DESTRUCTION OF LACTIC ACID STREPTOCOCCUS BACTERIOPHAGE BY VARIOUS BACTERICIDAL AGENTS

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INTRODUCTION

The use of rapidly acting virucides in dairy plant sanitation procedures is recommended as a means of reducing bacteriophage infection of starter cultures. At the present time there are two types of germicides commonly employed for this purpose, the quaternary ammonium compounds and the hypochlorites. Recently a third type of germicide has been considered for dairy plant sanitation. Compounds of this type, known as iodophors, are preparations of iodine solubilized in a wetting agent. The iodophors have been shown to be effective bactericidal agents; however, their virucidal activity has not been evaluated.

One of the objectives of this study was to compare the virucidal activity of quaternaries, hypochlorites and iodophors. By studying various factors which influence virucidal activity it also was hoped that basic information might be obtained on the mechanism of action of the virucides. Such information should enable more efficient formulation and application of these compounds.

HISTORICAL

Bacteriophage in the Dairy Industry

Bacteriophage (phage) destruction of the lactic acid streptococci has a long and active history in the dairy industry. Although several methods for the control of phage are well known, dairy plant operators still have difficulty with the phage problem.

Hadley and Dabney (26, pp.15-16) described the lysis of Streptococcus lactis and Streptococcus fecalis by phage and differentiated between those two species on the basis of phage specificity. Slow lactic acid fermentations were critically studied by Whitehead and Cox (58, p.197) in 1934. This work led to the isolation and characterization of phage as the causative agent of slow and erratic starter activity two years later (57, p.55). Whitehead and Hunter (60, p.403) in 1939 pointed out the common occurrence of bacteriophage in the dairy industry and advanced methods for the isolation and characterization of the phage particles. Since this time there have been numerous reports on the phage problem as experienced in dairy plants throughout the world.

In more recent years phage active against dairy organisms other than Streptococcus lactis and Streptococcus cremoris have been discovered. A phage capable

of lysing Leuconostoc citrovorum, the organism responsible for aroma production in starters, was discovered in 1946 (21, p.25). A phage specific for Streptococcus thermophilus has been discovered by Dean, Nelson, and Ryser (16, p.21). This phage was sufficiently active to seriously hamper the production of Swiss cheese in some plants. Two phage races specific for Streptococcus diacetilactis have been isolated by Czulak (14, p.464).

Methods of Controlling Bacteriophage Infection

The most valuable methods for keeping the phage problem at a minimum include choice of starter, starter rotation, isolation of mother culture from plant operations, and plant sanitation. The desirability of starter rotation has been emphasized by Hunter (29, p.295) in 1944 and Whitehead and Hunter (59, p.67) in 1945. In 1954 Zehren and Whitehead (64, pp.209-219) studied host phage relationships in starter cultures used for cheese manufacturing in New Zealand. They found that some cultures could not be used for more than 1 or 2 days without risk of starter failure whereas others might be used successfully over a period of several months. From these findings some general principles of starter failure due to phage were advanced. In cheese vats, phage is usually present, but not in high enough titer to cause immediate

starter failure. Under actual manufacturing conditions phage for the sensitive strains rapidly reach a high enough titer to cause failure in the vats. With the more stable strains the build-up takes place slower and the starter has a longer useful life in the plant. The occurrence of high phage titer in the cheese vat will result in greater numbers of air borne phage and a greater chance of reinfection. Laboratory studies were made on sensitive and resistant host-phage systems. It was found that the sensitive starters had homologous phage races with short latent period and large burst size. With the more stable starters a relatively longer latent period and smaller burst size was found.

Regardless of the culture used or the program of starter rotation, plant sanitation is of great importance in keeping bacteriophage under control. As Elliker (21, p.28) points out, whenever bacteriophage are present in a plant they may develop wherever there is a sensitive bacterial cell. This includes organisms on equipment, floors and walls and on dust in the plant atmosphere. Whey separators throw a fine atomized mist over a large portion of the plant and thus represent one of the most dangerous reservoirs of phage.

Destruction of Bacteriophage

The ubiquity of the lactic acid bacteriophage might, at first consideration, make sanitary measures seem rather futile. The phage problem, however, is not an all or none situation. Collins (13, p.901) emphasized the importance of sanitizing equipment just before the manufacture of products requiring lactic starters. It was found that as few as 3 phage particles per liter of skim milk were sufficient to cause mass lysis of the host, thus preventing normal coagulation. It was also found that after a titratable acidity of 0.56% was reached mass lysis was not caused by 2.5×10^5 bacteriophage particles per ml., over a period of 7 hours. These experiments clearly illustrate the need for efficient and rapidly acting sanitizing agents to aid in keeping phage out of the critical initial stage of fermentation.

In 1941 Whitehead and Hunter (61, p.64) recovered phage from air and in a later experiment they (59, p.71) exposed a liter flask, containing an active lactic culture, to the atmosphere of a dairy plant for ten seconds and recovered enough phage to lyse the culture overnight. This observation stimulated investigation on the practicability of using mists of germicides to destroy airborne phage particles. Wolf, Nichols and Ineson (62, p.315) in 1946 reported on the first experiments

employing hypochlorite mists to inactivate phage. More recent work on the application of mists has been carried out by Bennett and Nelson (6, p.846). Glycols and a commonly used quaternary were found to be ineffective in the reduction of air borne phage. The use of 1 g. available chlorine per 1000 cu. ft. of air in a fine aerosol was found to be effective. The sterilization of air is an expensive process and has not received general acceptance as a control measure. Sterilization of utensils including vats, and agitators may be carried out more easily and inexpensively and if properly done may be of considerable aid in reducing the phage problem.

The destruction of phage of the lactic streptococci in aqueous systems has been under study for a number of years. Hunter and Whitehead (30, p.64), in 1940, surveyed a number of compounds for activity against bacteriophage. Oxidizing agents were found to be the most effective compounds, with hypochlorite solutions being by far the most potent. Disinfectants such as formaldehyde, mercuric chloride and phenol were ineffective. Prouty (50, p.214), upon the results of virucidal trials with quaternary ammonium compounds, recommended their use at 200 ppm for 2 minutes as sufficient to inactivate bacteriophage on dairy equipment. Parker and Elliker (48, p.3) compared directly the activity of quaternaries and

a hypochlorite against bacteriophage of S. cremoris. Hypochlorites were found to completely inactivate phage in 15 seconds at a concentration of 50 ppm. Two hundred ppm of quaternary ammonium compounds were required to give this activity. Cahill (8, p.72) found that hydrogen peroxide in a concentration of 1000 ppm was sufficient to inactivate S. cremoris phage in phosphate buffer but not in milk. The most recent work on the destruction of bacteriophage of the lactic acid streptococci has been done by Bennett and Nelson (7, p.849). Calcium hypochlorite was found to be superior to a number of quaternaries tested. The presence of organic matter decreased the activity of both types of virucides. The hypochlorite was found to be more effective at lower pH levels and the quaternaries more effective at high pH levels. A final rinse of 100 ppm hypochlorite or 200 ppm quaternary were recommended by these workers for the destruction of phage on clean surfaces.

Quaternary Ammonium Compounds

Domagk (17, p.830) in 1935 showed that a quaternary ammonium compound (alkyldimethylbenzyl ammonium chloride) was germicidal to 9 pathogenic organisms.

The quaternaries are characterized by having the pentavalent nitrogen molecule and may be represented by the general formula $R_1R_2R_3R_4NX$. One of the R groups is a

long chain aliphatic ranging from 8 to 18 carbons. The other R groups are usually methyl, ethyl or benzyl groups, although many other possibilities of structure do exist. The X group represents a halide salt. All of the quaternary compounds are surface active and have been found to be exceptionally stable over a period of years.

The use of quaternaries in the dairy industry has been discussed by Elliker (20, pp.156-167). Some of the uses to which quaternaries have been applied include mastitis sanitation, general farm utensil and milking machine sanitation, and dairy plant and equipment sanitation. The use of quaternaries in respect to the bacteriophage problem has been discussed.

There have been numerous studies on the germicidal activity of quaternaries. Lawrence (36) and more recently Reddish (51) have summarized much of this work and offer excellent bibliographies. In general the quaternaries exhibit a high degree of germicidal activity against many microorganisms; however, factors such as bacterial species, pH of germicide solution, presence of organic matter and germicide concentration may modify activity. Some species of Pseudomonas have been found particularly resistant to quaternary action. Klein, Morton and Mudd (33, p.391) have found quaternaries to be active against the viruses of vaccinia and influenza

when used in vitro.

Some workers (3, pp.249-271; 5, pp.611-620; 4, pp. 621-637; 46, pp.332-336; 34, pp.444-449) believe that the germicidal activity of the quaternaries is due to the penetration of the quaternary into the cell where it acts to inactivate essential enzyme systems. Others (23, pp.77-85; 53, p.401; 54, pp.406-407; 15, pp.517-519) consider that the surface active nature of the molecule brings about death by accumulation of the germicide at cell surfaces in concentrations sufficient to disrupt the cell membrane.

Chlorine Compounds

The germicidal properties of chlorine compounds have been known for many years, since Koch's observation of their activity in 1881. The first commercial hypochlorite was prepared in 1799 by the action of chlorine gas on lime (41, p.327). Today hypochlorites are marketed as calcium and sodium hypochlorite. Recent formulations usually are buffered at pH levels which increase the stability of these compounds without seriously reducing germicidal activity.

Hypochlorites are the sanitizing agents most widely used in industry today. In the dairy industry they are used extensively in mastitis sanitation, to sanitize plant equipment and utensils, and to chlorinate water

used in the manufacture of cheese, butter, and other products.

Hypochlorites are more active against a broad spectrum of bacterial species than are the quaternary ammonium compounds. Parker et al. (47, pp.136-139) have shown the high activity of a sodium hypochlorite on a number of psychrophilic bacteria. Miller (42, p.141) showed that 50 ppm of a sodium hypochlorite completely killed in 15 seconds heavy suspensions of Escherichia coli, Aerobacter aerogenes, Pseudomonas aeruginosa, Sarcina sp., and Micrococcus pyogenes var. aureus. Recent studies by Hays[1] show the superiority of hypochlorites over quaternaries and iodine compounds against a variety of bacterial spores. Reference has already been made to the usage of hypochlorites in aerosols and in aqueous solutions for the destruction of bacteriophage. A residual free chlorine concentration of 0.2 ppm was shown to inactivate a 1:500 dilution of poliomyelitis virus in 10 minutes (52, p.644). Neefe et al. (44, pp.371-372) showed that a residual free chlorine content of 0.4 to 1.1 ppm inactivated the virus of infectious hepatites in 30 minutes.

The review article by Marks and Strandskov (39, pp.

[1] Personal communication.

164-166) on the mode of action of the halogens is very informative in respect to the activity of the various forms of halogens. The hydrolysis of elemental halogen and ionization of hypohalous acid are pH controlled. The rapid decrease of germicidal activity of chlorine with an increase of pH above 6 corresponds to the ionization of hypochlorous acid. Marks, Wyss and Strandskov (40, p.165) working with B. metiens spores showed that killing time is a function of the concentration of hypochlorous acid. This also was demonstrated by Chang (11, p.1192) using cysts of Endamoeba histolytica. The specific action of hypochlorous acid has been studied by Knox et al. (35, p.458), who found that chlorine in bactericidal amounts or less inhibited the sulfhydryl enzymes. Ingols et al. (31, p.999) in a more recent study also attribute chlorine activity to the action of hypochlorous acid in oxidizing the sulfhydryl groups to sulfonyl groups in enzyme systems. It is also pointed out that at high chlorine: nitrogen ratios fundamental damage to protein may occur through deamination reactions in amino acids. Conclusive evidence on the mode of action of chlorine on viruses has not been established.

Iodine Compounds

The discovery and early uses of iodine have been

outlined in a recent review article by Gershenfeld (51, pp.171-211). Its use in the treatment of wounds dates back to 1839. Since that time application has increased to include its use, in various forms, as an antiseptic for the skin, wounds, and mucous surfaces of the body; for the sterilization of catgut and surgical instruments. The use of iodine has also spread to the field of sanitation as a disinfecting agent in drinking water and in industrial plant sanitation.

