Evaluation of the Virucidal Activity of Commonly Used Disinfectants

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by

Murray Drulak, M.Sc.

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EVALUATION OF THE VIRUCIDAL ACTIVITY OF COMMONLY USED DISINFECTANTS

BY

MURRAY DRULAK

A dissertation submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

EVALUATION OF THE VIRUCIDAL ACTIVITY OF COMMONLY USED DISINFECTANTS

Virus in fetal calf serum was exposed to disinfectant, and the disinfecting activity was neutralized at the end of the exposure time by skim milk. This neuralized mixture was then assayed for virus using the plaque assay.

Coxsackievirus B5, echovirus 11, f2 bacteriophage, herpes simplex virus 2, reovirus 3 and A2 plaque virus were tested for resistance to commonly used disinfectants. In addition, three preparations of vesicular stomatitis virus, each grown in cells from a different vertebrate, were tested for resistance to inactivation by disinfectant.

The six disinfectants considered in this study were: Wescodyne, Javex, sodium hydroxide, One Stroke Ves-Phene, Sonacide and ethanol. The viruses (with the exception of A2 plaque virus) were exposed to dilutions of these disinfectants for ten, twenty and thirty seconds and a residual virus versus exposure time plot of

$\log_{10}$ PFU/ml versus square root minutes exposure time

was drawn for each virus-disinfectant combination. The square root transformation of exposure time yielded a linear

function for virus versus exposure time.

Slopes were used to:

(i) determine the relative resistance of different viruses to a given disinfectant

(ii) evaluate the relative virucidal activity of different disinfectants on each virus

Overall rankings of viral resistance to the group of disinfectants tested, in order of decreasing resistance to inactivation were as follows:

(i) f2 bacteriophage and echovirus 11

(ii) reovirus 3

(iii) coxsackievirus B5

(iv) herpes simplex virus 2

(v) vesicular stomatitis virus

with f2 bacteriophage and echovirus 11 being of the same overall resistance to the group of disinfectants previously listed and more so than the other viruses considered in the study. Enveloped viruses were generally more susceptible to inactivation by disinfectants than non-enveloped viruses. The kind of vertebrate vesicular stomatitis virus was grown in was a significant factor in determining its susceptibility to disinfectant inactivation.

Disinfectants are listed below in order of decreasing overall effectiveness against the previously listed viruses.

(i) 95% ethanol

(ii) 0.25% (w/v) sodium hydroxide

(iii) 1 - 100 Javex

(iv) 1 - 200 Wescodyne

(v) undiluted Sonacide

(vi) 1 - 50 Ves-Phene

Due to certain practical disadvantages in using ethanol and sodium hydroxide, for practical purposes Javex at dilutions less than 1-50, was the most effective disinfectant. Ethanol, sodium hydroxide and Javex were wide spectrum disinfectants inactivating both enveloped and nonenveloved viruses. Wescodyne, Ves-Phene and Sonacide inactivated enveloped viruses to a greater extent than non-enveloped viruses.

The linearity of the function relating log residual virus with square root exposure time made it possible by extrapolation to predict an exposure time to a certain disinfectant that would yield zero residual virus. Such an exposure time was termed an χ^2 value. χ^2 values for coxsackievirus B5 and reovirus 3, indicated the presence of virus when none was predicted, which meant extrapolation was not justified.

A2 plaque virus, reported to be antigenically related to hepatitis B virus, was exposed to dilutions of the six disinfectants and methanol, for one and ten minute periods. In this case, the presence of virus or lack of it

at the end of these exposure times provided the criterion of effectiveness of the disinfectants. Results indicated: A 1-50 dilution of One Stroke Ves-Phene, 1-50 Wescodyne and 1% (w/v) sodium hydroxide failed to completely inactivate the samples of a common pool of A2 plaque virus in ten minutes. The same was true for undiluted Sonacide. A 1-50 concentration of Javex totally inactivated A2 plaque virus in a ten-minute, but not a one-minute exposure time. A solution of 80% methanol was the only disinfectant tested that consistantly inactivated A2 plaque virus completely in one minute. One hundred per cent methanol and 95% ethanol completely inactivated A2 plaque virus in a ten-minute exposure time, but were not consistantly effective in a one-minute exposure time.

Ten per cent and 17.5 percent skim milk were demonstrated to be effective neutralizers of the toxicity of a wide variety of disinfectants used in testing a highly diverse group of viruses. In the case of herpes simplex virus 2, fetal calf serum was an adequate neutralizer of disinfectant activity.

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I received advice on statistical techniques used in this thesis from Dr. N. Stephens of the Department of Physiology.

I would also like to state at this time that the relationship of infectivity versus exposure time to the disinfectant

$\log_{10}$ PFU/ml versus square root minutes

was originally developed by Dr. A. Wallbank, Larry Werboski and Peter McEwan.

In closing, I would like to express my appreciation to Dr. J. C. Wilt and the Department of Medical Microbiology for financially supporting me in the initial months of the project.

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INTRODUCTION

INTRODUCTION

Since standard methods for growing and assaying bacteria have long been in use, the evaluation of action of chemical disinfectants on bacteria has been extensively studied, leading to development of standard methods for testing of disinfectants.

Unfortunately, in the case of animal viruses, the major quantitative test for detection of virus, the plaque assay, has been developed only since 1952 and is available for only a limited number of viruses.

This being the case, relatively few studies of the virucidal activity of commonly used disinfectants have been done.

Quite often when such studies are done, the virus is exposed to disinfectant for an impractical length of time, at the end of which a neutralizer substance of debatable efficiency is added to stop the action of the disinfectant. Since the ideal disinfectant should inactivate virus very quickly, a short exposure time should be incorporated into testing procedures for disinfectants. Complete and prompt neutralization of disinfectant activity is critical so that the ability of the disinfectant to inactivate virus can be realistically evaluated with reference to a given exposure time. Also, neutralization of disinfectant activity is important in preventing disinfectant toxicity from destroying cells used to assay for virus.

These factors prompted the development of the unique approach to disinfectant testing used in this study. Skim milk was used successfully in neutralizing a wide variety of disinfectant-virus reactions at the end of ten, 20 and 30 seconds. Six disinfectants, each representative of a chemically distinct group of disinfectants, were tested. They were Sonacide, Wescodyne, sodium hydroxide, Javex, One Stroke Ves-Phene and ethanol (Table 60). Test viruses of widely differing properties were used in this study. They were herpes simplex virus 2, echovirus 11, coxsackievirus B5, reovirus 3, f2 bacteriophage and vesicular stomatitis virus. Vesicular stomatitis virus was grown in three different cell types and the disinfectant susceptibility of the virus yields were compared.

A2 plaque virus, a virus antigenically similar to hepatitis B virus was tested using the six disinfectants previously mentioned and methanol.

CHAPTER I

REVIEW OF THE LITERATURE

INTRODUCTION

Viral diseases are the most common of human infections. Two-thirds of all human infections occur in the respiratory system. Of these, over 90% are associated with viruses and only about three to four per cent with bacteria (1). Unlike bacterial diseases, the great majority of viral diseases are resistant to any therapeutic treatment. This makes active immunization and disinfection two important weapons in the fight against viral disease. In the case of immunization, although immunized persons seldom become ill, sometimes they can be carriers of the virus. In addition, vaccines effective against all viruses are not presently available. All of these factors increase the importance of disinfection of the environment to break the chain of virus spread in a population (1).

Fortunately, even in the absence of disinfection, many viruses have been demonstrated not to survive in nature for prolonged periods of time after the host has died or when virus is separated from susceptible living cells. The rate at which infectivity is lost depends on the virus and the conditions of the surroundings, such as hydrogen ion concentration, the enzymes present, temperature and other conditions (2). For example, influenza A virus in normal chick embryo allantoic fluid became noninfective

when stored for 24 hours at 35°C (3), while mumps virus lost its infectivity in six hours at 25°C (4). The addition of normal serum usually decreased the rate of this type of inactivation (2). The humidity of the air was demonstrated to affect the survival of virus (5). A suspension of influenza A virus in serum and broth sprayed into an enclosed space remained infectious for 24 hours when the relative humidity was 24 per cent, but only for 30 minutes at 80 per cent humidity. The amount of domestic waste in river water had a marked effect on the survival of coxsackievirus (6). Coxsackievirus was added to samples of water and the amount of time required for a 1000 fold decrease in infectivity during storage at 20°C was determined. In clean water this occurred in less than two days, but in polluted water two weeks was required.

Factors such as temperature, presence of organic material and pH which determine the viability of a virus in a certain environment also determine the effectiveness of a disinfectant in inactivating virus. In this literature review, such factors along with the test methods used to determine the effectiveness of disinfectants in inactivating viruses and bacteria will be discussed. The mechanisms of action of disinfectants considered in this study will be defined to the limited extent present knowledge will allow. In addition, the kinetics of inactivation of viruses and bacteria by chemical disinfectants will be considered.

Definition of Terms:

Since there are so many different terms which have been used to describe in vitro inactivation of micro-organisms, the following terms will be defined.

Disinfectant

Reddish (7) defined a disinfectant as an agent which destroys bacteria or other microorganisms or inactivates virus. This term is most commonly used to designate chemicals that kill the growing forms but not necessarily the resistant spore forms of bacteria or viruses except when specifically mentioned as doing so. Proper use of a disinfectant is contingent on the purpose for which it is employed or the type of infectious agent that is suspected to be present.

The official definition of the word disinfection adopted by the American Public Health Association (8), the United States Public Health Service, British Ministry of Health and several other foreign national governments is

- the killing of pathogenic agents by chemical or physical means directly applied.

Specific synonyms have been used denoting a particular function of a disinfectant such as germicide, bactericide, fungicide and virucide (9).

Sterilization -- the complete destruction or removal of all forms of life (10, 7). The number of agents capable of achieving this is limited, with high temperature including saturated steam under pressure and certain types of filters being among them. Only a few chemicals are capable of achieving sterilization and often the term sterilization is used loosely and erroneously applied when disinfection is really implied (10).

Sanitizer -- an agent that reduces the bacterial count to safe levels as may be judged by public health requirements on food handling equipment, eating and drinking utensils (11). Sanitization is an awkward term referring to the process of rendering sanitary or of promoting health. It is akin to disinfection, but also implies cleansing as well as removal of infection (12). The term is often used in connection with the day-to-day control of bacterial contamination in equipment and utensils in dairies and food plants, dishes and glasses in restaurants and taverns where no specific infectious agent is known or suspected to be present. The term should be restricted to use in connection with cleaning operations (13).

Antiseptic -- a substance that opposes sepsis, putrefaction or decay by preventing or arresting the growth or action of microorganisms, either by inhibiting their activity or by destroying them; used especially in application to living tissue (14). Antiseptics had earlier been defined as

substances which, when applied to microorganisms, will render them innocuous, either by killing them or preventing their growth, according to the character of the preparation or method of application; used especially for preparations applied to living tissue (15).

A synonym used instead of antisepsis is bacteriostasis (16).

Mechanisms of Action of Disinfectants

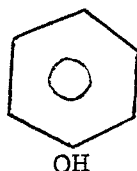
Although the chemistry of disinfectants and the structure of microorganisms is well known, the reactions of a disinfectant with microorganisms which result in inactivation are not known. Such information is slowly being evolved, not by those working in the field of disinfection but by those interested in biochemistry, enzymology, immunology, cytology, physiology and other related fields (17). In the following material the chemical reactions promoted by the various disinfectants to be investigated are assembled. One may speculate that various constituents of viruses such as lipid envelopes, protein capsids and nucleic acid cores can be targets for disinfectants according to the ability of the disinfectants to interact with them.

Phenolics

Phenol is probably the oldest chemical disinfectant and, although it is not extensively used today, many chemical derivatives of it are used.

The chemical structure of phenol (figure 1) offers five sites upon which substitutions can be made and at these locations benzene rings, aliphatic, aromatic or other groups can be substituted to make a family of disinfectant compounds termed phenolics (17).

figure 1



Phenol (18)

In studies done on the bactericidal effects of phenolics it is of particular interest that most substitutions on the phenolic structure increase its bactericidal activity often many hundredfold (17). The phenolics owe their antibacterial properties to their capacity to combine with and denature proteins (19). It would appear that the free hydroxyl group on phenol constitutes the site which reacts with and denatures protein (20). Substitutions on the phenolic ring affect its electrolyte dissociation and its solubility hence affecting its distribution ratio between aqueous and non-aqueous phases favoring the latter, thus increasing the amount of chemical picked up by the lipoid constituents of the bacterial cells (21, 22).

Phenolics are not particularly virucidal (23), but have been reported to exhibit marked virucidal activity on one category of virus termed lipophilic viruses. Lipophilic viruses were defined as those viruses which have an affinity for long chain lipid components. Such a category of virus would include the lipid enveloped viruses (24).

Phenol inactivated viruses but the concentration required to destroy infectivity depended on the virus and upon the nature and amount of other substances present. Numerous experiments have been conducted with phenol under a wide variety of conditions. In most instances, they indicated that in concentrations below one per cent inactivation was either slow or imperceptible (25).

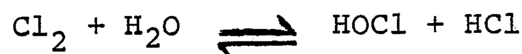
For example, it was necessary to expose rabies virus to a one per cent solution of phenol for 24 hours at 37°C to achieve complete inactivation (25). In the case of eastern equine encephalomyelitis virus, although a two and one-half per cent solution of phenol decreased the virus titer by 100,000 fold after one hour exposure at 20°C (27), a 0.5 per cent solution did not inactivate the viruses of either equine infectious anemia or rift valley fever in 30 days. In addition, although an 0.5 per cent solution of phenol eliminated bacterial contamination of lymph which contained vaccinia virus after a

two week exposure at 0°C, even a five per cent solution did not inactivate the virus (28). Influenza A virus behaved in a manner similar to vaccinia virus when exposed to two and one-half per cent and 0.5 per cent solutions of phenol at 4°C (20) but, unlike vaccinia virus, it was inactivated by a two per cent solution in ten minutes when the temperature was elevated to 25°C (30). These observations from various studies indicate that phenol has little usefulness as a virucide since it was effective only in a limited number of instances. This is further emphasized when it is realized that infectious nucleic acid is extracted from viruses by treatment with 70% w/w phenol which destroys the protein coat of the virus (223). Phenolic disinfectants are commonly used at concentrations considerably lower than 70%. The phenolic disinfectant used in this study was One Stroke Ves-Phene.

Chlorine

It was during the first half of the nineteenth century that the disinfecting and deodorizing properties of chlorine-containing compounds were first recognized. Koch (1881) reported on the bactericidal properties of hypochlorites and this was probably the first report of chlorine-containing compounds as disinfectants (31).

When chlorine is added to water at pH values above two, it is converted to hypochlorous acid which is the active germicidal compound (7).



$$Cl_2 + H_2O \rightleftharpoons HOCl + HCl$$

The hypochlorous acid dissociates according to the ionization equilibrium to give hypochlorite ion.

$$HOCl \rightleftharpoons {}^{-}OCl + H^+$$

The extent of this dissociation depends on pH. At alkaline pH dissociation is increased.

Chlorine in the form of hypochlorite compounds has been commonly used as a disinfectant (31). It has been suggested (32) that the active germicidal agent of hypochlorites is also hypochlorous acid. The hydrolysis reaction for sodium hypochlorite is

$$NaOCl + H_2O \rightleftharpoons HOCl + NaOH$$

Again, as in the case of HOCl formed from Cl_2, the HOCl formed from hypochlorite compounds dissociate

$$HOCl \rightleftharpoons {}^{-}OCl + H^+$$

and as noted previously, acid pH shifts the equilibrium to the left, while alkaline pH shifts it to the right.

In addition to HOCl, unhydrolized hypochlorite or the OCl-ion have germicidal power since alkaline

solutions of both sodium and calcium hypochlorites which possess only small amounts of HOCl exhibit germicidal properties, although with alkaline hypochlorites the disinfection is slow at the start and then increases (33).

Chlorine in its various forms acts primarily as an oxidant and can directly halogenate many positions in complex organic structures (17). For example, chlorine reacts with sulfhydryl groups and the halogen is an exceptionally active inhibitor of enzymes containing sulfhydryl groups (34).

Hypochlorite also reacted with proteins and related compounds by forming derivatives containing chlorine linked to nitrogen (35). Whether such reactions of chlorine-containing compounds with proteins of viruses destroyed these viruses was debatable since, in a study (36) considering f2 bacteriophage, it was shown that the alteration of viral protein by chlorine did not prevent f2 from absorbing to host bacteria even after a loss of infectivity. In fact, protein was necessary for the bacteriophage to absorb to host bacteria. It was reported that chlorine inactivated f2 bacteriophage by reacting with its RNA.

Although early reports indicated that chlorination was of little value in causing virus inactivation, these were subsequently disproved (37). Such reports failed to take into account the normal chlorine demand of the test system. One or two parts per million (ppm)

of free chlorine rapidly inactivated poliovirus (38), and so superchlorination was deemed an absolutely safe treatment.

Hepatitis virus, adenovirus, coxsackievirus and echovirus, although varying in their resistance to inactivation by disinfectants, were all reduced by 99 per cent in 30 minutes when exposed to 0.2 to two ppm of chlorine (39).

The virucidal activity of chlorine was greatly reduced by the presence of organic compounds such as proteins, since they were subject to oxidation or combination with chlorine. When the buffer action or acidity of such substances reduced the pH of an otherwise alkaline chlorine solution, the virucidal activity of such a solution was increased (40).

In a system devoid of extraneous material that would diminish free chlorine (41), it was found that 99.6 per cent of purified coxsackievirus was inactivated at 29°C in three minutes by 0.5 ppm of chlorine at pH 7 and by one ppm at pH 9. Comparison of the effect of chlorine on several enteroviruses (42) revealed that coxsackievirus, echovirus and the three types of poliovirus had almost identical activation energies for oxidative inactivation in the range of 0°C to 50°C and were equally sensitive to inactivation by chlorination.

One of the most valuable uses of chlorine is in the destruction of poliovirus in swimming pools. It is probable that infection can be acquired by swimming in pools even though the water is chlorinated. The period elapsing between contamination of the water and exposure of a swimmer obviously cannot be controlled. Crowding would decrease this period. During an outbreak of the disease the incidence of those shedding the virus would be increased. At such times, pools should be closed unless residual chlorine can be maintained at not less than 0.5 ppm (43). When partially purified poliovirus was suspended in water from lakes, rivers or wells and chlorine was added to yield 0.05 ppm, infectivity was destroyed in ten minutes provided the pH was within the range 7.9 to 8.3 (44). A factor shown to be a limitation on the amount of chlorine used in swimming pools was its irritant effect on the eyes of the swimmers. When free chlorine residues exceeded one ppm, unpleasant odors and significant eye irritation occurred (45). The toxic actions of chlorine were related to the pH of the water (46). Results in a prolonged study using a pool under controlled conditions showed that total available residual chlorine in a concentration of 0.5 ppm at pH eight was less irritating to the eyes than is 0.05 ppm at pH seven. The virucidal power of chlorine decreases as pH is increased (47).

The antiviral activity of bleach containing sodium hypochlorite was tested by laundering diapers soiled with feces containing Sabin poliovirus 2 in solutions of bleach containing 200 ppm available chlorine. Such a concentration of available chlorine was effective in destroying virus under test conditions representative of those present in the household (48).

Studies indicated that many minor illnesses are of viral origin (49) and the frequency with which infants spread such diseases was greater than that for older children and adults (50), which was partly due to the behavior of infants. In addition to considering the role of behavior in facilitating virus spread, an infant's garments also present a source of infection. Such a source of infection was effectively eliminated by washing the garments in chlorine bleach (48).

Since water has been recognized as an efficient vehicle for the spread of enteroviruses and the inadequacy of the coliform test to predict the presence of such viruses has been noted, a proposal has been put forward to develop a simple index of viral pollution analogous to the coliform index of bacterial water pollution (51). Since phage f2 was shown to be markedly more resistant to chlorine in water than either poliovirus or T2 coliphage, its potential use as an indicator of possible viral pollution was suggested. Phage f2 occurs naturally

in large numbers in waters polluted with human feces. Other studies (52) indicated that f2 bacteriophage should not be used as an indicator for evaluating the enteric viral quality of water and wastewater systems, since sufficient epidemiological and laboratory evidence is not available to support this use of f2 bacteriophage. It was suggested f2 should be used as a classical model to obtain basic information concerning defining fundamental parameters and reactions in virucidal testing, since it provides a much easier system to work with than animal viruses.

The chlorine disinfectant used in this study was Javex.

Iodine

Iodine has been used for various purposes in medicine for nearly 50 years, but its application as a germicide is of shorter duration. Iodine is a solid at normal temperatures, but it has a high vapor pressure and sublimes readily. It is only slightly soluble in water and its solubility is enhanced by the presence of iodide ions; it is readily soluble in alcohol. Solutions of iodine are normally brown in color and they stain (53).

Iodine has been reported to be a highly reactive element and it was this reactivity which makes it an effective germicide (54). Such reactivity was shown to

be similar to that of chlorine (17), but unlike chlorine its disinfecting action was the result of the direct intervention of free iodine molecules (55) which combine with the protein substances of the bacteria. In the case of f2 bacteriophage, iodine has been demonstrated to inactivate through iodination of the amino acid tyrosine in the protein moiety of the bacteriophage, but it had almost no effect on nucleic acid even at extremely high concentrations. Chlorine exerted its virucidal effect by inactivating viral nucleic acid (52). Hypoiodous acid did not take any part in reacting with protein except possibly at high pH values (56), and so the major reaction appeared to be one of direct halogenation.

In the presence of iodides, there is always a certain amount of periodide or triiodide formed in the reaction

$$I_2 + I^- \rightleftharpoons I_3^-$$

and such substances had only low activity against both bacterial spores (56) and vegetative bacteria (57).

Iodophores are not strictly compounds of iodine; they are mixtures of iodine with anionic, cationic or nonionic surface active agents. These agents act as carriers and solubilizers for iodine. Polyvinylpyrrolidone and polyethoxyethanol derivatives are examples of such surface active agents in commercial iodophore products such as Wescodyne, Povidone-Iodine, Betadine and Virac.

Since the iodine in these compounds is brought into solution with a solubilizer, there is no loss of iodine by its conversion to the inactive triiodide ion and this probably accounts for the activities of the iodophores being higher than those of the simpler iodine solutions (58).

Wescodyne, an iodophore containing polyetholy-ethanol iodine was found to be ineffective in inactivating poliovirus and other enteroviruses. In addition, organic material was demonstrated to rapidly deplete the active iodine in Wescodyne (59). Such results indicate Wescodyne has limited usefulness as a virucide.

The effectiveness of iodine as a virucide was determined in a number of studies done under different conditions. This made it difficult to compare results. It was reported that the application of tincture of iodine to skin before application of herpes simplex virus 1 prevented development of infection, whereas the application of iodine after inoculation of the scarified area with herpes virus did not interfere with the growth of virus (46). In other studies, low levels of iodine inactivated virus. For example, a 0.25 per cent solution of tincture of iodine inactivated the virus of fowl pox (60) and weak solutions of iodine (0.00055N) rapidly inactivated influenza virus (173). Iodine vapor in a

concentration of 0.1 ppm inactivated influenza virus (63). But in some instances even higher levels of iodine were not effective in inactivating virus. It was demonstrated that undiluted tincture of iodine did not have significant activity in in activating tobacco mosaic virus (61), and although it was initially proposed (64) that a few ppm of iodine will inactivate poliovirus in five to ten minutes, it was later demonstrated (65) that as much as 125 to 375 ppm were needed to accomplish this in one minute. It was recommended that disinfection of water with iodine should be based on enterovirus destruction since enteroviruses were even more resistant to inactivation by iodine than were cysts of amoeba (63).

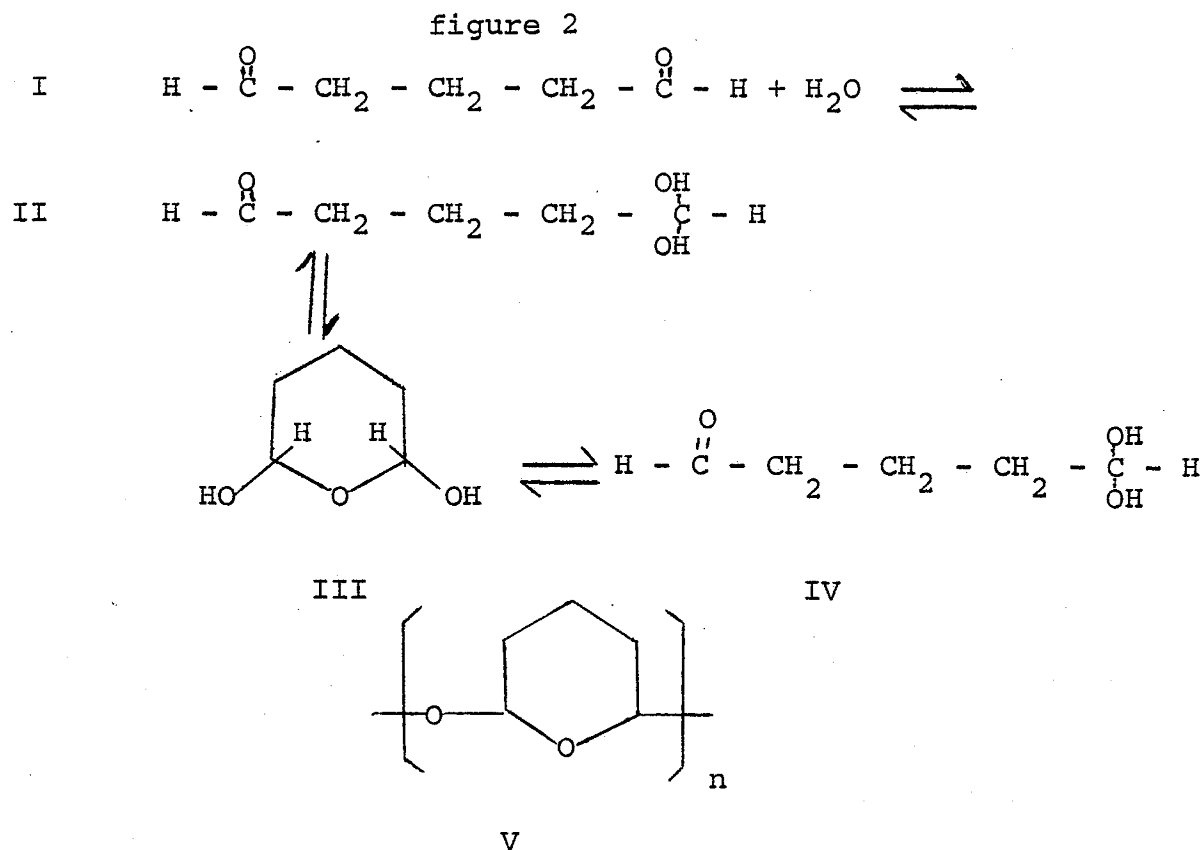
Aldehydes

Formaldehyde and glutaraldehyde (1,5 pentanedial) are two of the major disinfecting compounds from this group. It is believed that disinfectant activity of aldehyde-containing compounds is due to their alkylating properties (66). It is speculated that the aldehydes may attack the sulphydryl, hydroxyl, amino and carboxyl groups present in proteins (67). From studies conducted with *Escherichia coli* (*E. coli*) and *Bacillus megatherium* (68), it was concluded that the most likely sites for 1,5 pentanedial action would be amino groups in proteins, including cross linking reactions between these groups.

A neutral chemical by nature, 1,5 pentanedial is affected by pH with reference to its ability to inactivate microorganisms for although 1,5 pentanedial in monomeric form inactivates microorganisms (67), the pentanedial monomer molecule (I in figure 2) has a tendency to polymerize irreversibly into several more stable non-active hydrates of types II, III and IV (figure 2) at alkaline pH (69).

In the neutral or acid ranges, the pentanedial monomer has a tendency to form reversible polymers with an acetal-like structure (type V, figure 2).

figure 2



Pentanedial and Polymers in Aqueous Solution (10)

To maintain a large proportion of monomers in acid pentanedial solutions, one can use heat or ultrasonics since inactive polymer formation in the acid range is reversible (67).

The peak of pentanedial activity (70) is always observed when the maximum of CHO radicals can combine with the greatest number of reactive amines (NH_2) present in proteins and nucleotides. To increase reactive yield at NH_2 sites, one can use heat which exposes new sites through protein denaturation, nonionic agents which help aldehyde penetration to reactive sites or ultrasonics which accelerate diffusion of aldehydes and improve reaction time.

Since acid pentanedial solutions start losing their potency within six months at room temperature, and one month at $45^{o}C$, an additive was needed which would stabilize the acid pentanedial while also increasing its potency (67).

A new agent, potentiated acid 1,5 pentanedial was developed. It is an acid aqueous 2 per cent (w/w) solution of pentanedial potentiated with a specific mixture of nonionic ethoxylates of isomeric linear alcohols (0.25% w/w) (67).

Sonacide is a commercial preparation of this potentiated acid pentanedial, which is stable in its activity for at least 18 months (67).

In 1970, (71) (72), it was demonstrated that there was an increase in the killing rate of poliovirus when nonionic agents were added to acid pentanedial.

Coxsackievirus B3 was inactivated 100 fold in one minute at 25°C and pH 7.4 by two per cent 1,5 pentanedial. This reduction in infectivity was not inhibited by the presence of organic material, but rather facilitated by it (73). This may have been due to the organic material lowering the pH to a level at which 1,5 pentanedial was more effective.

A high degree of virucidal activity was observed when 1,5 pentanedial solutions were alkalinized with the appropriate buffer, although such virucidal activity was demonstrated for only up to two weeks after preparation of such solutions. Complete inactivation of the infectivity of poliovirus 1 and 2, echovirus 6, coxsackievirus B1, herpes simplex virus 1, vaccinia virus, influenza virus A2 Asian, adenovirus 2 and mouse hepatitis virus was demonstrated after a ten minute exposure time, although the titers of these viruses were not mentioned in the report (75). The infectivity of minute virus of mice was reduced by greater than $10^{5.7}$ TCID 50/ml when treated with Cidex (75), a commercial preparation containing two per cent 1,5 pentanedial at alkaline pH.

The aldehyde-containing disinfectant used in this study was Sonacide.

Alcohols

As a chemical group, the alcohols possess many desirable features of a disinfectant. They have a bactericidal rather than a bacteriostatic effect against vegetative bacteria. They evaporate readily after application and are colorless (76). The germicidal activity of the aliphatic alcohols increases regularly from methyl through octyl alcohol with each additional methyl group in the straight chain (77) (78). The most plausible explanation for the action of alcohols is that they denature proteins. In the absence of water, proteins are not denatured as readily as when water is present. This provides an explanation of why absolute ethanol, a dehydrating agent, is less bactericidal than mixtures of ethanol and water (79). The alcohols, particularly, have been widely used as skin disinfectants because of their germicidal action and their ability to remove lipids from surfaces (80).

Ethanol is active against certain viruses (80). Influenza virus type A in chick embryo matrix was inactivated within ten minutes at 25°C by 70 per cent ethanol (30). Exposure of eastern equine encephalitis virus to 70 per cent ethanol reduced its infectivity by four logs (56). The Lansing strain of poliovirus appeared to be resistant to the action of 70 per cent ethanol (83).

Results in a study of the bactericidal activity of ethanol (84) indicated the following:

1. Ethanol in proper concentrations was a powerful germicide when in contact with test organisms in suspension.
2. Ethanol destroyed (vegetative) bacteria in aqueous suspension at a constantly diminishing rate. The rate did not appear to be regularly logarithmic throughout; there was a sharp immediate reduction, after which the process took place more slowly.
3. Of the solutions tested, a 70 per cent (w/w) solution of ethanol was the most rapidly bactericidal, although even one per cent ethanol killed test bacteria slowly.
4. Susceptibility to inactivation by ethanol varied with different kinds of bacteria. The 70 per cent solution appeared to be toxic for *Staphylococcus aureus* in particular.

Disparity in the testing of the bactericidal ability of ethanol was attributed to the varying degree of contact between ethanol and the test bacteria. The bactericidal activity of ethanol was greatest against bacteria in suspension and less when test organisms were attached to threads and garnets.

Seventy per cent (w/w) ethanol was recommended for use, but exactly why 70 per cent should be more toxic for bacteria than any other concentration of ethanol remains an interesting biochemical problem.

Alkalies

The germicidal activity of alkali compounds depends on free hydroxyl ions although undissociated molecules may also influence activity. The addition of sodium chloride to solutions of sodium hydroxide, for example, increased the lethal activity of the alkali without altering the pH significantly.

In bacteria, a relatively high intracellular concentration of hydroxyl ions may interfere with the function of certain enzymes by altering the solubility of divalent or trivalent metallic prosthetic groups. High concentrations of hydroxyl ions may produce lethal damage to the surface components of the bacterial cell through hydrolytic reactions.

Alkalies have been employed for disinfection of excreta from patients, particularly those containing poliovirus. Hot solutions of sodium hydroxide have been used for disinfection of beverage and milk bottles (85).

Sodium hydroxide was recognized early as a practical and effective virucide and it was suggested that its low cost made it suitable for use in barns and stables (87).

Sodium hydroxide was reported to be effective in inactivating the virus of hog cholera. A ten per cent solution of sodium hydroxide added to virus-containing blood and giving final concentration of one per cent, quickly changed the blood to an almost clear syrupy liquid (88). The relevance of this observation with reference to inactivation of virus is not clear.

Fowl pox virus was inactivated by one per cent potassium hydroxide (86). It was reported (89) that sodium hydroxide was found to have relatively high germicidal efficiency against various types of nonsporulating bacteria, even in cases where one per cent solutions were used in the presence of organic material, such as skim milk, chicken feces or defibrinated blood. It was shown that there was no increase in germicidal efficiency of sodium hydroxide solutions as the temperature was increased from 25°C to 40°C (90). It was also found that the velocity of disinfection by sodium hydroxide solutions remained independent of temperature variations between 2°C and 25°C (91).

Factors Influencing the Testing of a Disinfectant

Temperature

The rate of disinfection was demonstrated to increase with temperature (92). For every 10°C rise in temperature, the disinfection velocity of silver nitrate increased three fold, while in the case of phenol the rate of disinfection increased eight times.

The increase in the rate of disinfection by a given rise in temperature varied for different classes of disinfectants and was influenced by the microorganism considered (93).

Although the accelerative effect of temperature on the rate of disinfection has been generally accepted (94), there are exceptions. No accelerative influence on disinfection by alkalies between 25°C and 40°C (95) took place, while in another study (91), an actual decrease resulted from increasing the temperature of sodium hydroxide solutions from 5°C to 15°C. An explanation for this was that the mobility of hydrogen ions was increased to a greater extent by increases in temperature as compared to the mobility of the hydroxyl ions, so that true alkalinity was decreased by increases in temperature. Through certain ranges, this more than compensated for by the additional efficiency of the alkali at the higher temperatures.

Increased temperature led to decreases in the disinfectant activity of alkaline solutions of glutaraldehyde, since increased irreversible polymerization into inactive forms occurred (67). In acid solutions, there is an increase in the disinfectant activity of glutaraldehyde, since the molecular energy level was raised and more active monomers were formed from the reversible acetal-like polymers. This explains the increase in sporicidal activity when acid solutions of glutaraldehyde were heated (96). In the 20°C to 55°C range, any 10°C increase in temperature reduced the time needed for sterilization of a preparation of spores by potentiated acid glutaraldehyde by more than a factor of four (67).

The bactericidal efficiencies of phenol, ortho-cresol, ethyl alcohol and n butyl-alcohol against *Staphylococcus aureus* and *Eberthella typhosa* (*Salmonella typhosa*) were determined (97). Tests were made with each disinfectant and test organism at temperatures 10°C apart, temperature coefficients were calculated from the results of these tests by dividing the disinfection time in minutes at the lower temperature by the disinfection time at the higher temperature. When results from individual experiments were averaged, it was possible to obtain average temperature coefficients of sufficient accuracy to permit their use in calculating disinfection time at one temperature using data obtained at another temperature. The influence of temperature on germicidal power of selected disinfectants was studied (98), resulting in the classification of germicides into three groups:

1. Germicidal activity unaffected by a rise in temperature: chiefly chemical substances possessing reducing properties.
2. Germicidal activity approximately doubled by a rise in temperature from 20°C to 37°C: phenols and alcohols belonged to this group.
3. Germicidal activity increased as much as ten to 20 fold by the same temperature rise: oxidizing agents belonged to this group.

