Further departure from optimal conditions may lead to death.

Micro-organisms differ widely in their ability to survive unfavourable conditions and the stage of development of the individual cell may vary its sensitivity to unfavourable environments. Generally a state of reduced metabolic activity, artificially or naturally induced, will increase the ability to survive unfavourable conditions.

Thus stationary phase bacteria will often survive environmental changes better than those in the exponential growth phase (Meynell, 1958; Christian & Ingram, 1959; Koch, 1959; Postgate & Hunter, 1962; Razin, 1963).

Deprivation of an essential factor in the environment or the inclusion in it of an injurious physical or chemical agent may be equally injurious to bacteria. Thus according to Elek (1968), antibacterial action becomes a relative matter. Many substances are toxic to bacteria in sufficiently high concentrations, while the extremely powerful disinfectants, if present in very low concentrations will so little change the environment that no toxic effect can be demonstrated.

Bacteria will be affected by any factor which interferes with their extremely complex enzyme systems. Enzyme inactivation however, whether chemically or thermally induced, is not the only mechanism of disruption: physical disruption of the integrity of the bacterial cell by surface-active agents, ultrasonic vibrations or mechanical means may be mentioned.

Possible changes in the bacterial environment attributable to variations in such factors as growth media, harvesting and resuspending methods and media and even the stage of growth of the cells must be borne in mind when considering the apparent reaction of a micro-organism to a disinfectant or sanitizer. An effect apparently due to one of the latter may actually result from a change in the environment of the organism before it is exposed to the agent. The findings of various workers as they relate to this problem are discussed here.

B. Pre-disinfection Conditions

1. Growth phase of cells

Traditionally biological activity of a bacterial population has been defined by referring to the growth phase of the cells at a specific time. Such a statement is particularly true of a population existing in a "closed system" (Herbert, 1961). A closed system exists when bacteria are inoculated in the traditional manner into a vessel containing a limited volume of culture medium.

Under these circumstances the following events occur in a fairly regular sequence (Herbert, 1961).

(a) A period of lag (lag phase). During this period, individual cells grow in size, which is indicated by an increase in the total dry mass of cells present. There is, however, no cell division and so no increase in the number of cells. The length of the lag phase depends on the condition of the cells inoculated - "young" resting cells put into fresh medium begin to grow in mass almost immediately. In such cultures, this period is characterised by a rapid increase in cell size and an even more rapid increase in RNA content until values typical of logarithmic phase cells are reached. This phase is characterised by two phenomena:

(i) the true lag phase - a period of adaptation to the environment during which little or no growth occurs and

(ii) a period of accelerating growth in size of the cell.

(b) A period of exponential growth (the "logarithmic" phase). Cell division occurs during this phase, starting slowly and then rapidly accelerating during a period that varies from only a few minutes to an hour or more. Once a maximum division rate has been reached, each successive division requires the same time as those preceding. The population doubles in each consecutive division interval, so that the number of bacteria increases in an exponential or logarithmic manner.

During this period extracellular nutrients are rapidly utilised. Dry mass and cell numbers increase, while cell size and RNA content remain consistently large and high until the end of this phase.

(c) A decelerating growth phase. The onset of this phase may be due to one or more of a variety of unfavourable factors. Growth rate decreases more rapidly than division rate; cell size decreases until it reaches that of resting cells. Due to slower synthesis the RNA content also declines until the resting phase values are reached.

The initiation of the decelerating phase is governed by a number of factors. Some of these factors are changes in hydrogen ion concentration (pH) as would occur in the dissimilation of fermentable

carbohydrates to acids, lowering the pH, the general depletion of nutrients and an accumulation of wastes. An elevated incubation temperature results in accelerated growth with a corresponding rapid depletion of nutrients and accumulation of wastes; it gives rise to an earlier inception of the stationary phase and often the maximum population is lower (Carpenter, 1968).

(d) A stationary or resting phase. Cells remain viable but no longer grow and division, if it occurs, takes place slowly. The cells are small in size with a low RNA value (Herbert, 1961). Complete cessation of division during the stationary phase results in a phase of increasing death rate.

(e) An accelerating death phase. This situation is the inverse of the exponential growth phase. It is seldom studied at optimum growth temperatures and conditions. This phase is of interest in studies concerned with heat sterilization and disinfection (Thimann, 1963). Survivor curves, constructed by plotting the logarithm of the number of survivors against the time of heating or exposure to a disinfectant, generally assume straight lines. Regression lines, fitted to this data, are often used for the statistical analysis of results.

Investigating cell turnover during the stationary phase of bacterial population growth, either on enriched or chemically defined media, Ryan (1959) found in enriched media, a constant level of viable cells for a relatively long period during which the mass of the culture increased gradually; the cell death rate being approximately equal to that of cell production. Conversely, in a defined medium, with the limitation of a growth factor, the stationary phase is characterised by a progressive decrease in viable cell numbers without a measurable decrease in total cell mass or numbers. Postgate and Hunter (1962) considered that the nutritional status of a culture was of greater importance than the growth phase in investigations into the death rate of starved bacteria.

Bacteria grown in closed systems are most commonly investigated during either the logarithmic phase or in the stationary phase. The evidence presented so far relative to the size and RNA content of the

cells leads one to expect that these two types of cells will react in very different ways to stress.

The traditional growth curve and the definition of a bacterial population by the particular growth phase in which it is found, is considered by Farwell and Brown (1971) to provide a vastly over-simplified approach. Their contention is particularly valid in mixed populations as would be found under natural conditions; for example, in soil, water and foodstuffs where competition exists for available nutrients, the production of toxins and antibiotics and changes in chemical reaction would probably favour one species to the suppression or exclusion of another. Brown (1964) found that, in mixed bacterial populations growing under natural conditions, as the salt concentration increased, the numbers of Gram-positive organisms decreased in relation to the Gram-negative types. Organisms grown axenically in a sterilized medium are unlikely to behave in the same way.

It has been repeatedly shown that, not only do stationary phase cells differ in size and composition from the logarithmic phase cells, but they are also more resistant to environmental change. They resist physical disruption and osmotic shock and maintain better viability during storage (Herbert, 1961). They also show increased resistance to the presence of sodium hydroxide (Watkins & Winslow, 1932); to radiation (Heinmets, Lehman, Taylor & Kathan, 1954b); to cold shock (Meynell, 1958; Koch, 1959); to osmotic lysis (Christian & Ingram, 1959; Razin, 1963); to starvation (Harrison & Lawrence, 1963; Postgate & Hunter, 1962) and enhanced temperatures (Harrison & Lawrence, 1963).

2. Growth medium

It is suggested by Herbert (1961) that the resistance of stationary phase cells to physical disruption and osmotic shock is only evidenced when the cells are grown in complex types of culture media. This is supported by the findings of Strange, Dark and Ness (1961) who investigated Aerobacter aerogenes grown either in tryptic meat digest broth containing tryptone and glucose, or in a carbon limited medium (a simple NH_4-salt medium with mannitol as the carbon limiting factor). Not only were differences in the protein content

of the cells found, but RNA and carbohydrate contents also differed. The cells grown on the complex medium also retained viability longer when aerated at 37°C and when unaerated at 20°C, compared with those cells from the limited medium. Strange and Ness (1963) working with the same organism but in an aerated saline phosphate solution, also found a higher viability with cells grown in complex medium compared with a simple salts medium.

On the other hand, Pseudomonas aeruginosa grown in a complex medium was more sensitive to storage in various diluents at various temperatures than a similar culture from a simple salt medium in which carbon or magnesium or both carbon and magnesium were limited (Farwell & Brown, 1971). This statement may confirm that of Smith and Wyss (1969) who, discussing Azotobacter and Escherichia coli, found that starved cells maintained viability longer than unstarved cells; however, they emphasised that the washing and suspension procedures were also of importance, and are two of the very few workers who do so.

Postgate and Hunter (1962) found a much greater variation in death rates with organisms from continuous growth cultures, than in stationary phase cells from closed culture systems. Strange and Ness (1963) also found that log phase bacteria from complex media were apparently more resistant to chilling than organisms grown in a defined medium.

Lemcke (1955) demonstrated that minimal thermal resistance occurred at three hours growth with cultures of Bacterium coli, while maximum resistance was to be found in 24 hour cultures after a slow but steady increase in resistance with age. Lemcke and White (1959) concluded that death rates due to heating were not uniform; this was particularly the case in young Escherichia coli cultures where a period of rapid death was frequently followed by the slower death of a relatively small number of survivors. Older cultures showed more uniform responses to heating. These results would confirm findings by Sherman and Cameron (1934) and Elliker and Frazier (1938) that young bacterial cells are generally less resistant to shock than older stationary phase cells.

Although Herbert (1961) decries the use of complex, chemically undefined media in studying the effects of environment on bacteria, it could be reasoned that these media are preferable in disinfectant testing and allied investigations. The overall greater resistance and apparently reduced variation in resistance found by a number of workers in cells derived from complex media would tend to offset the reproducibility of synthetic, chemically defined media. The use of synthetic chemically defined media would limit the variety of micro-organisms that could be used in disinfectant and sanitizer tests. Not all the organisms are chemosynthetic heterotrophs, such as Escherichia coli; others are comparable to Streptococcus faecalis which does not have the ability to grow on a glucose-salt medium, but requires vitamins, amino acids, purines, pyrimidines and peptides as well. The possibility also exists that though a synthetic medium does allow growth of the micro-organism, it might not be complete enough to elicit all the capabilities of the organism. It might also be pertinent that the recognised disinfectant, antiseptic and sanitizer test methods generally utilise complex organic ingredients in the test culture growth media. An exception to this is in the Association of Official Agricultural Chemists: Phenol Coefficient Method (Official, Final Action 1965) where a synthetic broth is described as well as a nutrient broth containing beef extract, NaCl and peptone. Either of these broths may be used for the daily transfer of test cultures. If the two broths are assumed to give comparable results then the practical inconvenience of making up the synthetic broth would nullify any theoretical advantages of that broth (A.O.A.C., 1965).

In the presence of disinfectants, the constituents of the growth medium may affect particular reactions of bacteria. Klimek and Bailey (1956) studying the influence of calcium and magnesium ions on the rate of killing of Escherichia coli by benzalkonium chloride, observed that the metallic ions prolong the exposure time required to achieve a given level of lethal results. The effect was on the cell; not on the quaternary ammonium compound. The effect was, however, dependent on the type of peptone used in the bacterial culture medium.

Greater stability of stored suspensions was obtained by Cook and

Wills (1958) from static, non-aerated media than from aerated media. These workers further found that growth in a liquid medium appeared to yield a cell suspension which retained its viability longer than a suspension prepared from cells grown on a solid medium.

3. Growth medium hydrogen ion concentration

Growth can only take place within a certain pH range, and rapid or luxuriant growth occurs only in a narrow optimum pH zone. Within this narrow optimum pH zone the hydrogen ion concentration of the growth medium does therefore not appear to exert a great effect on the microbial population's reaction to stress.

The nature of microbial metabolic activities is such that the reactions of the growth substrate does not remain constant. Breakdown of protein and other nitrogenous compounds often yields ammonia and other alkaline byproducts, while carbohydrate fermentations yield organic acids. Thus the reaction of the growth substrate can change until the maximum or minimum pH limits for the organism are reached. At this stage growth slows and may eventually stop. Death of the culture or cell population may occur unless the possibility of reaction reversion exists (Carpenter, 1968).

As far as can be determined, the effect of the pH of the growth medium on the reaction of the microbial population grown in or on it to subsequent stress, does not appear to have been studied. The practice has rather been to adjust the medium to a pH known to be optimal or nearly optimal for that species, and frequently to include buffers to retain the pH at that optimum for as long as possible.

4. Growth temperatures

Growth temperatures have been found to affect the response of micro-organisms to some forms of subsequent stress. Increased heat resistance was imparted to Streptococcus faecalis by enhanced growth temperatures except in extremely young cells subcultured for less than one hour (White, 1951). Elliker and Frazier (1938) found that growth at or above the optimum temperature resulted in Escherichia coli cultures, whose heat resistance during the maximum stationary phase was distinctly greater than that of cultures incubated below optimum growth temperatures. On the other hand, Hoffman, Valdina and Frank (1966)

determined that the growth of Escherichia coli at 45°C resulted in the production of cells affected by cell wall damage compared with cells grown at its optimum temperatue (37°C). The fragile cells grown at 45°C swelled in distilled water and disrupted when egg-white lysozyme was added. (It is possible, but unlikely, that these 'fragile' cells might have had greater heat resistance). The cells grown at the optimum temperature were unaffected.

When investigating factors affecting the anti-microbial activity of phenols, Bennett (1959) found that Staphylococcus aureus is more resistant to phenols when grown at elevated temperatures; further that Escherichia coli cultures derived from warm-blooded animals were more resistant than those from cold-blooded animals. However Pseudomonas strains sensitive to cold are more sensitive to phenolic activity than psychrotrophic strains.

5. Preparation of the test inoculum

Unaltered bacterial cultures may be exposed in their growth medium to a particular stress under conditions where other factors of the overall environment are not to be significantly altered. Such a procedure might be applied in investigations concerning the effect of heat, antibiotics, radiation or cold shock on actively growing or stationary phase cells. The degree of stress imposed may be assessed by determining changes in either viability or optical density (Farwell & Brown, 1971). Such trials would also include in situ assessments of pasteurisation, sterilization or disinfection efficiencies.

On the other hand, in many circumstances, harvesting of cells is a prerequisite for various studies of stress reactions. This is particularly the case when disinfectant and sanitizer efficiency is determined under standardised laboratory conditions.

Carpenter (1968) quoting various investigators, stated that non-sporeforming bacteria possess considerable resistance to mechanical and hydrostatic pressures, for example, pressures of 85 000 to 100 000 pounds per square inch for five minutes were required to produce lethal results on Streptococcus lactis. Other reports indicate that non-sporeforming bacteria will only be killed after 14 hours at 88 000 pounds per square inch. This was confirmed by Farwell and Brown (1971), who

stated that centrifugation and filtration have little effect on cells which possess a structural wall.

The most significant changes occur with the transfer of the organisms by various methods from the original growth culture medium to the suspending media. The transfer may involve changes in temperature, pH, tonicity, ionic composition and other environmental factors. These changes could occur whether the transfer involved washing from an agar slope, centrifugation or filtration.

The influences of suspending media are manifold and have been extensively investigated by innumerable workers. Research workers require media which will give a true reflection of the total population under examination. In mixed populations, the more sensitive cells may die or the hardy cells grow and multiply; neither condition would give a true reflection of the original population. According to Jayne-Williams (1963) the function of a diluent should be to "enable a true assessment to be made of the condition of a bacterial population, and death or revival of organisms should not take place during the dilution process".

Natural environments vary in their chemical composition and concentration of ions. One of the most important of these natural habitats is the sea, characterised by its content of the common inorganic salt, sodium chloride. A distribution curve plotting either the earth's total bacterial population or the number of bacterial species against the salt concentration of their habitats would probably peak in the freshwater-seawater region. Although species of bacteria can exist and thrive in concentrations of sodium chloride from about 0 to 6,2 molal, most occur naturally close to the bottom of the sodium chloride range (Brown, 1964). On this basis one could possibly justify the selection of sodium chloride solutions, ranging from 0 to 0,68 molal, as being natural suspending media for most bacteria. The sodium chloride molarity of seawater is approximately 0,6 to 0,68 molal.

The ionic composition of bacterial cytoplasm is usually different from that of the extracellular environment and without a regulatory mechanism the bacterium would either suffer expansion of the cell membrane to rupture point (plasmoptysis) in strongly hypotonic solutions

or plasmolysis in strongly hypertonic solution (Carpenter, 1968). That such a barrier exists has been demonstrated by a number of workers (Brown, 1964; Farwell & Brown, 1971). In any unicellular organism, capable of growing over a wide range of ionic conditions, a permeability barrier must be of great importance. This barrier is involved in regulating the internal composition while in direct contact with the external environment.

A difference exists between the Gram-positive and Gram-negative bacteria in the composition of the cell envelope which contains the ionic diffusion barrier. This is evidenced by the fact that Gram-positive bacteria become less plentiful in bacterial populations as the sodium chloride concentration of the environment increases. The Gram-positive bacteria studied have been shown to be bounded by a cytoplasmic lipoprotein membrane and a rigid cell wall consisting predominantly of mucopeptide, a carbohydrate-amino acid polymer. The barrier includes the cytoplasmic membrane. In the case of the Gram-negative organisms there is no evidence for a rigid cell wall together with a delicate membrane as in the Gram-positive bacteria. Electron micrographs of Gram-positive bacteria show the thicknesses of the cytoplasmic membrane and the cell wall to be about the same, and the cell wall to have the appearance and dimensions of a lipoprotein membrane (Brown, 1964). That such an osmotically active membrane exists not only in bacteria was demonstrated by Myers, Provost and Wisseman (1967) who found it to exist in Rickettsia mooseri, an intracellular parasite.

6. Selective permeability in bacteria

The fact that bacteria show selective permeability for nutrients and waste products is due to the cell membrane and this property varies from species to species. Bernheim (1964) demonstrated that this species' difference is very real; Pseudomonas aeruginosa has a barrier to sucrose that is not possessed by Escherichia coli, and further that the barrier in Ps. aeruginosa was stable against either heat, freezing or thawing. However the treatment of Ps. aeruginosa with either benzalkonium chloride or E.D.T.A. allowed an outward diffusion of ions accompanied by water, resulting in a shrinking of the organisms (Bernheim, 1963).

A bacterial cell placed in a more concentrated environment will lose water until thermodynamic equilibrium with the environment is reached; there is no known mechanism by which a cell could prevent this process from occurring. Entry of water into a cell can be opposed by osmotic pressure; there is no counterpart for loss of water (Brown, 1964). The intracellular concentration of inorganic ions varies not only directly in response to changes in composition of the environment, but also with the salt requirements or tolerances of organisms. Christian and Waltho (1961) investigated non-halophilic Gram-positive and/-negative bacteria grown under uniform conditions in a complex medium. They found that all species accumulated K^+, and that the intracellular K^+ was positively correlated with the maximum salt concentration tolerated by the organisms for growth.

Bacteria from a habitat having a naturally low ionic concentration are not normally very susceptible to osmotic swelling or rupture, since their environment cannot be diluted to any extent. However they may be susceptible to an increased concentration of salts resulting in plasmolysis. Adaptation to reduced osmotic pressure is assisted by bi- and multi-valent cations such as Mg^{++}, Ca^{++} and spermine (Brown, 1964). Organisms with higher osmotic requirements (marine bacteria) are more susceptible to dilution, with plasmoptysis often occurring. Rupture can be prevented by salts of Mg^{++} and Na^+.

The role of Mg^{++} was further investigated by Upadhyay and Wilks (1969) who found that Mg^{++} was better able to protect Clostridium botulinum at 80°C than Ca^{++}, perhaps due to Mg^{++} being involved in the stability of some heat-sensitive enzyme system. Winslow and Dolloff (1928) showed that Mg^{++} with either Na^+ or K^+ prolong bacterial viability. Klimek and Bailey (1956) found that Mg^{++} and Ca^{++} ions increase the exposure time necessary to kill Escherichia coli in benzalkonium chloride. The effect was on the cell and not on the compound.

The addition or presence of divalent cations is extremely important in the maintenance of bacterial structure and viability. The addition of magnesium sulphate decreased or eliminated the deletrious effects of phosphate buffer used to store several bacterial species.

Loss in viability of Aerobacter aerogenes due to cold shock was prevented by the presence of magnesium in phosphate buffer (Strange, 1964). Osmotic shock causing lysis in Mycoplasma (of the pleuro-pneumonia group) was reduced by extremely low concentrations of aluminium, ferrous, magnesium, zinc and cadmium cations (Razin, 1964), while death of Aerobacter aerogenes due to substrate limitation was delayed by magnesium, calcium and iron cations. Numerous other examples of cell membrane behaviour have been recorded. Razin (1963) demonstrated that osmotic lysis occurred only with organisms from actively growing cultures and in solutions of very low tonicity. Shaunessy and Winslow (1927), investigating the diffusion products of bacterial cells as influenced by various electrolytes, found a species difference in that Bact. coli survive practically undiminished in distilled water or dilute solution through a moderate pH range, while B. cereus dies out rapidly, 90 per cent of the cells being non-viable after an hour. Cold shock resulted in bacteria becoming permeable to large molecules e.g. ribonuclease (mol. wt. 14 000). At 20°C, distilled water allowed greater permeability than at 0°C compared to saline phosphate buffer (Strange & Postgate, 1964). Strange (1966) found Escherichia coli when starved in saline buffer, suffered a considerable loss of protein during a period when viability remained at 95 to 100 per cent of the original population.

7. Effect of diluent on bacterial viability

Evidence of suspending medium or diluent effect is extremely contradictory. As has already been stated, it is important in certain studies that changes in a bacterial population, either mixed or single species, should not occur. A complex medium causing cells to grow may mask subsequent stress particularly if the stress is of a relatively minor nature. A deficient suspending menstruum may cause changes in the cell structure or function which could affect additively results of subsequent stress such as the action of a disinfectant. The stressing of a population, damaged prior to treatment, would therefore give a false impression of the treatment causing the stress.

Winslow and Brooke (1927) noted that some organisms died almost immediately when centrifuged and washed in distilled water, while those suspended, but not washed, in distilled water survived. The survival

of the susceptible organisms was probably due to the carry over of growth medium. Washing in salt and sugar solutions gave similar results to distilled water. Escherichia coli however, survived centrifugation and washing in distilled water. The protective influence of the nutrient broth was ascribed to two of its ingredients: meat extract and peptone.

Ballantyne (1930), whose experiments covered periods of long duration, found that many bacterial suspensions remained viable for 5 to 32 months in 0,85 per cent NaCl solution and from 14 to 32 months in distilled water. He reported distilled water to be more favourable for the survival of B. typhosus than 0,85 per cent NaCl solution. This is also true for other organisms including B. coli, B. tuberculosis, B. diphtheriae, Strep. hemolyticus and Strep. viridans. Further he found that, uniformly, Gram-negative bacilli survived longer in the two diluents mentioned than on solid media. Of significance was his further finding that the supernatant fluid removed during washing prolongs the survival period of bacteria present in it. The so-called water of condensation on agar-slant media is an important factor for the survival of unwashed bacteria suspended in distilled water or NaCl solution, due to the carry over of 'albumen' and 'total solids' from the agar slant. Solutions of 0,4 per cent NaCl were better suited to bacterial survival than 0,85 per cent solutions, but no better than distilled water.

Winslow and Haywood (1931) investigated the effect of various cations and cation concentrations on bacterial populations of Escherichia coli. These workers found that as the concentration of a salt in the suspending medium was increased, one may observe first, a stimulative effect which rises to a peak, then gradually decreases until further increases have a toxic effect; very high concentrations of the salt may be so toxic that they bring about a condition of sterility. For NaCl the stimulating range lay between 0,005 M and 0,25 M with a maximum at 0,08 M. These results compared reasonably well with those of Winslow and Dolloff(1928) who reported stimulation extending up to 0,3 M with a maximum at 0,1 M. Winslow and Haywood (1931) also reported on concentrations for KCl, LiCl, $BaCl_2$, $MgCl_2$,

$CaCl_2$, $MnCl_2$, $ZnCl_2$ and $CdCl_2$. Compared with NaCl, $MgCl_2$ and $CaCl_2$ have stimulative ranges beginning near 0,0001 M and extending to about 0,02 M with maxima at 0,008 M while $CdCl_2$ is stimulatory between 0,00001 M and 0,0001 M. Winslow (1934) concluded that all cations in low concentration tend to stimulate growth and development, while stronger concentrations tend to inhibit growth and ultimately to exert a toxic action.

Bernheim (1965) observed that when washed cells were suspended in a salt solution, an immediate decrease in cell size occurs and the maximum reduction is reached at 60 seconds after suspension. Subsequent incubation results in a gradual swelling of cells due to the uptake of ions and their water of hydration. After 60 minutes the cell controls its intracellular concentration. In the presence of an anionic detergent, diffusion into the cell cannot be controlled and lysis may take place.

The viability of Pseudomonas aeruginosa was reduced by 99 per cent in 1 hour by distilled water, while in 0,9 per cent NaCl the cells remained viable for half an hour, the cells becoming rapidly inactivated when suspended for longer periods in this diluent. Skim milk as a diluent caused little if any reduction in viability during a 90 minute contact period (Wagenaar & Jezeski, 1952).

Vanderzant and Krueger (1968) studying the effect of dilution procedures on Pseudomonas aeruginosa, showed that the viable population from agar-slant cultures decreased in distilled water and in Standard Methods (1960) buffer to a greater degree, than that from cultures grown in skim milk. These authors agree with Ballantyne that unwashed cultures survived better than did washed cultures; this could be due to the transfer of growth medium with the cells or because washing increased their sensitivity to the diluent.

That organic matter, and particularly peptone, can minimise viability losses during dilution is substantiated by Straka and Stokes (1957), Jayne-Williams (1963) and King and Hurst (1963). All these authors found that peptone gives more protection to bacterial suspensions than do other diluents. Their results with distilled water were less consistent. Jayne-Williams (1963) found that it

killed Staph. aureus after 90 minutes, while Straka and Stokes observed only a slight kill. In the case of other organisms, Jayne-Williams (1963) recorded that 40-60 per cent may die in 20 minutes and >90 per cent in 1 hour but King and Hurst (1963) found no change in E. coli. All three groups of authors stated that the other diluents tested by them caused the rapid death of the suspended cultures.

Spangler and Winslow (1943), reporting on Bacillus cereus, claimed that this organism died rapidly in distilled water while NaCl at 0,00001 M to 0,3 M protected the cells from the action of distilled water. The most favourable concentrations of NaCl were at 0,001 M to 0,1 M while 0,4 M to 0,5 M concentrations were often more toxic than distilled water. NaCl solutions, 0,01 M to 0,1 M have also been reported favourable to Escherichia coli.

Carlucci and Pramer (1960) investigating Escherichia coli that had passed from sewage into seawater found that distilled water resulted in a more rapid loss of viability than 25 per cent seawater; the death rate increased either when the concentration of seawater in the diluent was increased towards 100 per cent seawater or reduced towards zero. Tonicity has an important role as shown by the increased loss in viability of Aerobacter aerogenes in buffered physiological saline if the tonicity of the medium was altered in either direction (Postgate & Hunter, 1962).

Many of the contradictory findings in connection with the suitability of distilled water as a diluent may be due to its quality. Butler and Knight (1960) found that washed suspensions of Mycoplasma died rapidly in 0,85 per cent NaCl prepared from steam condensate. Geldreich and Clark (1965) detailed suitability criteria for distilled water supplies and, of nine samples, found that only three satisfied their criteria. Their distilled water suitability test was incorporated into the Standard Methods for the Examination of Dairy Products, 11th ed. The criteria included toxic substances, electrolytic corrosion resulting in associated metal ion toxicity and the presence of growth promoting substances. Incidently, acceptable distilled water was produced by widely differing equipment viz. a commercial all glass unit

using previously distilled water, a small capacity standard glass double distillation unit and a large capacity tin-lined still used in conjunction with a carbon filter and deionizing column.

The influence of anions on bacterial cell viability has not been investigated as extensively as that of cations. Winslow (1934) concluded that the phosphate radicle exerts a specific stimulatory effect on bacterial viability while carbonate solutions appeared to have a specific inhibitory effect. Christian and Ingram (1959) also showed that the least anionic toxicity was found with HPO_4^- followed by NO_3^-, Cl_2^- and SO_4^-, while Gossling (1958) found that when bicarbonate ions were excluded from Ringer's solution, the viability of Escherichia coli transferred from Ringer's solution to sodium chloride was not so depressed. Bernheim (1963) found that the rate of optical density decrease was greater when organic anions (pyruvate, acetate etc.) were involved than was the case with nitrates, chlorides and bromides.

8. Effect of diluent hydrogen ion concentration

Butterfield (1932) attached little importance to the pH of dilution media provided the pH was less than 8,2 since he found that pH 9,0 in distilled water was bactericidal. Winslow (1934) was more specific and considered hydrogen ion concentration was one of the main influences and that optimum viability was to be found at pH 7,5 when coupled with a 0,1 M sodium chloride concentration. A lowering of pH and Na^+ concentration below these optimum levels decreased viability. Increases in Na^+ and pH above the optimum caused a very much more rapid change from stimulation to inhibition which was found with 0,6 M Na^+ concentration and pH 8,3.

Ballantyne (1930) did not consider pH among the factors influencing survival of bacteria though Cohen (1922), studying the effect of pH on Bact. coli and Bact. typhosum in water, stated that a pH lower than 7 favoured survival. Straka and Stokes (1957 and 1959) indicated that survival rates were higher at pH 7,0 than at lower or higher values. Carlucci and Pramer (1960) found that pH 5,0 was most suitable for Escherichia coli recovered from seawater, when 25 per cent seawater, or an equivalent sodium chloride solution, was used as the diluent.

Vanderzant and Krueger (1968) adjusted diluent to pH 5, pH 6, pH 7 or pH 8 with either HCl or NaOH. The results suggested that the viability of Pseudomonas strains might be very slightly greater at pH 7, but this was not confirmed by a statistical analysis which showed no significant differences between viabilities at the four pH values. They further suggested that the addition of acids and bases in order to adjust pH values of a diluent might have some additional effect on the characteristics of a bacterial population held in the adjusted diluents.

Sherman and Cameron (1934) found that, within the limits of pH 5,5 and pH 7,0 abrupt changes in hydrogen ion concentration of the medium did not have any lethal effects on growth of young cells of Bacterium coli. Cook and Wills (1958) found that Escherichia coli washed and suspended in phosphate (0,1 M) at pH 7,0 maintained viability and bactericide resistance better than washed or unwashed cells suspended in water. Winslow (1934) maintained that maximum viability was to be found at pH 7,5. A decrease in pH resulted in a corresponding decrease in viability. An increase in pH caused a much more rapid decrease in viability than a drop in pH below pH 7,5. Levels of pH above 8,3 were definitely inhibitory, particularly when accompanied by increased concentrations of solutes in the diluent. Spangler and Winslow (1943) restricted their observations of the influence of the sodium ion on viability of B. cereus to pH values between 6,6 and 6,8.

Gossling (1958) investigating the loss of viability in E. coli transferred from one diluent to another found the highest mortality at pH 7,2, with diminished lethal effect on either side of pH 7,2 but especially on the alkaline side. The lethal effect diminished particularly when the bicarbonate was omitted from the Ringer's solution.