Molecular iodine is the most germicidal form in which this element occurs. Wyss and Strandskov (63, p. 261) have shown that the maximum activity of iodine solution occurs within the range where only molecular iodine exists. Activity decreases as pH is increased due to the formation of less active hypoiodous acid. Due to the low solubility of molecular iodine in water, various types of solubilizing agents have been employed. Iodide ion, organic solvents and surface active agents have all been employed in commercial preparations. A considerable number of these preparations are mentioned in Gershenfeld's review article.

Molecular iodine closely approaches hypochlorous acid in germicidal activity. Its use has been considered for the disinfection of water supplies. Chambers et al. (10, p.163) found that under favorable conditions 0.6

ppm iodine was sufficient to destroy, in one minute, low concentrations of a number of bacterial species. Chang and Morris (12, p.1011) showed that 5 to 10 ppm iodine was effective in destroying large numbers of enteric bacteria, amoebic cysts, cercariae, leptospira and polio virus within 10 minutes at room temperature. Dilutions of Lugol's solution and tincture of iodine have been shown by Dunham and MacNeal (10, p.947) to inactivate vaccinia virus within 3 minutes. Activity of iodine against influenza virus has been demonstrated by these workers (18, p.123). In contrast to activity against animal viruses, Anson and Stanley (2, p.679) found that iodine at low concentrations displayed little activity against the tobacco mosaic virus. Sporicidal activity is described by Gershenfeld and Witlin (24, p.452).

Whenever solubilizing agents are used in iodine preparations, the formation of complexes and equilibrium reactions must be considered in the evaluation of a germicide. A sodium thiosulfate titration of an Iodine-Iodide solution indicating 200 ppm iodine does not mean that the iodine is present in that concentration in molecular form. Morris et al. (43, p.1013) have reviewed some of the reactions which take place when molecular iodine is reacted with iodide salts. In concentrated solutions the iodine is almost completely tied up in the

form of polyiodides, whereas, at concentrations of a few parts per million, dissociation into elemental iodine and iodide is essentially complete. Carroll (9, p.417) has demonstrated that the bactericidal activity of the triiodide ion toward M. pyogenes var. aureus and E. coli is negligible when compared to that of diatomic iodine.

The class of iodine compounds known as iodophors are relatively new and have considerable potential as industrial sanitizing agents. For these reasons, and their consideration in the experimental work to follow, they will be discussed in more detail than the older and more familiar hypochlorites and quaternary ammonium compounds.

Iodophors are compounds in which the iodine has been solubilized in a cationic, anionic, or nonionic surface active agent. Commercial preparations to date seem to be predominantly of the nonionic type. Allawala and Riegelman (1, pp.396-401) have studied the sporicidal and physio-chemical properties of iodine solubilized in a nonionic carrier. It was found that an equilibrium existed between solubilized and free iodine similar to that found in iodine-iodide preparations. The micellular or solubilized iodine was found to be devoid of any bactericidal activity. The concentration of free iodine in the water phase was considered to be the active agent. As in other iodine preparations employing solubilizing

agents, thiosulfate titrations which give total available iodine might become misleading. The following values selected from the data of Allawala and Riegelman illustrate this. If 12,000 ppm iodine are solubilized in 9.3% of the nonionic wetting agent, 330 ppm iodine will appear in the aqueous phase. If a total of 344 ppm iodine are solubilized in a 2% solution of nonionic wetting agent only 2.4 ppm iodine will be in the aqueous phase. These facts are important in the formulation procedures and also in the interpretation of germicidal activity. These workers also showed that the 99% killing time of the spores of B. metiens was a function of the free iodine concentration both in the presence and absence of added surface active agent.

The use of iodophors in the dairy industry has been studied by Lazarus (37, pp.144-147). When used in farm utensil sanitation iodophors were reported by him to receive a high degree of acceptance from the farmers who used them. An iodophor at a concentration of 10 ppm was found to act as rapidly as a 50 ppm solution of hypochlorite against a suspension of 100 million cells/cc of E. coli. The advantages of iodophors have been listed by Lazarus and also by Johns (32, pp.31, 48). As detergent sanitizers their cost is considered comparable to the combined cost of an alkaline cleaner and chlorine. In

addition to their rapidity of action against bacteria the iodophors were found to be less affected by organic matter than the hypochlorites. They are less corrosive to metals than hypochlorites and were reported to aid in the prevention of the accumulation of milk stone. They are easy to use in that as long as color remains germicidal activity is present, thus providing a built in concentration indicator. They are free rinsing and have sufficient wetting properties to insure contact on surfaces to be sanitized.

Some of the fundamentals of iodine reactions with biological substances have been reviewed by Olcott and Fraenkel-Conrat (45, pp.182-184). At low pH values iodine acts as an oxidizing agent attacking the sulfhydryl groups of enzymes and proteins in general. Under neutral or alkaline conditions iodine reacts with phenolic groups. The substitution of iodine on the tyrosine molecule is a reaction of considerable biological significance. Anson and Stanley (2, p.689) found that the oxidation of the sulfhydryl groups of tobacco mosaic virus beyond the disulfide stage did not affect the biological activity of the virus. If iodine concentration was increased to form diiodotyrosine, inactivation of the virus occurred. Molecular iodine acts as the oxidizing agent at low pH values and at high pH levels

it is thought that the hypoiodous acid molecule is the primary iodinating reagent. Unlike chlorine, iodine does not form amines. While the theory of the oxidation of sulfhydryl groups of enzymes may explain the rapidity of action of iodine against bacterial cells, still other mechanisms must be considered in the destruction of viruses. Before this information is forthcoming more studies are necessary in the correlation of activity of iodine and the chemical composition of the viral particles under study.

MATERIALS AND METHODS

Preparations of Bacteriophage Filtrates

Phage stocks of strains 144F and W of *S. cremoris* were prepared by inoculating 1 ml. of an active phage filtrate into 200 ml. of an actively growing skim milk culture of the sensitive host. A duplicate bottle of phage-free culture was inoculated to observe the time required for coagulation of milk in the absence of phage. After coagulation of the milk in the control culture, the phage inoculated culture was artificially coagulated with 10% sterile lactic acid. The precipitated protein was removed by centrifugation in a Sorval angle head centrifuge, Model SS-1, at about 10,000 r.p.m. for 30 seconds. The clear supernatant was then filtered through a Seitz bacteriological filter. The filtrate was transferred to a sterile bottle containing calcium carbonate to neutralize acidity of the whey. Titers of about 10^{10} were obtained in this manner and stock preparations maintained this titer over a period of several months when stored at 2°C. For virucidal studies 1:100 dilutions of these filtrates in distilled water were mixed with equal volumes of test virucide.

Preparation of Bacterial Suspensions

Twenty four hour cultures were streaked onto bottle

slants of TGY agar and incubated at 30°C for 24 hours. A 1:100 dilution of sterile whey was used to wash growth from the slants and to prepare the concentration needed in germicidal trials. The whey was used to parallel the medium used in phage suspensions. Bacterial preparations were filtered through #1 Whatman paper to remove any large particles of agar or other organic matter, and then standardized with a Beckman model B spectrophotometer at a wave length of 440 mμ, to give a concentration of 200 x 10^6 cells per ml.

Germicide Solutions

Stock solutions of germicides were prepared at a concentration of approximately 1000 ppm. These solutions were titrated to determine the actual concentration just prior to each run and appropriate dilutions were made for the trials in distilled water or buffer solution.

Quaternary ammonium compound. Alkyl dimethyl ethyl benzylammonium chloride was selected as a representative quaternary ammonium compound. The concentration was determined by the method developed by Furlong and Elliker (22, p.226).

Iodine compounds. Biopal was selected as a typical iodophor. This germicide is a liquid preparation containing 10% iodine solubilized in a nonionic wetting agent. Concentrations were determined by titration with

a standardized 0.0105 N sodium thiosulfate solution. Starch indicator could not be used satisfactorily with the iodophor as a sharp end point could not be reached. Titrations were made to a colorless end point with reproducible results. With iodine preparations other than the iodophor a starch indicator gave a satisfactory end point.

Iodine-potassium iodide and aqueous iodine solutions were prepared with reagent grade chemicals.

Chlorine compounds. A liquid sodium hypochlorite preparation containing 4.62% available chlorine was used as a typical hypochlorite. Various preparations of a new class of chlorine compounds also were used. They are the trichloro derivatives of cyanuric acid. These germicides were in powder form with the available chlorine content ranging from 16 to 80%. In water solutions hypochlorous acid is released as the active agent. Concentrations of all chlorine compounds were determined by the thiosulfate titration method (55, pp.98-100).

Buffers

In all experiments where the pH was controlled the following buffer systems were used at a final concentration of M/100 unless stated otherwise:

pH 4 and 5 .....sodium acetate-acetic acid

pH 6 and 7 .....monosodium phosphate-disodium phosphate

pH 8 and 9 .....sodium borate-boric acid

All pH determinations were made with the Beckman Model G glass electrode pH meter.

Inactivators

The inactivator for the quaternary ammonium compound consisted of 2.2 g. of asolectin and 15.8 ml. of Tween-80 per liter of M/100 phosphate buffer at pH 7.2. Inactivator solutions were sterilized in 8 oz. bottles for 20 minutes at 121°C. Just prior to each run 9 ml. aliquots were aseptically transferred into sterile test tubes. When large numbers of tubes were to be used the solutions were dispensed into tubes in 9.2 ml. amounts and autoclaved. This gave a final volume of 9 ml.

For inactivation of iodine and chlorine compounds a solution of 80 mg. of sodium thiosulfate per liter was used. This was also buffered at pH 7.2 with M/100 phosphate buffer.

In studies with chelating agents $CaCl_2$ was added to inactivator tubes in a concentration of 400 ppm.

Diluents

For dilutions of bacterial cells, 99 ml. sterile distilled water blanks were used. Serial dilutions of S. cremoris phage were made through 9 ml. dilution blanks of a buffered saline solution containing 8.5 g NaCl per

liter of M/100 phosphate buffer at pH 7.2.

Media

Tryptone-glucose-yeast extract media (TGY) of the following composition was used for the cultivation and assay of all bacterial species used in germicidal studies: Tryptone, 5 g; glucose, 1 g; yeast extract, 2.5 g; agar, 15 g; distilled water to make 1 liter. The pH was adjusted to 6.8 with NaOH and HCl.

For cultivation and assay of the S. cremoris host-phage system a medium developed by Hanneson (27, p.72) for the cultivation of lactics was employed. This medium, designated as T-19, has the following composition: tryptone, 20 g; gelatin, 5 g; yeast extract, 2.5 g; glucose, sucrose, and lactose, 5 g each; NaCl, 4 g; sodium acetate, 1.5 g; agar, 15 g; distilled water to make 1 liter. The pH was adjusted to 6.8. Broth and semisolid, 0.8%, agar were also used. Both strains of S. cremoris were also cultivated in 10% skim milk, reconstituted from Darigold spray dried skim milk powder for use in resazurin reduction trials.

Method for Determining Bactericidal and Virucidal Activity

The method developed by Weber and Black (56, pp.1406-1415) and modified by Johns (32, pp.1-2) was used in determining the activity of the various germicides. In

this method the bacteria or bacteriophage suspensions were pipetted into wide mouth, 30 ml. capacity screw-cap bottles (medication bottles) in 5 ml. aliquots. At 0 time a 5 ml. aliquot of the test germicide was transferred to a medication bottle and the contents mixed by shaking with a rotary motion. At the end of the desired exposure periods, 1 ml. aliquots were removed from the medication bottle and transferred to an appropriate inactivator tube. All germicides and suspensions of bacteria and bacteriophage were prepared double strength so that the desired concentration would be reached upon dilution in the medication bottle. Surviving bacteria were assayed by transfering a 1 ml. aliquot from the inactivator tube or appropriate dilution blank to a sterile petri dish and then pouring in 15 ml. of TGY agar previously cooled to 45°C. Plates were incubated for 48 hours at 30°C. for colony development.