It was reported (99) that almost twice the concentration of hypochlorite was required to kill *Eberthella typhosa* (*E. typhosa*) at 2°C as at 40°C. Increased temperatures

augmented the action of alcohol although the effect was less than in the case of phenol or formaldehyde (100).

pH

The inactivation of f2 bacteriophage by iodine decreased as the hydrogen ion concentration increased (52), which correlated with the finding (101) that increased hydrogen ion concentration inhibited the iodination of the amino acid tyrosine.

Numerous workers found that the germicidal action of phenol compounds diminished at alkaline pH (102). It was found that a 2.85% solution of phenol at pH ten had the equivalent germicidal activity activity to a 1.43% solution at pH seven (103). The germicidal efficiency of phenol and orthocresol decreased coincidentally with an increase in percentage of sodium hydroxide added to them. This indicated phenolates were less efficient germicidal agents than corresponding phenols (104).

Within the range of pH six to nine, free available chlorine was in the form of both hypochlorous acid and hypochlorite. With a rise in pH from six to nine, the proportion of chlorine in hypochlorous acid form declined from virtually 100% down to almost zero, while the proportion of hypochlorite ion increased. Hypochlorous acid was demonstrated to be approximately 80 times more powerful a bactericide than hypochlorite ion. Consequently, in free residual chlorination the higher the pH value the less active is the chlorine because of its lower proportion of hypochlorous acid. The

resistance of f2 bacteriophage to inactivation at alkaline pH by chlorine did not appear to result from a difference in the chemical reactivity of HOCl and OCl^- towards viral RNA. It was more likely due to the inability of chlorine as hypochlorite ion to penetrate the coat protein of the virus (106). As early as 1890, it was demonstrated that the addition of acid greatly increased the germicidal efficiency of hypochlorite solutions (107). In studies (108, 109) in which very resistant bacterial spores were used to evaluate the effectiveness of chlorine as a disinfectant at various pH, it was found that at pH values less than five the degree of acidity was less important than the amount of chlorine present for inactivation, with two ppm chlorine being sufficient to destroy anthrax spores.

In alkaline solutions the pH greatly affected the germicidal action of chlorine solution; 30 parts per million at pH nine was more actively germicidal than 100 ppm at pH ten. In another study (110) that yielded similar results, the kind of alkali present as well as the hydrogen ion concentration affected the germicidal efficiency of hypochlorites.

It was demonstrated (111) that a concentration of 25 ppm of available chlorine killed 99 per cent of resistant spores in two and a half minutes at pH six, three and three-fifths minutes at pH seven, in five minutes at pH eight, nineteen and a half minutes at pH

nine, while at pH ten this concentration of chlorine had little effect. From a practical standpoint, the differences between pH six and eight were minor, while the great increase in killing time at higher pH probably prevented effective disinfection.

A solution of calcium hypochlorite containing 1000 ppm available chlorine had a pH of 11.3 and required 64 minutes to kill 99 per cent of exposed spores. Addition of hypochlorous acid to this solution bringing the reaction to pH 7.3 reduced the killing time to less than 20 seconds (109).

Initially, glutaraldehyde was commercially available as a disinfectant only in acid aqueous solution, since at alkaline pH, glutaraldehyde had a tendency to convert irreversibly to stable, non-active polymers (69), while polymers at acid pH were the reversible acetal-like type. It should be noted that acid glutaraldehyde solutions started to lose activity within six months at room temperature and one month at 45°C (67).

Concentration of Disinfectant

Early work (112) indicated that concentration of a disinfectant enhanced its activity, with an exponential relationship being noted for concentration of disinfectant and rate of bacterial killing. This exponent varied with each type of disinfectant and expressed the effect dilution

had on the germicidal efficiency of disinfectants. A one per cent solution of phenol was demonstrated to be a relatively strong disinfectant capable of destroying cultures of *E. typhosa* within a few minutes while 0.5 per cent solutions were ineffective. In contrast, halving the concentration of chlorine only doubled the kill time for this disinfectant.

Results obtained against a suspension of *Bacillus metiens* spores indicated that increasing the concentration of available chlorine fourfold reduced the killing time to almost one-half (111). Changes in chlorine concentration do not affect inactivation rates to the same extent as in the case of other disinfectants such as the phenolics. This may be due to the fact that chlorine in aqueous disinfecting solutions is markedly affected by the pH with even slight changes toward alkalinity exerting much more influence upon the germicidal efficiency of chlorine solutions than do large changes in chlorine concentration. It is conceivable that a lesser concentration of chlorine is more active as a disinfectant at a lower pH than a greater concentration of chlorine is at an alkaline pH (113). A solution containing 1000 ppm of available chlorine was only slightly more germicidal than the same solution diluted to 100 ppm in distilled water (109). The reaction was changed by dilution from pH 11.3 to 10.4. The same solution was more germicidal when diluted to 20 ppm and with a pH of 8.3.

The death rate of bacterial spores at 60°C was increased by an increase in hydroxyl ion concentration when alkalies were tested for disinfectant activity (90). It was noted that sodium hydroxide solutions of differing concentrations but of the same pH due to the presence of various amounts of peptones had approximately equal germicidal activity. It was found that a 0.2 per cent solution of sodium hydroxide acting for ten minutes at 20°C was effective in inactivating fowl pox virus present in a concentration of 1000 infective doses per ml. Under the same conditions, a 0.17 per cent solution of sodium hydroxide was not effective (113).

Numerous experiments have shown that both absolute ethanol and ethanol in concentrations less than 50 per cent were practically useless as germicides. Much reliance has been placed on 70 per cent alcohol although critical tests have conclusively demonstrated that only the less resistant organisms were killed (100).

An equation was developed as the basic equation for the mechanism of disinfection (114) with C representing the disinfectant concentration, t the time for disinfection to take place and n, the concentration exponent for the disinfectant. Such an equation assumed all conditions in the disinfectant test other than concentration and time were constant. A later study in which this equation was modified ultimately provided a

mathematical formula to evaluate or predict the activity of a certain concentration of disinfectant (115). $C^{n} \cdot t = A$ was expressed in logarithmic form

$$n \log C + \log t = \log A$$

when disinfection times were determined for a series of disinfectant concentrations, with the results expressed in equations

$$n \log C_1 = \log A - \log t_1$$
$$n \log C_2 = \log A - \log t_2$$

Successive pairs of equations were combined by subtraction to yield the derived equations

$$n_1 = \frac{\log t_2 - \log t_1}{\log C_1 - \log C_2} \qquad n_2 = \frac{\log t_3 - \log t_2}{\log C_2 - \log C_3}$$

From such equations an average n was determined for a series of concentrations.

Alternately, in an earlier study (114) the average value of n in such a series of disinfectant concentrations was determined graphically. When successive values for log C and log t were plotted, a straight line was obtained. The value of n was then calculated by the equation

$$n = \frac{Y_2 - Y_1}{X_2 - X_1}$$

after selection of two suitable points (X_1 Y_1) and (X_2 Y_2) on the straight line drawn under guidance of the plotted points. The n values obtained graphically and by equation correlated well (115). After the average value n was

obtained values of log A were found by substitution in the equation

$$n \log C + \log t = \log A$$

and an average value of A was then calculated. With the average value of n and A known, the effect of concentration on exposure time could be predicted, provided other factors of the test such as pH were kept constant.

Organic Material

The efficiency of all disinfectants was reduced by the presence of organic matter to a degree dependent upon the chemical nature and physical condition of both the disinfectant and the organic material (116).

Several factors were proposed to affect the efficiency of the disinfectant in inactivation of bacteria (117), as follows:

1. The protective action of organic material on bacteria upon exposure to disinfectant was especially noticeable when the organic matter was particulate or viscous. Even though the disinfectant ultimately permeated the debris and reached the cells, it was present only in limited amounts. It was also possible for the protected bacterial cells to either adjust themselves to the unfavourable environment or to neutralize sufficient amounts of the disinfectant to prevent lethal exposures. This was especially true for acids, alkalies, oxidizing and reducing agents.

2. The organic material reacted with the germicide to form compounds which were less soluble or less toxic than the unaltered disinfectant. When insoluble compounds were formed, they acted as other particulate materials in affording mechanical protection for the bacteria. The disinfectant that reacted with the organic matter was removed from the solution, thus leaving it proportionately weaker and therefore less effective.
3. Organic matter neutralized a portion of the disinfectant. This occurred when the buffer action of proteins partially neutralized acid and alkaline solutions. Reducing substances in sewage and water heavily polluted with organic matter made disinfection with chlorine more difficult.
4. Particulate matter absorbed part of the disinfectant from the solution. This was expected to happen since the disinfectant often reduced the surface tension.
5. Even when none of the above-mentioned factors were operative to a significant degree, microorganisms were found to be more resistant to the action of disinfectants when proteins or other organic colloids were present. The presence of organic matter was believed to retard hydrolysis of cell proteins.
6. The cationic detergent type of disinfectant appeared to be inactivated by the presence of several natural substances such as lecithin (118).

Evaluation of the virucidal action of disinfectants in the presence of organic material is of the greatest importance, since many of the more dangerous viral pathogens are expelled from the body surrounded by a protective coat of organic material (119).

In a study of the inactivation of foot and mouth disease virus by disinfection (87), it was found chemicals which caused coagulation of proteins and consequent protection of virus by absorption to the formed particles, were not active as virucides. Bichloride of mercury, alcohol and the creosols formed large coagula with the virus containing material and either were entirely inactive or only very slowly active. Substances such as sodium hydroxide which did not form coagula but dissolved protective material inactivated the virus readily.

Acids, alkalies, oxidizing agents, detergents and other chemicals were tested for their virucidal activity against swine vesicular disease virus. Virus diluted in hard water was inactivated by nitric, sulphuric, acetic and formic acids, by sodium hydroxide, sodium metasilicate, sodium hypochlorite, peracetic acid, potassium permanganate, benzene sulphonic acid, formalin and ethyl alcohol and by iodophor disinfectants (120). These chemicals and disinfectants with the exception of sulphuric acid, potassium permanganate and sodium hypochlorite were virucidal in the presence of organic material in the form of pig faeces. As with foot and mouth disease virus, the

presence of organic material lessened the virucidal activity of all the disinfectants tested, with the exception of formalin, acetic acid and ethyl alcohol (120).

A few instances have been cited where it appeared that the disinfectant was more active in the presence of organic matter, but critical examination revealed that the introduction of organic matter was accompanied by some alteration of the environment which was injurious to the organisms or increased the activity of the germicide (119).

Glutaraldehyde, unlike formaldehyde, did not coagulate blood (67), which meant it made cleaning medical or dental instruments coated with blood easier, and also glutaraldehyde was shown to act against *E. coli* and *Streptococcus pyogenes* in the presence of large amounts of organic matter. In one study (73), the presence of organic material actually facilitated the virucidal activity of glutaraldehyde against enterovirus.

Test Methods for Determining the Bactericidal Activity of a Disinfectant

Standard methods are available for evaluating the bactericidal properties of a disinfectant (121). The section on test methods for bactericides will be divided into three areas:

1. Phenol Coefficient Tests
2. Use Dilution Tests
3. Other Tests

Phenol Coefficient Tests:

Recognition of the need for a standard precise method for comparing and evaluating disinfectants led to development of the Rideal-Walker Test in 1903 (122). The test was based on comparing the resistance of the test bacteria to a certain chemical with their resistance to a specific concentration of phenol adopted as a reference standard. This test was a tube dilution procedure in which the highest dilution of the disinfectant that killed the standard test organism, *Salmonella typhosa*, in seven and one-half minutes but not five minutes was divided by the highest dilution of a standard five per cent phenol solution that killed *Salmonella typhosa* in 7.5 but not five minutes, to yield what was termed the Rideal-Walker Coefficient. The test was performed at 15°C to 18°C (123).

Since the disinfectant tested was compared to a five per cent solution of phenol and not undiluted phenol, conversion of the Rideal-Walker Coefficient of that disinfectant to an actual use dilution was attained by multiplying the Rideal-Walker Coefficient by 20 (123, 124).

The Rideal-Walker method specified standard media, use of a specific test culture of Salmonella typhosa maintaining it according to a given routine and the use of specific apparatus such as a standard transfer loop for making uniform culture transfers.

The Rideal-Walker method has been revised and modified over the years. Such modifications and the reasons behind them will now be considered. Since the Rideal-Walker method did not include a determination of the activity of a disinfectant in the presence of organic material and since disinfectants are almost invariably required to act in the presence of organic material, a modified form of the Rideal-Walker test was introduced in 1908 which took this factor into account (125). It was termed the Chick-Martin test. In this test the disinfectant and the phenol dilutions contained three per cent of sterilized human faeces, previously dried and ground. The culture used was a Salmonella typhi (Salmonella typhosa) but it was different from the test strain used in the Rideal-Walker test. Upon exposure to disinfectant for a standard time period of 30 minutes, subcultures were made in duplicate into a peptone meat extract glucose medium. The Chick-Martin coefficient was calculated for a certain disinfectant as follows: The mean of the highest concentration of phenol permitting growth and the lowest

concentration preventing growth, is divided by the mean of the highest concentration of test disinfectant permitting growth and the lowest concentration preventing growth.

Later (126, 127) it was pointed out that dried faeces introduced variability into the test system since the organic content of such faeces would differ markedly from lot to lot. Also, the solubility of dried faeces was variable and poor. A standard preparation of yeast was advocated as the organic material for the test and the Chick-Martin test was subsequently modified (128).

In 1909, the Lancet appointed a commission of inquiry into the chemical and bacteriological standardization of disinfectants. The report (129) was published the same year and in it was described a new testing technique. It differed from the Rideal-Walker test in the following respects (1) it used *Bact. coli communis* (*E. coli*) as the test organism, stating that the use of *Salmonella typhi* was a fetish (2) it employed MacConkey medium (3) sampling was done with platinum spoons of three times the capacity of the normal loop (4) killing dilutions of phenol and the test disinfectant were found after 2.5 minutes and 30 minutes contact and the phenol coefficient calculated from the means of the two killing dilutions.

In 1911 further modifications of the Rideal-Walker test were proposed, resulting in the United States Hygienic Laboratory Method (130). It differed from the Rideal-Walker test in that a more dilute peptone-meat extract medium was used to culture the test organism, the temperature at which the test was carried out was raised to 20°C, a spiral inoculating loop was introduced and the method of calculating the phenol coefficient was altered.

A method for disinfectant testing based on the Rideal-Walker test, developed by Shippen and Reddish was adopted as the official United States Food and Drug Administration method (FDA) (131). The test organism used in the FDA method was a 22 to 26 hour culture of *Salmonella typhi* (Hopkins strain) grown in nutrient broth at 37°C. The broth contained ten grams of Armour's peptone, five grams of Liebigs' beef extract and five grams of sodium chloride per liter of distilled water. It had a final pH of 6.5. The test was performed at 20°C instead of 17°C to 18°C as was the case with Rideal-Walker tests, and the exposure times were five, ten and 15 minutes. The phenol coefficient was a figure obtained by dividing the numerical value of the greatest dilution of the disinfectant capable of killing *Salmonella typhi* in ten minutes but not in five minutes by the greatest dilution of phenol showing the

same results. This report also recognized that it was desirable to know the performance of disinfectants against microorganisms other than Salmonella typhosa and made provision for testing Staphylococcus aureus.

In recent years the FDA method has been replaced by the United States Association of Offical Agricultural Chemists (AOAC) Phenol Coefficient method (132). In principle it differed little from the FDA method, the main points of departure were as follows:

1. Subculture media were selected that would more effectively neutralize the action of disinfectant carried over when viability tests were done. Choice of culture medium depended on the disinfectant tested.
2. It specified the use of two test organisms, Salmonella typhosa and Staphylococcus aureus.

One of the weaknesses of the Rideal Walker test was that the disinfectant dilutions were made in distilled water. Many disinfectants and germicides can show extremely good results when run in use dilutions of distilled water. This does not mean that institutions in their actual use practises will achieve the same results. Hard water has an inactivating effect on many disinfectants, even at relatively low degrees of hardness (133). Distilled water must be used as a standard diluent, since the hardness of tap water may not only vary from source to source but from day to day in one source, which leads to variable results.

The AOAC test does not specify water hardness and such tests are often run in distilled water (134).

When phenol coefficient tests were first proposed, typhoid fever was an infectious disease claiming many fatalities. Since the causative agent of this disease, *Salmonella typhosa* was the infectious agent most likely to be the object of any disinfection procedure, it was selected as the test organism of choice. *Salmonella typhosa* has been used continuously for 63 years as the test organism and is employed in the current method for all initial evaluations. Attention has been called to the limited value inherent in test procedures restricted to use of a single test organism (81, 82, 182, 201) since in this test *Salmonella typhosa* would not be an index of effectiveness of disinfectants against such resistant pathogens as *Mycobacterium tuberculosis*, *Mycobacterium paratuberculosis*, *Clostridium tetani* and *Bacillus anthracis* (135). The adoption of *Staphylococcus aureus* as a secondary test organism in the FDA method (131) partially corrected this deficiency and it is employed with *Salmonella typhosa* in the present official AOAC procedure (132).

The size of the droplet picked up by the inoculating loop used in the phenol coefficient tests affected the number of organisms transferred from the disinfectant-organism mixture to the subculture medium

and consequently influenced the test result. Therefore the droplet must be a uniform size, being big enough to ensure a reasonable uniformity in volume, while being small enough to avoid a carryover of a bacteriostatic concentration of the disinfectant. Both spiral and single turn loops of different sizes were tried with no improvement in test results (135). The problem of transferring bacteriostatic quantities of disinfectant into the subculture media upon sampling is inherent in the design of phenol coefficient tests. A subtransfer method (137) in which four loopfuls of the first subculture were transferred to a second tube of subculture medium was employed to overcome this difficulty. This technique was an effective way of preventing bacteriostasis when testing many types of germicides. This problem has also been approached by employing subculture media containing materials capable of suppressing bacteriostasis by the disinfectant (138, 139).

The importance of the composition of medium used to grow the test organism in the phenol coefficient tests was noted early (140). It was observed that the greatest single factor influencing the resistance of bacteria to chemical germicides was the chemical composition of the medium in which they were grown (141). The variations in results obtained in the phenol coefficient procedure had been traced to variations in

the beef extract (142) and in the peptone (143) which were specified for use in preparing the standard medium employed in propagating the test culture. It was suggested that the following be specified in culture media (144)

i) the nature of the raw materials from which media were derived

ii) the type of preparation employed for media (for example acid hydrolysis, tryptic digestion of peptic digestion)

iii) chemical composition

iv) bacteriological properties

Although phenol coefficients were originally intended to signify the relative activity of phenolic derivatives of coal tar, they have been assigned to most groups of disinfectants. The coefficients were probably suitable measures of the practical value of the phenol-like disinfectants (135) but in comparing disinfectants unrelated to phenol these values may be entirely misleading. For example, the phenol coefficients for quaternary ammonium compounds were extremely high, probably due to inadequate neutralization[146], although corrections were later made by the introduction of more efficient inactivators in the AOAC tests for such compounds (147).

The water solubility of phenolic compounds was shown to be inversely related to their phenol coefficients (148). In a study where a large number of phenolics were

tested (149), it was found that the more active the phenolic, the closer the concentration needed to kill in ten minutes was to the saturation concentration of that compound in water. Such compounds were more difficult to dissolve in water and therefore for many purposes were less convenient. It followed from this that the more active the phenolic, the higher its lipoid solubilities. There was a relationship between biological activity and the partition coefficient of the compounds between lipoids and water (150). The biophase of the cell into which biologically active drugs were taken up consisted of a three-dimensional polar lipoid and the activity of phenolic was dependent on the concentration penetrated into the biophase (151). Therefore the more active phenolics had a higher cell/water partition coefficient and a greater proportion of the chemical was removed by cells (152).

Use Dilution Tests

While the phenol coefficient tests are often termed suspension tests, use dilution tests depend on carrier objects coated with test microorganisms which are dipped into a dilution of disinfectant for a certain fixed exposure time and subsequently residual virus is assayed (153). Several reports emphasized that no evidence has ever been found to show that the

maximum dilution of disinfectant determined to be effective by the phenol coefficient method is the same as the dilution of the product necessary for disinfection to occur (154, 155, 156). The carrier methods were shown to be a more accurate way of determining the actual use dilution of a disinfectant and are therefore termed use dilution tests (157).

In 1881, Koch (158) impregnated silk threads with *Bacillus anthracis* spores, which were exposed to chemical solutions for varying periods of time. After exposure to the disinfectant, such threads were washed to remove residual disinfectant, and dipped into nutrient gelatin or used for inoculating animals to assay residual virus. This was one of the first use dilution tests, for the results were often used as the basis of picking the dilution of disinfectant to use in practical situations.

This method was adopted later for use in testing the activity of a disinfectant on non-spore-forming bacteria. A method was also proposed in which the test bacteria were dried on the surfaces of small garnets of uniform size prior to exposure to disinfectant. The garnets were washed and treated with chemical neutralizer at the end of the exposure time. This prevented the bacteriostatic effect of disinfectant carried over from the test mixture to the subculture media of the garnet.

A standardized glass rod carrier method was proposed (159) as a direct measure of the maximum safe use dilution of disinfectants. This method made use of small glass rods on which the test organisms were dried for 30 minutes. The seeded rods were then exposed to disinfectant solutions at 20°C for one, five, ten and 30 minutes, rinsed with water or neutralizing solution and transferred to culture media. This test was the original Use Dilution Test. This procedure later proved to be a safe index to the practical disinfecting value of germicides used by veterinarians (160).

Another use dilution test was proposed (161) in which the test organism was suspended in a one to ten dilution of skim milk applied to microscopic slides, drained and partially dried. Such slides were immersed in the germicide, gently agitated, rinsed, placed in petri dishes, overlayed with agar medium and incubated. The destruction of 99.9 per cent or more of the test organism was taken as the endpoint. This method simulated conditions under which certain germicides, especially hypochlorites and quaternaries were used in dairies.

A multiple metal ring carrier method was proposed (162). This method included the interfering factor of organic material on the activity of the germicide. This carrier test was adopted as the standard carrier type dilution test method by the AOAC (132).

The use dilution of a disinfectant in this test is required under specified conditions to kill *Staphylococcus aureus* and *Salmonella choleraesius* on steel cylinders within a period of ten minutes (163). This test is satisfactory insofar as it defines a disinfectant as an agent that completely destroys designated infectious organisms on a steel cylinder in ten minutes. It does not, however, provide information as to

(a) what such a disinfectant will do on other surfaces common to the hospital environment

(b) the ability of a treated surface to continue to destroy pathogens that may recontaminate the surface.

The capacity of a surface treated with disinfectant to continue to destroy bacteria applied as a repeat contamination was evaluated (164). In the study four designated disinfectants and one sanitizer were subjected to the AOAC Use Dilution Test procedure up to the point of placing the series of ten treated steel cylinders into Letheen broth. The cylinders were allowed to drain for ten minutes on sterile filter paper placed on a petri plate after the ten minute exposure to the disinfectant. They were subsequently recontaminated by being placed in a 1-100 dilution of a 48-hour serial broth culture of *Staphylococcus aureus*. The carriers were then placed on a sterile filter paper in a petri plate and held for

four, six, eight hours and overnight before being placed into Letheen broth for incubation at 37°C for 48 hours.

In a similar study, the cylinders treated with disinfectants according to the AOAC Use Dilution Test were held after removal from the disinfectant solutions for varying periods of time, one hour, one day and longer before recontamination with a 1-100 dilution of a 48-hour serial broth culture of *Staphylococcus aureus*. All carriers were then held overnight and placed in Letheen broth for 48 hours at 37°C.

Disinfectant action on realistic surfaces was also studied. Linoleum, vinyl, asphalt, rubber, ceramic tile and painted plasterboard were considered with each material being cut into 5 x 30 mm samples. The AOAC Use Dilution procedure was used in slightly modified form, since surfaces could not be treated as steel cylinders for the following reasons

(a) their susceptibility to the high temperature of autoclaving

(b) The flooring materials used had thick backing or felt, making inoculation by total immersion impractical.

The samples of each material were inoculated with test organisms using a platinum loop calibrated to deliver 0.001 ml. This gave an inoculum of approximately 10^5 cells. This was the same number found on steel cylinders inoculated by the conventional AOAC Use Dilution technique.

The tile surfaces were dried for 20 minutes in a 37°C incubator after loop inoculation. Ten replicates of each tile sample were immersed in ten ml of the use dilution of disinfectant and subsequently transferred to two different subculture media. All tiles were incubated for 48 hours in subculture media. Results indicate that the efficiency of action of disinfectants varied with the surface to which they were applied. The importance of such findings is that a ten minute period for inactivation as required in the AOAC Use Dilution Test on steel cylinders was insufficient to kill *Staphylococcus aureus* on realistic porous surfaces. The penetration of porous surfaces by disinfectant is a very important consideration in the evaluation of disinfectant action (164).

A major deficiency in the current phenol coefficient method was the practise of converting the phenol coefficient number into safe use dilutions by multiplying it by 20. The formula 2.5 coef x (n + 1) was used. The coef. was the phenol coefficient found using *Salmonella typhosa* at 20°C and n was the disinfectant concentration exponent for the organism against which the disinfectant was employed. This was a better indicator of a use dilution for a disinfectant, since it was more in accord with established concepts of the dynamics of germicidal activity.

This was borne out when it was shown use dilutions obtained by this formula coincided more closely to the use dilutions determined by the AOAC Use Dilution Method which were considerably less than the use dilutions calculated by multiplying the phenol coefficient by 20 (165).

Other Tests:

In 1965 another approach for establishing use dilutions for germicides was developed (166, 167). It was termed the capacity test and was based on principles suggested by earlier workers in the field (168). The principle of the capacity test was to determine the lethal activity of a given concentration of disinfectant upon successive additions of a bacteria-organic material mixture. The organic material was a standard yeast preparation.

The test was used by another group on a wide range of commercial products (169), the conclusion being drawn that the capacity test imitated practical situations in which there were successive additions of bacteria together with organic material.

The mechanics of the capacity test were as follows: Three, one ml amounts of yeast/bacteria suspension were added to three ml of each disinfectant at ten minute intervals. Eight minutes after each addition, samples were taken with a Pasteur pipette and one drop was transferred to a nutrient agar plate and to each of two duplicate tubes of

nutrient broth with inactivator. The use of one drop samples indicates the test is unquantitative. The lowest disinfectant concentration considered safe was the one which permitted at least two incremental yeast/bacteria additions before a positive culture appeared.

An in use test for disinfectants in the hospital environment was developed (170). In this test, samples were taken from buckets containing disinfectant at the end of a cleaning session, from disinfectant wrung from mops, from disinfectant in which brushes or mops were stored, and from disinfectant in which urine bottles were stored or rinsed. One ml quantities of such samples were transferred to nine ml of inactivating solution appropriate for that disinfectant. Ten drops of this 1-10 dilution were placed on the surface of a dried nutrient agar plate. This was incubated for 72 hours and examined at intervals for growth. The failure of a dilution of disinfectant to act was defined as any growth on more than five drops out of the ten added to the nutrient agar plate. This corresponded to about 1000 organisms per ml of the liquid sampled.

The British Standard Quantitative Technique used for testing quaternary ammonium compounds (171) was adapted to test five commercial nonphenolic disinfectants. The test determined the concentration of each disinfectant needed to provide an inactivation factor of 10^4.

In this technique, five ml volumes of ten fold disinfectant dilutions in sterile tap water were distributed to large test tubes. Five ml of a one in four mixture of bacterial suspension (10^9 organisms per ml) in organic material was added to each tube. One ml of the reaction mixture was added to nine ml of broth containing neutralizing agent after periods of five minutes. Samples of this broth were subcultured in triplicate on nutrient agar.

A comparison of results obtained by this British Quantitative technique with those obtained by the capacity use dilution test (130) in the previous study mentioned (169) indicated that effective concentrations of quaternary ammonium compounds and halogens were deemed to be ten times greater by the capacity test as opposed to the British Quantitative technique, which supports the conclusion that the capacity test incorporated a considerable margin of safety.

One study on disinfectant testing concluded (175) that sufficient data was not yet available to enable laboratory tests to be designed which can represent the situation in practice accurately.

Neutralization of Disinfectant Activity

Proper neutralization of the action of a disinfectant at the end of an exposure time is necessary for meaningful quantitation of the activity of the disinfectant.

One group of investigators developed what they felt was a universal neutralizing medium for antimicrobial chemicals (176). The medium contained Bacto-tryptone, yeast extract, dextrose, sodium thioglycolate, sodium thiosulfate, sodium bisulfite, Tween 80 and lecithin. This neutralizing medium, in broth and agar forms, was described as being effective in neutralizing more concentrated amounts of chlorine, formaldehyde, glutaraldehyde, iodine, mercurials, phenolics and quaternary ammonium compounds than other media previously available. It was stated that this medium showed promise for studying and evaluating known and unknown compounds, mixtures and formulations in the liquid state and in environmental sampling where neutralization of the chemicals was important to differentiate between bacteriostatic activity and bactericidal action.

A review emphasizing the necessity of neutralization of disinfectant carried over into media used to grow residual bacteria is that of MacKinnon (177). Caution must be exercised in choosing a neutralizer since too powerful a neutralizer may complete the work the disinfectant tested started out to do. Bacteriostasis can also occur when disinfectant molecules become attached to the cell wall of the organism by weak chemical bonds (178).

The realization that many types of disinfectant were bacteriostatic at vastly greater dilutions than those at which they were bactericidal led to the use of a wide

range of chemical and physical neutralizers. The choice of a neutralizer is limited by certain criteria. It must, by definition, neutralize the disinfectant against which it is used. It should not give rise to bacteriostatic effect, either of its own or as a result of any products formed when it is combined with the disinfectant. The action of the neutralizer should be fairly rapid, for slow neutralization allows continued bactericidal effect after the exposure time is ended. Few, if any, of the commonly used neutralizers were found to be completely non-bacteriostatic (177). Neutralizers were employed either as an intermediate stage for diluting or neutralizing disinfectant before transfer to a growth medium or such substances were included in the growth medium itself. Both systems had their disadvantages. In the first, small numbers of survivors became less apparent through dilution. In the second, the bacteriostatic power of the neutralizer tended to have a greater effect on the growth of the microorganism. Organisms that have been physically or chemically damaged may require growth conditions very different from those needed by undamaged organisms. In one study, in which low concentrations of disinfectants had been employed to bring about mutations in bacteria, there were changes in nutritional requirements (179). It was recommended that selection of a suitable medium for recovery and neutralization can only be done when its effects on damaged microorganisms are known (180). As a result of damage to the organism the neutralizer may have exerted an effect different from that on undamaged micro-organisms (181).

Test Methods for Determining Virucidal Activity of Disinfectants

Standard methods are available for evaluating bactericides and fungicides, but there are no existing standard techniques available for evaluating virucides (121) Many of the reports on virucidal agents are fragmentary and widely scattered, with the data obtained under a wide variety of test conditions.

A study was undertaken to compare the virucidal activities of commonly employed agents against vaccinia and influenza A viruses. One-half ml amounts of undiluted viral inoculum were added to five ml of disinfectant and to five ml of saline, respectively. After an incubation period of ten minutes at room temperature or in a 37°C water bath, the mixture was diluted in serial ten fold steps to 10^{-8}, and each dilution was inoculated into eggs. The dilution of each disinfectant-virus mixture and the inoculation of eggs with this mixture was completed within a period of three minutes (30).

The data indicated that both influenza A virus and vaccinia virus were rendered noninfective by one per cent formaldehyde, 0.1 per cent mercuric chloride, two per cent phenol, and 0.1 M sodium oleate. These two viruses were inactivated more than 99.9 per cent by one per cent crystal violet, 70 per cent ethanol, 48.5 per cent isopropanol, and one per cent Lugols iodine solution. The most troublesome problem encountered in this study was the toxicity of the various preparations for the eggs used in the infectivity assay.

The problem of autointerference in the detection of infective virus when inactive virus was present might appear in such studies of virucides (183). Although autointerference had not been reported to be associated with vaccinia virus in chorioallantoic membrane (184), it was shown that preliminary injection of influenza B virus, previously irradiated with ultraviolet light prevented the growth of subsequently inoculated virus. Therefore, the possibility existed that some active influenza virus could still be present in mixtures yielding no hemagglutinin after inoculation into the allantoic cavity. Lipophilic qualities of viruses may influence their susceptibility to disinfectants (24). It was observed by Noll et al (185) that certain viruses, especially those containing lipid, combined with lipid. Such viruses were termed lipophilic. Viruses which did not combine with lipids were termed hydrophilic. While enveloped viruses such as influenza virus were termed lipophilic and viruses such as poliovirus, coxsackievirus and echovirus were termed hydrophilic, adenovirus virus which lacks a lipid envelope reacted with lipid and was classified as a lipophilic virus (185). The resistance of the hydrophilic viruses to some germicides was considerably greater than that of vegetative bacteria (24). This was ascribed to the failure of these viruses to react with germicides having lipophilic properties such as long carbon

chains. Lipophilic viruses were shown to be more susceptible to inactivation by germicides having lipophilic properties. No correlation between virus size or type of nucleic acid with germicide susceptibility was found. Inactivation of virus was determined by mixing 0.9 ml of the test germicide with 0.1 ml of undiluted virus. After contact at room temperature for one, three, five and ten minutes, viral inactivation was determined by assaying for residual virus and comparing the virus titer obtained from such assays with that of untreated controls.

Poliovirus 1, coxsackievirus B1, echovirus 6, adenovirus 2, herpes simplex virus, vaccinia virus and influenza (Asian) virus were tested with ten germicides(24). They were sodium hypochlorite, Weladyne (iodine complexed with nonionic detergent at low pH), bichloride of mercury, Cidex (active ingredient two per cent glutaraldehyde), ethyl alcohol, isopropyl alcohol, phenol, O-phenylphenol and Zephiran (a cationic detergent). The study revealed 200 ppm sodium hypochlorite, 150 ppm iodine in Weladyne and a 0.2 per cent solution of $HgCl_2$ inactivated the viruses completely by the end of ten minutes. Undiluted Cidex inactivated all of the test viruses in one minute. Ethyl alcohol was shown to be a potent virucidal agent and within the range of 70 per cent to 95 per cent the viruses were inactivated within one minute. Isopropyl alcohol was extremely effective against lipophilic viruses, while having limited and

irregular activity against hydrophilic viruses. Five per cent phenol inactivated the seven viruses in ten minutes. However, the compound was active over a rather narrow range of concentrations. Although O-phenylphenol was ten times as active as phenol against the lipophilic viruses, it failed to inactivate hydrophilic viruses in 12 per cent solution after ten minutes. Zephiran, a cationic detergent, readily inactivated lipophilic viruses in a one per cent solution, while a ten per cent solution of Zephiran was completely inert against hydrophilic viruses even after 24 hours at room temperature. Tests for infectivity were carried out by inoculating influenza virus into the allantoic cavity of the chick embryo and testing for viral hemagglutinin in the allantoic fluid. All other viruses were grown in cultures of HeLa cells except herpes simplex virus, which was grown in freshly trypsinized rabbit kidney cells. As in the test system previously described, germicide was carried over into the early dilutions and was frequently toxic for tissue culture cells and therefore whether or not complete viral inactivation took place could not be determined.

It was felt that wide variation in susceptibility of viruses to germicides precluded establishing a general figure of activity analogous to the phenol coefficient for bacteria (186). A test method for viruses analogous to the AOAC Use Dilution Test for bacteria was developed (187). Newcastle disease virus was used in this test. Modifications

in the test method from that used for bacteria were kept to a minimum. In the bacterial use dilution test, sterile steel carrier rings were placed in a standard, liquid culture of bacteria. After 15 minutes, the rings were removed and dried at 37°C for 20 to 60 minutes. Subsequently, the rings were placed in a tube of disinfectant. The disinfectant was diluted to the concentration the manufacturer specified. Each ring was transferred to a tube containing ten ml of standard broth after ten minutes. Ten carrier rings prepared in this manner constituted a bacterial use dilution test. If the ten tubes showed no evidence of bacterial growth, the disinfectant was judged effective at the use dilution prescribed by the manufacturer.