Standard Methods for the Examination of Dairy Products (1967) requires that the stock phosphate buffer solution should be adjusted to pH 7,2. In the A.O.A.C. (1965) "Available chlorine germicidal equivalent concentration - official final action" the stipulated pH for the sterile phosphate buffer solution is pH 8,0 using test organisms

S. typhosa ATCC No. 6539 and Staph. aureus ATCC No. 6538. In the case of "Germicidal and Detergent Sanitizers - official, final action" the phosphate buffer solution should be adjusted to pH 7,2 (A.O.A.C., 1965).

9. The effect of holding temperature

Yet another factor considered by Ballantyne (1931) was the temperature at which bacterial cells were held in diluents. Room temperatures, rather than 0-8°C, favoured the survival of most organisms in distilled water and in 0,85 per cent NaCl, but this concentration proved toxic at 37°C. Cook and Steel (1955) using Bacterium coli suspended in distilled water, found viability persisted for up to 8 weeks at ambient temperature with only negligible variation.

According to Harrison (1955), repeated freezing and thawing is much more lethal than a single treatment. An incidental finding was that the second freezing is generally more lethal than any other. Freezing appears to bring about some metabolic change. Straka and Stokes (1959) and Nakamura and Dawson (1962) found that the capability of growth of previously frozen organisms, on simple media, is governed by the length of the time the organisms were frozen. Organisms frozen for long periods and then thawed, required enriched media for recovery.

Straka and Stokes (1959) suggested that the presence of specific peptides in trypticase and other enzymatic digests of casein was essential for the recovery of Escherichia coli and some Pseudomonas species subjected to injury by cold. Tryptic soy agar was far superior to the simpler medium composed of inorganic salts, citrate and glucose.

Multiplication ceases below the minimum growth temperature, and hardy organisms frozen in dilute peptone or milk may be kept at -20°C for a year or two without complete loss of viability. Less hardy organisms may be kept for weeks or months under the same conditions (Carpenter, 1968).

Proom (1951) reported that apart from spore-forming bacteria, the Gram-positive were more resistant to cold shock than the Gram-negative organisms. This was partially confirmed by Farley, Roeser and

Konetzka (1961) in that cocci, e.g. Staph. aureus, were only slightly affected, if at all, while bacilli e.g. E. coli were highly susceptible to cold.

Meynell (1958) found that log-phase cells of E. coli, suspended in several different diluents, became non-reproducing when the temperature was decreased rapidly from 37°C to 4°C. In the case of Pseudomonas aeruginosa cells early in the log-phase, Gorrill and McNeil (1960) found a high loss in cold diluents, except with old cultures and in the case of Staph. aureus. The lethal effect of cold shock was reduced with slow temperature changes. Koch (1959) observed that the cold shock effect was negligible in cells harvested at the late exponential growth stage, whereas it was greatest during the early stage. Cold shock appears to be most serious during centrifugation but if this is done at growth temperature the release of intracellular material is diminished.

Mycoplasma, a pleuropneumonia-like organism, was resistant to osmotic shock at 0°C but lysed rapidly if dilution took place at a temperature of 37°C, (Razin, 1964), while at room temperature lysis only took place with organisms from actively growing cultures and in solutions of very low tonicity (Razin, 1963).

Gossling (1958) studied changes in ionic environment in the case of Mic. candidus. When this organism was transferred from phosphate buffer to Ringer's solution at 3°C to 4°C, a higher mortality ensued than followed a similar transfer at room temperature; the reverse transfer (Ringer's solution to phosphate buffer) had little effect at either temperature. Escherichia coli, under similar conditions, was only slightly more sensitive at the lower temperature than at room temperature.

Escherichia coli, throughout its log phase of growth is susceptible to an initial cold shock (that is abrupt change of temperature) from 37°C to 0°C (Hegarty & Weeks, 1940). Mature cells, however, are not affected by either an initial cold shock nor prolonged holding at 0°C. Indications are that cold shock in log phase cells generates changes within the cell, rather than exercising only a physical effect. These findings confirm those of Sherman and Cameron (1934) that younger cells

of Bacterium coli suffered about 95 per cent mortality in one hour when growth temperature was changed abruptly from 45°C to 10°C. On the other hand, cultures growing at 10°C adjusted, without much loss, to a change in temperature to 45°C, and growth commenced within one hour. When changes in environment (temperature or osmotic pressure) were made slowly, there was little effect on young cells.

While early log phase bacilli i.e. Escherichia coli are highly susceptible to cold shock on exposure to low temperatures (0°C-4°C), cocci i.e. Staph. aureus are only slightly affected (Farley, Roeser & Konetzka, 1961). Farrell and Rose (1968) give further evidence of log phase bacteria experiencing cold shock. The viability of Bacterium coli cells washed and suspended in quarter strength Ringer's solution, and held at 4°C, decreased by 11 to 13 per cent during 8-12 days storage. On the contrary, Farwell and Brown (1971) found that the viability of stored suspensions was enhanced by a reduction in temperature, particularly in stationary phase bacteria.

Raising the temperature of suspensions of Aerobacter aerogenes to 40°C increased the viability loss rate (Postgate & Hunter, 1962) while the permeability of bacteria, suspended in distilled water, to ribonuclease was greater at 20°C than at 0°C and was greater when the organisms were stored in saline phosphate buffer.

10. Effect of population density

The density of cells in test suspensions as a factor in the survival of bacteria has been noted by several workers. Ballantyne (1930), working with B. typhosus, found that a very high bacterial concentration favoured survival. In confirmation, he quoted the results of Fisher (1898) and Livingstone (1921). Winslow and Brooke (1927) also reported the same phenomenon with Bacillus cereus. Shaughnessy and Winslow (1927) noted that the cell wall of B. cereus was very permeable and that some of the bacterial cell contents were lost to the surrounding medium. These substances exercise a protective function and if they are liberated fast enough and in sufficient quantity their concentration in the suspending menstruum will reach a critical level at which further loss of cell contents is greatly retarded or prevented. The remaining viable cells either survive

indefinitely or else die off at a greatly reduced rate. The degree of leaching is also affected by the severity of the washing procedure (Spangler & Winslow, 1943). These workers also found that cell concentrations greater than 10^7 cells per ml were necessary to achieve such a protective effect. Gunter (1954) found that though cell concentration had no effect in the case of E. coli, greater mortality occured as the cell concentration decreased in the case of Azotobacter agile and Rhodopseudomonas spheroides.

An equilibrium which is set up by the death of some cells and the balance surviving or even reproducing by utilising the leakage materials was termed cryptic growth by Ryan (1959) and by Harrison (1960). Harrison (1960), using Bacterium lactis aerogenes, found that the survival effect of high cell concentrations was reversed when the density exceeded 10^9 cells per ml as then the organisms died faster. He suggested that this might be due to anoxia. The highest survival rate was achieved at 10^8 cells per ml. Postgate and Hunter (1963) also found that increased cell density led to improved survival, the optimum level coinciding with Harrison's figure of 10^8 cells per ml; they did not find a depression in survival rate at higher cell concentrations. That the protective influence of high cell concentrations might be due to magnesium leakage from the cells was proposed by Thomas and Batt (1968) in the case of Streptococcus lactis.

* * * * * *

A consideration of factors liable to affect the viability of bacteria prior to their exposure to stress in the form of disinfectants or sanitizers is essential. Stress prior to the actual test may affect the resistance characteristics of the selected micro-organisms. Strict attention is essential at all stages of the preparation of the bacterial cell suspension; from the selection of the strain of the bacterial species to its growth procedures, incubation, harvesting and suspension.

C. Post-disinfection Recovery Factors

In the evaluation of a disinfectant, the treatment of the cell suspension prior to its being stressed by the disinfectants, has a marked effect of the results obtained, but equally critical are the methods of handling the bacterial cells after stressing. Most of the survivors will be in a near-moribund state and any additional strain after the actual exposure to the disinfectant may further reduce the number of survivors and this additional reduction will apparently increase the efficiency of the disinfectant. It may not be seen as a fault in the evaluation technique.

1. Inactivation of the disinfectant

In any disinfectant tests, the time of action must be reasonably standardised according to the nature of the test. The aliquot removed from a test solution after the required exposure time will include, in addition to dead and surviving cells and diluent, some of the disinfectant. It is imperative that at the end-point of the test the anti-bacterial action is immediately stopped (Sykes, 1965).

Frequently, dilution of the culture/disinfectant solution is the sole means of inhibiting the bacteriostatic action of the disinfectant; such a procedure is adopted in the A.O.A.C. (1965) Phenol Coefficient Method and the A.O.A.C. (1965) Use-dilution Method. In the latter method however, it is recognised that the lack of growth "may be due to the bacteriostatic action of medicant absorbed on a carrier that has not been neutralised by the subculture medium employed" and the transfer of each ring to a new tube of sterile medium is required.

For certain disinfectants a positive neutralization of the carried-over disinfectant is necessary; this is particularly the case with quaternary ammonium compounds and the mercurials (Sykes, 1965). Various organic materials are known to inhibit the action of quaternary ammonium compounds. Klarmann and Wright (1948) used ox-gall, while Sherwood (1942) used agar. Valko and DuBois (1944) finally selected sodium dodecyl sulphate (Duponal PC) as a detoxifying agent. The addition of lecithin and Tween 80 to the normal beef extract-peptone broth is incorporated in the "Use-dilution Confirmation tests" of the A.O.A.C. (Stuart, Ortenzio & Friedl, 1953). In the case of the South African Bureau of Standards Specification, S.A.B.S. 636-1971, a complex

inactivator is specified containing mono-potassium phosphate, sodium citrate, sodium taurocholate, sodium thiosulphate and sorbitan mono-oleate complex (Tween 80). (This inactivator is also suitable for halogen disinfectants - South African Bureau of Standards, 1971). Lubrol W (a fatty alcohol-ethylene oxide condensate) together with lecithin and Suramine U.S.P. are extensively used (Sykes, 1965).

Inactivation of the halogens is normally accomplished by the use of sodium thiosulphate (Shaffer & Stuart, 1968) in spite of the report by Kayser and van der Ploeg (1964) that sodium thiosulphate itself inhibited the growth of a number of strains of Staphylococcus. This inhibitory effect was diminished by the addition of Tween 80.

Chambers (1956) stipulated toxicity and effectivity tests for neutralizers (inactivators) to be used in his modification of the Weber and Black Method for Sanitizers. These tests are important in order to determine whether the neutralizer or inactivator is not itself toxic to nor exerts any inhibitory effect on the micro-organisms used in the test.

D. Enumeration of Viable Survivors

Any method of culturing disinfectant-damaged micro-organisms should provide an environment suitable for the recovery and growth of the micro-organisms. There are two methods in common use, either sub culture into broth media or subculture after dilution, using agar media. Subculture into broth media is more commonly used, e.g. in the Phenol Coefficient test (A.O.A.C., 1965) where after contact of culture and disinfectant for the prescribed time, successive loopfuls of the culture-disinfectant mixture are transferred to subculture tubes for incubation and assessment of growth. Subculture into broth either gives a positive or negative result for growth or may be used for the determination of "most probable numbers" (Standard Methods for the Examination of Dairy Products- A.P.H.A., 1967).

The usual cultural procedure for determining numbers of viable survivors has been the agar plate method. Although this is an empirical method, influenced by the competence and accuracy of the analyst, it is of considerable value for estimating bacterial populations especially when bacterial densities are low (Standard Methods for the Examination of Dairy Products, 1967).

Nelson (1943) showed that when studying the effects of exposure to heat on micro-organisms, it is incorrect to assume that conditions suitable for growth of unheated organisms are equally satisfactory for growth after exposure as heat-treated bacteria are more demanding in their growth medium requirements than unheated control organisms. This finding was extended to antiseptic-damaged bacteria by Harris, Richards and Whitefield (1961) who also showed that the variation in survivor counts on different media is greater when the mortality of the suspension was high. The restoration of the viability of chemically inactivated Escherichia coli was enhanced by the incorporation of tricarboxylic acid cycle metabolites into the recovery medium (Heinmets, Lehman, Taylor & Kathan, 1954a). In the case of cold-injured bacteria, Straka and Stokes (1959) considered that the presence of specific peptides contributed to bacterial recovery. These peptides were those in trypticase and other enzymatic digests of casein. Tryptic soy agar was far superior to an agar medium containing inorganic salts, citrate and glucose. Clark (1968) comparing three general growth media for the enumeration of heat-treated staphylococci found tryptic soy agar to be the most suitable. Crisley, Peeler and Angelotti (1965) used this agar as the control medium in a comparative study of selective media for staphylococci. Tryptic soy agar has also been described as a general purpose medium for the cultivation of fastidious micro-organisms (Difco, 1966). A trypticase soy agar is one of the subculture media specified for the A.O.A.C. method for testing water disinfectants (A.O.A.C., 1965).

One of the main criticisms of the use of agar plating media for the recovery of damaged micro-organisms has been that the relatively high temperature at which agar must be poured may hamper the growth of the organisms. Jacobs and Harris (1960) found that temperatures above 37°C were unfavourable for the recovery of phenol damaged E. coli and Staph. aureus. Further, Vanderzant and Matthys (1965) caused a reduction of 25 per cent or more in the viable count in six of nine species of micro-organisms when the temperature of the plating medium was increased from 45° to 50°C. When the plating medium was held at 45°C. prior to pouring, they found that the maximum temperatures

reached at the time of pouring were 37°C in the case of a 0,1 ml culture sample incorporated into the plating medium and 35°C in the case of a 1,0 ml culture sample. As these temperatures are within the range of the incubation temperatures (35-37°C for 48 hours), no marked effect on viability should be expected. The temperature of the plating medium should, therefore, be controlled within narrow limits.

Determining a viable organisms count by the agar plating method invariably entails serial dilution and the selection of suitable plates after the incubation period. According to Standard Methods for the Examination of Dairy Products (1967), plates with between 30 and 300 colonies are selected for the greatest accuracy in assessing results. This publication also details observations on variations from normal.

E. Disinfectants Applicable to the Dairy Industry

Sykes (1967) asks the question "Why do we need to use chemical disinfectants?" Frequently the desired degree of physical cleaning can be achieved by simple washing or scrubbing with or without soaps or detergents in relatively large volumes of hot or cold water. These are at best only cleaning operations. They remove gross amounts of soiling material but leave behind large numbers of micro-organisms. Where there is a direct risk of the transmission of infective or spoilage micro-organisms as in hospitals, medical practice, the food, catering and dairy industries or in the domestic sphere, then disinfection or sanitization is an essential requirement. Heat, particularly moist heat, is undoubtedly one of the most effective disinfecting agents, but there are numerous situations where the application of heat is either impractical or impossible.

In England and in Wales until May 1943, the use of an oxidising or preservative agent was not permitted in the cleansing of farm dairy equipment, only heat could be used. Since that date however, the use of approved solutions of sodium hypochlorite was permitted by the Ministry of Agriculture and Fisheries. In the Milk and Dairies (General) Regulations 1959, the use of other chemicals such as quaternary ammonium compounds (QAC) and iodophors was approved (Ross, 1967).

Clegg (1967) considers that there are six general groups of disinfectants which can be used in dairying:

1. Chlorine-bearing disinfectants,
2. Quaternary ammonium compounds (QAC)
3. Iodophors
4. Strong alkalis
5. The phosphoric acid wetting agents (PAWA)
6. The amphoteric compounds.

Haffer (1965) considers the PAWA and the amphoteric compounds effective but somewhat expensive; as cost is an important consideration in the dairy industry, extensive use of these compounds will be limited until their price to the consumer is reduced.

Strong alkalis are generally considered as de-fatting agents as well as having a disinfectant effect. Their corrosiveness at use dilutions precludes them as general purpose disinfectants (Thiel, Clough & Clegg, 1955).

The chlorphenols and other disinfectants with marked odours are automatically excluded since milk is a powerful absorbent of odours (Davis, 1963).

The main groups of disinfectants commonly used as dairy disinfectants or sanitizers are therefore the halogen and the quaternary ammonium compounds. The halogen compounds are those containing chlorine, iodine, fluorine and bromine as the active ingredients. Bromine is strongly irritant and unpleasant to handle and has no advantage over chlorine as a disinfectant, while fluorine is limited in its application (Sykes, 1965). Chlorine, the most reactive of the halogens, has been the subject of investigation for many years and its application in the dairy, food and domestic spheres is widely known. Further, its application in water and sewage treatment remains one of its major uses.

The disinfecting abilities of chlorine and its compounds have been recognised ever since the early 19th century when they were used by Semmelweis and Alcock (Trueman, 1971). As a result this group of disinfectants has been one of the most widely studied and reported.

The discovery of a group of synthetic compounds possessing bactericidal properties was reported in 1916 by Jacobs, Heidelberger and their co-workers, but it was only in 1935 that Domagk again focussed attention on the bactericidal properties of a large number of quaternary ammonium salts (Hoogerheide, 1945).

In the case of the iodophors, it was in 1949 that Shelanski found that certain surface-active agents could form soluble complexes with iodine which retained the germicidal activity of iodine without its disadvantages (Trueman, 1971).

It would thus appear pertinent to investigate the widely divergent actions of iodophors and quaternary ammonium compounds - both formulated from surface active agents but with differing properties and anti-bacterial reactions.

Although the variety of QAC is proverbial, the iodophors are also produced in many variations of formulation for various fields of application.

This series of investigations has therefore been limited to examples of the quaternary ammonium compounds and iodophors.

1. The Iodophors

Although iodine in tincture or solution is not suitable for disinfecting dairy equipment, the iodophors are widely used (Sykes, 1965). An iodophor is a combination of iodine and a solubilizing agent. The resulting compound is a complex of indefinite composition which when dissolved in water, slowly liberates most of the iodine (Gershenfeld, 1968). The solubilizing agents most commonly used are non-ionic compounds (Lazarus, 1954; Mueller, 1955; Lawrence, Carpenter & Naylor-Foote, 1957), particularly the group whose iodine-solubilizing factor is a polyoxethylene ethanol chain (Lazarus, 1954), or a blend of nonylphenoxy polyoxethylene ethanol-type surfactants of different ethylene oxide percentages (Brost & Krupin, 1957). These authors state that, in order to prevent phase separation during storage at various temperatures, it is necessary to include a non-ionic surfactant, whose composition is based by weight on 75 to 86 per cent of ethylene oxide, in the mixture of different non-ionic surfactants. The foam normally

produced by the non-ionic surfactants may be reduced by incorporating a small proportion of nonylphenoxyethanol containing 23 per cent of ethylene oxide; the reduction of foam increases with the concentration of this type of surfactant. Foam restriction is an extremely important factor in circulation cleaning of dairy equipment.

Iodophor formulations intended for use as detergent-sanitizers must meet detergency as well as sanitizing requirements in use-dilution concentrations, and thus the iodine:detergent ratio becomes all important (Brost & Krupin, 1957).

Other surfactants have been used in iodophors, for instance Frisch, Davies and Krippaehne (1958) referred to a cationic "iodophore" [sic] for use in skin degerming applications. However the anionic and cationic surface active agents do not normally give the compatability and stability associated with the non-ionics (Gershenfeld, 1955a).

In order to maintain an optimum hydrogen ion concentration, iodophors are normally buffered with phosphoric acid. The extent to which phosphoric acid is added depends on the use dilution and the purpose of the iodophor (Gershenfeld & Witlin, 1955). Other acids may be substituted for phosphoric acid such as glycollic acid, lactic acid, sulphamic acid and citric acid (Rammell & Croft, 1971). The range of phosphoric acid addition in South Africa varies from <1 per cent to 30 per cent (Hathorn, 1972) depending on the end-use of the iodophor.

As free iodine is liberated slowly from the iodophor complex when it is diluted with water, the bactericidal properties of iodine are retained without some of its detrimental effects i.e. sensitivity inducing, staining, toxicity, irritation and low vapour pressure. Thus the desirable attributes ascribed to iodophors are essentially those of weak iodine solutions (Gershenfeld, 1968). Comparative tests against other bactericides such as other halogens, phenols, mercurials, dyes and QAC, revealed that iodine deserves to be rated among the most efficient antiseptics in use today (Gershenfeld, 1957).

In numerous publications reference is made to the advantages of free iodine solutions and of iodophors in many widely differing fields such as general sanitizing, disinfecting and antiseptic agents (Gershenfeld, 1955; Gershenfeld & Witlin, 1955; Elliker, 1955;

Gershenfeld & Witlin, 1958); for the treatment of water supplies (Chambers, Kabler, Malaney & Bryant, 1952; Gershenfeld, 1955c); for dairy hygiene (Johns, 1954a); in fruit and vegetable processing and packaging (Casolari, Campanini & Cicognani, 1967); opthalmology (Harris, Rowell & Beaudreau, 1958); surgery (Lawrence, Carpenter & Naylor-Foote, 1957); atmospheric disinfection (Cheli, Preite, Renzalli & Biolchinie, 1967) and sanitization of food utensils (Mueller, 1955).

Conversely, Cousins, Hoy and Clegg (1959) found that iodophors were no more effective than hypochlorite. They stated that the pH value of a 25 p.p.m. iodophor solution made up with hardwater (from a borehole) was pH 6,37 compared to pH 2,8 of a similar solution made up with distilled water. This rise in pH was in opposition to the report by Johns (1954b) that it was difficult to increase the pH of a 25 p.p.m. iodine iodophor solution above pH 2,8. Two possibilities exist for these comparative findings; either the phosphoric acid content of the Iodophor A used by Cousins et al (1959) was lower than that used by Johns (1954b) or, it was a question of alkalinity rather than a question of hardness. This can be considered in the light of the findings by Rammell and Croft (1971) regarding alkalinity versus water hardness.

(a) Bactericidal action of iodine

The bactericidal action of iodine is said to depend on the precipitation of proteins (Gershenfeld, 1955b, 1957), the iodine being partly absorbed, partly loosely bound and partly converted to iodide ions. The loosely bound iodine continues to exert its action. This reasoning was partially confirmed by Brandrick, Newton, Henderson and Vickers (1967); in an investigation of the action of iodine on E. coli they showed that 90 per cent of the iodine absorbed by the cells reappeared as iodide in the supernatant fluid, indicating an oxidative action on the cell material. In the case of chlorine and the hypochlorites it is the hypohalous acid that is most bactericidal, but with iodine the free element is more active.

The bactericidal activity of iodine in aqueous solution varies according to the hydrogen ion concentration. This is summarised below.

$$3I_2 + 3HOH \qquad \text{very acid solution}$$
$$H^+ \quad OH^-$$
$$3HIO + 3I^- + 3H^+ \qquad \text{progressively acid}$$
$$H^+ \quad OH^-$$
$$3IO^- + 3H^+ \qquad \text{progressively alkaline}$$
$$H^+ \quad OH^-$$
$$2I + IO_3^- \qquad \text{very alkaline solution}$$

Symbol	Name	Bactericidal Activity
I_2	Free iodine	Most active
HIO	Hypoiodous acid	Active
IO^-	Hypoiodous acid ion	Slightly active
IO_3^-	Iodate ion	Inactive

(from Marks & Strandskov, 1950; Trueman, 1971)

(i) Factors affecting the anti-microbial activity of iodophors

The desirable attributes ascribed to iodophors are essentially those of weak iodine solutions and similarly factors, affecting the anti-microbial activity of iodine, will also affect iodophors in use-dilution.

As these factors are of some importance it is necessary to examine them in broader detail.

(aa) Concentration Chambers, Kabler, Malaney and Bryant (1952) concluded that, under the most favourable conditions (pH 6,5 at 20-26°C), the minimum average concentrations of iodine to kill the tested organisms (*E. coli*, *S. typhosa*, *S. paratyphi*, *S. schottmuelleri*, *S. flexneri*, *S. dysenteriae*, *S. sonnei* and *S. faecalis*) in one minute was 0,6 p.p.m. The concentration required to kill under the most adverse conditions (pH 9,15 at 2-5°C) was 4,3 p.p.m.

Effective dilutions of iodophors recommended for use as sanitizers or detergent-sanitizers contain 25 p.p.m. to 75 p.p.m. of

free iodine. The lower concentration is equivalent to 200 p.p.m. free chlorine from hypochlorite (Gershenfeld, 1955c). Solutions containing 12,5 p.p.m. free iodine have been approved by sanitarians and health authorities for equipment and utensils in the dairy industry (Gershenfeld, 1968).

(bb) Hydrogen ion concentration As free iodine is the most bactericidal form, pH is extremely important. Chambers et al. (1952) confirmed that iodine exhibits a lower antibacterial activity at high pH ranges. Further, Rammell and Croft (1971) emphasised the need to maintain a low pH in iodophor solutions. Davis (1962) reported that the germicidal power of an iodophor declines above a pH of 4. Sykes (1965) set upper limits of pH 3 to 4 for maximum activity.

As mentioned, iodophors are buffered with weak acids; phosphoric acid is generally used as, besides being relatively non-corrosive, it complexes Ca^{++} and Mg^{++} ions that encourage milkstone formation in dairy utensils (Sykes, 1965; Rammell & Croft, 1971).

No reference to the effect of hard water on the activity of iodophors could be traced in the literature, although alkalinity, as opposed to hardness, has been considered (Rammell & Croft, 1971). These authors stated that at the phosphoric acid concentration generally encountered at working dilutions of iodophors, the pH of the solution is 2,8 when distilled water is used for dilution, pH 3,5 when a water of total alkalinity of 75 mg/l, as $CaCO_3$, is used. An increase of alkalinity of the water to 150 mg/l $CaCO_3$ results in a pH of about 6,0 for the working solution. In New Zealand, of the water samples tested 40 per cent have a total alkalinity > 75 mg/l, while 19 per cent exceeded 150 mg/l $CaCO_3$. Thus to maintain an effective pH the iodophor should contain approximately 10 per cent phosphoric acid, which would be suitable for waters with up to 75 mg/l $CaCO_3$. (Unfortunately this information came to hand after the present series of trials had already been initiated).

(cc) Organic matter Iodine, being a highly reactive element, suffers a marked depression of its bactericidal activity when organic matter is present. Variations in the minimum lethal levels of

iodine, reported in the literature, are probably due to the methods of testing employed and the carry over of organic matter with the culture into the medication mixture (Sykes, 1965).

Johns (1954b) was of the opinion that the iodophors are less affected by milk than chlorine compounds: a 25 p.p.m. iodophor dilution required a greater addition of milk than a 200 p.p.m. chlorine solution to become inactivated.

A further indication of the effect of organic matter is given by Sykes (1965) comparing results obtained by Gershenfeld and Witlin (1955) with those of Lawrence, Carpenter and Naylor-Foote (1957). Gershenfeld and Witlin found that considerable differences result from changes in the inoculum size. Reducing the inoculum from 0,2 to 0,05 ml caused a reduction in the minimum lethal concentration necessary from 6 to 2 parts per million of available iodine; on the other hand Lawrence et al. (1957) found the addition of 5 per cent serum resulted in up to a twenty fold reduction in iodine activity.

(dd) Temperature As with the majority of disinfectants, an increase in temperature is accompanied by a greater bactericidal effect. According to Chambers, Kabler, Malaney and Bryant (1952) a higher concentration of iodine was required to kill between 2°C and 5°C than between 20°C and 26°C.

It must be appreciated that there is a limit to the temperature than can be applied to iodine and iodophors. Even at room temperature iodine changes spontaneously from a solid into the vapour state without passing through the liquid phase; this is due to its very low vapour pressure. Loss of iodine as vapour from a concentrated iodophor is prevented by the solubilizing agent which holds it in solution. However free iodine vapour may be given off by an iodophor when the temperature is in excess of 43°C (Trueman, 1971).

(ee) Bacteriostatic and bactericidal activity Numerous substances have been reported to be capable of neutralizing the bactericidal effect of iodine. These include serum, glycerine, syrup, faeces, egg, milk, sputum, urine, sodium thiosulphate, metallic mercury and ammonia water (Gershenfeld, 1968).

Studies of iodine inactivation indicate that bacteriostatic iodine solutions are practically identical in strength to those showing a bactericidal effect. It appears therefore that iodine kills immediately rather than by a prolonged period of stasis. The highest dilution of iodine killing in one minute (1:20 000) was the same as that killing after ten minutes exposure; this is a property not shared by any other disinfectant. Sykes (1965) emphasises that two outstanding characteristics of iodine are its lack of selectivity against different bacteria; all types being killed at about the same level of concentration, and its exclusively bactericidal, rather than bacteriostatic, action.

(ii) Advantages and disadvantages of iodophors Many of the undesirable properties of iodine, such as skin irritation and sensitisation, allergic reactions, corrosive properties, and low solubility in water except in the presence of alcohol and potassium iodide, are not found in the case of the iodophors. The following comments are intended to summarise the advantages and disadvantages of the iodophors.

(aa) Surface activity The surface active agents used as solubilizers for the elemental iodine improve the wetting properties of the use-dilutions; helping to penetrate organic soil and emulsifying fat deposits. The detergency factor is enhanced by the further addition of surfactants to iodophors used as sanitizers (Sykes, 1965).

(bb) Activity range Iodophors, behaving very like aqueous solutions of iodine, are rapidly bactericidal to a broad spectrum of vegetative organisms (Gershenfeld, 1968) including the tubercle bacillus (Lawrence et al, 1957). Further, iodine is fungicidal, viricidal and sporicidal (Sykes, 1965), although the sporicidal effect only occurs when high concentrations (1 000 p.p.m.) act for relatively long periods (6-10 hours) (Lawrence et al, 1957).

(cc) Organic matter Iodine, being chemically less reactive than chlorine, is negligibly affected by organic matter when high concentrations are used, as in skin disinfection (Sykes, 1965). At low concentrations, however, the effect can be significant. Davis (1962) considers that iodophor solutions remain bactericidal in the presence of organic matter as long as the pH remains below 4.

(dd) Stability The solubilization of iodine in surfactants reduces the iodine vapour pressure, eliminates the odour and prevents irritation of the eyes provided the temperature does not exceed 43°C (Trueman, 1971).

(ee) Toxicity The gastric toxicity of iodophors is extremely low in comparison with that of iodine. Skin sensitisation is extremely rare, and staining does not usually occur (Trueman, 1971).

(ff) Visual strength indication Dilute iodophor solutions have a characteristic amber colour; when this pales to a straw yellow, it indicates that further iodophor is required (Davis, 1962).