Two methods commonly used for the assay of lactic phage are plaque count and serial dilution using resazurin reduction in tubes of milk. In both assay methods appropriate dilutions were prepared from the inactivator tubes. In the plaque count method 1 ml. of the phage dilution was added to 0.25 ml. of a sensitive broth grown culture and one minute allowed for adsorption of the phage onto the cells. At the end of this time 3 ml. of

semisolid agar, previously melted and cooled to 45°C. were poured into the adsorption tube, the contents mixed and then poured into a petri dish containing 15 ml. of previously poured and hardened solid agar. The contents were spread evenly over the surface of the solid layer and allowed to harden. After incubation (right side up) for 12 hours at 30°C. the plaques were fully developed and countable.

In the resazurin reduction method it is necessary to carry out serial dilutions to the point where surviving phage are diluted out. One ml. of each dilution was added to duplicate tubes containing 9 ml. of 10% skim milk, 0.5 ml. of a double strength resazurin solution and 1 drop of a sensitive culture. Control tubes were also prepared containing no phage. The reduction of resazurin proceeds from a bluish-purple through pink to white. Tubes which contain phage are recognized by a slower rate of reduction than in control tubes, usually not proceeding past the pink stage. Tubes were incubated in a 30°C. water bath and observations were made at hourly intervals through 8 hours. The last dilution showing inhibition gave the titer of the surviving phage.

In both plaque count and resazurin methods 5 hour old cultures were used.

The temperature of the virucides and test suspensions

were maintained at 25°C. within 1°C. by use of a cenco serological water bath. Control studies were made to insure the effectiveness of inactivators.

PART I

EVALUATION OF BACTERIOPHAGE COUNTING METHODS

In considering assay methods for phage several questions arose with respect to materials and methods. Although distilled water has been used as a diluent, this choice was questioned on the basis of early results. All of the assay methods used in virucidal studies with lactic phage to date have utilized some reaction in milk with the limiting dilution technique as the assay method for surviving phage. It was felt that the plaque count method would yield more quantitative results. A comparison of these two techniques was necessary before a definite choice of assay method could be made. Potter and Nelson (49, p.110) have reported on the necessity of calcium for the optimum proliferation and plaque formation of some strains of lactic phage. Trials were made to ensure the adequacy of the assay medium with respect to calcium.

Results

Selection of diluent. Six diluents were tested for their ability to prevent inactivation of suspended phage particles. A 1:100 dilution of a phage whey filtrate in distilled water was diluted serially through 9 ml. aliquots of solutions of the following composition:

(a) M/100 phosphate buffer at pH 7.1; (b) unbuffered saline containing 8.5 g. NaCl per liter; (c) one-fourth strength Ringer's solution consisting of 1.6 g NaCl, 0.03 g $CaCl_2$, 0.035 g KCl and 0.05 g $NaHCO_3$ per liter; (d) distilled water; (e) TGY broth. The plaque count method was used to evaluate surviving phage. Comparative counts of developing phage for each of these diluents are given in table 1.

The data from table 1 show that phage particles are inactivated when diluted serially through distilled water blanks. Buffered saline and TGY broth provided a comparable degree of protection in the dilution procedure.

Selection of plaquing medium. For optimum development a medium must provide nutrients necessary for good growth of the host culture and the proper ionic and nutritional environment for adsorption and penetration of phage particles into the sensitive host. The medium referred to as T-19 in the section on Materials and Methods has been shown to give excellent growth of S. cremoris and therefore was selected as the basic test medium. Modifications in the salt content, which might affect phage-host reactions were made and developing phage assayed by the double layer technique previously described. Table 2 gives the composition of the various test media and relative development of the sensitive host and phage

TABLE 1

Plaque counts obtained with various diluents using phage strain 144F

Diluent	Phage plaque counts per ml. $X10^6$	$X10^7$	$X10^8$
Trial 1			
Phosphate buffer	lysed*	TC**	164
Unbuffered saline	TC	185	25
Buffered saline	lysed	TC	176
One-fourth Ringer's	TC	192	23
Distilled water	12	0	0
Trial 2			
Distilled water	12	-	-
Buffered saline	-	-	78
TGY broth	-	-	85

* lysed denotes phage particles present in numbers great enough to cause lysis of all developing bacteria resulting in a clear plate.

** TC denotes plaques too numerous to count accurately but fewer in number than in lysed plates.

TABLE 2

Comparison of the abilities of different plating media to support growth of 144F strain of S. cremoris and development of its homologous phage

Medium	Phage plaque count per ml.	Bacterial plate count per ml.
T-19 unmodified	$100x10^8$	$506X10^6$
T-19 minus NaCl and NaAc	$64X10^8$	$118X10^6$
T-19 minus NaCl and NaAc with final $CaCl_2$ conc. of 0.0064 M	$82X10^8$	$249X10^6$

plaques thereon.

Results of trials with the various test media show that unmodified T-19 gave the largest number of bacterial colonies and phage plaques.

Comparison of plaque and resazurin reduction methods for counting bacteriophage. As previously mentioned the plaque count method was considered to be more desirable for studying degrees of inactivation of phage particles. The resazurin reduction method was considered to be sensitive and has the advantage of repropagating phage in their natural environment. Before turning exclusively to the use of plaque counts, a comparison was desired with the resazurin reduction method. Table 3 summarizes results obtained on the assay of phage filtrates by these two methods. A trial run also was made employing an

iodophor to determine the correlation between these two methods as they would be used in further studies. In the virucide trial, the pH was maintained at 50 by use of a M/100 acetate buffer. Results of this trial are tabulated in table 4.

With the exception of trial 2, as indicated in table 3, the plaque count method gave higher phage counts than the resazurin reduction method with strain 144F. The two assay methods correlated well when employed with W strain, however, as indicated in table 4, at the 15 and 300 second time intervals higher counts were obtained by the resazurin reduction method. Strain 144F of S. cremoris grew abundantly on the solid medium and the phage particles gave rise to clearly defined plaques. Growth on solid medium with strain W was somewhat scanty resulting in poor contrast between the developing plaques and host growth.

Discussion

Cations have been shown to be important stabilizing agents for most phage races. With many phages this stabilizing activity is effective over a narrow range of cation concentrations. Inactivation of phage due to adverse conditions such as heat and possibly chemical agents proceeds more rapidly outside this stabilizing concentration range. These observations have been

TABLE 3

Comparison of plaque count and resazurin reduction methods for determining numbers of phage

Trial	Phage Strain	Phage particles per ml assayed by: Plaque count	Resazurin reduction
1	144F	$116X10^7$	$1X10^6$
2	144F	$32X10^8$	$1X10^{10}$
3	144F	$356X10^8$	$1X10^{10}$
4	W	$14X10^8$	$1X10^9$
5	W	$98X10^7$	$1X10^{10}$

TABLE 4

Comparison of plaque count and resazurin reduction methods for determining numbers of phage surviving treatment with 100 ppm iodophor

Phage strain	Method of counting	Phage survivors per ml after: 15 secs.	60 secs.	300 secs.
144F	Plaque count	$80X10^4$	$105X10^3$	$20X10^1$
	Resazurin reduction	$1X10^4$	$1X10^2$	$1X10^2$
W	Plaque count	$18X10^4$	$34X10^3$	$<1X10^1$
	Resazurin reduction	$1X10^6$	$1X10^4$	$1X10^2$

Control counts of initial phage suspension:

Strain 144F:	Plaque count method:	$128X10^6$
	Resazurin reduction:	$49X10^5$
Strain W:	Plaque count method:	$49X10^5$
	Resazurin reduction:	$5X10^7$

explained by assuming equilibrium reactions between the cations and sites on the phage particles to form more stable units.

Surface active substances, such as proteins, also act as stabilizing agents. TGY broth and buffered saline proved to be satisfactory diluents. The latter was chosen for further studies because it is easier to prepare and gives less foaming in dilution procedures.

The highest number of phage plaques was developed on medium T-19. Equally important was the abundant host growth obtained on solid medium which provided an excellent background for contrast between developing plaques and bacterial growth. The ratio and amounts of sodium chloride and sodium acetate have been shown to be important factors in the development and growth of lactic acid streptococci. The increased number of plaques obtained with T-19 medium may be a reflection of better host growth, or the concentration of cations in the medium may be favorable for optimum adsorption and penetration of phage into the host. Calcium was probably present in adequate concentrations in the various components used in the preparation of T-19.

The plaque count and resazurin reduction methods for counting phage correlated well in these studies. In most cases the plaque count gave higher counts with phage

strain 144F. The growth characteristics of phage strains 144F and W in milk and on solid media were factors in the choice of the assay method.

On the basis of the preceding results it was decided to use the plaque count method as the means of counting phage surviving virucide treatment, with buffered saline as the diluent, and unmodified T-19 as the plaquing medium.

PART II

FACTORS AFFECTING THE VIRUCIDAL ACTIVITY OF A QUATERNARY AMMONIUM COMPOUND

There are many factors which may affect the activity of germicidal or virucidal agents. Many of these factors such as temperature, concentration of germicide, concentration of organic matter in a test solution, pH and exposure times may be controlled to a considerable degree under laboratory conditions. With the quaternary ammonium compounds changes in the aforementioned environmental conditions may be reflected in changes in germicidal and virucidal activity. Miller (42, pp.39-49) has studied a number of these factors with respect to various bacterial species. It has been found that species differ considerably in their resistance to quaternaries. Bacterial resistance, in many cases, also is a function of the pH of the solution. In general, quaternaries are more effective at higher pH values although some exceptions may be found to this, especially with Pseudomonas species. Organic matter has been found to hamper the activity of these compounds but to a lesser extent than with the hypochlorites. Hard water salts are very active in reducing activity of quaternaries. This may be overcome by the use of chelating agents.

The following section is devoted to the study of

such factors in relation to bacteriophage. It is difficult to devise an experiment to measure only one quaternary characteristic. For example, effective concentrations will vary with the pH used. Representative data have been selected which best emphasize the property under consideration and the interdependence on some other factor noted when significant.

Alkyl dimethyl ethyl benzylammonium chloride was selected as a representative quaternary and was employed in all studies in this section.

Results

Variations in resistance between phage strains. While the variations in resistance between phage strains to the virucidal action of quaternaries were not of primary concern in this work, it was desired to use a strain which was not highly sensitive. Strain W has been reported to be comparatively resistant to quaternary action and strain 144F has characteristics which make it desirable for use in the plaque count assay method. A direct comparison was made between these two strains. The results of strain differences in resistance to the quaternary, as a function of time, are given in table 5. The effect of various concentrations of quaternary, at a single exposure time, against these two strains are summarized in table 6.

TABLE 5

Comparative resistance of phage strains 144F and W to 25 ppm quaternary at various time intervals

Phage strain	Percent inactivation of phage at time intervals of:		
	15 secs.	30 secs.	60 secs.
144F	87.500	99.950	99.999
W	99.993	99.997	99.999

Initial counts of phage suspensions:
Strain 144F: $24X10^6$ particles per ml.
Strain W: $45X10^6$ particles per ml.

TABLE 6

Variations in resistance between phage strains 144F and W to 30 seconds exposure to various concentrations of quaternary

Phage strain	Quaternary concentration	Plaque count per ml.	Percent inactivation
	ppm		
144F	12.5	$110X10^5$	91.270
	25	$6X10^5$	99.524
	50	$6X10^1$	99.999+
	100	$<1X10^1$	100
	200	$2X10^1$	99.999+
W	12.5	$31X10^3$	99.295
	25	$2X10^3$	99.954
	50	$13X10^1$	99.999+
	100	$<1X10^1$	100
	200	$<1X10^1$	100

Initial counts of phage suspensions:
Strain 144F: $126X10^6$ particles per ml.
Strain W: $44X10^5$ particles per ml.

The greater resistance of phage strain 144F to the action of the quaternary is apparent at the 15 and 30 second exposure periods as illustrated in table 5. At the 60 second exposure period both strains showed the same order of resistance. Table 6 shows that strain 144F is more resistant at 12.5 and 25 ppm quaternary. At higher concentrations the resistance of these two strains is similar. A sharp increase in the percent inactivation occurred with both strains when the quaternary concentration was increased from 25 to 50 ppm.