In this test for virus inactivation, a standard allantoic fluid suspension of Newcastle disease virus was used instead of a standard bacterial culture. The carrier rings were placed in the virus suspension, after which they were dried and transferred to the disinfectant tubes in the exact manner described in the bacterial use dilution test. A difference between the viral and bacterial use dilution test was that, instead of transferring rings from the disinfectant to ten ml of broth, they were transferred to one ml of broth. Subsequently, 0.1 ml of this broth was inoculated into the allantoic sac of each of six, ten day old embryonated chicken eggs. If none of the inoculated embryos were killed by viral infection within five days

after inoculation, the disinfectant was judged effective at the dilution tested. Although the authors (187) stated that there was not appreciable viral inactivation during the 60 minute drying period, when this procedure was repeated using porcelain rings instead of the metal rings, and vesicular stomatitis virus instead of Newcastle disease virus, very few virus particles were recovered after 20 minutes. The amount of recoverable virus was reduced from $10^{9.5}$ LD50 per 0.1 ml to $10^{1.0}$ LD50 per 0.1 ml without any disinfectant. The data presented in the study (187) indicated that most commercial disinfectants tested were effective at the use dilution of the manufacturer.

The use dilution type of test method for virucides was subsequently modified by another group of investigators (188), who demonstrated the adaptability of the procedure to tissue culture. The method was modified as follows: A concentrated tissue culture preparation of virus was used; Hep-2 tissue culture cells were used as a visible test system; Letheen broth was substituted for nutrient broth to reduce the cytotoxic effect of the disinfectant on the tissue culture as well as to neutralize the activity of the disinfectant against the virus at the end of the exposure time. Controls were included to measure the virus titer end point and the toxicity of the disinfectant to the cell cultures. The comparison of control end points with those of the test proper was the criterion for measuring the effectiveness of the disinfectants against the viruses.

Subsequently, this group of investigators reported (189) that due to inconsistencies in results, this test protocol could not be adopted as a standard test procedure for potential virucides without further modifications. Some of the modifications proposed were: the virus-contaminated cylinders should be placed in a vacuum desiccator for 20 to 30 minutes at 37°C to facilitate more rapid and thorough drying and minimize virus loss; obtain a large number of collaborators in different laboratories to perform the same test to verify reproducibility; increase the number of test viruses and retain the quaternary ammonium compound, phenolic, and iodophor as test germicides but also add a hypochlorite and glutaraldehyde for evaluation.

A study (121) was done to develop an easily reproducible method for evaluating the effect of disinfectants on vesicular stomatitis virus. The liquid dilution test procedure proposed was as follows:

One vial with nine ml of disinfectant at 1.11 times the concentration to be tested was placed in a water bath at 20°C. A one ml amount of virus suspension was added to each vial of disinfectant with a syringe and hypodermic needle. The suspension was mixed when the virus was introduced and just before each portion of the virus-disinfectant mixture was removed. At five, ten and 30 minutes after introducing the virus into the disinfectant, one ml was removed and placed in a serum

bottle with nine ml of phosphate buffered saline (PBS) which was kept at $0^{o}C$. Five eggs were inoculated with 0.1 ml of virus-disinfectant mixture per egg on the chorioallantoic membrane for each contact time. The eggs were candled daily for three days, and on the sixth day. Embryos that died within 24 hours of inoculation were considered nonspecific injury deaths. Twelve disinfectants were tested by using the dilution procedure, of which seven inactivated the virus within ten minutes, whereas five failed to do so in 30 minutes. An effective disinfectant was defined as one that in ten minutes reduced the virus LD50 titer by 6.5 logs. Results indicated one per cent cresylic acid, substituted phenolic (0.5 per cent), 2.5 per cent phenol, 0.2 per cent chlorinated phenol, 0.4 per cent HCl, two per cent sodium orthophenylphenate and 0.645 per cent NaOCl were effective virucides while five per cent of the quaternary ammonium compound tested, ten per cent NaOH, ten per cent KOH, ten per cent Na_2CO_3, 70 per cent ethanol and 80 per cent ethanol were not.

The test method used to obtain these results with vesicular stomatitis virus was used in another study where disinfectants were tested against Newcastle disease virus (190). The toxicity of chemicals for the embryonated eggs used to assay for residual Newcastle disease virus was evaluated by injecting five eggs in the allantoic cavity with 0.1 ml of a 1-10 dilution of the

greatest concentration of each disinfectant. The disinfectants were diluted with 0.5 M PBS, pH seven as was done in the test method. Five disinfectants were considered and in each case the disinfectant inactivated the virus within ten minutes at 20°C when the recommended use dilution of the manufacturer was used. When the same five disinfectants were diluted to twice the recommended dilution of the manufacturer, three of the five inactivated all the detectable virus. The greatest concentration of disinfectant tested killed no embryos when injected into the allantoic cavity.

The virucidal ability of A-33 Dry, a quaternary based formulation with dimethylbenzyl ammonium chloride as the active ingredient, was determined using 11 test viruses. They were vaccinia virus, poliovirus 1, reovirus, respiratory syncytial virus, croup-associated virus and six influenza virus strains (191). Each test virus was exposed to the use dilution of 1-256 of A-33 Dry for ten minutes at room temperature. The virus-disinfectant mixture was neutralized in Letheen broth and serially diluted in trypticase soy broth. Influenza virus was assayed in embryonated eggs, while cell cultures were used for the other viruses. Reovirus, poliovirus 1, croup-associated virus and respiratory syncytial viruses were inoculated into monolayer cultures of a clonal derivative of HeLa cells. Vaccinia virus was assayed, using a continuous line of monkey kidney cells ($LLC-MK_2$).

Disinfectants were assayed for cytotoxic effects prior to testing. Final dilutions of neutralizer-disinfectant mixture were tested for toxicity. Toxicity was scored on the basis of either the death of the chick embryos or the detachment of tissue culture cells during a 48 hour period. A 1-256 dilution of A-33 Dry reduced virus titers by five to eight logs for influenza viruses, three logs for vaccinia virus and two to four logs for respiratory syncytial virus, croup-associated virus, poliovirus 1 and reovirus. Cytotoxic effects were usually absent in dilutions of 1-100 or greater of neutralized virus-disinfectant mixture.

In a study (192) to determine the virucidal activity of benzalkonium chloride on 13 viruses, it was found to inactivate influenza, measles, canine distemper, rabies, fowl laryngotracheitis, vaccinia, semliki forest, feline pneumonitis, meningopneumonitis and herpes simplex viruses when present at 1.33 mg/ml or less. Poliovirus and encephalomycarditis virus were not inactivated. It was concluded that all viruses tested were sensitive to benzalkonium chloride, with the exception of the picorna group. The presence of organic material tended to have an inhibitory effect on inactivation by benzalkonium chloride.

Acids, alkalies, sodium hypochlorite and phenolic disinfectants were tested for their virucidal activity against foot and mouth disease virus recovered during the

epidemic in Britain in 1967-68 (193). In the test method, the test virus was diluted 1-10 in disinfectant. In certain experiments ox serum, milk, feces, soil suspension or synthetic detergent were included. The pH was measured and the mixture was held at 20°C or 4°C for varying periods. The action of the disinfectant was stopped by ten fold dilution in buffer at 4°C. Residual virus was assayed either by mouse inoculation or by the plaque technique. Control preparations without chemical were treated similarly. The log virus inactivation was calculated from the formula: $\log_{10}$ titer of virus control minus $\log_{10}$ titer of the virus in the presence of chemical. The amount of chemical was expressed as the final dilution in the mixture. At pH values less than six and greater than 11, the rate of foot and mouth disease virus inactivation was rapid. In the presence of hydrochloric acid, phosphoric acid, citric acid, sodium hydroxide or sodium metasilicate a five log reduction in titer was produced in 15 seconds at 20°C and 4°C. In the case of sodium carbonate, a five log reduction in titer occurred in three minutes at 20°C and in 30 minutes at 4°C. Inactivation of five log units of foot and mouth disease virus occurred upon exposure to a 0.025 per cent solution of sodium hypochlorite in hard water. When sodium hypochlorite was added to virus in the presence of ox serum, the same degree of inactivation

occurred only when the strength of the solution was increased to two per cent. When virus was mixed with phenolic disinfectants in the presence of ten per cent ox serum, the rate of virus inactivation was slow and the reduction in titer was at the most 1.4 log units after two hours.

The outbreak of swine vesicular disease in Great Britain in 1972 led to disinfectant testing against the etiologic agent swine vesicular disease virus, using the previously described test method of the foot and mouth disease virus study (120). The results indicated swine vesicular disease virus was more resistant to chemical disinfection than foot and mouth disease virus. Acids, alkalies, oxidizing agents, detergents and other chemicals were tested for their virucidal activity. One per cent formic acid (pH 1.8) and 0.066 per cent nitric acid (pH 1.6) caused a reduction in titer of four log units or more, although ten per cent acetic acid (pH 2.6) caused a 2.3 log reduction and two per cent sulfuric acid (pH 1.4) caused a 3.3 log inactivation. A two per cent solution of sodium hydroxide (pH 12.7) and five per cent sodium metasilicate (pH 12.45) inactivated more than four logs of swine vesicular disease virus. Other chemicals causing greater than a four log inactivation were 90 per cent ethanol, ten per cent formalin, and oxidizing agents such as 0.2 per cent peracetic acid, 0.2 per cent potassium permanganate and a 0.5 per cent solution of sodium hypochlorite. In some instances, the presence of pig feces

required a higher concentration of the chemical in order to cause a four log reduction in titer.

A study was conducted to determine the virucidal activity of Wescodyne, an organic iodine preparation (polyethoxyethanol iodine complex) (59). Poliovirus of a titer of $10^{8.2}$ PFU/ml was diluted in two fold steps in tap water. Samples of such dilutions were mixed with equal volumes of various Wescodyne dilutions. The Wescodyne-virus mixtures were held at 27°C for 15 minutes and then diluted. This was necessary to obtain a Wescodyne concentration of not more than 1-200 so that cytotoxicity could be avoided in tissue culture used to assay infectivity. Virus which was diluted 1-2, when exposed to 1-4 Wescodyne for 15 minutes still contained poliovirus of titer 10^2 PFU/ml. A 1-256 dilution of Wescodyne did not completely inactivate poliovirus diluted 1-128 or less.

In another study (194), nine parts of each of 20 kinds of disinfectants, appropriately diluted in sterile distilled water, were mixed with one part of variola virus suspension at room temperature (18° to 23°C). At the end of the exposure time, portions of the mixture were diluted 100 fold with minimal essential medium. Volumes of 0.1 ml thereof were inoculated into wells of microculture plates containing an established monkey kidney cell line. Cytopathic effect and plaque formation were used as indicators of ineffective virus inactivation. Virus inactivation kinetic curves were determined.

Kinetics of Bacterial Inactivation by Chemicals

As long ago as 1908, it was realized (112) that when large numbers of microorganisms were subjected to chemical disinfection, heat, radiation or other lethal factors in vitro, the viable count of the microorganism dropped off approximately exponentially with time. This was equivalent to saying that in any given time period a definite fraction of the population died and in the next similar time period the same fraction of survivors died and so forth. The process is similar mathematically to a monomolecular chemical reaction or to the radioactive decay of an unstable chemical isotope (17).

Bacteriologists call this the logarithmic death rate, since if the logarithm of the number surviving in any experiment is plotted against the exposure time, a straight line can usually be drawn through the points. Since this treatment usually fitted observed facts within experimental error and was such a convenient way of treating disinfection data, scant attention was paid to cases of poor fit or to the fact that no good rationale had been developed as to why such a mathematical formula should be followed in the disinfection process (17).

When the logarithmic plot is used, it is possible to express the intrinsic activity of a disinfectant in terms of a D value (D_{10}), particularly when the test organism is a spore (195). The D_{10}, the decimal reduction time, is the time in minutes to effect a 90 per cent kill of the test organism which is the reciprocal of the slope of the logarithm survivors versus exposure time plot (196).

Many cases of poor exponential fit have been published. In such instances, small but significant numbers of surviving organisms were found at an exposure time where an extrapolation of the exponential death function would have indicated a very low probability of any infectious agent being found in the volume tested (17). Therefore, any attempt to extrapolate such data to predict time-concentration conditions which produce 100 per cent mortality is not without its dangers and should be undertaken with extreme care. It was reported (197) that 99.9 per cent of 10^9 cells/ml of E. coli were inactivated by 0.005 per cent benzylchlorophenol in five minutes, but 40 days later the viable count had been reduced no further. The explanation offered was that, after the reaction had been in progress for about seven hours and the mortality reached 99.999 per cent, the survivors had adapted themselves to the environment and commenced multiplication using exudates from dead cells as nutrient. A similar phenomenon has been observed in other bactericidal systems, and with logarithmically growing cultures treated with insufficient antibacterial agent to produce sterility (198, 199, 200).

Analyses of the bacterial mortality in aqueous solutions of bactericides have produced two major theories about the nature of the biological reaction. The mechanistic theory treated the bactericidal reaction as a chemical reaction in which the mortality rate of organisms approximated closely to that which would be expected if the bactericidal reaction was a monomolecular

one. This would imply a constant death rate throughout the entire course of the reaction (201). Significant departures from this exponential law were attributed to factors operating during the reaction (202).

The alternative theory, the vitalistic theory, is based on the distribution of resistances of the individual organisms in a population. It postulated that the deviations from the exponential law resulted from inherent heterogeneity of the microorganisms (197). Data supporting this theory demonstrated the existence of a rectilinear relation between probit mortality of microorganisms and a simple transformation of contact time such as the logarithm of the contact time (203).

Kinetics of Viral Inactivation by Chemicals

When analyzing the kinetic aspects of viral inactivation, it is worth noting some of the ways in which these processes differ from the comparatively simple chemical reactions upon which most kinetic concepts are based. One point of difference is the initial reactant concentration. Some viruses are available for inactivation experiments at concentrations as high as 10^{12} infectious units per ml, but concentrations of 10^{6} or less are more typical. If there are as many as 1000 virus particles per infectious unit (which is unlikely in most cases) the maximal concentration of active particles would be 10^{-6}M or less. By way of contrast, the molecular reactions which supply the main body of our knowledge of chemical kinetics are

not usually observed at reactant concentrations less than 10^{-4}M. Exceptions which come to mind are gaseous-phase reactions at very low pressures and certain catalytic processes, but in neither of these categories can one find much in common with the biological reactivity of viruses (202).

Viral inactivation experiments differ also in the range of concentration over which the results are meaningful. If a virus suspension containing 10^7 infectious units per ml is subjected to a treatment such that only one unit per million escapes destruction, the treated suspension will contain ten infectious units per ml and, when put into contact with its cellular host, may have no less effect than the untreated suspension. If the treatment is continued until there is on the average only one infectious unit per liter of suspension, this degree of survival is still more than negligible, for the probability that any given milliliter contains one or more infectious units is 1-1000. A probability this high would make the preparation unsatisfactory for use as a killed vaccine.

Another factor to consider with reference to viral inactivation kinetics is the experimental error in assaying residual virus. Under usual conditions, the fiducial limits for a single virus analysis are seldom less than ± 0.1 logarithmic unit and maybe ± 0.3 logarithmic unit, i.e. the expected error is from ± 10 per cent to ± 50 per cent. This amount of error is gross in comparison

with the ± one per cent or less which may be present in a precise chemical analysis. In summary, in viral inactivation a comparatively small number of particles are being dealt with. The reaction involving such particles is studied over a tremendously wide range of concentration. As a result, there is relatively great experimental error. Therefore, all of the inactivating processes in which the effect is time dependent should be treated graphically. The logarithm of the observed survival ratio is plotted against time. If the result is a straight line from the origin, the process is exponential as far as the data extend (202).

In a study of the kinetics of inactivation for poliovirus 1 with chlorine, results indicated that under the conditions employed, the same theories which were useful in explaining inactivation of other microorganisms also applied to poliovirus 1 and the rate of inactivation was independent of the virus concentration. This was especially true when experiments were conducted at pH 8.5, since a straight line was obtained which passed through the origin of a plot of logarithm of the fractional number of surviving organisms versus exposure time to chlorine. However, at pH six and pH seven results indicated an initial rapid decrease in virus titer during the first minute and then a straight line relationship as predicted. No explanation was offered for this variation (204).

In this study (204), variable results obtained in previous studies on the inactivation of various mammalian viruses were attributed to three sources of variation:

1. The presence of variable amounts of organic impurities in the virus suspensions.
2. The failure in previous studies to differentiate between the various forms of active chlorine, some of which were more effective disinfectants than others.
3. The dependence on static or endpoint methods of infectivity assay, which was not sufficiently precise for kinetic analysis as compared to an assay of virus by the plaque assay method.

The devitalization rates for the interaction between elemental iodine and three enteroviruses, poliovirus 1, coxsackievirus A9 and echovirus 7 were determined. It was found in a plot between the logarithm of infectivity (percentage of plaque forming units) and exposure time to iodine that the relationship was not linear (205). It appeared that the reaction between I_2 and the virion either was inconsistent with the assumption that devitalization of virion resulted from interaction with a single molecule of I_2 or that the virions were clumped in such a manner that curves of the multihit type were produced. The data in this study indicated that devitalization of the virion by I_2 resulted from a

bi-molecular reaction that was kinetically of the first order but was modified by the multihit effect as a result of virus clumping. The authors of the study felt the effect of virus aggregation on devitalization curves seemed to be given little consideration and believed that the path of the devitalization curve was very dependent upon the degree of aggregation. For example, if 90 per cent of the virions were separate particles and 10 per cent formed in large clumps, the curve up to 90 per cent devitalization would have been of the first order type and then would have leveled off until the clumps were reduced to single surviving virions, at which time the curve would again slope downward (205). The breaking up of such viral clumps was demonstrated to be difficult (206).

The kinetics of inactivation of poliovirus has probably been studied in greater detail than the kinetics of the inactivation process for any other virus, since this was the basis for production of the Salk poliovirus vaccine in the mid 1950's. The Salk vaccine was developed by interaction of formaldehyde with poliovirus virus. This resulted in destruction of infectivity in accordance with the laws that govern a first order chemical reaction (207). This meant that if conditions were constant throughout the reaction period, the rate at which virus was destroyed was constant. In such instances there was establishment of a linear relationship between residual

virus and exposure time. Therefore, it was possible, using a plot of residual virus versus exposure time to formaldehyde to predict the time necessary to prepare a poliovirus vaccine free of infectious particles. This was done by extrapolation to the exposure time at which zero infectivity would theoretically be present. An additional definable period of exposure time could be added as a margin of safety in vaccine production (207).

The variables that influenced the time required for inactivation of virus were

1. concentration of free formaldehyde
2. temperature at which the reaction took place
3. pH of the reaction mixture
4. concentration of virus initially present.

The conditions selected for vaccine production were

1. 1-4000 formalin solution
2. temperature of 36° - $37^{o}C$
3. pH seven.

If these conditions were kept constant, the time required for destruction of virus varied depending on the concentration of virus in the starting material. If the protein content of the suspending fluid of the virus varied, then the slope of the reaction-time line changed, since this protein bound some formaldehyde.

Earlier work indicated a $37^{o}C$ temperature yielded a significantly better linear relationship between residual

virus and exposure time than did a reaction temperature of 4°C. A temperature of 4°C was used earlier in the unfounded belief that higher temperatures destroyed viral antigenicity (208).

When the Salk poliovirus vaccine was released for widespread use in April 1955, despite assurances of safety a number of vaccinated children developed poliomyelitis. Since most of these cases were associated with lots of vaccine produced by the Cutter Laboratories, this event has come to be known as the Cutter incident (209). Salk attributed the improperly inactivated virus to not following the criteria for vaccine production accurately. Salk felt that some of the laboratories where the vaccine was mass produced assumed the purpose of the filtration step in vaccine production was only the removal of bacteria and molds and not the removal of virus aggregates and particulate matter that could interfere with the formaldehyde reaction. Since the virus fluid had been found to be bacteriologically sterile, a bacteria retaining filter was not used to remove the very fine particles, but simply a relatively coarse clarifying filter. This clarified fluid was treated with formalin. Infective virus was found in this fluid at a time when none was expected. Inactivation was demonstrated to be predictable in all other batches of vaccine filtered through the finer Seitz filter prior to treatment.

Salk's first order inactivation was challenged by Timm *et al* (210), who expressed the opinion that the data showed that first order kinetics were not operative and inactivation curve information could not be used reliably for predicting inactivation time. They claimed that inactivation rate curves plotted in accordance with the first order rate laws showed a disproportionately great drop in virus titer. This appeared on the curve as a sharp deviation from the theoretical straight line. Timm failed to prove that such "sharp" deviations were statistically significant. He also failed to define what he meant by the theoretical straight line.

Gard reported that systematic studies of the effect of formaldehyde on poliovirus had shown the rate of inactivation to decrease in a regular fashion with the time of treatment (211). A formula describing the reaction was proposed:

$$\log Y_o - \log y = a \log (1+bt)$$

where y_o was the virus activity at time 0; y, virus activity at time t; and a and b, parameters. This formula had the following characteristics. When logarithmic survival ratios were plotted against time, a continuously curving line was obtained. However, when plotted against log time, the curve approached a linear asymptote, providing a possibility of extrapolation. The slope of the asymptote was given by the parameter a of the formula, its position

by parameter b. When logarithmic survival rates were plotted against log (1+bt), the relationship was linear and the fit of experimental data was good. Gard felt the formula described the change in resistance of the virus to the action of formaldehyde in the course of treatment, presumably due to a change in the permeability of the virus protein membrane. Gard also emphasized that the term infectivity was relative to the assay system used to detect it.

The possibility of the reversibility of inactivation of the virus by disinfectant was discussed by Gard. Bacteriophage treated with formaldehyde seemed to lose activity at an approximately constant rate, if the virus was assayed immediately at the time of sampling. If the reaction was interrupted by addition of a neutralizer such as bisulfite, serum, amino acids or other formaldehyde acceptor, and the samples from such a mixture were kept at 37°C at varying intervals before being assayed, considerable gradual reactivation was demonstrable (212). Similar results were reported for $HgCl_2$ acting on influenza virus. Reactivation in this case was achieved by treatment with H_2S (213).

In experiments where virus was treated with formaldehyde (211), control virus produced more than 50 per cent of the final plaque count on the third day after inoculation and reached 100 per cent in five days, while

virus exposed to formaldehyde for 24 hours did not produce maximum plaques for a further two days. Longer periods of exposure of the virus to formaldehyde emphasized this phenomenon. For example, after three days of treatment, the virus was exceedingly slow in producing lesions, the first plaques appearing only after six days incubation. The final plaque count was attained on the twelfth day. The limitation on the time cell cultures could be maintained in vitro hampered further exploration of the increased plaquing time for chemically damaged virus.

Once cell cultures were infected by the chemically damaged virus, cytopathogenic changes occurred at normal rates irrespective of the specific nature of the previous treatment of the virus. Only the initiation of this process was delayed. The significance of this phenomenon was that, if the final readings of the plates had been taken on the fifth day, residual virus would have been detected, but if the readings were taken after three days, residual virus would have not yet manifested itself. An inactivation curve based on such findings would have had a steeper slope as well as a straighter course than one obtained by means of a less sensitive assay technique (211).

The sensitivity of three assay systems used to detect infectivity of poliovirus after formaldehyde treatment was studied. The three virus assays were: the quantal assay, in which virally induced cytopathogenic effect (CPE) was observed in roller tubes; the metabolic inhibition test (MIT) and the plaque assay. The quantal

assay was slightly more sensitive than the MIT in detecting poliovirus. The plaque assay was as sensitive as the quantal assay with respect to detecting untreated virus, but was not as sensitive as either the MIT test or the quantal assay in detecting residual virus after formaldehyde treatment (214). The relative lack of sensitivity of the plaque assay compared to the quantal assay and the MIT was attributed to the formaldehyde damaged virus requiring a longer time for absorption (215).

Charney indicated that a fraction of the formaldehyde treated poliovirus suffered a relatively rapid loss of infectivity compared with a more stable surviving fraction. The extent to which this took place was stated to be dependent on the previous history of the poliovirus preparation. The loss of such unstable infectivity also occurred if poliovirus was stored for long periods of time. Charney did not give data to support this statement in his paper (214), which is highly suspect since poliovirus is one of the most stable animal viruses studied. In addition, he did not define what he meant by tissue culture fluids, the length of time such fluids were stored, or the temperature of storage. Charney concluded that, with regard to the specific conditions employed and with appropriate interpretation of the inactivation kinetics, the inactivation curve for poliovirus treated with formaldehyde lent itself to the type of extrapolation from which a prediction of safety could be made (214).

Evidence was presented that indicated different batches of cells used to assay for poliovirus varied widely in sensitivity to infection (216). In addition, there was no information on the relative sensitivity of children to infection by the formaldehyde-treated poliovirus as compared to tissue culture preparations (209). This fact was emphasized when it was found that, although virus not treated with formaldehyde was more easily detectable in tissue culture than in cortisone-treated monkeys, upon formaldehyde treatment the reverse was true (209).

CHAPTER II

MATERIALS AND METHODS

Cells

Cells were prepared and maintained using the methods of Cooper (232).

BGM

BGM refers to African green monkey cells. The BGM cells used were obtained from Dr. A.L. Barron, Medical College, State University of New York at Buffalo, N.Y. 14212 (231).

L

The mouse L cells used were obtained from the American Type Culture Collection. This cell line was originally derived from clone 929 in March 1948 by K.K. Sanford, W.R. Earle and G. D. Likely (229).

Vero

The Vero cells were obtained from the American Type Culture Collection. This cell line was orginally derived from the kidney of a normal, adult, African green monkey on March 27, 1962 by T. Yasumura and Y. Kawakita at the Chiba University in Chiba, Japan.

Bovine Kidney Cells (BKC)

The BKC used were obtained from American Type Culture Collection. This cell line was originally derived from a kidney of a normal, adult steer, February 18, 1957 by S.H. Madin and N.B. Darby (227).

Chick Embryo Fibroblasts (CEF)

Chick embryo fibroblasts were prepared from fertilized eggs obtained from the Department of Animal Science, University of Manitoba by the method indicated in Cooper (233).

LM Cells

The mouse LM cells used were obtained from the American Type Culture Collection. The LM strain was originally derived from clone 929 by R. J. Kuchler and D.J. Merchant during the course of studies on its nutritional requirements (228).

E. coli K37+

The E. coli K37+ used was obtained from Dr. N. Zinder, Rockefeller University. E. coli K37+ was passaged three times on tryptone agar (Appendix 5) before being suspended in nutrient broth (Appendix 7) and used. The bacteria on the agar slants was 24 hours old when suspended in nutrient broth. There were approximately 4×10^{10} bacteria per ml in the nutrient broth suspensions.

Vesicular Stomatitis Virus

Vesicular stomatitis virus (VSV) Indiana strain was obtained from the American Type Culture Collection, and used in the testing of all virucides. It was isolated in cattle in Indiana in 1925. Three different VSV preparations were produced by passing virus through either Bovine Kidney cells (VSV/BKC) LM cells (VSV/LM) or chicken Embryo Fibroblasts (VSV/CEF) at least three times.

In each case, a one to five PFU input multiplicity was used to infect Blake bottles of cells. The virus was absorbed to cells for 30 minutes at 37°C before addition of R3 medium (Appendix 1). The virus was harvested 48 to 72 hours later when the cells exhibited extensive cytopathic effect. The tissue culture fluid was centrifuged at

1000 g for ten minutes to pellet out gross cellular debris. The virus was stored in 0.5 ml quantities at -85°C. The virus was thawed just before use.

<u>Reovirus 3</u>

Reovirus 3 was obtained from Dr. J. Levy of the Viral Carcinogenesis Branch, National Cancer Institute, Bethesda, Maryland, 20014.

The reovirus pool was prepared using the following method (217) in L cells:

L cells were grown on monolayers in Blake bottles in R3 medium. Virus was produced at 37°C in cells infected at a multiplicity of five to ten PFU/cell under a maintenance medium of 199 (Appendix 10) containing two per cent fetal calf serum. Reovirus was harvested 24 hours post infection. The medium was decanted from the infected monolayer and it was washed twice with PBS (Appendix 8). The washed cells were removed from the glass surface, using glass beads in a small volume of PBS. This cell suspension was pelleted at 250 g for ten minutes. The cells were resuspended in five ml of PBS and mixed with an equal volume of Freon 113. This mixture was homogenized in a Sovall Omnimixer for one minute at half speed. The two liquid phases were separated at low speed centrifugation (800 g) for ten minutes. The aqueous phase was made to 20 ml with PBS and stored in 0.5 ml quantities at -85°C. The virus was thawed just before use.

f2 Bacteriophage

The original f2 stock from which pools of f2 were grown was obtained from Dr. Norton Zinder, Rockefeller University, 66th Street and York Avenue, New York. f2 stocks were produced by the agar layer method (218) originally designated for the production of the T series phages. In this method, 0.05 ml of the host bacteria *E. coli* K37$^+$ and 0.5 ml of f2 virus dilution were added to 2.5 ml of melted 0.75 per cent tryptone agar. The melted agar was at 44°C - 46°C. This volume was quickly poured over a much thicker basal layer of 1.5 per cent tryptone agar in a petri dish and incubated at 37°C for 24 hours.

It was essential that the virus dilution used was such that enough virus was present to cause a semiconfluent layer of lysis of the host bacteria.

Twenty-four hours later, five ml of nutrient broth was poured on each plate exhibiting semiconfluent lysis and incubated at room temperature for four hours.

Such broth was subsequently removed with a Pasteur pipette, exposed to five per cent chloroform for ten minutes, periodically shaking vigorously to destroy bacteria and centrifuged at 1000 g for ten minutes. The supernatant was harvested.

The virus preparation was stored at 4°C.

Herpes Simplex Virus 2

The herpes simplex virus 2 from which stocks were prepared was the MS strain from American Type Culture Collection, with the original source being from the brain of a human with multiple sclerosis. An input multiplicity of one to five PFU herpes simplex virus 2 per cell was absorbed to BGM cells in 75 cm^2 plastic Falcon flasks for one hour at 37°C before being overlayed by 20 ml of 199 medium containing four per cent fetal calf serum (219). After 20 to 24 hours of incubation at 34°C, the infected cells were removed from the plastic and suspended in 199 medium. This suspension was centrifuged at 1000 g for ten minutes. The pelleted cells were suspended in five ml of sterile distilled water and lysed by 20 strokes with a tight-fitting Dounce homogenizer. This suspension was again centrifuged at 1000 g for ten minutes. The supernatant containing the herpes simplex 2 virus was drawn off, diluted with an equal volume of 199 medium and stored at -85°C in 0.5 ml quantities. Virus was thawed just before use.

Coxsackievirus B5

The original coxsackievirus B5 preparation was isolated by Dr. Sattar and Dr. Westwood at the University of Ottawa, Department of Microbiology, from raw sludge. It was designated as 24-R5. It was passaged in BGM cells three times before use. A pool of coxsackievirus B5 was prepared by absorbing an input multiplicity of one to five PFU per cell to BGM cells in plastic 25 cm^2 Falcon flasks for 30 minutes at 37°C. The infected cells were overlayed by

five ml of R3. Upon exhibition of extensive cytopathic effect by the BGM cells 24 to 48 hours later, the tissue culture fluids were removed and centrifuged at 1000 g for ten minutes. The supernatant was removed and stored in 0.5 ml quantities at -85°C. Virus was thawed just before use.

Echovirus 11

The original echovirus 11 preparation was a clinical isolation from the Provincial Laboratory of Manitoba. It was designated as 2491-75. It was passaged in BGM cells three times before use. A pool of echovirus 11 was prepared and stored, using the same method described above for coxsackievirus B5.

A2 Plaque Virus

The source of the A2 plaque virus used was Dr. A. C. Fassolitis of the United States Public Health Service, F.D.A., Cincinnati, Ohio. A2 plaque virus was passaged in Vero cells three times before use. The pool of A2 plaque virus used in my study was prepared by absorbing A2 plaque virus in 25 cm^2 plastic Falcon flasks containing Vero cells at 37°C for 30 minutes. Such Falcon flasks were then overlayed with five ml of R3 which contained eight per cent fetal calf serum. Upon exhibition of extensive cytopathic effect by the Vero cells 48 to 72 hours later, the tissue culture fluids were centrifuged at 1000 g for ten minutes. The supernatant which contained virus was removed and stored in 0.5 ml quantities at -85°C. Virus was thawed just before use.

Organic Material

Fetal calf serum purchased from the Flow Laboratories, 936 W. Hyde Park Blvd., Inglewood, California 90302 was used as organic load in virucide testing. The same batch of fetal calf serum in inactivated form (57°C for 30 minutes) was used in virucide testing for all test viruses considered, with the exception of vesicular stomatitis virus, for which an earlier batch was used.

Disinfectants

All disinfectants tested for usefulness as virucides were diluted in sterile distilled water and prepared on the same day testing was done.

Table 1

Disinfectants Tested

Commercial Name	Active Ingredients	Manufacturer
Javex	6% sodium hypochlorite	Bristol-Myers Canada Ltd.
Ethanol	95% ethanol	Commercial Alcohols
One Stroke Ves-Phene	O-phenylphenol 10% O-benzyl-p-chlorophenol 8.5% p-tertiary amylphenol 2.0%	Vestal Labs Division of W.R. Grace Co.
Wescodyne	Polyethoxy polypropoxy ethanol-iodine complex 9.10% Nonylphenoxypoly (ethyleneoxy) ethanol-iodine complex 8.74% (Provides 1.6% minimum titratable iodine)	West Chemical Products
Sodium Hydroxide	50% w/v NaOH	Fisher Scientific Ltd.
Sonacide	2% Glutaraldehyde	Ayerst Ltd.
Methanol	100% methanol	Commercial Alcohols

In all cases, solutions of methanol and ethanol were prepared as volume to volume (v/v) solutions.

Temperature

Experiments were done in a vertical flow biological safety cabinet where the mean temperature was 26°C. The temperature did not fluctuate more than 2°C.

Test Methods

Plaque Assay for Animal Viruses

Volumes of 0.5 ml of serial tenfold dilutions (Table 3) of the preparation on which an assay was desired were adsorbed to monolayer cultures of appropriate lines of cells (Table 2) in 52 x 15 mm tissue culture dishes for 30 minutes at 37°C. Such plates were overlayed by three to five ml of a mixture of equal volumes of 1.8% bacto-agar (Appendix 9) and the MEM overlay medium (Appendix 2). These plates were incubated in four per cent CO_2 in a CO_2 cabinet at 37°C for varying periods of time (Table 2), depending on the replicative cycle of the virus. When the virus produced isolated areas of cell destruction (termed plaques) under the agar-overlay mixture a neutral red dye preparation in the form of agar (Appendix 11) was added to each plate in five ml amounts. The plates were incubated for a 24 hour period at room temperature to give the dye a chance to diffuse throughout the plate and emphasize the plaques which, unlike areas that contained living cells, did not stain red. The plaques were counted and expressed in terms of plaque forming units per ml (PFU/ml).

Plaque Assay for f2 Bacteriophage

Volumes of 0.5 ml on which an assay was desired were added to a mixture of 2.5 ml of melted 0.75 per cent tryptone agar and 0.05 ml *E. coli* K37^{+} . These volumes were poured quickly over tryptone agar-containing petri dishes and allowed to harden. Such plates were incubated 24 hours and areas of bacterial destruction on the plates were readily observed, counted and expressed in terms of PFU/ml.

Testing the Virucidal Ability of a Disinfectant in the Presence of Organic Material on the Viruses Other than VSV Considered in the Study

A volume of 0.4 ml of disinfectant dilution to be tested was added to a mixture consisting of 0.05 ml of fetal calf serum and 0.05 ml of virus. Such 0.5 ml quantities were incubated for ten seconds, 20 seconds and 30 seconds. In each case, at the end of the specified incubation time an appropriate amount of 17.5 per cent skim milk (Appendix 6) was added to act as a neutralizer of the action of the disinfectant. Organic material was defined as ten per cent fetal calf serum, since 0.05 ml of fetal calf serum was present in the 0.5 ml virus-disinfectant mixture. In each experiment the effectiveness of the 17.5 per cent skim milk neutralizer was tested by the following control; 0.05 ml of fetal calf serum and 0.4 ml of disinfectant dilution tested were added to the same amount of 17.5 per cent skim milk as that used in the neutralization of ten, 20 and 30 second

reaction samples. A volume of 0.05 ml of virus was then added. The virus stayed in this skim milk preparation for a period of time equivalent to that which the ten, 20 and 30 second samples stayed in skim milk before being assayed. In both the case of the neutralized ten, 20 and 30 second samples and the neutralized control, an 0.5 ml amount was serially diluted tenfold in 4.5 ml dilution blanks with 0.5 ml amounts of the appropriate dilutions assayed for infectivity. The plaque assay method in 52 x 15 mm tissue culture dishes containing appropriate cell lines was used to assay for virus. These cell lines are listed for each virus in Table 2.