(iii) Iodophor dairy sanitizers The presence of phosphoric acid, or other suitable acids, will prevent the formation of milkstone on utensils and other equipment. If, however milkstone is already present prior to the introduction of iodophors, a more vigorous treatment will be required for its removal (Johns, 1954b).

Iodophors do possess some disadvantages amongst which are:

(aa) The high cost per unit volume of concentrate. This can only be countered by careful consideration of the actual cost per unit volume of use dilution solution.

(bb) Their ability to stain some types of plastics and to corrode metals such as silver and some ferrous alloys.

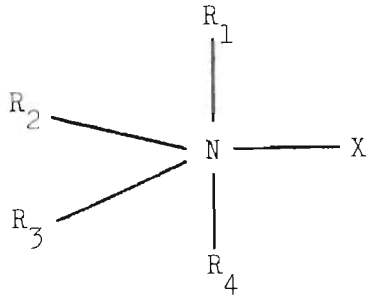
(cc) Their utilisation temperatures are limited. Above temperatures which range from 43-50°C, depending on the formulation, iodophors will release iodine vapour to the atmosphere rapidly.

(dd) Their tendency slowly to lose iodine by evaporation from use dilution concentrations standing in open containers. The loss of iodine increases with high ambient temperatures. "This is not normally a significant problem" (Trueman, 1971).

Hathorn (1972) however showed, that of eleven iodophors tested, all lost at least 95 per cent of their available iodine content in 24 hours when 1:640 concentration water dilutions were exposed to the air, and at least 97 per cent after holding for 48 hours. Effectively this limits the use of a dilute solution to one occasion; it cannot be held over for future use.

2. The Quaternary Ammonium Compounds (QAC)

The quaternary ammonium compounds comprise a very widely used and intensively studied group of the antibacterial surface-active ccmpounds. They have a general formula:



where X is usually a halogen. Those showing the most marked antibacterial activity have as one of the four organic radicals (R), a chain length of between 8 and 18 carbon atoms (Hamilton, 1971).

From this general formula, it is evident that an almost unlimited variety of compounds of different constitution and structure is possible, each compound exhibiting its own specific antibacterial and other properties. Some of the variables are: the halogen; length of carbon chain; whether branched or unbranched; whether a benzene ring is present or not. A further complication results from the fact that any specific QAC may be marketed under a wide variety of trade names sometimes without reference to the actual compound present. Any attempts to generalise about QAC must therefore be carefully ccnsidered and it must be accepted that there may possibly be individual compounds to which certain generalisations may not refer. Like other surface-active compounds, the QAC ccntain hydrophobic and hydrophilic groups and tend to migrate to surfaces and interfaces. In the case of QAC the hydrophilic group carried a positive charge when ionised (Sykes, 1965).

(a) Antibacterial mechanism

Gale and Taylor (1946) noted that in the presence of QAC, lysis of lysine takes place with extreme rapidity. In 1947, the same workers reported the disruption of the cell wall in the case of *Streptococcus faecalis*, *Staphylococcus aureus* and *Saccharomyces carlsbergensis*.

Salton, Horne and Cosslett (1951) noted that cetyltrimethyl-ammonium bromide (CTAB) solutions caused cytoplasmic contractions away from the cell wall. High concentrations of CTAB appeared to strip off the cell envelopes. Salton (1951) found a relationship between the proportion of cells killed, the 260 μm -absorbing material released into the suspending medium and the amount of CTAB used.

The ruptures in the membrane resulting from CTAB treatment are thought to be relatively small, since only substances of low molecular weight are released from the cells. The largest molecule so far observed released from CTAB treated organisms is cytochrome C_3 (Postgate, 1956).

At low concentrations, QAC compounds inhibit the energy releasing reactions of the bacterial cell; this particular action of the compounds appears to be more important than the non-specific mechanical leakage of cellular material found in the presence of high QAC concentrations. The velocity of the reaction is a function of the weight ratio of germicide to cells and of the absolute cell density (Stedman, Kravitz & King, 1957). This latter statement confirms the contention by Knox, Auerbach, Zarudnaya and Spirtes (1949) that the effects of killing and inhibition with cationic detergents are proportional to the detergent-bacterial ratio and not to the detergent concentration alone.

Roberts and Rahn (1946) demonstrated complete inactivation of the cell dehydrogenase systems by lethal concentrations of QAC. Dawson, Lominski and Stern (1953) described how lysis of Staphylococcus aureus cells occurs at optimal or low ratios of the QAC to micro-organisms. Lysis did not occur at high QAC concentrations and the authors concluded that the enzymes causing autolysis become denatured, whereas at the low concentrations the autolytic enzymes are free to act on the split lipoprotein within the cell.

Chaplin (1952) inferred that acquisition of resistance by micro-organisms to QAC might be due to the increased lipid content of the cell, possibly explaining the higher resistance of Gram-negative compared with Gram-positive micro-organisms. MacGregor and Elliker (1958) gave two possible explanations for the observed difference in

sensitivity among various Pseudomonas aeruginosa strains:

(i) The resistant cells contain a large amount of inert material which does not react with QAC, and,

(ii) the normal strain contains more of 'some non rate limiting QAC sensitive enzyme or co-factor and therefore shows a lower apparent percentage inhibition during substrate oxidation.' They further suggested that the QAC resistance in P. aeruginosa might be similar to that described by Chaplin (1952) for Serratia marcescens - a lipid or lipid-like complex bound together by multivalent inorganic cations.

Referring to the action of various inorganic cations, Mueller and Seeley (1951) demonstrated the hypothesis that the metallic cation could inactivate the QAC by association with it to form some type of chemical complex, was incorrect, and Meggison and Mueller (1956) concluded that the higher the valence of the metallic cation, the lower was the electronegativity of the suspended bacterial particles. Adding QAC to these suspensions lowered the electronegativity still further. The higher the valency of the metallic cation, the more depressed the effect of the QAC on the bacteria.

Newton (1960) concludes a summary of the mode of action of surface active bactericides by recording that it is a very controversial subject.

(b) Compatability and incompatability with other compounds

Lawrence (1968a) presents a comprehensive list of a variety of compatabilities and incompatabilities of QAC with various compounds, including that of the antagonism between QAC and soap.

In addition, Ridenour and Armbruster (1948) noted that when solutions of a QAC were made up with waters containing the cations, Ca^{++} and Mg^{++}, which are responsible for hardness in water, the concentration of QAC required to achieve a 99,9 per cent kill with Eberthella typhosa had to be almost trebled in comparison with the same QAC made up in distilled water. They concluded that some sort of neutralisation of the disinfectant occurred when hard water was used to make up the sanitizer bath. Klimek (1955) investigating the influence of hard water on QAC effectivity concluded that the attenuation of a QAC

(benzolkonium chloride) is not attributable to hardness per se. The site of action of antagonistic waters is the bacterial cell and maximum interference with the germicide requires exposure of the cells to the antagonistic water prior to exposure to the germicide. They further determined that only with those organisms grown in nutrient agar prepared with one of the following peptones - Casitone, Polypeptone, Phytone or Peptonum siccum was there an interfering action on the QAC by certain waters. Other peptones do not produce organisms with the observed hard water effect.

Klimek and Bailey (1956) later confirmed Klimek's observation and added that in alkaline medium, iron up to 5 p.p.m. and aluminium salts did not interfere with QAC activity, unless aluminium salts were added in acid medium. This contradicted Mueller and Seeley's (1948) findings that not only Ca^{++}, Mg^{++} decreased the germicidal effect of QAC but also ferric iron.

It is difficult to reconcile the findings of these workers as Klimek only found the antagonistic effect when the bacterial cells (E. coli) were exposed to the hard water prior to exposure to germicide, whereas Ridenour and Armbruster had found the antagonistic effect without a prior exposure of the cells to hard water. Klimek concludes by stating that the so-called 'hard water effect' on the germicidal efficiency of benzolkonium chloride (and presumably related quaternary ammonium germicides) 'is a laboratory phenomenon of questionable significance in the evaluation of germicidal efficiency'. Suffice to mention that most recognised test procedures still require that the possible affect of hard water on QAC be taken into account.

(c) Bactericidal and bacteriostatic action

These actions may be considered along with that of 'reversibility' of the antibacterial effect whether it be bacteriostatic or bactericidal. Valko and DuBois (1944) claimed to have obtained results which, for the first time, indicated that the antibacterial effect of surface active agents could, under certain conditions, be reversed. They observed the protective action on bacteria of relatively harmless cations against toxic cations, and suggested that this was a question of ion exchange. The harmless cation in these trials was that of sodium dodecyl sulphate (Duponol P.C.).

Various workers, such as Chaplin (1952), showed that bacteria can readily adapt themselves to QAC and develop resistance to it. MacGregor and Elliker (1958) found that QAC tolerance of Pseudomonas aeruginosa could be raised from 100 to 700 parts per million after nine transfers in a liquid medium and eventually to 2 000 parts per million. Similar results were obtained with E. coli and Serratia marcescens (Dvorkovitz, 1952). This author noted the fear that repeated applications of a sanitizer insufficient to effect a complete kill might allow the development of resistant organisms under practical conditions. He continued by stating that QAC are bacteriostatic and not bactericidal. This statement is repudiated by Newton (1960) who states that certain substances which will complex with QAC, and so prevent it from exercising a bactericidal action, will only do so if added to the bacterial culture before or simultaneously with the surface active agent, but are not effective after the combination of the agent with the cells. The use of neutralizers in antibacterial tests has shown that no marked bacteriostasis is involved provided lethal, and not sub-lethal, concentrations are involved (Mallman & Harley, 1950).

Generally QAC are more active against Gram-positive than against Gram-negative bacteria, though the differences may not be great (Sykes, 1965). This may be due to the differences in type and content of phospholipids in the two types of organisms (Brown, 1964), however Hoogerheide (1945) had found no difference in the sensitivity of Staph. aureus and E. coli to CTAB. It appears that the differences in sensitivity might be due to the bacterial strains tested and/or the QAC type.

(i) Factors affecting bactericidal and bacteriostatic action

(aa) Influence of pH Alkalinity increases the efficiency of many quaternaries. Considerably higher activity was noted from CTAB at pH 7,8 than at pH 5,0 (Hoogerheide, 1945). Mueller and Seeley (1951) found that to obtain a 99,99 per cent kill in five minutes, 8 p.p.m. of a QAC were required at pH 10, while a reaction of pH 3, required 100 p.p.m. of the same QAC.

(bb) Influence of temperature Enhanced bactericidal effects are obtained with increased temperatures. Hoogerheide (1945) found a phenol coefficient for CTAB at 20°C and pH 8 of 830 while at the same pH a coefficient of 1 250 was obtained at 37°C. Sykes (1965) states that the temperature coefficients of the various QAC vary somewhat, but that about half the concentration is required for the same bactericidal effect at 37°C as at 20°C.

(cc) Organic matter The importance of organic matter contamination in the dairy and food industries hardly requires emphasis, and that its presence in various forms such as milk, serum, etc., may affect the efficiency of disinfectants, has been widely reported.

Hoogerheide (1945) showed that the bacteriostatic efficiency of CTAB is not subject to the same inhibition as its bactericidal properties. Quisno and Foter (1946) found that the addition of 10 per cent of ox serum required an increase in the lethal concentration of a QAC from 1 in 66 000 to 1 in 1 000 in the case of E. coli and from 1 in 127 000 to 1 in 17 000 with Streptococcus pyogenes.

Mallman and Harley (1950) showed that QAC was actually neutralized or absorbed by suspended solids and grease as well as soiled equipment surfaces. They also found that extremely high bacterial populations could decrease the availability of QAC per bacterial cell to sub-lethal concentration even though the solution had contained 200 p.p.m. of the sanitizer.

The reduction to sub-lethal concentrations during use, has resulted in attempts to develop field methods for quantitative determination of QAC. Unfortunately, while laboratory methods give accurate results, no precise procedure is apparently available for tests in the field (Ridenour & Armbruster, 1948; Lawrence, 1968a).

(dd) Toxicity Quaternary ammonium compounds have been shown to have a very low toxicity level. Finnegan and Dienna (1954) concluded that concentrations of 200 p.p.m. of two QAC represented no hazard to workers, even to those exposed to extended contact time as would be found with dishwashers. The same workers found that animals tolerated a 1 in 1 000 solution of QAC as their only source of drinking water without impairment to weight gain or other untoward reactions.

(ee) Adsorption of QAC Whereas chlorine and iodine are inactivated by chemically combining with organic matter; the QAC are inactivated by an adsorption action. By adsorption it is implied that the QAC are 'removed' from further action. Thus quaternaries, being surface active agents, may be removed by materials, such as filter-pads, cotton wool and charcoal, from solution (Quisno, Gibby & Foter, 1946). As a result the effective QAC concentration is reduced. When QAC are adsorbed onto bacterial surfaces the impression gained is that the bacteria have been killed, when perhaps only a bacteriostatic action has taken place. Thus effective neutralization of the QAC is required to determine the actual effect on the bacterial cell (Sykes, 1965).

Agar is also capable of neutralizing QAC. Sherwood (1942) reported that the agar cup method was unsuitable for QAC assessment because of this factor.

(d) Applications of QAC in the dairy industry

It would appear that the majority of bacteria contaminating milk and utensils are of the Gram-positive types; thus the QAC should be eminently suitable for dairy sanitization purposes. This would include the reduction in numbers of the thermoduric types of micro-organisms. Krog and Marshall (1942) considered that QAC have a definite application in the sanitization of milk-handling equipment, although they are only capable of a 90 per cent reduction in bacteria.

Puhle (1950) reporting on field trials with QAC sanitizers concluded that the dairy industry had at its disposal a means of improving the standard of dairy farm milking equipment. He indicated that 93 per cent of 1 168 producers using QAC detergent sanitizers had a thermoduric count of under 10 000 organisms per ml over a ten month period.

Although, as already stated, the toxicity of QAC is estimated to be low for higher animals, Swartling (1968) states that many authors have shown that residual traces of these sanitizers in milk can decrease the rate of acid formation by starter organisms. A concentration of 0,1 to 2 mg per litre will interfere with the making of certain types of cheese. The quoted concentration is of the same order of magnitude as that reported to have been found in milk delivered to dairies (0 to

3 mg per litre) - thus a thorough rinsing is essential after their use on dairy equipment. Disinfectants of other types, chlorine, iodophors and hexachlorophenols (used for udder washing), have not been shown to be deletrious in their normal residual quantities to the use of milk, for any purpose (Swartling, 1968).

* * * * * *

The disinfectants applicable to the dairy industry are relatively limited. Those disinfectants that might affect the flavour or odour of milk are completely unsuitable. Chlorine and its derivatives, were the first commonly accepted dairy disinfectants and have thus been subjected to a considerable amount of investigation. The iodophors and quaternary ammonium compounds have relatively recently been accepted in the dairy industry. As their mode of action differs, these two detergent-sanitizers would appear to be suitable for assessing the influence of diluents on the reaction of bacteria to disinfectants or detergent-sanitizers.

F. Test Methods

Numerous disinfectant and sanitizer test procedures have been devised and recommended. It must be apparent, however, that no single test procedure can be expected to assess performance characteristics of a disinfectant under all conditions of application. Disinfectants and sanitizers are used in diverse application against organisms in suspension, on inanimate surfaces, on skin and living tissue, in the presence of organic matter. Further, the times during which the material will act are extremely variable, ranging from a momentary dip to a prolonged soak. At best a test procedure can only simulate one particular set of use conditions, but in doing so it attempts to standardise as many variables as possible.

The majority of established test procedures utilise unwashed test suspensions of the selected micro-organisms. The carry over of broth or 'water of condensation' from agar slopes has been shown to have a protective effect on bacterial suspensions (Ballantyne, 1930). However, Cook and Wills (1958) showed that E. coli washed and suspended in phosphate buffer maintained its viability and bactericidal resistance

better than did unwashed or washed cells suspended in water. This is a somewhat unusual finding. As the degree of culture medium carry over in unwashed cells in uncontrollable, it would appear that washing combined with centrifugation would ensure more constant conditions, and should make it possible to achieve more accurate test results.

The utilisation of broth-tube culture methods for determination of survivors on a 'growth'/'no growth' basis, as used in the Phenol Coefficient Method (A.O.A.C., 1965), would not be applicable to circumstances where actual survivor counts are required. On the other hand, the Chambers' modification of the Weber and Black Method for Sanitizers requires the determination of survivors based on plating dilution aliquots in agar media (Chambers, 1956). Thus, where actual survivors are to be assessed, the most suitable test methods would be those adopted for the testing of sanitizers, or other similar tests. Furthermore, consideration should be given to any official testing system in vogue in the Republic of South Africa. Fortunately certain similarities exist between the Chambers' modification of the Weber and Black Method for Sanitizers recommended for use in dairies, restaurants and food plants (Chambers, 1956) and the Standard Specification for Quaternary Ammonium Compound Disinfectants (South African Bureau of Standards, SABS 636-1971).

In order that the influence of diluents on the outcome of a disinfectant or sanitizer test may be assessed, a test procedure must be developed that would include the normal procedures, but that would also exclude those factors possibly contributing to variations in results.

The following table allows a comparison to be made between the Chambers' modification and the South African Bureau of Standards' Specification. As may be seen in the table there are areas of both similarity and of disparity.

COMPARISON OF THE CHAMBERS' MODIFICATION OF THE WEBER AND BLACK METHOD FOR SANITIZERS AND THE SOUTH AFRICAN BUREAU OF STANDARDS' STANDARD SPECIFICATION FOR QUATERNARY AMMONIUM COMPOUNDS

ITEM	CHAMBERS	S.A.B.S.
TEST ORGANISMS	Escherichia coli ATCC - 112299 Staphylococcus aureus ATCC - 6538	Escherichia coli SATCC Esc 25 Staphylococcus aureus SATCC STA 53 Pseudomonas aeruginosa SATCC PSE 2
CULTURE MEDIUM	Nutrient agar or any other equivalent medium	E. coli MacConkey medium S. aureus Staphylococcus medium P. aeruginosa nutrient medium
TEST SUSPENSION PREPARATION	Washing off agar slopes	Washing off agar slopes
AGE OF CULTURE	18-24 hours	24 $\pm$ 1 hour
SUSPENDING METHOD	Shaking and filtration through filter paper	Shaking and filtration through filter paper
TEST SUSPENSION DILUENT	Phosphate buffer	Distilled water
BACTERIAL DENSITY OF SUSPENSION	1×10^{10}/ml	1×10^{5}/ml
VOLUME OF TEST SOLUTION	99 ml	99 ml
TEMPERATURE OF TEST SOLUTION	22,2-23,9°C	22°C
VOLUME OF INOCULUM	1 ml	1 ml
BACTERIAL DENSITY OF TEST SOLUTION	1×10^{8}/ml	1×10^{3}/ml
MAXIMUM USE LIFE OF TEST SUSPENSION	Not specified	3 hours
HOLDING TEMPERATURE FOR TEST SUSPENSION	Cracked ice	22°C

ITEM	CHAMBERS	S.A.B.S.
NEUTRALIZER	Tween 80, Asolecithin with/without sodium thiosulphate	Complex neutralizer (also suitable for halogens)
VOLUME OF NEUTRALIZER	9 ml	1 ml
VOLUME FROM TEST SOLUTION INTO NEUTRALIZER	1 ml	1 ml
EXPOSURE PERIOD	0,25; 0,5; 1,0; 2,0; 5,0; 10,0; 20,0; 30,0; minutes	5,0 minutes
RECOVERY MEDIUM	Tryptone glucose extract agar or other	Nutrient agar
TEMPERATURE OF AGAR	45°C	45°C
DILUTIONS PLATED	Yes	No
DILUTION DILUENT	Phosphate buffer	Nil
INCUBATION TEMPERATURE	35°C	32°C
INCUBATION TIME	48 hours	72 hours
SATISFACTORY RESULT PER CENT KILL AND TIME	>99,999% in 30 seconds	>99,9% in 5 minutes

A comparison of the two test methods shows differences of procedure in a number of cases:

1. Culture Media

The use of selective media in the SABS procedure for the propagation of E. coli and Staph. aureus versus a general growth medium.

2. Test Suspension Diluent

The use of phosphate buffer in the Chambers' modification compared to distilled water in the SABS.

3. Bacterial Density

The Chambers' modification requires a very much higher bacterial density not only in the suspension but also in the test solution. It might appear that the higher suspension would give a

very much greater degree of accuracy than that of the SABS method. On the other hand the SABS method requires no dilution which is often accompanied by variations in bacterial count.

4. Holding Temperature for Test Suspension

As both test suspensions are derived from stationary phase culture, it would appear from the opinions already considered, that a low temperature rather than room temperature should favour the viability of the bacterial test suspension.

5. Neutralizer Volume

The SABS neutralizer is a highly efficient neutralizer and is not generally required in greater volumes in relation to the aliquot transferred from the test solution.

6. The Exposure Period

The Chambers' range of exposure periods allows a comparison to be made with the effectiveness of various sanitizer/disinfectant formulations. No comparison is possible in the SABS method as the only exposure period is five mintues; thus no degrees of killing ability can be assessed as in the Chambers' modification.

7. Percentage Kill and Time of Exposure

As sanitizers are called upon to act within relatively short periods, the Chambers' modification requirements appear to be slightly more realistic in both percentage kill and exposure time. The SABS method would appear to be the more lenient of the two methods.

It would appear that, although these methods differ in certain aspects, there is enough general similarity between them to use them as a basis from which to develop a procedure to determine the effect of various diluents in which the bacterial suspension is prepared, on the outcome of an otherwise standardised test to determine the effectiveness of sanitizers.

It is of interest that a number of diluents are used for the bacterial test suspensions in various tests. Besides the two diluents just mentioned, phosphate buffer is used in the A.O.A.C. Method for testing water disinfectants for swimming pools (**Official, First Action**); sterile physiologic saline in the Mallman **Carrier Method as modified** for regulatory sanitizer evaluation; sterile skim milk in the Hoffman,

Yeager, Kaye Method for self-sanitizing surfaces; and sterile physiological saline for the Latlief, Goldsmith, Friedl and Stuart Method for detecting bacteriostasis significant in preventing ammonia formation from urea. In none of the tests mentioned are the test organisms washed before suspension in the specific diluent (A.O.A.C., 1965; Lawrence & Block, 1968; Sykes, 1965).

G. Discussion of the Literature and Conclusions

The bacterial cell has remarkable powers of adaptation to its environment due to its enzymic constitution. However there are factors which influence its growth and other activities. Perhaps of the greatest importance are the circumstances under which the cells are grown in the laboratory in a nutrient environment limited in mass with either the cells being of a single species or the cells being of a mixed population. The latter situation would be that generally found under natural conditions. The investigation of such a mixed culture would present innumberable problems of maintaining proportional numbers. Traditionally therefore, axenically grown cultures have been the subject of investigation.

The stage of growth of the bacterial cells influences the reaction to stress factors; the older stationary phase cells appear to be more resistant than young cells. Thus imposing stress on exponential phase cells may not give a true indication of the resistance capabilities of the species under investigation.

In disinfectant testing it has always been a requisite to select particularly resistant strains of bacteria; this requisite has been based on the premise that death rates of resistant bacterial strains would be reliable indicators of disinfectant potential with respect to other strains of the same type.

Not only must resistant strains be considered, but the treatment of the cells prior to exposure to the disinfectant must not decrease the resistance of the cells, nor must the cells be damaged thereby giving a false indication of disinfectant efficiency.

The growth medium itself should not impose any limitation on the development and growth of the bacterial cell, thus decreasing the cell's ability to withstand stress whether imposed in the form of a disinfectant

diluent or any other factor. It can be assumed therefore that stationary phase cells, grown in nutritionally satisfying complex media, will have a greater resistance to osmotic shock (Herbert, 1961) and to chilling (Strange & Ness, 1963) than younger cells.

Growth at optimum growth temperatures appears to be in the majority of cases reported, a requisite for optimum resistance against heat and disinfectant treatment. Evidence against this is that by Bennett (1959) in the case of Staphylococcus aureus and by White (1951) with Streptococcus faecalis when enhanced resistance to phenols and enhanced temperatures respectively, were described. Growth temperatures below the optimum resulted in Escherichia coli being less resistant to enhanced temperatures (Elliker & Frazier, 1938).

Investigating specific factors influencing the reactions of bacteria invariably requires the harvesting and preparation of bacterial cells. The harvesting of the cells may involve the use of either centrifugation or filtration, or both. These physical stresses have apparently little effect on bacterial cells (Carpenter, 1968; Farwell & Brown, 1971). More significant are the environmental changes involving temperature, pH, tonicity, ionic composition and other factors. These are all involved in the suspension of concentrated cells in a diluent. The function of the diluent being primarily to enable a true assessment to be made of the bacterial population without death or growth of cells taking place during the dilution process and the holding period.

The ability of bacterial cells to survive changes in environment must initially depend on the cell membrane with its powers of selective permeability, and this function varies according to the species and sub-species variation. Under certain circumstances magnesium and calcium ions are able to stabilize cells against changes in osmotic pressures and changes in temperature (Brown, 1964; Upadhyay & Wilks, 1969).

Reports on the effect of diluent on bacterial viability are extremely confusing. It is perhaps in this field that most of the contradictory evidence is to be found; particularly when one considers the various diluents used for disinfectant testing. Theoretically, sodium chloride, because of its prevalence in nature, should in low

concentrations be a highly suitable diluent. The optimum range of NaCl concentration appears to lie between 0,005 M and 0,3 M with a maximum between 0,08 M and 0,1 M (Winslow & Dolloff, 1928; Winslow & Haywood, 1931; Spangler & Winslow, 1943).

Distilled water, of all the diluents, has given the most contradictory results. Ballantyne (1930) found that many bacterial suspensions remained viable for long periods in distilled water. This contradicted Winslow and Brooke's (1927) findings of the lack of viability in distilled water. However Wagenaar and Jezeski (1952) and Vanderzant and Krueger (1968) and others confirmed the original results obtained by Winslow and Brooke. Ballantyne's results were probably due to a carry over of growth medium with the cells, thereby imparting to the distilled water the characteristics of an extremely dilute growth medium. On the other hand, the quality of the distilled water used by the other workers might have included some toxic or growth inhibitory substance as indicated by Geldreich and Clark (1965). The advantages of distilled water as a diluent for bacterial cell suspensions used in a disinfectant-sanitizer test are considerable in that interactions between either diluent and disinfectant or diluent and test solution would be minimised. If distilled water in fact does impose stress on the bacterial cells, then the reliability of a test result would be questionable.

Maintenance of viability can be promoted by the use of suitably diluted organic suspensions, particularly peptone (Jayne-Williams, 1963). However as this diluent may act as a growth medium with an increase in cell numbers at ambient temperatures it would be essential to reduce the holding temperature so that cell division could not occur.

Numerous other diluents besides distilled water and solutions of sodium chloride and peptone have been described, these include Ringer's solution, quarter strength Ringer's solution and phosphate buffered distilled water. These have been subjected to investigation by various workers (Cook & Wills, 1958; Gossling, 1958; Farrell & Rose, 1968) with somewhat contradictory results.

The hydrogen ion concentration of the diluent apparently influences the viability of bacteria. Levels of pH above 8,3 seem to be inhibitory (Winslow, 1934), while survival rates appear to be highest at pH 7

(Straka & Stokes, 1957; 1959). An increase in pH above 7,5 appears to be more deletrious than a decrease below that level (Winslow, 1934).

The temperature at which bacterial cells are held in the diluent has little influence on viability provided the temperature lies between the optimum growth temperature and 0°C and the cells are derived from stationary phase cultures. A low holding temperature is a requisite in the case of diluents with a growth promoting potential such as peptone. In order to eliminate one cause of differences in results, the holding temperature for all bacterial suspensions should be the same and be below optimum growth temperatures.

The density of cells in suspension is a highly significant factor with regard to cell viability. Increased cell density leads to improved cell survival (Ryan, 1959; Harrison, 1960; Postgate & Hunter, 1963), apparently due to the permeability of the cell membrane; thus the loss of the contents of some of the bacterial cells to the surrounding medium, exerts a protective action on the remainder. The remaining viable cells either survive or die at a reduced rate. The cell concentration required to achieve this protective effect appears to be not less than 10^7 cells per ml (Spangler & Winslow, 1943).

The formulation of the test solution, to which the disinfectant-sanitizer is added, should give conditions comparable with those that might exist under practical use conditions. Controlled quantities of organic matter and of the salts that contribute to water hardness, should be added to distilled water.

For the purposes of this study a compromise is made between the Chambers' Modification of the Weber and Black Method for Sanitizers and that of the South African Bureau of Standards' Standard Specification for Quaternary Ammonium Compounds; further in order to obviate certain aspects of these two methods which are contrary to the literature - such as harvesting direct from slopes with the inherent carry over of media constituents - certain changes will be made to the accepted procedures.

In order to minimise the delay between harvesting and the initiation of the test, the determination of optical density and the filtration of the suspension through filter paper will not be carried out.

The post-disinfection recovery factors are as critical as the pre-disinfection procedures. These post-disinfection factors include the immediate and complete inactivation or neutralization of the disinfectant at the end of the exposure period. This inactivation may be accomplished by a simple dilution procedure which reduces the disinfectant concentration to non-toxic levels. The still viable cells are then in a position to recover and grow. This procedure is not applicable to some disinfectants however, which require active neutralization. The neutralizers used may be either of a simple or complex nature; the main criterion being that the neutralizer shall not impose further stress on near-moribund cells thereby reducing the apparent number of survivors.

Two general methods of survivor determination are in vogue: a qualitative and a quantitative method. Where the mere presence of survivors is to be determined, the qualitative broth culture method will show evidence that survivors are present. To determine actual numbers of survivors, an agar plating procedure must be adopted.

Due to the varying degree of damage suffered by treated cells, the recovery-growth medium must provide optimum conditions, so that all cells, except those actually killed, may recover and multiply. These conditions generally appear to be found when a complex medium is utilised which provides specific peptides contributing to bacterial recovery. The temperature of the recovery medium is extremely critical in that high temperatures (in excess of incubation temperatures) may retard recovery, thus control of the plating medium temperature must be within narrow limits; further, the recovery incubation period and temperatures should be such as to allow growth of the still viable cells.