Concentration and pH of quaternary solutions. Since the pH of quaternary solutions have been shown to modify germicidal activity it becomes necessary to consider this factor in concentration studies. Tables 7 through 10 summarize the results of a series of trials in which various concentrations of quaternary were employed at controlled pH levels. Taken collectively these tables show the relationship between virucidal concentration and pH. Table 11 shows the effect of pH on a single concentration of quaternary. The results of control studies employing buffers alone are given in table 12. The pH values are those of the virucide-phage mixture in the medication bottles.

Tables 7 through 10 indicate an increase in virucidal activity of the quaternary with increasing pH values.

A concentration of 25 ppm at pH 9 was found to be equivalent in activity to 50 ppm concentrations at lower pH levels. A sharp decrease in phage survivors occurred at all pH levels when concentrations were increased from 25 to 50 ppm. This was particularly evident in the 15 and 30 second exposure periods. Extended exposure times reduced the effects of concentration and pH on virucidal activity. Results from table 11 show a steady decline in surviving phage between pH values of 4 through 7. The slight increase in resistance between pH 7 and 8 was not apparent in other exposure times. Maximum phage inactivation occurred at pH 9. At pH 4 the buffer alone inactivated more phage than did the virucide. It should be pointed out that the buffer was employed for a period of 60 seconds whereas the quaternary was used for only 15 seconds. This does, however, emphasize the sensitivity of the phage particles to low pH values. No inactivation due to buffers occurred at other pH levels. Figure 1 shows that the rate of phage inactivation by the quaternary increased with increases in pH.

Organic matter. Under ideal conditions quaternaries as sanitizing agents are applied to previously clean surfaces. These conditions may not always be found and inadequate cleaning procedures may result in the accumulation of considerable amounts of organic matter in

TABLE 7

Effect of quaternary concentration on activity at pH 5.0 against phage strain 144F

Quaternary concentration	Final pH	Plaque count X10 per ml. at various exposure times in seconds				
		15	30	60	120	300
ppm						
25	5.0	lysed*	lysed	231	300	26
50	5.0	35	16	0	2	1
100	5.0	3	1	1	0	0
200	5.0	0	1	1	0	0

* Lysed denotes phage present in sufficient numbers to lyse bacterial growth resulting in a clear plate. Initial count of phage suspension: $59X10^5$ particles per ml.

TABLE 8

Effect of quaternary concentration on activity at pH 6.0 and 7.0 against phage strain 144F

Quaternary concentration	Final pH	Plaque count X10 per ml. at various exposure times in seconds				
		15	30	60	120	300
ppm						
25	6.1	lysed*	88	11	3	0
50	6.2	38	2	0	1	0
25	7.1	lysed	79	9	2	0
50	7.0	6	1	0	1	0

* Lysed denotes phage present in sufficient numbers to lyse bacterial growth resulting in a clear plate. Initial count of phage suspension: $76X10^5$ particles per ml.

TABLE 9

Effect of quaternary concentration on activity at pH 8.0 against phage strain 144F

Quaternary concentration	Final pH	Plaque count X10 per ml. at various exposure times in seconds				
		15	30	60	120	300
ppm						
25	8.15	TC*	9	3	1	1
50	8.1	19	4	5	1	2
100	8.1	1	1	0	2	0
200	8.0	2	1	0	0	0

*TC denotes plaques too numerous to count accurately.
Control count of initial phage suspension: $82X10^5$ particles per ml.

TABLE 10

Effect of concentration on quaternary activity at pH 9.0 against phage strain 144F

Quaternary concentration	Final pH	Plaque count X10 per ml. at various exposure times in seconds				
		15	30	60	120	300
ppm						
25	9.05	15	1	1	1	1
50	9.0	1	3	0	1	0
100	9.0	0	0	1	2	1
200	8.9	0	0	0	0	0

Control count of initial phage suspension: $71X10^5$ particles per ml.

TABLE 11

Effect of pH on the activity of 25 ppm quaternary against phage strain 144F

Final pH	Plaque counts per ml. at various exposure times		
	15 secs.	30 secs.	60 secs.
4.2	$159X10^4$	$15X10^4$	$58X10^1$
5.0	$127X10^4$	$198X10^2$	$5X10^2$
6.15	$10X10^3$	$4X10^1$	$6X10^1$
7.1	$153X10^1$	$11X10^1$	$4X10^1$
8.1	$28X10^2$	$<1X10^1$	$<1X10^1$
9.0	$10X10^1$	$6X10^1$	$<1X10^1$

Initial count of phage suspension: $61X10^5$ particles per ml.

TABLE 12

Effect of M/100 buffers at various pH levels on phage strain 144F during a 60 second exposure period

Buffer	Final pH	Plaque count per ml. $X10^5$
Acetate	4.3	7.1
Acetate	4.9	67
Phosphate	5.95	67
Phosphate	7.05	72
Borate	8.1	83
Borate	8.9	78

Initial count of phage suspension: $72X10^5$ particles per ml.

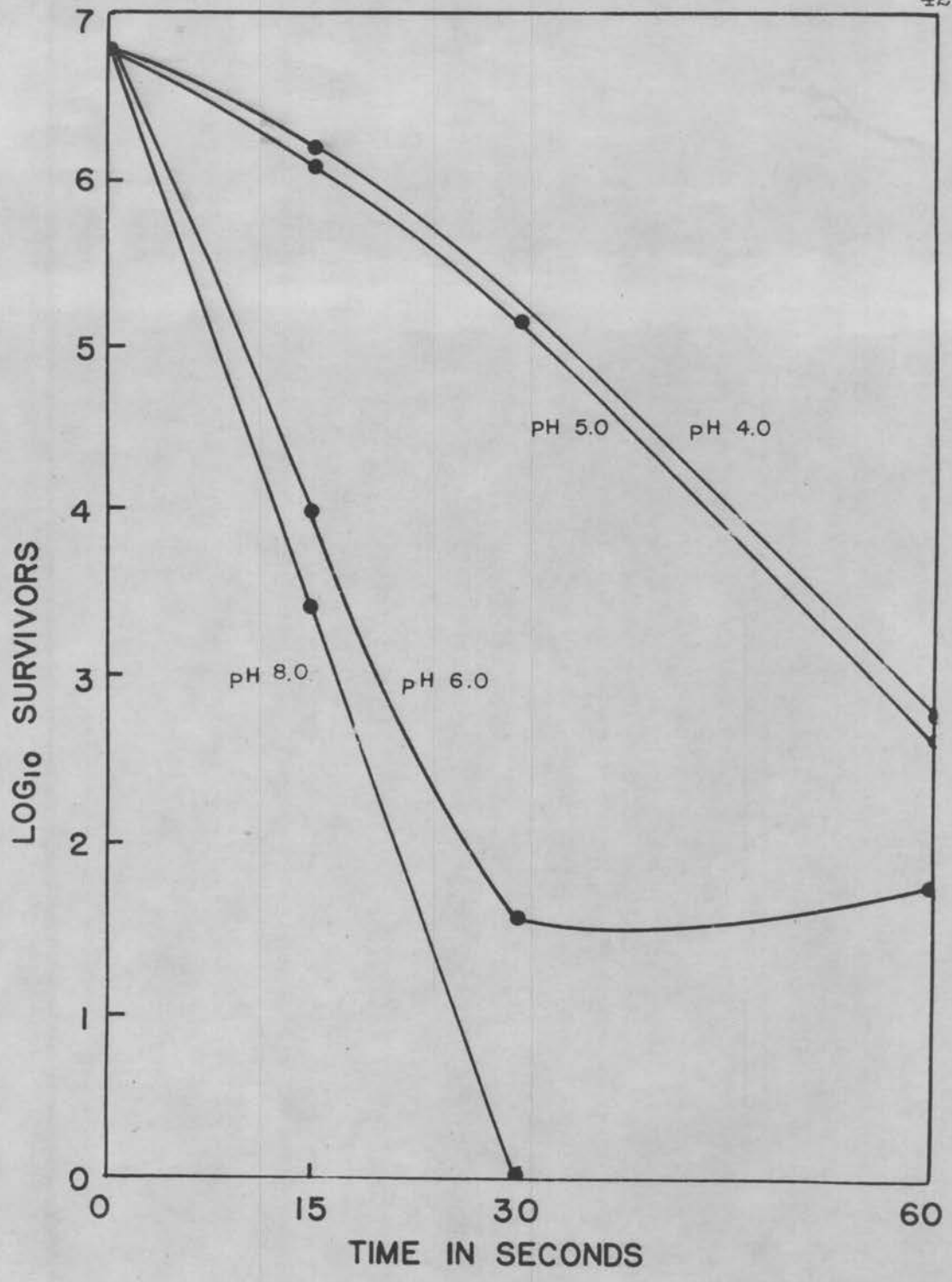

Figure 1. Effect of pH on the rate of inactivation of phage strain 144F by 25 ppm quaternary.

sanitizing solutions. The following studies were made to show what might be expected of the quaternary in the presence of organic matter. Various dilutions of a phage whey filtrate, in distilled water, were used to simulate the type of organic matter which might be found on dairy equipment. It was felt that differences in the number of phage particles in the various filtrate dilutions would not affect the overall activity of the test virucide in the presence of added organic matter.

The data in table 13 show the effect of increasing amounts of organic matter on the activity of various concentrations of quaternary acting for 1 minute. A quaternary concentration of 200 ppm and an exposure period of 2 minutes has been recommended for the destruction of phage on dairy equipment. A trial was made using this concentration of quaternary in the presence of 0.05% organic matter, to determine if organic matter reactions could be overcome. Results over a 2 minute exposure period are given in table 14.

As indicated in table 13, the organic matter present in a 1:20 dilution of a whey filtrate was sufficient to greatly reduce the virucidal activity of quaternary solutions ranging from 25 ppm to 100 ppm. When the whey filtrate was used undiluted the quaternary showed little virucidal activity even at 100 ppm. By increasing the

TABLE 13

Effect of organic matter on the activity of various concentrations of quaternary against phage strain 144F during a 60 second exposure period

Quaternary concentration	Dilution of whey filtrate	Percent organic matter	Final pH	Plaque count per ml.
ppm				
25	1:2	0.5	5.7	$152X10^7$
25	1:20	0.05	6.4	$238X10^5$
25	1:200	0.005	6.7	$23X10^1$
50	1:2	0.5	5.7	$139X10^7$
50	1:20	0.05	6.4	$229X10^3$
50	1:200	0.005	6.8	$<1X10^1$
100	1:2	0.5	5.7	$112X10^7$
100	1:20	0.05	6.4	$29X10^3$
100	1:200	0.005	6.8	$<1X10^1$

Initial count of 1:2 phage suspension: $165X10^7$ per ml.
Initial count of 1:20 phage suspension: $165X10^6$ per ml.
Initial count of 1:200 phage suspension: $165X10^5$ per ml.

TABLE 14

Activity of 200 ppm quaternary, in the presence of organic matter, against phage strain 144F

Dilution of whey filtrate	Percent organic matter	Final pH	Plaque count per ml. at exposure periods of: 30 secs.	120 secs.
1:20	0.05	6.4	$11X10^1$	$<1X10^1$

Initial count of phage suspension: $35X10^7$ particles per ml.

quaternary concentration to 200 ppm and extending the exposure period to 120 seconds the organic matter effect in a 1:20 dilution of a whey filtrate was overcome, as indicated in table 14.

Calcium ions. The germicidal activity of quaternaries is reduced in the presence of hard water salts. To study this effect in relation to phage inactivation, quaternary solutions were prepared containing various amounts of calcium ions added as $CaCl_2$. The data in table 15 illustrate the effect of increasing amounts of $CaCl_2$ on quaternary virucidal activity. There was a steady decline in quaternary activity with increasing concentrations of $CaCl_2$. The controls showed no inactivation of phage due to calcium or testing procedures. Other trials were made with various dilutions of whey filtrates and at pH 5.0 and 8.0. Under all these conditions the inhibitory action of added $CaCl_2$ on quaternary activity was apparent.

Chelating agents. In many instances the activity of quaternaries against bacteria may be increased by the addition of a chelating agent to the germicide. Some commercially marketed quaternaries contain chelating agents. Studies on the effect of chelating agents in bacteriophage sanitation have not been reported. In the present study two types of chelating agents were studied.