The virus used in each experiment was titrated at the time of the experiment. The effectiveness of the 17.5 per cent skim milk neutralizer was determined by comparing this titer with the neutralized control results.

Another control, termed the cell susceptibility control was needed to determine whether toxicity of the disinfectant affected the ability of the cell line used in the plaque assay to support virus growth. Virus was diluted in serial tenfold dilutions to the dilution preceding the one calculated to yield between 30 and 300 PFU per 0.5 ml. Then 0.5 ml of this preceding dilution was added to 4.5 ml of a mixture of disinfectant - skim milk identical in proportion to that found when the virus-disinfectant mixture was neutralized by a certain volume of skim milk in actual

disinfectant testing. Such a mixture was assayed using the plaque assay. The cell susceptibility test results were compared to the titer using a t test at the 95% confidence level. Results on page 135 and 137 for neutralized controls and cell susceptibility controls indicated skim milk is an effective neutralizer for a wide variety of disinfectants in testing on a diverse group of viruses.

Table 2

Cell Line Used in Plaque Assay for the Virus

Virus	Cell Line	Plate Incubation Time in Plaque Assay
coxsackievirus B5	BGM	24 hours
echovirus 11	BGM	48 hours
reovirus 3	L	7 days
herpes simplex virus 2	BGM	6 days
f2 bacteriophage	*E. coli* K37^{+}	24 hours

Table 3

Diluent Used in the Plaque Assay for Detection of Different Viruses

Virus	Diluent
coxsackievirus B5	PBS-A (234)
echovirus 11	PBS-A (234)
reovirus 3	PBS-A (234)
A2 plaque virus	17.5 per cent skim milk
herpes simplex virus 2	199 Medium (219)
VSV	R3

Testing of the Virucidal Ability of a Disinfectant on Vesicular Stomatitis Virus in the Presence of Organic Material

Four ml of disinfectant dilution to be tested was added to a mixture consisting of 0.5 ml of fetal calf serum and 0.5 ml of virus. Samples of a 0.5 ml volume were removed from this five ml reaction mixture at ten, 20, and 30 seconds after virucide addition, with the reaction suspension being mixed between each sampling. These 0.5 ml samples were added to 4.5 ml of ten per cent skim milk immediately after they were taken. The skim milk acted as a neutralizer of the action of the disinfectant. Organic material was defined as ten per cent fetal calf serum since there was 0.5 ml fetal calf serum in the five ml virus-virucide mixture. In each experiment, the effectiveness of ten per cent skim milk as a neutralizer was tested by the following control: 0.5 ml of fetal calf serum and 0.5 ml of the dilution of disinfectant tested were added to four ml of ten per cent skim milk. This five ml volume was mixed and 4.5 ml of it was removed to another tube. A volume of 0.5 ml of virus was added to this 4.5 ml volume. The virus in this five ml volume, termed the neutralized control, was incubated in this tube for a time period equivalent to that the ten, 20 and 30 second samples were incubated in the skim milk neutralizer before being assayed.

In both the case of the neutralized ten, 20 and 30 second samples and the neutralized control, an 0.5 ml amount was serially diluted in 4.5 ml R3 dilution blanks and 0.5 ml amounts of the appropriate dilutions were assayed for residual virus, using the plaque assay. The VSV/LM was assayed on LM cell monolayers, the VSV/BKC on bovine kidney cells and the VSV/CEF on chick embryo fibroblasts. The VSV used in each experiment was titrated at the time of the experiment. The effectiveness of the ten per cent skim milk neutralizer was determined by comparing this titer with the neutralized control results.

CHAPTER III

PROCEDURES AND EXPERIMENTAL RESULTS

The Relationship between Residual Virus and Exposure Time to the Virucide

In virucide testing, although the relationship of $\log_{10}$ residual virus (PFU/ml) versus exposure time yields a linear function in many cases, a significant number of instances occur when this is not so. In order to obtain a linear relationship, previous workers in our laboratory transformed experimental data in a number of ways. This was desired so that data could be converted into a form where statistical evaluation would be convenient and also to determine the validity of the relationships for predicting the exposure time necessary to inactivate a given titre of virus by extrapolation. The data conformed best to a linear regression relationship where $\log_{10}$ PFU/ml was plotted against square root minutes exposure time. Examples are presented in Figures 3 and 7. Each plotted point is the mean of four to eight replicate observations. The lines are the statistically fitted expected regression relationships. In these figures, 95 per cent confidence levels for the mean dependent variable are used. An example of the $\log_{10}$ PFU/ml versus square root minutes relationship as the best linear fit is seen in the case of VSV treated with 1-100 Javex (Figure 3). An example of the experimental protocol can be seen in Appendix 19.

Table 4 shows the fit of experimental data to a linear relationship when it was transformed in various ways. In each case, the correlation coefficient squared (r^2) which is the proportion of the variation of the dependent variable explained by the regression equation and the F value which is the ratio of

$\frac{\text{mean square regression}}{\text{mean square deviation}}$ is listed with df designating the degrees of freedom for the F value. The data conformed best to a linear regression relationship where $\log_{10}$ PFU/ml was plotted against square root minutes, since F values and r^2 values were larger for this particular relationship than the others listed in Table 4.

Further examples of this will be found in table 5 and the remainder of this data can be consulted in Appendix 12, tables 25 to 31. These tables compare the results for $\log_{10}$ PFU/ml vs time and $\log_{10}$ PFU/ml vs square root time.

With the exception of 70 per cent ethanol (Figure 4) the consistently larger F values and r^2 values when $\log_{10}$ PFU/ml versus square root minutes exposure time was plotted indicated the better fit to a linear function. Such listings were not made for those cases where no significant inactivation took place (Figure 6) and where there were less than three points on the plot (Figure 5). When either of these conditions were the case, "N.D." signifying not done was listed. The reproducibility of the linear $\log_{10}$ PFU/ml versus square root minutes relationship for VSV exposed to 1-100 Javex was determined by comparing slopes of the different experimental trials (Table 6). The statistical comparison was at the 95 per cent confidence level. The nonsignificant F value in Table 6 indicates

that the slopes of the different experimental trials when VSV was exposed to 1-100 Javex are not significantly different. The reproducibility of the $\log_{10}$ PFU/ml versus square root minutes exposure relationship for each virus-disinfectant combination tested in this study are listed in Table 6 and Tables 32 to 38 (Appendix 13).

In all cases, with the exception of echovirus 11 exposed to 1-100 Javex, the regression relationship is reproducible at the 95 per cent confidence level. The F value for echovirus 11 treated with 1-100 Javex is significant at the 95 per cent confidence level (Table 32) but not at the 99 per cent confidence level.

Table 4

Determination of the Fit to Linearity of Different Residual Virus versus Exposure Time Relationships for Vesicular Stomatitis Virus Treated with 1-100 Javex[a]

Relationship	Trial	r^2 [b]	df [c]	F
$\log_{10}$ PFU/ml vs Minutes	1	0.441	1,26	20.481
	2	0.490	1,23	22.072
$\log_{10}$ PFU/ml vs Square root minutes	1	0.932	1,26	354.005
	2	0.952	1,23	455.491
$\log_{10}$ PFU/ml vs $\log_{10}$ Minutes	1	0.776	1,22	76.259
	2	0.905	1,19	180.206
$\log_{10}$ PFU/ml vs $\log_e$ Minutes	1	0.776	1,22	76.268
	2	0.905	1,19	180.235
$\log_{10}$ PFU/ml vs 1/ Minutes	1	0.664	1,22	43.506
	2	0.823	1,19	88.477
$\log_{10}$ PFU/ml vs Square minutes	1	0.503	1,26	26.343
	2	0.532	1,23	26.156

a - as determined by analysis of regression

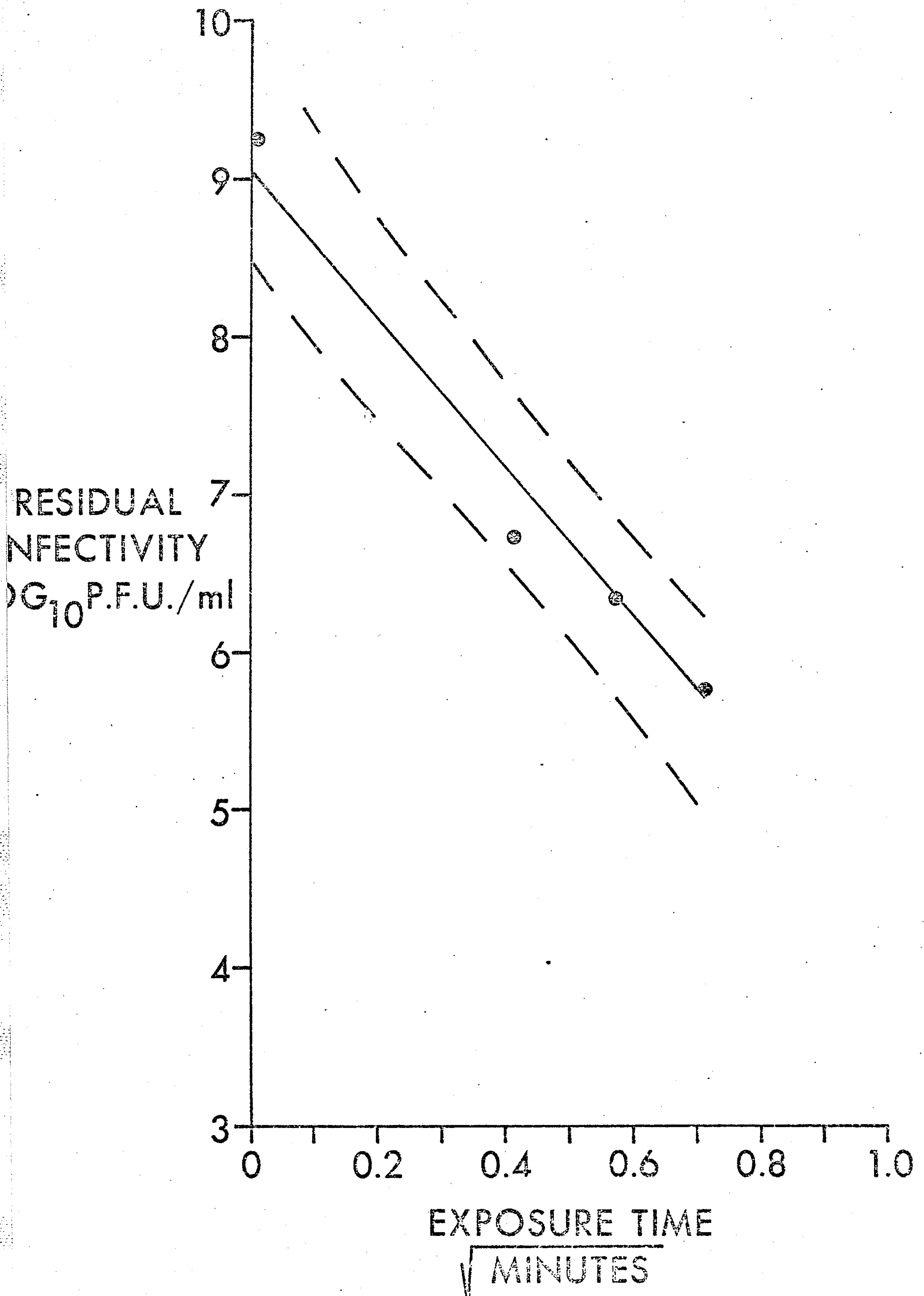
b - correlation coefficient squared

c - degrees of freedom for the F value

FIGURE 3

LM cell grown vesicular stomatitis virus in fetal calf serum treated with 1-100 Javex

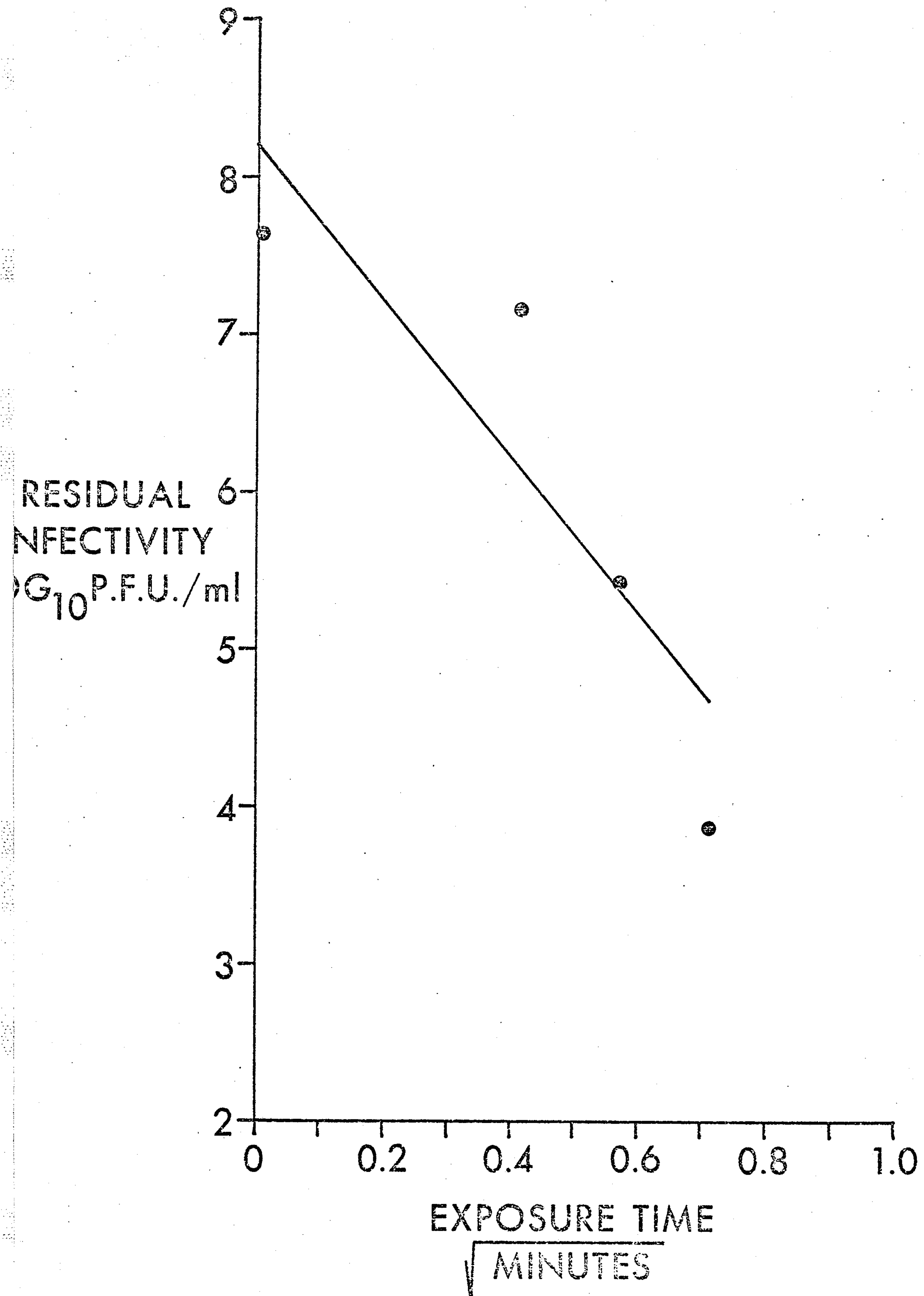
$\log_{10}$ PFU/ml versus square root minutes exposure time



RESIDUAL
NFECTIVITY
OG10P.F.U./ml
10
9
8
7
6
5
4
3
0
0.2
0.4
0.6
0.8
1.0
EXPOSURE TIME
√ MINUTES

FIGURE 4.

Trial 1; coxsackievirus B5 in fetal calf serum treated with 70% Ethanol log_{10} PFU/ml versus square root minutes exposure time.



RESIDUAL
NFECTIVITY
G10P.F.U./ml
9
8
7
6
5
4
3
2
0
0.2
0.4
0.6
0.8
1.0
EXPOSURE TIME
√MINUTES

FIGURE 5.

Trial 1; Bovine kidney cell grown vesicular stomatitis virus in fetal calf serum treated with undiluted Sonacide $\log_{10}$ PFU/ml versus square root minutes exposure time.

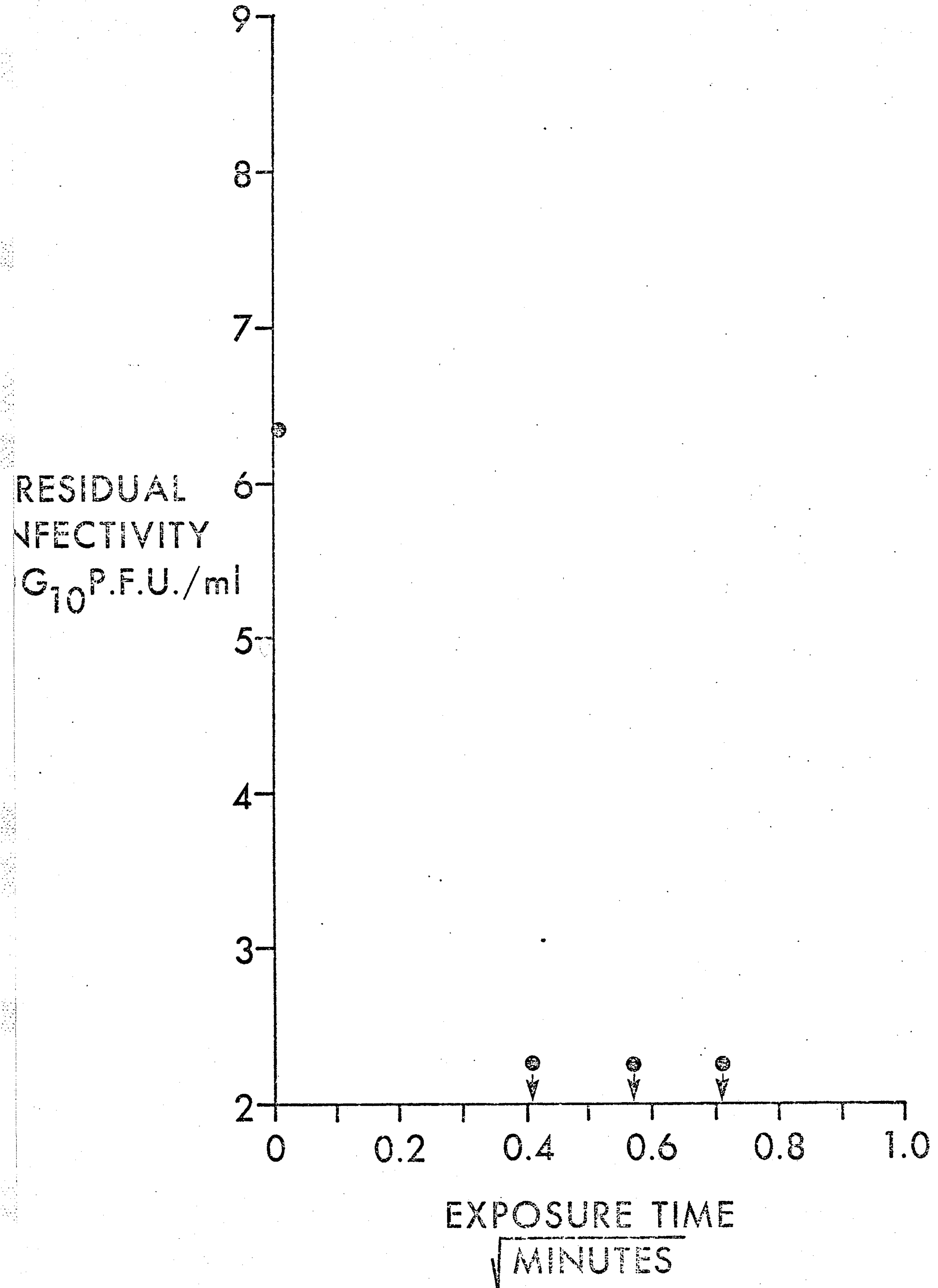
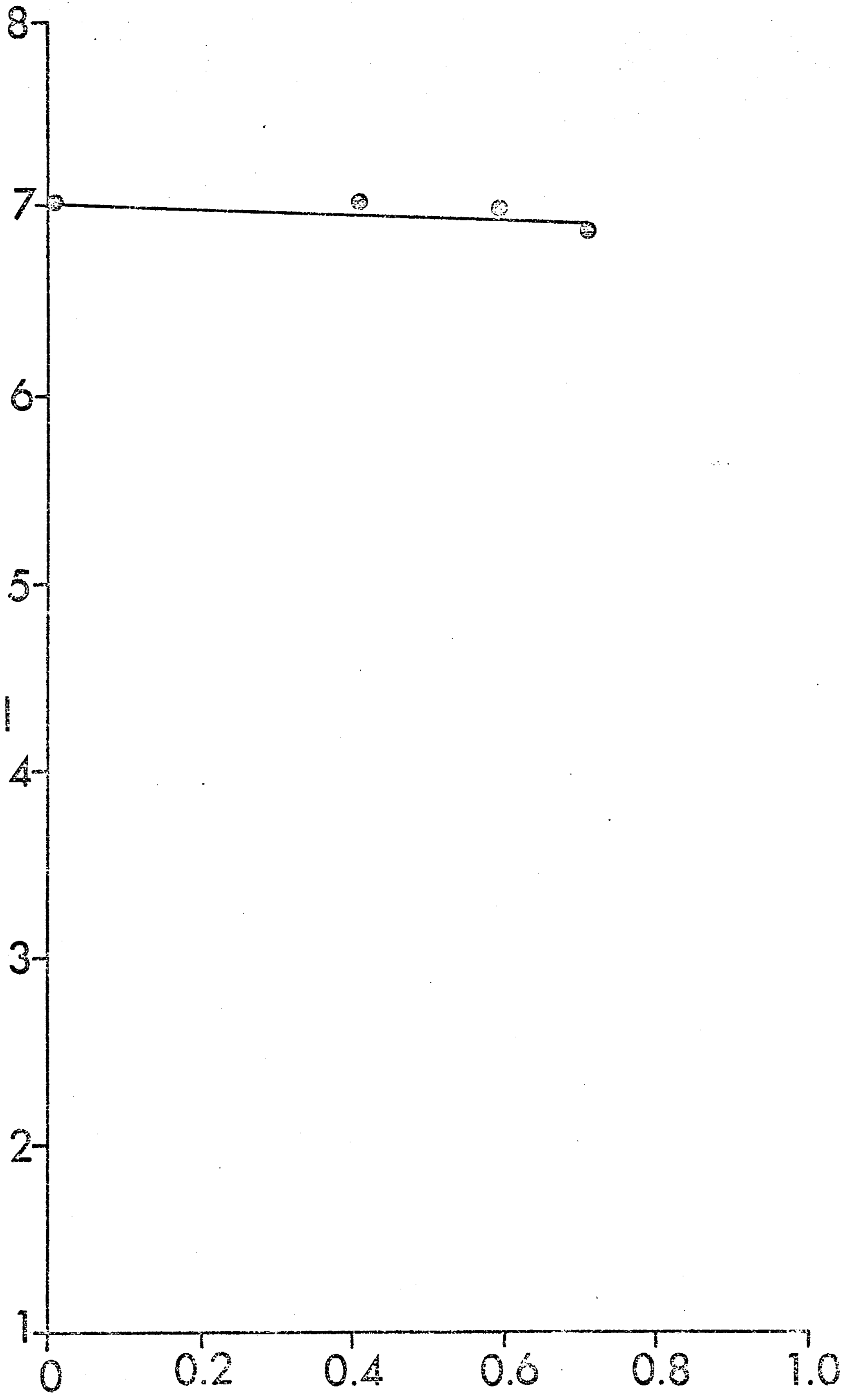

RESIDUAL
NFECTIVITY
G10P.F.U./ml
9
8
7
6
5
4
3
2
0
0.2
0.4
0.6
0.8
1.0
EXPOSURE TIME
√MINUTES

FIGURE 6.

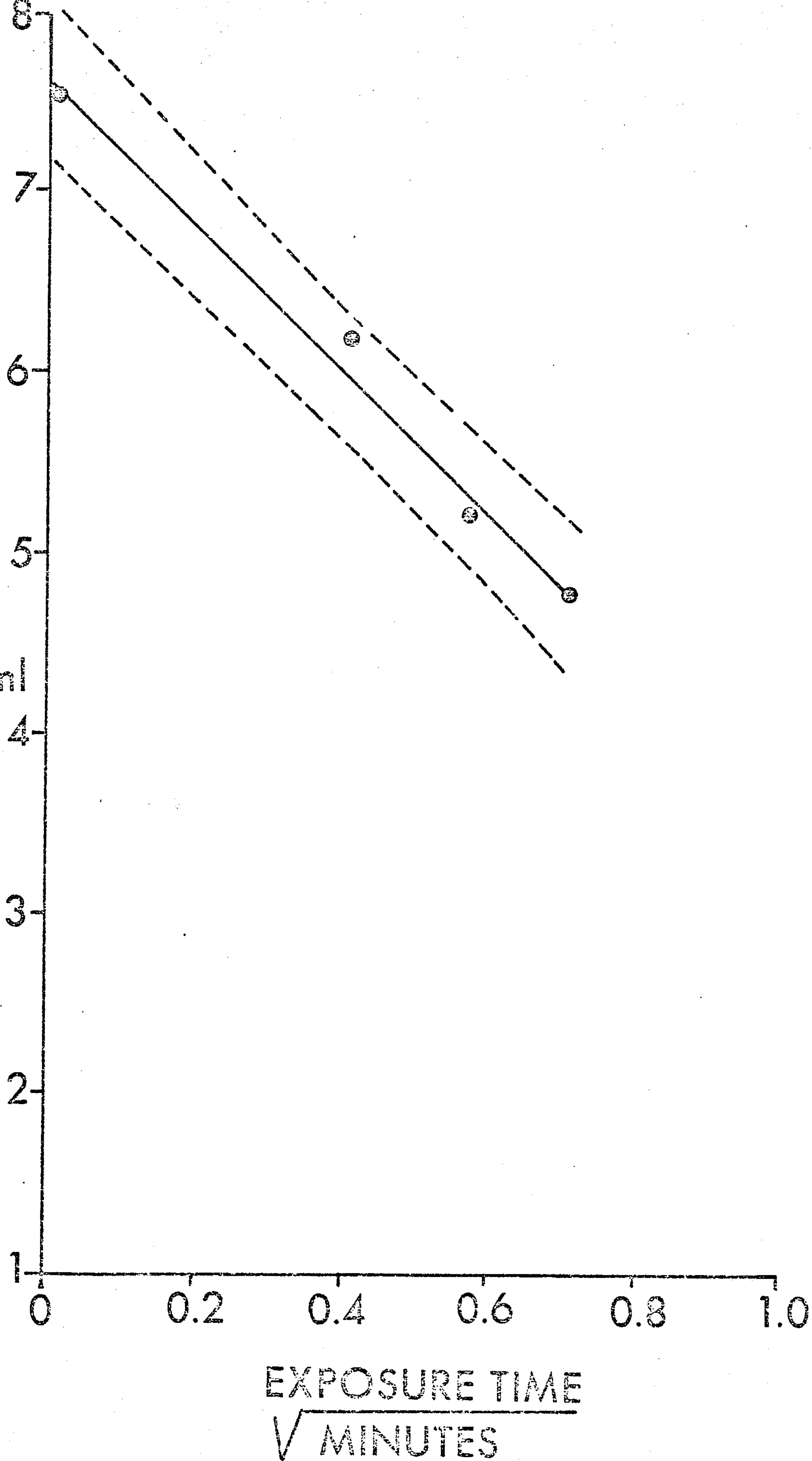
Trial 1; echovirus 11 in fetal calf serum exposed to 1-50 Ves-Phene $\log_{10}$ PFU/ml versus square root minutes exposure time.



8
7
6
5
4
3
2
1
RESIDUAL
NFECTIVITY
OG10 P.F.U./ml
0
0.2
0.4
0.6
0.8
1.0
EXPOSURE TIME
√MINUTES

FIGURE 7.

Trial 2; reovirus 3 in fetal calf serum treated with undiluted Sonacide $\log_{10}$ PFU/ml versus square root minutes exposure time.


RESIDUAL
INFECTIVITY
LOG10 P.F.U./ml
8
7
6
5
4
3
2
1
0
0.2
0.4
0.6
0.8
1.0
EXPOSURE TIME
√ MINUTES

The mean slope values calculated from data of the different experimental trials in Table 6 and Tables 32 to 38 (Appendix 13) are listed in Tables 7, 21 and Tables 39 to 44 (Appendix 14). Such mean slopes were involved in two types of statistical comparison at the 95 per cent confidence level.

1) the relative resistance of a virus to inactivation by different disinfectants (Tables 8 to 13)
2) the relative effectiveness of a disinfectant in inactivating different viruses (Tables 14 to 20).

An example of a statistical comparison of mean slopes is when the slope (Table 21) of the inactivation of echovirus 11 treated with undiluted Sonacide is compared to the slope (Table 21) of echovirus 11 treated with 1-50 Javex. The F value is 127.108 (df 1,20),is significant at the 95 per cent confidence level and the slopes are significantly different. The conclusion that can be drawn from such a comparison is that since the echovirus 11-1-50 Javex inactivation has a greater slope than that of echovirus 11-undiluted Sonacide inactivation, 1-50 Javex is more effective than undiluted Sonacide in inactivating echovirus 11 (Table 8). Although in comparisons involving instances where no significant amount of inactivation occurred (Figure 6) and where the inactivation was so rapid that less than three points were plotted on the graph (Figure 5), nonstatistical comparisons had to be made.

For example, undiluted Sonacide inactivated four logs of VSV/BKC in ten seconds (Figure 5) and undiluted Sonacide destroyed 1.5 logs of reovirus 3 in ten seconds (Figure 7), so undiluted Sonacide was considered more effective in inactivating VSV/BKC than it was in inactivating reovirus 3 (Table 17).

General trends seen in Tables 8 to 13 where the relative resistance of a virus to different disinfectants is listed indicate that 95 per cent ethanol,0.25 per cent (w/v) sodium hydroxide and Javex at dilutions less than 1-75 were wide spectrum disinfectants, since they were effective in inactivating a wide variety of viruses whereas undiluted Sonacide, 1-200 Wescodyne and 1-50 Ves-Phene were comparably effective only in the case of enveloped viruses such as VSV and herpes simplex virus 2. Tables 14 to 20 indicate the relative effectiveness of a disinfectant on different viruses. The nonenveloped viruses(f2 bacteriophage, coxsackievirus, echovirus and and reovirus) were more resistant to inactivation by disinfectants than the enveloped viruses (herpes simplex virus 2 and VSV). These tables also indicate that the host cell in which VSV was grown affected resistance to inactivation by disinfectant.