The disinfectants applicable to the dairy industry are relatively limited. The majority of common disinfectants such as the phenol and coal-tar derivatives cannot be considered as they affect the flavour and odour of milk. Applicable disinfectants are those based on chlorine, iodine and quaternary ammonium compounds. Other suitable disinfectants such as the amphoteric compounds, strong alkalies and phosphoric acid wetting agents have only limited application due either to problems of use or, of cost.

Chlorine was the first commonly accepted dairy disinfectant and remains widely used to this day. Chlorine and its derivative compounds have been developed to deal with problems associated with organic matter and the corrosive tendencies, and have for these reasons been the subject of considerable investigation and research. The other two disinfectants which may be basically considered as detergent-sanitizers, are the iodophors and the quaternary ammonium compounds. Their application in the general disinfection-sanitization field developed mainly during the period 1935 to 1950, whereas chlorine has been used from the early 19th Century. In view of these considerations it was deemed appropriate that these investigations should be based on representative samples of the iodophor and the QAC types of detergent-sanitizer. The mode of action of these two disinfectants is totally different and would therefore be suitable for assessing the factors involved in this series of investigations.

A perusal of the literature suggests that the most contraversial aspect of disinfectant or sanitizer tests appears to be that involving the suspension of the bacterial cells in diluent after harvesting. In order to derive the most information regarding the effects of diluents on the reaction of the bacterial cell to the disinfectants, it is essential that the conditions of harvesting and suspension should be uniform throughout the trials, and that all other factors be as strictly controlled as possible.

It is considered relevant to investigate the role of diluent in disinfectant trials because of the widely differing diluents at present in use in 'official' disinfectant test procedures. The fact that a common diluent is not used for all test procedures leads to the obvious conclusion that the diluent effect on the outcome of the tests must be of some consequence. Further, that the use of a 'universal' diluent would probably lead to greater accuracy and to a reduction in inter-laboratory variation. In addition, if diluents have an effect, then the diluent that is the most consistent under the majority of conditions should be the one of choice.

The diluents to be subjected to investigation are those most often stipulated in disinfectant and sanitizer test procedures - phosphate buffered water and distilled water - as well as some of the traditional diluents such as sodium chloride solution, Ringer's solution, quarter strength Ringer's solution and peptone water.

CHAPTER IV

MATERIAL AND GENERAL EQUIPMENT

A. Glassware

1. Preparation of Glassware

(a) New glassware

Glassware on arrival was washed in a detergent[1], rinsed twice in tap water followed by boiling submerged in distilled water in a stainless steel container for 30 minutes. This was followed by rinsing twice in distilled water. The glassware was then drained and air dried while inverted on suitable trays.

(b) Used glassware

Immediately after use, all glassware was rinsed in tapwater, soaked for two hours in a hot detergent solution[1] and thoroughly brushed and washed. Glassware that had been in contact with unneutralized QAC was boiled in the detergent.

Following washing, or washing and boiling in detergent, the glassware was rinsed twice in tapwater and twice in distilled water, then allowed to drain and air dry.

This procedure was developed after preliminary trials to determine whether possible QAC or iodophor residues could result in the inhibition of Aerobacter aerogenes and Staphylococcus aureus. The use of glassware, rinsed with 2 g/l of cetyltrimethylammonium bromide, then washed, rinsed, drained and air dried in the described procedure, resulted in no reduction in plate counts compared with counts of the organisms cultured in new glassware prepared as in (a) above.

2. Sterilization of Glassware

Sterilization of glassware was either by hot air at 180°C for 2 hours after attaining 180°C, or in the autoclave at 121°C for 15 minutes. Glassware was either covered by aluminium foil or placed in suitable containers e.g. pipettes in pipette canisters, and then kept covered until used.

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[1] Wyandotte Poma. S.A.Scale Co.

3. Description of Glassware

Borosilicate glassware was used, except in the case of media, diluent, test and culture bottles which were of soda glass.

(a) Bottles

All bottles were fitted with rubber lined metal screw caps.

(i) Media bottles Medical flats with a capacity of ± 454 ml (16 fl. ozs) were partially filled with not more than 300 ml medium prior to autoclaving.

(ii) Test and diluent bottles Medical flats with a capacity of ± 230 ml (8 fl. ozs) were partially filled with not more than 105 ml liquid prior to autoclaving.

(iii) Culture bottles Wide mouth McCartney bottles of 28 ml (1 fl. oz) capacity were partially filled with 10 ml broth or agar medium prior to autoclaving.

(b) Pipettes

Undamaged bacteriological type pipettes, calibrated to deliver the various capacities required, when the last drop was blown out or touched out, were used.

(c) Other glassware

Other glassware, such as volumetric flasks, measuring cylinders, were used as required after treatment as described in A. 1.

B. Petri Dishes

For the major studies only pre-sterilised plastic single service petri dishes were used. Plastic petri dishes were found to be more suitable than glass dishes, as they gave a greater number of countable plates. This was due to a remarkable reduction in spreader colonies. Glass petri dishes were used in the initial studies not involving disinfectants.

C. Autoclave

A large capacity autoclave supplied by a saturated steam line was available. Instrumentation allowed semi-automatic operation which contributed to the standardization of autoclaving procedure. This was important in the production of standardized volumes of test solutions after autoclaving.

D. Hot-air Sterilizing Oven

A fan equipped, hot-air sterilizing oven was used for the sterilization or drying of glassware. The temperature-time combination for sterilization was 180°C for 2 hours after the attainment of 180°C, while for drying, a maximum temperature of 80°C for 6-8 hours was used for bottles and rubber-lined screw caps.

E. Liquid Dispenser

An automatic liquid dispenser[1] was used to dispense pre-set volumes of liquid with a 0,1 per cent accuracy.

F. Colony Counter

Counting was done manually on an illuminated colony counter[2] fitted with an electronic tally device actuated by a handheld probe.

G. Incubators

Three top-vented incubators were maintained at 35°C ± 2°C. Shelves were arranged for the stacking of not more than three layers of petri dishes, allowing at least 2,5 cm around, above and below each stack of 3 plates.

H. Water Baths

A thermostatically controlled water bath maintained at 45°C ± 1°C was provided to temper melted media prior to plate pouring; another maintained at 22,5°C ± 0,5°C was provided for the disinfectant-test solution bottles.

I. pH Meter

This meter, fitted with an extended scale and an mv scale was used for pH and certain titration procedures.

J. Stop-clock

A laboratory type stop-clock calibrated in minutes and seconds, was used for timing experiments.

K. Centrifuge

A centrifuge[3] was used at a resultant centrifugal force of 6 to 9,75 kN/kg for 5 minutes in the preparation and washing of the bacterial suspensions. Autoclavable polypropylene centrifuge tubes of 50 ml capacity were used when sterile conditions were required.

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[1] Baltimore Biological Laboratory Inc., Maryland, U.S.A.
[2] Model C-110; New Brunswick Scientific Co., New Jersey, U.S.A.
[3] Sorval SS-1.

CHAPTER V

MEDIA, SOLUTIONS AND SUSPENSIONS

A. Distilled Water

Distilled deionised water with resistivity exceeding 4 MΩ/cm was used when distilled water was specified. It complied with the requirements laid down by Geldreich and Clark (1965) and the American Public Health Association (1967).

B. Reagents

All reagents used were of analytical reagent grade.

C. Dehydrated Media

Difco dehydrated media[1] were used, and whenever possible, material from the same batch was used throughout the work.

1. Tryptic Soy Broth Difco (TSB)

This broth, prepared according to the manufacturers' instructions, was used for the preparation of experimental cultures.

2. Tryptic Soy Agar Difco (TSA)

This agar was used for the determination of bacterial survivor counts, and for the preservation of stock cultures by freezing.

D. Hard Water

Hard water was prepared according to the method prescribed by the South African Bureau of Standards (S.A.B.S., 636-1971). A solution of 280 mg anhydrous calcium chloride in 100 ml distilled water, was diluted to 1 000 ml. When 97 ml of this water was diluted to 100 ml, i.e. by the addition of 3 ml distilled water, the final dilution had a hardness of approximately 280 p.p.m., calculated as calcium carbonate. Hard water was dispensed in 101 ml volumes to yield a final volume of 97 ml $\pm$ 0,1 ml after standardized autoclaving.

E. Skimmed Milk

In a high speed blender, 50 g spray-dried skimmed milk powder was blended with 400 ml distilled water for 5 minutes, and then diluted to 500 ml. The solubility of the milk powder was acceptable when the

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[1] Difco Laboratories, Detroit, Michigan, U.S.A.

reconstituted skimmed milk showed no visible centrifugate after centrifuging at a resultant centrifugal force of 6 kN/kg for 5 minutes.

The reconstituted skimmed milk was dispensed in 10 ml volumes into 28 ml McCartney bottles; autoclaved for 10 minutes at 121°C, then stored at 4°C.

1. Determination of Skimmed Milk Suitability

The skimmed milk was assessed for inhibitory substances by the following procedure: A volume of 0,1 ml of an 18 hour broth culture of Staph. aureus was spread with an alcohol-flamed glass "hockey stick" over the surface of a previously poured TSA plate. A 13 mm sterile antibiotic assay disc (Whatman) was dipped into the sterilized reconstituted skimmed milk, allowed to drain, and placed on the surface of the cultured agar. The inverted plate was incubated at 35°C for 24 hours. Inhibition of growth around the assay disc would indicate the presence of an inhibitory substance and the rejection of that skimmed milk powder batch. This procedure was repeated for E. coli. The assay was triplicated in each case.

F. Diluents

In all, six diluents were prepared for the suspension of the micro-organisms used in these studies. The diluents were dispensed in 104 ml volumes by the automatic liquid dispenser, the screw caps were loosely applied and the bottles were autoclaved at 121°C for 15 minutes. Immediately after autoclaving the screw caps were tightened and the bottles were stored at room temperature on shelves out of direct sunlight.

1. Distilled Water

This was prepared as described.

2. Physiological Saline

The saline diluent was prepared by dissolving 8,5 g NaCl in 500 ml distilled water, then diluting to 1 000 ml with distilled water.

3. Ringer's Solution

The composition of the Ringer's solution used was:

NaCl	9,00 g
KCl	0,42 g
$CaCl_2$	0,24 g
$NaHCO_3$	0,20 g
Distilled water	1 000 ml

(Davis, 1963)

4. Quarter Strength Ringer's Solution ($\frac{1}{4}$ Ringer's)

The formulation was similar to that for Ringer's solution except that the distilled water was increased from 1 000 ml to 4 000 ml.

5. Phosphate Buffered Distilled Water

(a) Phosphate buffer stock solution, 0,25 M

A mass of 34 g KH_2PO_4 was dissolved in 500 ml distilled water, adjusted to pH 7,2 with 1 N NaOH and diluted to 1 litre. The solution was dispensed in 15 ml volumes into 28 ml McCartney bottles and sterilized at 121°C for 15 minutes. The bottles were then stored in a refrigerator.

(b) Phosphate buffered distilled water

A volume of 1,25 ml phosphate buffer stock solution was diluted to 1 litre with distilled water.

6. Peptone Water

A mass of 1 g Difco Peptone was dissolved in 500 ml distilled water and diluted to 1 000 ml with distilled water.

G. Neutralizer Solution

The neutralizer solution was prepared from the following reagents:-

Mono-potassium phosphate (KH_2PO_4)	0,5 g
Sodium citrate ($Na_3C_6H_5O_7.3H_2O$)	0,5 g
Sodium taurocholate	8,0 g
Sodium thiosulphate ($Na_2S_2O_3.5H_2O$)	1,5 g
Sorbitan mono-oleate complex (Difco Tween 80)	8,0 g

(S.A.B.S., 1971)

The reagents were dissolved in 500 ml distilled water by heating, the pH was adjusted to 7,2 and finally the volume was diluted to 1 000 ml. Volumes of 100 ml were dispensed into 230 ml bottles, and after autoclaving, the neutralizer was stored at room temperature on shelves out of direct sunlight.

1. Toxicity of Neutralizer

A volume of 1 ml of an 18 hour culture of Staphyloccus aureus in TSB was pipetted into a 100 ml of neutralizer. After incubation at 35°C for 72 hours, the solution was assessed for any growth that might have taken place. The procedure was repeated with Escherichia coli. In both cases growth was apparent at the end of the period.

2. Effectivity of the Neutralizer

The effectivity of the neutralizer was determined for the QAC and the iodophor sanitizers as follows:

(a) Neutralization of QAC

A bacterial suspension was prepared by centrifuging and washing 1 ml of an 18 hour Staphyloccus aureus culture in phosphate buffered distilled water. The washed cells were then suspended in 100 ml phosphate buffered distilled water and shaken vigorously.

At the same time a 200 mg QAC/l solution was prepared in distilled water. A volume of 5 ml of the QAC solution was added to 5 ml of neutralizer. Within 15 seconds, 1 ml of the Staphyloccus aureus suspension was added to the neutralizer-sanitizer mixture. The solution was mixed by gentle shaking, and serial dilutions were plated in TSA. After 5 minutes a further series of serial dilutions were plated in TSA.

The procedure was repeated with Escherichia coli.

(b) Neutralization of Iodophor

A 25 mg available iodine/litre solution was prepared in distilled water. The procedure followed was as detailed in (a) above. No significant differences were found between the first and second platings.

H. Test Solutions

Test solutions were dispensed into 230 ml bottles, in volumes such that the final volume after autoclaving was 97 ml $\pm$ 1 ml. The following test solutions were dispensed:-

1. Distilled Water

Prepared as previously described.

2. Hard Water

Prepared as described.

I. Sanitizer Solutions

Sanitizer solutions were prepared in distilled water such that the final concentration, when 1 volume was diluted to 100 volumes in the test solution, would be that required for the specific sanitizer.

1. Quaternary Ammonium Compound Solutions (QAC)

The QAC used in these trials was not a commercial formulation containing various additives, but the 'pure substance' N-cetyl-N_1N_1N-trimethylammonium bromide ($C_{19}H_{42}BrN$) Guaranteed Reagent[1] M M 364,46, with a minimum assay of 99 per cent.

2. Iodophor Sanitizer

A commercially prepared Iodophor[2] was obtained with the following characteristics:

Composition: Polyoxyethylated polyoxyalkylated Alkyl Phenol Iodine Complex.

"Quoted" Available Iodine: 1,75%

Determined Available Iodine: 1,68% m/m (1,69% m/v)

Strong acid content expressed as sulphuric acid: 0,3% m/m.

This iodophor had high stability characteristics in concentrate form and was relatively low foaming.

After being well mixed, the iodophor concentrate was decanted from the shipping container into a ground glass stoppered $2\frac{1}{2}$ litre bottle. It was presumed that a more stable concentrate could be maintained in a glass rather than in a plastic container.

(a) Analytical Methods

(i) Available iodine Available iodine was determined by potentiometric titration with standard sodium thiosulphate using a pH meter provided with a millivolt scale, a platinum/calomel electrode system and a magnetic stirring device (Vogel, 1953).

For determining the available iodine on a mass/mass basis, about 1 gram of the iodophor was weighed accurately into a 100 ml beaker,

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[1] E. Merck, Darmstadt

[2] Cooper & Nephews, (S.A.) Ltd.

50 ml distilled water was added and the resulting solution titrated with 0,02 N sodium thiosulphate.

For determinations on a mass/volume basis, 1 ml of the sample was pipetted out as accurately as possible. Due to the usually high viscosity of iodophors, pipetting was not easily performed.

The available iodine was calculated according to the formula:

$$\text{percentage available iodine} = \frac{\text{volume} \times \text{normality of } Na_2S_2O_3 \times 12.69}{\text{mass of sample (or volume of sample)}} \%$$

(ii) Acid content Acidity was determined by potentiometric titration. It was necessary to use two equivalent points: pH 4,4 for the estimation of strong acids as sulphuric acid and pH 8,8 for the estimation of weak acids as phosphoric acid. (pH 8,8 corresponds to the conversion of monosodium dihydrogen phosphate to disodium monohydrogen phosphate).

About 1 gram of iodophor was weighed accurately into a beaker to which 50 ml distilled water was added and the solution was potentiometrically titrated against 0,1 N sodium hydroxide solution. The titrant volume at pH 4,4 (T1) was noted and the titration continued to pH 8,8 (T2). The weak acid content was calculated acccrding to the formula:

$$\text{percentage weak acid (m/m) (as phosphoric acid)} = \frac{T_2 - T_1 \times \text{normality of NaOH} \times 0,98}{\text{mass of sample}} \%$$

Where T_1 exceeded $(T_2 - T_1)$ the presence of strong acids was assumed. The strong acid content was calculated according to the formula:

$$\text{percentage strong acid (as sulphuric acid) (m/m)} = \frac{2(T_1 - T_2) \times \text{normality of NaOH} \times 0,98}{\text{mass of sample}} \%$$

CHAPTER VI

BACTERIAL CULTURES AND PROCEDURES

A. Test Organisms

1. Origin of test organisms

The test organisms used in these studies were obtained as freeze-dried cultures from the South African Type Culture Collection maintained by the South African Bureau of Standards, Private Bag 191, Pretoria.

The cultures were as follows:-

(a)	*Aerobacter aerogenes*	SATCC AER 5
(b)	*Escherichia coli*	SATCC ESC 25
(c)	*Micrococcus flavus*	SATCC MCR 25
(d)	*Pseudomonas aeruginosa*	SATCC PSE 2
(e)	*Staphylococcus aureus*	SATCC STA 53

2. Stock cultures of the test organisms

From the freeze-dried cultures, tryptic soy broth cultures were propagated and incubated at 35°C for 24 hours. After two further transfers into the same medium and incubation for the same period, a 0,1 ml volume of a 24 hour culture was inoculated into 10 ml TSB or onto TSA slopes, then stored and frozen in a chest-type deep-freeze cabinet at -12°C.

3. Verification of organisms

All the organisms were subjected to microscopic examination under phase-contrast and stained conditions. The Gram reaction was determined for all the organisms.

Various biochemical tests were carried out on the organisms to determine if they were 'true to type'. A number of these tests were repeated at varying periods to assess whether contamination had taken place. Further, representative colonies were removed from plates counted, to ensure that the colonies were derived from test organisms and not from contaminants.

The identification characteristics were as follows:

Aerobacter aerogenes: A Gram-negative rod; Simmon's citrate positive; methyl red negative; indole negative; Voges-Proskauer positive and Eijkman negative.

Escherichia coli: A Gram-negative rod; Simmon's citrate negative; methyl red positive; Voges-Proskauer negative; indole positive and Eijkman positive.

Micrococcus flavus: A Gram-positive coccus; oxidises glucose and produces acid from glucose; no reduction of mannitol in salt agar; coagulase negative; catalase positive.

Pseudomonas aeruginosa: A motile Gram-negative rod, producing a yellow-green pigment on agar.

Staphylococcus aureus: A Gram-positive coccus; coagulase positive; catalase positive; ferments mannitol in salt agar; SATCC Sta 53 is phage type 80-81 (human) (Stiles, 1969).

It is not considered that the characteristics enumerated above are exhaustive; however they were deemed sufficient for the identification of the micro-organisms used in these trials. The characteristics mentioned were capable of ready testing in this laboratory, and procedures were as described by Harrigan and McCance (1966).

4. Experimental cultures and suspensions of the test organisms

Frozen stock cultures were rapidly thawed when required, then incubated for 24 hours. These cultures were subcultured, in TSB twice, before use. The experimental culture was prepared by inoculating 1 loopful of the final 24 hour culture from the frozen stock culture, into 10 ml TSB and this was incubated for 20 hours $\pm \frac{1}{2}$ hour at 35°C. This time of incubation was chosen because growth studies had shown that, at 20 hours, the organisms had entered the stationary (rest) phase, but had not advanced so far within that phase that any significant change in viable population would have occurred.

Half an hour before an experiment was commenced either 1 ml or 5 ml of the 20 hour TSB culture was pipetted into a sterile centrifuge tube; to the centrifuge tube was added 40 ml of the selected diluent. A volume of diluent equal to 40 ml plus the volume of culture was

pipetted into the opposing centrifuge tube for balancing purposes. After centrifuging for 5 minutes at a resultant centrifugal force of 9,75 kN/kg the supernatant was poured off and fresh diluent was added. A further period of 5 minutes centrifugation was allowed. The supernatant was again poured off and the cells were suspended in approximately 100 ml of the same diluent. The suspension was vigorously shaken making at least 25 complete up and down movements of about a foot in not more than 7 seconds.

Initially sterile glass beads were included in the diluent bottle to facilitate obtaining a clump-free suspension of cells. However it was found that equally good results were obtained when the glass beads were excluded.

In order to minimise the time between suspension of the cells and the inception of the experimental period, it was necessary to forego filtration of the suspension and its optical density determination.

A 1 ml volume of the 20 hour TSB culture after centrifugation and suspension in the diluent, furnished a suspension in the region of 2 to 3 x 10^5 organisms/ml while the suspension derived from 5 ml of TSB culture generally gave a suspension in the region of 2 to 4 x 10^7 organisms/ml.

The suspension was stored in a cracked ice/water mixture for the duration of each experiment.

For each replication of an experiment an experimental culture of the appropriate organism was subcultured and prepared, as detailed from a fresh frozen stock culture. This was considered to minimise possible changes in the characteristics of the organisms due to continuous subculturing in broth. Frozen stock cultures were prepared in sufficient quantities to conclude a series of experiments. The frozen stock cultures of all the organisms remained viable for over a year.

5. Dilution procedure

In all cases where dilution was required to prepare plates with a total number of colonies lying between 30 and 300 per plate, the procedures were as described in the Standard Methods for the Examination of Dairy Products, 12th Edition (A.P.H.A., 1967).

6. Identification of plates

All plates were completely identified with details of the experiment, the micro-organism, the time and dilution, as well as any other pertinent information that might be required in a particular experiment.

7. Plating methods

All dilutions were plated in triplicate. Melted TSA maintained at 45°C ± 1°C was poured in volumes of 10-12 ml immediately after the respective dilutions had been pipetted, into the plate, while slightly lifting the lid of the Petri dish. As each plate was poured the TSA and the pipetted aliquot were mixed by swirling the plates in a series of figure-of-eight movements, carefully avoiding splash on the lid or over the edge. The plates were allowed to solidify on a level surface and after solidification, they were inverted and placed in the incubator in three-plate stacks.

8. Incubation of plates

All plates were incubated at 35°C for 48 hours in the incubators previously described.

9. Colony counting

The selection and counting of the triplicate plates was carried out as detailed in the Standard Methods for the Examination of Dairy Products (Ed. 12) (A.P.H.A., 1967). The arithmetic mean of the three appropriate counts were used in the subsequent calculations and statistical analyses.

10. Replication of experiments

Each individual experiment was replicated at least three times; each replicate experiment was conducted on separate days in order to avoid any specific experimental error.

CHAPTER VII

THE INFLUENCE OF DILUENT ON THE VIABILITY OF SMALL BACTERIAL POPULATIONS

A. Introduction

The contradictory reports on the survival of bacterial populations in various diluents suggest an avenue of investigation, especially as certain disinfectant testing procedures require the use of low bacterial numbers; hence small differences in actual numbers, which are attributable to the effects of diluents, may have a marked effect on the test results. One such testing method is that of the South African Bureau of Standards, SABS 636-1971, in which the bacterial test suspension is diluted until it contains 100 000 $\pm$ 10 000 organisms per millilitre. The final test solution would thus initially contain 1 000 $\pm$ 100 organisms per millilitre on the basis that 1 ml of bacterial suspension is added to 99 ml of test solution.

The majority of workers showed that small bacterial populations are unable to survive without loss of viability in most of the common diluents.

It was decided therefore to investigate the influence of six commonly recommended diluents - distilled water, Ringer's solution, quarter strength Ringer's solution, ($\frac{1}{4}$ Ringer's), phosphate buffered distilled water, physiological saline, and peptone water - on the viability of the following organisms:-

Aerobacter aerogenes
Escherichia coli
Micrococcus flavus
Pseudomonas aeruginosa
Staphylococcus aureus

The hydrogen ion concentration having been considered pertinent in the survival of bacterial suspensions (Winslow, 1934; Spangler & Winslow, 1943), representative samples of the diluents were subjected to pH determination. The mean pH values are given in Table 7.1. together with the ranges found during the series of trials.

TABLE 7.1. Mean and range of pH values of diluents

Diluent	Mean pH	Range
Distilled Water	6,8	6,67 - 6,98
¼ Ringer's	7,0	6,89 - 7,23
Ringer's	7,2	7,11 - 7,29
Phosphate buffer	6,9	6,83 - 6,98
Peptone	7,2	7,17 - 7,28
Saline	7,0	6,88 - 7,15

The pHs involved would appear to conform very closely to the optima discussed by Cook and Wills (1958) and Straka and Stokes (1957 & 1959). Although the pH of distilled water was uniformly below pH 7,0 and below the mean of the other diluents, it is unlikely that the lower pH of distilled water would be the factor involved in the generally lower survival rates of the micro-organisms in this diluent.

B. Experimental Procedure

A volume of 1 ml of a 20-hour culture (propagated as already described), was centrifuged and washed in the diluent under consideration. Dilutions in the same diluent were made in order to produce a test suspension containing approximately 100 000 to 200 000 bacterial cells per millilitre. The test suspension was placed in cracked ice and water for the duration of the six-hour experiment.

Immediately after preparation, a 1 ml aliquot of the well-shaken test suspension was pipetted into 99 ml of the same diluent held at ambient temperature (20-23°C). Aliquots of 1 ml and 0,1 ml, from the 1 in 100 dilution, were each plated in triplicate.

At 3 hours after the preparation of the test suspension, a further 1 ml aliquot was pipetted from the test suspension into 99 ml of the same diluent. This procedure was repeated again at 6 hours.

For each diluent and each bacterial species, the above procedure was repeated on three separate days. There were thus 9 readings at zero hours, 9 at 3 hours and 9 at 6 hours.

C. Calculations

The arithmetic mean count for each species in each diluent after 3 and 6 hours was expressed as a percentage of the arithmetic mean

of the initial count at 0 hours.

An analysis of variance utilising the percentage survivors, as input data, was calculated for each organism. Each analysis of variance consisted of six 'diluents' and two 'times' (3 and 6 hours after original suspension). The values of the initial suspension density were not included as, by definition, they were equivalent to 100 per cent.

In those analyses of variance where the application of the F-test indicated the presence of means of discrepant magnitude either at the $P < 0,01$ or $P < 0,05$ levels, Duncan's Multiple Range Test was applied. The results of these tests of significance and the analyses of variance are given in Appendix A, Computations 1-5.

D. Results and Discussion

These trials investigated the survival of several bacterial species in the selected diluents over a period of six hours.

TABLE 7.2. Bacterial population of test suspensions determined at initial time (0 hours)

Micro-organism	Bacteria/ml Mean	S.D.
Aerobacter aerogenes	245 516	± 81839
Staphylococcus aureus	236 950	± 69162
Pseudomonas aeruginosa	294 194	± 79530
Micrococcus flavus	198 666	± 36567
Escherichia coli	187 039	± 62502

The populations enumerated have a mean density of $2,3 \times 10^5$/ml, which in consideration of the populations mentioned in the literature could be considered relatively small. This population, bacteriologically speaking, is comparable with that required in the test suspension of the Standard Specification for Quaternary Ammonium Compounds (South African Bureau of Standards). It is considerably less than that specified by the Chambers' Modification of the Weber and Black Method for Sanitizers (Chambers, 1956), viz. 1×10^{10}/ml.

In order to reduce the time between harvesting and the initiation of the trial, the determination of optical density was not considered. Though the variation in population/ml might have been reduced in the

test suspension by adopting such a procedure and then diluting the suspension to a constant density, it was considered that control of pre-test procedures would provide suitable test suspensions, and provide a more accurate assessment of diluent effect. The results of Table 7.2. show that an acceptably consistent population level was achieved, Variation in the test solution population was minimised by strict control of the following pre-test procedures:

age of culture,
incubation temperature,
volume of culture aliquot centrifuged,
centrifuging procedure,
washing procedure,
suspension procedure,
dilution procedure, and,
plating procedure.

Initially variations in these factors resulted in plates with either insufficient colonies or plates with colonies too numerous to count i.e. outside the range of 30 to 300 colonies per plate. Once the procedure had been standardised, laboratory errors seldom caused the rejection of trial results.

The results (Appendix A, Calculation 1-5) demonstrate that the survival of relatively low cell populations is closely related to the diluent used for the suspension of bacteria after harvesting, except in the case of Staph. aureus.

Table 7.3. and Appendix A show that the diluent most consistently giving the highest survival rate was 0,1% peptone which is in agreement with the findings of Jayne-Williams (1963). This is not surprising as peptone is of organic origin and capable of promoting growth at ambient temperatures. Though not statistically different from any other diluent in the case of Staph. aureus, peptone still ranked second to Ringer's solution.

Distilled water, except in the case of Aer. aerogenes and Staph. aureus, appeared to be the least suitable for the survival of the other three micro-organisms tested. Although the distilled water, used in this series of experiments, was of a consistently high quality, and was

therefore very unlikely to contain any growth promoting or toxic substances, it was not capable of maintaining the viability of low density bacterial populations. The results are in general agreement with those of Winslow and Brooke (1927), Wagenaar and Jezeski (1952) and Vanderzant and Krueger (1968).

Of the other diluents, no conclusion could be made as their mean efficiencies were distributed irregularly between those of peptone and distilled water. Surprisingly, saline, although not significantly different from the other diluents, had the lowest mean survival value for Staph. aureus, a relatively salt tolerant organism.

Ringer's and ¼Ringer's were very similar in effectivity. In only one instance (Aer. aerogenes) did these diluents differ statistically at either P <0,01 or P <0,05. It would thus appear that either of these diluents could be selected with little material significance.

Phosphate buffer ranked equal to peptone in the case of three of the organisms, and once with distilled water (with E. coli). Saline as a diluent would appear to be slightly inferior to phosphate buffer.

In all cases the bacterial test suspension was held in cracked ice and water for the six hour duration of each experiment. Experience had demonstrated that the ability of a number of micro-organisms to grow in peptone at ambient temperature would have precluded its use as a diluent if this precaution had not been taken. As this was necessary in the case of peptone all the suspensions were maintained in containers surrounded by cracked ice and water for comparative purposes.

In all cases there were significant changes in the percentages of viable survivors between 3 and 6 hours after initial suspension. The ability of low density bacterial populations to remain fully viable for a lengthy period would appear to be limited in the generally recommended diluents. Peptone might be suitable at ambient temperature, provided that the suspension was not held long enough to allow significant growth to occur.