TABLE 15

Effect of calcium ions on the activity of 50 ppm quaternary against phage strain 144F during a 30 second exposure period

Concentration of $CaCl_2$	Final pH	Plaque count of phage survivors per ml.
ppm		
0 (dist. water)	6.4	$96X10^4$
25	6.2	$222X10^4$
50	6.2	$238X10^4$
100	6.2	$358X10^4$

Initial count of phage suspension: $36X10^7$ per ml.

TABLE 16

Effect of EDTA on the activity of 50 ppm unbuffered quaternary against phage strain 144F in the presence of 0.05 percent organic matter

Concentration of EDTA	Final pH	Plaque count per ml. after 30 secs. exposure
ppm		
0	6.2	$189X10^4$
25	6.0	$378X10^4$
50	5.75	$575X10^4$
100	5.4	$1700X10^4$

Initial plaque count of phage suspension: $30X10^7$ per ml.

Ethylene diamine tetraacetate (EDTA) was selected as a typical organic chelating agent. Sodium pyrophosphate was used as a representative inorganic chelating agent. Preliminary studies showed that 100 to 200 ppm chelating agent could be used alone without significantly inactivating phage.

Table 16 summarizes results obtained using EDTA in various concentrations with 50 ppm quaternary. These results, obtained in the presence of organic matter in a 1:20 dilution of a whey filtrate, show that a decrease in virucidal activity occurred with increasing concentrations of chelating agent. It also was noted that the addition of EDTA to the quaternary lowered the pH of the solutions. Trials were made in the presence of the same amount of organic matter but with buffered solutions. Under these conditions virucidal activity was again found to be reduced with the addition of EDTA, but to a lesser degree than in unbuffered solutions. To avoid organic matter and pH effects, higher dilutions of a whey filtrate were used in succeeding trials and the quaternary solutions were buffered. Table 17 shows the effect of EDTA on quaternary activity under these conditions. The virucidal activity of the quaternary was increased by the addition of EDTA at pH 5 and 8, with a higher degree of phage inactivation occurring at pH 8. The controls showed no significant phage inactivation due to the

TABLE 17

Effect of EDTA on the activity of 25 ppm buffered quaternary in the absence of organic matter against phage strain 144F

Concentration of EDTA	Final pH	Plaque count per ml. after 30 secs. exposure
ppm		
0	5.2	$1170X10^4$
25	5.1	$1140X10^4$
50	5.05	$1255X10^4$
100	5.05	$173X10^4$
200	5.0	$112X10^4$
0	8.0	$71X10^4$
25	7.9	$66X10^4$
50	7.95	$10X10^4$
100	7.9	$33X10^2$
200	7.9	$4X10^1$

Initial count of phage suspension: $65X10^6$ per ml.

chelating agent alone. Figure 2 shows graphically the potentiating action of EDTA on the virucidal activity of the quaternary.

It was felt that a study of the relationship between EDTA and various concentrations of calcium ions might provide information of practical importance and possibly indicate the nature of calcium ion attachment to phage particles. A series of phage suspensions were prepared with varying concentrations of $CaCl_2$. Each of these suspensions were exposed to 25 ppm quaternary with added EDTA. Results of this experiment are given in table 18. EDTA was found to be very effective in potentiating quaternary action in the absence of $CaCl_2$. With addition of $CaCl_2$ to the phage suspensions this potentiating activity was reduced. A $CaCl_2$ concentration of 100 ppm completely blocked the potentiating effect of 200 ppm EDTA.

The results obtained with studies on quaternary potentiation by the inorganic chelating agent, sodium pyrophosphate, are given in table 19. This compound showed a potentiating effect similar to that obtained with EDTA. A marked increase in quaternary activity occurred when 100 ppm sodium pyrophosphate were incorporated into the virucide. The control showed no inactivation of phage exposed to 200 ppm chelating agent.

TABLE 18

Effect of various concentrations of EDTA and $CaCl_2$ on the virucidal activity of 25 ppm quaternary, against phage strain 144F

Initial plaque count per ml.	Conc. of $CaCl_2$	Conc. of EDTA	Plaque count per ml. after 30 seconds
	ppm	ppm	
$62X10^6$	0	0	$10X10^5$
$62X10^6$	0	200	$2X10^1$
$57X10^6$	25	200	$102X10^1$
$46X10^6$	50	200	$368X10^3$
$55X10^6$	100	200	$19X10^5$
$39X10^6$	200	200	$16X10^5$

TABLE 19

Effect of sodium pyrophosphate on the activity of 25 ppm quaternary against phage strain 144F

Conc. of $Na_4P_2O_7$	Final pH	Plaque count per ml. after 30 secs. exposure
ppm		
0	7.85	$276X10^3$
25	7.95	$180X10^3$
50	8.0	$112X10^3$
100	8.0	$22X10^1$
200	8.0	$4X10^1$

Initial count of phage suspension: $74X10^6$ per ml.

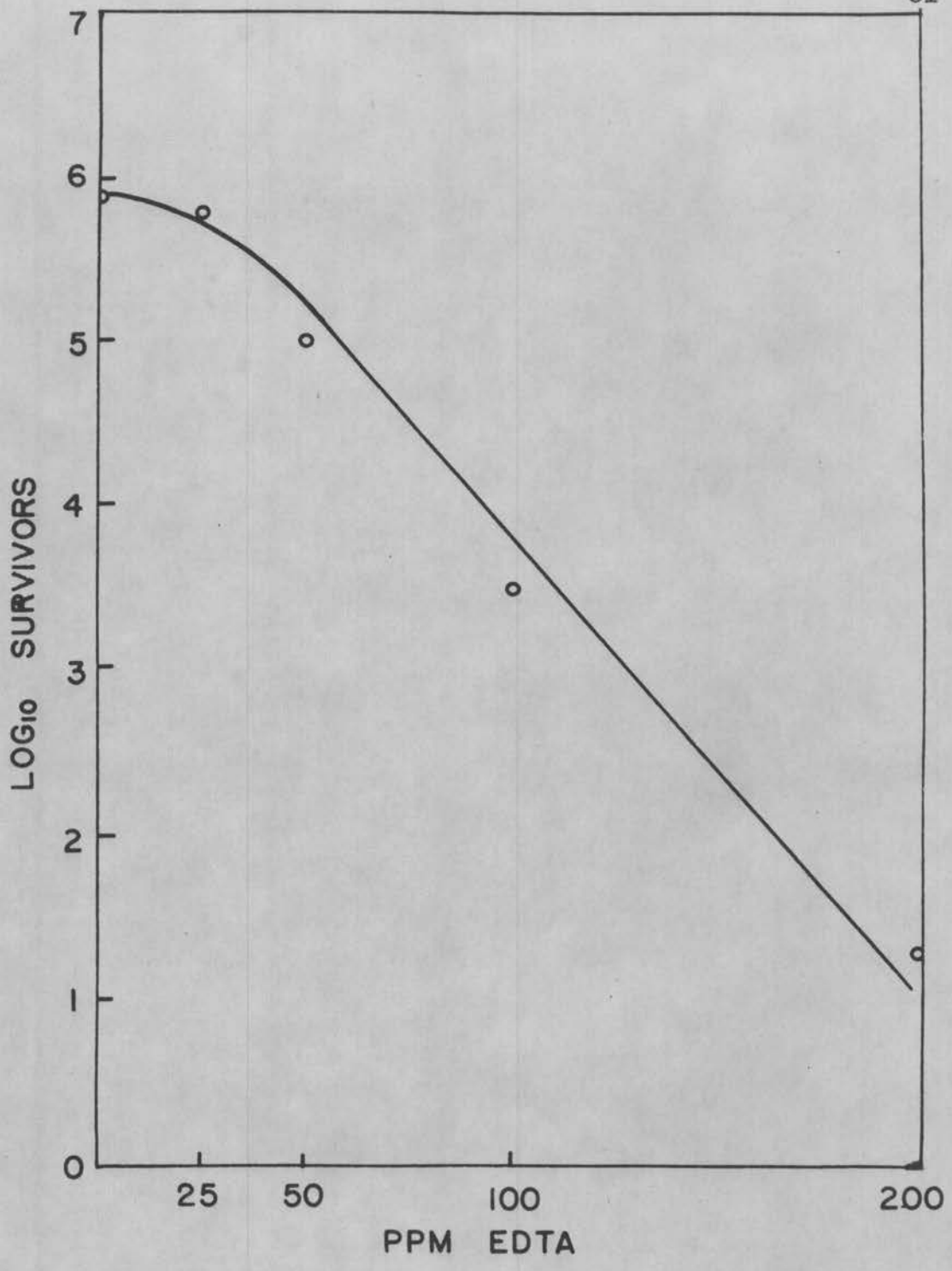

Figure 2. Effect of EDTA on virucidal activity of 25 ppm quaternary, at pH 8.0, against phage strain 144F during a 30 second exposure period.

A series of control studies were made to determine if the quaternary activity could be increased by other forms of phosphate compounds. Results of these studies, as tabulated in table 20, show that of the various combinations of phosphate preparations tested, only sodium pyrophosphate significantly increased quaternary activity.

TABLE 20

Effect of type of phosphate on activity of 25 ppm quaternary against phage strain 144F

Type of phosphate	Final pH	Plaque count per ml. after 30 seconds
M/200 borate buffer plus no phosphate	7.9	$18X10^5$
M/200 borate buffer plus 200 ppm Na_3PO_4	7.8	$13X10^5$
M/200 borate buffer plus 200 ppm $Na_4P_2O_7$	7.6	$34X10^1$
M/200 phosphate buffer	7.8	$2X10^5$
M/200 phosphate buffer plus 200 ppm Na_3PO_4	7.8	$13X10^5$

Initial count of phage suspension: $95X10^6$ per ml.

Discussion

Under most conditions, strain 144F proved to be more resistant to the quaternary than strain W. This was particularly apparent during short exposure periods. As the length of exposure time to the virucide was increased the two strains exhibited similar resistance. Parker (48,

p.1) has reported that W strain has yielded more resistant particles. Under the conditions of his test a 100% inactivation end point was used. In the present study the presence of a few resistant phage particles within a population was not under study. Under practical plant conditions, however, the presence of such resistant particles would assume great importance.

As mentioned earlier the activity of quaternary ammonium compounds has been attributed either to penetration into a bacterial cell and reaction with enzymes or to the disruption of bacterial surface due to high adsorption. In theorizing about the mode of action of these compounds on bacteriophage, limitations are imposed by the lack of knowledge of bacteriophage structure in general and also the lack of fundamental studies with the lactic acid streptococci host-phage systems.

The data obtained on the effect of concentration and pH suggest that virucidal activity of quaternaries can be explained on the basis of surface adsorption. The outer layer of the phage particle consists of protein material. If the pH of the solution containing phage particles is increased it would be expected that there would be an increasing number of acidic groups ionized which would react with the positively charged quaternary molecule. Glassman (25, pp.91-104) has reviewed some of the reactions which may occur between surface active agents and

proteins. Among these reactions are precipitation, denaturation and complex formation. Bacteriophage particles must have highly oriented surface groups to account for their high degree of host specificity. Even slight modifications of these groups could result in loss of activity for the phage particle.

It is true that some species of bacteria are more sensitive to quaternaries in solutions of low pH values. MacGregor, (38, p.66) in a recent study of quaternary action on Pseudomonas aeruginosa, suggested that the greater susceptibility of these cells in acidic solutions was due to a permeability factor and that greater adsorption of the germicide occurred at higher pH values in spite of the reduced effectiveness displayed. Increased susceptibility of phage particles to quaternaries at low pH values has not been reported and it may not be necessary to presume penetration beyond the protein coat to account for inactivation of biological activity.

The presence of organic matter in the form of whey proteins was found to markedly decrease the activity of the quaternary against bacteriophage. A 1:20 dilution of whey is representative of a comparatively heavy concentration of organic matter which may be found under conditions where inadequate cleaning has been carried out. The desirability of clean surfaces is emphasized

by these results. From theoretical considerations it is not surprising that organic matter lowers the efficiency of these compounds. The proteins as biological materials contain the same or similar groups as bacterial or phage particles which react with the quaternary molecule.