Table 5

Determination of the Fit of Different LM Cell Grown Vesicular Stomatitis Virus Infectivity Versus Exposure Time Relationships To a Linear Function [a]

DISINFECT-ANT	TRIAL	LM CELL GROWN VESICULAR STOMATITIS VIRUS: LOG 10 PFU/ml vs MINUTES			LOG 10 PFU/ml vs $\sqrt{\text{MINUTES}}$		
		r^2 [b]	df [c]	F	r^2 [b]	df [c]	F
1-100 JAVEX	1	0.441	1,26	20.481	0.932	1,26	354.005
	2	0.490	1,23	22.072	0.952	1,23	455.491
0.25% NaOH	1	0.502	1,17	17.111	0.954	1,17	349.068
	2	0.524	1,22	24.170	0.906	1,22	211.787
1-350 WESCODYNE	1	0.534	1,21	24.019	0.840	1,21	110.363
	2	0.499	1,23	22.914	0.826	1,23	109.174
1-350 VES-PHENE	1	0.465	1,25	21.689	0.873	1,25	171.775
	2	0.599	1,14	20.899	0.943	1,14	230.730

a - as determined by analysis of regression
b - correlation coefficient squared
c - degrees of freedom for the F value

Table 6

Reproducibility of the $\log_{10}$ PFU/ml versus Square Root Minutes Relationship for LM cell Grown Vesicular Stomatitis Virus Inactivation[a]

LM CELL GROWN VESICULAR STOMATITIS VIRUS

DISIN-FECTANT	TRI-AL	N[e]	INDEPENDENT VARIABLE[b]		DEPENDENT VARIABLE[c]		SLOPE	df	F
			MEAN	S.D.[d]	MEAN	S.D.[d]			
1-100 JAVEX	1	28	0.484	0.231	2.25	1.18	-4.939	2,71	0.127
	2	25	0.480	0.243	2.45	1.21	-4.867		
0.25% NaOH	1	19	0.436	0.264	1.80	1.11	-4.100	1,39	1.258
	2	24	0.477	0.244	1.78	0.952	-3.710		
1-350 WESCO-DYNE	1	23	0.500	0.270	2.61	0.590	-1.990	1,44	0.079
	2	25	0.470	0.240	2.58	0.550	-2.070		
1-350 VES-PHENE	1	27	0.476	0.231	2.22	0.600	-2.43	1,39	3.065
	2	16	0.423	0.274	2.59	0.830	-2.92		

a - as determined by comparison of slopes at the 95 per cent confidence level
b - square root minutes exposure time
c - $\log_{10}$ PFU/ml
d - standard deviation
e - total number of experimental observations

Table 7

Mean Slope Data For LM Cell Grown Vesicular Stomatitis Virus Inactivation Regressions

	LM CELL GROWN VESICULAR STOMATITIS VIRUS					
DISINFECTANT	N[c]	MEAN INDEPENDENT VARIABLE[a]		MEAN DEPENDENT VARIABLE[b]		MEAN SLOPE
		MEAN	S.D.	MEAN	S.D.	
1-100 JAVEX	27	0.482	0.237	2.35	1.20	-4.903
0.25% (w/v) NaOH	22	0.457	0.254	1.79	1.03	-3.905
1-350 WESCODYNE	24	0.485	0.255	2.60	0.570	-2.030
1-350 VES-PHENE	22	0.450	0.253	2.41	0.715	-2.675

a - square root minutes exposure time
b - log_{10} PFU/ml
c - total number of experimental observations

Table 8

Relative Resistance of echovirus 11 to the Six Disinfectants

	1-50 JAVEX	UNDILUTED SONACIDE	95% ETHANOL	0.25% NaOH	1-50 WESCODYNE	1-50 VES-PHENE	1-100 JAVEX	1-75 JAVEX	1-350 WESCODYNE	1-200 WESCODYNE
UNDILUTED SONACIDE IS	LESS EFFECTIVE THAN									
95% ETHANOL IS	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN								
0.25% NaOH IS	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	NOT DIFFERENT FROM							
1-50 WESCODYNE IS	LESS EFFECTIVE THAN	MORE EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN						
1-50 VES-PHENE IS	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN					
1-100 JAVEX IS	LESS EFFECTIVE THAN	MORE EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	NOT DIFFERENT FROM	MORE EFFECTIVE THAN				
1-75 JAVEX IS	LESS EFFECTIVE THAN	MORE EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN			
1-350 WESCODYNE IS	LESS EFFECTIVE THAN	NOT DIFFERENT FROM	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	MORE EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN		
1-200 WESCODYNE IS	LESS EFFECTIVE THAN	MORE EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	NOT DIFFERENT FROM	MORE EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	MORE EFFECTIVE THAN	
70% ETHANOL IS	LESS EFFECTIVE THAN	NOT DIFFERENT FROM	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	MORE EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	NOT DIFFERENT FROM	LESS EFFECTIVE THAN

Table 9

Relative Resistance of coxsackievirus B5 to the Six Disinfectants

	1-75 JAVEX	1-100 JAVEX	0.25% NaOH	UNDILUTED SONACIDE	1-350 WESCODYNE	1-200 WESCODYNE	1-50 VES-PHENE	95% ETHANOL
1-100 JAVEX IS	LESS EFFECTIVE THAN							
0.25% NaOH IS	NOT DIFFERENT FROM	MORE EFFECTIVE THAN						
UNDILUTED SONACIDE IS	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN					
1-350 WESCODYNE IS	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	MORE EFFECTIVE THAN				
1-200 WESCODYNE IS	NOT DIFFERENT FROM	MORE EFFECTIVE THAN	NOT DIFFERENT FROM	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN			
1-50 VES-PHENE IS	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN		
95% ETHANOL IS	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	
70% ETHANOL IS	LESS EFFECTIVE THAN	NOT DIFFERENT FROM	LESS EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	LESS EFFECTIVE THAN	MORE EFFECTIVE THAN	LESS EFFECTIVE THAN

Table 10

Relative Resistance of f2 Bacteriophage to the Six Disinfectants

		1-50 JAVEX	1-100 JAVEX	0.25% NaOH	UNDILUTED SONACIDE	1-20 WESCODYNE	1-20 VES-PHENE
1-100 JAVEX	IS	LESS EFFECTIVE THAN					
0.25% NaOH	IS	NOT DIFFERENT FROM	MORE EFFECTIVE THAN				
UNDILUTED SONACIDE	IS	LESS EFFECTIVE THAN	NOT DIFFERENT FROM	LESS EFFECTIVE THAN			
1-20 WESCODYNE	IS	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	NOT DIFFERENT FROM		
1-20 VES PHENE	IS	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	
95% ETHANOL	IS	LESS EFFECTIVE THAN	MORE EFFECTIVE THAN	LESS EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN

Table 11

Relative Resistance of reovirus 3 to the Six Disinfectants

	1-75 JAVEX	1-100 JAVEX	0.25% NaOH	UNDILUTED SONACIDE	1-350 WESCODYNE	1-200 WESCODYNE	1-50 VES-PHENE	95% ETHANOL
1-100 JAVEX IS	LESS EFFECTIVE THAN							
0.25% NaOH IS	LESS EFFECTIVE THAN	MORE EFFECTIVE THAN						
UNDILUTED SONACIDE IS	LESS EFFECTIVE THAN	MORE EFFECTIVE THAN	NOT DIFFERENT FROM.					
1-350 WESCODYNE IS	LESS EFFECTIVE THAN	NOT DIFFERENT FROM	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN				
1-200 WESCODYNE IS	LESS EFFECTIVE THAN	MORE EFFECTIVE THAN	NOT DIFFERENT FROM	LESS EFFECTIVE THAN	MORE EFFECTIVE THAN			
1-50 VES-PHENE IS	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN		
95% ETHANOL IS	NOT DIFFERENT FROM	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	
70% ETHANOL IS	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN

Table 12

Relative Resistance of herpes simplex virus 2 to the Six Disinfectants

		1-100 JAVEX	1-200 JAVEX	0.25% NaOH	UNDILUTED SONACIDE	1-350 WESCODYNE	1-350 VES-PHENE	95% ETHANOL
1-200 JAVEX	IS	LESS EFFECTIVE THAN						
0.25% NaOH	IS	NOT DIFFERENT FROM	MORE EFFECTIVE THAN					
UNDILUTED SONACIDE	IS	NOT DIFFERENT FROM	MORE EFFECTIVE THAN	NOT DIFFERENT FROM				
1-350 WESCODYNE	IS	LESS EFFECTIVE THAN	NOT DIFFERENT FROM	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN			
1-350 VES-PHENE	IS	LESS EFFECTIVE THAN	MORE EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	MORE EFFECTIVE THAN		
95% ETHANOL	IS	NOT DIFFERENT FROM	MORE EFFECTIVE THAN	NOT DIFFERENT FROM	NOT DIFFERENT FROM	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	
70% ETHANOL	IS	NOT DIFFERENT FROM	MORE EFFECTIVE THAN	NOT DIFFERENT FROM	NOT DIFFERENT FROM	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	NOT DIFFERENT FROM

Table 13

Relative Resistance of Bovine Kidney Cell Grown vesicular stomatitis virus to the Six Disinfectants

		70% ETHANOL	1-100 JAVEX	0.25% NaOH	UNDILUTED SONACIDE	1-350 WESCODYNE	1-350 VES-PHENE
1-100 JAVEX	IS	NOT DIFFERENT FROM					
0.25% NaOH	IS	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN				
UNDILUTED SONACIDE	IS	NOT DIFFERENT FROM	NOT DIFFERENT FROM	MORE EFFECTIVE THAN			
1-350 WESCODYNE	IS	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN		
1-350 VES-PHENE	IS	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	NOT DIFFERENT FROM	
95% ETHANOL	IS	NOT DIFFERENT FROM	NOT DIFFERENT FROM	MORE EFFECTIVE THAN	NOT DIFFERENT FROM	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN

Table 14

Relative Viral Resistance to Javex

IS	f2 bacteriophage	reovirus 3	echovirus 11	coxsackievirus B5	herpes simplex virus 2	VSV/BKC	VSV/LM
reovirus 3 IS	LESS RESISTANT THAN						
echovirus 11 IS	MORE RESISTANT THAN	MORE RESISTANT THAN					
coxsackievirus B5 IS	LESS RESISTANT THAN	MORE RESISTANT THAN	LESS RESISTANT THAN				
herpes simplex virus 2 IS	LESS RESISTANT THAN	LESS RESISTANT THAN	LESS RESISTANT THAN	LESS RESISTANT THAN			
VSV/BKC[1] IS	LESS RESISTANT THAN	LESS RESISTANT THAN	LESS RESISTANT THAN	LESS RESISTANT THAN	NOT DIFFERENT FROM		
VSV/LM[2] IS	LESS RESISTANT THAN	LESS RESISTANT THAN	LESS RESISTANT THAN	LESS RESISTANT THAN	MORE RESISTANT THAN	MORE RESISTANT THAN	
VSV/CEF[3] IS	LESS RESISTANT THAN	LESS RESISTANT THAN	LESS RESISTANT THAN	LESS RESISTANT THAN	MORE RESISTANT THAN	MORE RESISTANT THAN	LESS RESISTANT THAN

1 VSV grown in bovine kidney cells
2 VSV grown in LM cells
3 VSV grown in chick embryo fibroblasts

Table 15

Relative Viral Resistance to Wescodyne

	f2 bacteriophage	reovirus 3	echovirus 11	coxsackievirus B5	herpes simplex virus 2	VSV/BKC	VSV/LM
reovirus 3 IS	LESS RESISTANT THAN						
echovirus 11 IS	NOT DIFFERENT FROM	MORE RESISTANT THAN					
coxsackie-virus B5 IS	LESS RESISTANT THAN	LESS RESISTANT THAN	LESS RESISTANT THAN				
herpes simplex virus 2 IS	LESS RESISTANT THAN	NOT DIFFERENT FROM	LESS RESISTANT THAN	LESS RESISTANT THAN			
VSV/BKC[1] IS	LESS RESISTANT THAN	LESS RESISTANT THAN	LESS RESISTANT THAN	LESS RESISTANT THAN	LESS RESISTANT THAN		
VSV/LM[2] IS	LESS RESISTANT THAN	NOT DIFFERENT FROM	LESS RESISTANT THAN	NOT DIFFERENT FROM	NOT DIFFERENT FROM	MORE RESISTANT THAN	
VSV/CEF[3] IS	LESS RESISTANT THAN	LESS RESISTANT THAN	LESS RESISTANT THAN	LESS RESISTANT THAN	LESS RESISTANT THAN	NOT DIFFERENT FROM	LESS RESISTANT THAN

1 VSV grown in bovine kidney cells
2 VSV grown in LM cells
3 VSV grown in chick embryo fibroblasts

Table 16

Relative Viral Resistance to Ves-Phene

	f2 bacteriophage	reovirus 3	echovirus 11	coxsackievirus B5	herpes simplex virus 2	VSV/BKC	VSV/LM
reovirus 3 IS	LESS RESISTANT THAN						
echovirus 11 IS	NOT DIFFERENT FROM	MORE RESISTANT THAN					
coxsackievirus B5 IS	NOT DIFFERENT FROM	MORE RESISTANT THAN	NOT DIFFERENT FROM				
herpes simplex virus 2 IS	LESS RESISTANT THAN	LESS RESISTANT THAN	LESS RESISTANT THAN	LESS RESISTANT THAN			
VSV/BKC[1] IS	LESS RESISTANT THAN	LESS RESISTANT THAN	LESS RESISTANT THAN	LESS RESISTANT THAN	NOT DIFFERENT FROM		
VSV/LM[2] IS	LESS RESISTANT THAN	LESS RESISTANT THAN	LESS RESISTANT THAN	LESS RESISTANT THAN	MORE RESISTANT THAN	MORE RESISTANT THAN	
VSV/CEF[3] IS	LESS RESISTANT THAN	LESS RESISTANT THAN	LESS RESISTANT THAN	LESS RESISTANT THAN	MORE RESISTANT THAN	MORE RESISTANT THAN	MORE RESISTANT THAN

1 VSV grown in bovine kidney cells
2 VSV grown in LM cells
3 VSV grown in chick embryo fibroblasts

Table 17

Relative Viral Resistance to Sonacide

	f2 bacteriophage	reovirus 3	echovirus 11	coxsackievirus B5	herpes simplex virus 2
reovirus 3 IS	LESS RESISTANT THAN				
echovirus 11 IS	MORE RESISTANT THAN	MORE RESISTANT THAN			
coxsackievirus B5 IS	MORE RESISTANT THAN	MORE RESISTANT THAN	NOT DIFFERENT FROM		
herpes simplex virus 2 IS	LESS RESISTANT THAN	LESS RESISTANT THAN	LESS RESISTANT THAN	LESS RESISTANT THAN	
VSV/BKC[1] IS	LESS RESISTANT THAN	LESS RESISTANT THAN	LESS RESISTANT THAN	LESS RESISTANT THAN	NOT DIFFERENT FROM

1 VSV grown in bovine kidney cells

Table 18

Relative Viral Resistance to Sodium Hydroxide

IS	f2 bacterio-phage	reovirus 3	echovirus 11	coxsackievirus B5	herpes simplex virus 2	VSV/BKC	VSV/LM
reovirus 3 IS	MORE RESISTANT THAN						
echovirus 11 IS	NOT DIFFERENT FROM	LESS RESISTANT THAN					
coxsackie-virus B5 IS	NOT DIFFERENT FROM	LESS RESISTANT THAN	MORE RESISTANT THAN				
herpes simplex virus 2 IS	LESS RESISTANT THAN	LESS RESISTANT THAN	LESS RESISTANT THAN	LESS RESISTANT THAN			
VSV/BKC[1] IS	NOT DIFFERENT FROM	LESS RESISTANT THAN	MORE RESISTANT THAN	MORE RESISTANT THAN	MORE RESISTANT THAN		
VSV/LM[2] IS	MORE RESISTANT THAN	NOT DIFFERENT FROM	MORE RESISTANT THAN	MORE RESISTANT THAN	MORE RESISTANT THAN	MORE RESISTANT THAN	
VSV/CEF[3] IS	MORE RESISTANT THAN	MORE RESISTANT THAN	MORE RESISTANT THAN	MORE RESISTANT THAN	MORE RESISTANT THAN	MORE RESISTANT THAN	MORE RESISTANT THAN

1 VSV grown in bovine kidney cells
2 VSV grown in LM cells
3 VSV grown in chick embryo fibroblasts

Table 19

Relative Viral Resistance to 95% Ethanol

	f2 bacteriophage	reovirus 3	echovirus 11	coxsackievirus B5	herpes simplex virus 2
reovirus 3 IS	LESS RESISTANT THAN				
echovirus 11 IS	LESS RESISTANT THAN	LESS RESISTANT THAN			
coxsackievirus B5 IS	LESS RESISTANT THAN	LESS RESISTANT THAN	NOT DIFFERENT FROM		
herpes simplex virus 2 IS	LESS RESISTANT THAN	LESS RESISTANT THAN	LESS RESISTANT THAN	LESS RESISTANT THAN	
VSV/BKC[1] IS	LESS RESISTANT THAN	LESS RESISTANT THAN	LESS RESISTANT THAN	LESS RESISTANT THAN	NOT DIFFERENT FROM.

1 VSV grown in bovine kidney cells

Table 20

Relative Viral Resistance to 70% Ethanol

	VSV/BKC[1]	reovirus	echovirus	coxsackievirus B5
reovirus 3 IS	MORE RESISTANT THAN			
echovirus 11 IS	MORE RESISTANT THAN	LESS RESISTANT THAN		
coxsackievirus B5 IS	MORE RESISTANT THAN	LESS RESISTANT THAN	LESS RESISTANT THAN	
herpes simplex virus 2 IS	NOT DIFFERENT FROM	LESS RESISTANT THAN	LESS RESISTANT THAN	LESS RESISTANT THAN

1 VSV grown in bovine kidney cells

Table 21

Mean Slope Data For Echovirus 11 Inactivation Regressions

		Echovirus 11				
DISINFECTANT	N[c]	MEAN INDEPENDENT VARIABLE [a]		MEAN DEPENDENT VARIABLE [b]		MEAN SLOPE
		MEAN	S.D.	MEAN	S.D.	
1-100 JAVEX	25	0.473	0.240	1.69	0.477	-1.954
1-75 JAVEX	20	0.453	0.261	1.43	0.782	-2.910
1-50 JAVEX	22	0.463	0.252	2.43	1.27	-5.024
0.25% NaOH	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
UNDILUTED SONACIDE	22	0.462	0.249	1.47	0.215	-0.789
1-350 WESCODYNE	24	0.481	0.237	1.32	0.232	-0.849
1-200 WESCODYNE	20	0.448	0.255	1.90	0.422	-1.576
1-50 WESCODYNE	22	0.462	0.249	1.85	0.460	-1.781
1-50 VES PHENE	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
95% ETHANOL	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
70% ETHANOL	22	0.460	0.252	1.54	0.325	-1.037

a - square root minutes exposure time
b - $\log_{10}$ PFU/ml
c - total number of experimental observations

Total Inactivation of A2 Plaque Virus in One and Ten Minutes by Seven Common Disinfectants

In preliminary testing of disinfectants on A2 plaque virus, the standard technique previously described yielded non-reproductible results for some unknown reason. Therefore, the following modified method was adopted. A volume of 0.4 ml of virucide dilution to be tested was added to a mixture of 0.05 ml of fetal calf serum and 0.05 ml of a pool of A2 plaque virus.

After the virus in serum was exposed to the virucide for either one or ten minutes, an appropriate volume of 17.5 per cent skim milk was added to neutralize the activity of the virucides. This entire volume was assayed in 0.5 ml quantities for residual virus on Vero cells, using the plaque assay. In certain instances, the previously stated quantities of materials used in the test were halved. This was done since the large volume of skim milk needed to neutralize disinfectant activity in these instances, had to be completely assayed for residual virus.

Neutralized and cell susceptibility controls were set up as previously described and listed in Tables 53 and 59, respectively.

Dilutions of virucides that were capable of completely inactivating A2 plaque virus by the end of the exposure time were termed effective, while those that did not were termed ineffective. Table 22 is a summary of results, including the dilution by 17.5 per cent skim milk

needed to neutralize the activity of the virucides. It is based on data from duplicate experimental trials for each concentration of each disinfectant.

Table 22

A Summary of the Effectiveness of Different Disinfectants in the Inactivation of A2 Plaque Virus[d]

Disinfectant Dilution Tested	Time[a]	Conclusion[b]	Dilution of 17.5% Skim Milk Used for Neutralization
1-50 Wescodyne	10	Ineffective	1-80
1-50 Ves-Phene	10	Ineffective	1-80
1-50 Javex	1	Ineffective	1-10
	10	Effective	1-10
1-75 Javex	10	Ineffective	1-10
Undiluted Sonacide	1	Ineffective	1-100
	10	Ineffective	1-100
1% (w/v)	1	Ineffective	1-10
NaOH	10	Ineffective	1-10
100% Methanol	1	Ineffective[c]	1-20
	10	Effective	1-20
80% Methanol	1	Effective	1-20
95% Ethanol	1	Ineffective[c]	1-20
	10	Effective	1-20
80% Ethanol	1	Ineffective	1-20
	10	Ineffective	1-20
70% Ethanol	10	Ineffective	1-20

a - minutes
b - a disinfectant was termed ineffective if at least one PFU was detected by the plaque assay
c - in these cases, only one PFU was detected in one of the two experimental trials
d - A2 plaque virus used were samples from a common pool whose titer ranged from 1×10^5 PFU/ml to 2.5×10^6 PFU/ml mean

It is noted from Table 22 that 80% ethanol was the only disinfectant that was consistently effective using a one minute exposure time, although 100% methanol and 95% ethanol were effective in one out of the two experimental trials, with only one PFU residual infectivity being present in the other trial.

A dilution of 1-50 Javex was effective after a ten minute exposure time, but not after one minute.

The other four disinfectants, 1-50 Wescodyne, 1-50 Ves-Phene, two per cent (w/v) NaOH and undiluted Sonacide were not effective, even after a ten minute exposure time.

χ^2Value Testing

Considering the kinetics of inactivation of microorganisms exposed to heat, the following relationship was derived from a survivor curve plot with the Y axis being the logarithm of the number of organisms surviving the heat treatment and the X axis being the heating time (221).

$$\frac{U}{D} = \log No - \log Nu$$

where U is the exposure time at which there are Nu microorganisms left viable. No was the number of microorganisms in the population before heat exposure.

D is the exposure time necessary to inactivate one log or 90% of the population of the microorganism.

Rearranging this equation, the following was derived

equation 1 $$D = \frac{U}{\log No - \log Nu}$$

or

equation 2 $$D = \frac{1}{\frac{\log No - \log Nu}{U}}$$

since $\frac{\log No - \log Nu}{U}$ = the slope (b) of the survivor curve

equation 3 $$D = \frac{1}{b}$$

If the exposure time (R_A) needed to inactivate A logs of the microorganism is desired, then

$$R_A = \frac{1}{b} \times A$$

equation 4 ∴ $$R_A = \frac{A}{b}$$

In my work, the survivor curve plots for viral infectivity versus exposure time took the form of $\log_{10}$ PFU/ml versus square root exposure time.

Such a relationship would yield the modified form of equation 4 where $\sqrt{R_A} = \chi$

$$\chi = \frac{A}{b}$$

or

equation 5 $$\chi^2 = \left(\frac{A}{b}\right)^2$$

since the exposure time a given number of logs of virus were inactivated was expressed in square root minutes.

The practical significance of equation 5 for predicting the time necessary to inactivate A logs of virus was tested using the following procedure:

A volume of 0.4 ml of the dilution of disinfectant to be tested was added to a mixture consisting of 0.05 ml of either reovirus 3 or coxsackievirus B5 and 0.05 ml of fetal calf serum. This was mixed well and allowed to react for the time predicted by the extrapolation of the infectivity versus exposure time relationship which would yield zero infectively. This time, termed the χ^2 value, was determined by equation 5. The χ^2 values in Tables 23 and 24 are calculated using the mean slopes from Tables 39 and 41, respectively.

Subsequently, a certain volume of 17.5 per cent skim milk was added to the 0.5 ml reaction mixture. The volume of milk added was dependent on the amount necessary to neurtalize the action of the given concentration of the disinfectant considered.

This entire volume was then assayed in 0.5 ml quantities for virus, using the plaque assay method.

Results

Residual reovirus 3 was present at the time equation 5 predicted total viral inactivation. Also, residual virus was present for coxsackievirus B5 in all cases with the exception of 95% ethanol. Duplicate experimental trials were carried out.

These results clearly indicated extrapolation of the plot $\log_{10}$ PFU/ml versus square root minutes exposure time in order to predict an exposure time to a certain disinfectant that would completely inactivate virus of a certain titer was not valid. The titer of virus was arbitrarily multiplied by 200 to add a safety factor on for exposure time. The rationale behind this was to emphasize the fallacy of using the X^2 value for predicting zero infectivity since residual virus was present.

Table 23

χ^2 Values Calculated for Coxsackievirus B5

DISINFECTANT DILUTION	MEAN SLOPE	LOG 10 MEAN TITER[a]	LOG 10 MEAN TITER PLUS SAFETY FACTOR[b]	χ^2 VALUE[c]	DILUTION OF SKIM MILK[d] NEUTRALIZED IN
Undiluted SONACIDE	-0.677	5.990	8.291	150.0	1-100
1-75 JAVEX	-6.525	5.553	7.853	1.448	1-10
1-100 JAVEX	-4.224	6.006	8.306	3.870	1-10
0.25% NaOH	-7.244	6.154	8.454	1.360	1-10
1-350 WESCODYNE	-1.791	6.154	8.454	22.28	1-10
1-200 WESCODYNE	-7.393	5.553	7.853	1.128	1-10

a $\log_{10}$ PFU/0.05 ml
b $\log_{10}$ (PFU/0.05 x 200)
c the χ^2 value in minutes calculated by equation 5 using the log titer value plus the x 200 safety factor
d 17.5 per cent skim milk

Table 24

χ^2 Values Calculated for Reovirus 3

DISINFECTANT DILUTION	MEAN SLOPE	LOG 10 MEAN TITER[a]	LOG 10 MEAN TITER PLUS SAFETY FACTOR[b]	χ^2 VALUE[c]	DILUTION OF SKIM MILK[d] NEUTRALIZED IN
Undiluted SONACIDE	-3.935	6.60	8.900	5.115	1-100
1-50 VES-PHENE	-1.670	6.146	8.446	25.580	1-20
0.25% (w/v) NaOH	-3.269	6.146	8.446	6.675	1-10
95% ETHANOL	-7.191	6.146	8.446	1.380	1-20

a $\log_{10}$ PFU/0.05 ml
b $\log_{10}$ PFU/0.05 x 200
c the χ^2 value in minutes calculated by equation 5 using the log titer value plus the x 200 safety factor.
d 17.5 per cent skim milk

Controls

Neutralized Control

In each experiment the effectiveness of 17.5 per cent skim milk[1] as a neutralizer for the activity of the virucide was tested by the following control, termed the neutralized control.

A volume of 0.05 ml of fetal calf serum and 0.4 ml of disinfectant dilution tested were added to the same amount of 17.5 per cent skim milk as that used in the neutralization of the ten, 20 and 30 second reaction samples. Then 0.05 ml virus was added. The virus stayed in the skim milk dilution blank for a period of time equivalent to that which the ten, 20 and 30 second samples stayed in skim milk before being diluted.

Both in the case of the neutralized ten, 20 and 30 second samples and the neutralized control, a 0.5 volume was serially diluted tenfold in 4.5 ml dilution blanks with 0.5 ml amounts of the appropriate dilutions assayed for virus,using the viral plaque assay.

The virus was titered at the time of the experiment. The effectiveness of the 17.5 per cent skim milk neutralizer was determined by comparing the titer of the virus with the neutralized control results, using the t test at the 95% confidence level in those cases where the neutralized control values were less than the titer (Tables 45 to 53, Appendix 15).

1 in the case of herpes simplex virus 2, heat inactivated fetal calf serum was used as a neutralizer instead of skim milk because skim milk inactivated herpes simplex virus 2

Such a control was set up for all viruses studied except VSV.

In the case of VSV, 0.5 ml of fetal calf serum and 0.5 ml of the dilution of disinfectant tested were added to four ml of ten per cent skim milk. This five ml volume was mixed and 4.5 ml of it was removed and added to another tube. Then 0.5 ml of virus was added to the 4.5 ml volume. The virus in this five ml volume was incubated in this tube for a time period equivalent to that the ten, 20 and 30 second samples were incubated in the skim milk neutralizer before being diluted.

This neutralized control was subsequently diluted serially in tenfold dilutions with 0.5 ml amounts of the appropriate dilutions being assayed for virus, using the plaque assay method.

The VSV used in each experiment was titered at the time of the experiment. The effectiveness of the ten per cent skim milk neutralizer was determined by comparing the titer with the neutralized control results.

Results

Out of 146 experiments, involving a wide variety of disinfectants and virus combinations neutralized in skim milk, only six had neutralized controls significantly less than the titer using the t test at the 95% confidence level (one tailed test) (Tables 45 to 53, Appendix 15). Of these six experiments, in one the

neutralized control was not significantly different from the titer at the 97.5% confidence level, in four the neutralized control was not significantly different from the titer at the 99% confidence level, and in the remaining one instance the neutralized control was not significantly different from the titer at the 99.5% confidence level. In experiments involving herpes simplex virus 2, in which fetal calf serum was used as the neutralizer, neutralized controls were significantly less than titer controls in two out of 20 experiments, as determined by the t test at the 95% confidence level (Table 49). Of these two experiments, the neutralized control was not significantly different from the titer when the t test was considered at the 99% level in one instance and at the 99.5% level in the other instance.

Testing for Cell Susceptibility to the Toxic Effects of Disinfectants

In addition to the neutralized control, another control was set up to test the ability of skim milk[1] to neutralize the activity of the disinfectant. The main emphasis in this control, termed the cell susceptibility control, was to determine whether or not the disinfectant was sufficiently neutralized and that toxicity of the disinfectant did not affect the ability of the virus to multiply in the cell line used in the plaque assay.

1 in the case of herpes simplex virus 2, heat inactivated fetal calf serum was used as a neutralizer instead of skim milk.

In this control, virus was diluted in serial tenfold dilutions to the dilution preceding the one calculated of yielding between 30 and 300 PFU per 0.5 ml quantity assayed. Then 0.5 ml of this preceding dilution was added to 4.5 ml of a mixture of disinfectant-skim milk identical in proportion to that found when the virus-disinfectant mixture was neutralized by a certain volume of skim milk in actual disinfectant testing.

Two, 0.5 ml samples of this five ml volume were assayed, using the plaque assay.

The effectiveness of the 17.5 per cent skim milk was determined by comparing the titer of the virus with the cell susceptibility control results, using the t test at the 95% confidence level in those cases where the cell susceptibility values were less than the titer (Tables 54 to 59, Appendix 16).

Results

Out of 107 instances when skim milk was evaluated as a neutralizer of disinfectant there were only four cases where the cell susceptibility controls were significantly less than the titer at the 95% confidence level (Tables 54 to 59, Appendix 16). Of these four cases, in two the cell susceptibility control did not possess significantly less virus as compared to the titer at the

97.5% confidence level and in the other two instances this was true at the 99% confidence level and the 99.5% confidence level, respectively.

In herpes simplex virus 2 experiments, where fetal calf serum was used as the neutralizer, cell susceptibility controls were not significantly less than the titer in 18 of 20 instances at the 95% confidence level (Table 57). In the other two cases, the cell susceptibility control was not significantly less than the titer at the 99% level.

CHAPTER IV

DISCUSSION

The inactivation of virus relative to the exposure time to a disinfectant was studied in a unique way. Fetal calf serum was added to virus to simulate actual use conditions where the virus is protected from inactivation by organic material. Also, the exposure times considered in this study were only ten, 20 and 30 seconds with addition of skim milk at the end of such exposure times, neutralizing disinfectant activity. Residual virus versus exposure time was plotted in such a way that a linear relationship was obtained. The slope of such a relationship was used as a measure of the efficiency of the disinfectant in inactivating virus. In studying the inactivation of a virus by disinfectant, quite frequently the residual virus versus exposure time relationship ($\log_{10}$ PFU/ml versus exposure time in minutes) has been reported to be a linear function (224). However, actual experimental data fitted a linear function to varying degrees (204, 205).

Plots of experimental data may yield any one of three possible kinetic relationships as illustrated below in figure 8.

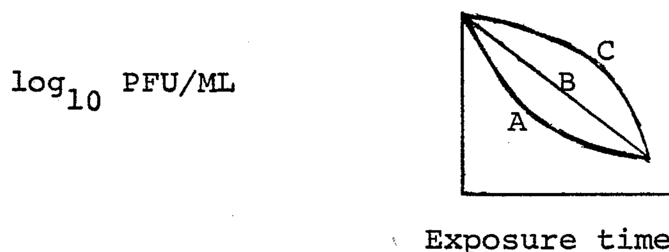


figure 8. Possible Kinetic Relationships for Inactivation Curves

Most experimental data obtained in the present study yielded a type A (figure 8) concave line. Explanations of this are numerous and, unfortunately, speculative. It has been suggested that this non-linear type of inactivation is due to aggregation of virus particles which results in protection of a certain proportion of the virus particles from disinfectant action (205). Another explanation ascribes this type of inactivation to heterogeneity in the virus population with respect to its susceptibility to inactivation by disinfectant (214). An alternative explanation is that at the beginning of virus interaction with disinfectant, there is a linear function which gradually flattens out due to an increasing proportion of disinfectant reacting with already inactivated virus particles (which are now in the vast majority) before getting a chance to react with the remaining few viable virus particles. In such an instance, the "tailing effect" of line A is inherent in the test system. A large excess of disinfectant will minimize it.

As stated earlier in the test system reported in this thesis, a sample from a standard batch of heat inactivated fetal calf serum was mixed with the test virus before inactivation so that there would be more stringent criteria for disinfectant evaluation with reference to actual disinfectant use situations. Such serum reacts with disinfectant and decreases its action on virus, thus possibly setting the stage for this tailing effect.

One possibility to consider also is that "tailing" occurs at the end of line A simply due to all the disinfectant being used up by the inactivation that takes place in the upper part of curve A.

Other factors, such as pH, conditions of storage for the virus and temperature, may play major roles in determination of a linear function with appropriate adjustments in them yielding such a function (204, 208, 214).

It is important to note initially that the data obtained in this study were not statistically analyzed for linearity if they fell into one of the following categories:

i) the plot of residual virus versus exposure time had less than three numerical points due to rapid complete viral inactivation by the disinfectant tested (figure 5);

ii) the plot yielded a nonsignificant linear regression relationship due to minimal viral inactivation by the disinfectant tested (figure 6).

It was deemed important to obtain a linear function for the residual virus versus exposure time relationship so that a numerical slope could be obtained. Such a slope value was used to compare the relative resistance of different test viruses to a given disinfectant and to determine the relative efficiency of different disinfectants in inactivating a given virus. This approach in evaluating disinfectants is unique.

In the instances previously mentioned, when the data was not conducive to statistical analysis, non-statistical comparisons were made. For example, undiluted Sonacide completely inactivated four logs of VSV/BKC in ten seconds (figure 5) and undiluted Sonacide destroyed two logs of reovirus 3 in ten seconds (figure 7), so undiluted Sonacide was considered more effective in inactivating VSV/BKC than reovirus 3.

Development of a linear function for the residual virus versus exposure time relationship to the disinfectant left open the possibility that extrapolation could lead to a prediction of exposure time necessary to obtain zero residual virus, as was the case in other studies on inactivation of microorganisms by various chemical and physical agents (221).

Initially, inactivation data obtained using the standard experimental method was transformed in different ways to obtain the consistently best fit to a linear function for the residual virus versus exposure time relationship (Table 4). In each case, the correlation coefficient squared (r^2) value (which is the proportion of the variation of the dependent variable, residual virus, explained by the regression equation (225) and the F statistic which is the ratio of $\frac{\text{mean square regression}}{\text{mean square deviation}}$ were used as criteria for the best fit of data to linearity.

It was found that the relationship

$$\log_{10} \text{PFU/ml versus square root of exposure time minutes}$$

consistently gave the best linear relationship with the one exception being 70 per cent ethanol (Figure 4). These trends are further illustrated in Table 5 and Tables 25 to 31, Appendix 12, in which the $\log_{10}$ PFU/ml versus square root minutes relationship was compared to the often used $\log_{10}$ PFU/ml versus exposure time (minutes) relationship for each virus. In each instance, the r^2 value for the $\log_{10}$ PFU/ml versus square root minutes relationship is consistently larger than the r^2 value for the $\log_{10}$ PFU/ml versus minutes relationship. The F values are also consistently larger in the case of the $\log_{10}$ PFU/ml versus square root minutes relationship denoting a better fit of data to linearity. In another study (211), the plot approached linearity when log inactivation of poliovirus was plotted versus log exposure time. Thus, the square root transformation of the independent variable, exposure time, straightens out the concave type curve (type A, figure 8) so commonly encountered, giving the data a better fit to a linear function.

A solution of 70 per cent ethanol acts on different viruses in such a way that a convex (type C, figure 8) curve was obtained. A different transformation would be needed to fit this data best to linearity. For

the sake of uniformity in comparison, all data including that obtained for 70 per cent ethanol was plotted as

$\log_{10}$ PFU/ml versus square root minutes

Why ethanol yields a convex curve instead of the commonly encountered concave curve or straight line remains a mystery, although it may be because ethanol must react with the fetal calf serum in the test system and saturate it before it can react with the virus and inactivate it to any measurable extent, thus causing an initial shoulder on the inactivation curve.

There are two experimental trials for each viral inactivation. The reproducibility of each inactivation relationship was determined by comparing the slopes of the different experimental trials statistically at the 95% confidence level. In all cases listed in Table 6 and Tables 32 to 38 (Appendix 13) the slopes of the two experimental trials were not significantly different at the 95% confidence level, with the exception of echovirus 11 in fetal calf serum treated with 1-100 Javex (Table 32). In this case, the slopes were significantly different at the 95% confidence level but not at the 99% confidence level.

In this study, the six disinfectants were selected such that each was representative of a certain class of disinfectant. Table 60 illustrates this.

Table 60

Classes of Disinfectant Tested

Disinfectant	Class Representative Of	
Wescodyne	Iodophor (organic iodine)	Halogens
Javex	Chlorine	Halogens
Sonacide	Aldehyde (glutaraldehyde)	
Sodium Hydroxide	Alkali	
Ethanol	Alcohol	
Ves-Phene	Phenolic	

A method of comparing the effectiveness of the disinfectants was required. The method will be described by referring to Table 61. The results in the table describe the effectiveness of 11 standard disinfectant preparations (left-hand column of the table) compared to 1-100 Javex (see title of table). Other tables (Tables 62-68) will list the effects of the same 11 standard preparations relative to other disinfectants than 1-100 Javex. The results in Table 61 were summarized by calculating the following:

$$\frac{\text{Number of entries "less effective than 1-100 Javex"}}{\text{total number of entries}} \times 100$$

This per cent figure gives some notion of the overall effectiveness of 1-100 Javex compared with the 11 standard preparations. There are tables comparing the following disinfectants to these 11 standard preparations:

95% ethanol, 0.25% w/v NaOH, 1-100 Javex, 1-200 Wescodyne, 1-350 Wescodyne, undiluted Sonacide, 1-50 Ves-Phene and 1-350 Ves-Phene.

With reference to Tables 61 to 68, 95% ethanol had the highest percentage value of the eight disinfectants, being more effective than 76% of the standard disinfectants listed in Table 68.

In my test system, four parts of disinfectant were mixed with one-half part of serum and one-half part of virus. This dilutes the disinfectant to four-fifths of its original strength. In the case of ethanol, 95% is diluted to 76% and 70% to 56%. Table 68 indicates, in the cases of echovirus 11, coxsackievirus B5 and reovirus 3, that the final dilution of 76% is more virucidal than the final dilution of 56%. This emphasizes the fact that, although ethanol may be very virucidal, it is only so in a very narrow range of concentrations with it rapidly losing its activity when diluted below 70% (100). This is a major disadvantage in using ethanol in practical situations where it is difficult to know what the final dilution is.

In the cases of herpes simplex virus 2 and VSV, inactivation by both 70% and 95% ethanol was too rapid for numerical data to be obtained. f2 bacteriophage was not treated with 70% ethanol.

A 0.25% solution of sodium hydroxide had the second highest percentage of the eight disinfectants, being more effective than 54% of the standard disinfectants listed in Table 67. Unfortunately, since 0.25% sodium

hydroxide has a pH of 12.5, it is toxic in many situations and its use is therefore limited to uses such as disinfection of barns and stables (87). Hot solutions of sodium hydroxide have also been used for disinfection of beverage and milk bottles (85).

A 1-100 solution of Javex had the third highest percentage of the eight disinfectants, being more effective in virus inactivation than 44% of the disinfectants listed in Table 61. Such a solution contains 600 ppm of chlorine. This solution of Javex was chosen to be tested, since it was active against a wide variety of viruses. Preliminary experiments indicated Javex lost its inactivating activity rapidly with reference to some of the more resistant viruses at dilutions greater than 1-100 in the presence of the fetal calf serum of the test system. Javex is relatively less toxic than sodium hydroxide, as well as being less expensive to use and active over a greater range of concentrations than ethanol. On this basis, it could be argued Javex is the most practical of the disinfectants tested, to use in a laboratory or commercial setting. Such a conclusion is supported in a practical study (48) in which hypochlorite bleach successfully inactivated poliovirus shed by infected babies into soiled diapers. The concentration of Javex which should be used depends on the individual use situation, but judging from the rates of inactivation exhibited by the more

resistant viruses such as f2 bacteriophage and echovirus 11, some concentration greater than 1-50 (1200 ppm chlorine) is recommended. A bleach solution containing 200 ppm available chlorine has been recommended as a disinfectant (24).