The diluents investigated by no means exhaust the variety of diluents available; milk for example, would probably be equally as effective as peptone. As stated, of the diluents investigated, peptone was indicated to be the most suitable for the micro-organisms

represented. The least suitable diluent under the experimental conditions was distilled water. The question therefore arises whether distilled water can be considered the most suitable medium for the preservation of the viability of the specific bacterial species utilised in the South African Bureau of Standards test procedure. In the case of Staphylococcus aureus no problem should arise as there was no significant difference between diluents. In the case of Escherichia coli, distilled water, though not differing significantly from either phosphate buffer or saline ($P < 0,05$) was inferior to quarter strength Ringer's solution, Ringer's solution and peptone.

TABLE 7.3. The influence of diluent on five micro-organisms over periods of 3 and 6 hours expressed as percentages of initial numbers.(1)

ORGANISMS	TIME (hours)	DILUENTS (2)					
		D.W.	R.	¼R.	P.B.	Sal	Pep
		%	%	%	%	%	%
Aer. aerogenes	0	100	100	100	100	100	100
	3	71,8	83,3	56,6	90,6	73,6	111,1
	6	60,5	81,4	42,7	78,4	57,4	77,7
Staph. aureus	0	100	100	100	100	100	100
	3	84,5	77,0	67,9	74,8	77,9	69,2
	6	43,4	56,7	61,0	55,6	47,2	62,3
Pseud. aeruginosa	0	100	100	100	100	100	100
	3	13,1	78,9	62,0	36,3	40,3	114,1
	6	9,9	70,2	48,1	29,9	33,0	65,5
Micrococcus flavus	0	100	100	100	100	100	100
	3	60,2	72,6	66,1	87.5	82,5	102,9
	6	19,2	70,9	59,5	86,8	77,4	96,8
E. coli	0	100	100	100	100	100	100
	3	57,8	92,8	77,3	75,9	76,2	100,7
	6	41,1	78,5	74,4	59,9	60,0	115,6

(1) vide Appendix A, Computations 1-5 for analysis of variance.

(2) D.W. = Distilled Water
¼R = ¼ Ringer's
Sal = Saline
R. = Ringer's
P.B. = Phosphate Buffer
Pep = Peptone

CHAPTER VIII

THE INFLUENCE OF DILUENT ON THE REACTION OF BACTERIA TO DISINFECTANTS AND SANITIZERS

A. Introduction

The results of the previous experiment (Chapter VII) showed that the diluents investigated were unable to maintain the viability of low density bacterial populations over a period of six hours. They indicated that, at three hours after suspension, the mean viability of the micro-organisms was considerably less than at initial time.

It is apparent from the literature reviewed, that the loss of viability may be reduced when the population density is increased. However a certain degree of stress may still be present, and though viability might not be affected, the level of resistance of the bacterial cells to other forms of stress may well be affected.

The effect of diluent on the reaction of the micro-organisms to a disinfectant could be due either to an interaction between the diluent and the disinfectant or between diluent and bacterium. Should there be an interaction between diluent and disinfectant, then the possibility exists of an increase or a decrease in the efficacy of the disinfectant. A reaction between the diluent and bacterial cell could either result in a protective system developing in or around the bacterial cell against the disinfectant, or the diluent could be directly detrimental to the cell.

As previously stated, various diluents are specified for disinfectant or sanitizer tests; could it be that diluents affect the outcome of the tests? If the diluent plays no role in the result of a test, then it would be logical to restrict the selection of a diluent to the simplest type available.

The choice of micro-organisms for the tests also varies, but it would appear that at least two species are normally required. The species utilised are apparently of widely divergent character. One of the species differences often applied is the Gram-stain reaction; Gram-negative species often reacting differently to those that are Gram-

positive. This factor is obviously pertinent in the case of many Quaternary Ammonium Compounds.

In order that a test may carry the stamp of validity, the conditions imposed during the test must approximate those found under use conditions; for example, the presence of organic matter is generally a condition found in the application of disinfectants under practical conditions. Another situation often found in practice is that the water, used for making up the disinfectant solutions, may contain certain compounds which contribute to water hardness. These compounds may affect the efficacy of the disinfectant.

Thus for the purposes of disinfectant tests a number of factors must be considered; these include:

1. the bacterial species,
2. the disinfectant or sanitizer,
3. the test solution, and
4. the diluent.

The following trials were designed to investigate the influence of six diluents on the reaction of Escherichia coli and Staphylococcus aureus, over a period of three hours, to either a Quaternary Ammonium Compound or an iodophor in test solutions of distilled water, hard water and hard water plus skimmed milk. The six diluents used were distilled water, Ringer's solution, quarter strength Ringer's solution ($\frac{1}{4}$ Ringer's), phosphate buffered distilled water, physiological saline and peptone solutions.

B. Experimental Procedure

1. Bacterial test suspensions

The test suspensions of Escherichia coli and Staphylococcus aureus were prepared as described in Chapter VI. A suspension prepared by centrifugation, washing and suspension of the cell concentrate of E. coli in 99 ml of the selected diluent, normally provided 6×10^7 cells per ml and in the case of Staph. aureus, 2×10^7 cells per ml.

2. Disinfectant/Sanitizer Solutions

The disinfectant solutions were prepared as described in

Chapter V. The concentration of the disinfectant solution was such that, when 1 ml was diluted to 100 ml in a test solution, bacterial survivors would be present after 5 minutes exposure to the disinfectant solution. The disinfectant concentration required was ascertained by preliminary trials using distilled water as the diluent, the other circumstances being those existing under actual test conditions. It was necessary that survivors be present after exposure to the disinfectant so that death rates could be calculated.

3. The test solutions

The test solutions consisted of either distilled water, hard water, or hard water plus skimmed milk prepared as described in Chapter V.

(a) The distilled water test solution consisted of

97 ml sterile distilled water,
1 ml sterile distilled water, and,
1 ml disinfectant solution.

The test control solution consisted of

97 ml sterile distilled water, and,
2 ml sterile distilled water.

(b) The hard water test solution consisted of

97 ml sterile hard water,
1 ml sterile distilled water, and,
1 ml disinfectant solution.

The test control solution consisted of

97 ml sterile hard water, and,
2 ml sterile distilled water.

(c) The hard water plus skimmed milk test solution consisted of

97 ml sterile hard water,
1 ml sterile skimmed milk, and,
1 ml disinfectant solution.

The test control solution consisted of

97 ml sterile hard water,
1 ml sterile skimmed milk, and,
1 ml sterile distilled water.

4. The diluents

The diluents used were those described in Chapter V.

5. Preparation of control and test solutions

The control and test solutions utilised in a particular experiment were prepared at least 30 minutes prior to testing. The solutions were tempered for 30 minutes in a water bath at 22,5°C $\pm$ 0,5°C prior to the test at 0 hours.

6. Preparation of test suspension

After centrifugation, washing and dilution in the selected diluent, the suspension was placed in cracked ice and water prior to the first test at 0 hours. The suspension was retained in the ice and water for 3 hours for use in the second test conducted $1\frac{1}{2}$ hours after the first test and in the third test at 3 hours after the first test.

7. Test procedure

After tempering the test and control solutions for 30 minutes, 1 ml of the bacterial test suspension was added to the test solution and simultaneously, a stop clock was started. At the same time, an assistant added 1 ml of the bacterial test suspension to the control solution. The bottles were closed and shaken gently, to minimise foaming.

About 20 seconds before the expiry of 1 minute from initial time, the solution was again shaken gently; 5 ml of the test solution was drawn into a suitable pipette, and at the exact end of the 1 minute exposure period, was expelled into 5 ml of neutralizer solution. A further 5 ml of the test solution was transferred to 5 ml volumes of neutralizer solutions at 2, 3, 4 and 5 minutes, after initial time. Simultaneously with the transfer of the 5 ml test solution into the neutralizer at 5 minutes after initial time, 5 ml of the control solution was pipetted into a further 5 ml neutralizer solution.

Dilutions from the test-neutralizer and control-neutralizer mixtures were always prepared in distilled water irrespective of the diluent originally selected. Appropriate aliquots were plated and poured in triplicate.

After incubation, plates containing 30 to 300 colonies were counted. Where counts showed that less than 30 organisms had survived in the sample plated, directly from the neutralizer solution with no dilution, these counts had to be incorporated in the results, even though variations between replicate plates were often excessive.

Each trial was repeated on three separate days.

C. Calculations

Survivor curves for each micro-organism, diluent, disinfectant and test solution combination were constructed by plotting the logarithm of the number of survivors against the time of exposure of the micro-organism to the disinfectant (0 to 5 minutes).

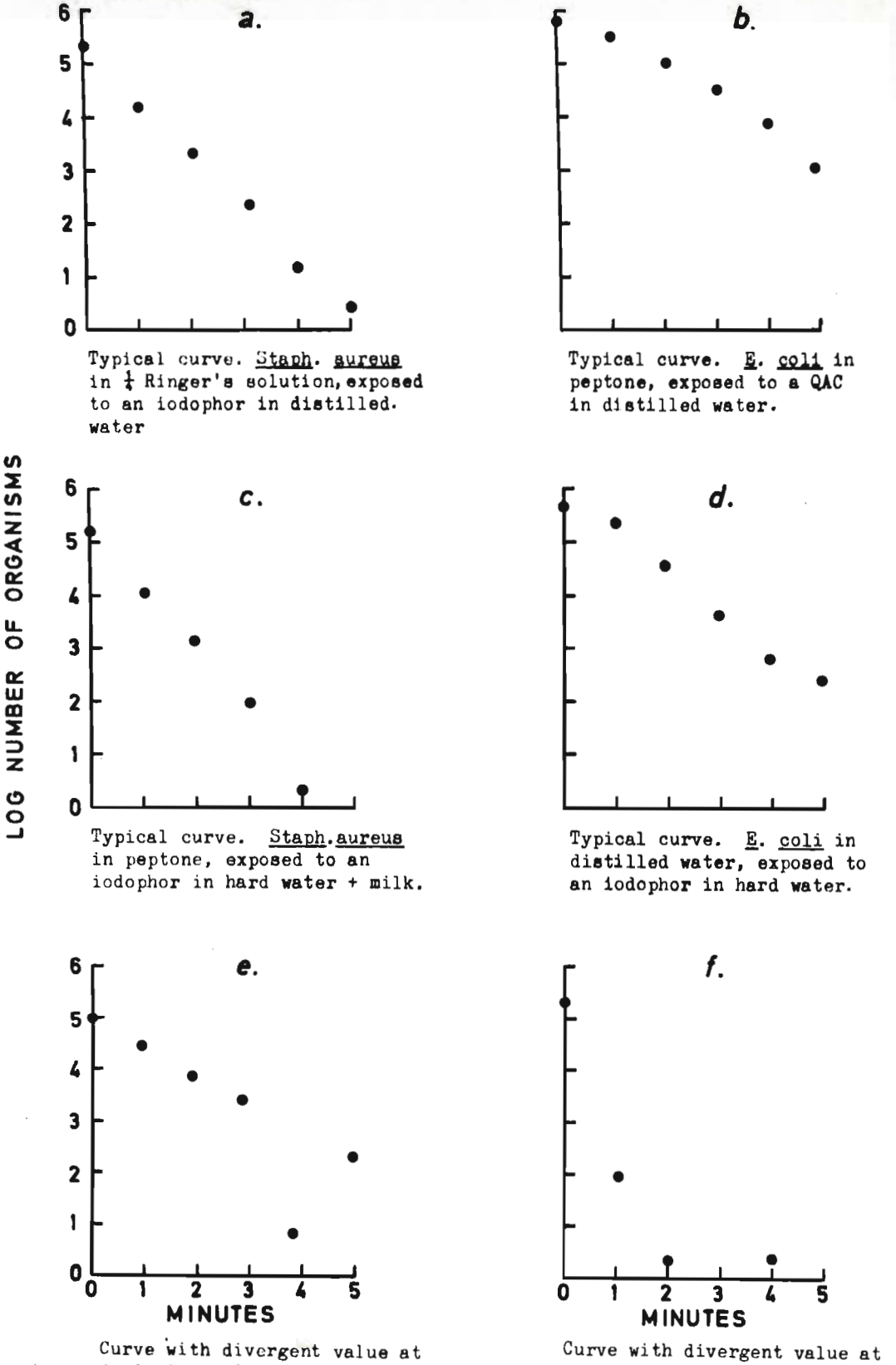
Regression lines were calculated using the logarithm of the mean of the triplicate plate survivor counts at each minute from 0 to 5 minutes. The survivor counts were deemed to be the dependent, random variable (y) and the independent non-random variable to be time in minutes (x). Each regression line Y was tested for linearity and the correlation coefficient r calculated.

The calculations of the regression line Y, tests for linearity and the correlation coefficient r were calculated using a computer programme based on the formulae given in Geigy Scientific Tables (Diem & Lentner, 1970).

Invariably, when regression lines were found to be non-linear, the deviation from linearity was due to either variable counts obtained from plates with less than 30 colonies counter per plate (15 of 216 regressions) or widely divergent values obtained from survivor counts at various times (17 of 216 regressions). Elimination of these few divergent values produced regression lines with correlation coefficients of more than 0,9 except in the case of 1 regression with a correlation coefficient of 0,895. Examples of typical and divergent curves are illustrated in Figure 8.1.

Utilising the b_{yx} values of each regression equation $Y = a_{yx} + b_{yx} x$ (b_{yx} being the tangent of the inclination B_{yx} of the regression line Y), analyses of variance were performed utilising two computer programmes developed at the University of California (Dixon, 1970):

FIG : 8.1. Examples of bacterial survival curves



Typical curve. Staph. aureus in ¼ Ringer's solution, exposed to an iodophor in distilled water

Typical curve. E. coli in peptone, exposed to a QAC in distilled water.

Typical curve. Staph. aureus in peptone, exposed to an iodophor in hard water + milk.

Typical curve. E. coli in distilled water, exposed to an iodophor in hard water.

Curve with divergent value at 4 minutes. Staph. aureus in distilled water, exposed to a QAC in distilled water.

Curve with divergent value at 4 minutes. E. coli in saline exposed to an iodophor in hard water + milk.

The first was for the analysis of variance for a factorial design using diluents and time, after initial suspension of the specific micro-organisms, as the two variables being studied. In those cases when the test quotient attained or exceeded the significance limit F, indicating that among the means there were some of discrepant magnitude, Duncan's multiple range test was used to analyse such a situation (Diem & Lentner, 1970).

The second programme was for the analysis of variance for a one-way design, which incorporated the multiple range test of Duncan (1955). In the latter analysis, each diluent at each of the 3 times after suspension was considered as an independent group.

Each analysis of variance for a micro-organism, disinfectant and test-solution combination consisted of six 'diluents' and three 'times' (0, $1\frac{1}{2}$ and 3 hours after initial suspension) and 3 replications. It must be emphasised that the results of each experiment were based on the mean of triplicate plate counts for the survivors at each minute, over 5 minutes, from the beginning of each test period; and that each experiment was repeated three times on separate days in order to minimise laboratory variation (see B - Experimental Procedure above).

The results of these analyses of variance are presented in Appendix B, Computations 1-12.

In the text, the results are presented in the form of decimal reduction time values (DRT) which may be defined as: the time, in minutes, necessary to reduce the bacterial population, under the circumstances of each test, by 90%, i.e. 1 logarithm cycle (Tables 8.4.-8.15.).

D. Results and Conclusions

1. Disinfectant concentrations

Prior to the initiation of the series of experiments discussed in this chapter, it was necessary to determine the disinfectant/sanitizer concentrations which, under the test circumstances, would yield approximately comparable decimal reduction

time values. For this purpose the diluent selected was distilled water since this was considered to be the diluent least likely to react with the disinfectant/test solutions.

Based on these preliminary trials the disinfectant concentrations were calculated, which, when diluted on the basis of 1 ml disinfectant solution in 100 ml test solution, would give the required concentration. This concentration was such that there would be some surviving micro-organisms present at the end of the five minute exposure period (vide part B of this chapter).

In order to compare the results of these preliminary trials, the logarithm of the number of survivors (Y) was plotted against the time, 0-5 minutes (X). After replicating these preliminary trials the regression equation was calculated. The decimal reduction time (DRT) was calculated from the reciprocal of b_{yx}. The DRT so calculated may be defined as: the time, in minutes, necessary to reduce by 90 per cent (1 log. cycle) the bacterial population under the conditions of that particular trial.

The results of these preliminary investigations are given in Table 8.1.

Although the decimal reduction times show some variation, they are close enough to allow the following conclusions to be made regarding the variable effects of disinfectant/sanitizers on the two micro-organisms used in these trials. There can be no doubt that the strain of E. coli used had a far greater resistance to QAC than Staph. aureus under all the test conditions. This would tend to confirm the statements of Chaplin (1952) that Gram-negative organisms display a greater resistance to QAC than Gram-positive organisms. This was later confirmed by the results of other trials in this laboratory using Pseudomonas aeruginosa. To achieve a decimal reduction time of 2,02 minutes with the last-named organism, an equivalent of 180 mg QAC/litre concentration had to be incorporated in the hard water plus milk test solution.

Contrary to these findings with QAC, no selectivity could be shown in the case of the iodophor. Even Pseudomonas aeruginosa required very similar decimal reduction times to those produced by

TABLE 8.1.

The disinfectant concentrations required to achieve comparable DRT[1] values under various test conditions

Test conditions			Initial disinfectant concentration mg active ingredient/l	Disinfectant test concentration active ingredient mg/100 ml test soln.	DRT[1] (mean for all diluents) minutes
Bacterium	Test solution	Disinfectant			
E. coli	Distilled water	Iodophor	75 mg av. iod/l [2]	0,075 mg av. iod [2]	1,479
E. coli	Hard water	Iodophor	80 mg av. iod/l	0,08 mg av. iod	1,026
E. coli	Hard water + milk	Iodophor	800 mg av. iod/l	0,80 mg av. iod	0,792
E. coli	Distilled water	QAC	1500 mg QAC/l	1,5 mg QAC	1,241
E. coli	Hard water	QAC	4500 mg QAC/l	4,5 mg QAC	2,053
E. coli	Hard water + milk	QAC	15000 mg QAC/l	15 mg QAC	0,875
Staph. aureus	Distilled water	Iodophor	75 mg av. iod/l	0,075 mg av. iod	1,283
Staph. aureus	Hard water	Iodophor	80 mg av. iod/l	0,08 mg av. iod	1,522
Staph. aureus	Hard water + milk	Iodophor	800 mg av. iodEl	0,8 mg av. iod	0,966
Staph. aureus	Distilled water	QAC	200 mg QAC/l	0,2 mg QAC	1,401
Staph. aureus	Hard water	QAC	375 mg QAC/l	0,375 mg QAC	1,058
Staph. aureus	Hard water + milk	QAC	4000 mg QAC/l	4,0 mg QAC	1,348

[1]Decimal Reduction Time

[2]Available iodine

TABLE 8.2. pH Values for iodophor test solutions

Composition of test solution	pH of Test solution
98 ml distilled water + 1 ml iodophor + 1 ml bacterial suspension in distilled water	5,7 - 5,8
97 ml hard water + 1 ml iodophor + 1 ml distilled water + 1 ml bacterial suspension in distilled water	6,4 - 6,5
97 ml hard water + 1 ml iodophor + 1 ml skimmed milk + 1 ml bacterial suspension in distilled water	4,3 - 4,4

TABLE 8.3. The ability of milk solids to bind iodine

	Titration no.		
	1	2	3
	mg	mg	mg
Milk solids added to 100 ml of iodophor solution	0,0	0,05	0,10
Available iodine in initial iodophor solution	1,953	1,953	1,953
Iodine available after tempering at 22 °C for 30 minutes	1,953	1,592	1,223
Available iodine bound by milk solids	-	0,361	0,730

Staph. aureus and E. coli; 1,01 minutes in a hard water, milk and iodophor test solution (the iodophor concentration being 8 mg available iodine/100 ml), compared with 0,966 and 0,792 minutes in this trial.

The effect of hard water, as used in these experiments, on the efficacy of iodophor would appear to be negligible with both micro-organisms. The difference of 0,5 mg available iodine/l between distilled water and hard water test solutions had but a minor effect on the E. coli and Staph. aureus DRT values. A possible reason for the slight increase in iodine required could have been due to the different pH values of the distilled and the hard water test solutions which contained 0,075 and 0,08 mg available iodine respectively. The pH rose from 5,7 for the distilled water to 6,4 for the hard water test solutions. The slight increase in available iodine was probably required to offset the rise in pH. The pH values of the iodophor test solutions are given in Table 8.2.

There can be no doubt that the presence of milk solids, representing organic matter, has a profound result on both disinfectants. A reason for the effect of milk solids on the iodophor is that iodine is a highly reactive element combining with the milk casein to produce thyroproteins. Table 8.3. indicates the inactivation of iodine by milk solids.

The results in Table 8.3. indicate that 1 ml of skimmed milk containing 10% solids can bind approximately 0,7 mg available iodine. Thus a solution containing less than this amount of iodine would have no disinfecting properties whatsoever. In the case of the hard water plus skimmed milk test solution it was found that at least 0,8 mg available iodine had to be present in order to achieve a decimal reduction time of approximately 1 minute. In fact the time was 0,792 minutes (Table 8.1.). Taking into account that the milk would inactivate approximately 0,7 mg available iodine only 0,1 mg would be available for disinfecting purposes. This value is comparable with 0,075 and 0,08 mg available iodine used in the distilled water and hard water test solutions. The decimal reduction time was decreased to 0,792 minutes, either due to the increased residual amount of iodine available or to a lower pH, or both.

The practical implications of milk residues in dairy hygiene can not be overlooked. The importance of efficient rinsing prior to the sanitization stage must be stressed from the point of view of economy as well as efficiency.

In the case of QAC, the effect of milk was additive to that of hard water. The effect of hard water on the efficacy of QAC is quite apparent in Table 8.1. Even though hard water is more suitable than distilled water from a point of view of pH, a detrimental effect is still found. Whether the effect of the hard water is one that is detrimental to the QAC as suggested by Ridenour and Armbruster (1948) or whether the site of hard water influence is on the micro-organisms (Klimek, 1955) is difficult to ascertain. The fact that the QAC was tempered in the hard water prior to the addition of the bacterial suspension may tend to support the opinion of Ridenour and Armbruster (1948).

2. The effect of diluent on the reaction of STAPHYLOCOCCUS AUREUS and ESCHERICHIA COLI, suspended in various diluents over a 3 hour period, to an iodophor or QAC in either distilled water, or hardwater, or hardwater + milk test solution.

The statistical analyses of this series of experiments is given in Appendix B, Computations 1-12. The reasons for dealing with each micro-organism/disinfectant/test solution combination separately have been discussed in Section C of this chapter; the results will therefore be discussed accordingly.

Reference will be made to the influence of diluent on the micro-organism concerned and, though in actual fact, the results pertain to the micro-organism in the particular diluent, mention will only be made of the diluent, thus avoiding repetition of the name of the species in combination with the diluent.

(a) Staphylococcus aureus exposed to an iodophor in a distilled water test solution.

The statistical analysis is given in Appendix B, Computation 1 and the relevant decimal reduction times (DRT) are given in Table 8.4.

Peptone, with a mean DRT of 2,762 minutes was the only diluent

TABLE 8.4. The decimal reduction time (DRT) values for Staph. aureus, suspended in various diluents over a period of 3 hours, subjected to an iodophor in a distilled water test solution.

D.R.T. values (minutes)

Diluent	Time after initial suspension			Diluent mean values
	0 hours	1½ hours	3 hours	
	mins	mins	mins	mins
Peptone	2,899	1,992	4,167	2,762
Dist. water	1,018	1,350	3,571	1,497
Ringer's	0,929	1,330	3,425	1,416
¼ Ringer's	1,241	0,894	1,208	1,091
Phos. Buffer	0,861	0,954	1,285	1,004
Saline	0,904	0,953	1,082	0,975
Time mean values	1,095	1,152	1,795	1,284

TABLE 8.5. The decimal reduction time (DRT) values for Staph. aureus, suspended in various diluents over a period of 3 hours, subjected to an iodophor in a hard water test solution.

D.R.T. values (minutes)

Diluent	Time after initial suspension			Diluent mean values
	0 hours	1½ hours	3 hours	
	mins	mins	mins	mins
Phos. Buffer	2,976	3,436	2,347	2,849
Ringer's	2,004	1,425	1,466	1,592
Dist. water	1,297	1,520	1,838	1,520
¼ Ringer's	1,961	1,479	1,178	1,475
Peptone	4,032	1,011	0,840	1,236
Saline	1,156	1,089	1,475	1,220
Time mean values	1,859	1,416	1,374	1,522

which was statistically different from all the other diluents ($P = <0,05$). Saline solution and phosphate buffered distilled water differed statistically from distilled water and Ringer's solution but not from ¼ Ringer's solution. Distilled water and Ringer's solution, also, did not differ statistically from ¼ Ringer's.

However, the death rates of Staph. aureus in both distilled water and Ringer's solution at 3 hours after suspension differed statistically from that organism's death rates in these diluents at initial suspension (0 hours) but not from the 1½ hour after suspension death rate. Thus neither distilled water nor Ringer's can be considered suitable diluents for Staph. aureus over a period of 3 hours after suspension under these test conditions.

Three of the six diluents, ¼ Ringer's, phosphate buffered distilled water and saline solution may therefore be considered as suitable diluents under the circumstances of this section of the experiments.

It is possible that peptone exerted a protective influence on Staph. aureus against the iodophor thereby producing abnormally high decimal reduction times (mean DRT value = 2,762 minutes versus an overall mean value of 1,284 minutes).

(b) Staphylococcus aureus exposed to an iodophor in a hard water test solution

The statistical analysis is given in Appendix B, Computation 2 and the relevant decimal reduction times (DRT) are given in Table 8.5.

In this series of experiments, phosphate buffered distilled water differed statistically from all the other diluents ($P = <0,05$). The ability of phosphate buffer to extend the decimal reduction time of Staph. aureus in this experiment (mean DRT = 2,849 minutes) compared with the overall mean DRT value of 1,522 minutes may be due to the phosphate radicle exerting a specific stimulatory effect as suggested by Winslow (1934), although the mechanism of a possible stimulation was not investigated in this study.

Peptone, although it did not differ from the other four diluents, was not a consistent diluent in that the DRT of Staph. aureus at 0 hours in peptone (DRT = 4,032 minutes) differed statistically from that at

$1\frac{1}{2}$ hours (DRT = 1,011 minutes) and at 3 hours (DRT = 0,840 minutes) after suspension. The probability exists that the peptone only exerts a protective influence on Staph. aureus over a relatively short period.

(c) Staphylococcus aureus exposed to an iodophor in a hard water + milk test solution

The statistical analysis is given in Appendix B, Computation 3 and the relevant decimal reduction times (DRT) are given in Table 8.6.

Peptone was again statistically different from all the other diluents. Staph. aureus in peptone had a mean decimal reduction time of 3,610 minutes, varying from 6,250 minutes at 0 hours to 5,650 minutes at $1\frac{1}{2}$ hours and only 2,024 minutes at 3 hours after suspension. Thus the influence of peptone on Staph. aureus would appear to diminish, and peptone could not be considered a suitable diluent under these circumstances.

Phosphate buffered distilled water and distilled water differed statistically from $\frac{1}{4}$ Ringer's but not from saline or Ringer's solution. Neither did $\frac{1}{4}$ Ringer's solution differ from the latter two diluents.

Therefore, apart from peptone, the remaining five diluents, although showing some differences, could all be considered suitable diluents for Staph. aureus under the circumstances pertaining; none of the five diluents varied statistically over 3 hours after suspension of the organism.

(d) Staphylococcus aureus exposed to a QAC in a distilled water test solution

The statistical analysis is given in Appendix B, Computation 4 and the relevant decimal reduction times (DRT) are given in Table 8.7.

Although saline solution gave the longest decimal reduction times of all the diluents (mean DRT = 4,184), it did not differ statistically from the other diluents except phosphate buffered distilled water. This was due to the wide variation in the results obtained with saline solution.

Phosphate buffer was statistically different from all the diluents except $\frac{1}{4}$ Ringer's; $\frac{1}{4}$ Ringer's, in turn, did not differ from any of the five diluents.

TABLE 8.6. The decimal reduction time (DRT) values for Staph. aureus, suspended in various diluents over a period of 3 hours, subjected to an iodophor in a hard water + milk test solution.

D.R.T. values (minutes)

Diluent	Time after initial suspension			Diluent mean values
	0 hours	1½ hours	3 hours	
	mins	mins	mins	mins
Peptone	6,250	5,650	2,024	3,610
¼ Ringer's	1,011	1,577	0,923	1,109
Saline	0,848	0,728	1,066	0,860
Ringer's	0,784	1,033	0,739	0,834
Phos. Buffer	0,833	0,678	0,818	0,770
Dist. water	0,985	0,680	0,614	0,729
Time mean values	1,031	0,984	0,893	0,966

TABLE 8.7. The decimal reduction time (DRT) values for Staph. aureus, suspended in various diluents over a period of 3 hours, subjected to a QAC in a distilled water test solution.

D.R.T. values (minutes)

Diluent	Time after initial suspension			Diluent mean values
	0 hours	1½ hours	3 hours	
	mins	mins	mins	mins
Saline	6,250	7,576	2,358	4,184
Peptone	1,873	1,312	2,155	1,704
Dist. water	2,475	1,639	1,253	1,656
Ringer's	1,815	3,300	0,992	1,610
¼ Ringer's	1,101	1,185	1,236	1,171
Phos. Buffer	0,420	0,816	1,898	0,726
Time mean values	1,215	1,548	1,488	1,401

The one-way analysis of variance was non-significant under the conditions of this experiment. No deductions from this analysis could be made.

(e) Staphylococcus aureus exposed to a QAC in a hard water test solution

The statistical analysis is given in Appendix B, Computation 5 and the relevant decimal reduction times (DRT) are given in Table 8.8.

Saline solution, Ringer's solution and distilled water as diluents for Staph. aureus did not differ statistically from ¼ Ringer's solution and phosphate buffered distilled water, though they did differ from peptone. On the other hand, peptone solution did not differ statistically from ¼ Ringer's and phosphate buffered distilled water.

The highest decimal reduction time values with Staph. aureus in this experiment was with peptone solution, and the lowest with saline.

As far as the individual diluent time death rate values were concerned, none was statistically different; therefore preference can not be given to any particular diluent.