Calcium ions were shown to decrease the activity of the quaternary against phage, when added either to the virucide solution or phage suspension. The most striking results were obtained with various combinations of calcium ions and chelating agents. The chelating agents EDTA and sodium pyrophosphate showed a much greater effect in potentiating quaternary activity than did the calcium ions in retarding activity. However, the potentiating activity of the chelating agents could be almost completely overcome by the addition of calcium ions. From these reactions a theory of phage-calcium ions relationship may be proposed. The phage particle may hold calcium ions in a complex which is more resistant to quaternary action than is the phage particle alone. With the addition of chelating agents these calcium ions may be removed from the phage particle thus exposing more sensitive sites for the quaternary to act upon.

In the presence of organic matter it was found that EDTA reduced the virucidal activity of the quaternary. Under these conditions it is possible that the chelating

agent was also reacting with cations, introduced with the organic matter, in free form and also those associated with whey protein. This may account for the failure of the chelating agent to potentiate quaternary activity but the inhibitory action remains unexplained.

PART III

FACTORS AFFECTING THE VIRUCIDAL ACTIVITY OF CHLORINE COMPOUNDS

The hypochlorites, as mentioned in the Historical section, have been shown to be very effective against the bacteriophage for the lactic acid streptococci. These compounds were included in this study to compare their activity with other virucides using the same test methods and to determine some of the fundamental factors affecting their activity against a bacteriophage of S. cremoris.

The trichlorocyanuric acid compounds are a new class of organic chlorine germicides. In solution these compounds hydrolyze to yield hypochlorous acid as the active agent. It was considered desirable to evaluate germicidal and virucidal properties of these new preparations along with sodium hypochlorite.

Results

Effect of concentration and pH. In the previous section on quaternaries it was possible to show critical concentration levels at which inactivation of phage particles was sharply increased. A relationship between these concentration levels and the pH of virucide solutions was apparent. It was desired to show this type of relationship with the chlorine compounds.

A series of trials was made using concentrations as low as 10 ppm sodium hypochlorite against a 1:200 dilution of a whey filtrate of phage strain 144F. Complete inactivation of the phage was obtained in a 60 second exposure period at pH values ranging from 4.0 through 9.0. To establish conditions permitting some phage survival, the exposure time was reduced to 30 seconds and a 1:20 dilution of a phage filtrate was employed, which introduced additional organic matter into the test system. Under these conditions sufficient phage survival was obtained to allow evaluation of pH effects. Table 21 and figure 3 illustrate the results obtained on the effect of pH on sodium hypochlorite activity. It was noted that maximum virucidal activity occurred at pH 4.0 and rapidly decreased with increasing pH values through pH 7.0. Between pH 7.0 and 9.0 there was relatively little difference in virucidal activity.

Bactericidal and virucidal properties of trichlorocyanuric acid compounds (TCU). Bactericidal trials against Micrococcus caseolyticus were included with these compounds as a control measure and to evaluate their general activity against a representative test organism as well as against phage. As indicated in table 22, phage were completely inactivated by all 6 of the TCU compounds in 15 seconds at a concentration of 10 ppm available

TABLE 21

Effect of pH on virucidal activity of 10 ppm sodium hypochlorite against phage strain 144F

Final pH	Plaque count per ml. after 30 secs.
4.4	$<1X10^1$
5.15	$2X10^1$
6.05	$130X10^3$
7.0	$441X10^5$
7.8	$385X10^5$
8.85	$950X10^5$

Initial count of phage suspension: $35X10^7$ per ml.

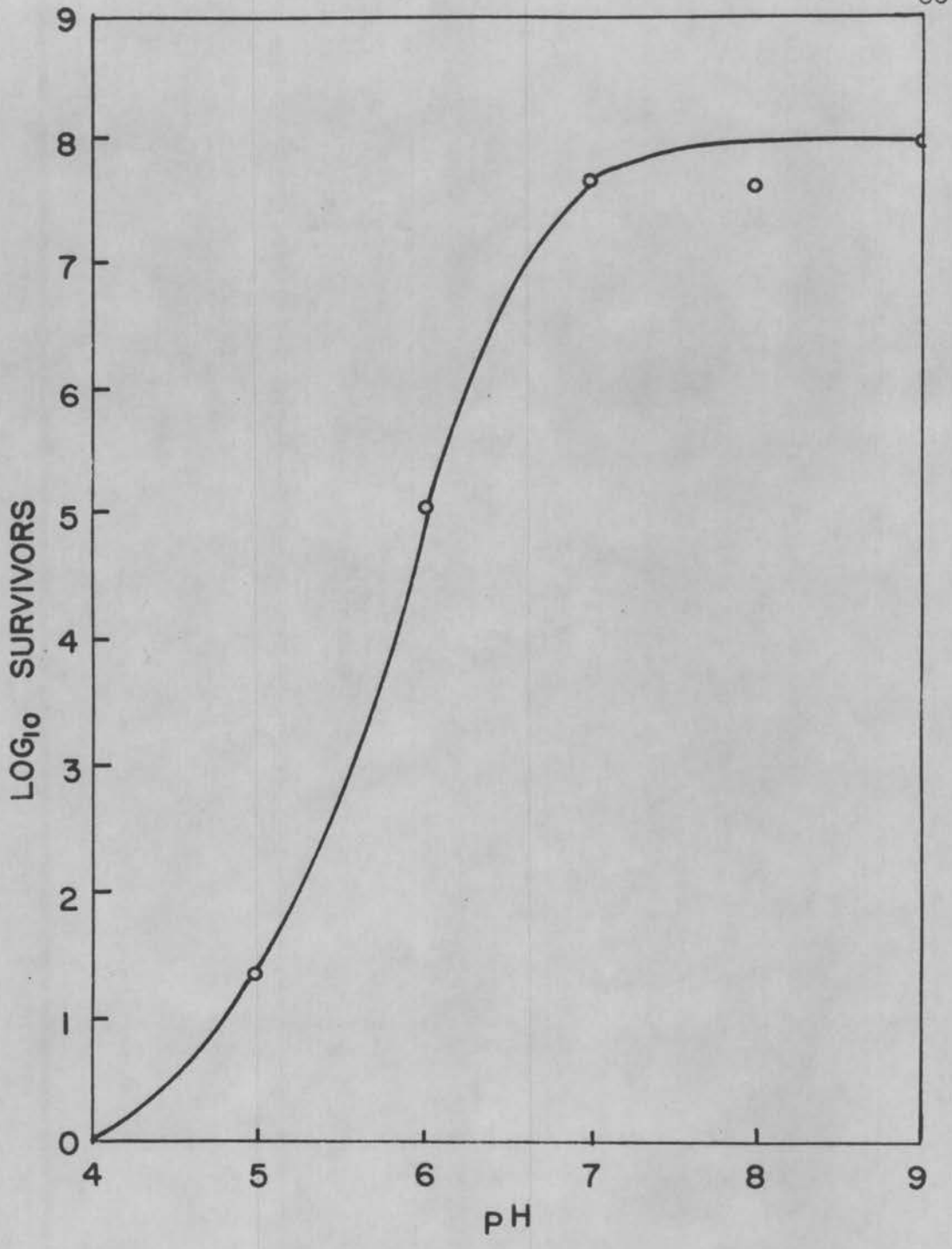

Figure 3. Effect of pH on virucidal activity of 10 ppm sodium hypochlorite against phage strain 144F during a 30 second exposure period.

TABLE 22

Activity of trichlorocyanuric acid (TCU) preparations against *Micrococcus caseolyticus* and phage strain 144F

Composition of virucide			Survivors after 15 secs.	
Available chlorine	Additional components	Final pH	Bacterial plate count per ml.X10	Phage plaques per ml.X10
ppm				
10	None	7.6	138	<1
25	None	7.6	<1	<1
10	Acid salts	6.6	15	<1
25	Acid Salts	6.7	<1	<1
10	Polyphosphate	7.7	12	<1
25	Sodium bicarb.	7.7	<1	<1
10	Polyphosphate	7.6	416	<1
25	Sodium carb.	7.8	<1	<1
10	Sodium	7.8	131	<1
25	Carbonate	7.8	<1	<1
10	Na_2CO_3 plus	7.8	<1	<1
25	Wetting agent	7.7	<1	<1

Initial count of phage suspension: $111X10^6$ per ml.
Initial count of bacterial suspension: $117X10^6$ per ml.

chlorine. Variations in activity against M. caseolyticus were displayed by the various preparations with only one compound giving complete kill. At 25 ppm all of the preparations were 100 percent effective against both phage and bacteria in a 15 second exposure time.

Organic matter. Hypochlorites, as well as other forms of chlorine germicides, are known to be adversely affected by the presence of organic matter. The following experiments were made to determine the concentration of sodium hypochlorite necessary to overcome the effects of organic matter, introduced into the test system by various dilutions of a phage whey filtrate in an effort to simulate practical use conditions. As mentioned in the section on quaternaries, the various dilutions of whey filtrate will contain different numbers of phage particles. It was not believed that this would significantly affect the overall evaluation of virucidal activity in the presence of organic matter. The results from table 23 indicate that solutions of 25 and 50 ppm hypochlorite were effective in overcoming the 0.05% organic matter. The amount of organic matter introduced in a 1:2 dilution of whey could not be overcome by 100 ppm hypochlorite. The degree of survival obtained when a solution of 10 ppm hypochlorite was employed against a 1:20 dilution of the phage suspension suggested a means

TABLE 23

Effect of organic matter on the virucidal activity of sodium hypochlorite against phage strain 144F

Virucide conc.	Final pH	Dilution of filt.	% organic matter	Plaque count per ml. after 30 seconds
ppm				
10	6.8	1:200	0.005	$1X10^1$
25	6.6	1:200	0.005	$1X10^1$
10	6.4	1:20	0.05	$334X10^3$
25	6.5	1:20	0.05	$4X10^1$
50	6.5	1:20	0.05	$1X10^1$
100	6.0	1:2	0.5	$536X10^5$

Initial count of 1:2 dilution: $18X10^8$ per ml.
Initial count of 1:20 dilution: $18X10^7$ per ml.
Initial count of 1:200 dilution: $18X10^6$ per ml.

TABLE 24

Rate of inactivation of phage strain 144F by 10 ppm sodium hypochlorite in the presence of 0.05% organic matter

Final pH	Plaque counts per ml. after exposure times of: 15 secs.	30 secs.	60 secs.	120 sec.	300 sec.
6.4	$173X10^3$	$174X10^3$	$189X10^3$	$140X10^3$	$120X10^3$

Initial count of phage suspension: $18X10^7$ per ml.

of studying phage inactivation rates of hypochlorites. An experiment was made employing these conditions but increasing the number of exposure intervals over a period of 5 minutes. The data from table 24 point out that most of the phage destruction occurred within the first 15 seconds, with only a slight amount of inactivation occurring between 1 and 5 minutes.

Discussion

Both sodium hypochlorite and the trichlorocyanuric acid (TCU) preparations proved to be very effective virucidal agents. It was of interest that concentrations of the TCU compounds which completely inactivated phage permitted some survival of bacteria. Only one preparation, containing a wetting agent, was equally effective against phage and bacteria. The wetting agent may have functioned to disperse clumps of bacteria making individual cells more vulnerable to germicidal action.

The studies on chlorine activity against B. metiens spores by Marks and Strandskov was referred to in the Historical section. It was found by these workers that maximum sporicidal activity of hypochlorous acid occurred at pH 6.0 with no further increase in activity at lower pH values. At pH values above 6.0 activity decreased sharply. The sporicidal action was attributed to the unionized hypochlorous acid molecule and it was considered

to be almost completely in this form at pH 6.0. At pH values above 6.0 sporicidal activity decreased sharply. This latter reaction was attributed to the ionization of hypochlorous acid.

In the present study it was found that maximum virucidal activity occurred at pH 4.0 and rapidly decreased through pH 7.0. These results suggest that hypochlorous acid is not completely undissociated at pH 6.0 and that equilibrium reactions continue to form additional undissociated hypochlorous acid at least through pH 4.0. It is also possible that the increased activity of the hypochlorite at these low pH values may be due to a more favorable pH level for oxidation reactions to occur or possibly due to an increased sensitivity of the phage particle itself. The rapid decline in virucidal activity between pH 6.0 and 9.0 may be due to the ionization of hypochlorous acid to the relatively inactive hypochlorite ion.