The use concentration recommended by the manufacturer for Wescodyne for use in the presence of organic material is approximately 1-200 (226). This prompted the use of 1-200 Wescodyne as one of the standard reference disinfectants. A concentration of 1-200 Wescodyne was more effective in virus inactivation than 44% of the disinfectants listed in Table 63. The value for Wescodyne (44%) is not differen from the 44% value for 1-100 Javex, and the Wescodyne was tested at twice the dilution. But, as will be seen later in comparisons of virus susceptibility to disinfectants, unlike ethanol, sodium hydroxide and Javex, Wescodyne does not have a broad spectrum of action against all viruses tested but is rather selective, being effective against viruses possessing lipid envelopes and coxsackievirus B5, and relatively ineffective against others such as echovirus 11. This was especially emphasized in the case of f2 bacteriophage, where even an exposure of 1-20 Wescodyne was only effective to a limited extent. These results correlated with a study in which another nonenveloped enterovirus, poliovirus, was not completely inactivated when exposed to 1-4 Wescodyne for 15 minutes at room temperature (59).

It is interesting to note, though, that even 1-350 Wescodyne, a solution almost one-half of the recommended disinfecting strength, was shown to be more effective than 21% of the other disinfectants listed in Table 62.

Undiluted Sonacide was shown to be more effective than 28% of the other disinfectants listed in Table 66. This is a relatively low percentage value, and since the manufacturer recommends use of undiluted Sonacide, it is a relatively expensive disinfectant to use. Sonacide is selective in its action on the test viruses, being effective on enveloped viruses such as VSV/BKC (figure 5) and herpes simplex virus 2, and totally ineffective in inactivating others, notably the nonenveloped viruses such as coxsackievirus B5 and echovirus 11.

The least effective disinfectant of the six considered in the study was Ves-Phene, with 1-50 and 1-350 concentrations being more effective than 3% and 2% respectively of the disinfectants listed in Tables 65 and 64. In the case of f2 bacteriophage, even a 1-20 dilution of Ves-Phene was ineffective. The use concentration recommended by the manufacturer for Ves-Phene is 1-256.

In evaluating the virucidal ability of a disinfectant, it should be noted that the recommended use concentration has at least five-minute exposure time stipulated along with it. Often a five minute exposure time is too long in practical situations, and in this study a 30 second

exposure time was used in order to apply a more rigorous criterion for disinfectant evaluation.

Comparison of the resistance of each test virus to the six disinfectants considered in this study relative to the other test viruses is presented in Tables 69 to 74 (Appendix 18). Such a comparison is summarized in the form of a percentage value, which signifies that a certain virus is more resistant to inactivation by that percentage of the disinfectants tested as compared to the other test viruses listed. These percentage values are as follows:

f2 bacteriophage	68%
echovirus 11	67%
reovirus 3	62%
coxsackievirus B5	55%
herpes simplex virus 2	12%
VSV/BKC	9%

f2 bacteriophage and echovirus 11 were approximately equal in resistance to disinfectants and more resistant than the other test viruses. VSV/LM and VSV/CEF were not included in these comparisons, since data was not obtained for the action of Sonacide and ethanol on them. An obvious trend to note is that the nonenveloped viruses had percentage values over 50% and the enveloped viruses well under 20%, which is in agreement with Klein _et al_ (24) who found that enveloped viruses such as influenza virus were more susceptible to inactivation by a wide variety of disinfectants as compared to nonenveloped viruses such as echovirus, poliovirus and coxsackievirus.

In this study,the difference in susceptibility to inactivation by enveloped and nonenveloped viruses may be attributed to the fact that, although Javex, ethanol and sodium hydroxide are wide spectrum disinfectants in that they act effectively on all viruses regardless of whether they possess a lipid envelope or not, other disinfectants such as Sonacide, Ves-Phene and Wescodyne act predominantly better on enveloped viruses, thus lowering the percentage values for herpes simplex virus 2 and VSV/BKC.

Relative viral resistance to each disinfectant will now be discussed. In considering Javex, the nonenveloped viruses are listed below in order of resistance to inactivation by Javex, with the most resistant virus listed first and the least resistant last, as determined from the conclusions in Table 14.

i) echovirus 11

ii) f2 bacteriophage

iii) coxsackievirus B5

iv) reovirus 3

Echovirus 11 was the most resistant virus to Javex inactivation, in a 30-second exposure time, since 1-50 Javex inactivated three logs of virus as compared to four logs for f2 bacteriophage, and the even more susceptible coxsackievirus B5 and reovirus 3 of which four and one-half and six logs of virus were inactivated respectively by 1-75 Javex. In the instances where enveloped virus

was exposed to 1-100 Javex, either complete viral inactivation occurred before the first virus assay at ten seconds as was the case with herpes simplex virus 2 and VSV/BKC resulting in inactivation of five logs of virus, or in the cases of VSV/LM and VSV/CEF at least a three and one-half log inactivation was observed in 30 seconds. Statistically, VSV/LM was more resistant than VSV/CEF to Javex (Table 14). The enveloped viruses are listed below in order of resistance to inactivation by Javex, with the more resistant viruses listed first:

i) VSV/LM

ii) VSV/CEF

iii) VSV/BKC and herpes simplex virus 2

The fact that in each case at least three logs of virus were inactivated in the first 30 seconds of exposure to the disinfectant emphasizes that Javex is an effective disinfectant at the reported dilutions.

In a manner similar to Javex, the nonenveloped viruses are listed below in order of decreasing resistance to inactivation by Wescodyne (Table 15):

i) echovirus 11 and f2 bacteriophage

ii) reovirus 3

iii) coxsackievirus B5

As far as the data obtained in this study is concerned, echovirus 11 and f2 bacteriophage are not significantly different in their resistance to inactivation by Wescodyne and are more resistant than reovirus 3 and coxsackievirus B5

to inactivation (Table 15). f2 bacteriophage treated with 1-20 Wescodyne and echovirus 11 treated with 1-50 Wescodyne each resulted in one log inactivation in 30 seconds. Unfortunately, echovirus 11 was not exposed to 1-20 Wescodyne, so no further conclusions can be made. Reovirus 3 and coxsackievirus B5 exposed to 1-200 Wescodyne suffered two and one-half logs and five logs inactivation in 30 seconds, respectively. Wescodyne is an ineffective disinfectant therefore, since in certain instances only one log inactivation was obtained upon a 30 second exposure to 1-50 Wescodyne, which is four times the use concentration recommended by the manufacturer (59).

Preliminary experiments indicated 1-200 Wescodyne was very effective against enveloped viruses and therefore to further test the virucidal activity of Wescodyne, it was diluted to 1-350 and tested. The results are listed below for enveloped viruses in order of decreasing resistance to inactivation by 1-350 Wescodyne:

i) herpes simplex virus 2 and VSV/LM

ii) VSV/BKC and VSV/CEF

Conclusions were obtained from Table 15. Herpes simplex virus 2 and VSV/LM were the most resistant viruses of the four, and were inactivated two logs upon exposure to 1-350 Wescodyne for 30 seconds. VSV/BKC and VSV/CEF were inactivated by three logs upon exposure to 1-350 Wescodyne for 30 seconds.

In the case of Sonacide, the nonenveloped viruses are again listed in decreasing order of resistance to inactivation by undiluted Sonacide, as determined from Table 17:

coxsackievirus B5, echovirus 11
f2 bacteriophage
reovirus 3

With the exception of reovirus 3, the other viruses were inactivated by only one log or less in 30 seconds. Reovirus 3 was inactivated by two logs in 30 seconds. The two enveloped viruses, VSV/BKC and herpes simplex virus 2, were very rapidly inactivated by undiluted Sonacide and therefore a numerical plot was not obtained (Figure 5). Sonacide inactivated four logs of VSV/BKC and three logs of herpes simplex virus 2 in 30 seconds. In summary, Sonacide is an ineffective disinfectant for general use, since it is inactive against the nonenveloped viruses, although it is highly effective against enveloped viruses.

When viral resistance to inactivation by sodium hydroxide is considered (Table 18), somewhat of a paradox appeared, in that although sodium hydroxide was effective against all test viruses, it was especially effective against the nonenveloped viruses. The general trend of viral resistance to inactivation has been demonstrated to be the reverse. Therefore, in listing the viruses in decreasing order of resistance to inactivation by sodium

hydroxide below, the enveloped and nonenveloped viruses are listed together. All viruses were treated with 0.25% sodium hydroxide.

i) VSV/CEF

ii) VSV/LM, reovirus 3

iii) VSV/BKC, f2 bacteriophage

iv) coxsackievirus B5

v) echovirus 11

vi) herpes simplex virus 2

VSV/CEF, the most resistant of the viruses to the action of sodium hydroxide, was inactivated two logs in 30 seconds.

VSV/LM and reovirus 3 were inactivated three logs in 30 seconds. VSV/BKC and f2 bacteriophage were inactivated five logs in 30 seconds. Coxsackievirus B5 was inactivated five logs by 0.25% (w/v) sodium hydroxide in 30 seconds. Echovirus 11 was inactivated six logs by 0.25% (w/v) sodium hydroxide in 20 seconds. Herpes simplex virus 2 was inactivated four logs in less than ten seconds by 0.25% (w/v) sodium hydroxide.

One explanation why enveloped viruses tended to be more resistant to sodium hydroxide, as compared to non-enveloped virus, is that hydroxyl ion reacted with viral protein and/or nucleic acid and the lipid envelope interfered with this reaction. Enveloped viruses were more susceptible to inactivation by other agents, which could

have been due to inactivation by direct removal of the lipid envelope by these agents. Sodium hydroxide tended to be a wide spectrum disinfectant, since at least two logs of each virus were inactivated in the first 30 seconds of exposure.

When different viruses were compared for resistance to inactivation by 95% ethanol (Table 19), the viruses lined up in the following order of decreasing resistance to inactivation:

i) f2 bacteriophage

ii) reovirus 3

iii) coxsackievirus B 5 and echovirus 11

iv) VSV/BKC and herpes simplex virus 2

f2 bacteriophage, the most resistant of the test viruses, was inactivated three logs in 30 seconds by 95% ethanol. Reovirus 3 was inactivated five logs in 30 seconds upon exposure to 95% ethanol; coxsackievirus B5 and echovirus 11 were inactivated six logs each in 20 seconds; VSV/BKC and herpes simplex virus 2 were inactivated four and one-half logs in less than ten seconds. In all cases, 95% ethanol proved to be the most reliable disinfectant, with the most resistant virus, f2 bacteriophage, being inactivated three logs in 30 seconds.

When virus resistance to 70% ethanol was compared (Table 20), the viruses lined up in an order of decreasing resistance to inactivation by 70% ethanol that

was similar to the order for the viruses when exposed to 95% ethanol:

i) reovirus 3

ii) echovirus 11

iii) coxsackievirus B5

iv) VSV/BKC and herpes simplex virus 2

f2 bacteriophage was not exposed to 70% ethanol. The inactivation of reovirus 3 by 70% ethanol was not significant, while echovirus 11 was inactivated less than one log upon a 30 second exposure. The most susceptible non-enveloped virus to 70% ethanol inactivation was coxsackievirus B5, which was inactivated three logs in 30 seconds. VSV/BKC and herpes simplex virus 2 were inactivated five and four logs respectively by 70% ethanol in 30 seconds. Comparing the extents of inactivation by 70% ethanol to those of 95% ethanol, it is clear that 95% was the better disinfectant.

The nonenveloped viruses are listed below in decreasing order of resistance to 1-50 One Stroke Ves-Phene (as determined by Table 16):

i) f2 bacteriophage, echovirus 11, coxsackievirus B5

ii) reovirus 3

Inactivation of f2 bacteriophage, coxsackievirus B5 and echovirus 11 by 1-50 One Stroke Ves-Phene was negligible after 30 seconds exposure. Reovirus 3 was inactivated one log upon exposure to 1-50 One Stroke Ves-Phene for 30 seconds.

Preliminary experiments indicated 1-50 One Stroke Ves-Phene was very effective against enveloped viruses and therefore, to further test the limits of the disinfecting ability of One Stroke Ves-Phene, it was diluted to 1-350 and tested. The results are listed below for enveloped viruses in the order of decreasing resistance to inactivation by 1-350 One Stroke Ves-Phene. These conclusions were obtained from Table 16.

i) VSV/CEF

ii) VSV/LM

iii) VSV/BKC and herpes simplex virus 2

VSV/CEF was inactivated one log in 30 seconds by 1-350 One Stroke Ves-Phene. VSV/LM was inactivated two logs in 30 seconds by 1-350 One Stroke Ves-Phene. VSV/BKC and herpes simplex virus 2 proved to be the least resistant of the enveloped viruses, with exposure to 1-350 One Stroke Ves-Phene for 30 seconds leading to a three log inactivation. The results indicated that One Stroke Ves-Phene had also no activity against nonenveloped viruses, making it a very poor disinfectant and the least effective one of the six disinfectants studied. It did inactivate enveloped viruses quite well though.

Results indicated that the cell type in which VSV was grown significantly influenced its resistance to the action of disinfectants. VSV was passaged three times in each of chick embryo fibroblasts, LM cells

and bovine kidney cells. Each type of VSV was exposed to

1-350 Wescodyne

1-350 One Stroke Ves-Phene

1-100 Javex

0.25% (w/v) NaOH

and the relative resistances to each of the chemicals was determined and listed in Tables 14, 15, 16 and 18.

In the cases of exposure to 1-350 One Stroke Ves-Phene and 0.25% (w/v) sodium hydroxide, VSV/CEF was the most resistant to inactivation, followed by VSV/LM and VSV/BKC, which was the least resistant of the three.

Exposure to 1-350 Wescodyne and 1-100 Javex, though, indicated VSV/LM was the most resistant of the three types of VSV, with VSV/CEF and VSV/BKC being less resistant to inactivation by Javex and Wescodyne.

In summary, general trends indicated VSV/CEF as the most resistant type of VSV with reference to sodium hydroxide and One Stroke Ves-Phene, VSV/LM as the most resistant type to inactivation by Javex and Wescodyne and VSV/BKC as the least resistant type in all instances. These results show that the cell type VSV was grown in at least in part determined resistance to inactivation. More specifically, since the only component of the virus acquired from the host cell is the lipid of the envelope (230), this indicates that such lipid is the factor influencing inactivation.

Tables 23 and 24 indicated χ^2 values calculated for coxsackievirus B5 and reovirus 3, respectively. In each case, the actual virus titer on which the χ^2 value was calculated was multiplied arbitrarily by a factor of 200 to add a safety factor and therefore increase the χ^2 value exposure time. The χ^2 value was previously designated to be that time determined by extrapolation to yield zero residual virus in plots of

$$\frac{\log_{10} \text{PFU/ml versus square root minutes}}{\text{exposure time}}$$

to various disinfectants. This was determined using equation five in the χ^2 value testing section of the procedures and results section of this thesis. The mean slope value was used in this equation as an indicator of the rate of viral inactivation in the kinetic plot.

Results indicated extrapolation of the plot $\log_{10}$ PFU/ml versus square root minutes exposure time in order to predict an exposure time to a certain disinfectant which would completely inactivate virus of a certain titer, was not valid. This was the case since residual virus was detected in virus preparations exposed to disinfectant for the extrapolated exposure time. This may have been due to the "tailing" effect speculated on earlier in the discussion.

Since A2 plaque virus was reported to be antigenically similar to hepatitis B virus (220), it was considered as a virus in my study, although it should be stated at the

at the outset that disinfectant inactivation results obtained for A2 plaque virus may not apply to hepatitis B virus. Experiments were originally designed to obtain standard kinetic relationships for residual A2 plaque virus versus square root exposure time, but results were consistently not reproducible. The reasons why reproducibility was not obtained for A2 plaque virus and was for the other viruses remain unresolved. Therefore, in evaluating the action of disinfectants on A2 plaque virus, a modified approach was taken. Samples of a common pool of A2 plaque virus in ten per cent fetal calf serum were exposed to disinfectants for either one or ten minutes, and assays were performed to detect any residual virus at the end of such exposure times. In instances where residual virus was detected, the disinfectant was termed ineffective. Table 22 indicated that both Ves-Phene and Wescodyne were ineffective disinfectants, since residual virus was detected when A2 plaque virus was exposed to 1-50 Wescodyne for ten minutes and 1-50 One Stroke Ves-Phene for ten minutes. The manufacturers recommend a 1-200 use concentration for Wescodyne and a 1-128 use concentration for One Stroke Ves-Phene. Sonacide was tested in undiluted form, since this was recommended by the manufacturer. It also failed to completely inactivate A2 plaque virus in a ten minute exposure time. A 1-50 concentration of Javex proved to be effective with a

ten minute exposure time, since it consistently completely inactivated A2 plaque virus, although it was unable to do so in a one minute exposure time. A 1-75 concentration of Javex was ineffective in completely inactivating A2 plaque virus in a ten minute exposure time. The alcohols proved to be the most effective of the disinfectants tested, which was consistent with kinetic results discussed earlier for other viruses. For example, 80% methanol consistently inactivated A2 plaque virus completely in a one minute exposure time, something no other disinfectant tested could do. Inconsistent results were obtained for inactivation of A2 plaque virus by 95% ethanol and 100% methanol. Although one of the two experimental trials yielded complete inactivation in an exposure time of one minute, the other experimental trial indicated the presence of one PFU. In contrast, when the exposure time was increased to ten minutes for 95% ethanol and 100% methanol, A2 plaque virus was totally inactivated consistently.

Both 80% and 70% ethanol were ineffective in inactivating A2 plaque virus, even after a ten minute exposure time. These results indicated that

i) A one minute exposure to disinfectant was unrealistic, since residual virus was detected in all cases except 80% methanol

ii) alcohols such as 100% methanol, 80% methanol and 95% ethanol were effective disinfectants.

iii) Javex was effective at dilutions of 1-50 or less.

A 17.5 per cent or ten per cent solution of skim milk was used as the neutralizer for disinfectant activity for all viruses except herpes simplex virus 2. Since skim milk inactivated herpes simplex virus 2, alternative substances were tested for their ability to neutralize disinfectant activity, but at the same time not inactivate herpes simplex virus 2. MacKinnon (177) emphasized the importance of using a neutralizing substance which itself does not act as a virucide. Heat inactivated fetal calf serum proved to be the substance that best fulfilled this criterion in preliminary testing. It was therefore used as the neutralizer in all experiments involving herpes simplex virus 2.

In order to evaluate the efficiency of skim milk, or, in the case of herpes simplex virus 2 fetal calf serum, as a neutralizer of disinfectant activity, a neutralized control as described in the procedures and results section was set up with each experiment.

Residual virus in the neutralized control was compared statistically to the titer of the virus used in the experiment. The titer and the neutralized control were prepared at the time the experiment was done. The Student's t test at the 95% confidence level was

the statistical procedure used for the neutralized control, titer comparison.

In Tables 45 to 53 the amount of residual virus in the neutralized control and titer are listed for each experiment done in this thesis. The results of the t test comparisons are listed.

Out of 146 experiments, involving a wide variety of disinfectants and virus combinations, only six had neutralized controls significantly less than the titer using the t test at the 95% confidence level (one tailed test). Of these six experiments, in one the neutralized control was not significantly different from the titer at the 97.5% confidence level, in four the neutralized control was not significantly different from the titer at the 99% confidence level, and in the remaining one instance the neutralized control was not significantly different from the titer at the 99.5% confidence level.

In the experiments involving herpes simplex virus 2, in which fetal calf serum was used as the neutralizer, neutralized controls were significantly less than titer controls in two out of 20 experiments as determined by the t test at the 95% confidence level. Of these two experiments, the neutralized control was not significantly different from the titer when the t test was considered at the 99% level in one instance and at the 99.5% level in the other instance.

These results indicated skim milk was an effective neutralizer of the activity of a wide variety of disinfectants in the testing of a diverse group of viruses. Fetal calf serum was also an effective neutralizer, but less effective than skim milk.

In addition to the neutralized control, another control was set up to test the ability of skim milk (or, in the case of herpes simplex virus 2, fetal calf serum) to neutralize the activity of the disinfectant. The main emphasis in this control, termed the cell susceptibility test control, was to determine whether or not the disinfectant was sufficiently neutralized so that toxicity of the disinfectant did not affect the ability of the cell line used in the plaque assay to be infected by the virus or support virus replication. The cell susceptibility control was prepared in the manner described in the procedures and results section of this thesis. The amount of residual virus in the cell susceptibility control was compared statistically to the virus titer done at the same time, using the t test at the 95% confidence level (one tailed test). The results are listed in Tables 54 to 59.

Out of the 107 instances when skim milk was evaluated as a neutralizer of disinfectant, there were only four cases where the cell susceptibility controls were significantly less than the titer at the 95% level.

Of these four cases, in two the cell susceptibility control did not possess significantly less virus as compared to the titer at the 97.5% level, and in the other two instances this was true at the 99% level and the 99.5% level, respectively.

In herpes simplex virus 2 experiments, where fetal calf serum was used as the neutralizer, cell susceptibility controls were not significantly less than the titer in 18 of 20 instances at the 95% level. In the other two cases, the cell susceptibility control was not significantly less than the titer at the 99% level.

These results further emphasize that skim milk is an effective neutralizer for a wide variety of disinfectant-virus mixtures and inactivated fetal calf serum is a reliable neutralizer for herpes simplex virus 2 exposed to disinfectant. In another study (176), it was proposed that a medium containing Bacto-Tryptone,yeast extract, dextrose, sodium thioglycolate, sodium thiosulphate, sodium bisulfite, Tween 80 and lecithin could be used as a universal neutralizing substance. Previous workers in this laboratory indicated such a medium was not as effective as skim milk in neutralizing the concentrations of disinfectants tested.

This study took a unique approach in testing the virucidal activity of a disinfectant. Skim milk was used successfully in neutralizing a wide variety of disinfectant-virus reactions at the end of ten, 20 and 30 seconds. Six

disinfectants, each representative of a chemically distinct group of disinfectants, were evaluated. They were Sonacide, Wescodyne, sodium hydroxide, Javex, One Stroke Ves-Phene and ethanol, with it being concluded that Javex at a dilution of 1 - 50 (1200 ppm) or less is the most effective disinfectant to use in practical situations. Test viruses of widely differing properties were used in this study. They were herpes simplex virus 2, echovirus 11, coxsackievirus B5, reovirus 3, f2 bacteriophage and vesicular stomatitis virus. In general, nonenveloped viruses were more resistant to inactivation than enveloped viruses, with f2 bacteriophage and echovirus 11 being of equal resistance to inactivation and greater than the other viruses. Vesicular stomatitis virus was grown in the cells from three different animals, with the resistance to inactivation by disinfectants being different for the three VSV preparations. A2 plaque virus, a virus antigenically similar to hepatitis B virus was tested using the six disinfectants previously mentioned and methanol.

CHAPTER V

SUMMARY

SUMMARY

From experimental results obtained by exposure of virus to disinfectant in the presence of fetal calf serum the following conclusions were drawn:

i) In the vast majority of experiments which consisted of residual virus being assayed at varying exposure times to disinfectant, a plot of

$\log_{10}$ PFU/ml versus exposure time (minutes)

yielded a concave type of curve. Such curves were successfully converted to linear relationships by using the square root transformation for exposure time (minutes), thus yielding the relationship

$\log_{10}$ PFU/ml versus square root minutes exposure time

ii) Slopes of such a relationship were used to compare the relative effectiveness of different disinfectants in inactivating a given virus. Disinfectants are listed below in order of decreasing overall effectiveness against the battery of test viruses (f2 bacteriophage, echovirus 11, coxsackievirus B5, VSV, reovirus 3 and herpes simplex virus 2) employed in this thesis

i) 95% ethanol

ii) 0.25% sodium hydroxide

iii) 1-100 Javex

iv) 1-200 Wescodyne

v) undiluted Sonacide

vi) 1-50 Ves-Phene

Due to certain drawbacks in using ethanol and sodium hydroxide previously discussed, for practical purposes Javex, at dilutions less than 1-50, was the most effective disinfectant. Ethanol, sodium hydroxide and Javex were wide spectrum disinfectants, inactivating both enveloped and nonenveloped viruses. Wescodyne, Ves-Phene and Sonacide inactivated enveloped viruses to a greater extent than nonenveloped viruses.

iii) Slopes of such a relationship were used to compare the relative resistance of different viruses to a given disinfectant. Overall rankings of viral resistance to the group of disinfectants tested, in order of decreasing resistance were as follows:

i) f2 bacteriophage and echovirus 11
ii) reovirus 3
iii) coxsackievirus B5
iv) herpes simplex virus 2
v) VSV/BKC

with f2 bacteriophage and echovirus 11 being of the same overall resistance to the group of disinfectants, and more so than the other viruses considered in the study.

Enveloped viruses were generally more susceptible to inactivation by disinfectants than nonenveloped viruses, although the reverse was true when sodium hydroxide was employed.

The cell type in which VSV was grown was a significant factor in determining its suscept bility to disinfectant inactivation.

iv) The validity of extrapolating the relationship

$\log_{10}$ PFU/ml versus square root minutes exposure time

to a point where the exposure time was such that zero residual virus would be predicted was tested for considering coxsackievirus B5 and reovirus 3. Since residual virus was consistently found when none was predicted, this indicated prediction of zero residual virus by extrapolation was not justified.

v) A2 plaque virus,reported to be antigenically related to hepatitis B virus, was exposed for one and ten minutes to disinfectants in the presence of fetal calf serum. Results indicated:

A 1-50 dilution of One Stroke Ves-Phene, Wescodyne and 1% w/v sodium hydroxide failed to completely inactivate the samples of a common pool of A2 plaque virus in ten minutes. The same was true for undiluted Sonacide.

A 1-50 concentration of Javex totally inactivated A2 plaque virus in a ten minute but not a one minute exposure time.

A solution of 80% methanol was the only disinfectant tested that consistently inactivated A2 plaque virus completely in one minute.

100% methanol and 95% ethanol completely inactivated A2 plaque virus in a ten minute exposure time, but were not consistently effective in a one minute exposure time.

vi) Ten per cent and 17.5 per cent skim milk were demonstrated to be effective neutralizers of the toxicity of a wide variety of disinfectants used in testing a highly diverse group of viruses.

In the case of herpes simplex virus 2, fetal calf serum was an adequate neutralizer of disinfectant activity.

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CHAPTER VI

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CHAPTER VII

APPENDICES

APPENDIX 1

R3 Medium

Cell line stocks in glass bottles and monolayers in tissue culture dishes were maintained in R3 medium. R3 medium in certain instances was used as a virus diluent in the tenfold serial dilutions of the plaque assay.

R3 was prepared aseptically in 10,000 ml amounts and dispensed in 500 ml quantities.

R3 consisted of the following:

Component	Amount
Medium 199 (10X Schwartz/Mann)	820 ml
MEM amino acids (50X Gibco)	164 ml
MEM vitamins (100X Gibco)	82 ml
MEM non-essential amino acids (100X, Gibco)	82 ml
MEM l-glutamine (100X)	82 ml
Sodium pyrurate (100X Gibco)	100 ml
Tryptose phosphate broth (Difco)	1000 ml
Bovine Serum (Connaught Lab., Heat inactivated at 57°C for 30 minutes)	800 ml
Sterile distilled water made up to	10000 ml

After being dispensed in 500 ml quantities, a five ml volume of the penicillin, streptomycin, and Amphotericin B mixture was added to each bottle.

Before use, 5.5 ml of 7.5 per cent sodium bicarbonate solution was added to 500 ml of medium and the pH was adjusted to pH 7.3.

APPENDIX 2

Minimal Essential Medium (MEM) Overlay

34.8 grams of MEM Auto-Pow powder (Flow Laboratories) was added to 1850 ml of distilled water. This MEM preparation was autoclaved for 30 minutes at 121°C.

The entire 1850 ml volume of MEM was mixed with 368 ml of heat inactivated fetal calf serum, 70 ml of glutamine solution and 46 ml of PSF (penicillin, streptomycin, fungizone) solution and aseptically dispensed in 57 ml quantities.

The overlay was stored at -20°C and thawed before use.

APPENDIX 3

MEM Glutamine Solution (100X)

29.2 grams of L-glutamine was added to a liter of distilled water. This solution was filtered, using a 0.22 μ millipore filter. The L-glutamine solution was stored at -20°C and thawed before use.

APPENDIX 4

Penicillin, Streptomycin, Fungizone (PSF) Solution

The PSF solution prepared aseptically contained the following:

100 units of penicillin/ ml of solution

100 μg of streptomycin/ ml of solution

2.5 μg of Amphotericin B (fungizone/ ml of solution

APPENDIX 5

Tryptone Agar

Tryptone overlay and plating agars were prepared by adding seven and 15 grams per liter of Bacto-agar respectively to tryptone broth. Tryptone broth consisted of Bacto-tryptone ten grams per liter, Bacto-yeast extract ten grams per liter, glucose one gram per liter, NaCl eight grams per liter, and $CaCl_2$ 0.22 grams per liter (222).

APPENDIX 6

Skim Milk Neutralizer

A solution of 17.5 per cent skim milk was prepared in two liter lots by gradually adding 350 grams of skim milk powder to 1600 ml of distilled water in a volumetric flask. This was made up to two liters exactly. Then it was stirred well and autoclaved for exactly 20 minutes at 121°C. An additional ten minute time period was allowed for the autoclave to depressurize. Ten per cent skim milk was prepared in a similar manner. Carnation brand skim milk was used.

APPENDIX 7

Nutrient Broth

Eight grams of Difco nutrient broth powder and five grams of NaCl were added to one liter of distilled water. This was autoclaved at 121°C for 30 minutes.

APPENDIX 8

Dulbecco's Phosphate Buffer Solution (PBS) pH 7.5

Components:

Solution A (10X)

NaCl		80.0 grams
KCl		2.0 grams
Na_2HPO_4 (anhydrous)	Dibasic	11.5 grams
KH_2PO_4	Monobasic	2.0 grams

The solution was made up to one liter by adding distilled water.

Solution B (100X)

$CaCl_2$ 2.5 grams

The solution was made up to 250 ml by adding distilled water.

Solution C (100X)

$MgCl_2$ 2.5 grams

The solution was made up to 250 ml by adding distilled water.

Solutions A, B and C were stored at room temperature.

PBS-A Preparation

A volume of 100 ml of solution A was placed in a one liter flask about half full with distilled deionized water. If a pH indicator was desired, four ml of phenol red was added. The contents of the flask were made to one liter with distilled deionized water. The solution was dispensed in 500 ml quantities and autoclaved at 121°C for 30 minutes.

PBS Preparation

A 100 ml volume of solution A was added to a one liter volumetric flask, which was subsequently filled about three quarters full with distilled deionized water. A ten ml volume of each of solutions B and C were slowly added to the flask with stirring. The volume in the flask was made up to one liter with distilled deionized water.

The PBS was sterilized by filtering through an 0.22 µ millipore filter. PBS should not be autoclaved.

APPENDIX 9

1.8% Bacto-agar

1.8% Bacto-agar was prepared by mixing 18 grams of Difco Bacto-agar with one liter of distilled water. This solution was autoclaved at 121°C for 20 minutes and subsequently dispensed in 46 ml amounts.

APPENDIX 10

199 Medium

1.11 grams of Gibco 199 Medium powder was added to one liter of distilled water. The medium was filtered through a 0.22 µ millipore membrane.

APPENDIX 11

Neutral Red Agar

Initially, a one per cent solution of neutral red was prepared. It was filtered through a 33 cm Grade 230 Reeve Angel filter paper into a one liter reagent bottle

and stored in the cold room. Neutral red agar consisted of:

100 ml	one per cent neutral red solution
800 ml	distilled water
100 ml	10X PBS-A solution
9 grams	Difco Bacto-agar

This mixture was boiled to dissolve the agar and dispensed in 200 ml quantities.