(f) Staphylococcus aureus exposed to a QAC in a hard water + milk test solution

The statistical analysis is given in Appendix B, Computation 6 and the relevant decimal reduction times (DRT) are given in Table 8.9.

As a diluent for Staph. aureus, peptone solution with the lowest mean decimal reduction time of 0,694 minutes, was statistically different from all the other diluents.

Phosphate buffered distilled water and ¼ Ringer's solution did not differ meaningfully from either distilled water or Ringer's and saline solutions, though distilled water was statistically different from Ringer's and saline solution.

In the one-way analysis of variance it is apparent that peptone solution cannot be considered a suitable diluent for Staph. aureus under the conditions of this experiment, as its death rate at 3 hours after suspension differed statistically from the values at 0 and 1½ hours after suspension.

TABLE 8.8. The decimal reduction time (DRT) values for Staph. aureus, suspended in various diluents over a period of 3 hours, subjected to a QAC in a hard water test solution.

D.R.T. values (minutes)

Diluent	Time after initial suspension			Diluent mean values
	0 hours	1½ hours	3 hours	
	mins	mins	mins	mins
Saline	1,178	1,209	1,497	1,538
Ringer's	1,005	1,626	1,447	1,304
Dist. water	1,613	1,121	1,119	1,247
¼ Ringer's	1,220	0,823	0,957	0,974
Phos. Buffer	1,233	0,825	0,917	0,963
Peptone	0,714	0,612	0,883	0,720
Time mean values	1,092	1,000	1,087	1,058

TABLE 8.9. The decimal reduction time (DRT) values for Staph. aureus, suspended in various diluents over a period of 3 hours, subjected to a QAC in a hard water + milk test solution.

D.R.T. values (minutes)

Diluent	Time after initial suspension			Diluent mean values
	0 hours	1½ hours	3 hours	
	mins	mins	mins	mins
Ringer's	2,778	2,114	2,445	2,404
Saline	2,404	2,151	2,132	2,222
Phos. Buffer	1,626	1,742	2,101	1,802
¼ Ringer's	1,087	2,053	1,698	1,504
Dist. water	0,943	1,080	1,242	1,074
Peptone	0,542	0,558	1,462	0,694
Time mean values	1,151	1,272	1,754	1,348

It would thus appear under the circumstances of this experiment, that the diluent could be any one of the following - Ringer's, ¼ Ringer's, saline, phosphate buffered distilled water or distilled water. Peptone solution is not suitable since it differed statistically from the other diluents and was not statistically consistent over the period of 3 hours.

(g) Escherichia coli exposed to an iodophor in a distilled water test solution

The statistical analysis is given in Appendix B, Computation 7 and the relevant decimal reduction times (DRT) are given in Table 8.10.

Both peptone solution and distilled water differed statistically from one another and from the other diluents. None of the four remaining diluents, Ringer's, ¼ Ringer's, saline and phosphate buffered distilled water, differed from one another on an overall basis.

It can also be seen from the one-way analysis of variance, that all the diluents of E. coli were consistent as far as the death rates at 0, 1½ and 3 hours are concerned. Therefore there are no diluents that can actually be considered unsuitable for this micro-organism if they are also found to be consistent under the other test conditions.

(h) Escherichia coli exposed to an iodophor in a hard water test solution

The statistical analysis is given in Appendix B, Computation 8 and the relevant decimal reduction times (DRT) are given in Table 8.11.

Distilled water and saline solution, from the analysis of variance, differed statistically from the other diluents, and had mean DRT values of 0,726 minutes and 0,676 minutes respectively.

Saline solution was the least suitable diluent in that the initial time (0 hours) DRT value of 1,862 minutes differed statistically from the 1½ hours (DRT = 0,520 minutes) and 3 hours (DRT = 0,505 minutes) values respectively.

Distilled water may still be considered a suitable diluent and the DRT values of E. coli in this diluent were 0,565 minutes at 0 hours, 0,874 minutes at 1½ hours and 0,821 minutes at 3 hours after initial suspension.

TABLE 8.10. The decimal reduction time (DRT) values for E. coli, suspended in various diluents over a period of 3 hours, subjected to an iodophor in a distilled water test solution.

D.R.T. values (minutes)

Diluent	Time after initial suspension			Diluent mean values
	0 hours	1½ hours	3 hours	
	mins	mins	mins	mins
Peptone	9,009	2,591	14,286	5,291
¼ Ringer's	1,524	1,453	2,041	1,637
Saline	1,838	1,425	1,675	1,629
Phos. Buffer	1,022	1,330	1,661	1,287
Ringer's	1,094	1,477	1,217	1,242
Dist. water	0,867	0,880	1,136	0,946
Time mean values	1,377	1,381	1,733	1,479

TABLE 8.11. The decimal reduction time (DRT) values for E. coli, suspended in various diluents over a period of 3 hours, subjected to an iodophor in a hard water test solution.

D.R.T. values (minutes)

Diluent	Time after initial suspension			Diluent mean values
	0 hours	1½ hours	3 hours	
	mins	mins	mins	mins
¼ Ringer's	0,984	2,227	1,560	1,425
Peptone	1,072	1,795	1,443	1,374
Ringer's	1,168	1,042	2,088	1,307
Phos. Buffer	2,088	0,980	1,117	1,253
Dist. water	0,565	0,874	0,821	0,726
Saline	1,862	0,520	0,505	0,676
Time mean values	1,073	0,991	1,016	1,026

(i) Escherichia coli exposed to an iodophor in a hard water + milk test solution

The statistical analysis is given in Appendix B, Computation 9 and the relevant decimal reduction times (DRT) are given in Table 8.12.

Peptone again was statistically different from all the other diluents for E. coli under the conditions of these experiments. However, though peptone differed from the other diluents, its DRT values at 0 hours, 1½ hours and 3 hours after initial suspension of E. coli were consistent, unlike those of distilled water since the death rates of E. coli in distilled water at 0 hours differed statistically from those at 3 hours after suspension; neither differed significantly from the 1½ hour after suspension values.

Peptone, therefore, can be considered as apparently offering a protective medium to E. coli against the action of iodophor.

(j) Escherichia coli exposed to a QAC in the presence of a distilled water test solution

The statistical analysis is given in Appendix B, Computation 10 and the relevant decimal reduction times (DRT) are given in Table 8.13.

None of the diluents was statistically different from any other though there were variations in the DRT values of E. coli over a period of 3 hours in the case of peptone and saline solutions.

The DRT values of E. coli in peptone at initial suspension were statistically different from the values at 1½ and 3 hours after suspension: 4,808 minutes at 0 hours, 0,978 minutes at 1½ hours and 0,738 minutes at 3 hours after suspension.

In the case of saline solution the results at 1½ hours after suspension were statistically different from both those at 0 and 3 hours after suspension of E. coli.

Thus neither peptone nor saline solutions can be considered as suitable diluents for E. coli when exposed to a QAC made up in a distilled water test solution.

TABLE 8.12. The decimal reduction time (DRT) values for E. coli, suspended in various diluents over a period of 3 hours, subjected to an iodophor in a hard water + milk test solution.

D.R.T. values (minutes)

Diluent	Time after initial suspension			Diluent mean values
	0 hours	1½ hours	3 hours	
	mins	mins	mins	mins
Peptone	1,381	2,242	5,405	2,212
Saline	0,812	0,672	1,030	0,813
Phos. Buffer	0,891	0,707	0,842	0,805
Ringer's	0,669	0,696	0,705	0,690
Dist. water	0,965	0,779	0,428	0,644
¼ Ringer's	0,546	0,598	0,698	0,608
Time mean values	0,806	0,775	0,796	0,792

TABLE 8.13. The decimal reduction time (DRT) values for E. coli, suspended in various diluents over a period of 3 hours, subjected to a QAC in a distilled water test solution.

D.R.T. values (minutes)

Diluent	Time after initial suspension			Diluent mean values
	0 hours	1½ hours	3 hours	
	mins	mins	mins	mins
Saline	1,431	3,623	1,364	1,757
Dist. water	1,054	1,362	1,377	1,245
Ringer's	1,252	1,304	1,124	1,221
Peptone	4,808	0,978	0,738	1,160
Phos. Buffer	1,255	1,307	0,962	1,153
¼ Ringer's	0,970	1,259	1,087	1,093
Time mean values	1,339	1,377	0,059	1,241

(k) Escherichia coli exposed to a QAC in the presence of a hard water test solution

The statistical analysis is given in Appendix B, Computation 11 and the relevant decimal reduction times (DRT) are given in Table 8.14.

This series of experiments presented a considerable variation in the reaction of the micro-organism to the various test conditions. There were four subsets of diluents which differed statistically from one another. As can be seen from the analysis of variance, saline solution differed from all the other diluents with the exception of peptone solution. Peptone solution, distilled water, phosphate buffer in turn differed from ¼ Ringer's. Ringer's solution differed statistically from peptone but did not differ from either distilled water, phosphate buffered distilled water or ¼ Ringer's solution.

An examination of the one-way analysis of variance indicates that there are 3 diluents whose effects on E. coli are such that they can not be considered suitable for the suspension of E. coli over a period of three hours. E. coli suspended in ¼ Ringer's solution differed statistically at 3 hours after initial suspension from the 0 and 1½ hour death rate values. The variation in the DRT values for ¼ Ringer's may be seen in Table 8.14.

Phosphate buffered distilled water at 3 hours after suspension (DRT = 4,566 minutes) differed statistically from the 1½ and the 0 hour DRT values which had the identical value of 1,441 minutes.

Peptone solution as a diluent for E. coli was unsuitable because the 0 and 1½ hour values differed statistically from the 3 hour values.

The other three diluents may therefore be considered as suitable, but because saline solution was statistically different from distilled water and Ringer's solution, it would appear that the latter diluents are the ones of choice.

(l) Escherichia coli exposed to a QAC in the presence of hard water + milk test solution

The statistical analysis is given in Appendix B, Computation 12 and the relevant decimal reduction times (DRT) are given in Table 8.15.

In the analysis of variance it appears that distilled water as a

TABLE 8.14. The decimal reduction time (DRT) values for E. coli, suspended in various diluents over a period of 3 hours, subjected to a QAC in a hard water test solution.

D.R.T. values (minutes)

Diluent	Time after initial suspension			Diluent mean values
	0 hours	1½ hours	3 hours	
	mins	mins	mins	mins
Saline	4,673	4,630	3,257	4,065
Peptone	4,000	3,968	1,580	2,646
Dist. water	2,105	1,751	2,500	2,075
Phos. Buffer	1,441	1,441	4,566	1,866
Ringer's	1,984	2,123	1,374	1,764
¼ Ringer's	2,028	1,468	1,029	1,399
Time mean values	2,283	2,079	1,842	2,053

TABLE 8.15. The decimal reduction time (DRT) values for E. coli, suspended in various diluents over a period of 3 hours, subjected to a QAC in a hard water + milk test solution.

D.R.T. values (minutes)

Diluent	Time after initial suspension			Diluent mean values
	0 hours	1½ hours	3 hours	
	mins	mins	mins	mins
Dist water	0,875	0,978	2,132	1,139
Phos. Buffer	0,739	1,099	0,983	0,914
¼ Ringer's	0,788	0,740	1,272	0,881
Saline	0,961	0,724	0,925	0,856
Peptone	0,814	0,807	0,901	0,839
Ringer's	0,761	0,700	0,695	0,718
Time mean values	0,816	0,818	1,017	0,875

diluent for E. coli differed from all the other diluents except for phosphate buffered distilled water. In turn phosphate buffer and ¼ Ringer's solution differed statistically from Ringer's solution but not from saline and peptone solutions. Further that there were divergent time means within the diluents.

The one-way analysis of variance indicates that the distilled water and ¼ Ringer's solution were responsible for the divergent time means. In both these diluents, the 3 hour values differed statistically from those at 0 and 1½ hours after suspension.

Thus the other four diluents could be selected for the suspension of E. coli over 3 hours under the conditions of the test.

* * * * *

From the results and the discussion of each series of experiments it becomes apparent that there are no diluents that are suitable as a general diluent for the two organisms used in these trials over a period of three hours under all the test conditions described.

Comparing the results obtained from the analyses of variance, it is apparent that three diluents differed from all other diluents more than once. Peptone differed statistically on five occasions from all other diluents. (Staph. aureus exposed to an iodophor in either distilled water or hard water + milk test solutions; Staph. aureus exposed to a QAC in hard water + milk test solutions; and, E. coli exposed to an iodophor in either distilled water or hard water + milk).

Phosphate buffered distilled water differed once from the other diluents (as a diluent for Staph. aureus exposed to an iodophor in a hard water test solution).

Distilled water was statistically different from the other diluents for E. coli exposed to an iodophor in a distilled water test solution.

Without considering the period after initial suspension the three diluents most suitable for the test conditions discussed in this chapter are saline, Ringer's and ¼ Ringer's solutions.

Combining the results of the analyses of variance with those of the two-way analyses of variance, the situation becomes more lucid. Ringer's solution remains the most consistent of the diluents, being only unsuitable when Staph. aureus is exposed to an iodophor in a distilled water test solution. In all the other situations Ringer's solution was a suitable diluent for the two organisms discussed.

Saline and ¼ Ringer's solutions were unsuitable under two situations each - ¼ Ringer's at 3 hours after suspension of E. coli differed statistically from 0 and 1½ hours after suspension when E. coli was exposed to a QAC in the presence of either hard water or hard water + milk test solutions. Saline, suspending E. coli, differed statistically at 0 hours from 1½ hours after suspension (QAC in distilled water test solution) and, in addition, from 3 hours after suspension in the case of the exposure of E. coli to an iodophor in hard water.

Ideally the reaction of the micro-organism/diluent combination, to a disinfectant or sanitizer, should be such that there are no obvious extremes; the reaction should not show a protective influence of diluent on organism, nor an inactivating effect on the disinfectant. On the other hand the reaction should not be fast enough to indicate that stress has been imposed on the micro-organism by the diluent as well as by the disinfectant. All things considered, and with the reservations already expressed. Ringer's solution appears to be the most suitable diluent under the conditions of these experiments.

There can be no doubt that there is sufficient statistical evidence available from the results to show clearly that diluents definitely have an influence on the outcome of a disinfectant or sanitizer test where a micro-organism is exposed to a disinfectant or sanitizer under various test conditions.

A further conclusion that can be made is that the time, after initial suspension, at which a test is carried out may be very pertinent when certain diluents are to be used.

No attempt has been made to explain a number of the results. This could only be done by conjecture as no specific investigations in this regard were made.

The term 'disinfectant' has been used in preference to 'sanitizer' as both terms relate to the reduction of vegetative micro-organisms on inanimate surfaces. A sanitizer includes, by definition, a cleansing action. As no evaluation of the cleaning ability of the iodophor/sanitizer was made, these experiments were concerned with the disinfecting quality. The iodophor was formulated as a sanitizer/detergent, while the QAC was the A.R. substance. It is therefore possible that the surface-active additives in the iodophor could have improved (or lessened) the contact between iodophor and cell. This aspect is only of minor importance, as the basic study involved the influence of diluent on the reaction of a micro-organism to disinfectants. The efficacy of the disinfectants selected were not under consideration - they were the vehicles for the investigation.

CHAPTER IX

THE INFLUENCE OF DILUENT ON THE VIABILITY OF RELATIVELY LARGE BACTERIAL POPULATIONS

A. Introduction

In Chapter VII the influence of diluent on relatively small bacterial populations (mean density $2,3 \times 10^5$ cells/ml) over a period of six hours, was discussed. The general conclusions were that in these small populations, significant changes occurred in the percentages of viable survivors between 3 and 6 hours after initial suspension, and that the ability of low density bacterial populations to remain fully viable for a lengthy period in the generally recommended diluents would appear to be limited.

Various workers found that large bacterial populations showed greater viability than small ones. This survival is possible due to the protection afforded to some cells by the leakage of cell contents from a number of other cells into the surrounding medium (Shaughnessy & Winslow, 1927; Winslow & Brooke, 1927; Ballantyne, 1930; Spangler & Winslow, 1943).

During the experiments investigating the influence of diluent on the reaction of Escherichia coli and Staphylococcus aureus exposed to an iodophor or QAC in three test solutions (Chapter VIII), an opportunity existed to evaluate the effect of diluent on relatively large bacterial populations.

B. Experimental Procedure

As discussed in Chapter VIII, Section B, control test solutions (in which distilled water was substituted for the disinfectant-sanitizer solutions) were used to provide values for the initial bacterial populations exposed to the conditions of the experiment. The same bacterial suspension was used for tests conducted at 0, 1½ and 3 hours after suspension.

A volume of 1 ml of the bacterial suspension, in the selected diluent, was added to the control test solution at the same time as 1 ml of the suspension was added to the test solution containing the disinfectant. Five minutes after adding the suspension to the control solution, a 5 ml aliquot was pipetted from the control solution into 5 ml neutralizer solution. (This coincided with the 5th aliquot removed from the test solution). From the control-neutralizer solution combination, relevant dilutions were plated in triplicate.

This procedure was performed at 0, $1\frac{1}{2}$ and 3 hours after initial suspension and was repeated on 3 separate days. The bacterial plate counts so obtained, were utilised in this experiment.

C. Calculations

The arithmetic mean counts for the two bacterial species suspensions, in the three control test solutions, at $1\frac{1}{2}$ and 3 hours were expressed as a percentage of the arithmetic mean of the initial count at 0 hours.

An analysis of variance utilising the percentage survivors, as input data, was calculated for each organism-control test solution combination. The values of the initial suspension densities were not included in the calculations as, by definition, they were each equivalent to 100 per cent.

In those analyses of variance where the application of the F-test indicated the presence of means of discrepant magnitude either at $P<0,01$ or $P<0,05$ levels, Duncan's Multiple Range Test was applied. The results of these tests of significance and the analyses of variance are given in Appendix C, Calculations 1-6.

D. Results and Discussion

1. Initial bacterial cell densities of STAPHYLOCOCCUS AUREUS and ESCHERICHIA COLI

These experiments investigated the survival of two bacterial species, in six diluents, over a period of three hours. The fact that the bacterial cells were further exposed to one of three test solutions (without added disinfectant) and neutralizer solutions necessitated six

analyses of variance.

Table 9.1. indicates the mean population densities of the two bacterial species involved. The mean cell densities are approximately 100 times greater than those discussed in Chapter VII and may therefore be considered as relatively large. The means given in Table 9.1. for each micro-organism were calculated from the combined values for the six diluents used for suspending the bacteria in each case.

2. The influence of diluent on the viability of STAPHYLOCOCCUS AUREUS and ESCHERICHIA COLI exposed for 5 minutes to either distilled water or hard water or hard water + milk at $1\frac{1}{2}$ and 3 hours after initial suspension

(a) Staph. aureus exposed to a distilled water control test solution

The analysis of variance is given in Appendix C, Computation 1.

There was no statistical difference between the percentage survival of Staph. aureus under the conditions of the experiment; however, the percentage viability at 3 hours was statistically less than at $1\frac{1}{2}$ hours ($P<0,05$). The overall percentage survival of the micro-organisms at 3 hours after suspension was 82 per cent of the initial numbers, while at $1\frac{1}{2}$ hours the survival was 92 per cent.

The greatest reduction of this bacterial species was found with distilled water as diluent - 87 per cent at $1\frac{1}{2}$ hours and 67 per cent at 3 hours, - followed by Ringer's solution with a mean percentage survival of 80 per cent over the 3 hour period.

Phosphate buffer and peptone were the most successful diluents for the maintenance of Staph. aureus under the conditions imposed. The mean survival in phosphate buffered distilled water and peptone solution was 104 and 92 per cent respectively over 3 hours.

The overall survival for all diluents and the two specified times were 87 per cent. This was the only one of the series of six experiments that yielded an overall survivor percentage of less than 90 per cent of the initial suspension cell densities.

TABLE 9.1. Bacterial populations of suspensions determined at initial time (0 hours)

Micro-organism	Control test solution	Mean bacterial cells/ml ($\times 10^6$)	Standard deviation ($\times 10^6$)
Staph. aureus	Dist. water	25,728	$\pm$ 9,80
	Hard water	25,708	$\pm$ 12,83
	Hard water + milk	29,894	$\pm$ 14,81
E. coli	Dist. water	55,649	$\pm$ 20,22
	Hard water	68,437	$\pm$ 34,66
	Hard water + milk	69,992	$\pm$ 19,87

(b) Staph. aureus exposed to a hard water control test solution

The analysis of variance is given in Appendix C, Computation 2.

The results indicate that there were no statistical differences between the diluents, or between the $1\frac{1}{2}$ and 3 hour periods from initial suspension. The overall mean survival of Staph. aureus for diluents and time was 95 per cent.

Saline solution and phosphate buffered distilled water maintained the highest viability, whereas the lowest viability resulted from Ringer's solution as diluent (mean percentage viability was 82 per cent of the initial suspension cell density at 0 hours).

(c) Staph. aureus exposed to a hard water + milk control test solution

The statistical analysis is presented in Appendix C, Computation 3.

No statistical differences were established for either the influence of diluents or the time from initial suspension. The overall mean percentage viability for all diluent-time combinations was 91 per cent. Again, distilled water produced the lowest viability (71 per cent at 3 hours), while peptone had a 110 per cent average viability over the 3 hour period.

(d) E. coli exposed to a distilled water control test solution

The analysis of variance is presented in Appendix C, Computation 4.

Three diluents, distilled water, $\frac{1}{4}$ Ringer's solution and phosphate buffered distilled water had significantly lower viability percentages than the other diluents for E. coli under the conditions of these specific experiments.

Ringer's, saline and peptone solutions had viability figures of over 100 per cent for their mean percentages over 3 hours. The overall mean value was 97 per cent of the initial population density at 0 hours after suspension.

(e) E. coli exposed to a hard water control test solution

The results for this analysis of variance are presented in Appendix C, Computation 5.

A greater discrepancy of results was obtained in this series of experiments than in the one previously reported.

Saline and $\frac{1}{4}$ Ringer's solutions were statistically different from peptone and phosphate buffered distilled water. None of the four mentioned diluents were statistically different from Ringer's solution and distilled water.

The lowest viability was established with saline with a mean value of 76 per cent of the initial population density; while the highest viabilities were recorded with phosphate buffered distilled water and peptone with mean viabilities of 109 and 107 per cent respectively.

(f) E. coli exposed to hard water + milk control test solution

The analyses of this series of experiments are recorded in Appendix C, Computation 6.

The analysis of variance of the percentage viabilities of E. coli suspensions in the specified diluents indicates no significant differences between either diluents or times.

The overall mean percentage of viable organisms was 95 per cent of the cell population densities at 0 hours after suspension.

* * * * *

The results of this series of experiments are in contrast with those reported in Chapter VII.

The main variations between the two experiments are:

(i) the differences in densities of bacterial populations,

(ii) the use of only two bacterial species in these experiments compared with five species in the previously mentioned experiments, and

(iii) the fact that aliquots of the suspensions in this series of experiments were exposed to control test solutions at $1\frac{1}{2}$ and 3 hours after suspension and to neutralizer solutions subsequent to each exposure to the control test solutions.

In only one of the present series of experiments did the percentage of survivors at $1\frac{1}{2}$ hours after suspension of the bacterial cells, differ statistically from the 3 hour percentages. In four series there were no statistical differences between diluents able to preserve a reasonable percentage of viable cells over a period of 3 hours. In the two experiments where statistical differences were found between diluents, phosphate buffered distilled water and peptone solution were the most successful diluents.

Overall, phosphate buffered distilled water (twice), saline solution (twice) and peptone solution (twice) preserved the viability of the bacterial suspensions better than the other three diluents; however, it must be mentioned again that, in only two series of experiments were statistical differences recorded between diluents.

CHAPTER X

DISCUSSION AND CONCLUSION

This series of studies concerning the influence of diluents on the reactions of micro-organisms, was initiated as a result of an examination of a number of disinfectant and sanitizer test procedures (A.O.A.C., 1965; Sykes, 1965; Lawrence & Block, 1968). The diluents used for recognised test procedures vary; the inference would therefore be either that diluents are of no concern; that diluents are acknowledged to affect the outcome of the test or that the diluent which ensures the maximum viability of the micro-organism should be selected. Only one of the disinfectant or sanitizer test procedures investigated, stipulated the maximum period that a bacterial suspension can be used for consecutive tests (S.A.B.S., 1971).

The bacterial cell population densities required for disinfectant or sanitizer test suspensions are also a matter of interest. The bacterial suspension densities vary from 1×10^{10} cells/ml in the Chambers' Modification of the Weber and Black Method for Sanitizers (Chambers, 1956) to 1×10^{5} cells/ml for the South African Bureau of Standards Specification for Quaternary Ammonium Compounds (S.A.B.S., 1971).

It has been stated that low density bacterial cell populations are more susceptible to a loss of viability when suspended in diluents than large populations (Ballantyne, 1930; Spangler & Winslow, 1943; Postgate & Hunter, 1963).

The influence of diluent was investigated using six commonly prescribed diluents - 0,85% m/v sodium chloride, 0,1% m/v peptone, quarter strength Ringer's solution, Ringer's sclution, phosphate buffered distilled water and distilled water - to suspend five bacterial species over a period of six hours after initial suspension.

The results of this series of experiments (Chapter VII) showed that relatively small bacterial populations do react differently to the various diluents investigated. Only in the case of Staphylococcus aureus were there no significant differences between the effects of the

diluents, but, as with the other micro-organisms tested, there was a highly significant decrease in the viability of the bacterial population over a period of six hours. The mean decrease in the viable populations of Staphylococcus aureus was 46 per cent with a range of between 57 per cent and 38 per cent.

In the case of the other micro-organisms, Pseudomonas aeruginosa suffered the greatest mean loss of viability - 50 per cent loss. The loss of viability at six hours after initial suspension varied between 89 per cent in the case of distilled water as diluent and only 10 per cent with peptone solution.

Aerobacter aerogenes suffered the least mean loss of viability (26 per cent) of all the micro-organisms; the variation in viability loss was from 50 per cent ($\frac{1}{4}$ Ringer's solution) to 6 per cent in the case of peptone.

Of the six diluents investigated, peptone proved to be the diluent that best maintained the viability of the population sizes investigated, while distilled water was the poorest in the case of three micro-organisms - E. coli, Micrococcus flavus and Pseudomonas aeruginosa; as already mentioned, $\frac{1}{4}$ Ringer's solution caused the greatest loss in viability in the case of Aerobacter aerogenes. No statistical differences existed with Staphylococcus aureus in the various diluents.

The results, further discussed in Chapter VII, therefore indicate that diluents do affect the viability of bacterial cells, particularly of low density populations. The apparent degree of stress varies with the bacterial species and with the diluent used. The mean population size used in the described experiments was $2,3 \times 10^5$ cells/ml.

In order to ascertain the effect of diluent on relatively large populations, results derived from investigations into the effect of diluent on the reaction of micro-organisms to disinfectants, were utilised. The circumstances of the experiments reported in Chapters VII and IX mean that the results are not directly comparable, but considering the additional stresses imposed in the latter series of experiments, the results indicate that large populations are more resistant than small populations.

The bacterial population densities in Chapter IX were approximately 2 x 10^7 cells/ml in the case of Staphylococcus aureus and 6 x 10^7 cells/ml in the case of Escherichia coli. In the case of Staph. aureus there were no statistical differences between the effects of diluent on the viability of the bacterial cells under the three experimental conditions. Only when time, after initial suspension, was considered were there diluents that reduced the viability of Staph. aureus between $1\frac{1}{2}$ and 3 hours after suspension. (This was found when Staph. aureus suspended in the diluent was subjected to a control test solution of distilled water and neutralizer solution).

With Escherichia coli, the results were different from those results obtained with Staph. aureus. In two cases, diluent did affect the percentage viability of E. coli; once when E. coli was exposed to a distilled water control test solution and then neutralizer and once when the control test solution was hard water. No statistical differences were due to the period after initial suspension.

Though the bacteria in the control test solutions were exposed to greater stress than the smaller bacterial populations, the variation in viability was greater in the case of the smaller populations of Staph. aureus and E. coli.

The fact that the type of diluent, used to suspend a bacterial population, can affect the viability of bacteria suggested that a diluent may affect the bacterial reaction to disinfectants.

The series of experiments, reported in Chapter VIII, was concerned with six selected diluents for two micro-organisms - Staph. aureus and E. coli. The disinfectants investigated were a QAC and an iodophor. Although QAC and iodophors may be categorised as sanitizers, the term 'disinfectant' is used (in preference to 'sanitizer') as no assessment of cleansing action was made. It is appreciated that the two disinfectants selected for these experiments are but selections from a vast number of formulations of both the QAC and iodophor disinfectants. However it is contended that this aspect is only of minor importance, as the basic study was concerned with the influence of diluent on the reaction of a micro-organism to disinfectants, and not the mode of action of the disinfectants.

Disinfectants are used under varying conditions: disinfection may be of clean articles with the disinfectant solution made up with a soft water, or the water used may be relatively hard. A further situation would be the presence of organic matter. Normally, disinfectant test procedures require a test solution of hard water with milk representing organic matter.

The experiments were also designed to determine the test solution influences, if any, on the disinfectants. There is no doubt that the presence of organic matter requires a considerable increase in the proportion of available active disinfectant ingredient. Both disinfectants were affected by the presence of organic matter (in this case, milk solids). The concentration of the QAC and iodophor had to be increased considerably in order to maintain decimal reduction times (DRT) comparable with the other test conditions of distilled water and hard water (Table 8.1.).

The iodophor was only slightly affected by the hardness of water, whereas the concentration of QAC had to be increased from 1 500 mg QAC/l in a distilled water test solution to 4 500 mg QAC/l in a hard water test solution in the case of E. coli. The effect was not so pronounced with Staph. aureus. Hard water either affected the QAC causing a decrease in the efficacy of its action, as proposed by Ridenour and Armbruster (1948), or the hard water could have acted on the micro-organism, to increase its resistance to the QAC as suggested by Klimek (1955). This latter suggestion is highly improbable as the bacterial suspension was added after a half-hour tempering period for the disinfectant-test solution combination. Further investigations with commercial QAC sanitizers would be advantageous so that recommendations could be made regarding the variations in sanitizer concentrations required to correspond with the type of water available for the preparation of effective economical solutions. Unfortunately, the investigations did not include the influence of alkalinity, as opposed to mere hardness of water, on the iodophor. This aspect was suggested by Cousins, Hoy and Clegg (1959) and extensively discussed by Rammell and Croft (1971).