In the presence of organic matter a considerable amount of phage inactivation occurred within the first 15 seconds of exposure to the hypochlorite, with no significant decrease in phage after this period. This suggests that the hypochlorite reacts very rapidly with both the phage particles and organic matter. If the hypochlorite concentration is increased to provide a margin

of free chlorine complete inactivation of the phage would be expected to occur. Under the test conditions employed, 25 ppm hypochlorite was effective in the presence of moderate amounts of organic matter. It should be pointed out, however, that the pH levels of the test solutions were lower than those usually encountered in hypochlorite solutions used for sanitation procedures, and higher concentrations would probably be required under practical conditions.

PART IV

FACTORS AFFECTING THE VIRUCIDAL ACTIVITY OF IODINE COMPOUNDS

As mentioned in the Historical section, iodine has been recognized for many years as a very active bactericide. The work done with the iodophors, including numerous studies in this laboratory, have shown them to be rapidly acting germicides against a wide variety of bacterial vegetative cells. To date there have been no results published on the activity of these compounds against bacteriophage. For this reason and also because iodophors are being introduced into the dairy industry as sanitizing agents, the following studies were made. All of the factors shown to affect the activity of the quaternary and chlorine compounds were considered in relation to the iodophor. Other forms of iodine were included for purposes of comparison with the iodophor.

The following results are representative and were obtained by methods described in previous sections. Controls with the iodine compounds were made by neutalizing the iodine content with powdered sodium thiosulfate until the disappearance of color. In this manner any possible virucidal activity of other components of the iodine preparations could readily be detected.

Results

Variations in resistance between phage strains. Before studying the factors affecting the virucidal activity of iodine compounds, a comparison of iodine resistance between phage strains 144F and W was made to select the most resistant strain. The two phage strains were exposed to 100 ppm iodophor over a period of 5 minutes. Results of this trial, as indicated in table 25, showed that strain W was more resistant at 15 and 60 second exposure periods, however after 5 minutes exposure W phage was completely inactivated while 144F showed some survival. In actual numbers more phage particles of strain 144F survived at all time intervals due to an initially higher titer. Because of its ability to give higher titer stocks and more desirable plaquing characteristics, phage strain 144F was employed in succeeding experiments.

Concentration of iodine solutions. Iodophor concentrations of 10 to 25 ppm have been recommended for use in sanitizing procedures. Results from experiments in this laboratory with heavy suspensions of bacterial vegetative cells showed these concentrations to be effective. However, when employed against the lactic phage, they proved to be inadequate. As the iodophors have a potential use for dairy sanitation, it was desired to determine the concentration necessary to inactivate phage during short

exposure periods. A trial was made employing concentrations ranging from 10 ppm to 200 ppm. As shown in table 26, these concentrations were all ineffective during a 60 second exposure period. Furthermore, substantial increases in concentration were not reflected in corresponding increases of virucidal activity. These results were surprising, considering the bactericidal properties of the iodophors. Therefore it was decided to test the iodophor against phage and bacterial cells under the same experimental conditions. Results from a trial comparing iodophor activity against M. pyogenes var. aureus and phage strain 144F are given in tables 27 and 28. These data show that concentrations which were effective against the bacterial cells failed to significantly inactivate phage. A similar trial was made comparing iodophor activity against phage strain 144F and E. coli. Concentrations ranging from 10 ppm to 200 ppm iodophor completely killed bacterial cells in 60 seconds but were ineffective against the phage. The question arose as to whether this concentration effect and lack of virucidal activity against lactic phage was characteristic of iodophors or of iodine compounds in general. Further studies on concentration effects were made with iodine-iodide and aqueous iodine preparations.

Results of trials on the virucidal activity of

TABLE 25

Comparative resistance of phage strains 144F and W to 100 ppm iodophor at various time intervals

Phage strain	Percent inactivation of phage at time intervals of: 15 secs.	60 secs.	300 secs.
144F	99.375	99.918	99.999+
W	96.245	99.306	100

Initial counts of phage suspensions:
Strain 144F: $128X10^6$ particles per ml.
Strain W: $49X10^5$ particles per ml.

TABLE 26

Effect of iodophor concentration on activity at pH 5.0 against phage strain 144F

Iodophor concentration	Plaque count per ml. after 60 seconds
ppm	
10	$334X10^3$
25	$320X10^3$
50	$200X10^3$
100	$20X10^3$
200	$68X10^3$

Initial count of phage suspension: $181X10^6$ per ml.

TABLE 27

Virucidal activity of iodophor at pH 5.0 against phage strain 144F

Conc. of Iodophor	Final pH	Plaque count per ml. at following exposure times in seconds: 15	30	60	120	300
ppm						
12.5	5.15	$22X10^3$	$9X10^3$	$383X10^1$	$44X10^1$	$1X10^1$
25	5.15	$40X10^3$	$10X10^3$	$312X10^1$	$18X10^1$	$4X10^1$
50	5.15	$20X10^3$	$9X10^3$	$171X10^1$	$20X10^1$	$1X10^1$

Initial count of phage suspension: $48X10^6$ per ml.

TABLE 28

Bactericidal activity of iodophor at pH 5.0 against *Micrococcus pyogenes* var. *aureus*

Conc. of iodophor	Final pH	Colony count per ml. at following exposure times in seconds 15	30	60	120	300
ppm						
12.5	5.05	332	36	3	1	0
25	5.05	6	5	2	1	0
50	5.05	3	1	0	0	0

Initial count of bacterial suspension: $22X10^7$

TABLE 29

Effect of concentration of iodine-iodide solutions on activity against phage strain 144F

Concentration of iodine	Plaque count per ml. after 60 seconds
ppm	
12.5	$6X10^5$
25	$4X10^5$
50	$150X10^3$
100	$107X10^3$
200	$169X10^3$

Initial count of phage suspension: $88X10^6$ per ml.

iodine-iodide solutions of varying concentrations are summarized in table 29. These solutions contained potassium iodide and elemental iodine in the ratio of 2:1. Concentrations given represent titratable iodine. Reference to these data reveal that the iodine-iodide solutions were ineffective virucidal agents in concentrations of 12.5 to 200 ppm in an exposure period of 60 seconds. As with the iodophor, little gain in virucidal activity occurred when virucide concentration was increased.

As shown in table 30, increasing concentrations of an aqueous iodine solution, free of solubilizing agents, resulted in definite increases in virucidal activity.

Almost complete inactivation of the phage occurred at 100 ppm during a 60 second exposure period.

A comparison of the effect of concentration on virucidal activity of these three types of iodine solutions is given in figure 4.

pH of iodine solutions. Results obtained from virucidal trials with the iodophor at various pH levels are illustrated in table 31 and figure 5. It was found that virucidal activity increased slightly when the pH of the test solutions was increased from pH 4.4 to pH 6.9. With further increases of pH, virucidal activity was sharply increased and at pH 9.0 complete inactivation of the phage occurred during the 60 second exposure period. Results from similar trials with aqueous iodine are summarized in table 32. These data also show that virucidal activity was increased by raising the pH values of the virucide solutions. One outstanding difference occurred between the iodophor and aqueous iodine solutions. At pH 9.0 a sharp reduction in virucidal activity occurred in the aqueous system whereas with the iodophor maximum activity was obtained at this pH level. It was noted that the iodine color of the aqueous solution disappeared when the pH of the solution was raised from 8.0 to 9.0. In the studies with the iodophor some iodine color remained in the solution at pH 9.0 at the time of use but

TABLE 30

Effect of iodine concentration in aqueous solutions on activity against phage strain 144F

Concentration of iodine	Plaque count per ml. after 60 seconds
ppm	
12.5	$45X10^3$
25	$13X10^3$
50	$25X10^1$
100	$1X10^1$

Initial count of phage suspension: $46X10^6$ per ml.

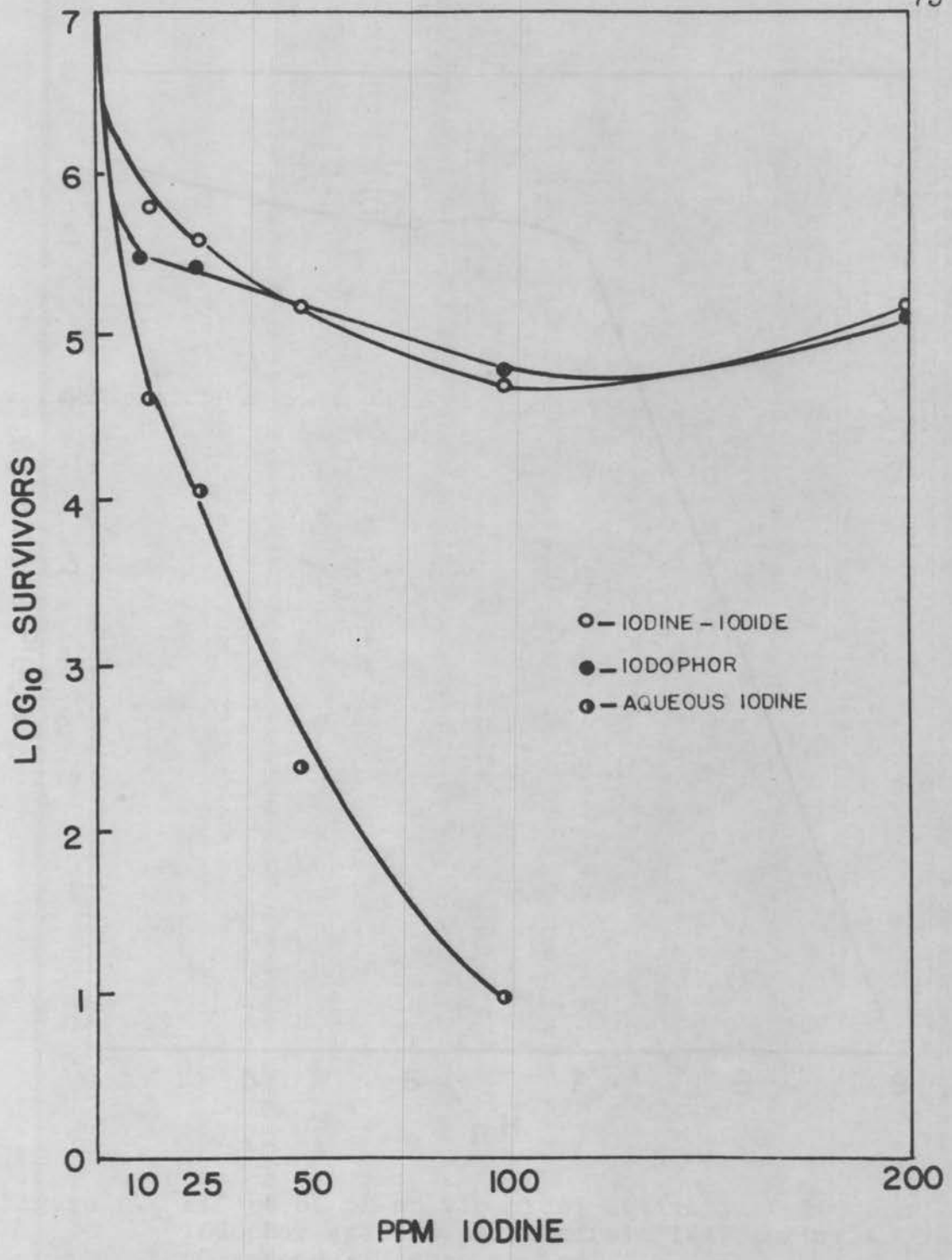

Figure 4. Effect of concentration on virucidal activity of various forms of iodine against phage strain 144F during a 60 second exposure period.