APPENDIX 12

Table 25

Determination of the Fit of Different Echovirus 11 Versus Exposure Time Relationships to a Linear Function [a]

DISINFECTANT	TRIAL	Echovirus 11: $\log_{10}$ PFU/ml vs MINUTES			Echovirus 11: $\log_{10}$ PFU/ml vs $\sqrt{MINUTES}$		
		r^2 [d]	df [e]	F	r^2 [d]	df [e]	F
UNDILUTED SONACIDE	1	0.959	1,20	469.986	0.806	1,20	81.497
	2	0.865	1,20	127.852	0.869	1,20	132.80
0.25% NaOH	1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D. [b]
	2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
1-50 VES-PHENE	1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D. [c]
	2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
1-350 WESCODYNE	1	0.803	1,25	101.743	0.814	1,25	109.528
	2	0.568	1,23	30.255	0.668	1,23	46.191
1-200 WESCODYNE	1	0.681	1,17	36.337	0.912	1,17	176.391
	2	0.596	1,19	28.076	0.905	1,19	181.579
1-50 WESCODYNE	1	0.627	1,20	33.573	0.976	1,20	805.422
	2	0.545	1,20	23.968	0.885	1,20	154.230
1-100 JAVEX	1	0.713	1,20	49.781	0.952	1,20	393.883
	2	0.574	1,26	35.001	0.965	1,26	710.601
1-75 JAVEX	1	0.595	1,17	25.023	0.930	1,17	224.878
	2	0.649	1,18	33.267	0.954	1,18	373.154
1-50 JAVEX	1	0.563	1,19	24.501	0.996	1,19	4259.250
	2	0.535	1,20	23.051	0.987	1,20	1489.046
95% ETHANOL	1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D. [b]
	2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
70% ETHANOL	1	0.724	1,20	52.562	0.628	1,20	33.769
	2	0.767	1,19	62.536	0.669	1,19	38.359

a - as determined by analysis of regression
b - N.D. denotes not done, due to a rapid viral inactivation yielding a relationship of less than three numerical points
c - N.D. denotes not done, due to no significant viral inactivation taking place
d - correlation coefficient squared
e - degrees of freedom for the F values

Table 26

Determination of the Fit of Different Coxsackievirus B5 Versus Exposure Time Relationships to a Linear Function [a]

DISINFECTANT	TRIAL	Coxsackievirus B5: $\log_{10}$ PFU/ml vs MINUTES			Coxsackievirus B5: $\log_{10}$ PFU/ml vs $\sqrt{\text{MINUTES}}$		
		r^2 [d]	df [e]	F	r^2 [d]	df [e]	F
UNDILUTED SONACIDE	1	0.763	1,23	74.109	0.647	1,23	42.216
	2	0.824	1,16	74.655	0.858	1,16	96.366
1-75 JAVEX	1	0.513	1,20	21.054	0.967	1,20	577.910
	2	0.562	1,17	21.827	0.974	1,17	627.739
1-100 JAVEX	1	0.517	1,23	24.580	0.990	1,23	2171.55
	2	0.585	1,17	23.960	0.964	1,17	449.916
0.25% NaOH	1	0.532	1,17	19.314	0.926	1,17	211.341
	2	0.521	1,20	21.723	0.967	1,20	582.474
1-200 WESCODYNE	1	0.549	1,17	20.667	0.903	1,17	157.885
	2	0.519	1,20	21.578	0.909	1,20	199.667
1-350 WESCODYNE	1	0.646	1,16	29.153	0.950	1,16	302.153
	2	0.586	1,20	28.260	0.844	1,20	108.465
1-50 VES-PHENE	1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D. [b]
	2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
95% ETHANOL	1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D. [c]
	2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
70% ETHANOL	1	0.749	1,19	56.838	0.713	1,19	47.120
	2	0.708	1,20	48.440	0.546	1,20	24.092

a - as determined by analysis of regression
b - N.D. denotes not done, due to no significant viral inactivation taking place
c - N.D. denotes not done, due to a rapid viral inactivation yielding a relationship of less than three numerical points
d - correlation coefficient squared
e - degrees of freedom for the F value

Table 27

Determination of the Fit of Different f2 Bacteriophage Versus Exposure Time Relationships To A Linear Function[a]

DISINFECTANT	TRIAL	f2 Bacteriophage: $\log_{10}$ PFU/ml vs MINUTES			f2 Bacteriophage: $\log_{10}$ PFU/ml vs $\sqrt{\text{MINUTES}}$		
		r^2 [c]	df [d]	F	r^2 [c]	df [d]	F
UNDILUTED SONACIDE	1	0.677	1,14	29.336	0.931	1,14	188.393
	2	0.790	1,14	52.684	0.876	1,14	98.922
1-20 WESCODYNE	1	0.545	1,20	23.882	0.836	1,20	101.822
	2	0.505	1,20	20.393	0.811	1,20	85.903
1-20 VES-PHENE	1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D. [b]
	2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
95% ETHANOL	1	0.543	1,18	21.413	0.894	1,18	152.597
	2	0.648	1,17	31.232	0.882	1,17	126.993
1-100 JAVEX	1	0.499	1,25	24.892	0.865	1,25	160.321
	2	0.510	1,14	14.550	0.807	1,14	58.658
1-50 JAVEX	1	0.620	1,14	21.193	0.946	1,14	245.472
	2	0.594	1,14	20.503	0.943	1,14	230.786
0.25% NaOH	1	0.582	1,14	19.498	0.916	1,14	152.004
	2	0.589	1,14	20.074	0.849	1,14	78.948

a - as determined by analysis of regression
b - N.D. denotes not done, due to no significant viral inactivation taking place
c - correlation coefficient squared
d - degrees of freedom for the F value

Table 28

Determination of the Fit of Different Reovirus 3 Versus Exposure Time Relationships to a Linear Function [a]

		Reovirus 3					
		log_{10} PFU/ml vs MINUTES			log_{10} PFU/ml vs /MINUTES		
DISINFECT-ANT	TRIAL	r^2 [c]	df [d]	F	r^2 [c]	df [d]	F
UNDILUTED SONACIDE	1	0.502	1,20	20.196	0.954	1,20	416.439
	2	0.632	1,18	30.971	0.970	1,18	581.408
0.25% NaOH	1	0.474	1,25	22.513	0.837	1,25	128.590
	2	0.485	1,20	18.840	0.873	1,20	137.909
1-50 VES-PHENE	1	0.744	1,20	58.183	0.867	1,20	130.830
	2	0.587	1,17	24.169	0.851	1,17	96.864
1-350 WESCODYNE	1	0.489	1,24	22.929	0.800	1,24	95.988
	2	0.479	1,23	21.156	0.808	1,23	96.887
1-200 WESCODYNE	1	0.558	1,17	21.483	0.777	1,17	59.308
	2	0.523	1,23	25.179	0.967	1,23	673.922
1-100 JAVEX	1	0.479	1,23	21.106	0.811	1,23	98.664
	2	0.469	1,26	23.003	0.837	1,26	133.595
1-75 JAVEX	1	0.630	1,17	28.912	0.993	1,17	2478.065
	2	0.733	1,12	32.480	0.932	1,12	163.594
95% ETHANOL	1	0.445	1,25	20.070	0.987	1,25	1927.693
	2	0.499	1,20	19.926	0.898	1,20	176.784
70% ETHANOL	1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D. [b]
	2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

a - as determined by analysis of regression
b - N.D. denotes not done, due to no significant viral inactivation taking place
c - correlation coefficient squared
d - degrees of freedom for the F value

Table 29

Determination of the Fit of Different Herpes Simplex Virus 2 Versus Exposure Time Relationships To A Linear Function [a]

		Herpes Simplex Virus 2					
		$\log_{10}$ PFU/ml vs MINUTES			$\log_{10}$ PFU/ml vs $\sqrt{\text{MINUTES}}$		
DISINFECTANT	TRIAL	r^2 [c]	df [d]	F	r^2 [c]	df [d]	F
1-200 JAVEX	1	0.550	1,26	31.771	0.890	1,26	210.812
	2	0.579	1,16	21.999	0.872	1,16	109.202
1-100 JAVEX	1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D. [b]
	2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
UNDILUTED SONACIDE	1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D. [b]
	2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
1-350 VES-PHENE	1	0.524	1,19	20.917	0.874	1,19	131.916
	2	0.520	1,20	21.673	0.866	1,20	128.982
0.25% NaOH	1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D. [b]
	2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
1-350 WESCODYNE	1	0.557	1,16	20.145	0.899	1,16	143.007
	2	0.473	1,25	22.403	0.869	1,25	165.116
95% ETHANOL	1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D. [b]
	2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
70% ETHANOL	1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D. [b]
	2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

a - as determined by analysis of regression
b - N.D. denotes not done, due to a rapid viral inactivation yielding a relationship of less than three numerical points
c - correlation coefficient squared
d - degrees of freedom for the F value

Table 30

Determination of the Fit of Different Bovine Kidney Cell Grown Vesicular Stomatitis Virus versus Exposure Time Relationships To A Linear Function

DISINFECTANT	TRIAL	Bovine Kidney Cell Grown Vesicular Stomatitis Virus					
		$\log_{10}$ PFU/ml vs MINUTES			$\log_{10}$ PFU/ml vs $\sqrt{MINUTES}$		
		r^2 [c]	df [d]	F	r^2 [c]	df [d]	F
1-350 WESCODYNE	1	0.445	1,26	20.873	0.932	1,26	356.379
	2	0.399	1,26	16.566	0.908	1,26	255.130
1-350 VES-PHENE	1	0.494	1,25	24.374	0.958	1,25	565.738
	2	0.398	1,26	17.176	0.882	1,26	193.587
0.25% NaOH	1	0.464	1,26	22.546	0.902	1,26	238.773
	2	0.473	1,23	20.651	0.902	1,23	212.551
1-100 JAVEX	1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D. [b]
	2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
UNDILUTED SONACIDE	1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D. [b]
	2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
95% ETHANOL	1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D. [b]
	2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
70% ETHANOL	1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D. [b]
	2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

a - as determined by analysis of regression
b - N.D. denotes not done, due to a rapid viral inactivation yielding a relationship of less than three numerical points
c - correlation coefficient squared
d - degrees of freedom for the F value

Table 31

Determination of the Fit of Different Chick Embryo Fibroblast Grown Vesicular Stomatitis Virus Versus Exposure Time Relationships To A Linear Function [a]

DISINFECTANT	TRIAL	CHICK EMBRYO FIBROBLAST GROWN VESICULAR STOMATITIS VIRUS					
		$\log_{10}$ PFU/ml vs MINUTES			$\log_{10}$ PFU/ml vs $\sqrt{MINUTES}$		
		r^2 [b]	df [c]	F	r^2 [b]	df [c]	F
1-100 JAVEX	1	0.494	1,23	22.417	0.942	1,23	375.013
	2	0.517	1,21	22.459	0.937	1,21	314.474
1-350 VES PHENE	1	0.584	1,20	28.027	0.905	1,20	190.145
	2	0.543	1,20	23.758	0.840	1,20	105.254
0.25% NaOH	1	0.514	1,23	24.324	0.881	1,23	170.816
	2	0.514	1,16	16.910	0.894	1,16	134.456
1-350 WESCODYNE	1	0.554	1,20	24.881	0.899	1,20	177.368
	2	0.465	1,26	22.558	0.8951	1,26	222.492

a - as determined by analysis of regression
b - correlation coefficient squared
c - degrees of freedom for the F value

APPENDIX 13

Table 32

Reproducibility of the $\log_{10}$ PFU/ml versus square root minutes relationship for Echovirus 11 Inactivation [a]

DISIN-FECTANT	TRIAL	N[g]	INDEPENDENT VARIABLE[d]		DEPENDENT VARIABLE[e]		SLOPE	df	F
			Mean	S.D.[f]	Mean	S.D.[f]			
1-100 JAVEX	1 2	22 28	0.462 0.484	0.249 0.231	1.83 1.55	0.464 0.492	-1.815 -2.092	1,46	5.220[b]
1-75 JAVEX	1 2	19 20	0.446 0.459	0.261 0.261	1.20 1.65	0.844 0.720	-3.121 -2.698	1,35	3.018
1-50 JAVEX	1 2	21 22	0.464 0.462	0.255 0.249	2.53 2.32	1.25 1.29	-4.904 -5.143	1,39	3.013
0.25% NaOH	1 2	N.D. N.D.	N.D. N.D.	N.D. N.D.	N.D. N.D.	N.D. N.D.	N.D. N.D.	N.D.	N.D.[h]
UNDILUTED SONACIDE	1 2	22 22	0.462 0.462	0.249 0.249	1.60 1.34	0.222 0.208	-0.799 -0.778	1,40	0.035
1-350 WESCODYNE	1 2	22 25	0.461 0.474	0.249 0.239	1.28 1.37	0.478 0.189	-0.875 -0.645	1,43	0.372
1-200 WESCODYNE	1 2	19 21	0.446 0.450	0.261 0.249	1.90 1.89	0.412 0.431	-1.506 -1.645	1,36	0.676
1-30 WESCODYNE	1 2	22 22	0.462 0.462	0.249 0.249	1.78 1.92	0.478 0.442	-1.895 -1.667	1,40	2.230
1-50 VES-PHENE	1 2	N.D. N.D.	N.D. N.D.	N.D. N.D.	N.D. N.D.	N.D. N.D.	N.D. N.D.	N.D.	N.D.[c]
95% ETHANOL	1 2	N.D. N.D.	N.D. N.D.	N.D. N.D.	N.D. N.D.	N.D. N.D.	N.D. N.D.	N.D.	N.D.[h]
70% ETHANOL	1 2	22 21	0.462 0.457	0.249 0.254	1.57 1.50	0.367 0.282	-1.167 -0.906	1,39	1.085

a - as determined by comparison of slopes at the 95% confidence level
b - significant at the 95% but not the 99% confidence level
c - N.D. denotes not done, due to no significant viral inactivation taking place
d - square root minutes exposure time
e - $\log_{10}$ PFU/ml
f - standard deviation
g - total number of experimental observations
h - N.D. denotes not done due to a rapid inactivation yielding a relationship of less than three numerical points

Table 33

Reproducibility of the $\log_{10}$ PFU/ml versus Square Root Minutes Relationship for Coxsackievirus B5 Inactivation[a]

			Coxsackievirus B5							
DISIN-FECTANT	TRIAL	N[e]	INDEPENDENT VARIABLE[b]		DEPENDENT VARIABLE[c]		SLOPE	df	F
			MEAN	S.D.[d]	MEAN	S.D.[d]			
1-100 JAVEX	1 2	25 19	0.474 0.446	0.239 0.261	2.28 1.51	1.05 1.08	-4.374 -4.073	1,40	2.648
1-75 JAVEX	1 2	22 19	0.462 0.446	0.249 0.261	1.58 2.16	1.72 1.65	-6.791 -9.258	1,37	2.166
0.25% NaOH	1 2	19 22	0.446 0.462	0.261 0.249	1.04 1.78	2.05 1.75	-7.565 -6.922	1,37	1.306
UNDILUTED SONACIDE	1 2	25 18	0.474 0.448	0.239 0.268	1.93 1.98	0.209 0.190	-0.703 -0.655	1,39	0.126
1-350 WESCODYNE	1 2	18 22	0.448 0.462	0.268 0.249	2.39 1.81	0.446 0.532	-1.621 -1.961	1,36	2.363
1-200 WESCODYNE	1 2	19 22	0.446 0.462	0.261 0.249	1.33 0.954	2.01 1.95	-7.336 -7.449	1,37	0.021
1-50 VES-PHENE	1 2	N.D. N.D.	N.D. N.D.	N.D. N.D.	N.D. N.D.	N.D. N.D.	N.D. N.D.	N.D.	N.D.[f]
95% ETHANOL	1 2	N.D. N.D.	N.D. N.D.	N.D. N.D.	N.D. N.D.	N.D. N.D.	N.D. N.D.	N.D.	N.D.[g]
70% ETHANOL	1 2	21 22	0.450 0.462	0.249 0.249	2.68 2.75	1.47 1.41	-4.992 -4.166	1,39	0.537

a - as determined by comparison of slopes at the 95% confidence level
b - square root exposure time minutes
c - $\log_{10}$ PFU/ml
d - standard deviation
e - total number of experimental observations
f - N.D. denotes not done, due to no significant viral inactivation taking place
g - N.D. denotes not done, due to a rapid viral inactivation yielding a relationship of less than three numerical points

Table 34

Reproducibility of the $\log_{10}$ PFU/ml Versus Square Root Minutes Relationship for f2 Bacteriophage Inactivation[a]

DISIN-FECTANT	TRI-AL	f2 BACTERIOPHAGE: N[e]	INDEPENDENT VARIABLE[b] MEAN	INDEPENDENT VARIABLE[b] S.D.[d]	DEPENDENT VARIABLE[c] MEAN	DEPENDENT VARIABLE[c] S.D.[d]	SLOPE	df	F
1-20 WESCODYNE	1	22	0.461	0.249	2.62	0.35	-1.28	1,40	2.063
	2	22	0.461	0.249	2.65	0.44	-1.59		
1-20 VES-PHENE	1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.[f]
	2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.		
95% ETHANOL	1	20	0.421	0.244	1.50	1.20	-4.65	1,35	2.190
	2	19	0.446	0.261	1.97	1.08	-3.91		
UNDILUTED SONACIDE	1	16	0.424	0.275	1.38	0.535	-1.878	1,28	0.543
	2	16	0.424	0.275	1.25	0.504	-1.717		
1-100 JAVEX	1	27	0.487	0.235	1.90	0.513	-2.031	1,39	0.222
	2	16	0.423	0.275	2.19	0.664	-2.171		
1-50 JAVEX	1	16	0.424	0.275	2.05	1.90	-6.735	1,28	1.823
	2	16	0.424	0.275	2.56	1.69	-5.988		
0.25% NaOH	1	16	0.424	0.275	2.00	1.95	-6.795	1,28	1.374
	2	16	0.424	0.275	2.31	1.73	-5.805		

a - as determined by comparison of slopes at the 95% confidence level
b - square root minutes exposure time
c - $\log_{10}$ PFU/ml
d - standard deviation
e - total number of experimental observations
f - N.D. denotes not done, due to no significant viral inactivation taking place

Table 35

Reproducibility of the $\log_{10}$ PFU/ml Versus Square Root Minutes Relationship for Reovirus 3 Inactivation [a]

		Reovirus 3							
			INDEPENDENT VARIABLE [c]		DEPENDENT VARIABLE [d]				
DISIN-FECTANT	TRI-AL	N [f]	MEAN	S.D. [e]	MEAN	S.D. [e]	SLOPE	df	F
1-100 JAVEX	1	25	0.474	0.239	2.13	0.729	-2.740	1,49	3.632
	2	28	0.484	0.231	2.60	0.542	-2.075		
1-75 JAVEX	1	19	0.385	0.217	1.39	1.88	-8.617	1,29	3.261
	2	14	0.352	0.242	1.76	1.90	-7.588		
0.25% NaOH	1	27	0.476	0.231	1.90	0.814	-3.221	1,45	0.056
	2	22	0.462	0.249	1.78	0.885	-3.317		
UNDILUTED SONACIDE	1	22	0.449	0.243	2.81	0.969	-3.900	1,38	0.095
	2	20	0.467	0.262	1.43	1.05	-3.970		
1-350 WESCODYNE	1	26	0.483	0.239	2.43	0.609	-2.280	1,47	0.015
	2	25	0.474	0.239	2.40	0.597	-2.240		
1-200 WESCODYNE	1	19	0.446	0.261	1.83	0.982	-3.320	1,40	0.150
	2	25	0.474	0.239	2.00	0.770	-3.161		
1-50 VES-PHENE	1	22	0.462	0.249	1.52	0.483	-1.810	1,37	1.605
	2	19	0.439	0.255	2.15	0.423	-1.530		
95% ETHANOL	1	27	0.481	0.235	1.71	1.67	-7.055	1,45	0.266
	2	22	0.462	0.249	1.57	1.93	-7.337		
70% ETHANOL	1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D. [b]
	2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.		

a - as determined by comparison of slopes at the 95% confidence level
b - N.D. denotes not done, due to no significant viral inactivation taking place in this case
c - square root minutes exposure time
d - $\log_{10}$ PFU/ml
e - standard deviation
f - total numbers of experimental observations

Table 36

Reproducibility of the $\log_{10}$ PFU/ml Versus Square Root Minutes Relationship for Herpes Simplex Virus 2 Inactivation[a]

DISIN-FECTANT	TRI-AL	N[f]	Herpes Simplex Virus 2: INDEPENDENT VARIABLE[b] MEAN	INDEPENDENT VARIABLE[b] S.D.[d]	DEPENDENT VARIABLE[c] MEAN	DEPENDENT VARIABLE[c] S.D.[d]	SLOPE	df	F
1-200 JAVEX	1	28	0.484	0.231	1.53	0.589	-2.403	1,42	1.864
	2	18	0.431	0.260	1.34	0.784	-2.812		
1-100 JAVEX	1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.[e]
	2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.		
UNDILUTED SONACIDE	1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.[e]
	2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.		
1-350 VES-PHENE	1	21	0.450	0.249	1.89	1.00	-3.765	1,39	0.010
	2	22	0.462	0.249	1.75	1.02	-3.812		
0.25% NaOH	1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.[e]
	2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.		
1-350 WESCODYNE	1	18	0.448	0.268	2.44	0.744	-2.629	1,41	0.413
	2	27	0.481	0.235	2.55	0.616	-2.443		
95% ETHANOL	1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.[e]
	2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.		
70% ETHANOL	1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.[e]
	2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.		

a - as determined by comparison of slopes at the 95% confidence level
b - square root minutes exposure time
c - $\log_{10}$ PFU/ml
d - standard deviation
e - N.D. denotes not done, due to a rapid viral inactivation yielding a relationship of less than three numerical points
f - total number of experimental observations

Table 37

Reproducibility of the $\log_{10}$ PFU/ml versus square root minutes Relationship for Bovine Kidney Cell Grown Vesicular Stomatitis Virus Inactivation [a]

DISIN-FECTANT	TRI-AL	N [f]	BOVINE KIDNEY CELL GROWN VESICULAR STOMATITIS VIRUS: INDEPENDENT VARIABLE [b] MEAN	INDEPENDENT VARIABLE [b] S.D. [e]	DEPENDENT VARIABLE [c] MEAN	DEPENDENT VARIABLE [c] S.D. [e]	SLOPE	df	F
1-100 JAVEX	1 2	N.D. N.D.	N.D. N.D.	N.D. N.D.	N.D. N.D.	N.D. N.D.	N.D. N.D.	N.D.	N.D. [d]
1-350 VES-PHENE	1 2	27 28	0.510 0.484	0.249 0.231	1.47 2.02	1.18 0.988	-4.638 -4.014	1,51	3.236
0.25% NaOH	1 2	28 25	0.484 0.474	0.231 0.239	1.03 1.19	1.28 1.44	-5.242 -5.727	1,49	0.857
1-350 WESCODYNE	1 2	28 28	0.484 0.530	0.231 0.252	0.910 1.04	0.98 1.19	-4.097 -4.503	1,52	1.287
UNDILUTED SONACIDE	1 2	N.D. N.D.	N.D. N.D.	N.D. N.D.	N.D. N.D.	N.D. N.D.	N.D. N.D.	N.D.	N.D. [d]
95% ETHANOL	1 2	N.D. N.D.	N.D. N.D.	N.D. N.D.	N.D. N.D.	N.D. N.D.	N.D. N.D.	N.D.	N.D. [d]
70% ETHANOL	1 2	N.D. N.D.	N.D. N.D.	N.D. N.D.	N.D. N.D.	N.D. N.D.	N.D. N.D.	N.D.	N.D. [d]

a - as determined by comparison of slopes at the 95% confidence level
b - square root minutes exposure time
c - $\log_{10}$ PFU/ml
d - N.D. denotes not done, due to a rapid viral inactivation yielding a relationship of less than ~~three~~ numerical points
e - S.D., standard deviation
f - total number of experimental observations

Table 38

Reproducibility of the log10 PFU/ml versus square root minutes Relationship for Chick Embryo Fibroblast Grown Vesicular Stomatitis Virus Inactivation [a]

		CHICK EMBRYO FIBROBLAST GROWN VESICULAR STOMATITIS VIRUS							
			INDEPENDENT VARIABLE [b]		DEPENDENT VARIABLE [c]				
DISIN-FECTANT	TRI-AL	N[e]	MEAN	S.D.[d]	MEAN	S.D.[d]	SLOPE	df	F
1-100 JAVEX	1	25	0.474	0.240	1.73	1.56	-6.318	1,44	0.059
	2	23	0.473	0.249	0.715	1.66	-6.438		
1-350 VES-PHENE	1	22	0.462	0.249	2.42	0.304	-1.160	1,40	0.794
	2	22	0.462	0.249	2.30	0.[illegible]53	-1.297		
0.25% NaOH	1	25	0.474	0.239	2.53	0.529	-2.072	1,39	1.991
	2	18	0.439	0.267	2.65	0.686	-2.434		
1-350 WESCODYNE	1	22	0.475	0.254	2.76	1.10	-4.096	1,46	2.952
	2	28	0.484	0.231	2.13	0.840	-3.440		

a - as determined by comparison of slopes at the 95% confidence level
b - square root minutes exposure time
c - log10 PFU/ml
d - standard deviation
e - total number of experimental observations

APPENDIX 14

Table 39

Mean Slope Data for Coxsackievirus B5 Inactivation Regressions

		Coxsackievirus B5				
DISINFECTANT	N[c]	MEAN INDEPENDENT VARIABLE[a]		MEAN DEPENDENT VARIABLE[b]		MEAN SLOPE
		MEAN[d]	S.D.	MEAN[d]	S.D.	
UNDILUTED SONACIDE	22	0.461	0.254	1.960	.199	-0.679
1-75 JAVEX	21	0.454	0.255	1.875	1.685	-8.025
1-100 JAVEX	22	0.460	0.250	1.895	1.07	-4.224
0.25% NaOH	21	0.454	0.255	1.41	1.900	-7.244
1-200 WESCODYNE	21	0.454	0.255	1.142	1.98	-7.393
1-350 WESCODYNE	20	0.455	0.259	2.10	.489	-1.791
1-50 VES-PHENE	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.[e]
95% ETHANOL	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
70% ETHANOL	22	0.456	0.249	2.72	1.44	-4.579

a - square root minutes exposure time
b - $\log_{10}$ PFU/ml
c - number of experimental observations
d - all means are based on two trials
e - N.D. - not done

Table 40

Mean Slope Data for f2 Bacteriophage Inactivation Regressions

DISINFECTANT	N [c]	MEAN INDEPENDENT VARIABLE [a]		MEAN DEPENDENT VARIABLE [b]		MEAN SLOPE
		MEAN [d]	S.D.	MEAN [d]	S.D.	
1-20 WESCODYNE	22	0.461	0.249	2.64	0.40	-1.435
1-20 VES-PHENE	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.[e]
95% ETHANOL	20	0.434	0.253	1.74	1.14	-4.280
UNDILUTED SONACIDE	16	0.424	0.275	1.32	0.520	-1.798
1-100 JAVEX	22	0.455	0.255	2.05	0.589	-2.101
1-50 JAVEX	16	0.424	0.275	2.35	1.78	-6.361
0.25% NaOH	16	0.424	0.275	2.16	1.84	-6.300

(All columns under the spanning header: f2 Bacteriophage)

a - square root minutes exposure time
b - $\log_{10}$ PFU/ml
c - number of experimental observations
d - means were based on two experimental trials
e - not done

Table 41

Mean Slope Data for Reovirus 3 Inactivation Regressions

		Reovirus 3				
DISINFECTANT	N [c]	MEAN INDEPENDENT VARIABLE [a]		MEAN DEPENDENT VARIABLE [b]		MEAN SLOPE
		MEAN [d]	S.D.	MEAN [d]	S.D.	
1-100 JAVEX	27	0.479	0.235	2.365	0.636	-2.408
1-75 JAVEX	17	0.369	0.230	1.580	1.89	-8.103
0.25% NaOH	25	0.469	0.240	1.840	0.850	-3.269
UNDILUTED SONACIDE	21	0.458	0.253	2.12	1.01	-3.935
1-350 WESCODYNE	26	0.479	0.239	2.42	0.603	-2.260
1-200 WESCODYNE	22	0.460	0.250	1.92	0.876	-3.241
1-50 VES-PHENE	21	0.451	0.252	1.84	0.453	-1.670
95% ETHANOL	25	0.471	0.242	1.64	1.80	-7.191
70% ETHANOL	N.D.	N.D.	N.D.	N.D.	N.D.	N.D. [e]

a - square root minutes exposure time
b - log_{10} PFU/ml
c - number of experimental observations
d - means were based on two experimental trials
e - not done

Table 42

Mean Slope Data for Herpes Simplex Virus 2 Inactivation Regressions

		Herpes Simplex Virus 2				
DISINFECTANT	N [c]	MEAN INDEPENDENT VARIABLE [a]		MEAN DEPENDENT VARIALLE [b]		MEAN SLOPE
		MEAN [d]	S.D.	MEAN [d]	S.D.	
1-200 JAVEX	23	0.458	0.246	1.435	0.687	-2.608
1-100 JAVEX	N.D.	N.D.	N.D.	N.D.	N.D.	N.D. [e]
UNDILUTED SONACIDE	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
1-350 VES-PHENE	22	0.465	0.249	1.82	1.01	-3.789
0.25% NaOH	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
1-350 WESCODYNE	23	0.465	0.252	2.495	0.680	-2.536
95% ETHANOL	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
70% ETHANOL	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

a - square root minutes exposure time
b - $\log_{10}$ PFU/ml
c - number of experimental observations
d - means were based on two experimental trials
e - not done

Table 43

Mean Slope Data for Bovine Kidney Cell Grown Vesicular Stomatitis Virus Inactivation Regressions

DISINFECTANT	Bovine Kidney Cell Grown Vesicular Stomatitis Virus					
	N [c]	MEAN INDEPENDENT VARIABLE [a]		MEAN DEPENDENT VARIABLE [b]		MEAN SLOPE
		MEAN [d]	S.D.	MEAN [d]	S.D.	
1-100 JAVEX	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.[e]
1-350 VES-PHENE	28	0.497	0.240	1.75	1.08	-4.326
0.25% NaOH	27	0.479	0.235	1.11	1.36	-5.485
1-350 WESCODYNE	28	0.507	0.242	0.975	1.09	-4.300
UNDILUTED SONACIDE	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
95% ETHANOL	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
70% ETHANOL	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

a - square root minutes exposure time
b - $\log_{10}$ PFU/ml
c - number of experimental observations
d - means were based on two experimental trials
e - not done

Table 44

Mean Slope Data for Chick Embryo Fibroblast Grown Vesicular Stomatitis Virus Inactivation Regressions

	Chick Embryo Fibroblast Grown Vesicular Stomatitis Virus					
		MEAN INDEPENDENT VARIABLE [a]		MEAN DEPENDENT VARIABLE [b]		MEAN SLOPE
DISINFECTANT	N [c]	MEAN [d]	S.D.	MEAN[d]	S.D.	
1-100 JAVEX	24	0.474	0.245	1.22	1.61	-6.378
1-350 VES-PHENE	22	0.462	0.249	2.36	0.329	-1.229
0.25% NaOH	22	0.457	0.253	2.59	0.608	-2.253
1-350 WESCODYNE	25	0.480	0.243	2.45	0.970	-3.768

a - square root minutes exposure time
b - $\log_{10}$ PFU/ml
c - number of experimental observations
d - means were based on two experimental trials

APPENDIX 15

Table 45

Comparison of Neutralized Controls with Titers for Echovirus 11 Experiments

DISINFECTANT DILUTION	TRIAL	TITER[a]	NEUTRALIZED CONTROLS[a]	t TEST RESULTS[c]	DILUTION OF VIRUCIDE IN SKIM MILK[b] FOR NEUTRALIZATION
1-100	1	94, 62	80, 86	No Difference	1-10
JAVEX	2	68, 80	74, 78	"	
1-75	1	96, 82	88, 84	"	1-10
JAVEX	2	136,122	132,118	"	
1-50	1	102,110	136,124	"	1-10
JAVEX	2	92, 96	100, 98	"	
0.25% NaOH	1	98,104	80, 96	"	1-10
	2	106,110	120, 92	"	
UNDILUTED	1	158,164	148,162	"	1-100
SONACIDE	2	92, 92	80, 94	"	
1-350	1	86, 98	110, 86	"	1-10
WESCODYNE	2	92, 92	94,124	"	
1-200	1	56, 92	64, 76	"	1-20
WESCODYNE	2	110,106	100, 80	"	
1-50	1	92, 96	88, 98	"	1-100
WESCODYNE	2	118,120	148,150	"	
1-50	1	86, 98	102, 72	"	1-20
VES-PHENE	2	92, 92	96, 88	"	
95%	1	94, 62	128, 80	"	1-20
ETHANOL	2	98,104	96, 96	"	
70%	1	154,170	168,172	"	1-20
ETHANOL	2	102,110	128,168	"	

a - expressed in PFU/ml x 10^5
b - 17.5 per cent skim milk
c - expressed at the 95% confidence level unless otherwise specified (one tailed test)

Table 46

Comparison of Neutralized Controls with Titers for Coxsackievirus B5 Experiments

DISINFECTANT DILUTION	TRIAL	TITER[a]	NEUTRALIZED COLTROLS	t TEST RESULTS[c]	DILUTION OF VIRUCIDE IN SKIM MILK[b] FOR NEURALIZATION
1-100	1	411,390	386,358	No difference	1-10
JAVEX	2	396,390	302,344	No difference[d]	
1-75	1	180,124	188,178	No difference	1-10
JAVEX	2	284,252	260,270	"	
0.25%	1	198,150	112,132	"	1-10
NaOH	2	316,248	326,290	"	
UNDILUTED	1	270,276	386,378	"	1-100
SONACIDE	2	398,390	356,320	No difference[d]	
1-350	1	280,320	280,336	No difference	1-10
WESCODYNE	2	94,114	114,146	"	
1-200	1	284,252	188,258	"	1-20
WESCODYNE	2	180,124	200,148	"	
1-50	1	198,150	180,192	No difference	1-20
VES-PHENE	2	158,150	120,122	No difference[e]	
95%	1	140,132	94,156	No difference	1-20
ETHANOL	2	372,342	320,346	"	
70%	1	266,236	240,250	"	1-20
ETHANOL	2	160,140	156,128	"	

a - expressed in PFU/ml x 10^5
b - 17.5 per cent skim milk
c - expressed at the 95% confidence level unless otherwise indicated (one tailed test)
d - significant at the 95% level but not at the 99% level
e - significant at the 99% level but not at the 99.5% level

Table 47

Comparison of Neutralized Controls with Titers for f2 Bacteriophage Experiments

DISINFECTANT DILUTION	TRIAL	TITER [a]	NEUTRAL-IZED CONTROLS[a]	t TEST RESULTS[c]	DILUTION OF VIRUCIDE IN SKIM MILK[b] FOR NEURALIZATION
1-100	1	2580,1760	2480,2760	No differ-	1-10
JAVEX	2	2100,2600	5400,4900	ence	
1-50	1	1090, 950	1020, 950	"	1-10
JAVEX	2	1120,1140	840,1190	"	
0.25%	1	3600,3000	3800,2400	"	1-10
NaOH	2	35200, 35800	25600, 32200	"	
UNDILUTED	1	35200, 35800	25600, 32800	"	1-100
SONACIDE	2	18400, 16000	17600 10400	"	
1-20	1	16.2,13.6	12.2, 12.2	"	1-100
VES-PHENE	2	4.0, 4.76	4.54, 5.96	"	
1-20	1	38.4,42.0	49.0, 4.40	"	1-100
WESCODYNE	2	6.0, 6.2	9.6, 7.2	"	
95%	1	3080,2420	2420,2720	"	1-20
ETHANOL	2	4.0, 4.76	6.60, 5.56	"	

a - expressed in PFU/ml x 10^7
b - 17.5 per cent skim milk
c - expressed at the 95% confidence level unless otherwise specified (one tailed test)

Table 48

Comparison of Neutralized Controls with Titers for Reovirus 3 Experiments

DISINFECTANT DILUTION	TRIAL	TITER [a]	NEUTRALIZED CONTROLS [a]	t TEST RESULTS [c]	DILUTION OF VIRUCIDE IN SKIM MILK[b] FOR NEUTRALIZATION
1-100 JAVEX	1	8.2,13.4	11.8,13.2	No difference	1-10
	2	11.6,13.2	15.0,16.6		
1-75 JAVEX	1	68, 80	116, 80	"	1-10
	2	84, 88	94, 76	"	
0.25% NaOH	1	12.6,13.2	13.0,12.6	"	1-10
	2	7.2, 5.0	9.6,10.8	"	
UNDILUTED SONACIDE	1	460,560	760,740	"	1-100
	2	360,380	380,480	"	
1-350 WESCODYNE	1	11.6,13.2	15.4,12.6	"	1-10
	2	8.2,13.4	10.2,12.4	"	
1-200 WESCODYNE	1	42, 44	42, 50	"	1-20
	2	34, 40	34, 30	"	
1-50 VES-PHENE	1	334,404	400,340	"	1-100
	2	190,150	176,172	"	
95% ETHANOL	1	540,580	640,760	"	1-20
	2	606,396	704,720	"	
70% ETHANOL	1	34, 26	14, 22	"	1-20
	2	72, 68	70, 68	"	

a - expressed in PFU/ml x 10^5
b - 17.5 per cent skim milk
c - expressed at the 95% confidence level unless otherwise specified (one tailed test)

Table 49

Comparison of Neutralized Controls with Titers for Herpes Simplex Virus 2 Experiments

DISINFECTANT DILUTION	TRIAL	TITER[a]	NEUTRAL-IZED CONTROLS[a]	t TEST RESULTS[c]	DILUTION OF VIRUCIDE IN SKIM MILK[b] FOR NEURALIZATION
1-200	1	182,174	130,136	No difference[d]	1-10
JAVEX	2	164,138	106,104	No difference[e]	
1-100	1	50, 80	40, 50	No difference	1-10
JAVEX	2	34, 30	36, 42	"	
UNDILUTED	1	34, 30	28, 32	"	1-200
SONACIDE	2	18, 32	20, 22	"	
1-350	1	128,180	150,170	"	1-10
VES-PHENE	2	134,168	128,132	"	
0.25%	1	18, 32	16, 12	"	1-10
NaOH	2	18, 8	12, 19	"	
1-350	1	134,168	90,220	"	1-10
WESCODYNE	2	66,204	158,156	"	
95%	1	50, 80	48, 60	"	1-20
ETHANOL	2	34, 30	32, 16	"	
70%	1	50, 80	56, 72	"	1-20
ETHANOL	2	34, 30	26, 20	"	

a - expressed in PFU/ml x 10^4
b - fetal calf serum was heat inactivated
c - expressed at the 95% confidence level unless otherwise specified (one tailed test)
d - significant at the 99% level but not at the 99.5% level
e - significant at the 95% level but not at the 99% level

Table 50

Comparison of Neutralized Controls with Titers for Bovine Kidney Cell Grown Vesicular Stomatitis Virus Experiments

DISINFECTANT DILUTION	TRIAL	TITER[a]	NEUTRAL-IZED CONTROLS[a]	t TEST RESULTS[d]	DILUTION OF VIRUCIDE IN SKIM MILK[b] FOR NEUTRALIZATION
1-100	1	260, 80	220,200	No difference	1-10[b]
JAVEX	2	100,128	84,118	"	
UNDILUTED	1	200,318	160,200	"	1-100[c]
SONACIDE	2	118, 60	120, 80	"	
1-350	1	2500,2500	2440,2300	"	1-10[b]
VES-PHENE	2	260,380	760,500	"	
0.25%	1	216,190	214,232	"	1-10[b]
NaOH	2	350,392	560,540	"	
1-350	1	142,108	224,182	"	1-10[b]
WESCODYNE	2	80,140	100,220	"	
95%	1	200,318	320, 80	"	1-20[c]
ETHANOL	2	118, 60	88, 96	"	
70%	1	28, 30	8, 40	"	1-20[c]
ETHANOL	2	224,252	208,168	"	

a - expressed in PFU/ml x 10^4
b - ten per cent skim milk
c - 17.5 per cent skim milk
d - expressed at the 95% confidence level unless otherwise specified (one tailed test)

Table 51

Comparison of Neutralized Controls with Titers for LM Cell Grown Vesicular Stomatitis Virus Experiments

DISINFECTANT DILUTION	TRIAL	TITER[a]	NEUTRALIZED CONTROLS[a]	t TEST RESULTS[c]	DILUTION OF VIRUCIDE IN SKIM MILK[b] FOR NEUTRALIZATION
1-100	1	212,232	114,210	No difference	1-10
JAVEX	2	134,136	94,136	"	
0.25%	1	140,112	144,120	"	1-10
NaOH	2	134,136	156,126	"	
1-350	1	104, 84	82, 60	"	1-10
VES-PHENE	2	142,162	202,202	"	
1-350	1	134,136	148,144	"	1-10
WESCODYNE	2	140,112	128,122	"	

a - expressed in PFU/ml x 10^7
b - ten per cent skim milk
c - expressed at the 95% confidence level unless otherwise specified (one tailed test)