Suggestions have been made that Gram-positive species are not as

resistant to QAC as are Gram-negative species (Chaplin, 1952). The present experiments do indicate a considerable difference in the concentrations of the particular QAC selected, required to impose comparable decimal reduction times on Staph. aureus and E. coli - representing Gram-positive and -negative organisms respectively. In contrast to the results with the QAC, the iodophor was not selective.

The statistical analyses of the influences of diluents on the two micro-organisms, under the stated test conditions, indicate that diluents do have an effect on the reaction of micro-organisms to disinfectants.

In the experiments concerning the iodophor, there was an indication that peptone imparts a greater resistance to both bacterial species. In four of the six experiments, the influence of peptone solution was statistically different from that of all the other diluents and produced relatively longer decimal reduction times than other diluents. In the other two experiments, peptone did not differ from the majority of the other diluents in its effect on the reaction of both Staph. aureus and E. coli to iodophors. The reaction of saline was distinctly different from that of peptone. In the iodophor experiments, although the results with saline were not statistically different from those of the majority of diluents, this solution generally gave the shortest decimal reduction times with the two micro-organisms tested.

In the experiments concerning QAC, saline reversed the role it had presented with the iodophor, and tended to lengthen the decimal reduction times of the organisms suspended in it. Though never statistically different from other diluents, it did give the largest DRT values in four of the six experiments.

When the analysis of variance concerning times is not considered, only three diluents did not differ statistically from other diluents in their effect on the two micro-organisms — these diluents were saline, ¼ Ringer's and Ringer's solution. The other three diluents differed from all the other diluents at least once in the twelve experiments e.g. peptone solution differed statistically five times.

When the results of the one-way analysis of variance are taken into account, a very different picture emerges, since this analysis takes into account the effects not only of diluent but also of the time that had elapsed from the initial suspension, in the diluent, of Staph. aureus and E. coli under the various test conditions.

There are no diluents that are genuinely suitable for all the test conditions applied to the two bacterial species over a period of three hours. Reference to Appendix B indicates that Ringer's solution was the most suitable diluent, and only deviated in the case of Staph. aureus exposed to an iodophor in a distilled water test solution, where the reaction at 3 hours differed statistically from that at the initial time of suspension; neither result, however, differed statistically from the reaction at $1\frac{1}{2}$ hours after suspension. Ringer's solution would therefore be suitable for all the contingent circumstances over a period of $1\frac{1}{2}$ hours from original suspension.

Assuming a maximum period of $1\frac{1}{2}$ hours after suspension to be a suitable period for conducting consecutive disinfectant or sanitizer tests with the micro-organism/bactericide/test solution combinations used in these experiments, then only two diluents would be suitable. They are $\frac{1}{4}$ Ringer's solution and, as mentioned, Ringer's solution. The other diluent, the effect of which had not differed statistically from all the diluents in any of the experiments, was saline. However, saline would not be suitable even over a $1\frac{1}{2}$ hour period because the reaction of E. coli at $1\frac{1}{2}$ hours after suspension differed statistically from its reaction immediately after suspension (0 hours).

On the basis of the work carried out in this study, it would not be possible to correctly select and recommend a diluent or diluents for use in accepted disinfectant or sanitizer test procedures. Such a recommendation would be invalid as no accepted test procedure requires the centrifugation and washing of the micro-organisms prior to suspension. This was an important part of the preparative procedure for the present trials. There can be no doubt that the carry-over of growth medium with the cells must affect the reaction of the micro-organism to a disinfectant. Although a cautionary phrase is normally included in the standard test procedures to the effect that disturbance

of the agar slope surface should be minimal, this precaution alone may not minimize inter-laboratory and inter-worker variation as successfully as a procedure in which centrifugation and washing of the cell suspension is applied.

Peptone, as it is of organic origin and is commonly included in growth media, as well as forming an excellent diluent for bacterial suspensions, was included to investigate its effect on the reaction of bacterial cells to disinfectants. Each millilitre of bacterial suspension in 0,1% peptone would contain approximately 0,001 g peptone which would be added with the bacterial cells to the tempered disinfectant test solution. Whether such a quantity would be comparable with the mass of growth medium carried over with unwashed bacterial cells is difficult to assess.

Although it is not possible to recommend a particular diluent or series of diluents for use in recognised test procedures, there can be no doubt that the hypothesis that diluents do affect the reaction of a micro-organism to a disinfectant under various test circumstances, has been amply demonstrated in this series of experiments. The fact that not a single one of the diluents considered was capable of producing consistent decimal reduction times for all the test conditions indicates that diluent influences vary.

A possible criticism of the experiments would be that each of the test conditions consisted of only three replications. However the fact that statistical differences were obtained at $P<0,05$ is indication enough that increased replications would probably have only produced more powerful test circumstances and would not have increased the number of results that were statistically significant.

No attempt can be made to explain the varied reactions of the micro-organisms to the diluents, as these aspects were not investigated. A consideration of the literature quoted indicates that the reaction of micro-organisms to their environment is extremely complex. Throughout these experiments, all procedures were controlled as rigidly as possible, so that the results could with confidence be attributed to the effects of the variables being investigated.

The reported experiments can only be considered to be preliminary investigations into the effect of diluents on micro-organisms in their reaction to disinfectants. Consideration should be given to the evaluation of the effects of centrifugation and washing in diluent of the test micro-organism prior to suspension, against suspension in diluent directly from growth media. The diluents used in these experiments by no means exhaust the diluents available for bacterial suspension; thus other diluents should be investigated to determine their suitability under specific test conditions, and further, the specific influences of diluents on the reaction of micro-organisms to disinfectant test conditions would be worth of study.

CHAPTER XI

SUMMARY

The reaction of the bacterial cell to its environment is determined by a large number of factors. A consideration of the factors influencing the reaction of a micro-organism to a disinfectant or sanitizer suggests that the suspension diluent could affect

(i) the viability of the bacterial cell suspension, and
(ii) the resistance of the micro-organism to a disinfectant.

Investigating the influence of six commonly specified diluents on a selection of bacterial species, it was found that relatively small bacterial cell suspensions ($2{,}3 \times 10^5$ cells/ml) were affected to a greater degree than larger bacterial populations ($2\text{-}6 \times 10^7$ cells/ml).

Suspensions of Staphylococcus aureus and Escherichia coli were prepared by centrifugation and washing in one of the following diluents: 0,1% peptone, 0,85% saline, Ringer's and ¼ Ringer's solutions, phosphate buffered distilled water and distilled water. The suspensions were exposed to concentrations of an iodophor and a QAC sufficient to allow survivors to be present after an exposure period of five minutes. The iodophor and QAC solutions were prepared either in distilled water, or hard water or hard water plus milk test solutions. Test procedures were carried out immediately after preparation of the bacterial suspension and also at 1½ hours and 3 hours after initial suspension.

Statistical analyses of the results indicated that, under the specified conditions, Ringer's solution and ¼ Ringer's solution were the only diluents suitable for the preparation of the bacterial test suspensions because they, compared with the other diluents, were the most consistent in their affect on the reaction of the bacterial cells to disinfectants; further, that bacterial suspensions prepared from these diluents were only satisfactory for consecutive tests over a period of 1½ hours.

None of the other diluents were suitable as the reactions of the bacterial suspensions to the disinfectant test conditions were either not consistent or comparable.

The findings presented are not of direct application to recognised disinfectant or sanitizer procedures, as such procedures do not include centrifugation and washing of bacterial cells prior to suspension. The results suggest that the validity of some test procedures could be improved by the adoption of centrifugation and washing procedures and the selection of a more suitable diluent or diluents.

CHAPTER XII

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CHAPTER XIII

ACKNOWLEDGEMENTS

I acknowledge the Department of Agricultural Technical Services for the provision of facilities to undertake this study and my sincere appreciation to the following persons for their contribution to this thesis.

Professor C. W. Abbott, Head of the Department of Dairy Science, University of Natal, for his constructive criticism, guidance and support.

Dr. M. E. Stiles, previously Senior Lecturer, Department of Dairy Science, University of Natal, and now of the University of Alberta, at Edmonton, Canada, for initially suggesting the avenue of research, for his objective guidance during the early stages of the work and for his interest, support and suggestions after he had left the University of Natal.

Professor A. A. Rayner, Head of the Department of Biometry, University of Natal, Dr. R. M. Pringle, Senior Lecturer, Department of Biometry and Mr. S. Minnaar for their assistance in the initial analysis of data.

Dr. M. K. S. Hathorn of the London Hospital Medical College for his inestimable assistance with the preparation of the major portion of the data for computer analysis.

Miss J. Dormer for her technical assistance during the initial stages of this work.

Mrs. S. Volans, without whose conscientious help and attention to technical detail, the major portion of this work would not have been possible.

Mr. Solomon Sibisi who carried out the vast task of cleaning glassware, autoclaving and heat sterilization and whose devotion to duty played no small role in the quality of results obtained in these experiments.

Colleagues and friends for their co-operation and assistance.

My wife and family for their constant support and encouragement.

APPENDIX A

COMPUTATION 1

The analysis of variance applied to the percentage survivors of Aerobacter aerogenes, suspended in various diluents, over periods of three and six hours after initial suspension.

1. Means - 3 replications

Diluents	Time		Diluent Means
	3 hours	6 hours	
¼ Ringer's (¼R)	56,567	42,667	49,617
Saline (Sal)	73,600	57,433	65,517
Dist. Water (D.W.)	71,767	60,500	16,133
Ringer's (R)	83,767	81,433	82,600
Phos. Buffer (P.B.)	90,600	78,367	84,483
Peptone (Pep)	111,100	77,733	94,417
Time means	81,233	66,356	73,794

2. Analysis of Variance

Source of Variation	Degree of Freedom	Mean Squares	F. Ratio	P
Between diluents	5	1594,612	17,382	<0,01
Between time	1	1992,129	21,715	<0,01
Diluents x time	5	156,549	1,706	N.S.
Within replicates	24	91,738		

3. Results (significance probability = 0,05)

(a) Diluent Means in Ranked Order

Rank:	1	2	3	4	5	6
Diluent:	¼R	Sal.	D.W.	R.	P.B.	Pep
Means:(1)	49,617	65,517	66,133	82,600	84,483	94,417

(b) Time Means in Ranked Order

Rank:	1	2
Time:	6 hours	3 hours
Means:(1)	66,356	81,233

(1) NOTE: Any two means not underscored by the same line are significantly different (significance probability = 0,05)

Any two means underscored by the same line are not significantly different (significance probability = 0,05)

COMPUTATION 2

The analysis of variance applied to the percentage survivors of Staphylococcus aureus, suspended in various diluents, over periods of three and six hours after initial suspension.

1. Means - 3 replications

Diluents	Time		Diluent Means
	3 hours	6 hours	
Saline (Sal)	77,867	42,200	62,533
Dist. Water (D.W.)	84,500	43,367	63,933
¼ Ringer's (¼R)	67,900	61,000	64,450
Phos. Buffer (P.B.)	74,833	55,633	65,233
Peptone (P)	69,200	62,267	65,733
Ringer's (R)	77,000	56,700	66,850
Time means	75,217	54,361	64,789

2. Analysis of Variance

Source of Variation	Degree of Freedom	Mean Squares	F. Ratio	P
Between diluents	5	13,527	0,115	N.S.
Between time	1	3914,589	33,425	<0,01
Diluents x time	5	269,716	2,303	N.S.
Within replicates	24	117,116		

3. Results (significance probability = 0,05)

a) Time Means in Ranked Order

Rank:	1	2
Time:	6 hours	3 hours
Means:(1)	54,361	75,217

(1) NOTE: Any two means not underscored by the same line are significantly different (significance probability = 0,05)

COMPUTATION 3

The analysis of variance applied to the percentage survivors of Pseudomonas aeruginosa, suspended in various diluents, over periods of three and six hours after initial suspension.

1. Means - 3 replications

Diluents	Time 3 hours	Time 6 hours	Diluent Means
Dist. Water (D.W.)	13,067	9,900	11,483
Phos. Buffer (P.B.)	36,267	29,933	33,100
Saline (Sal)	40,333	32,967	36,650
¼ Ringer's (¼R)	62,000	48,067	55,033
Ringer's (R)	78,900	70,233	74,567
Peptone (Pep)	114,100	65,467	89,783
Time means	57,444	42,761	50,103

2. Analysis of Variance

Source of Variation	Degree of Freedom	Mean Squares	F. Ratio	P
Between diluents	5	4990,641	18,530	<0,01
Between time	1	1940,400	7,205	<0,05
Diluents x time	5	433,577	1,610	N.S.
Within replicates	24	269,321		

3. Results (significance probability = 0,05)

a) Diluent Means in Ranked Order

Rank:	1	2	3	4	5	6
Diluent:	D.W.	P.B.	Sal	¼R	R	Pep
Means:(1)	11,483	33,100	36,650	55,033	74,567	89,783

b) Time Means in Ranked Order

Rank:	1	2
Time:	6 hours	3 hours
Means:(1)	42,761	57,444

(1) NOTE: Any two means not underscored by the same line are significantly different (significance probability = 0,05)

Any two means underscored by the same line are not significantly different (significance probability = 0,05)

COMPUTATION 4

The analysis of variance applied to the percentage survivors of Micrococcus flavus, suspended in various diluents, over periods of three and six hours after initial suspension.

1. Means - 3 replications

Diluents	Time 3 hours	Time 6 hours	Diluent Means
Dist. Water (D.W.)	60,200	19,233	39,717
¼ Ringer's (¼R)	66,133	59,500	62,817
Ringer's (R)	72,633	70,933	71,783
Saline (Sal)	82,500	77,400	79,950
Phos. Buffer (P.B.)	87,467	86,767	87,117
Peptone (Pep)	102,867	96,800	99,833
Time means	78,633	68,439	73,536

2. Analysis of Variance

Source of Variation	Degree of Freedom	Mean Squares	F. Ratio	P
Between diluents	5	2614,612	19,228	<0,01
Between time	1	935,340	6,879	<0,05
Diluents x time	5	349,461	2,570	N.S.
Within replicates	24	135,977		

3. Results (significance probability = 0,05)

(a) Diluent Means in Ranked Order

Rank:	1	2	3	4	5	6
Diluent:	D.W.	¼R	R	Sal	P.B.	Pep
Means:(1)	39,717	62,817	71,783	79,950	87,117	99,833

(b) Time Means in Ranked Order

Rank:	1	2
Time:	6 hours	3 hours
Means:(1)	68,439	78,633

(1) NOTE: Any two means not underscored by the same line are significantly different (significance probability = 0,05)

Any two means underscored by the same line are not significantly different (significance probability = 0,05)

COMPUTATION 5

The analysis of variance applied to the percentage survivors of Escherichia coli, suspended in various diluents, over periods of three and six hours after initial suspension.

1. Means - 3 replications

Diluents	Time 3 hours	Time 6 hours	Diluent Means
Dist. Water (D.W.)	57,800	41,133	49,467
Phos. Buffer (P.B.)	75,867	59,900	67,883
Saline (Sal)	76,167	59,967	68,067
¼ Ringer's (¼R)	77,333	74,367	75,850
Ringer's (R)	92,800	78,533	85,667
Peptone (Pep)	100,700	115,633	108,167
Time means	80,111	71,589	75,850

2. Analysis of Variance

Source of Variation	Degree of Freedom	Mean Squares	F. Ratio	P
Between diluents	5	2353,031	28,121	<0,01
Between time	1	653,653	7,812	<0,05
(1) Diluents x time	5	238,409	2,849	<0,05
Within replicates	24	83,675		

(1) The interaction between diluents x time - significant at P<0,05 - is entirely due to the increase in viable numbers in peptone.

3. Results (significance probability = 0,05)

(a) Diluent Means in Ranked Order

Rank:	1	2	3	4	5	6
Diluent:	D.W.	P.B.	Sal	¼R	R	Pep
Means:(2)	49,467	67,883	68,067	75,850	85,667	108,167

(b) Time Means in Ranked Order

Rank:	1	2
Time:	6 hours	3 hours
Means:(2)	71,589	80,111

(2) NOTE: Any two means not underscored by the same line are significantly different (significance probability = 0,05)

Any two means underscored by the same line are not significantly different (significance probability = 0,05)

APPENDIX B

COMPUTATION 1

The analysis of variance applied to the death rate data of Staphylococcus aureus, suspended in various diluents over a period of 3 hours, subjected to an iodophor in a test solution of distilled water (3 replications).

1. Means (b_{yx}) - 3 replications

TIME AFTER INITIAL SUSPENSION

Diluents	0 hours	1½ hours	3 hours	Diluent Means
Peptone (Pep)	0,345	0,502	0,240	0,362
Dist. water (D.W.)	0,982	0,741	0,280	0,668
Ringer's (R.)	1,076	0,752	0,292	0,706
¼ Ringer's (¼R.)	0,806	1,118	0,828	0,917
Phos. Buffer (P.B.)	1,161	1,048	0,778	0,996
Saline (Sal)	1,106	1,049	0,924	1,026
Time mean values	0,913	0,868	0,556	0,779

2. Analysis of Variance

Source of Variation	Degree of Freedom	Mean Squares	F. Ratio	P
Between diluents	5	0,57299	9,082	<0,01
Between time	2	0,67622	10,718	<0,01
Diluents x time	10	0,09118	1,445	N.S.
Within replicates	36	0,06309		

3. Results: (significance probability = 0,05)

a) Diluent Means in Ranked Order

Rank:	1	2	3	4	5	6
Diluent:	Pep	D.W.	R.	¼R.	P.B.	Sal
Means:(1)	0,362	0,668	0,706	0,917	0,996	1,026

b) Time Means in Ranked Order

Rank:	1	2	3
Time:	3 hours	1½ hours	0 hours
Means:(1)	0,556	0,868	0,913

(1) NOTE: Any two means not underscored by the same line are significantly different (significance probability = 0,05)
Any two means underscored by the same line are not significantly different (significance probability = 0,05)

4. One-way Analysis of Variance

Source of Variation	Degree of Freedom	Mean Squares	F. Ratio	P
Between groups	17	0,30172	4,7824	<0,01
Within groups	36	0,06309		
Total	53			

5. Treatment Means in Ranked Order

Mean (b_{yx})	Diluent/Time (hrs)		Rank
0,240	Peptone	3	1
0,280	Dist. Water	3	2
0,292	Ringer's	3	3
0,345	Peptone	0	4
0,502	Peptone	1½	5
0,741	Dist. Water	1½	6
0,752	Ringer's	1½	7
0,778	Phos. Buffer	3	8
0,806	¼ Ringer's	0	9
0,828	¼ Ringer's	3	10
0,924	Saline	3	11
0,982	Dist. Water	0	12
1,048	Phos. Buffer	1½	13
1,049	Saline	1½	14
1,076	Ringer's	0	15
1,106	Saline	0	16
1,118	¼ Ringer's	1½	17
1,161	Phos. Buffer	0	18

Note: Two means linked by a common line do not differ from one another significantly (significance probability = 0,05).

Two means not linked by a common line differ from one another significantly (significance probability = 0,05).

6. Results

There are 6 homogeneous subsets (subsets of elements, no pair of which differ by more than the shortest significant range for a subset of that size) which are listed as follows:

Distilled Water, 1½ hours to Phosphate Buffer, 0 hours;

Peptone, 1½ hours to Saline, 3 hours;

Peptone, 0 hours to ¼ Ringer's, 0 hours;

Peptone, 3 hours to Peptone, 1½ hours;

Ringer's, 3 hours to Ringer's 1½ hours;

Distilled Water, 3 hours to Distilled Water, 1½ hours.

COMPUTATION 2

The analysis of variance applied to the death rate data of Staphylococcus aureus, suspended in various diluents over a period of 3 hours, subjected to an iodophor in a test solution of hard water (3 replications).

1. Means (b_{yx}) - 3 replications

TIME AFTER INITIAL SUSPENSION

Diluents	0 hours	1½ hours	3 hours	Diluent Means
Phos. Buffer (P.B.)	0,336	0,291	0,426	0,351
Ringer's (R.)	0,499	0,702	0,682	0,628
Dist. water (D.W.)	0,771	0,658	0,544	0,658
¼ Ringer's (¼R.)	0,510	0,676	0,849	0,678
Peptone (Pep)	0,248	0,989	1,191	0,809
Saline (Sal)	0,865	0,918	0,678	0,820
Time mean values	0,538	0,706	0,728	0,657

2. Analysis of Variance

Source of Variation	Degree of Freedom	Mean Squares	F. Ratio	P
Between diluents	5	0,26069	6,812	<0,01
Between time	2	0,19451	5,083	<0,05
Diluents x time	10	0,15407	4,026	<0,01
Within replicates	36	0,03827		

3. Results: (significance probability = 0,05)

Diluent Means in Ranked Order

Rank:	1	2	3	4	5	6
Diluent:	P.B.	R.	D.W.	¼R.	Pep	Sal
Means:(1)	0,351	0,628	0,658	0,678	0,809	0,820

Time Means in Ranked Order

Rank:	1	2	3
Time:	0 hours	1½ hours	3 hours
Means:(1)	0,538	0,706	0,728

(1) NOTE: Any two means not underscored by the same line are significantly different (significance probability = 0,05).

Any two means underscored by the same line are not significantly different (significance probability = 0,05).

4. One-way Analysis of Variance

Source of Variation	Degree of Freedom	Mean Squares	F. Ratio	P
Between groups	17	0,19019	4,9697	<0,01
Within groups	36	0,03827		
Total	53			

5. Treatment Means in Ranked Order

Mean (b_{yx})	Diluent/Time (hrs)		Rank
0,248	Peptone	0	1
0,291	Phos. Buffer	1½	2
0,336	Phos. Buffer	0	3
0,426	Phos. Buffer	3	4
0,499	Ringer's	0	5
0,510	¼ Ringer's	0	6
0,544	Dist. Water	3	7
0,658	Dist. Water	1½	8
0,676	¼ Ringer's	1½	9
0,678	Saline	3	10
0,682	Ringer's	3	11
0,702	Ringer's	1½	12
0,771	Dist. Water	0	13
0,849	¼ Ringer's	3	14
0,865	Saline	0	15
0,918	Saline	1½	16
0,989	Peptone	1½	17
1,191	Peptone	3	18

Note: Two means linked by a common line do not differ from one another significantly (significance probability = 0,05).

Two means not linked by a common line differ from one another significantly (significance probability = 0,05).

6. Results

There are 8 homogeneous subsets (subsets of elements, no pair of which differ by more than the shortest significant range for a subset of that size) which are listed as follows:

Phosphate Buffer, 3 hours to Distilled Water, 0 hours;
Distilled Water, 1½ hours to Peptone, 1½ hours;
Ringer's, 0 hours to Saline, 0 hours;
Distilled Water, 3 hours to Saline, 1½ hours;
Phosphate Buffer, 0 hours to Ringer's, 1½ hours;
Peptone, 0 hours to Distilled Water, 3 hours;
Phosphate Buffer, 1½ hours to Distilled Water, 1½ hours;
¼ Ringer's, 3 hours to Peptone, 3 hours.

COMPUTATION 3

The analysis of variance applied to the death rate data of Staphylococcus aureus, suspended in various diluents over a period of 3 hours, subjected to an iodophor in a test solution of hard water + milk (3 replications).

1. Means (b_{yx}) - 3 replications

TIME AFTER INITIAL SUSPENSION

Diluents	0 hours	1½ hours	3 hours	Diluent Means
Peptone (Pep)	0,160	0,177	0,494	0,277
¼ Ringer's (¼R.)	0,989	0,634	1,084	0,902
Saline (Sal)	1,179	1,373	0,938	1,163
Ringer's (R.)	1,276	0,968	1,354	1,199
Phos. Buffer (P.B.)	1,200	1,474	1,223	1,299
Dist. water (D.W.)	1,015	1,471	1,629	1,371
Time mean values	0,970	1,016	1,120	1,035

2. Analysis of Variance

Source of Variation	Degree of Freedom	Mean Squares	F. Ratio	P
Between diluents	5	1,47333	14,366	<0,01
Between time	2	0,10701	1,043	N.S.
Diluents x time	10	0,16190	1,579	N.S.
Within replicates	36	0,10255		

3. Results: (significance probability = 0,05)

Diluent Means in Ranked Order

Rank:	1	2	3	4	5	6
Diluent:	Pep	¼R.	Sal	R.	P.B.	D.W.
Means:(1)	0,277	0,902	1,163	1,199	1,299	1,371

(1) NOTE: Any two means not underscored by the same line are significantly different (significance probability = 0,05).

Any two means underscored by the same line are not significantly different (significance probability = 0,05).

4. One-way Analysis of Variance

Source of Variation	Degree of Freedom	Mean Squares	F. Ratio	P
Between groups	17	0,54115	5,2767	<0,01
Within groups	36	0,10255		
Total	53			

5. Treatment Means in Ranked Order

Mean (b_{yx})	Diluent/Time (hrs)		Rank
0,160	Peptone	0	1
0,177	Peptone	1½	2
0,494	Peptone	3	3
0,634	¼ Ringer's	1½	4
0,937	Saline	3	5
0,968	Ringer's	1½	6
0,989	¼ Ringer's	0	7
1,015	Dist. Water	0	8
1,084	¼ Ringer's	3	9
1,179	Saline	0	10
1,200	Phos. Buffer	0	11
1,223	Phos. Buffer	3	12
1,276	Ringer's	0	13
1,354	Ringer's	3	14
1,373	Saline	1½	15
1,471	Dist. Water	1½	16
1,474	Phos. Buffer	1½	17
1,629	Dist. Water	3	18

Note: Two means linked by a common line do not differ from one another significantly (significance probability = 0,05).

Two means not linked by a common line differ from one another significantly (significance probability = 0,05).

6. Results

There are 5 homogeneous subsets (subsets of elements, no pair of which differ by more than the shortest significant range for a subset of that size) which are listed as follows:

Saline, 3 hours to Phosphate Buffer, 1½ hours;

¼ Ringer's, 1½ hours to Phosphate Buffer, 3 hours;

Distilled Water, 0 hours to Distilled Water, 3 hours;

Peptone, 3 hours to ¼ Ringer's, 3 hours;

Peptone, 0 hours to ¼ Ringer's, 1½ hours.

COMPUTATION 4

The analysis of variance applied to the death rate data of Staphylococcus aureus, suspended in various diluents over a period of 3 hours, subjected to a QAC in a test solution of distilled water (3 replications).

1. Means (b_{yx}) - 3 replications

TIME AFTER INITIAL SUSPENSION

Diluents	0 hours	1½ hours	3 hours	Diluent Means
Saline (Sal)	0,160	0,132	0,424	0,239
Peptone (Pep)	0,534	0,762	0,464	0,587
Dist. water (D.W.)	0,404	0,610	0,798	0,604
Ringer's (R.)	0,551	0,303	1,008	0,621
¼ Ringer's (¼R.)	0,908	0,844	0,809	0,854
Phos. Buffer (P.B.)	2,382	1,226	0,527	1,378
Time mean values	0,823	0,646	0,672	0,714

2. Analysis of Variance

Source of Variation	Degree of Freedom	Mean Squares	F. Ratio	P
Between diluents	5	1,30247	2,942	<0,05
Between time	2	0,16491	0,373	N.S.
Diluents x time	10	0,62541	1,413	N.S.
Within replicates	36	0,44270		

3. Results: (significance probability = 0,05)

a) Diluent Means in Ranked Order

Rank:	1	2	3	4	5	6
Diluent:	Sal	Pep	D.W.	R.	¼R.	P.B.
Means:(1)	0,239	0,587	0,604	0,621	0,854	1,378

(1) NOTE: Any two means not underscored by the same line are significantly different (significance probability = 0,05). Any two means underscored by the same line are not significantly different (significance probability = 0,05).

4. One-way Analysis of Variance

Source of Variation	Degree of Freedom	Mean Squares	F. Ratio	P
Between groups	17	0,77038	1,7402	N.S.
Within groups	36	0,44270		
Total	53			

COMPUTATION 5

The analysis of variance applied to the death rate data of Staphylococcus aureus, suspended in various diluents over a period of 3 hours, subjected to a QAC in a test solution of hard water (3 replications).

1. Means (b_{yx}) - 3 replications

TIME AFTER INITIAL SUSPENSION

Diluents	0 hours	1½ hours	3 hours	Diluent Means
Saline (Sal)	0,849	0,433	0,668	0,650
Ringer's (R.)	0,995	0,615	0,691	0,767
Dist. water (D.W.)	0,620	0,892	0,894	0,802
¼ Ringer's (¼R.)	0,820	1,215	1,045	1,027
Phos. Buffer (P.B.)	0,811	1,212	1,091	1,038
Peptone (Pep)	1,401	1,633	1,132	1,389
Time mean values	0,916	1,000	0,920	0,945

2. Analysis of Variance

Source of Variation	Degree of Freedom	Mean Squares	F. Ratio	P
Between diluents	5	0,63269	4,523	<0,01
Between time	2	0,04040	0,289	N.S.
Diluents x time	10	0,14387	1,028	N.S.
Within replicates	36	0,13989		

3. Results: (significance probability = 0,05)

a) Diluent Means in Ranked Order

Rank:	1	2	3	4	5	6
Diluent:	Sal	R.	D.W.	¼R.	P.B.	Pep
Means:(1)	0,650	0,767	0,802	1,027	1,038	1,389

(1) NOTE: Any two means not underscored by the same line are significantly different (significance probability = 0,05).

Any two means underscored by the same line are not significantly different (significance probability = 0,05).

4. One-way Analysis of Variance

Source of Variation	Degree of Freedom	Mean Squares	F. Ratio	P
Between groups	17	0,27546	1,9692	<0,05
Within groups	36	0,13989		
Total	53			

5. Treatment Means in Ranked Order

Mean (b_{yx})	Diluent/Time (hrs)		Rank
0,433	Saline	$1\frac{1}{2}$	1
0,615	Ringer's	$1\frac{1}{2}$	2
0,620	Dist. Water	0	3
0,668	Saline	3	4
0,691	Ringer's	3	5
0,811	Phos. Buffer	0	6
0,820	$\frac{1}{4}$ Ringer's	0	7
0,849	Saline	0	8
0,892	Dist. Water	$1\frac{1}{2}$	9
0,894	Dist. Water	3	10
0,995	Ringer's	0	11
1,045	$\frac{1}{4}$ Ringer's	3	12
1,091	Phos. Buffer	3	13
1,132	Peptone	3	14
1,212	Phos. Buffer	$1\frac{1}{2}$	15
1,215	$\frac{1}{4}$ Ringer's	$1\frac{1}{2}$	16
1,401	Peptone	0	17
1,633	Peptone	$1\frac{1}{2}$	18

Note: Two means linked by a common line do not differ from one another significantly (significance probability = 0,05).