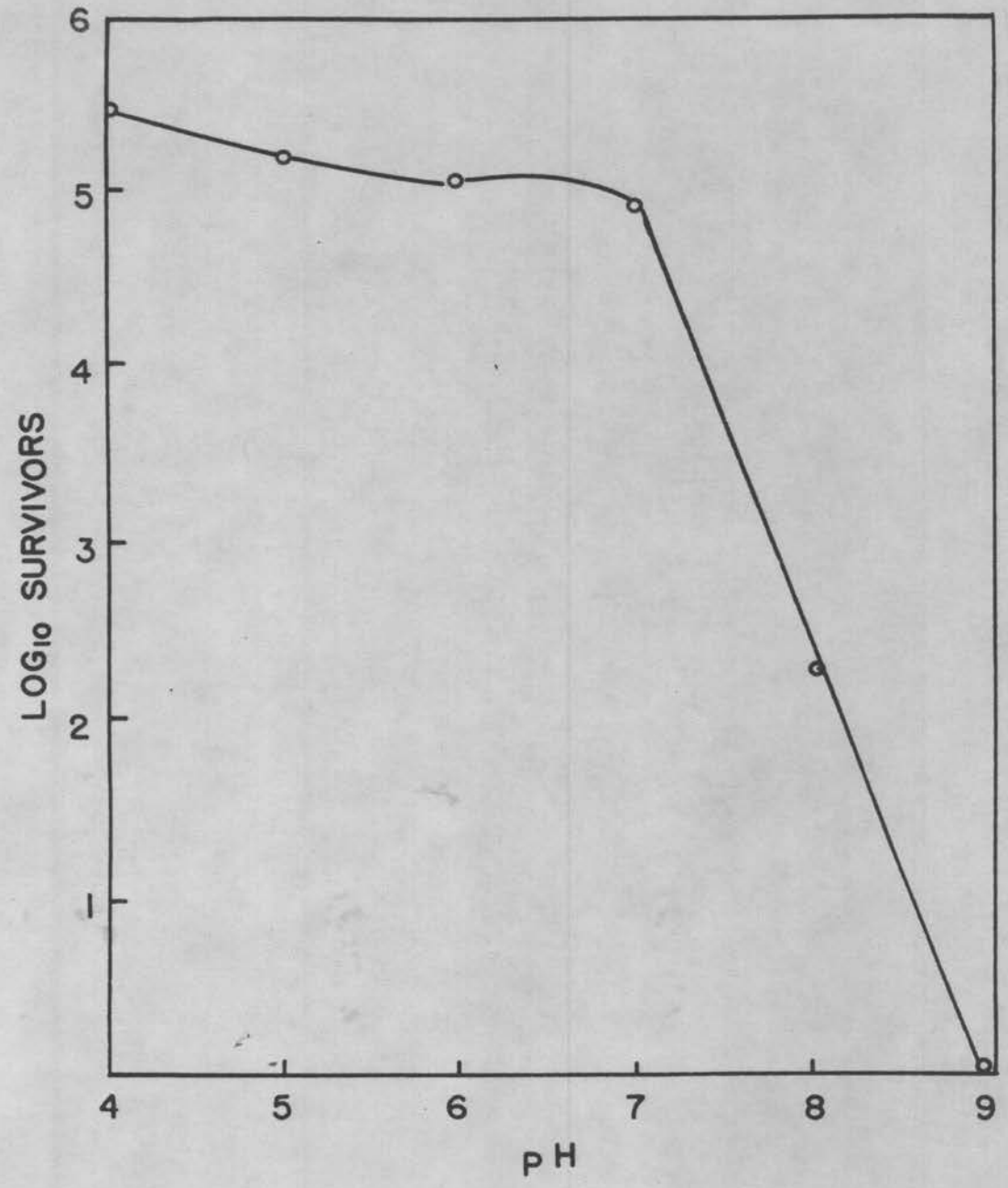

Figure 5. Effect of pH on virucidal activity of 100 ppm iodophor against phage strain 144F during a 60 second exposure period.

TABLE 31

Effect of pH on the activity of 100 ppm iodophor against phage strain 144F

Final pH	Plaque count per ml. after 60 seconds
4.4	$303X10^3$
5.15	$145X10^3$
6.05	$118X10^3$
6.9	$89X10^3$
7.95	$19X10^1$
8.9	$<1X10^1$

Initial count of phage suspension: $148X10^6$ per ml.

TABLE 32

Effect of pH on the activity of 50 ppm aqueous iodine against phage strain 144F

Final pH	Plaque count per ml. after 60 seconds
4.1	$26X10^3$
5.0	$48X10^1$
6.0	$22X10^1$
6.95	$15X10^1$
7.8	$2X10^1$
8.95	$12X10^3$

Initial count of phage suspension: $45X10^6$ per ml.

later faded. Control studies showed significant phage inactivation, due to pH effects, only at the pH 4.0 level.

Organic matter. As in previous trials, organic matter was added in the form of various dilutions of a phage whey filtrate. Results from experiments employing varying dilution of iodophor in the presence of organic matter are indicated in table 33. Reference to this table shows that increases in organic matter reduced virucidal activity of every iodophor concentration tested. Organic matter effects were not overcome by increasing the concentration of the virucide. This is especially noticeable at the 0.05% level of organic matter where 25, 50, and 100 ppm iodophor showed the same degree of phage inactivation.

Nature of iodine solution. In the preceding experiments various iodine solutions were studied separately, and under different conditions. Table 34 shows results obtained from a study of the virucidal activity of various forms of iodine solutions tested under the same conditions. Aqueous iodine proved to be more virucidal than equal concentrations of iodophor or iodine-iodide preparations during a 60 second exposure period.

Characteristics of phage surviving iodine treatment. In the virucidal studies made with the quaternary and

TABLE 33

Effect of organic matter on the activity of various concentrations of iodophor against phage strain 144F during a 60 second exposure period

Iodophor conc.	Dilution of whey filtrate	Percent organic matter	Final pH	Plaque count per ml.
ppm				
25	1:2	0.5	5.75	$113X10^7$
25	1:20	0.05	5.8	$24X10^5$
25	1:200	0.005	6.0	$220X10^2$
50	1:2	0.5	5.7	$10X10^7$
50	1:20	0.05	5.7	$24X10^5$
50	1:200	0.005	5.7	$161X10^2$
100	1:2	0.5	5.6	$6X10^7$
100	1:20	0.05	5.5	$20X10^5$
100	1:200	0.005	5.0	$163X10^2$

Initial count of 1:2 phage suspension: $165X10^7$ per ml.

Initial count of 1:20 phage suspension: $165X10^6$ per ml.

Initial count of 1:200 phage suspension: $165X10^5$ per ml.

TABLE 34

Effect of solubilizing agents on the activity of 32 ppm iodine against phage strain 144F

Solubilizing agent	Final pH	Plaque count per ml. after 60 secs.
Distilled water	6.0	$24X10^1$
Potassium iodide	6.1	$105X10^3$
Nonionic wetting agent	6.05	$12X10^3$

Initial count of phage suspension: $30X10^6$ per ml.

chlorine compounds, phage surviving treatment gave rise to plaques which could not be distinguished from those produced by untreated phage. A large proportion of the phage surviving treatment with iodine compounds were found to give rise to plaques which were considerably smaller than those produced by untreated phage. Many of them were of pinpoint size as compared to a diameter of 1.5 mm in the untreated phage. To determine if the small plaque size was a stable characteristic, a number of these plaques were picked into broth cultures of strain 144F for repropagation. Phage particles resulting from these trials gave rise to plaques of normal size.

Discussion

The iodophor displayed three interesting characteristics when employed as a virucidal agent. It proved to

be relatively ineffective against the phage particles and its activity could not be significantly changed by increasing the concentration. Virucidal activity was increased with increases in pH values of the solution. Activity against bacteria has been greatest at low pH values. The third characteristic of interest is the nature of the phage particle surviving iodine treatment with respect to plaque forming ability.

The small difference in virucidal activity of the iodophor with substantial increases in concentration may be explained if the free iodine is considered to be the active agent. When aqueous iodine is employed, differences in concentration are reflected in differences in activity. The free iodine in solution is directly proportional to the added iodine in the aqueous solution. With the iodophor a different situation exists. The equilibrium reactions taking place with these compounds were described in the review of literature. A total iodine concentration of 200 ppm might provide only a few parts per million in the free form, the rest being in combination with the non-ionic solubilizing agent. Apparently bacteria are sensitive to very low concentrations of iodine whereas the bacteriophage particles are not. Equilibrium reactions prevent the attainment of free iodine concentrations which would be effective

against the phage. A similar situation occurs with the iodine-iodide system.

With both aqueous iodine and the iodophor, increases in pH resulted in increased activity. It was also noted in the preceding paragraph that increases in free iodine resulted in increased activity against phage particles. It would be difficult to explain these results as due to the pH effect on the virucide alone. Raising the pH of an iodine solution tends to shift the equilibrium to favor the formation of iodides and hypoiodates at the expense of molecular iodine. The possibility exists then that the phage particle itself is rendered more sensitive to the action of iodine at higher pH values. When the iodophor was employed at pH 9.0 some iodine color remained and 100% inactivation of phage suspension was attained. With the aqueous iodine preparation color remained at pH 8.0 and essentially 100% inactivation was obtained; however, when the pH was raised to 9.0 the color faded until it was almost white. Concurrent with the color change there was a marked decrease in activity, indicating the need for molecular iodine for virucidal activity.

The small plaques produced by the phage surviving iodine treatment suggest that the iodine reacted with the phage and modified it in some manner without destroying the property of infectivity. The small plaque

characteristic is not stable and is lost on the first repropagation. The underlying cause for the formation of small plaques was not determined in these studies and reasons for them may be advanced only on theoretical grounds. Some of the factors which might conceivably lead to small plaque formation would include a slow rate of adsorption and penetration of the phage particles into the host cell, and an extended latent period and small burst size.

SUMMARY AND CONCLUSIONS

A method was developed for counting S. cremoris bacteriophage by plaquing procedures. This technique was applied to a study of various factors affecting virucidal activity of a quaternary ammonium compound (alkyl dimethyl ethyl benzyl ammonium chloride), two forms of chlorine (sodium hypochlorite and trichlorocyanuric acid), an iodophor (iodine in combination with a nonionic carrier), iodine-iodide solutions and aqueous iodine.

In trials employing no added organic matter the quaternary ammonium compound proved an effective virucide in concentrations of 25 to 50 ppm, dependent on the pH of the solutions. In the presence of 0.05% organic matter 200 ppm were required for phage inactivation during a 2 minute exposure period. This concentration may be more desirable under practical conditions where quaternary compounds are used as the sanitizing agent.

Chelating agents in the form of ethylene diamine tetraacetate and sodium pyrophosphate were effective in potentiating virucidal activity of the quaternary, possibly by removal of calcium ions from phage sites which react with the quaternary molecule. The incorporation of chelating agents in quaternary formulations for phage destruction appears desirable.

The activity of the quaternary against S. cremoris

phage was studied over a pH range of 4.0 through 9.0. Highest virucidal activity was exhibited at pH 8.0 and 9.0.

The chlorine compounds, when employed with no added organic matter, were shown to rapidly inactivate phage in concentrations as low as 10 ppm over a pH range of 4.0 to 9.0. The inhibition of virucidal activity of sodium hypochlorite by 0.05% organic matter could be overcome by increasing the virucide concentration to 50 ppm in a solution of pH 6.5.

Organic matter was added to some test systems to retard virucidal action of hypochlorites sufficiently to allow a study of pH effects. Under such conditions sodium hypochlorite was most effective when employed at pH 4.0. Virucidal activity rapidly decreased as the pH of the solutions was raised through pH 7.0. Little change in activity occurred between pH 7.0 and 9.0. This behavior suggests that undissociated hypochlorous acid is responsible for virucidal activity.

The rapidity and completeness of phage destruction by chlorine compounds qualifies them as excellent sanitizing agents for use in phage control.

Aqueous iodine was shown to be an effective virucide in concentrations of 50 ppm or more. The iodophor and solutions of iodine-potassium iodide were ineffective

against phage in concentrations as high as 200 ppm at pH levels near neutrality. Equilibrium reactions between molecular iodine and solubilizing agents such as potassium iodide and nonionic wetting agent may prevent the attainment of effective virucidal concentrations of free iodine.

The virucidal activity of the iodophor and aqueous iodine was studied over a pH range of 4.0 through 9.0. Both types of solutions were more effective in the alkaline range. The mechanism of action of iodine was not determined in these studies, however the greater activity of iodine solutions in the alkaline range suggests that some mechanism other than oxidation by molecular iodine is responsible for phage inactivation.

The iodophor was not shown to be sufficiently effective against S. cremoris phage to recommend its use as a virucidal agent.

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