Table 52

Comparison of Neutralized Controls with Titers for Chick Embryo Fibroblast Grown Vesicular Stomatitis Virus Experiments

DISINFECTANT DILUTION	TRIAL	TITER[a]	NEUTRAL-IZED CONTROLS[a]	t TEST RESULTS[c]	DILUTION VIRUCIDE IN SKIM MILK[b] FOR NEUTRALIZATION
1-100 JAVEX	1	222,210	240,236	No difference	1-10
	2	240,220	180,210	"	
0.25% NaOH	1	100, 96	108, 94	"	1-10
	2	228,158	108,130	"	
1-350 VES-PHENE	1	240,220	200,204	"	1-10
	2	222,210	226,204	"	
1-350 WESCODYNE	1	222,210	220,180	"	1-10
	2	28.4,25.4	22.8,19.8	"	

a - expressed in PFU/ml x 10^5
b - ten per cent skim milk
c - expressed at the 95% confidence level unless otherwise specified (one tailed test)

Table 53

Comparison of Neutralized Controls with Titers for A2 Plaque Virus Experiments

DISINFECTANT DILUTION	TRIAL	TITER[a]	NEUTRALIZED CONTROLS[a]	t TEST RESULTS[b]	DILUTION OF VIRUCIDE IN SKIM MILK[b] FOR NEUTRALIZATION
1-50 (1 minute) JAVEX (experiments)	1	142,116	118, 94	No difference	1-10
	2	85, 90	81, 89	"	
1-50 (ten minute) JAVEX (experiments)	1	142,116	118, 94	"	1-10
	2	182,176	162,166	No difference[d]	
1-75 JAVEX	1	142,116	118, 94	No difference	1-10
	2	85, 90	81, 89	"	
UNDILUTED SONACIDE	1	60, 66	68, 58	"	1-100
	2	110,102	100,120	"	
1-50 VES-PHENE	1	40, 56	40, 44	"	1-80
	2	148,118	138,198	"	
1-50 WESCODYNE	1	142,118	192,196	"	1-80
	2	176,182	178,168	"	
1% (w/v) NaOH	1	40, 56	54, 48	"	1-10
	2	148,118	110,158	"	
80% METHANOL	1	10, 24	16, 12	"	1-20
	2	84, 72	96, 80	"	
100% (1 minute) METHANOL (experiments)	1	72, 84	64, 92	"	1-20
	2	20, 34	40, 44	"	
100% (ten minute) METHANOL (experiments)	1	20, 34	40, 44	"	1-20
	2	170,138	118,114	"	
70% ETHANOL	1	84, 72	72, 52	"	1-20
80% (1 minute) ETHANOL (experiments)	1	84, 72	76, 64	"	1-20
	2	10, 24	10.4,15.6	"	
80% (10 mins.) ETHANOL	1	256,206	208,184	"	1-20
95% (1 minute) ETHANOL (experiments)	1	84, 72	80, 68	"	1-20
	2	10, 24	12,15.4	"	
95% (10 min. experiments) ETHANOL	1	206,256	200,192	"	1-20
	2	20, 34	84, 88	"	

a - expressed in PFU/ml x 10^4
b - expressed at the 95% confidence level unless otherwise indicated (one tailed test)
c - 17.5 per cent skim milk
d - significant at the 95% level but not at the 97.5% level

APPENDIX 16

Table 54

Comparison of Cell Susceptibility Controls with Titers For Echovirus 11 Experiments

DISINFECTANT DILUTION	TRIAL	TITER[a]	CELL SUSCEPT-IBILITY CONTROLS[a]	t TEST RESULTS[c]	DILUTION OF VIRUCIDE IN SKIM MILK[b] FOR NEUTRALIZATION
1-100	1	94, 62	86, 78	No difference	1-10
JAVEX	2	68, 80	84, 72	"	
1-75	1	96, 82	90, 80	"	1-10
JAVEX	2	136,122	182,184	"	
1-50	1	102,110	126,128	"	1-10
JAVEX	2	92, 96	118,108	"	
0.25%	1	82, 90	72, 78	"	1-10
NaOH	2	106,110	106,106	"	
UNDILUTED	1	158,164	262,228	"	1-100
SONACIDE	2	92, 92	158,114	"	
1-350	1	86, 98	72, 96	"	1-10
WESCODYNE	2	92, 92	132,144	"	
1-200	1	56, 92	106, 96	"	1-20
WESCODYNE	2	90, 82	92, 96	"	
1-50	1	92, 96	126,140	"	1-100
WESCODYNE	2	118,120	168,192	"	
1-50	1	86, 98	96, 66	"	1-20
VES-PHENE	2	92, 90	82, 83	No difference[d]	
95%	1	94, 62	102,132	No difference	1-20
ETHANOL	2	68, 80	62, 78	"	
70%	1	154,170	220,172	"	1-20
ETHANOL	2	102,110	118,136	"	

a - expressed in PFU/ml x 10^5
b - 17.5 per cent skim milk
c - expressed at the 95% confidence level unless otherwise specified (one tailed test)
d - significant at the 99% level but not at the 99.5% level

Table 55

Comparison of Cell Susceptibility Controls with Titers for Coxsackievirus B5 Experiments

DISINFECTANT DILUTION	TRIAL	TITER[a]	CELL SUSCEPTIBILITY CONTROLS[a]	t TEST RESULTS[c]	DILUTION OF VIRUCIDE IN SKIM MILK[b] FOR NEUTRALIZATION
1-100	1	218,244	218,196	No difference	1-10
JAVEX	2	140,142	172,172	"	
1-75	1	180,124	188,178	"	1-10
JAVEX	2	284,252	260,270	"	
0.25%	1	140,142	198,150	"	1-10
NaOH	2	218,244	228,276	"	
UNDILUTED	1	218,244	236,222	"	1-100
SONACIDE	2	140,142	192,136	"	
1-350	1	218,244	244,278	"	1-10
WESCODYNE	2	140,142	148,142		
1-200	1	284,252	198,294	"	1-20
WESCODYNE	2	180,124	180,174	"	
1-50	1	182,200	218,244	"	1-20
VES-PHENE	2	140,142	142,118		
95%	1	218,244	246,210	"	1-20
ETHANOL	2	140,142	166,118	"	
70%	1	266,236	324,296	"	1-20
ETHANOL	2	160,140	184,148	"	

a - expressed in PFU/ml x 10^5
b - 17.5 per cent skim milk
c - expressed at the 95% confidence level unless otherwise specified (one tailed test)

Table 56

Comparison of Cell Susceptibility Controls with Titers for Reovirus 3 Experiments

DISINFECTANT DILUTION	TRIAL	TITER[a]	CELL SUSCEPT-IBILITY CONTROLS[a]	t TEST RESULTS[c]	DILUTION OF VIRUCIDE IN SKIM MILK[b] FOR NEUTRALIZATION
1-100	1	380,342	400,470	No difference	1-10
JAVEX	2	482,560	590,620	"	
1-75	1	68, 80	141,148	"	1-10
JAVEX	2	84, 88	180, 96	"	
0.25%	1	380,342	446,430	"	1-10
NaOH	2	482,560	562,702	"	
UNDILUTED	1	380,342	410,422	"	1-100
SONACIDE	2	482,560	666,710	"	
1-350	1	380,342	434,424	"	1-10
WESCODYNE	2	482,560	594,600	"	
1-200	1	42, 44	101,108	"	1-20
WESCODYNE	2	34, 40	96,127	"	
1-50	1	380,342	390,480	"	1-100
VES-PHENE	2	482,560	610,604	"	
95%	1	80, 68	62, 78	"	1-20
ETHANOL	2	72, 68	112,184	"	
70%	1	26, 34	88, 78	"	1-20
ETHANOL	2	72, 68	128, 96	"	

a - expressed in PFU/ml x 10^5
b - 17.5 per cent skim milk
c - expressed at the 95% confidence level unless otherwise specified (one tailed test)

Table 57

Comparison of Cell Susceptibility Controls with Titers for Herpes Simplex Virus 2 Experiments

DISINFECTANT DILUTION	TRIAL	TITER[a]	CELL SUSCEPT-IBILITY CONTROLS[a]	t TEST RESULTS[c]	DILUTION OF VIRUCIDE IN SKIM MILK[b] FOR NEUTRALIZATION
1-200 JAVEX	1	N.D.[d]	N.D.[d]	N.D.[d]	N.D.[d]
	2	N.D.[d]	N.D.[d]	N.D.[d]	
1-100 JAVEX	1	50, 80	40, 48	No difference	1-10
	2	34, 30	22, 18	No difference[e]	
UNDILUTED SONACIDE	1	32, 28	18, 18	No difference[e]	1-200
	2	18, 32	40, 32	No difference	
1-350 VES-PHENE	1	24, 42	24, 30	"	1-10
	2	5, 6	5, 4	"	
0.25% NaOH	1	18, 32	24, 20	"	1-10
	2	18, 8	14, 19	"	
1-350 WESCODYNE	1	24, 42	36, 26	"	1-10
	2	5, 6	5, 5	"	
95% ETHANOL	1	50, 80	38, 60	"	1-20
	2	34, 30	36, 32	"	
70% ETHANOL	1	50, 80	96, 66	"	1-20
	2	34, 30	20, 32	"	

a - expressed in PFU/ml x 10^4
b - fetal calf serum was heat inactivated
c - expressed at the 95% confidence level unless otherwise specified (one tailed test)
d - not done
e - significant at the 95% level but not at the 99% level

Table 58

Comparison of Cell Susceptibility Controls with Titers for Bovine Kidney Cell Grown Vesicular Stomatitis Virus Experiments

DISINFECTANT DILUTION	TRIAL	TITER[a]	CELL SUSCEPT-IBILITY CONTROLS[b]	t TEST RESULTS[d]	DILUTION OF VIRUCIDE IN SKIM MILK[b] FOR NEUTRALIZATION
1-100	1	42, 52	78, 52	No difference	1-10
JAVEX	2	124,126	120,122	"	
UNDILUTED	1	200,318	190,140	"	1-100
SONACIDE	2	118, 60	100, 40	"	
1-350	1	42, 52	78, 76	"	1-10
VES-PHENE	2	124,126	128,136	"	
0.25%	1	42, 52	78, 66	"	1-10
NaOH	2	124,126	156,148	"	
1-350	1	42, 52	46, 50	"	1-10
WESCODYNE	2	124,126	132,124	"	
95%	1	200,318	350,440	"	1-20
ETHANOL	2	59, 30	98, 96	"	
70%	1	14, 15	8, 12	"	1-20
ETHANOL	2	224,252	330,342	"	

a - expressed in PFU/ml x 10^4
b - ten per cent skim milk
c - 17.5 per cent skim milk
d - expressed at the 95% confidence level unless otherwise specified (one tailed test)
e - significant at the 99% level but not at the 99.5% level

Table 59

Comparison of Cell Susceptibility Controls with Titers for A2 Plaque Virus Experiments

DISINFECTANT DILUTION	TRIAL	TITER[a]	CELL SUSCEPTIBILITY CONTROLS[a]	t TEST RESULTS[c]	DILUTION OF VIRUCIDE IN SKIM MILK[b] FOR NEUTRALIZATION
1-50	1	142,116	118,116	No difference	1-10
JAVEX	2	176,182	256,370	"	
UNDILUTED	1	60, 66	56, 82	"	1-100
SONACIDE	2	110,102	120,118	"	
1-50	1	40, 50	38, 40	"	1-80
VES-PHENE	2	148,118	184,230	"	
1% NaOH	1	40, 56	242,220	"	1-10
	2	148,118	310,250	"	
1-50	1	142,118	148,112	"	1-80
WESCODYNE	2	176,182	180,162	"	
80%	1	84, 72	64, 54	"	1-20
ETHANOL	2	10, 24	22, 10	"	
95%	1	84, 74	40, 74	"	1-20
ETHANOL	2	20, 34	22, 22	"	
80%	1	84, 74	64, 60	No difference[d]	1-20
METHANOL	2	10, 24	12, 16	No difference	
100%	1	84, 74	62, 44	"	1-20
METHANOL	2	20, 34	62, 44	"	
70% ETHANOL	1	84, 72	60, 58	No difference[d]	1-20

a - expressed in PFU/ml x 10^4
b - 17.5 per cent skim milk
c - expressed at the 95% confidence level unless otherwise specified (one tailed test)
d - significant at the 95% level but not at the 97.5% level

APPENDIX 17

Table 61

Virucidal Activity of Different Disinfectants on Different Viruses Relative to 1-100 Javex

DISINFECTANT	TEST VIRUSES					
	reo-virus	cox-sackie-virus	echo-virus	f2	herpes simplex virus 2	VSV/BKC
0.25% NaOH IS	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	NOT DIFFERENT FROM	LESS EFFECTIVE THAN
UNDILUTED SONACIDE IS	MORE EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	NOT DIFFERENT FROM	NOT DIFFERENT FROM	NOT DIFFERENT FROM
1-350 WESCODYNE IS	NOT DIFFERENT FROM	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN
1-200 WESCODYNE IS	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	----	----
1-50 WESCODYNE IS	----	----	NOT DIFFERENT FROM	LESS EFFECTIVE THAN	----	----
1-350 VES-PHENE IS	----	----	----	----	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN
1-50 VES-PHENE IS	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	----	----
1-75 JAVEX IS	LESS EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	----	----	----
1-50 JAVEX IS	----	----	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	----	----
95% ETHANOL IS	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	NOT DIFFERENT FROM	NOT DIFFERENT FROM
70% ETHANOL IS	LESS EFFECTIVE THAN	NOT DIFFERENT FROM	LESS EFFECTIVE THAN	----	NOT DIFFERENT FROM	NOT DIFFERENT FROM

Table 61 is a partial summary of tables 8 to 13 for the purpose of easy reference during discussion.
1-100 Javex is more effective than $\frac{20}{46}$ or 44% of the disinfectants listed in table 61.

Table 62

Virucidal Activity of Different Disinfectants on Different Viruses Relative to 1-350 Wescodyne

DISINFECTANT	TEST VIRUSES					
	reo-virus	cox-sackie-virus	echo-virus	f2	herpes simplex virus 2	VSV BKC
0.25% NaOH IS	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN
UNDILUTED SONACIDE IS	MORE EFFECTIVE THAN	LESS EFFECTIVE THAN	NOT DIFFERENT FROM	----	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN
1-200 WESCODYNE IS	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	----	----	----
1-50 WESCODYNE IS	----	----	MORE EFFECTIVE THAN	----	----	----
1-50 VES-PHENE IS	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	----	----
1-350 VES-PHENE IS	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	MORE EFFECTIVE THAN	NOT DIFFERENT FROM
1-100 JAVEX IS	NOT DIFFERENT FROM	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN
1-75 JAVEX IS	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	----	----
1-50 JAVEX IS	----	----	MORE EFFECTIVE THAN	----	----	----
95% ETHANOL IS	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN
70% ETHANOL IS	LESS EFFECTIVE THAN	MORE EFFECTIVE THAN	NOT DIFFERENT FROM	----	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN

Table 62 is a partial summary of tables 8 to 13 for the purpose of easy reference during discussion.
1-350 Wescodyne is more effective than $\frac{10}{47}$ or 21% of the disinfectants listed in table 62.

Table 63

Virucidal Activity of Different Disinfectants on Different Viruses Relative to 1-200 Wescodyne

DISINFECTANT	TEST VIRUSES			
	reo-virus	cox-sackie-virus	echo-virus	f2
0.25% NaOH IS	NOT DIFFERENT FROM	NOT DIFFERENT FROM	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN
UNDILUTED SONACIDE IS	MORE EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	----
1-350 WESCODYNE IS	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	----
1-50 WESCODYNE IS	----	----	NOT DIFFERENT FROM	----
1-50 VES-PHENE IS	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN
1-350 VES-PHENE IS	----	----	----	----
1-100 JAVEX IS	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN
1-75 JAVEX IS	MORE EFFECTIVE THAN	NOT DIFFERENT FROM	MORE EFFECTIVE THAN	----
1-50 JAVEX IS	----	----	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN
95% ETHANOL IS	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN
70% ETHANOL IS	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	----

Table 63 is a partial summary of tables 8 to 13 for the purpose of easy reference during discussion.

1-200 Wescodyne is more effective than $\frac{14}{31}$ or 44% of the disinfectants listed in table 63.

Table 64

Virucidal Activity of Different Disinfectants on Different Viruses Relative to 1-350 Ves-Phene

DISINFECTANT	TEST VIRUSES					
	reo-virus	cox-sackie-virus	echo-virus	f2	herpes simplex virus 2	VSV/BKC
0.25% NaOH IS	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN
UNDILUTED SONACIDE IS	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN
1-350 WESCODYNE IS	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	LESS EFFECTIVE THAN	NOT DIFFERENT FROM
1-200 WESCODYNE IS	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	----	----
1-50 WESCODYNE IS	----	----	MORE EFFECTIVE THAN	----	----	----
1-50 VES-PHENE IS	----	----	----	----	----	----
1-100 JAVEX IS	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN
1-75 JAVEX IS	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	----	----	----
1-50 JAVEX IS	----	----	----	----	----	----
95% ETHANOL IS	MORE EFFECTIVE	MORE EFFECTIVE	MORE EFFECTIVE	MORE EFFECTIVE	MORE EFFECTIVE	MORE EFFECTIVE
70% ETHANOL IS	----	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	----	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN

Table 64 is a partial summary of tables 8 to 13 for the purpose of easy reference during discussion.

1-350 Ves-Phene is more effective than $\frac{1}{42}$ or 2% of the disinfectants listed in table 64.

Table 65

Virucidal Activity of Different Disinfectants on Different Viruses Relative to 1-50 Ves-Phene

DISINFECTANT	TEST VIRUSES			
	reo-virus	cox-sackie-virus	echo-virus	f2
0.25% NaOH IS	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN
UNDILUTED SONACIDE IS	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN
1-350 WESCODYNE IS	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	----
1-200 WESCODYNE IS	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	----
1-50 WESCODYNE IS	----	----	MORE EFFECTIVE THAN	----
1-350 VES-PHENE IS	----	----	----	----
1-100 JAVEX IS	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN
1-75 JAVEX IS	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	----
1-50 JAVEX IS	----	----	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN
95% ETHANOL IS	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN
70% ETHANOL IS	LESS EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	----

Table 65 is a partial summary of tables 8 to 13 for the purpose of easy reference during discussion.

1-50 Ves-Phene is more effective than $\frac{1}{31}$ or 3% of the disinfectants listed in table 65.

Table 66

Virucidal Activity of Different Disinfectants on Different Viruses Relative to Undiluted Sonacide

DISINFECTANT	TEST VIRUSES					
	reo-virus	cox-sackie-virus	echo-virus	f2	herpes simplex virus 2	VSV/BKC
0.25% NaOH IS	NOT DIFFERENT FROM	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	NOT DIFFERENT FROM	MORE EFFECTIVE THAN
1-350 WESCODYNE IS	LESS EFFECTIVE THAN	MORE EFFECTIVE THAN	NOT DIFFERENT FROM	----	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN
1-50 WESCODYNE IS	----	----	MORE EFFECTIVE THAN	----	----	----
1-50 VES-PHENE IS	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	----	----
1-350 VES-PHENE IS	----	----	----	----	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN
1-100 JAVEX IS	LESS EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	NOT DIFFERENT FROM	NOT DIFFERENT FROM	NOT DIFFERENT FROM
1-75 JAVEX IS	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	----	----	----
1-50 JAVEX IS	----	----	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	----	----
95% ETHANOL IS	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	NOT DIFFERENT FROM	MORE EFFECTIVE THAN
70% ETHANOL IS	LESS EFFECTIVE THAN	MORE EFFECTIVE THAN	NOT DIFFERENT FROM	----	NOT DIFFERENT FROM	NOT DIFFERENT FROM

Table 66 is a partial summary of tables 8 to 13 for the purpose of easy reference during discussion.
Undiluted Sonacide is more effective than $\frac{11}{40}$ or 28% of the disinfectants listed in table 66.

Table 67

Virucidal Activity of Different Disinfectants on Different Viruses Relative to 0.25% (w/v) NaOH

DISINFECTANT	TEST VIRUSES					
	reo-virus	cox-sackie-virus	echo-virus	f2	herpes simplex virus 2	VSV/BKC
UNDILUTED SONACIDE IS	NOT DIFFERENT FROM	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	NOT DIFFERENT FROM	MORE EFFECTIVE THAN
1-350 WESCODYNE IS	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN
1-50 WESCODYNE IS	----	----	NOT DIFFERENT FROM	LESS EFFECTIVE THAN	----	----
1-350 VES-PHENE IS	----	----	----	----	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN
1-50 VES-PHENE IS	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	----	----
1-100 JAVEX IS	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	NOT DIFFERENT FROM	LESS EFFECTIVE THAN	NOT DIFFERENT FROM	MORE EFFECTIVE THAN
1-75 JAVEX IS	MORE EFFECTIVE THAN	NOT DIFFERENT FROM	MORE EFFECTIVE THAN	----	----	----
1-50 JAVEX IS	----	----	LESS EFFECTIVE THAN	NOT DIFFERENT FROM	----	----
95% ETHANOL IS	MORE EFFECTIVE THAN	MORE EFFECTIVE THAN	NOT DIFFERENT FROM	LESS EFFECTIVE THAN	NOT DIFFERENT FROM	MORE EFFECTIVE THAN
70% ETHANOL IS	LESS EFFECTIVE THAN	NOT DIFFERENT FROM	LESS EFFECTIVE THAN	----	NOT DIFFERENT FROM	MORE EFFECTIVE THAN

Table 67 is a partial summary of tables 8 to 18 for the purpose of easy reference during the discussion.

0.25% (w/v) NaOH is more effective than $\frac{23}{42}$ or 54% of the disinfectants listed in table 67.

Table 68

Virucidal Activity of Different Disinfectants on Different Viruses Relative to 95% Ethanol

DISINFECTANT	TEST VIRUSES					
	reo-virus	cox-sackie-virus	echo-virus	f2	herpes simplex virus 2	VSV/BKC
0.25% NaOH IS	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	NOT DIFFERENT FROM	MORE EFFECTIVE THAN	NOT DIFFERENT FROM	LESS EFFECTIVE THAN
UNDILUTED SONACIDE IS	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	NOT DIFFERENT FROM	NOT DIFFERENT FROM
1-350 WESCODYNE IS	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN
1-200 WESCODYNE IS	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	----	----
1-50 WESCODYNE IS	----	----	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	----	----
1-50 VES-PHENE IS	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	----	----
1-350 VES-PHENE IS	----	----	----	----	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN
1-100 JAVEX IS	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	NOT DIFFERENT FROM	NOT DIFFERENT FROM
1-75 JAVEX IS	NOT DIFFERENT FROM	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	----	----	----
1-50 JAVEX IS	----	----	LESS EFFECTIVE THAN	MORE EFFECTIVE THAN	----	----
70% ETHANOL IS	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	LESS EFFECTIVE THAN	----	NOT DIFFERENT FROM	NOT DIFFERENT FROM

Table 68 is a partial summary of tables 8 to 12 for the purpose of easy reference during discussion.

95% ethanol is more effective than $\frac{35}{46}$ or 76% of the disinfectants listed in table 68.

APPENDIX 18

Table 69

Number of Disinfectants Echovirus 11 Is More Resistant To Than the Other Viruses

	DISINFECTANT	reovirus 3	coxsackie-virus B5	f2 bacterio-phage	herpes simplex virus 2	VSV/BKC	VSV/LM	VSV/CEF
1-200	WESCODYNE	1[a]	1	0[b]	1	1	1	1
UNDILUTED	SONACIDE	1	0	1	1	1	X	X
	70% ETHANOL	0	1	X[c]	1	1	X	X
	95% ETHANOL	0	0	0	1	1	X	X
1-100	JAVEX	1	1	1	1	1	1	1
1-50	VES-PHENE	1	0	0	1	1	1	1
0.25% (w/v)	NaOH	0	0	0	1	0	0	0

a 1 signifies echovirus 11 is more resistant to a certain disinfectant than the virus indicated

b 0 signifies echovirus 11 is less resistant or not different in resistance to disinfectant than the virus indicated

c X not included in comparisons, because not done

Echovirus 11 is more resistant to $\frac{28}{42}$ of 67% of the disinfectants tested as compared to the other test viruses listed.

Table 70

Number of Disinfectants Coxsackievirus B5 Is More Resistant To Than Other Viruses

	DISINFECTANT	reovirus 3	echovirus 11	f2 bacteriophage	herpes simplex virus 2	VSV/BKC	VSV/LM	VSV/CEF
1-200	WESCODYNE	0	0	0[b]	1[a]	1	0	1
UNDILUTED	SONACIDE	1	0	1	1	1	X	X
	70% ETHANOL	0	0	X[c]	1	1	X	X
	95% ETHANOL	0	0	0	1	1	X	X
1-100	JAVEX	1	0	0	1	1	1	1
1-50	VES-PHENE	1	0	0	1	1	1	1
0.25% (w/v)	NaOH	0	1	0	1	0	0	0

a 1 signifies coxsackievirus is more resistant to a certain disinfectant than the virus indicated

b 0 signifies coxsackievirus is less resistant or not different in resistance to disinfectant than the virus indicated

c X not included in complarisons, because not done

Coxsackie B5 is more resistant to $\frac{23}{42}$ or 55% of the disinfectants tested as compared to the other test viruses listed.

Table 71

Number of Disinfectants f2 Bacteriophage Is More Resistant To Than the Other Viruses

	DISINFECTANT	echovirus 11	coxsackie-virus B5	reovirus 3	herpes simplex virus 2	VSV/BKC	VSV/LM	VSV/CEF
1-200	WESCODYNE	0[b]	1[a]	1	1	1	1	1
UNDILUTED	SONACIDE	0	0	1	1	1	X	X
	70% ETHANOL	X	X[c]	X	X	X	X	X
	95% ETHANOL	1	1	1	1	1	X	X
1-100	JAVEX	0	1	1	1	1	1	1
1-50	VES-PHENE	0	0	1	1	1	1	1
0.25% (w/v)	NaOH	0	0	0	1	0	0	0

a 1 signifies f2 is more resistant to a certain disinfectant than the virus indicated

b 0 signifies f2 is less resistant or not different in resistance to disinfectant than the virus indicated

c X not included in comparisons, because not done

f2 is more resistant to $\frac{26}{38}$ or 68% of the disinfectants tested as compared to the other test viruses listed.

Table 72

Number of Disinfectants Reovirus 3 Is More Resistant To Than the Other Viruses

	DISINFECTANT	echovirus 11	coxsackie-virus B5	f2	herpes simplex virus 2	VSV/BKC	VSV/LM	VSV/CEF
1-200	WESCODYNE	0[b]	1[a]	0	0	1	0	1
UNDILUTED	SONACIDE	0	0	0	1	1	X	X
	70% ETHANOL	1	1	X[c]	1	1	X	X
	95% ETHANOL	1	1	0	1	1	X	X
1-100	JAVEX	0	0	0	1	1	1	1
1-50	VES-PHENE	0	0	0	1	1	1	1
0.25% (w/v)	NaOH	1	1	1	1	1	0	0

a 1 signifies reovirus 3 is more resistant to a certain disinfectant than the virus indicated

b 0 signifies reovirus 3 is less resistant or not different in resistance to than the viruses indicated

c X not included in comparisons, because not done

Reovirus 3 is more resistant to $\frac{26}{42}$ or 62% of the disinfectants tested as compared to the other test viruses listed.

Table 73

Number of Disinfectants Herpes Simples Virus 2 Is More Resistant to Than the Other Viruses

	DISINFECTANT	reovirus 3	echovirus 11	coxsackie-virus B5	f2	VSV/BKC	VSV/LM	VSV/CEF
1-200	WESCODYNE	0[a]	0	0	0	1[b]	0	1
UNDILUTED	SONACIDE	0	0	0	0	0	X	X
	70% ETHANOL	0	0	0	X[c]	0	X	X
	95% ETHANOL	0	0	0	0	1	X	X
1-100	JAVEX	0	0	0	0	0	0	0
1-50	VES-PHENE	0	0	0	0	0	1	1
0.25% (w/v)	NaOH	0	0	0	0	0	0	0

a 1 signifies herpes simplex virus 2 is more resistant to a certain disinfectant than the virus indicated

b 0 signifies herpes simples virus 2 is less resistant or not different in resistance to disinfectant than the viruses indicated

c X not included in comparisons, because not done

Herpes simplex virus 2 is more resistant to $\frac{5}{42}$ or 12% of the disinfectants tested as compared to the other test viruses listed.

Table 74

Number of Disinfectants VSV/BKC Is More Resistant To Than the Other Viruses

	DISINFECTANT	echovirus 11	coxsackie-virus B5	reovirus 3	f2	herpes simplex virus 2	VSV/LM	VSV/CEF
1-200	WESCODYNE	0[a]	0	0	0	0	1[b]	0
UNDILUTED	SONACIDE	0	0	0	0	0	X[c]	X
	70% ETHANOL	0	0	0	0	0	0	0
	95% ETHANOL	0	0	0	0	0	X	X
1-100	JAVEX	0	0	0	0	0	0	0
1-50	VES-PHENE	0	0	0	0	0	0	0
0.25% (w/v)	NaOH	1	1	0	0	1	0	0

a 1 signifies VSV/BKC is more resistant to a certain disinfectant than the virus indicated

b 0 signifies VSV/BKC is less resistant or not different in resistance to disinfectant than the virus indicated

c X not included in comparisons, because not done

VSV/BKC is more resistant to $\frac{4}{44}$ or 9% of the disinfectants tested as compared to the other test viruses listed.

APPENDIX 19

AN EXAMPLE OF EXPERIMENTAL PROTOCOL

Four ml of 1-100 Javex was added to a mixture consisting of 0.5 ml of heat-inactivated fetal calf serum and 0.5 ml of vesicular stomatitis virus (grown in LM cells). Samples of a 0.5 ml volume were removed from this five ml reaction mixture at ten, 20, and 30 seconds after addition of 1-100 Javex, with the reaction suspension being mixed between each sampling. These 0.5 ml samples were added to 4.5 ml of ten per cent skim milk immediately after they were taken. The skim milk acted as a neutralizer of the action of the disinfectant. After a delay ranging from 1 to 3 minutes (which was noted in each experiment) such skim milk mixtures were serially diluted tenfold in R3 medium, and dilutions were assayed for residual VSV in a variable number (four to eight) tissue cultures dishes containing LM cells using the plaque assay as described in the materials and methods section.

An untreated control was set up in which the stock preparation of vesicular stomatitis virus was titrated in duplicate tissue culture dishes containing LM cells at the time of the experiment. A control, termed the neutralized control, was also prepared at the time of the experiment as follows: 0.5 ml of fetal calf serum and 0.5 ml of 1-100 Javex were added to 4 ml of ten per cent skim milk. This five ml volume was mixed and 4.5 ml of it was removed to another tube. A volume of 0.5 ml of VSV was added to this 4.5 ml volume. The virus was incubated in this tube for a time equivalent to the delay period noted in the actual test, and then serially

diluted in tenfold steps and assayed. The effectiveness of the ten per cent skim milk neutralizer was determined by comparing the untreated control with the neutralized control results using a one-tailed t test at the 95% confidence level.

Actual data for an experiment where VSV was exposed to 1-100 Javex are listed below along with the two untreated control values and two neutralized control values and the t test result which indicated the neutralized control values were not significantly different from the untreated control at the 95% confidence level.

PFU/0.5 ml
for VSV in serum treated
with 1-100 Javex

DIL'N	10 SECONDS	20 SECONDS	30 SECONDS
10^{-3}	222, 201	111, 113	26, 28
	235, 245	130, 124	32, 28
	260, 262	124, 119	35, 38
	237		30, 34

PFU/0.5 ml
VSV CONTROLS

DIL'N	NEUTRALIZED CONTROL	UNTREATED CONTROL
10^{-7}	120, 102	113, 118

t test

df = 2, t = -0.482

In a plot of residual virus versus exposure time to disinfectant it was desirable to obtain a linear function (as indicated in Figure 9). This would convert data into a form where statistical evaluation would be convenient.

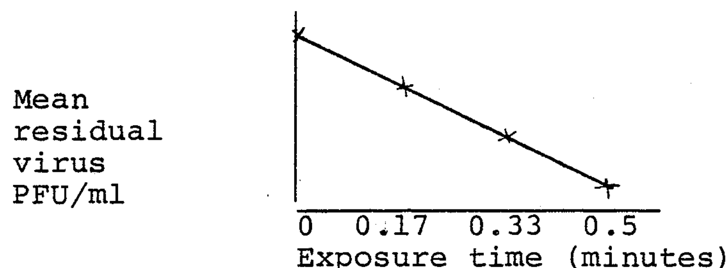

FIGURE 9

In the case of the data previously listed for VSV exposed to 1 - 100 Javex, the zero point on the graph consists of the mean of the two untreated control values and the two neutralized control values (since there was no significant difference between the untreated control and neutralized control). The ten, 20 and 30 second plotted values are the means of replicate observations at each of those points.

The data was transformed in various ways (as illustrated in Table 4) to obtain the relationship between residual virus and exposure time which best conformed to a linear function. This was determined by running the transformed replicate values in program ST 34 (the linear regression and correlation components of the ST 34 Rankit transformation and analysis were used) of the University of Manitoba on-line computer system. The results of program ST 34 for the data transformed in different ways is listed in Table 4. In each case, the correlation coefficient squared (r^2) which is the proportion of the variation of

the dependent variable explained by the regression equation and the F value which is the ratio of $\frac{\text{mean square regression}}{\text{mean square deviation}}$ are listed with df designating the degrees of freedom for the F value. The data conformed best to a linear regression relationship where $\log_{10}$ PFU/ml was plotted against square root minutes, since F values and r^2 values were larger for this relationship than the others listed in Table 4. The previously listed data for VSV exposed to 1 - 100 Javex is therefore plotted

$\log_{10}$ PFU/ml versus square root minutes exposure time with the line being plotted from the expected values listed from the ST 34 program results and the points plotted being the means of the replicate values at zero, ten, 20 and 30 seconds (Figure 3). The confidence limits were at the 95% confidence level.

Two trials of VSV exposed to 1 - 100 Javex were done, with the r^2 and F values listed in Table 5. The reproducibility of the linear regression relationship for VSV exposed to 1 - 100 Javex was determined by comparing the slopes of the two trials statistically at the 95% confidence level using program ST 46 (ST 46 is analysis of variance with one covariate) of the on-line computer system as indicated in Table 6. In Table 6, N is the total number of experimental observations forming the regression relationship. The N for the data previously listed for VSV exposed to 1 - 100 Javex is 25. The N value for the other trial of VSV exposed to 1 - 100 Javex for which the data is not listed is 28. The mean of the

dependent variable is the sum of the replicate ($\log_{10}$ PFU/ 0.5 ml residual virus) at 0, 0.4, 0.57, and 0.7 square root minutes divided by N. The mean independent variable is the sum of the replicate time values divided by N. The N value, means of the dependent and independent variables, their standard deviations and slopes, for each of the two experimental trials for VSV exposed to 1 - 100 Javex can be obtained from program ST 34 program results and subsequently put in program ST 46 which compares slopes to determine reproducibility The result from ST 46, in the form of an F value of 0.127 (df 2, 71) (Table 6) indicates that there is no significant difference between the slopes of the two trials. Table 7 illustrates the mean values of the two trials listed in Table 6. For example, in Table 6 for VSV exposed to 1 - 100 Javex

		INDEPENDENT VARIABLE		DEPENDENT VARIABLE				
TRIAL	N	MEAN	S.D.	MEAN	S.D.	SLOPE	df	F
1	28	0.484	0.231	2.25	1.18	-4.939	2, 71	0.127
2	25	0.480	0.243	2.45	1.21	-4.867		
TOTAL	53	0.964	0.474	4.70	2.39	-9.806		

Dividing these total values by two yields

27	0.482	0.237	2.35	1.20	4.903

as seen in Table 7. Different virus-disinfectant regression relationships were compared by slopes using these mean values in program ST 46. Conclusions from such comparisons are listed in Tables 8 - 20.