Two means not linked by a common line differ from one another significantly (significance probability = 0,05).

6. Results

There are 4 homogeneous subsets (subsets of elements, no pair of which differ by more than the shortest significant range for a subset of that size) which are listed as follows:

Ringer's, $1\frac{1}{2}$ hours to $\frac{1}{4}$ Ringer's, $1\frac{1}{2}$ hours;

Saline, 3 hours to Peptone 0 hours;

Saline, $1\frac{1}{2}$ hours to Peptone 3 hours;

Ringer's, 0 hours to Peptone $1\frac{1}{2}$ hours.

COMPUTATION 6

The analysis of variance applied to the death rate data of Staphylococcus aureus, suspended in various diluents over a period of 3 hours, subjected to a QAC in a test solution of hard water + milk (3 replications).

1. Means (b_{yx}) - 3 replications

TIME AFTER INITIAL SUSPENSION

Diluents	0 hours	1½ hours	3 hours	Diluent Means
Ringer's (R.)	0,360	0,473	0,395	0,409
Saline (Sal)	0,416	0,465	0,469	0,450
Phos. Buffer (P.B.)	0,615	0,574	0,476	0,555
¼ Ringer's (¼R.)	0,920	0,487	0,589	0,665
Dist. water (D.W.)	0,060	0,926	0,805	0,931
Peptone (Pep)	1,844	1,792	0,684	1,440
Time mean values	0,869	0,786	0,570	0,742

2. Analysis of Variance

Source of Variation	Degree of Freedom	Mean Squares	F. Ratio	P
Between diluents	5	1,36710	9,169	<0,01
Between time	2	0,43021	2,885	N.S.
Diluents x time	10	0,21780	1,461	N.S.
Within replicates	36	0,14910		

3. Results: (significance probability = 0,05)

a) Diluent Means in Ranked Order

Rank:	1	2	3	4	5	6
Diluent:	R.	Sal	P.B.	¼R.	D.W.	Pep
Means:(1)	0,409	0,450	0,555	0,665	0,931	1,440

(1) NOTE: Any two means not underscored by the same line are significantly different (significance probability = 0,05).

Any two means underscored by the same line are not significantly different (significance probability = 0,05).

4. One-way Analysis of Variance

Source of Variation	Degree of Freedom	Mean Squares	F. Ratio	P
Between groups	17	0,58082	3,8954	<0,01
Within groups	36	0,14910		
Total	53			

5. Treatment Means in Ranked Order

Mean (b_{yx})	Diluent/Time (hrs)		Rank
0,360	Ringer's	0	1
0,395	Ringer's	3	2
0,416	Saline	0	3
0,465	Saline	1½	4
0,469	Saline	3	5
0,473	Ringer's	1½	6
0,476	Phos. Buffer	3	7
0,487	¼ Ringer's	1½	8
0,574	Phos. Buffer	1½	9
0,589	¼ Ringer's	3	10
0,615	Phos. Buffer	0	11
0,684	Peptone	3	12
0,805	Dist. Water	3	13
0,920	¼ Ringer's	0	14
0,926	Dist. Water	1½	15
1,060	Dist. Water	0	16
1,792	Peptone	1½	17
1,844	Peptone	0	18

Note: Two means linked by a common line do not differ from one another significantly (significance probability = 0,05).

Two means not linked by a common line differ from one another significantly (significance probability = 0,05).

6. Results

There are 2 homogeneous subsets (subsets of elements, no pair of which differ by more than the shortest significant range for a subset of that size) which are listed as follows:

Ringer's, 0 hours to Distilled Water, 0 hours;

Peptone, 1½ hours to Peptone, 0 hours.

COMPUTATION 7

The analysis of variance applied to the death rate data of Escherichia coli, suspended in various diluents over a period of 3 hours, subjected to an iodophor in a test solution of distilled water (3 replications).

1. Means (b_{yx}) - 3 replications

TIME AFTER INITIAL SUSPENSION

Diluents	0 hours	1½ hours	3 hours	Diluent Means
Peptone (Pep)	0,111	0,386	0,070	0,189
¼ Ringer's (¼R.)	0,656	0,688	0,490	0,611
Saline (Sal)	0,544	0,702	0,597	0,614
Phos. Buffer (P.B.)	0,978	0,752	0,602	0,777
Ringer's (R.)	0,914	0,677	0,822	0,805
Dist. water (D.W.)	1,154	1,137	0,880	1,057
Time mean values	0,726	0,724	0,577	0,676

2. Analysis of Variance

Source of Variation	Degree of Freedom	Mean Squares	F. Ratio	P
Between diluents	5	0,75108	16,286	<0,01
Between time	2	0,13140	2,849	N.S.
Diluents x time	10	0,04630	1,004	N.S.
Within replicates	36	0,04612		

3. Results: (significance probability = 0,05)

a) Diluent Means in Ranked Order

Rank:	1	2	3	4	5	6
Diluent:	Pep	¼R.	Sal	P.B.	R.	D.W.
Means:(1)	0,189	0,611	0,614	0,777	0,805	1,057

(1) NOTE: Any two means not underscored by the same line are significantly different (significance probability = 0,05).

Any two means underscored by the same line are not significantly different (significance probability = 0,05).

4. One-way Analysis of Variance

Source of Variation	Degree of Freedom	Mean Squares	F. Ratio	P
Between groups	17	0,26360	5,7159	<0,01
Within groups	36	0,04612		
Total	53			

5. Treatment Means in Ranked Order

Mean (b_{yx})	Diluent/Time (hrs)		Rank
0,070	Peptone	3	1
0,111	Peptone	0	2
0,386	Peptone	1½	3
0,490	¼ Ringer's	3	4
0,544	Saline	0	5
0,597	Saline	3	6
0,602	Phos. Buffer	3	7
0,656	¼ Ringer's	0	8
0,677	Ringer's	1½	9
0,688	¼ Ringer's	1½	10
0,702	Saline	1½	11
0,752	Phos. Buffer	1½	12
0,822	Ringer's	3	13
0,880	Dist. Water	3	14
0,914	Ringer's	0	15
0,978	Phos. Buffer	0	16
1,137	Dist. Water	1½	17
1,154	Dist. Water	0	18

Note: Two means linked by a common line do not differ from one another significantly (significance probability = 0,05).

Two means not linked by a common line differ from one another significantly (significance probability = 0,05).

6. Results

There are 6 homogeneous subsets (subsets of elements, no pair of which differ by more than the shortest significant range for a subset of that size) which are listed as follows:

¼ Ringer's, 3 hours to Distilled Water, 3 hours;

Saline, 3 hours to Phosphate Buffer, 0 hours;

Phosphate Buffer, 1½ hours to Distilled Water, 0 hours;

Peptone, 1½ hours to Phosphate Buffer, 1½ hours;

Saline, 0 hours to Ringer's 0 hours;

Peptone, 3 hours to Peptone, 1½ hours.

COMPUTATION 8

The analysis of variance applied to the death rate data of Escherichia coli, suspended in various diluents over a period of 3 hours, subjected to an iodophor in a test solution of hard water (3 replications).

1. Means (b_{yx}) - 3 replications

TIME AFTER INITIAL SUSPENSION

Diluents	0 hours	1½ hours	3 hours	Diluent Means
¼Ringer's (¼R.)	1,016	0,449	0,641	0,702
Peptone (Pep)	0,933	0,557	0,693	0,728
Ringer's (R.)	0,856	0,960	0,479	0,765
Phos. Buffer (P.B.)	0,479	1,020	0,895	0,798
Dist. water (D.W.)	1,771	1,144	1,218	1,378
Saline (Sal)	0,537	1,922	1,979	1,479
Time mean values	0,932	1,009	0,984	0,975

2. Analysis of Variance

Source of Variation	Degree of Freedom	Mean Squares	F. Ratio	P
Between diluents	5	1,12925	6,946	<0,01
Between time	2	0,02757	0,170	N.S.
Diluents x time	10	0,62338	3,835	<0,01
Within replicates	36	0,16257		

3. Results: (significance probability = 0,05)

a) Diluent Means in Ranked Order

Rank:	1	2	3	4	5	6
Diluent:	¼R.	Pep	R.	P.B.	D.W.	Sal
Means:(1)	0,702	0,728	0,765	0,798	1,378	1,479

(1) NOTE: Any two means not underscored by the same line are significantly different (significance probability = 0,05).

Any two means underscored by the same line are not significantly different (significance probability = 0,05).

4. One-way Analysis of Variance

Source of Variation	Degree of Freedom	Mean Squares	F. Ratio	P
Between groups	17	0,70207	4,3186	<0,01
Within groups	36	0,16257		
Total	53			

5. Treatment Means in Ranked Order

Mean (b_{yx})	Diluent/Time (hrs)		Rank
0,449	¼ Ringer's	1½	1
0,479	Phos. Buffer	0	2
0,479	Ringer's	3	3
0,537	Saline	0	4
0,557	Peptone	1½	5
0,641	¼ Ringer's	3	6
0,693	Peptone	3	7
0,856	Ringer's	0	8
0,895	Phos. Buffer	3	9
0,933	Peptone	0	10
0,960	Ringer's	1½	11
1,016	¼ Ringer's	0	12
1,020	Phos. Buffer	1½	13
1,144	Dist. Water	1½	14
1,218	Dist. Water	3	15
1,771	Dist. Water	0	16
1,922	Saline	1½	17
1,979	Saline	3	18

Note: Two means linked by a common line do not differ from one another significantly (significance probability = 0,05).

Two means not linked by a common line differ from one another significantly (significance probability = 0,05).

6. Results

There 3 homogeneous subsets (subsets of elements, no pair of which differ by more than the shortest significant range for a subset of that size) which are listed as follows:

¼ Ringer's, 1½ hours to Distilled Water, 3 hours;

Distilled Water, 1½ hours to Distilled Water, 0 hours;

Distilled Water, 0 hours to Saline, 3 hours.

COMPUTATION 9

The analysis of variance applied to the death rate data of Escherichia coli, suspended in various diluents over a period of 3 hours, subjected to an iodophor in a test solution of hard water + milk (3 replications).

1. Means (b_{yx}) - 3 replications

TIME AFTER INITIAL SUSPENSION

Diluents	0 hours	1½ hours	3 hours	Diluent Means
Peptone (Pep)	0,724	0,446	0,185	0,452
Saline (Sal)	1,231	1,487	0,971	1,230
Phos. Buffer (P.B.)	1,122	1,415	1,188	1,242
Ringer's (R.)	1,495	1,436	1,419	1,450
Dist. water (D.W.)	1,036	1,284	2,339	1,553
¼ Ringer's (¼R.)	1,832	1,672	1,433	1,646
Time mean values	1,240	1,290	1,256	1,262

2. Analysis of Variance

Source of Variation	Degree of Freedom	Mean Squares	F. Ratio	P
Between diluents	5	1,66558	5,188	<0,01
Between time	2	0,01174	0,037	N.S.
Diluents x time	10	0,40757	1,270	N.S.
Within replicates	36	0,32102		

3. Results: (significance probability = 0,05)

a) Diluent Means in Ranked Order

Rank:	1	2	3	4	5	6
Diluent:	Pep	Sal	P.B.	R.	D.W.	¼R.
Means:(1)	0,452	1,230	1,242	1,450	1,553	1,646

(1) NOTE: Any two means not underscored by the same line are significantly different (significance probability = 0,05).

Any two means underscored by the same line are not significantly different (significance probability = 0,05).

4. One-way Analysis of Variance

Source of Variation	Degree of Freedom	Mean Squares	F. Ratio	P
Between groups	17	0,73101	2,2772	<0,05
Within groups	36	0,32102		
Total	53			

5. Treatment Means in Ranked Order

Mean (b_{yx})	Diluent/Time (hrs)		Rank
0,185	Peptone	3	1
0,446	Peptone	$1\frac{1}{2}$	2
0,724	Peptone	0	3
0,971	Saline	3	4
1,036	Dist. Water	0	5
1,122	Phos. Buffer	0	6
1,188	Phos. Buffer	3	7
1,231	Saline	0	8
1,284	Dist. Water	$1\frac{1}{2}$	9
1,415	Phos. Buffer	$1\frac{1}{2}$	10
1,419	Ringer's	3	11
1,433	$\frac{1}{4}$ Ringer's	3	12
1,436	Ringer's	$1\frac{1}{2}$	13
1,487	Saline	$1\frac{1}{2}$	14
1,495	Ringer's	0	15
1,672	$\frac{1}{4}$ Ringer's	$1\frac{1}{2}$	16
1,832	$\frac{1}{4}$ Ringer's	0	17
2,339	Dist. Water	3	18

Note: Two means linked by a common line do not differ from one another significantly (significance probability = 0,05).

Two means not linked by a common line differ from one another significantly (significance probability = 0,05).

6. Results

There are 4 homogeneous subsets (subsets of elements, no pair of which differ by more than the shortest significant range for a subset of that size) which are listed as follows:

Distilled Water, $1\frac{1}{2}$ hours to Distilled Water, 3 hours;

Peptone, 3 hours to Saline, 0 hours;

Peptone, 0 hours to $\frac{1}{4}$ Ringer's, 0 hours;

Peptone, $1\frac{1}{2}$ hours to Ringer's 0 hours.

COMPUTATION 10

The analysis of variance applied to the death rate data of Escherichia coli, suspended in various diluents over a period of 3 hours, subjected to a QAC in a test solution of distilled water (3 replications).

1. Means (b_{yx}) - 3 replications

TIME AFTER INITIAL SUSPENSION

Diluents	0 hours	1½ hours	3 hours	Diluent Means
Saline (Sal)	0,699	0,276	0,733	0,569
Dist. water (D.W.)	0,949	0,734	0,726	0,803
Ringer's (R.)	0,799	0,767	0,890	0,819
Peptone (Pep)	0,208	1,022	1,355	0,862
Phos. Buffer (P.B.)	0,797	0,765	1,040	0,867
¼ Ringer's (¼R.)	1,031	0,794	0,920	0,915
Time mean values	0,747	0,726	0,944	0,806

2. Analysis of Variance

Source of Variation	Degree of Freedom	Mean Squares	F. Ratio	P
Between diluents	5	0,13479	2,414	N.S.
Between time	2	0,25963	4,649	<0,05
Diluents x time	10	0,22974	4,114	<0,01
Within replicates	36	0,05584		

3. Results: (significance probability = 0,05)

a) Time Means in Ranked Order

Rank:	1	2	3
Time:	1½ hours	0 hours	3 hours
Means:(1)	0,726	0,747	0,944

(1) NOTE: Any two means not underscored by the same line are significantly different (significance probability = 0,05).

Any two means underscored by the same line are not significantly different (significance probability = 0,05).

4. One-way Analysis of Variance

Source of Variation	Degree of Freedom	Mean Squares	F. Ratio	P
Between groups	17	0,20533	3,6769	<0,01
Within groups	36	0,05584		
Total	53			

5. Treatment Means in Ranked Order

Mean (b_{yx})	Diluent/Time (hrs)		Rank
0,208	Peptone	0	1
0,276	Saline	1½	2
0,699	Saline	0	3
0,726	Dist. Water	3	4
0,733	Saline	3	5
0,734	Dist. Water	1½	6
0,765	Phos. Buffer	1½	7
0,767	Ringer's	1½	8
0,794	¼ Ringer's	1½	9
0,797	Phos. Buffer	0	10
0,799	Ringer's	0	11
0,890	Ringer's	3	12
0,920	¼ Ringer's	3	13
0,949	Dist. Water	0	14
1,022	Peptone	1½	15
1,031	¼ Ringer's	0	16
1,040	Phos. Buffer	3	17
1,355	Peptone	3	18

Note: Two means linked by a common line do not differ from one another significantly (significance probability = 0,05).

Two means not linked by a common line differ from one another significantly (significance probability = 0,05).

6. Results

There are 3 homogeneous subsets (subsets of elements, no pair of which differ by more than the shortest significant range for a subset of that size) which are listed as follows:

Saline, 0 hours to Phosphate Buffer, 3 hours;

¼ Ringer's, 3 hours to Peptone, 3 hours;

Peptone, 0 hours to Saline 1½ hours.

COMPUTATION 11

The analysis of variance applied to the death rate data of Escherichia coli, suspended in various diluents over a period of 3 hours, subjected to a QAC in a test solution of hard water (3 replications).

1. Means (b_{yx}) - 3 replications

TIME AFTER INITIAL SUSPENSION

Diluents	0 hours	1½ hours	3 hours	Diluent Means
Saline (Sal)	0,214	0,216	0,307	0,246
Peptone (Pep)	0,250	0,252	0,633	0,378
Dist. water (D.W.)	0,475	0,571	0,400	0,482
Phos. Buffer (P.B.)	0,694	0,694	0,219	0,536
Ringer's (R.)	0,504	0,471	0,728	0,567
¼ Ringer's (¼R.)	0,493	0,681	0,972	0,715
Time mean values	0,438	0,481	0,543	0,487

2. Analysis of Variance

Source of Variation	Degree of Freedom	Mean Squares	F. Ratio	P
Between diluents	5	0,23552	9,430	<0,01
Between time	2	0,05005	2,004	N.S.
Diluents x time	10	0,11701	4,685	<0,01
Within replicates	36	0,02498		

3. Results: (significance probability = 0,05)

a) Diluent Means in Ranked Order

Rank:	1	2	3	4	5	6
Diluent:	Sal	Pep	D.W.	P.B.	R.	¼R.
Means:(1)	0,246	0,378	0,482	0,536	0,567	0,715

(1) NOTE: Any two means not underscored by the same line are significantly different (significance probability = 0,05).

Any two means underscored by the same line are not significantly different (significance probability = 0,05).

4. One-way Analysis of Variance

Source of Variation	Degree of Freedom	Mean Squares	F. Ratio	P
Between groups	17	0,14399	5,7652	<0,01
Within groups	36	0,02498		
Total	53			

5. Treatment Means in Ranked Order

Mean (b_{yx})	Diluent/Time (hrs)		Rank
0,214	Saline	0	1
0,216	Saline	1½	2
0,219	Phos. Buffer	3	3
0,250	Peptone	0	4
0,252	Peptone	1½	5
0,307	Saline	3	6
0,400	Dist. Water	3	7
0,471	Ringer's	1½	8
0,475	Dist. Water	0	9
0,493	¼ Ringer's	0	10
0,504	Ringer's	0	11
0,571	Dist. Water	1½	12
0,633	Peptone	3	13
0,681	¼ Ringer's	1½	14
0,694	Phos. Buffer	1½	15
0,694	Phos. Buffer	0	16
0,728	Ringer's	3	17
0,972	¼ Ringer's	3	18

Note: Two means linked by a common line do not differ from one another significantly (significance probability = 0,05).

Two means not linked by a common line differ from one another significantly (significance probability = 0,05).

6. Results

There are 5 homogeneous subsets (subsets of elements, no pair of which differ by more than the shortest significant range for a subset of that size) which are listed as follows:

Ringer's, 1½ hours to Ringer's, 3 hours;

Distilled Water, 3 hours to Phosphate Buffer 0 hours;

Saline, 0 hours to Ringer's, 0 hours;

Saline, 3 hours to Distilled Water, 1½ hours;

Phosphate Buffer, 1½ hours to ¼ Ringer's, 3 hours.

COMPUTATION 12

The analysis of variance applied to the death rate data of Escherichia coli, suspended in various diluents over a period of 3 hours, subjected to a QAC in a test solution of hard water + milk (3 replications).

1. Means (b_{yx}) - 3 replications

TIME AFTER INITIAL SUSPENSION

Diluents	0 hours	1½ hours	3 hours	Diluent Means
Dist. water (D.W.)	1,143	1,022	0,469	0,878
Phos. Buffer (P.B.)	1,354	0,910	1,017	1,094
¼ Ringer's (¼R.)	1,269	1,351	0,786	1,135
Saline (Sal)	1,041	1,382	1,081	1,168
Peptone (Pep)	1,228	1,239	1,110	1,192
Ringer's (R.)	1,314	1,428	1,438	1,393
Time mean values	1,225	1,222	0,983	1,143

2. Analysis of Variance

Source of Variation	Degree of Freedom	Mean Squares	F. Ratio	P
Between diluents	5	0,24922	4,747	<0,01
Between time	2	0,34604	6,592	<0,01
Diluents x time	10	0,12330	2,349	<0,05
Within replicates	36	0,05250		

3. Results: (significance probability = 0,05)

a) Diluent Means in Ranked Order

Rank:	1	2	3	4	5	6
Diluent:	D.W.	P.B.	¼R.	Sal	Pep	R.
Means:(1)	0,878	1,094	1,135	1,168	1,192	1,393

b) Time Means in Ranked Order

Rank:	1	2	3
Time:	3 hours	1½ hours	0 hours
Means:(1)	0,983	1,222	1,225

(1) NOTE: Any two means not underscored by the same line are significantly different (significance probability = 0,05).

Any two means underscored by the same line are not significantly different (significance probability = 0,05).

4. One-way Analysis of Variance

Source of Variation	Degree of Freedom	Mean Squares	F. Ratio	P
Between groups	17	0,18654	3,5534	<0,01
Within groups	36	0,05250		
Total	53			

5. Treatment Means in Ranked Order

Mean (b_{yx})	Diluent/Time (hrs)		Rank
0,469	Dist. Water	3	1
0,786	¼ Ringer's	3	2
0,910	Phos. Buffer	1½	3
1,017	Phos. Buffer	3	4
1,022	Dist. Water	1½	5
1,041	Saline	0	6
1,081	Saline	3	7
1,110	Peptone	3	8
1,143	Dist. Water	0	9
1,228	Peptone	0	10
1,239	Peptone	1½	11
1,269	¼ Ringer's	0	12
1,314	Ringer's	0	13
1,351	¼ Ringer's	1½	14
1,354	Phos. Buffer	0	15
1,382	Saline	1½	16
1,428	Ringer's	1½	17
1,438	Ringer's	3	18

Note: Two means linked by a common line do not differ from one another significantly (significance probability = 0,05).

Two means not linked by a common line differ from one another significantly (significance probability = 0,05).

6. Results

There are 4 homogeneous subsets (subsets of elements, no pair of which differ by more than the shortest significant range for a subset of that size) which are listed as follows:

Phosphate Buffer, 3 hours to Ringer's, 3 hours;

Phosphate Buffer, 1½ hours to Phosphate Buffer, 0 hours;

¼ Ringer's, 3 hours to Distilled Water, 0 hours;

Distilled Water, 3 hours to ¼ Ringer's, 3 hours.

APPENDIX C

COMPUTATION 1

The analysis of variance applied to the percentage survivors of Staphylococcus aureus, suspended in various diluents, over periods of $1\frac{1}{2}$ and 3 hours after initial suspension, and exposed to a test control solution of distilled water and neutralizer solution.

1. Means - 3 replications

Diluents	TIME $1\frac{1}{2}$ hours	3 hours	Diluent Means
Dist. Water (D.W.)	86,550	67,000	76,775
Ringer's (R.)	83,467	76,400	79,933
Saline (Sal)	85,683	77,033	81,358
$\frac{1}{4}$ Ringer's ($\frac{1}{4}$R.)	96,517	79,167	87,842
Peptone (Pep)	86,583	96,550	91,567
Phos. Buffer (P.B.)	114,317	92,933	103,625
Time means	92,186	81,514	86,850

2. Analysis of Variance

Source of Variation	Degree of Freedom	Mean Squares	F. Ratio	P
Between diluents	5	1161,922	2,302	N.S.
Between time	1	2050,131	4,061	<0,05
Diluents x time	5	408,719	0,810	N.S.
Within replicates	60	504,832		

3. Results: (significance probability = 0,05)

a) Time Means in Ranked Order

Rank:	1	2
Time:	$1\frac{1}{2}$ hours	3 hours
Means:(1)	92,186	81,514

(1) NOTE: Any two means not underscored by the same line are significantly different (significance probability = 0,05).

Any two means underscored by the same line are not significantly different (significance probability = 0,05).

COMPUTATION 2

The analysis of variance applied to the percentage survivors of Staphylococcus aureus, suspended in various diluents, over periods of $1\frac{1}{2}$ and 3 hours after initial suspension, and exposed to a test control solution of hard water and neutralizer solution.

1. Means - 3 replications

Diluents	TIME $1\frac{1}{2}$ hours	3 hours	Diluent Means
$\frac{1}{4}$ Ringer's ($\frac{1}{4}$R.)	81,067	83,700	82,383
Dist. Water (D.W.)	89,717	89,683	89,700
Peptone (Pep)	97,217	88,283	92,750
Ringer's (R.)	103,700	93,117	98,408
Phos. Buffer (P.B.)	104,650	96,533	100,592
Saline (Sal)	106,799	105,283	106,042
Time means	97,192	92,767	94,979

2. Analysis of Variance

Source of Variation	Degree of Freedom	Mean Squares	F. Ratio	P
Between diluents	5	857,116	1,625	N.S.
Between time	1	352,450	0,668	N.S.
Diluents x time	5	89,667	0,170	N.S.
Within replicates	60	527,559		

COMPUTATION 3

The analysis of variance applied to the percentage survivors of Staphylococcus aureus, suspended in various diluents, over periods of 1½ and 3 hours after initial suspension, and exposed to a test control solution of hard water + milk and neutralizer solution.

1. Means - 3 replications

Diluents	TIME 1½ hours	TIME 3 hours	Diluent Means
Dist. Water (D.W.)	86,733	71,100	78,917
Saline (Sal)	85,567	87,650	86,608
¼ Ringer's (¼R.)	92,717	84,400	88,558
Phos. Buffer (P.B.)	94,217	87,250	90,733
Ringer's (R.)	87,050	97,217	92,133
Peptone (Pep)	115,317	105,583	110,450
Time means	93,600	88,867	91,233

2. Analysis of Variance

Source of Variation	Degree of Freedom	Mean Squares	F. Ratio	P
Between diluents	5	1321,407	1,930	N.S.
Between time	1	403,280	0,588	N.S.
Diluents x time	5	258,067	0,376	N.S.
Within replicates	60	685,764		

COMPUTATION 4

The analysis of variance applied to the percentage survivors of Escherichia coli, suspended in various diluents, over periods of $1\frac{1}{2}$ and 3 hours after initial suspension, and exposed to a test control solution of distilled water and neutralizer solution.

1. Means - 3 replications

Diluents	TIME $1\frac{1}{2}$ hours	3 hours	Diluent Means
Dist. Water (D.W.)	89,717	80,367	85,042
$\frac{1}{4}$ Ringer's ($\frac{1}{4}$R.)	90,950	82,233	86,592
Phos. Buffer (P.B.)	96,267	94,500	95,383
Ringer's (R.)	104,999	102,033	103,517
Saline (Sal)	102,800	107,950	105,375
Peptone (Pep)	104,450	108,167	106,308
Time means	98,197	95,875	97,036

2. Analysis of Variance

Source of Variation	Degree of Freedom	Mean Squares	F. Ratio	P
Between diluents	5	1087,665	3,747	<0,01
Between time	1	97,070	0,334	N.S.
Diluents x time	5	109,987	0,379	N.S.
Within replicates	60	290,303		

3. Results: (significance probability = 0,05)

a) Diluent Means in Ranked Order

Rank:	1	2	3	4	5	6
Diluent:	D.W.	$\frac{1}{4}$ R.	P.B.	R.	Sal	Pep
Means:(1)	85,042	86,592	95,383	103,517	105,375	106,308

(1) NOTE: Any two means not underscored by the same line are significantly different (significance probability = 0,05).

Any two means underscored by the same line are not significantly different (significance probability = 0,05).

COMPUTATION 5

The analysis of variance applied to the percentage surivivors of Escherichia coli, suspended in various diluents, over periods of 1½ and 3 hours after initial suspension, and exposed to a test control solution of hard water and neutralizer solution.

1. Means - 3 replications

Diluents	TIME 1½ hours	TIME 3 hours	Diluent Means
Saline (Sal)	66,100	84,950	75,525
¼ Ringer's (¼R.)	88,450	83,800	86,125
Ringer's (R.)	96,783	92,117	94,450
Dist. Water (D.W.)	95,450	94,133	94,972
Peptone (Pep)	99,067	115,600	107,333
Phos. Buffer (P.B.)	116,933	101,900	109,417
Time means	93,797	95,417	94,607

2. Analysis of Variance

Source of Variation	Degree of Freedom	Mean Squares	F. Ratio	P
Between diluents	5	1961,784	3,683	<0,01
Between time	1	47,207	0,089	N.S.
Diluents x time	5	530,449	0,996	N.S.
Within replicates	60	532,686		

3. Results: (significance probability = 0,05)

a) Diluent Means in Ranked Order

Rank:	1	2	3	4	5	6
Diluent:	Sal	¼ R.	R.	D.W.	Pep	P.B.
Means:(1)	75,525	86,125	94,450	94,972	107,333	109,417

(1) NOTE: Any two means not underscored by the same line are significantly different (significance probability = 0,05).

Any two means underscored by the same line are not significantly different (significance probability = 0,05).

COMPUTATION 6

The analysis of variance applied to the percentage survivors of Escherichia coli, suspended in various diluents, over periods of $1\frac{1}{2}$ and 3 hours after initial suspension, and exposed to a test control solution of hard water + milk and neutralizer solution.

1. Means - 3 replications

Diluents	TIME $1\frac{1}{2}$ hours	3 hours	Diluent Means
Ringer's (R.)	96,433	77,833	87,133
$\frac{1}{4}$ Ringer's ($\frac{1}{4}$R.)	96,400	87,583	91,992
Peptone (Pep)	92,767	93,950	93,358
Phos. Buffer (P.B.)	100,533	91,500	96,017
Dist. Water (D.W.)	105,733	97,183	101,458
Saline (Sal)	102,667	102,567	102,617
Time means	99,089	91,769	95,429

2. Analysis of Variance

Source of Variation	Degree of Freedom	Mean Squares	F. Ratio	P
Between diluents	5	415,875	0,823	N.S.
Between time	1	964,339	1,908	N.S.
Diluents x time	5	155,018	0,307	N.S.
Within replicates	60	505,416		